Cytotoxic and Genotoxic Potential of *Trigonostemon reidioides* Extract on Human Caco-2 Cells

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Background: *Trigonostemon reidioides roots have been used as a Thai traditional medicine for the treatment of drug addiction; however, the cytotoxic and genotoxic potential of the herbal extract on human cells have not been clarified.*
Objective: *To evaluate the cytotoxicity and genotoxicity of the ethanolic extract of T. reidioides roots (TR) on human intestinal epithelial Caco-2 cells.*
Material and Method: *Cell viability of TR (0.005 to 0.5 mg/mL)-treated Caco-2 cells was measured by MTT assay after 24 and 48 h exposure. DNA fragmentation was evaluated by Hoechst 33342 staining and comet assay was conducted in the cells treated with TR at the concentrations equal to and higher than the IC_{50}. The protein expressions of ERK1/2 as well as pERK1/2 were investigated by immunoblotting. Densitometric analysis of thin layer chromatography (TLC) fingerprint of TR was performed to identify scopoletin content.*
Results: *Exposure of the human Caco-2 cells to TR resulted in decreased cell viability with an IC_{50} value of approximately 0.2 mg/mL. Treatment of the cells with TR (0.01 mg/mL) for 24 h and 48 h significantly increased ($p<0.05$) cell proliferation. TR at the concentration of IC_{50} induced DNA fragmentation, and TR (0.2 to 0.5 mg/mL) significantly increased the percentage of DNA in the tail in a concentration-dependent manner as revealed by comet assay. Immunoblotting revealed that the phosphorylation of ERK1/2 was significantly increased ($p<0.05$) in the cells treated with TR (0.5 mg/mL) when compared with the control. Scopoletin content in TLC was approximately 271 ng per mg of extract.*
Conclusion: *The present study contributes current evidence that caution must be practiced concerning the dose and duration of T. reidioides root medication. Moreover, further study in animal models concerning its safety application is required to confirm the finding in the cellular level of the present study.*

Keywords: *T. reidioides, Cytotoxicity, Apoptosis, DNA damage, ERK1/2*

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Trigonostemon reidioides (Kurz) Craib (Euphorbiaceae) roots have been used in Thai traditional medicine. The ingestion of the fine powder of *T. reidioides* roots with water has been used as an emetic and antidote for the detoxification of poisonous substances in body such as those found in mushrooms, shellfish as well as for the treatment of drug addiction[1]. Phytochemical constituents in *T. reidioides* roots include rediocides, stigmasterol, tomentin and scopoletin[2,3]. Rediocides A and G also exhibited cytotoxicity against numerous carcinoma cells such as HeLa cells[1]. Scopoletin has been shown to protect against cell death in various conditions such as ethanol-induced liver injury[4]; moreover, it was shown to act as a teratogen[5]. Since rediocides and scopoletin are found in *T. reidioides* roots, *T. reidioides* roots extract should exhibit genotoxic effects conferred by those phytochemicals. The aim of the present study was to investigate the cytotoxicity and genotoxicity of the ethanolic extract of *T. reidioides* roots (TR) in human colon adenocarcinoma Caco-2 cells which were commonly used as *in vitro* human intestinal epithelial cell models for permeability studies[6]. Furthermore, Caco-2 cells have been used as a model for genotoxicity

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in numerous studies[7,8]. In addition to the examination of genotoxicity and cytotoxity in Caco-2 cells, the present study further investigated the potential molecular pathway effectors such as ERK1/2 in the treated cells. Although, ERK1/2 is usually activated by mitogenic agents[9], activation of phosphorylated extracellular signal-regulated kinase (ERK) 1/2 has been demonstrated in both cytotoxic and apoptotic induction[10,11]. Hence, the effect of the extract on the activation of ERK 1/2 on the Caco-2 cells was also investigated.

