Anti-Proliferation and Apoptosis Induction in Breast Cancer Cells by *Cratoxylum cochinchinense* Extract

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Background: *Breast cancer is the most common invasive cancer in females worldwide. It was found about 37.5% in Thai females and is one of the leading causes of death-related cancers in women. Therefore, new finding of anti-cancer compound as a therapeutic candidate in breast cancer is necessary.*
Objective: *To investigate the effect of Cratoxylum cochinchinense extract on anti-proliferation and apoptosis induction in breast cancer cells.*
Material and Method: *Cell proliferation and cell viability assay were determined by MTT assay. Hoechst 33342 and JC-1 staining were used to determined nuclear morphological changes and mitochondrial membrane potential, respectively.*
Results: *C. cochinchinense extract showed anti-proliferation in MDA-MB-468 treated cells in a time- and dose-dependent manner with IC_{50} value of 19.19±0.8 μg/ml. In addition, C. cochinchinense extract induced nuclear condensation and apoptotic bodies in MDA-MB-468 treated cells. JC-1 staining revealed that C. cochinchinense extract induced mitochondrial membrane dysfunction.*
Conclusion: *C. cochinchinense extract showed anti-proliferation and apoptosis induction properties in MDA-MB-468 treated cells. These results suggested that C. cochinchinense extract may be a potential candidate for anti-cancer drug developing. The underlying mechanisms of apoptosis induction should be further studied.*

Keywords: *Breast cancer cells, C. cochinchinense extract, Apoptosis, Anti-proliferation*

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Breast cancer is the most common cancer and is the leading cause of death among females worldwide. Breast cancer developed from breast tissue originating from ducts is known as ductal carcinomas, while originating from lobules is known as lobular carcinomas. The normal cells divide continuously, attach to other normal cells and stay in place in tissues. Then cells become cancerous because they lose their ability to stop dividing. In Thailand, Kotepui M, et al reported that 7,711 breast cancer cases were included in 10 year-period (2002-2011). The breast cancer incidence of people under age 40 years was relatively low while those over 40 was very high[1].

Apoptosis or programmed cell death is an essential physiological process in multiple cellular. This process plays a critical role including normal cell turnover, development of embryo, functioning of the immune system, tissue homeostasis and elimination of damaged cells[2]. During the early process of apoptosis, cells were initially described by its morphological characteristics including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation[3].

Inappropriate apoptosis or altered regulation of apoptosis processing has also been linked to many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. Abnormal apoptosis induces the processes of oncogenesis including initiation, progression and metastasis in abnormal cell[4].

Therefore, the compound that induces apoptosis is necessary for development as an anti-cancer drug. Natural compound treatment was used as anti-cancer therapy and prevention in many types of cancer. *Cratoxylum cochinchinense* is found in Thailand and is known by the local name ''tuegliang''. This plant was used in traditional medicine including fevers, coughs, diarrhoea, itches, ulcers and abdominal pain. Moreover, natural compound extracted from *C. cochinchinense* has been reported to possess cytotoxicity against many types of cancer cell lines[5]. *C. cochinchinense* extract is a natural

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compound composes of bisanthraquinone and cytotoxic xanthones extracted from the stems of *C. cochinchinense*[6]. Therefore, this study aims to investigate the effect of *C. cochinchinense* extract on apoptosis induction in breast cancer MDA-MB-468.

Material and Method

Cell culture

Breast cancer cell line MDA-MB-468 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, and after reaching ~90% confluences, cells were subcultured and the medium was replaced 2-3 times/week.

Natural compound and chemical reagent

C. cochinchinense extract was obtained from Associate Professor Wilawan Mahabusarakam, Faculty of Science, Prince of Songkla University, Thailand in purified powder form. It was extracted from the stems of *C. cochinchinense*[6]. *C. cochinchinense* extract was dissolved and diluted in DMSO at the desired concentrations for the assays. 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 *C. cochinchinense* extract was determined by MTT assay, a colorimetric assay for assessing cell viability, measuring mitochondrial dehydrogenase enzyme activity. In living cells the enzyme can reduce yellow MTT to purple formazan[7]. MDA-MB-468 cells were seeded in a 96-well plate ($5x10^3$ cells/well) and allowed to grow for 24 h. Then, cells were treated with *C. cochinchinense* extract at various concentrations, whereas the control group was treated with DMSO. After incubation for 24 h, 100 µL of 0.5 mg/ml MTT solution was added to each well, and the plate was further incubated for 2 h at 37°C. The supernatant was removed, and DMSO was added to solubilize the water insoluble 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).

