Apoptosis Induction in Breast Cancer Cells by Cowanin

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Background: *Breast cancer is a common cancer occurs in women worldwide. In Thailand and United States, it is the first and second leading cause of cancer-related death among females, respectively. Therefore, a novel bioactive compound with high selectivity for cancer cell death is important in cancer research.*
Objective: *To investigate the effect of cowanin on anti-proliferation and apoptosis induction of breast cancer cell, MDA-MB-468.*
Material and Method: *MTT assay was used for cell proliferation and viability assay. Nuclear morphological changes and mitochondrial membrane potential was determined by Hoechst 33342 staining and JC-1 staining, respectively.*
Results: *Cowanin showed anti-proliferation and cell viability reduction in MDA-MB-468 cells in a time- and dose-dependent manner with IC_{50} value of 16.27±0.88 µg/mL. Furthermore, cowanin induced chromatin condensation, apoptotic bodies and mitochondrial membrane dysfunction in MDA-MB-468 cells.*
Conclusion: *Cowanin showed anti-proliferation and apoptosis induction in MDA-MB-468 cells. These results are basic knowledge for developing the chemotherapeutic agent for breast cancer in the future.*

Keywords: *Breast cancer cells, Cowanin, Apoptosis, Cells proliferation*

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Breast cancer is a malignant tumor that starts in the breast tissue and can occur in both males and females. It is the second leading cancer in the world[1]. In Thailand, breast cancer is the first leading cause of cancer-related deaths among females. The American Cancer Society estimated 230,480 cases were diagnosed in women for 2011 in the USA alone, resulting in 39,970 deaths. Since 1990, death rates from breast cancer have decreased by over 25% as patients with breast cancer were treated with tamoxifen and chemotherapy[2,3]. However, the chemotherapy still has some side effects on the patients. Therefore, novel bioactive compound with high selectivity for cancer cell death is crucial for cancer research.

Apoptosis is a programmed cell death that Kerr, Currie and Wyllie defined for the first time in 1972[4]. Apoptosis occurs normally during development, aging and as a homeostatic mechanism to maintain cells in tissues. Apoptosis has been divided to two signaling pathways: first, the extrinsic or death receptor pathway which is activated by pro-apoptotic receptor signals at the cellular level. Then the activated caspase-8 leads to apoptosis. The second is intrinsic or mitochondrial pathway which involves a dysfunction of mitochondrial membrane potential. The characteristics of apoptosis includes morphological changes, cell shrinkage, membrane blebbing, chromatin condensation and apoptotic bodies. Apoptosis has been reported to associate with stroke, myocardial infarction, reperfusion injury, arteriosclerosis, heart failure, infertility, diabetes, AIDS, hepatitis, renal failure, Alzheimer's, Huntington's, Parkinson's diseases and especially cancer cells[5-7].

Cowanin is a bioactive compound extracted from several parts of *Garcinia cowa* Roxb found in Thailand and known as "Cha-muang", also found in Malaysia and Myanmar. Several studies have reported about the activities of Cha-muang including increased blood circulation, treatment of coughs, indigestive, anti-HIV, anti-oxidant and anti-tumor activity. Cowanin also showed anti-bacterial and anti-malarial activity. In addition, cowanin was cytotoxic to the NCl-H187, KB and MCF-7 cell lines[8-12]. However, the effects of cowanin on apoptosis induction in human breast cancer have not yet been reported.

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In the present study, we investigated the effect of cowanin on induction of apoptosis and inhibition of cell proliferation in MDA-MB-468 cells.

Material and Method

Compounds and Chemicals reagents

Fetal bovine serum (FBS), MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], Hoechst 33342 and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMSO was purchased from Merck Calbiochem, San Diego, CA. Dulbecco's Eagle's medium (DMEM) was purchased from Gibco Grand Island, NY. Cowanin was obtained from Associate Professor. Dr. Surat Laphookhieo, Mae Fah Luang University. Cowanin was isolated and purified from the twig of *Garcinia cowa* Roxb. and dissolved in DMSO.

Cell culture

Breast cancer cell line MDA-MB-468 was obtained from American Type Culture Collection (ATCC). MDA-MB-468 cells was maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS, 100 U/mL pencillin, 100 µg/mL streptomycin (PAA Laboratories, Pasching, Austraria). Cells were cultured at 37°C in a humidified incubator in an atmosphere of 5% CO_2. The medium was refreshed every 2 to 3 days.

