Apoptotic Induction of Skin Cancer Cell Death by Plant Extracts

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Objective: *The aim of the present study was to investigate the effects of plant extracts on cancer apoptotic induction.*
Material and Method: *Human epidermoid carcinoma A431 cell line, obtained from the American Type Culture Collection (ATCC, Manassas, VA), was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% carbon dioxide (CO_2). Plant extract solutions were obtained from S & J international enterprises public company limited. These plant extracts include 50% hydroglycol extracts from Etlingera elatior (Jack) R.M.Smith (torch ginger; EE), Rosa damascene (damask rose; DR) and Rafflesia kerrii Meijer (bua phut; RM). The cell viability, time and dose dependency were determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A431 cells were treated with the plant extracts and stained with Hoechst 33342 fluorescent staining dye.*
Results: *Cell viability was demonstrated by the inhibitory concentration 50% (IC_{50}). The anti-proliferative effects were shown to be dependent on time and dose. Typical characteristics of apoptosis which are cell morphological changes and chromatin condensation were clearly observed.*
Conclusion: *The plant extracts was shown to be effective for anti-proliferation and induction of apoptosis cell death in skin cancer cells. Therefore, mechanisms underlying the cell death and its potential use for treatment of skin cancer will be further studied.*

Keywords: *Etlingera elatior, Rosa damascena, Rafflesia kerrii, Skin cancer, Apoptosis*

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Skin cancer is becoming an increasingly important public health problem worldwide. Ultraviolet radiation from the sun, the main cause of skin cancer, is absorbed by skin thereby causing DNA damage and induces reactive oxygen species (ROS) in the cells. Skin cancers are classified into two type, non-melanoma skin cancer (NMSC) and cutaneous melanoma (CM). NMSC arises from other epidermal cells were subdivided into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), while melanoma originates through the transformation of melanocytes[1-4]. The latter is known to mediate the mitochondrial-dependent apoptotic cell death. Multiple studies have already demonstrated the relationship between ultraviolet exposure and increased risk of developing skin cancer[5-7]. Moreover, there are an increasing number of studies that implemented the use of medicinal plants in treatment of cancer. Among the medicinal plants, Etlingera elatior (Jack) R.M.Smith (torch ginger; EE), Rosa damascene (damask rose; DR) and Rafflesia kerrii Meijer (bua phut; RM), these plants were shown to have both antioxidant and cytotoxicity activities in the cells but no data were available on its activities on any cancer cells[8-13]. The present study, therefore, concentrated on investigation of the apoptotic effects of EE, DR and RM in skin cancer cells.

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Material and Method

Chemical reagents

Plant extract solutions were obtained from S & J international enterprises public company limited. The plant extracts included 50% hydroglycol extracts

from flowers of Etlingera elatior (Jack) R.M.Smith (torch ginger; EE), flowers of Rosa damascene (damask rose; DR) and flowers of Rafflesia kerrii Meijer (bua phut; RM).

Cell culture

Human epidermoid carcinoma cell line A431 was obtained from the American Type Culture Collection, CRL-1555 (ATCC, Manassas, VA). Cell line was maintained as a monolayer in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% Carbon dioxide (CO_2).

Cell proliferation and viability assays

Cytotoxicity of EE, DR and RM was initially determined by cell proliferation analysis using MTT assay, a colorimetric method for measuring the activity of living cells[14]. Cells were seeded at a density of 1 x 10^4 cells/well in a 96-well plate and allowed to grow for 24 hour (h). Cells were then treated with EE, DR and RM at various concentrations from 0.001-30 µg/µL, whereas the control group was treated with 50% hydroglycol for 24 h. At the end of the stipulated time, 100 microliters of 0.5 mg/mL MTT solution was added to each well and the plate was further incubated at 37°C. Supernatant was aspirated and 100 microliters of dimethyl sulfoxide (DMSO) was added to each well to solubilize water-insoluble purple formazan crystals. The absorbance at 570 nm was measured using a microplate reader (Multiskan EX, Thermo electron corporation, Finland) and the inhibitory concentration 50% (IC_{50}) value was calculated using GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA).

Time and dose dependent assay

Cells were seeded in a 96-well plate at 1 x 10^4 cells/well and incubated for 24 h. The cells were then treated with various concentrations (0, 3, 6, 12, 24 and 48 µg/µL) of EE, DR and RM for 3, 6, 9, 12 and 24 h. Cell survival was expressed as percentage of viable cells of treated cells compared to control cells. Effect of the EE, DR and RM extracts on cell viability was initially determined by MTT assay as previously described.