The effect of *C. cochinchinense* extract on cell viability at different times and doses was determined. The cells were treated with *C. cochinchinense* extract at various concentrations of 5, 10, 15, 20, 25, 30 and 35 µg/ml, whereas the control group was treated with DMSO. After incubation for 3, 6, 9, 12 and 24 h, cell viability was determined by the MTT assay. Survival percentage (%) of the cells was calculated relative to the control. Cell viability was assessed in three independent experiments.

Nuclear morphological staining with Hoechst 33342

MDA-MB-468 cells were seeded at $4x10^5$ cells/ 35 mm^3 for 24 h. The cells were treated with 30 µg/ml *C. cochinchinense* extract for 3, 6, 9, 12 and 24 h. As control, the cells were treated with DMSO for 24 h and subsequently stained with 10 µM Hoechst 33342 for 30 min at 37°C and examined under a fluorescence microscope (IX73; Olympus, Tokyo, Japan).

Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

The $\Delta\Psi m$ was determined using the potential sensitive dye JC-1, which is a lipophilic cation that is incorporated into the mitochondrial membrane. Cells were seeded at $3x10^5/mm^3$ for 24 h and treated with 30 µg/ml *C. cochinchinense* extract for 3, 6, 9 and 12 h, and the control cells were treated with DMSO. The cells were then 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.

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 SPSS statistical software package (version 11.5) and also carried out using the software GraphPad Prism 3.03 (GraphPad Software, Inc.).

Results

C. cochinchinense extract inhibits cell viability in MDA-MB-468 breast cancer cells

The anti-proliferation activity of *C. cochinchinense* extract in the MDA-MB-468 cells was determined by MTT assay. The IC_{50} value was 19.19$\pm$0.8

μg/ml compared with normal epithelial cell line Vero cells (about 25 μg/ml, data not show). *C. cochinchinense* extract inhibited cell viability in a time- and dose- dependent manner. Treatment of MDA-MB-468 cells with 30 μg/ml of *C. cochinchinense* extract for 24 h reduced cell viability to about 20% comparing with that noted in the control cells (Fig. 1).

Effect of C. cochinchinense extract on nuclear morphological changes

To determine the anti-proliferation and cell death induction mediated by *C. cochinchinense* extract, Hoechst 33342 staining was carried out. Hoechst 33342 is a fluorescence dye and a cell-permeable DNA stain used for labeling DNA, although the dyes can bind to all nucleic acid enhancing fluorescence considerably[8]. The characteristic of morphological changes in apoptotic cell including cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies were determined. Hoechst 33342 stain revealed condensed chromatin and apoptotic bodies in the MDA-MB-468 cells following treatment with *C. cochinchinense* extract (Fig. 2). The results showed that *C. cochinchinense* extract induced chromatin condensation and apoptotic bodies, characteristics of apoptotic cells.

Effect of C. cochinchinense extract on mitochondrial membrane potential (ΔΨm)

During apoptosis, several key events occur in the mitochondria. One of these events is mitochondrial membrane potential (ΔΨm). JC-1 is a lipophilic cationic dye that can selectively enter into mitochondria and reversibly change color from green to red when the membrane potential was increased. In healthy cells with high membrane potential, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. In contrast, in apoptotic or unhealthy cells with low ΔΨm, JC-1 remains in the monomeric form, which shows only green fluorescence[9]. The ratio of green to red fluorescence is dependent only on the ΔΨm. The results showed that MDA-MB-468 cells treated with *C. cochinchinense* extract for 3, 6, 9 and 12 h had an increased green/red ratio, while the control cells showed red fluorescence (Fig. 3) indicating that *C. cochinchinense* extract induced the loss of ΔΨm in the MDA-MB-468 cells.

Discussion

In this study, MDA-MB-468 cell line was used as a model for triple-negative breast cancer (TNBC). The term of TNBC is used to describe tumors with estrogen receptor (ER) and progesterone receptor (PR) negative and human epidermal growth factor receptor 2 (HER2) normal[10]. For the treatment, chemotherapy remains the core treatment option for patients with TNBC because this type of cancer doesn't have any specific receptor. However, possible side effects of chemotherapy treatment due to attacking cells that are dividing quickly. This side effect depends on the type of drugs, the amount taken, and the length of treatment[11].