Material and Method

Drugs and chemicals

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and non-essential amino acid were provided by Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), SYBR® Safe DNA gel stain, and low melting-point agarose were purchased from Invitrogen (New Zealand). Primary antibodies against ERK1/2 and phospho- (p)ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA). Fetal bovine serum (FBS) (HyClone®) was from Perbio Science bvba (Belgium). All other reagents and solvents were of analytical grade.

Plant material and extraction

The roots of *T. reidioides* were collected from Kanchanaburi, Thailand in May 2008; and were identified by Associate Professor Dr. Rapeepol Bavovada. A voucher specimen (RB 05-08) was deposited at the herbarium of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Dried roots of *T. reidioides* (200 g) were grounded and passed through a No. 40 sieve (Retsch, Haan, Germany). The powder was mixed with absolute ethanol (1 L) and heated at 60°C on water-bath for 1 h. The marc was repeatedly extracted twice. Total filtrates were combined and evaporated to dryness using a rotary evaporator to obtain a crude ethanolic *T. reidioides* extract (TR) with 2.46% yield. The extract was kept in amber vials at -20°C. Before testing, the extract was dissolved in 100% DMSO and diluted in medium to keep the final concentration at 0.25% DMSO.

Cell culture

Human colon adenocarcinoma Caco-2 cells (HTB-37, American Type Culture Collection, Rockville, MD, USA), were maintained in DMEM supplemented with 15% FBS, 1% nonessential amino acids, 0.5 mM L-glutamine and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were subcultured once a week by 0.25% trypsin-EDTA, and the medium was changed twice a week.

Assessment of cell viability

To assessment whether TR induced cell proliferation and/or cytotoxicity in Caco-2 cells, cell viability was assessed by a dye staining method using MTT reduction assay as described previously[12]. The tetrazolium MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals, which is solubilized and the absorbance can be read by a spectrophotometer. The cells were incubated with TR (0.005 to 0.5 mg/mL) in a 96-well plate for 24 and 48 h. Treated cells were incubated with MTT solution at the final concentration of 1 mg/mL for 4 h at 37°C. The reaction was stopped by adding lysis buffer containing 50% (v/v) N,N-dimethyl formamide and 20% sodium dodecyl sulphate, pH 4.7, and maintained overnight at 37°C. The optical density was measured by a microplate reader (Sunrise Classic, Tecan, Austria) using a wavelength of 595 nm. The results were expressed as the percentage of cell viability of treated cells relative to the vehicle control cells [(mean absorbance of treated cells/mean absorbance of control cells) x100]. The IC_{50} value was also calculated.

Hoechst 33342 staining

To assess whether TR induced apoptotic cell death, nuclear morphological changes were examined by Hoechst 33342 staining. DNA condensation, nuclear fragmentation, and apoptotic bodies are the significant morphological changes of apoptotic cells. Briefly, Caco-2 cells were treated with TR (0.1 to 0.5 mg/mL) or hydrogen peroxide (H_2O_2, a positive control) (100 µg) for 24 h. The cells were then stained with Hoechst 33342 (12 µg/mL) for 15 min at 37°C in the dark. After washing, the cells were immediately observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Comet assay

The single cell gel electrophoresis assay known as the comet assay is a versatile, sensitive, simple and economical technique used to assess the DNA strand breaks. Comet assay was performed according to Tice et al[13] with minor modifications. Caco-2 cells were exposed to TR (0.01 to 0.5 mg/mL) for 24 h, or 100 µg H_2O_2 for 30 min. Cell suspensions ($1x10^5$

cells/mL) was mixed with 1% low-melting point agarose in equal volume (100 µL) at 37°C, and then applied onto slides pre-coated with 0.8% normal-melting point agarose. After solidification, the embedded cells were immersed in a cold alkaline lysis buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), 1% (v/v) Triton X-100 and 10% (v/v) DMSO for 1 h at 4°C in the dark. After lysis, the slides were placed in cold alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13) and DNA was allowed to unwind for 5 min at 4°C. Electrophoresis was run in the same buffer for 10 min at 25 V and approximately 300 mA, and the slides were rinsed gently with neutralizing buffer (0.4 M Tris buffer, pH 7.5) for 5 min. The slides were dehydrated in absolute methanol and dried at room temperature. The slides were then stained with SYBR® Safe DNA gel stain (1: 10,000), and DNA damage was observed with a fluorescent microscope (Olympus Bx41, Japan). Cells with damaged DNA displayed a bright head and tail (high migration of DNA fragment from the nucleus). Comet Score Freeware version 1.5 (TriTek, Summerduck, VA, USA) was used to calculate % DNA in tail.