Cell proliferation and cell viability assay

Cytotoxicity of cowanin was determined by using MTT (3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT assay is a colorimetric assay for measuring the activity of living cells, detected by dehydrogenase enzymes that reduce yellow tetrazolium MTT to purple formazan in the mitochondria of living cells[13]. Cells were seeded at a density of $1x10^4$ cells/well and grown for 24 h. Cells were treated with cowanin at various concentrations (2-fold dilution) and the control group was treated with 0.5% DMSO. After incubation for 24 h, 0.5 mg/mL MTT solution was added. The supernatant was removed and DMSO was added to solubilize insoluble purple formazan crystal. The absorbance at 570 nm was measured using a microplate reader (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).

The effect of cowanin on cell viability was analyzed by MTT assay which is a colorimetric assay for measuring the activity of living cells, detected by dehydrogenase enzymes that reduce yellow tetrazolium MTT to purple formazan in the mitochondria of living cells[13]. Cells were seeded at $1x10^4$ cells/well and grown for 24 h, then treated with cowanin at various concentrations (10, 15, 20, 25, 30 and 35 µg/mL). After incubation for 3, 6, 9, 12 and 24 h, MTT solution was added and DMSO was finally added to solubilize insoluble purple formazan crystal. The absorbance was measured at 570 nm using a microplate reader (Multiskan EX; Thermo Electron Corp., Vantaa, Finland). Cell survival was expressed as percentage (%) of viable cells to control cells using Microsoft Excel. Cells were treated in triplicates and the experiments were repeated three times.

Detection of apoptotic cells by Hoechst 33342 staining

Hoechst 33342 stains are blue fluorescent dyes commonly used to stain DNA[14]. The MDA-MB-468 cells were seeded at $3x10^5$ cells/well and incubated for 24 h. After incubation, cells were treated with 20 µg/mL cowanin for 3, 6, 9 and 12 h, respectively and 0.5% DMSO was used as a control. After that, cells were washed and fixed with paraformaldehyde for 30 min. Then cells were stained with 5 µg/mL of Hoechst 33342. After treatment, cells were washed and observed under fluorescence microscope.

Detection of mitochondrial membrane potential ($\Delta\psi m$)

MDA-MB-468 cells were seeded at $3x10^5$ cells/well and incubated for 24 h. Then, cells were treated with 20 µg/mL of cowanin for 3, 6, 9 and 12 h, respectively and 0.5% DMSO was used as a control. After incubation, the JC-1 staining solution was added to each well, mixed gently then observed under fluorescence microscopy. The cells can be analyzed directly in the culture medium since phenol red does not interfere with fluorescent staining. Healthy cells with mainly JC-1 (J-aggregates) can be detected with red fluorescence whereas apoptotic cells with mainly JC-1 monomers can be detected with green fluorescence.

Statistical analysis

All data presented were obtained from at least three independent experiments and were presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using the software GraphPad Prism 3.03 (GraphPad Software, Inc.).

Results

Cowanin inhibits breast cancer cells MDA-MB-468 proliferation

Initially, anti-proliferative activity of cowanin in MDA-MB-468 cells was screened by using MTT assay and the IC_{50} value was 16.27±0.88 µg/mL (Fig. 1A). As shown in Fig. 1B, inhibition of cell viability by cowanin occurred in a time- and dose-dependent manner. Treatment of MDA-MB-468 cells with cowanin at various concentrations (10, 15, 20, 25, 30 and 35 µg/mL), respectively for 3, 6, 9, 12 and 24 h reduced cell viability in a time and dose dependent manner as compared with the control group. The result indicated that cowanin inhibited cell proliferation and reduced cell viability in MDA-MB-468 cells.

The effect of cowanin on morphological changes and nuclear condensation in MDA-MB-468 cells

In the presence of 20 µg/mL cowanin, MDA-MB-468 cells showed round morphology with cell shrinkage and nuclear condensation. These are the characteristics of apoptotic cells (Fig. 2A). Representative images of Hoechst 33342 staining were shown in Fig. 2B, the results showed that 20 µg/mL of cowanin induced nuclear condensation and simultaneously morphological changes in MDA-MB-468 cells at 3, 6, 9 and 12 h, respectively. The results suggested that cowanin-induced apoptosis contributed to reduced cell viability of MDA-MB-468 cells. Thus, the results indicated that cowanin induced apoptosis of MDA-MB-468 cells in a time-dependent manner.

