Anti-Proliferation and Apoptosis Induction in Epidermoid Carcinoma A431 Cells by Artonin E

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Background: *Skin cancer is the type of cancer that is becoming an increasingly important public health problem worldwide. The treatment for skin cancer depends on the stage and location of cancer cells. Therefore, the new finding of anti-cancer compound as a therapeutic candidate for skin cancer is necessary.*
Objective: *To investigate the effect of artonin E on anti-proliferation and apoptosis induction in skin cancer epidermoid carcinoma A431 cells.*
Material and Method: *Cell viability and cell proliferation were determined by MTT assay. Nuclear morphological changes, mitochondrial membrane potential and protein expression were determined by Hoechst 33342 and JC-1 staining, respectively. Protein expression was determined by Western blot analysis.*
Results: *Artonin E showed anti-proliferation in A431 treated cells in a dose-dependent manner with an IC_{50} value of 9.05±6.15 μg/ml. In addition, artonin E induced chromatin condensation and apoptotic bodies in A431 treated cells. JC-1 staining showed that artonin E induced loss of mitochondrial membrane potential. Western blot analysis showed the upregulation of cleaved-caspaase-7 and cleaved-PARP in A431 treated cells.*
Conclusion: *In this study, artonin E showed anti-proliferation and apoptosis induction in A431 treated cells. Moreover, artonin E induced cleaved caspase-7 and cleaved-PARP activation in A431 cells, resulting in apoptosis cell death. Our results indicated that artonin E may be further developed as an anti-cancer drug and the underlying mechanisms of apoptosis induction in A431 cells should be studied.*

Keywords: *Skin cancer, Artonin E, Apoptosis, Anti-proliferation*

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Skin cancer is one of the most common types of cancer diagnosed in the United Kingdom involving exposure to ultraviolet (UV) light or sunlight. Skin cancer is the uncontrolled growth of abnormal skin cells due to the unrepaired DNA damage in normal cells[1]. Skin cancer was divided into two main groups including malignant melanoma (MM) and a non-melanoma skin cancer (NMSC). The NMSC consists of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)[2]. In this study, A431 was used as a SCC model. A431 is an epidermoid carcinoma cell line, which express abnormally high levels of epidermal growth factor receptor (EGFR). This cancer cell type was used in studies of the cell cycle and cell signaling pathways in skin cancer[3].

Apoptosis or programmed cell death is a type of cell death, which is an essential physiological process that occurs in multiple cellular including normal cell turnover, development of an embryo, tissue homeostasis as well as elimination of damaged cells[4]. The morphological characteristics changes in early process of apoptosis including cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation called "apoptotic bodies"[5]. Caspases are family of protease enzymes play an important roles in apoptosis process. Activation of caspase-9, -7 and caspase-3 causes the cleavage vital substrate PARP, resulting in cellular death[6]. Caspase deficiency has been linked to the development of many cancers as well as cancer metastasis.

Artonin E is an active flavonoid isolated from the stem bark of the *Artocarpus* family. The plant in

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this family was mostly found in Southeast Asian and Pacific origin, which are cultivated throughout the tropics[7]. Previously, artonin E showed variety of properties such as cancer cell growth inhibition[8], cancer cell apoptosis induction[9], anti-microbial and anti-malarial activity[10]. However, the effects of artonin E on human epidermoid carcinoma skin cancer, which EGFR overexpression, have not yet been studied. Therefore, we aimed to verify the hypothesis that artonin E could inhibit the growth of the human skin cancer A431 cell line through induction of apoptosis.

Material and Method

Cell culture

Epidermoid carcinoma cell line A431 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained as a monolayer in DMEM medium (Invitrogen Life Science, USA) supplemented with 10% FBS (GE Healthcare, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories, Pasching, Austria). The cells were cultured in 5% CO_2 at 37°C.

Natural compound

Artonin E extract was obtained from Associate Professor Dr. Wilawan Mahabusarakam, Prince of Songkhla University. It was purified from the fruit of *Artocarpus elasticus*. The powder of artonin E was dissolved and diluted in DMSO.