Recent research has demonstrated that *C. cochinchinense* extract showed cytotoxicity, anti-tumor

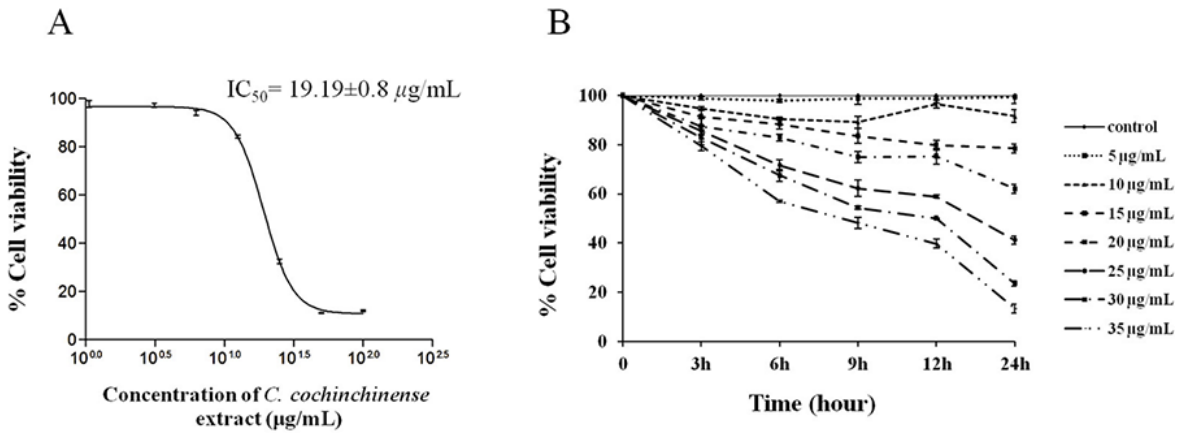

Fig. 1 Effect of *C. cochinchinense* extract on cell viability assay. (A) The MTT assay with IC_{50} value of *C. cochinchinense* extract against MDA-MB-468 cells at 24 h. (B) Time- and dose-dependent effect of *C. cochinchinense* extract on MDA-MB-468 cell viability following treatment with different concentrations of *C. cochinchinense* extract at several time points. The IC_{50} value were expressed as mean ± SD, n = 3.

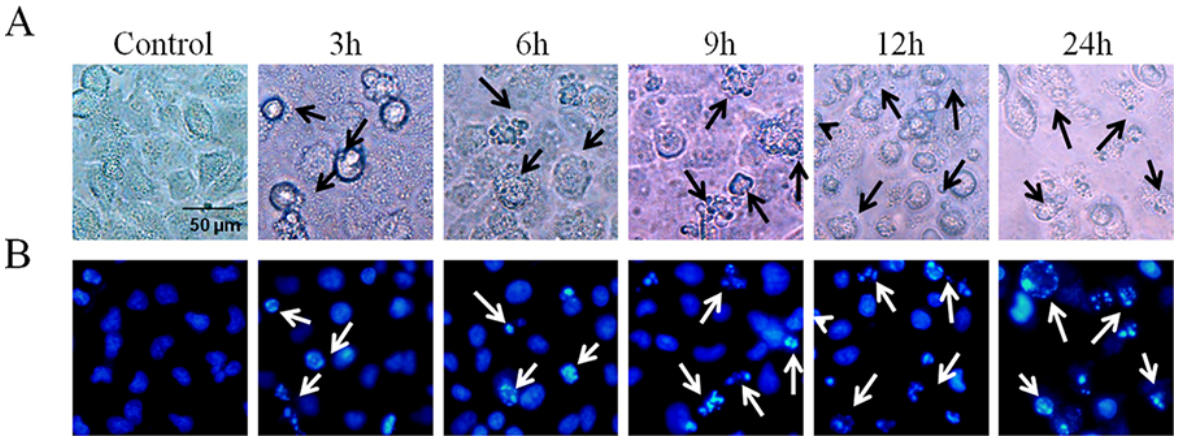

Fig. 2 Effects of *C. cochinchinense* extract on nuclear condensation of MDA-MB-468 cells. Cells were treated with 30 µg/ml *C. cochinchinense* extract for 3, 6, 9, 12 and 24 h and then stained with Hoechst 33342 and examined under a fluorescent microscope (magnification, x40). (A) Morphological changes in MDA-MB-468 treated cells were observed under phase contrast microscope. (B) Chromatin condensation and apoptotic bodies were observed under a fluorescent microscope.

