การศึกษากลไกของสารสกัดกระชายดำในการต้านการแบ่งตัวของเซลล์มะเร็งเต้านม

The study mechanism of *Kaempferia parviflora* extract in anti-proliferation of breast cancer

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นักศึกษาระดับปริญญาโท สาขากายวิภาคศาสตร์มหาบัณฑิต คณะแพทยศาสตร์ศิริราชพยาบาล
มหาวิทยาลัยมหิดล

บทคัดย่อ

ที่มาและความสำคัญ ในปัจจุบันมะเร็งที่พบมากที่สุดในเพศหญิง คือมะเร็งเต้านมและนำไปสู่การเสียชีวิตด้วยเช่นกัน ซึ่งการรักษามะเร็งเต้านมในปัจจุบันเป็นการรักษาแบบองค์รวมขึ้นกับระยะและความรุนแรงของมะเร็ง มีการศึกษาอย่างแพร่หลายเกี่ยวกับสมุนไพรในการยับยั้งการแบ่งตัวของเซลล์มะเร็ง ในการศึกษาครั้งนี้เราต้องการทดสอบผลของสารสกัดจากกระชายดำในการต้านการแบ่งตัวของเซลล์มะเร็งเต้านม วิธีการดำเนินการ ทดสอบผลของสารสกัดกระชายดำในเซลล์มะเร็งเต้านม (MCF-7, MDA-MB-231 และ SKBR-3) โดยทดสอบดูความเป็นพิษต่อเซลล์มะเร็งเต้านมโดยใช้ MTT assay, ดูการกระจายของปริมาณ DNA ภายในวัฏจักรเซลล์ (Cell cycle) ด้วยการย้อมโพรพิเดียมไอโอไดต์ (PI) และเพื่อดูรูปแบบการตายอะพอพโทซิส (Apoptosis) โดยการย้อมแอนเนคซินไฟร์และโพรพิเดียมไอโอไดต์ (AnnexinV/PI) แล้ววิเคราะห์ด้วยวิธีการโฟลไซโทเมทรี (Flow cytometry) ผลการทดลอง ผลที่ได้จากการทดลองครั้งนี้พบว่าสารสกัดกระชายดำสามารถออกฤทธิ์เป็นพิษต่อเซลล์มะเร็งเต้านมชนิด MCF-7 ได้ดีที่สุดเมื่อเทียบกับเซลล์มะเร็งเต้านมชนิดอื่นโดยแสดงค่า IC50 470 μg/ml และผลการวิเคราะห์ด้วยด้วยโฟลไซโทเมทรี (Flow cytometry) พบว่าสารสกัดกระชายดำสามารถเหนี่ยวนำให้เกิดการกระจายของเซลล์ในช่วง sub G0/G1 phase และเหนี่ยวนำให้ MCF-7 เกิดการตายแบบอะพอพโทซิส สรุป สารสกัดจากกระชายดำออกฤทธิ์ออกฤทธิ์ได้ดีอย่างจำเพาะต่อเซลล์มะเร็งเต้านม MCF-7 นอกจากนี้สารสกัดกระชายดำกระตุ้นให้เกิดการกระจายของปริมาณ DNA ภายในวัฏจักรของเซลล์และเหนี่ยวนำให้เกิดอะพอพโทซิส

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Abstract

Background Breast cancer is the most common cancer and leading cause of cancer mortality in female. The treatment of breast cancer is combination with several treatment. This research project focuses on the KP for anti-proliferation on breast cancer cell lines. Methods Breast cancer cell lines, MCF-7, MDA-MB-231, and SKBR-3 were used to investigate the anti-proliferation effect of KP extract. Cell viability was determined by MTT assay. DNA distribution in cell cycle was determined by PI staining and cell apoptosis assay was determined by AnnexinV/PI staining. Results KP extract was reduced MCF-7 cell viability in a time and dose dependent manner. IC50 value was 470 μg/ml of MCF-7 cancer cell lines. KP extract was accumulated of cell cycle at subG0/G1 phase and induced apoptosis on MCF-7 cancer cell line. Conclusions KP extract was highly effective on MCF-7 cancer cell line. Treated cells were induced cell cycle distribution and induced cell apoptosis.

Keywords Apoptosis, Breast cancer, *Kaempferia parviflora*

Introduction

Cancer is a noncommunicable disease (NCDS) as the leading cause of death and the most essential barrier to increasing life anticipation in every country of the world. According to approximately from World Health Organization (WHO) in 2015, cancer is the first or second leading cause of death before age 70 years in 91 of 172 countries. Cancer incidence and mortality are rapidly growing worldwide (1). In Thailand, breast cancer is the most common cancer in female with the incidence of 28.5% of new cancer cases (2). Cancer is a heterogeneous disease, which comprises of many biologically different entities with apparent pathological features and clinical implication. Carcinogenesis of breast cancer depends on multi-factors including mutated genes that control cell proliferation, differentiation and survival. Based on gene expression profiles, breast cancer can be classified into five subtypes including the luminal subtypes (luminal A and luminal B), the HER-2 enriched subtype, and the triple negative subtype. Each subtype has different biological and genetic pathways leading to different cell behaviors and response to treatment (3).