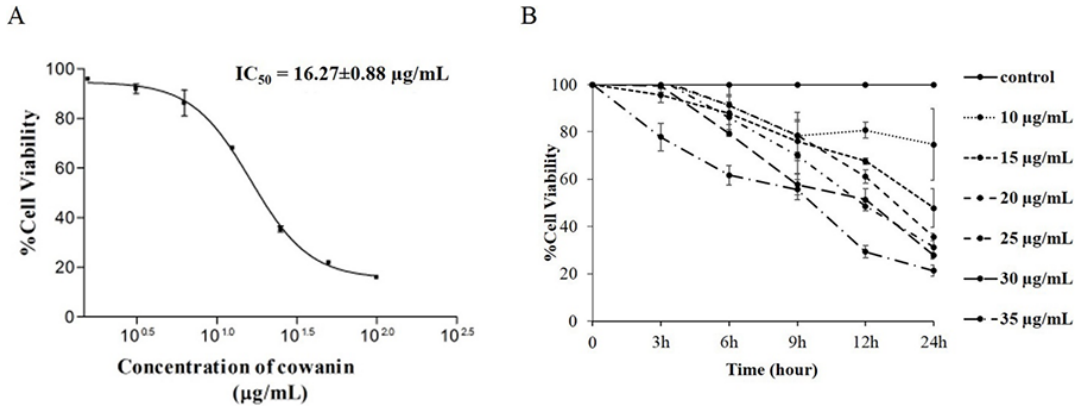

Fig. 1 Cowanin inhibited cells growth in MDA-MB-468 cells. (A) Effect of cowanin on cell viability by MTT assay. (B) Time- and dose-dependent effect of cowanin in MDA-MB-468 cells treated with various concentrations of cowanin at different time points. The IC_{50} value were expressed as mean ± SD, n = 3.

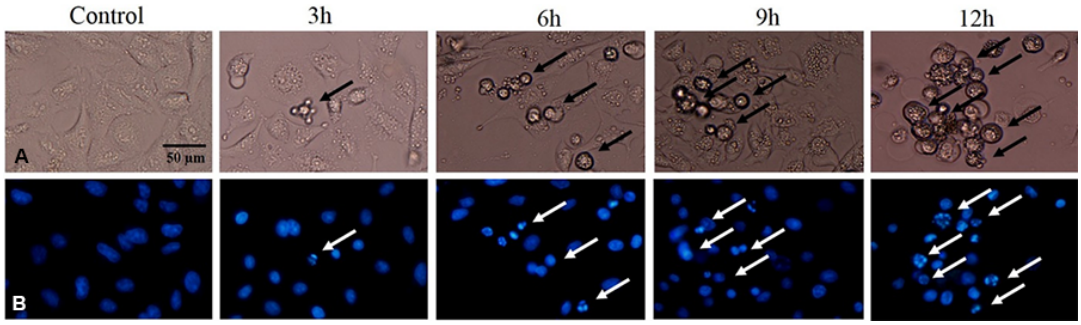

Fig. 2 Effects of cowanin extract on morphological changes and nuclear condensation. MDA-MB-468 cells were treated with 20 µg/mL cowanin for 3, 6, 9, and 12 h, respectively. Cells were stained with Hoechst 33342 and examined under a phase contrast and fluorescent microscope (magnification, x40). (A) Morphological changes in MDA-MB-468 cells observed under phase contrast microscope. Morphological changes is indicated by black arrows. (B) Chromatin condensation and apoptotic bodies in MDA-MB-468 treated cells observed under fluorescent microscope. Chromatin condensation is indicated by white arrows. Data presented were obtained from at least three independent experiments, n = 3.