Western blot analysis

To evaluate the mechanism underlying TR-induced apoptosis or genotoxicity in Caco-2 cells, pERK1/2 and ERK1/2 stress signaling proteins were measured by immunoblotting. The cells were exposed to TR (0.05, 0.1 and 0.5 mg/mL) for 24 h. H_2O_2 (100 µM) treated cells for 30 min were used as a positive control. After washing with cold PBS, the cells were resuspended in lysis buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1% NP-40, 0.2% NaN_3 supplemented with 1 mM phenylmetanesulfonyl fluoride, 2 µg/mL leupeptin and 2 µg/mL aprotinin. Equal amounts of protein samples mixed with Laemmli sample buffer were resolved by 12% SDS-polyacrylamide gel electrophoresis. The protein bands were transferred onto PVDF membranes, blocked with 5% skimmed milk in TBST (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl and 0.01% Tween-20) for 2 h at room temperature, and probed with the specific primary antibody against ERK1/2 and pERK1/2 (1: 1,000) in TBST with 5% BSA overnight at 4°C. The membranes were incubated with goat anti-rabbit IgG horseradish peroxidase-linked antibodies (1: 5,000) for 2 h at room temperature. Protein bands were visualized using the chemiluminescence assay (ECL kit, GE Healthcare) in Gel Documentation System, GeneGnome5 (Syngene, Cambridge, USA), and the relative density was quantified by using Image J.

Thin layer chromatography (TLC)-densitometric analysis

To determine a fingerprint profile, TLC-densitometric method was performed to evaluate the scopoletin content in TR as modified from a previous report[14]. Various samples 10 µL (2 mg each) of TR were spotted on TLC plate, which was further developed in the mobile phase toluene: diethylether (1:1, v/v, triple development) saturated with 10% acetic acid. The plates were then air-dried, scanned at 344 nm either in absorption or fluorescence mode using a Camag TLC system (Switzerland), comprising a Camag Linomat 5 semiautomatic sample applicator, Camag TLC Scanner 3, Camag win CATS 1.3.5 software.

Statistical analysis

Results are expressed as mean ± SEM, n = 3 to 4. A statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by Turkey's HDS post hoc test for multiple sets of data. A *p*-value <0.05 was considered statistically significant.

Results

TR-induced cytotoxicity and cell proliferation

MTT assay revealed a reduction in the viability of TR-treated Caco-2 cells in a concentration- and time-dependent manner (Fig. 1). TR at concentrations lower than 0.1 mg/mL were not toxic to the cells after up to 48 h incubation (Fig. 1) and significantly exerted cell proliferation ($p<0.05$). Meanwhile, TR at concentrations higher than 0.1 mg/mL decreased the percentage of cell viability after 24 h exposure, with the IC_{50} value at approximately 0.2 mg/mL.

TR-induced apoptosis

Chromatin condensation and nuclear fragmentation indicating apoptotic cell death were observed in Caco-2 cells treated with TR (0.1 to 0.5 mg/mL) and 100 µM H_2O_2 (Fig. 2). On the other hand, the vehicle-treated cells (control) showed round blue nuclei of viable cells.

TR-induced genotoxicity

TR at concentrations lower than the IC_{50} (0.1 mg/mL) did not show DNA damage. Meanwhile TR at concentrations beyond IC_{50} (0.2 to 0.5 mg/mL) significantly induced DNA damage in a concentration-dependent manner after 4 h treatment, as observed by an increase in % DNA in tail (Fig. 3) when compared with the vehicle control.