Detection of nuclear morphology by Hoechst 33342 nuclear staining

Hoechst 33342 is a fluorescent dye with the ability to bind DNA, thereby causing the nuclei and mitochondria to emit fluorescent light when observed under a UV light[15]. A431cells were seeded at a density of 1 x 10^6 cells/well in a 6-well plate and allowed to grow for 24 h. The cells were then treated with 30 µg/µL of EE, 10 µg/µL of DR and 1 µg/µL of RM for 6 h. After trypsinization, cells were washed with 1X PBS once and stained with 3 µg/mL of Hoechst 33342 for 15 min. Stained cells were washed with PBS once and the emitted light was examined by using fluorescent microscope with an ultraviolet filter.

Results

Cytotoxic potential of the plant extracts

The cytotoxic effect of various concentrations of 0-30 micrograms/ml EE, DR and RM extracts on A431 cells after 3, 6, 9, 12 and 24 h incubation to reveal a time and dose dependent inhibition of cell growth and proliferation (Fig. 1). It was found that IC_{50} of EE, DR and RM was estimated to be 6.18, 3.22 and 0.3 µg/µL, respectively. Based on time and dose dependent values apparent from growth inhibition curve of EE, DR and RM (Fig. 2), 30 µg/µL of EE, 10 µg/µL of DR and 1 µg/µL of RM treatments for 6 h were selected for further studies.

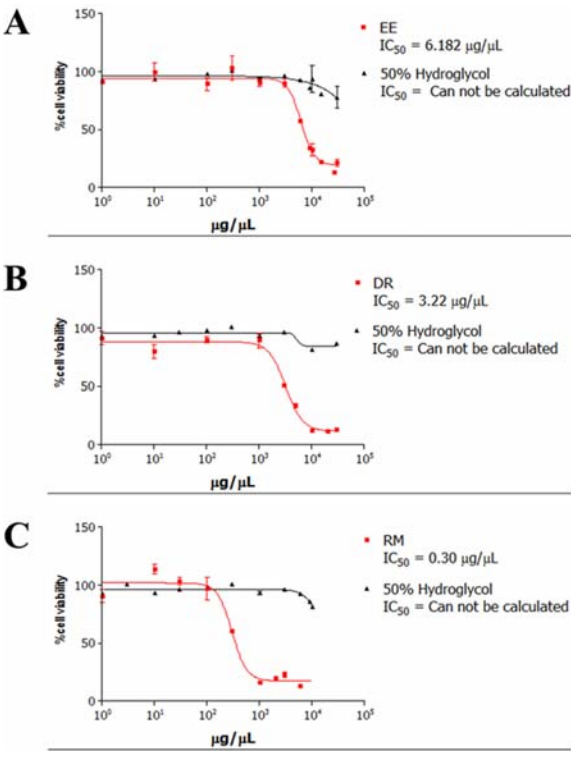

Fig. 1 The effect of EE (A), DR (B) and RM (C) extracts on cell viability (IC_{50}). A431 cells were treated with various concentrations of the extracts 0.001-30 µg/µL for 24 h

Induction of apoptosis by plant extracts

Upon treatment with EE, DR and RM extracts, shrinkage and fused cells were observed comparing with control cells. Following incubation of the A431 cell line with cytotoxic doses of EE (30 µg/µL), DR (10 µg/µL) and RM (1 µg/µL) extracts, cell morphological changes and chromatin condensation were observed by using Hoechst 33342 nuclear staining (Fig. 3).

Hoechst 33342-stained nucleus pulpous cells demonstrated typical apoptotic morphology. Condensation of the nuclear materials was followed by formation of the apoptotic bodies (Fig. 4).

Discussion

Cell proliferation and cell viability tests by MTT assay revealed reduction in the viability of A431 cells upon various concentrations of EE, DR and RM extracts after 24h incubation. It implied the effectiveness of plant extracts to inhibit cell proliferation and induce A431 cell death. Moreover, cell viability was shown to be in a time and dose dependent manner. The data accumulated from these observations were used to determine the doses of the plant extracts and time of incubation for further mechanistic studies which were 30 µg/µL of EE, 10 µg/µL of DR and 10 µg/µL of RM