Reagent

Chemicals for cell viability assay, 3-(4,5-dimethylthaiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) was purchased from Merck Calbiochem (San Diego, CA, USA). Chemicals for fluorescence microscope observation, Hoechst 33342 dye and 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl-imidacarbocyanine iodide (JC-1) dye were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell proliferation and cell viability assays

The cytotoxicity of artonin E was determined by MTT assay. A431 cells were seeded at $5x10^3$ cells/well for 24 h. Then, cells were treated with artonin E at various concentrations (0.5, 1, 3, 5, 10, 30 and 50 µg/ml), whereas the control group was treated with 0.5% DMSO for 24 h. After incubation, MTT solution was added to each well, and the plate was further incubated for 2 h at 37°C. Then MTT solution was removed and DMSO was added to solubilize purple formazan crystals. The absorbance was measured using a microplate reader at 570 nm (Multiskan EX; Thermo Electron Corp., Vantaa, Finland), and the IC_{50} value was calculated using the GraphPad Prism 3.03 (GraphPad Software, Inc., San Diego, CA, USA).

Nuclear morphological staining

Hoechst 33342 is a fluorescence dye used for labeling DNA, it's a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to double stranded DNA[11].

A431 cells were seeded at $3x10^5$cells/well for 24 h. The cells were treated with 3, 10 and 30 µg/ml artonin E for 24 h as well as DMSO as a control. After incubation time, Hoechst 33342 (final concentration 10 µM) was added to stain nucleic acid for 30 min at 37°C and examined under a fluorescence microscope (IX73; Olympus, Tokyo, Japan).

Measurement of mitochondrial membrane potential (Δψm)

One of the key events in apoptosis induction is the loss of mitochondrial membrane potential (Δψm). JC-1 dye is commonly used in apoptosis studies to monitor mitochondrial health with selectively enter into mitochondria. In healthy cells with high Δψm, JC-1 forms complex as J-aggregated form with intense red fluorescence. During apoptosis with low Δψm, JC-1 remains in the monomeric form with green fluorescence[12]. JC-1 staining was used to monitor the Δψm which is specific to mitochondria. Cells were seeded at $3x10^5$cells/well for 24 h and treated with 10 µg/ml artonin E for 3, 6 and 9 h. Then, cells were stained with 5 µg/ml of JC-1 in the dark at 37°C for 15 min and washed with PBS for 3 times before analysis by fluorescence microscopy.

Western blot analysis

To detect the caspase and c-PARP expression in treated cells Western blot analysis was used to determine. Cells were treated with 3, 10 and 30 µg/ml artonin E for 24 h, harvested and washed with ice-cold PBS. After that cell lysates were lysed RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.5% Triton X-100) containing complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Pall Corporation, USA) for 1 h at 100 V with the use of a Mini Trans-Blot Cell® (Bio-Rad). After blocking with 5% nonfat milk in TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween 20),

the blots were incubated overnight with caspase-7, c-PARP and β-Actin at 4°C (Cell Signaling Technology, Beverly, MA). The membranes were washed in TBST and incubated with appropriate secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. The specific protein bands were detected by chemiluminescent HRP substrate (Merck Millipore Corp., Merck KGaA).

Statistical analysis

All data presented were obtained from at least three independent experiments and were presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS statistical software package (version 11.5) and also carried out using the software GraphPad Prism 3.03 (GraphPad Software, Inc.). Statistical significance was assessed by one-way analysis of variance (ANOVA). The western blotting band intensity was quantified by ImageJ densitometer. An asterisk indicates that the experimental values are significantly different from those of the control ($p \leq 0.05$).

Results

Artonin E inhibits cell viability in A431 skin cancer cells

The anti-proliferation activity of artonin E in the A431 cells was determined by MTT assay. The IC_{50} value of artonin E was 9.05±6.15 μg/ml, which inhibited cell viability in a dose-dependent manner. Artonin E at 30 μg/ml reduced cell viability to about 20% comparing with control cells upon 24 h incubation (Fig. 1B).

Artonin E induce nuclear morphological changes in A431 cells

To determine whether artonin E induces nuclear morphological changes in A431 treated cells, Hoechst 33342 staining was carried out. Hoechst 33342 stain revealed condensed chromatin as well as apoptotic bodies in the A431 treated cells (Fig. 2). The results suggested that artonin E induced chromatin condensation and apoptotic bodies in A431 cells, characteristics of apoptotic cells.

Artonin E induce the loss of mitochondrial membrane potential (Δψm)

The results showed that A431 cells treated with 10 μg/ml artonin E for 3, 6 and 9 h showed an increased green fluorescence, while the control cells showed only red fluorescence (Fig. 3) indicating that 10 μg/ml artonin E induced the loss of Δψm in the A431 cells.