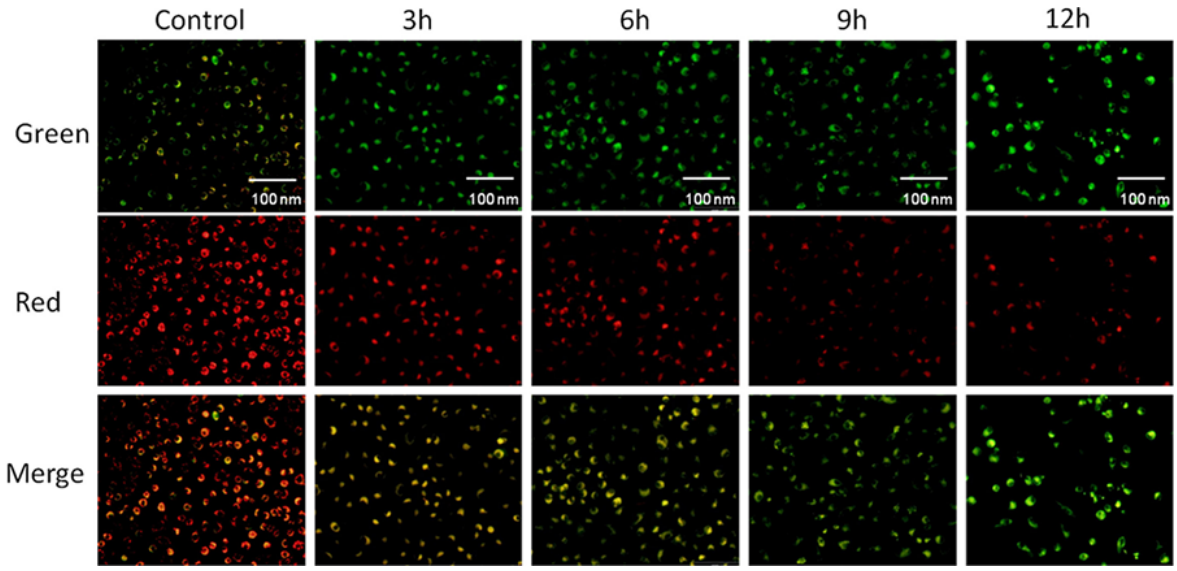

Fig. 3 Effect of *C. cochinchinense* extract on mitochondrial membrane potential in MDA-MB-468 cells. Cells were treated with 30 µg/ml *C. cochinchinense* extract for 3, 6, 9 and 12 h. Red fluorescence in the control cells indicated high membrane potential and green fluorescence in the *C. cochinchinense* extract treatment indicated loss of membrane potential. The *C. cochinchinense* extract treatment showed an increased green/red fluorescence intensity ratio in a time dependent manner (magnification, x40).

and anti-oxidant properties against various human tumor cell lines. Rattanaburi S, et al reported that *C. cochinchinense* extract exhibited strong cytotoxicity against human epidermoid carcinoma A341 cell line and human breast cancer SKBR-3cell line[6]. Cochinxanthone D, a xanthone from stem of *C. cochinchinense* showed antioxidant activity in both the DPPH radical scavenging and the lipid peroxidation inhibition assays[12]. Moreover, mangiferin from *C. cochinchinense* showed antioxidant properties leading to apoptosis induction in Jurkat T cells[13]. However, the mechanisms of apoptosis induction in breast cancer MDA-MB-468 cells with triple negative receptor have not yet been reported. The results showed that *C.*

cochinchinense extract inhibited MDA-MB-468 cell growth in a time- and dose-dependent manner with an IC_{50} value of 19.19±0.8 μg/ml (Fig. 1). To confirm apoptosis induction, we investigated characteristic morphological changes including cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies. Hoechst 33342 stain revealed condensed chromatin and apoptotic bodies in the MDA-MB-468 cells following treatment with *C. cochinchinense* extract (Fig. 2). Furthermore, the effect of *C. cochinchinense* extract on the ΔΨm dysfunction in MDA-MB-468 cells was detected by increased green/red fluorescence ratio at 3 h (Fig. 3). Loss of the ΔΨm and release of sequestered pro-apoptotic proteins from the intermembranous space into the cytosol stimulates apoptosome formation followed by activation of caspase-9[14]. The caspase-9 causes the activation of the effector caspases (-3, -6, -7), which cleave vital substrates, resulting in cellular death[15]. However, the mechanism and involving protein of apoptosis induction in MDA-MB-468 cell lines by *C. cochinchinense* extract need to be further determined.

Conclusion

C. cochinchinense extract showed the effect of anti-proliferation and apoptosis induction in TNBC MDA-MB-468 cell line. This compound showed apoptosis induction through decreased of mitochondrial potential. Finally, morphological changes such as chromatin condensation and apoptotic bodies were detected in apoptosis cells. Thus, *C. cochinchinense* extract may be used as an anti-cancer agent in TNBC to inhibit cell proliferation via apoptosis-associated cell death induction. Thus, the mechanism of proliferation inhibition and apoptosis induction in animal model need to be further studied for clinical application in the future.