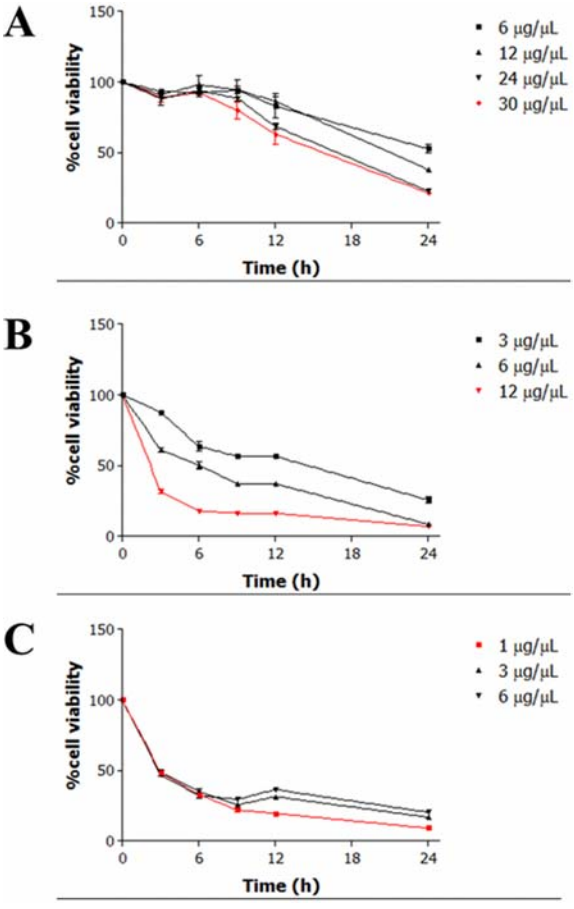

Fig. 2 Time and dose dependent relations of A431cells upon treatment with various concentrations of EE (A), DR (B) and RM (C) 0, 1, 3, 6, 12, 24 and 30 µg/µL for 3, 6, 9, 12 and 24 h incubation

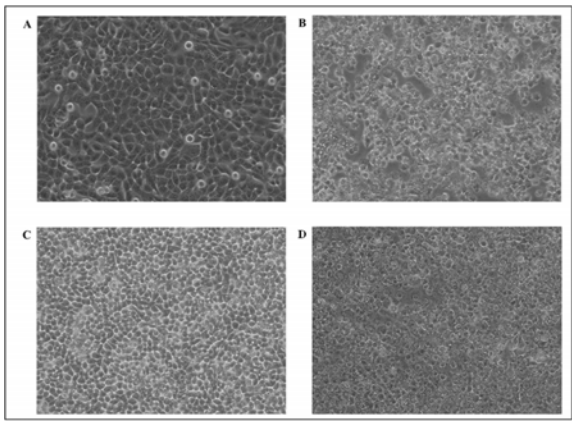

Fig. 3 Morphological changes in A431 cells upon treatment with EE, DR and RM for 6 h of incubation. (A) non-treated A431 cells as control (B) A431 cells treated with 30 µg/µL of EE (C) A431 cells treated with 10 µg/µL of DR (D) A431 cells treated with 1 µg/µL of RM

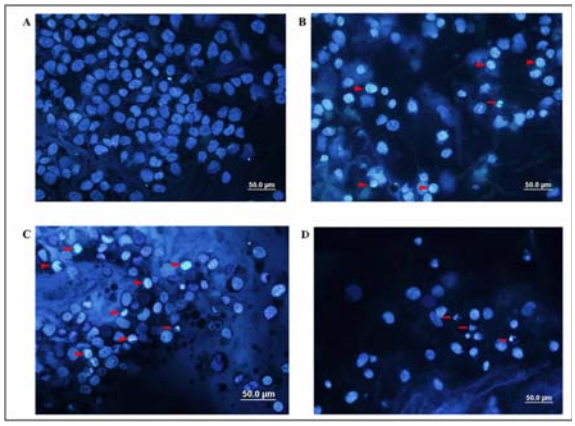

Fig. 4 Apoptotic effect of A431 cells upon treatment with EE, DR and RM for 6 h incubation. Hoechst 33342-stained nucleus of the cells demonstrated typical apoptotic morphology. Nuclear condensations (arrow) of the nuclear material were followed by formation of the apoptotic bodies (arrowheads). (A) Non-treated A431 cells as control (B) A431 cells treated with 30 µg/µL of EE (C) A431 cells treated with 10 µg/µL of DR (D) A431 cells treated with 1 µg/µL of RM

and the incubation time was 6 h.

Cell morphological changes could be obviously seen in Fig. 3 as shrunk and fused cells upon treatment with EE, DR and RM compared with the controlled untreated cells. Moreover, Hoechst 33342 DNA staining revealed condensation of chromatin and nucleus pulpous cells which demonstrated typical apoptotic nuclear morphology (Fig. 4).

The present study demonstrated the effect of plant extracts from EE, DR and RM could inhibit growth and proliferation of A431 cell line. Furthermore, cell morphological changes and chromatin condensation, which led to the induction of apoptosis were also observed.