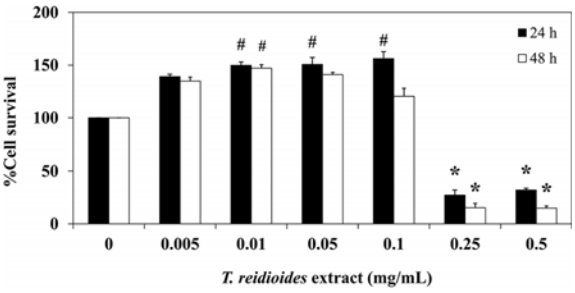

Fig. 1 Cytotoxicity of TR on Caco-2 cells. The cells were incubated with various concentrations of TR (0.005 to 0.5 mg/mL), for 24 and 48 h. Data are expressed as % of control cell survival, measured by the MTT assay. Each point represents the mean ± SEM of four independent experiments, each performed in triplicate. # $p<0.05$, significantly different (increment) compared to the corresponding control. * $p<0.05$, significantly different (decrement) compared to the corresponding control.

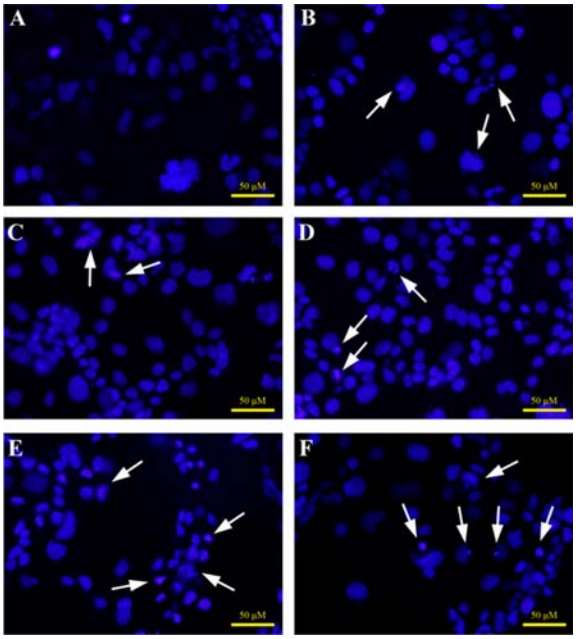

Fig. 2 Morphological changes in TR-treated Caco-2 cells for 24 h. Representative microphotographs of Caco-2 cells stained with Hoechst 33342 of (A) control, (B) H_2O_2 (100 µM), (C) TR 0.1 mg/mL, (D) TR 0.2 mg/mL, (E) TR 0.4 mg/mL and (F) TR 0.5 mg/mL treatments (magnification of x200) of three independent experiments. Arrow marks indicated the apoptotic cells.

TR-regulated the ERK1/2 signaling pathway

H_2O_2 (100 µM) was used as a positive control. TR (0.5 mg/mL) significantly activated the phosphorylation of ERK1/2 to levels of about higher than control (Fig. 4).