Artonin E induce the apoptosis through caspase and cleave-PARP activation

To determine whether artonin E induced apoptosis through caspase activation, we examined the expression of caspase-7 and cleave-PARP. As shown in Fig. 4A, 10 μg/ml artonin E increased cleave-caspase-7 and cleave-PARP expression compared with the control group. These results indicate that artonin E induced apoptosis through caspase activation.

Discussion

In this study, A431 cell line was used as a

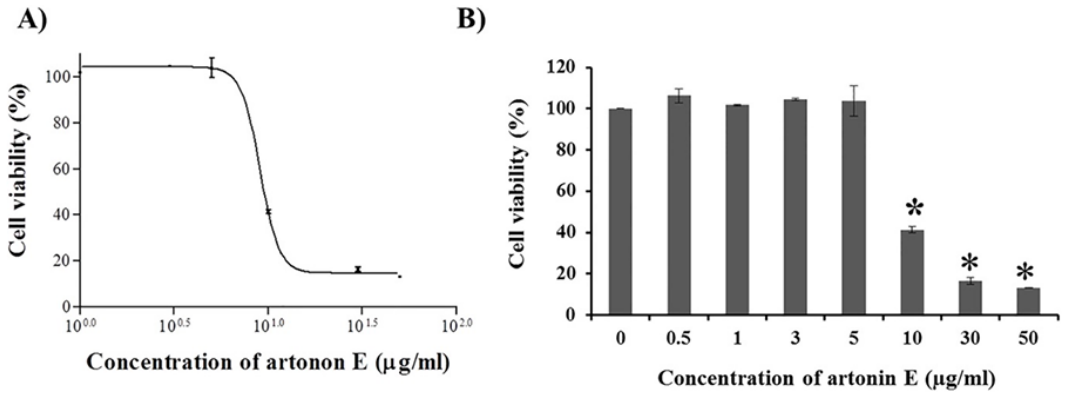

Fig. 1 Effect of artonin E on cell viability assay. (A) The MTT assay with IC_{50} value of artonin E against A431 cells at 24 h. (B) Effect of artonin E on A431 cell viability following treatment with different concentrations of artonin E at 24 h. The IC_{50} value was expressed as mean ± SD from at least three independent experiments. *p<0.05, vs. control.

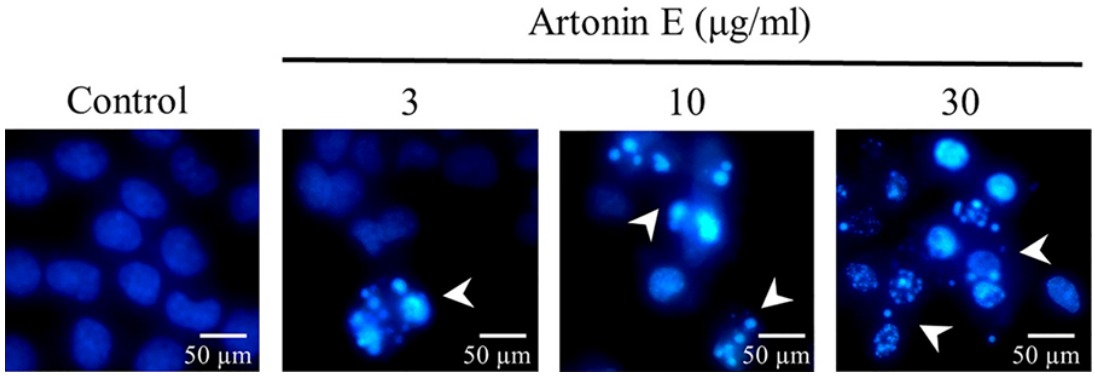

Fig. 2 Effects of artonin E on nuclear condensation in A431 cells. Cells were treated with 3, 10 and 30 µg/ml artonin E for 24 h and then stained with Hoechst 33342 and examined under a fluorescent microscope (magnification, x40). Apoptotic bodies are indicated by white arrow.