Apoptosis is known to be the most potent defense mechanism against cancer. Some evidence showed that [6]-gingerol, a ginger extract could induce apoptosis of A431 cell line[16]. [6]-Gingerol was shown to exert its regulatory effects on the activation of apoptotic machinery in A431 cells by increasing ROS which led to decrease in mitochondrial membrane potential (MMP) and deregulation of Bax/Bcl-2 ratio at gene transcriptional level as well as protein expression level. It also induced the up-regulation of cytochrome-c and apoptotic protease activating factor 1 (Apaf-1) which subsequently culminating in triggering of caspase cascade to induction of apoptosis.

In addition, resveratrol (trans-3, 40, 5-trihydroxystilbene), a polyphenolic phytoalexin present mainly in grapes, red wine and berries, was known to possess strong chemopreventive and anticancer properties in several cancer types[17]. Resveratrol has cytotoxic effects through inhibiting cellular proliferation, which led to the induction of apoptosis, as evident by an increase in the fraction of cells in the sub-G1 phase of the cell cycle and regulation of the JAK/STAT pathway. Furthermore, resveratrol treatment actively stimulated ROS and mitochondrial membrane depolarization. Consequently, an imbalance in the Bax/Bcl-2 ratio triggered the caspase cascade and subsequent cleavage of PARP, thereby shifting the balance in favor of apoptosis in A431 cells.

Conclusion

Torch ginger, damask rose and bua phut extracts have the potential to be used as skin cancer treatment. Moreover, it is propitious enough that further characterization of pathways regulating cell cycle progression and apoptosis will facilitate the novel findings of biochemical compounds that have potential use for treatment of skin cancer.

Acknowledgement

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Potential conflicts of interest

None.

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การเหนี่ยวนำการตายในเซลล์มะเร็งผิวหนังโดยใช้สารสกัดสมุนไพร

วลัยรัตน์ ตันเจริญ, มาลิน จุลศิริ, สิรินันท์ นิลวรางกูร, ยูคิโอ นาคามูระ, รมิดา วัฒนโภคาสิน

วัตถุประสงค์: *ศึกษาผลของสารสกัดสมุนไพรที่ในการเหนี่ยวนำการตายแบบ apoptosis ในเซลล์มะเร็งผิวหนัง*
วัสดุและวิธีการ: *เพาะเลี้ยงเซลล์มะเร็งผิวหนัง A431 (human epidermoid carcinoma) ด้วยอาหารเลี้ยงเซลล์ DMEM ในตู้ควบคุมอุณหภูมิ 37°C, 5% CO_2 จากนั้นนำมาทดสอบกับสารสกัดสมุนไพรจากดอกดาหลา (EE), กุหลาบมอญ (DR) และบัวผุด (RM) ซึ่งละลายใน 50% hydroglycol ศึกษาการเหนี่ยวนำการตายของเซลล์มะเร็ง ผิวหนังโดยหาค่า IC_{50} และหาความเข้มข้นและเวลาที่เหมาะสมในการเหนี่ยวนำการตายของเซลล์มะเร็งผิวหนังของสารสกัดสมุนไพรแต่ละชนิดด้วยวิธี MTT assay ศึกษาการเปลี่ยนแปลงรูปร่างของเซลล์มะเร็งผิวหนัง โดยใช้กล้องจุลทรรศน์แบบหัวกลับสังเกตและถ่ายภาพการเปลี่ยนแปลงรูปร่างของเซลล์ ศึกษาการเปลี่ยนแปลงลักษณะนิวเคลียสของเซลล์มะเร็งผิวหนังโดยนำมาย้อมด้วยสีย้อม Hoechst 33342 แล้วถ่ายภาพโดยใช้กล้องจุลทรรศน์แบบเรืองแสง (fluorescent microscope)*
ผลการศึกษา: *สารสกัดจากสมุนไพร EE, DR และ RM มีฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์ A431 ที่ความเข้มข้นแตกต่างกันพบว่าความเข้มข้นของ EE 30 μg/μL, DR 10 μg/μL และ RM 1 μg/μL ทำให้เซลล์มีขนาดและรูปร่างของเซลล์เปลี่ยนแปลงไป เมื่อเปรียบเทียบเทียบกับ control จากนั้นเมื่อทำการย้อมสีฟลูออเรสเซนส์ด้วยสีย้อม Hoechst 33342 พบว่านิวเคลียสมีการหดตัว (chromatin condensation) และเกิด apoptotic bodies ซึ่งเป็นลักษณะหนึ่งของกระบวนการตายของเซลล์แบบ apoptosis*
สรุป: *สารสกัดสมุนไพรจากดอกดาหลา (EE), กุหลาบมอญ (DR) และบัวผุด (RM) แสดงฤทธิ์ในการยับยั้งการเจริญเติบโตและเหนี่ยวนำให้มีการตายของเซลล์ A431*