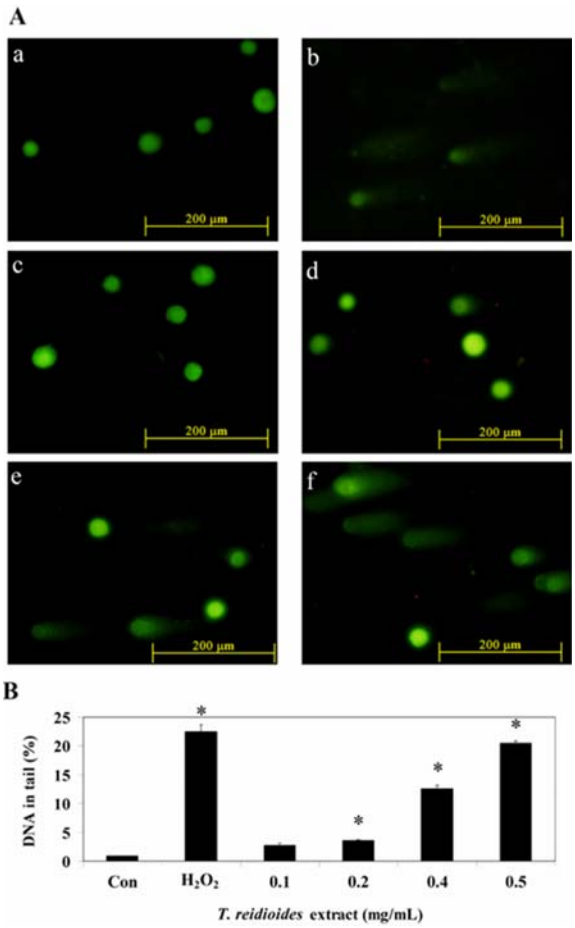

Fig. 3 Assessment of DNA damage by comet assay on TR treated Caco-2 cells. (A) Representative microphotographs of (a) vehicle control, (b) H_2O_2 (100 µM), (c) TR 0.1 mg/mL, (d) TR 0.2 mg/mL, (e) TR 0.4 mg/mL and (f) TR 0.5 mg/mL showed broken DNA (magnification of x20). (B) Mean values of comet parameters % DNA in tail were showed. Results are indicated as mean ± SEM of three independent pictures captured on three independent slides. *$p<0.05$, significantly different to the control.

Phytochemical analysis of TR by TLC-densitometric analysis

Scopoletin in TR showed good separation as compared with standard scopoletin (*Rf* 0.55) (Fig. 5). Scopoletin content in TR was equal to 6.66±1.05 ng per g of dried roots, approximately 271 ng per mg of TR.

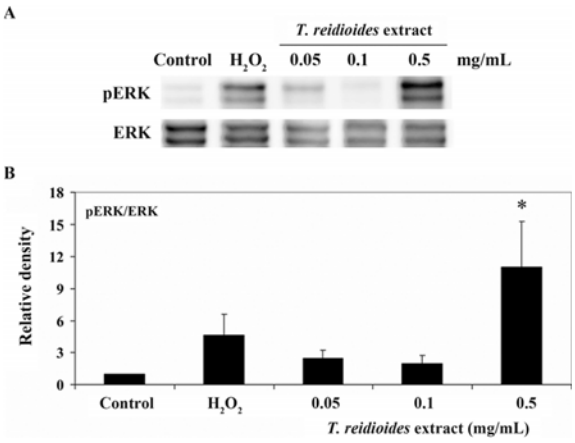

Fig. 4 (A) Representative immunoblots of pERK1/2 and ERK1/2 expression in Caco-2 cells treated with TR and (B) their relative density of pERK1/2 and ERK1/2 ratio. Caco-2 cells were treated with TR (0.05, 0.1 and 0.5 mg/mL) or H_2O_2 (100 μM) for 24 h, phosphorylated ERK1/2 and ERK1/2 were performed by western blotting, and relative density of three independent samples were analysed by using Image J. *p<0.05, significantly different to the control.

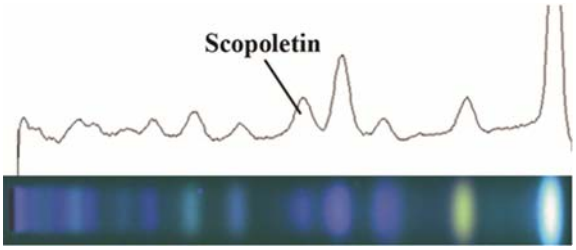

Fig. 5 TLC image and densitogram of TR.