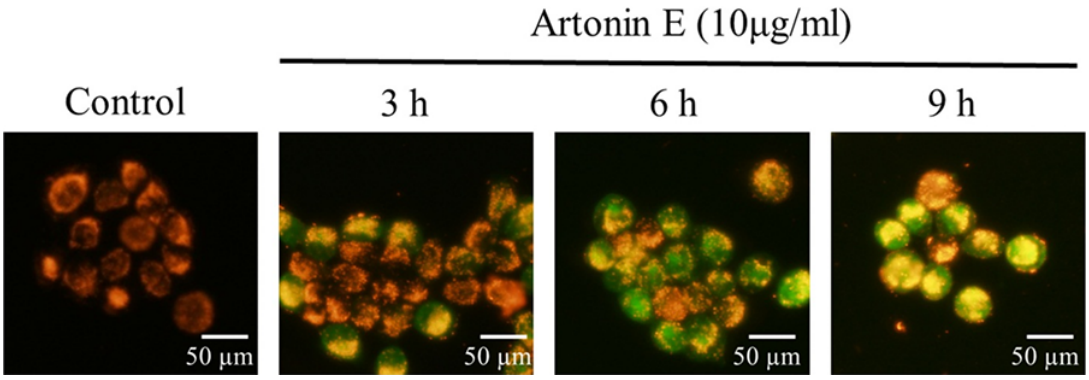

Fig. 3 Effect of artonin E on mitochondrial membrane potential in A431 cells. Cells were treated with 10 µg/ml artonin E for 3, 6 and 9 h. Red fluorescence in the control cells indicated high membrane potential and green fluorescence in the artonin E treatment indicated loss of membrane potential. The artonin E treatment showed an increased green fluorescence intensity in A431 cells (magnification, x40).

model for epidermoid carcinoma skin cancer. Recent study has demonstrated that artonin E showed several activities including anti-migration and anti-invasion against lung cancer cell lines. Plaibua et al reported that artonin E exhibited anti-migration and anti-invasion in human lung cancer H292 cell line, which decreased of phosphorylated FAK (Tyr397), phosphorylated Akt (S473) and CDC42[8]. Artonin E was shown to enhance anoikis (anoikis is a form of apoptosis) by down regulation of the anti-apoptotic protein MCL-1 protein in lung cancer cell line H460[13]. Recently, Molecular docking employing Glide was used to study the binding affinities of artonin E with estrogen receptor (ER). The best glide scored ligand of artonin E was -12.72 Kcal and inhibit growth of an ER positive breast cancer cells at low concentration 3.8 to 6.9 µM compared with standard drugTamoxifen (18.9 to 24.1 µM)[14]. In case of TRIAL resistance, artonin E induced apoptosis via caspase-3 and caspase-7 activation with TRAIL combination as well as enhanced the protein levels of p53 and DR5 upregulation[15]. Moreover, Rahman et al reported that artonin E inhibited the growth of human ovarian cell line SKOV-3 with an IC_{50} value of 12.83±0.28 µg/ml at 24h. Artonin E induced apoptosis through caspase-3, -8 and caspase-9 as well as induced S phase arrest. On the other hand, artonin E showed less toxicity to immortalized normal human ovarian surface epithelial cell line T1074 (IC_{50} 44.8±0.76 µg/ml), normal Chinese hamster ovary cell line CHO (IC_{50} 57.6±2.0 µg/ml) and human periodontal ligament fibroblasts (IC_{50} 67.0±1.0 µg/ml)[9]. However, the mechanisms of apoptosis induction in skin cancer A431 cells have not yet been reported. Our study showed that artonin E inhibited A431 cell growth in a dose-dependent manner with an IC_{50} value of 9.05±6.151 µg/ml (Fig. 1). Then we confirm apoptosis induction by investigated