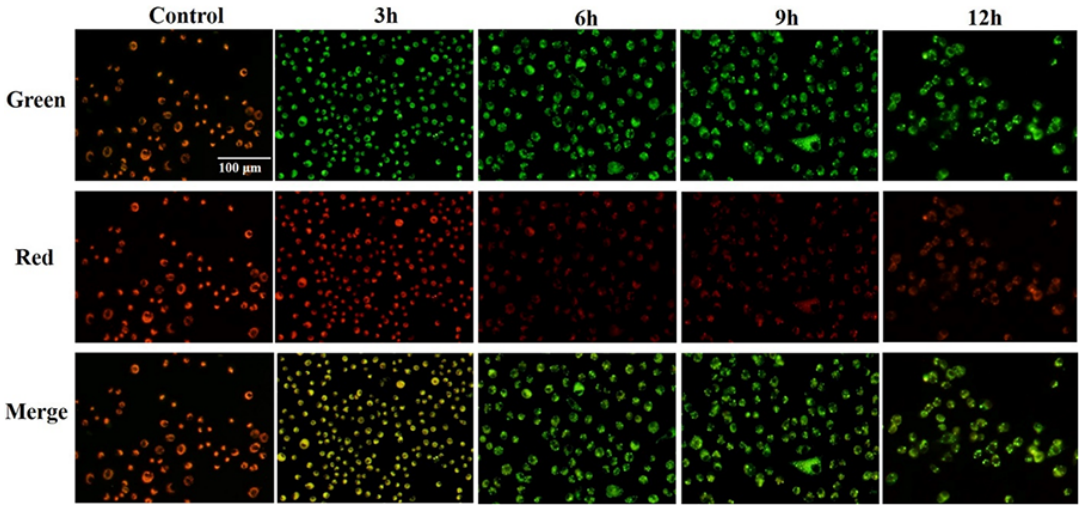

Fig. 3 Effect of cowanin on mitochondrial membrane potential in MDA-MB-468 cells. Cells were treated with 20 µg/mLcowanin for 3, 6, 9 and 12 h. The control cells showed red fluorescence indicated high mitochondria membrane potential whereas in cowanin-treated cells showed only green fluorescence indicated the loss of mitochondria membrane potential. Cowanin treatment showed an increased green/red fluorescence intensity ratio in a time dependent manner (magnification, x20). Data presented were obtained from at least three independent experiments, n = 3.

The effect of cowanin on mitochondria membrane potential (Δψm) in MDA-MB-468 cells

To determine mitochondrial membrane potential (Δψm) associated with apoptosis, MDA-MB-468 cells were stained with JC-1 after exposure to cowanin. JC-1 is a cationic dye that accumulates in mitochondria. Under normal conditions, JC-1 accumulation in the mitochondria leads to aggregates of dye as the red fluorescence. Whereas, loss of mitochondrial membrane potential leads to loss of aggregation and will be detected as green fluorescence of JC-1 monomer[15]. The result was shown in Fig. 3, the untreated cells showed red fluorescence indicating the normal mitochondrial membrane potential, while in the presence 20 µg/mL of cowanin led to the loss of mitochondria membrane potential at 3, 6, 9, and 12 h showing green fluorescence. These results indicated that cowanin induced apoptosis by reduced mitochondrial membrane potential of MDA-MB-468 cells in a time-dependent manner.

Discussion

Breast cancer is the most common malignancy in women with increased incidence worldwide[16]. Poor prognosis of breast cancer is partially attributed to multiple-drug resistance and anti-apoptosis of cancer cells[16]. Thus, we have demonstrated that cowanin inhibited cell proliferation and also induced apoptosis in human breast cancer cells MDA-MB-468. Cowanin is a new bioactive compounds extracted from the twig of *Garcinia cowa* Roxb. Previous reports indicated anti-bacterial and anti-malarial activity of cowanin from *Garcinia cowa* Roxb[9,10]. Ritthiwigrom *et al*[11] reported that cowanin showed cytotoxicity against NCl-H187, KB and MCF-7 cell line whereas have not been reported in MDA-MB-468. Our results demonstrated that cowanin inhibited cell proliferation of MDA-MB-468 cells in a time- and dose-dependent manner with IC_{50} value of 16.27±0.88 µg/mL (Fig. 1A and 1B).

Apoptosis is a programmed cell death which is an attractive target in cancer therapy. Apoptosis can be induced via two signaling pathway[17]. To confirm that cowanin induced apoptosis in MDA-MB-468 cells, characteristic morphological changes including membrane blebbling, cell shrinkage, chromatin condensation and formation of apoptotic bodies were detected by Hoechst 33342 staining. The result showed chromatin condensation in MDA-MB-468 cells after cowanin treatment in a time-dependent manner (3, 6, 9 and 12 h, Fig. 2A and 2B). In addition, changes in mitochondrial membrane potential were detected by JC-1 staining (Fig. 3) confirming mitochondrial membrane dysfunction. Loss of the mitochondrial membrane potential will release the pro-apoptotic proteins from the mitochondria to the cytosol and activated caspase-9 and then effector caspases

(caspase-3 or -7), resulting in apoptosis induction and cellular death[18]. The results suggested that cowanin induced apoptosis in MDA-MB-468 cells.

This new finding indicated the potential of cowanin as an anti-cancer therapy in human breast cancer MDA-MB-468 cells.

Conclusion

Cowanin extracted from *Garcinia cowa* Roxb. showed anti-proliferation and apoptosis induction in breast cancer MDA-MB-468 cells. These results suggested that cowanin may be a potential candidate for anticancer drug and it is the basic knowledge for developing chemotherapeutic agent of breast cancer in the future. However, the mechanism of apoptosis induction in animal models needs to be further studied for clinical application in the future.