Discussion

According to epidemiologic evidence in Thailand, the ingestion of one-teaspoonful of TR root powder (1 g of dried powder approximately equal to 24.6 mg of TR extract, 2.46% yield) with water has been the formula used for the treatment of drug addiction by stimulating nausea and vomiting. Nevertheless, the appropriate dosage of the extract and the effects on the human body have not been adequately studied. To study the effects of the TR, we used Caco-2 cells as *in vitro* model. Human colonic carcinoma Caco-2 cells are undifferentiated cells that have been used for the determination by comet assay of genotoxic risk from food contaminations such as mycotoxins, nivalenol and fusarenon X[8]. In addition, comet assay has been used to investigate DNA damage caused by substances at the toxic concentrations as it is a rapid, simple, high sensitive and reliable tool for detection of double-stranded DNA breaks originated from genotoxicity[15,16].

The present study showed that 24 h incubation of Caco-2 cell in TR (0.01-0.1 mg/mL equal to 0.00041 to 0.0041 mg of dried roots, respectively) increased cell survival in Caco-2 cells detected by MTT assay. The increased cell survivability in TR may be explained by a previous study demonstrating that TR at lower concentrations increased the activity of mitochondrial dehydrogenase activity[12] in Caco-2 cells. In contrast, TR at concentrations equal or higher than the IC_{50} (0.2 mg/mL) were toxic to the cells as determined by MTT assay, caused DNA fragmentation as measured by Hoeschst 33342 and comet assay in our model system. Therefore, the beneficial or the deleterious effect of the TR is time- and concentration-dependent.

As shown in this study, TR (0.1 mg/mL) increased the activity of mitochondrial dehydrogenase as described above; it did not cause DNA fragmentation to be detected by comet assay. Hence, DNA damage was suggested when comet tail was significant detected at toxic concentrations equal to or higher than the IC_{50}. The present study showed that TR (0.2 to 0.5 mg/mL) caused DNA damage in a concentration-dependent manner detected by comet assay suggesting TR-induced DNA damage.

It has been recognized that the severity of cytotoxicity and genotoxicity of herbal extracts depend on concentrations, incubation time and cell types[17]. However, studies into the toxic effects of herbal extracts must take into consideration the action of their individual compounds, as some previous studies have demonstrated the net actions of crude extracts resulted from the combined biological action of their active ingredients[18]. In fact, some active ingredients of TR such as rediocides had been shown to be cytotoxic against cancer cell lines HeLa and HepG2[1], while other active ingredients including stigmasterol exhibited antiperoxidative effects in mice[19]. Additionally, beta-sitosterol was demonstrated to prevent genotoxic damage induced by doxorubicin[20] and showed potent anti-proliferative effects against human lung adrenocarcinoma A549 cells through cell cycle arrest and the induction of the mitochondrial pathway[21]. Moreover, scopoletin prevented the decrease of antioxidant activity in ethanol-treated HepG2 cells[4]. These reports can thus explain the paradoxical effects of TR in some experimental settings, as the extracts used have various constituents that have differing effects on cell viability.

Our data demonstrated that phosphorylation of ERK1/2 in Caco-2 cells was found after treatment of TR (0.5 mg/mL) as well as H_2O_2 (100 μM), which was used as a positive control. The increment of activated ERK1/2 in association of cell death after exposure of H_2O_2 at cytotoxic concentrations was demonstrated on various cell lines such as human SK-N-MC neuroblastoma cells[10] and human urinary bladder cancer J82 cells[22], and after induction of active substances in HCT116[23] and in Caco-2 human colon cancer cells[24]. The present data possibly suggests that ERK1/2 signaling pathway could require in TR-induced cytotoxicity and apoptosis in Caco-2 cells.

In conclusion, the present study has demonstrated that the TR at low concentrations (0.01-0.1 mg/mL) increased cell survival, and did not cause genotoxicity. In contrast, TR at higher concentrations (0.2 mg/mL) induced apoptosis and DNA damage which may be partly via phosphorylation of ERK1/2. The present study, using Caco-2 cells as a model system, suggests that ingestion of *T. reidioides* roots as herbal medicine should be of concern regarding dose of treatment.

What is already known on this topic?