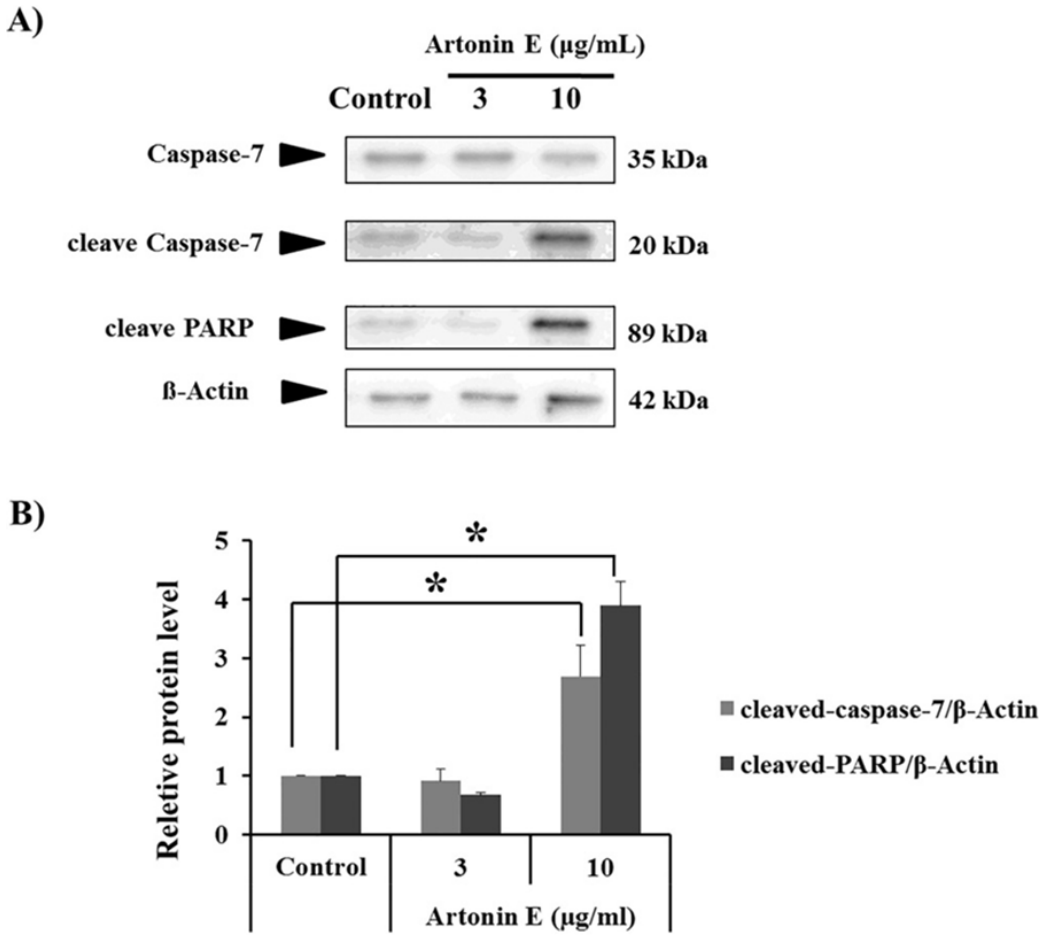

Fig. 4 Effect of artonin E on apoptotic protein expression. Cells were treated with 3 and 10 μg/ml artonin E for 24 h. (A) Effect of artonin E on cleaved-caspase-7 and cleave-PARP in A431 cells was determined by Western blot analysis. (B) Artonin E treated cells showed a significant increase in the cleave-caspase-7/β-Actin ratio and cleave-PARP/β-Actin ratio at 10 μg/ml artonin E in the A431 cells using β-Actin as an internal control. The quantitative data are expressed as the mean ± SD from at least three independent experiments. **p*<0.005, vs. control.

characteristic morphological changes in A431 treated cells. Nuclear stain revealed condensed chromatin and apoptotic bodies in the A431 cells after treated with artonin E (Fig. 2). Furthermore, the effect of artonin E on the mitochondrial membrane potential in A431 cells was monitored by increased green fluorescence at 3 h (Fig. 3). The loss of the Δψm and release of some pro-apoptotic proteins into the cytosol stimulated apoptosome formation followed by activation of caspase-9[16]. Then active caspase-9 can activate the effector caspases (-3, -6, -7), which cleave vital substrates including PARP, resulting in apoptosis induction[17]. In this study, cleaved caspase-7 and cleaved PARP were increased by 10 μg/ml artonin E (Fig. 4), but at 30 μg/ml artonin E was more toxic to A431. In addition, 10 μg/ml of artonin E induced apoptosis in A431 cells indicated by nuclear morphological changes, decreased Δψm and mediator protein activation. However, the mechanism of apoptosis induction and anti-proliferation in A431 cell lines by artonin E need to be further determined.

Conclusion

Artonin E showed anti-proliferation and apoptosis induction in epidermoid carcinoma A431 cell line through growth inhibition, nuclear morphological changes including chromatin condensation and decreased mitochondrial membrane potential. Therefore, artonin E may be used as an anti-cancer agent in epidermoid carcinoma A431 to inhibit cell proliferation through apoptosis-associated cell death induction. Thus, the mechanism of apoptosis induction

as well as cell proliferation inhibition in animal models need to be further studied.