What is already known on this topic?

Breast cancer is a common cancer worldwide. Death rates from breast cancer have decreased due to tamoxifen and chemotherapy treatment. However, chemotherapy has side effects on the patients. Therefore, bioactive compound with low toxicity and high selectivity for cancer cell apoptosis is important. Previous study showed anti-bacterial, anti-malarial activity and cytotoxicity of cowanin in various cell lines, but not in human breast cancer MDA-MB-468 cells.

What this study adds?

This new finding indicated the potential of cowanin as an anti-cancer therapy in human breast cancer MDA-MB-468 cells by inducing apoptosis including morphological changes, nuclear condensation and mitochondrial membrane dysfunction in MDA-MB-468 cells.

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Potential conflicts of interest

None.

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การเหนี่ยวนำการตายแบบอะพอพโทซิสในเซลล์มะเร็งเต้านมโดยสารโควานิน

นิตติญา ชาวชายโขง, สิรินันท์ นิลวรางกูร, นุจรี ตัญจพัฒนกุล, สุรัตน์ ละภูเขียว, รมิดา วัฒนโภคาสิน

ภูมิหลัง: *มะเร็งเต้านมเป็นมะเร็งชนิดหนึ่งที่พบมากในเพศหญิงทั่วโลก ในประเทศสหรัฐอเมริกาพบอัตราการเสียชีวิตจากมะเร็งเต้านมในเพศหญิงเป็นอันดับ 2 ส่วนประเทศไทยพบเป็นสาเหตุการเสียชีวิตอันดับ 1 ดังนั้นการศึกษา สารสกัดจากธรรมชาติที่มีประสิทธิภาพในการทำลายเซลล์มะเร็งสูง จึงเป็นสิ่งสำคัญในการทำวิจัยเกี่ยวกับมะเร็ง*

วัตถุประสงค์: *ศึกษาผลของสาร cowanin ในการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสและการยับยั้งการเจริญเติบโตในเซลล์มะเร็งเต้านม*

วัสดุและวิธีการ: *ทดสอบฤทธิ์ของสาร cowanin ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งเต้านมชนิด MDA-MB-468 ในช่วงเวลาและความเข้มข้นที่แตกต่างกันด้วยวิธี MTT assay นอกจากนี้ศึกษาฤทธิ์ในการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสโดยสาร cowanin ด้วยการวิเคราะห์การเปลี่ยนแปลงรูปร่างของเซลล์และการหดตัวของโครมาติน ด้วยวิธีการย้อมสี Hoechst 33342 รวมทั้งการวิเคราะห์ความสมบูรณ์ของเยื่อหุ้มไมโตคอนเดรียด้วยวิธีการย้อมสี JC-1*

ผลการศึกษา: *สาร cowanin มีฤทธิ์ในการยับยั้งการเจริญเติบโตต่อเซลล์มะเร็งเต้านมชนิด MDA-MB-468 โดยแสดงค่า IC_{50} เท่ากับ 16.27±0.88 μg/mL นอกจากนี้พบว่า cowanin เหนี่ยวนำให้เซลล์มะเร็งเต้านมชนิด MDA-MB-468 เกิดการตายแบบอะพอพโทซิสโดยเหนี่ยวนำให้เกิดการเปลี่ยนแปลงรูปร่างของเซลล์เกิดการหดตัวของโครมาติน และเกิดเป็นชิ้นส่วนเศษเซลล์นอกจากนี้พบว่าสาร cowanin มีฤทธิ์ในการเหนี่ยวนำให้เยื่อหุ้มเมมเบรนของไมโตคอนเดรียสูญเสียการทำงานและส่งผลให้เซลล์มะเร็งเต้านมชนิด MDA-MB-468 เกิดการตายแบบอะพอพโทซิส*

สรุป: *สาร cowanin แสดงคุณสมบัติในการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสและยับยั้งการเจริญเติบโตของเซลล์มะเร็งเต้านมชนิด MDA-MB-468 ซึ่งผลการศึกษาสาร cowanin ในมะเร็งเต้านมนี้อาจเป็นทางเลือกในการพัฒนาสารสกัดจากธรรมชาติไปเป็นยาสำหรับรักษาโรคมะเร็งร่วมกับการรักษาด้วยเคมีบำบัดเพื่อลดผลข้างเคียงที่เกิดกับผู้ป่วย*