What is already known on this topic?

Breast cancer is the most common type of cancer and is the second leading cause of cancer death following lung cancer among women worldwide. *C. cochinchinense* extract is an effective bioactive compound, which has been used in traditional medicine including anti-cancer and anti-oxidant treatment. *C. cochinchinense* extract showed strong inhibition on cell growth in various cell lines.

What this study adds?

C. cochinchinense extract exhibited cell proliferation in TNBC MDA-MB-468 cell line. In addition, *C. cochinchinense* extract induced apoptosis cell death resulting in 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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การยับยั้งการเจริญเติบโตและการเหนี่ยวนำการตายแบบอะพอพโทซิสในเซลล์มะเร็งเต้านมโดยสารสกัดจาก Cratoxylum cochinchinense

สุกานดา อินนาจักร, สิรินันท์ นิลวรางกูร, วิลาวัลย์ มหาบุษราคัม, รมิดา วัฒนโภคาสิน

ภูมิหลัง: *มะเร็งเต้านมเป็นมะเร็งที่พบได้มากในผู้หญิงทั่วโลกและสำหรับในประเทศไทยมีอัตราการเกิดของมะเร็งเต้านมในผู้หญิงอยู่ที่ร้อยละ 37.5 มะเร็งเต้านมถือเป็นสาเหตุหลักในการเสียชีวิตในผู้ป่วยโรคมะเร็งที่พบในผู้หญิง ดังนั้นการค้นหาสารที่มีคุณสมบัติในการต้านมะเร็งสำหรับมะเร็งเต้านมจึงมีความสำคัญเป็นอย่างยิ่ง*

วัตถุประสงค์: *ศึกษาผลของสารสกัดจาก C. cochinchinense ต่อการยับยั้งการเจริญเติบโตและการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสในเซลล์มะเร็งเต้านม*

วัสดุและวิธีการ: *ทดสอบฤทธิ์ของสารสกัดจาก C. cochinchinense ในการยับยั้งการเจริญเติบโตของเซลล์ โดยเซลล์มะเร็งเต้านมที่ใช้ คือ MDA-MB-468 และทดสอบฤทธิ์ในการยับยั้งการเจริญเติบโตของสารต่อเซลล์ในช่วงเวลาและความเข้มข้นที่แตกต่างกันด้วยวิธี MTT assay ศึกษาฤทธิ์ในการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิส โดยสารสกัดจาก C. cochinchinense ด้วยการวิเคราะห์การเปลี่ยนแปลงรูปร่างของเซลล์และการหดตัวของโครมาติน รวมทั้งการวิเคราะห์ความสมบูรณ์ของเยื่อหุ้มไมโตคอนเดรียด้วยวิธีการย้อมสี Hoechst 33342 และ JC-1 ตามลำดับ*

ผลการศึกษา: *สารสกัดจาก C. cochinchinense มีฤทธิ์ในการยับยั้งการเจริญเติบโตต่อเซลล์ที่ค่า IC_{50} เท่ากับ 19.19±0.8 μg/mg โดยที่ฤทธิ์ในการยับยั้งของสารสกัดจาก C. cochinchinense นั้นจะขึ้นอยู่กับระยะเวลาและความเข้มข้นของสารที่ใช้ ผลจากการศึกษาการเปลี่ยนแปลงรูปร่างของเซลล์และการแตกหักของโครมาตินรวมทั้งการวิเคราะห์ความสมบูรณ์ของเยื่อหุ้มไมโตคอนเดรียพบว่าสารสกัดจาก C. cochinchinense เหนี่ยวนำให้เกิดการหดตัวของโครมาตินและเหนี่ยวนำให้เกิดความเสียหายของเยื่อหุ้มไมโตคอนเดรีย ซึ่งเป็นสาเหตุในการนำไปสู่การตายของเซลล์แบบอะพอพโทซิสในเซลล์มะเร็งเต้านม MDA-MB-468*

สรุป: *สารสกัดจาก C. cochinchinense แสดงคุณสมบัติในการยับยั้งการเจริญเติบโตและเหนี่ยวนำให้เกิดการตายของเซลล์แบบอะพอพโทซิสในเซลล์มะเร็งเต้านม MDA-MB-468 ดังนั้นผลการศึกษาสารสกัดจาก C. cochinchinense อาจเป็นทางเลือกในการพัฒนาไปเป็นยาสำหรับรักษาโรคมะเร็ง แต่ทั้งนี้กลไกในการเหนี่ยวนำให้เกิดการตายของเซลล์แบบอะพอพโทซิสควรมีการศึกษาในเชิงลึกต่อไป*