What is already known on this topic?

Artonin E is an effective bioactive compound, which has been reported in several activities including anti-cancer, anti-malarial and anti-microbial as well as artonin E showed strongly inhibit cell growth in various cancer cell lines.

What this study adds?

Artonin E inhibited cell proliferation in epidermoid carcinoma A431 cell line. Moreover, artonin E induced apoptosis cell death resulting in nuclear morphological changes, decreased mitochondrial membrane potential and increased cleaved caspase-7 as well as cleaved-PARP in epidermoid carcinoma cell line A431.

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Potential conflicts of interest

None.

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การยับยั้งการเจริญเติบโตและการเหนี่ยวนำการตายแบบอะพอพโทซิสในเซลล์มะเร็งผิวหนัง A431 โดยสาร Artonin E

สุกานดา อินนาจักร, ศุภกร ตั้งจิรคภัณฑ์, สิรินันท์ นิลวรางกูร, นุจรี ตัญจพัฒนกุล, วิลาวัลย์ มหาบุษราคัม, รมิดา วัฒนโภคาสิน

ภูมิหลัง: มะเร็งผิวหนังเป็นมะเร็งชนิดหนึ่งที่เป็นปัญหาสาธารณสุขที่สำคัญทั่วโลกสำหรับการรักษานั้นจะขึ้นอยู่กับระยะและบริเวณที่เกิดมะเร็ง ดังนั้นการค้นหาสารที่มีคุณสมบัติในการต้านมะเร็งสำหรับมะเร็งผิวหนัง จึงมีความสำคัญเป็นอย่างยิ่ง

วัตถุประสงค์: ศึกษาฤทธิ์ของสารartonin E ต่อการยับยั้งการเจริญเติบโตและการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสในเซลล์มะเร็งผิวหนัง

วัสดุและวิธีการ: ทดสอบฤทธิ์ของสาร artonin E ในการยับยั้งการเจริญเติบโตของเซลล์ด้วยวิธี MTT assay ศึกษาการเปลี่ยนแปลงรูปร่างของนิวเคลียสวิเคราะห์ความสมบูรณ์ของเยื่อหุ้มไมโตคอนเดรียด้วยวิธีการย้อมสี Hoechst 33342 และ JC-1 ตามลำดับการแสดงออกของโปรตีนนั้นจะใช้วิธี Western blot ในการศึกษา

ผลการศึกษา: สาร artonin E มีฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งผิวหนังชนิด A431 ซึ่งขึ้นอยู่กับความเข้มข้นของสารที่ใช้โดยมีค่า IC_{50} อยู่ที่ 9.05±6.15 μg/ml และสาร artonin E ยังกระตุ้นให้เกิดการหดตัวของโครมาตินรวมถึงการเกิดรูปแบบของ apoptotic bodies ในเซลล์มะเร็ง A431 ผลจากการวิเคราะห์ความสมบูรณ์ของเยื่อหุ้มไมโตคอนเดรียพบว่าสาร artonin E เหนี่ยวนำให้เกิดความเสียหายของเยื่อหุ้มไมโตคอนเดรีย สำหรับผลจาก Western blot analysis แสดงให้เห็นว่ามีการแสดงออกของโปรตีน cleaved-caspase-7 และ cleaved-PARP ในเซลล์มะเร็งผิวหนังชนิด A431 ที่ถูกทดสอบด้วยสาร artonin E

สรุป: ในการวิจัยครั้งนี้พบว่าสาร artonin E แสดงคุณสมบัติในการยับยั้งการเจริญเติบโตและเหนี่ยวนำให้เกิดการตายของเซลล์แบบอะพอพโทซิสในเซลล์มะเร็ง A431 นอกจากนี้สาร artonin E ยังสามารถกระตุ้น caspase-7 และ cleaved-PARP เพื่อนำไปสู่กระบวนการตายแบบอะพอพโทซิส ดังนั้นผลการศึกษาอาจเป็นทางเลือกในการพัฒนาไปเป็นยาสำหรับรักษาโรคมะเร็ง แต่ทั้งนี้กลไกในการเหนี่ยวนำให้เกิดการตายในเซลล์มะเร็งชนิดนี้ควรมีการศึกษาในเชิงลึกต่อไป