T. reidioides extract and active ingredient rediocides were cytotoxic to cancer cell lines HeLa and HepG2.

What this study adds?

The present study is to demonstrate the cytotoxic and genotoxic potential as well as ERK1/2 protein expression of *T. reidioides* extract on human colon adrenocarcinoma Caco-2 cells.

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Potential conflicts of interest

None.

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แนวโน้มความเป็นพิษต่อเซลล์และจีนของโลดทะนงแดงในเซลล์ Caco-2

พรรณี หนูซื่อตรง, เมยานี บุญมาเลิศ, ธงชัย กูบโคกกรวด, บุญรัตน์ จันทร์ทอง, นนท์ธเนศ นลินรัตน์, ดวงเดือน เมฆสุริเยนทร์

ภูมิหลัง: รากโลดทะนงแดง (Trigonostemon reidioides) เป็นยาสมุนไพรไทยที่ใช้รักษาอาการติดยาเสพติด แต่ยังไม่เคยมีการศึกษาพิษต่อเซลล์และต่อจีน

วัตถุประสงค์: เพื่อประเมินความเป็นพิษต่อเซลล์และจีนของสารสกัดเอทานอลจากรากของโลดทะนงแดง (TR) ต่อเซลล์เพาะเลี้ยงจากเยื่อบุลำไส้ของมนุษย์ Caco-2

วัสดุและวิธีการ: บ่มเซลล์ Caco-2 ด้วย TR ที่ความเข้มข้นระหว่าง 0.01 ถึง 0.5 มิลลิกรัมต่อมิลลิลิตร เป็นเวลา 24 และ 48 ชั่วโมง เมื่อครบกำหนดเวลาทำการวัดอัตราการอยู่รอดของเซลล์ด้วยวิธี MTT assay ประเมินการแตกของดีเอ็นเอโดยการย้อมสีด้วย Hoechst 33342 และวัดความหนาแน่นของ DNA damage ด้วยวิธี comet ศึกษาการแสดงออกของโปรตีน ERK1/2 และ pERK1/2 โดยวิธี western blot โดยใช้ไฮโดรเจนเปอร์ออกไซด์เป็นสารควบคุมบวก และศึกษาลายนิ้วมือของโลดทะนงแดงด้วยวิธี Thin layer chromatography (TLC) โดยวัดปริมาณ scopoletin ใน TR

ผลการศึกษา: ค่า IC_{50} (24 ชั่วโมง) เท่ากับ 0.2 มิลลิกรัมต่อมิลลิลิตร การบ่ม TR ที่ความเข้มข้น 0.01 มิลลิกรัมต่อมิลลิลิตร เป็นเวลา 24 และ 48 ชั่วโมง พบว่าเพิ่มจำนวนเซลล์อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ในทางตรงกันข้าม การบ่มเซลล์ที่ความเข้มข้น 0.2 มิลลิกรัมต่อมิลลิลิตร พบการแตกหักของดีเอ็นเอ การศึกษาโดยวิธี comet พบว่า TR (0.2, 0.4 และ 0.5 มิลลิกรัมต่อมิลลิลิตร) ทำให้ดีเอ็นเอถูกทำลายมากขึ้นเป็นลำดับตามความเข้มข้นที่มากขึ้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) และ TR ที่ความเข้มข้น 0.5 มิลลิกรัมต่อมิลลิลิตร เพิ่มการแสดงออกของโปรตีน pERK1/2 เมื่อเปรียบเทียบกับกลุ่มควบคุมผล TLC พบปริมาณ scopoletin เท่ากับ 271 นาโนกรัมต่อ TR 1 มิลลิกรัม

สรุป: การศึกษาครั้งนี้ให้ข้อเสนอแนะว่าการใช้รากโลดทะนงแดงควรคำนึงถึงขนาดและระยะเวลาที่ได้รับ และควรมีการศึกษาความปลอดภัยในการใช้สมุนไพรชนิดนี้เพิ่มเติมในสัตว์ทดลองต่อไป