Kaempferia parviflora (KP) known as Kra-chai-dam in Thai or black ginger. This plant is a member of Zingiberaceae family. The KP has been traditional used as folk medicine in various disease. The recent study has been shown the properties of this plant including anti-gastric ulcer, sexual supplement activity, anti-allergic, anti-inflammatory, cardioprotective activity and

effect on osteoarthritis (4-9) The property of KP extract for anti-proliferation of cancer had been reported on a few cancer type such as human promyelocytic leukemia HL-60 cells, human promonocytic leukemic u937 cells, human cholangiocarcinoma HUCCA and RMCCA-1 cells, human cholangiocarcinoma B16 cells, human HeLa cell 229 and human ovarian cancer SKOV-3 cells (10-15). The several studies were demonstrated the anti-proliferation effect, inhibitory effect on cell migration and induce cell apoptosis. The previous studies had reported KP extract can reduce the matrix metalloproteinase (MMP-2) activity involve cell migration and loosed of mitochondrial transmembrane potential (MPT). The effect KP on caspase-3 had been reported suppressed caspase-3 after treated cells with KP, caspase-3 related to cell apoptosis mechanism. This research project selects the representative breast cancer cell lines for each subtype including MCF7 (luminal subtype), SKBR3 (HER-2 enriched subtype) and MDA-MB231 (triple negative subtype) in order to study possible different effects of KP on each breast cancer subtype (3).

Methods

Plant material and extraction of KP

The fresh rhizome of KP was collected from Loei province, Thailand. The rhizome was extracted with ethanol. Then, the ethanolic extract was analyzed by thin layer chromatography at Faculty of Pharmacy, Mahidol University, Thailand.

Cell culture condition

The human breast cancer cell lines, MCF-7 (ATCC® HTB-22™), MDA-MB-231 (ATCC® HTB-26™), SKBR-3 (ATCC® HTB-30™), and HeLa (ATCC® TCP-1022™) were cultured in complete DMEM (Gibco, USA) in T75 flask. These cells were harvested and washed twice by 1X PBS (Amresco®, USA), trypsinized with 0.5% Trypsin in EDTA (Gibco, USA.) for 2 min. The reaction was stopped by 0.2 ml FBS (Gibco, USA.) and transferred to the new culture medium flask. Human dermal fibroblast was incised from breast cancer patient and used as a control group. It was cultured in DMEM high glucose supplemented with 10% FBS plus 1% Penicillin and streptomycin.

Cell viability assay

The effect of KP on cell viability in MCF-7, MDA-MB-231, and SKBR-3 were determined by MTT assay [(3, 4, 5-dimethlthiazole2-yl) 2-5-diphenyltetrazodium bromide] (Sigma). Breast cancer cell lines were seeded at a density of $5x10^3$ cells per well. The human dermal fibroblast

was used as a negative control and seeded at a density of $1x10^4$ cells per well. HeLa was used as a positive control was seeded at a density of $5x10^3$ cells. Cells were treated with KP extract at different concentrations; 0, 100, 200, 300, 400, and 500 µg /ml. A final concentration of 0.1% DMSO and doxorubicin were used a negative control. These cells were incubated for different time intervals (24, 48 and 72 hours) and 50 µl of 1 mg/ml in PBS of MTT [(3, 4, 5-dimethlthiazole2-yl) 2-5-diphenyltetrazodium bromide] (Sigma) was added into well plates. After incubation with MTT solution, the supernatant was decanted and 100 µl of DMSO was added to 96 well plates. The 96 well plates were incubated in the dark for 10 min and checked for complete solubilization of purple formazan crystals. These cells were analysed by ELISA reader at 595 nm (Biotek Laboratories®, USA). The assay was done in triplicate with three independent experiments, Cell viability calculated by the following formula.

Cell viability (%) = [(control O.D – sample O.D)/ control O.D] ×100

Cell cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) staining base on measurement of the DNA content of the cell by flow cytometry. MCF-7, HeLa, and, human dermal fibroblast were seeded at a density of $7x10^3$, $5x10^3$, and $5x10^3$; respectively in 6 well plate for 24 hours. The KP extract was added into each plate at different concentrations: 470, 340, and 650 µg/ml, respectively for 24 hours. The cells were harvested and resuspended with cold PBS. Cell suspension at a density of $5x10^5$ cells/ml was centrifuged at 400 g for 5 min at RT. Cells were incubated in trypsin solution, and followed by trypsin inhibitor, and RNase solution for 10 min. Cells were stained with 200 µl of propidium iodide (PI) and incubated 10 min in the dark. The cells were analyzed by flow cytometry with Cell Quest software (BD, USA) within 1 hour.

Apoptosis assay

Cell apoptosis was assessed by FICT AnnexinV/PI staining. MCF-7, HeLa, and human dermal fibroblast were seeded at a density of $7x10^3$, $5x10^3$, and $5x10^3$ cells; respectively in 6 well plate for 24 hours. The KP extract was added in each plate at different concentrations; 470, 340, and 650 µg/ml; respectively for 24, and 48 hours. Cells were harvested, and resuspended 1X Binding. Then, Annexin V and PI (BD, Bioscience, San Jose, CA, USA) were added in cell suspension, and cells were incubated at RT for 15 min in the dark. The flow cytometry was performed using Cell Quest software (BD, USA) within 1 hour.

Results

The effect of ethanolic KP extract to cell viability

To investigate the effect of KP extract on cell viability, MCF-7, MDA-MB-231, SKBR-3, HeLa, and human dermal fibroblast were treated with KP extract at different concentration 0, 100, 200, 300, 400, and 500 μg/ml for 24, 48, and 72 hours and measured by MTT assay. The result from MTT assay showed reduction in cell viability in a dose and time dependent manner. Half-maximal inhibitory concentration (IC50) of KP extract were 470, 900, and 1180 μg /ml (MCF-7, MDA-MB-231, and SK-Br3; respectively) (**Figure 1**). The IC50 values of HeLa and fibroblast were 330, and 650 μg/ml; respectively. However, treated cells were showed highly effective on cells cytotoxicity when compared with control cell. The results from MTT assay were showed less responsiveness of MDA-MB-231, and SKBR-3 when compared with other subtype of breast cancer cell lines.

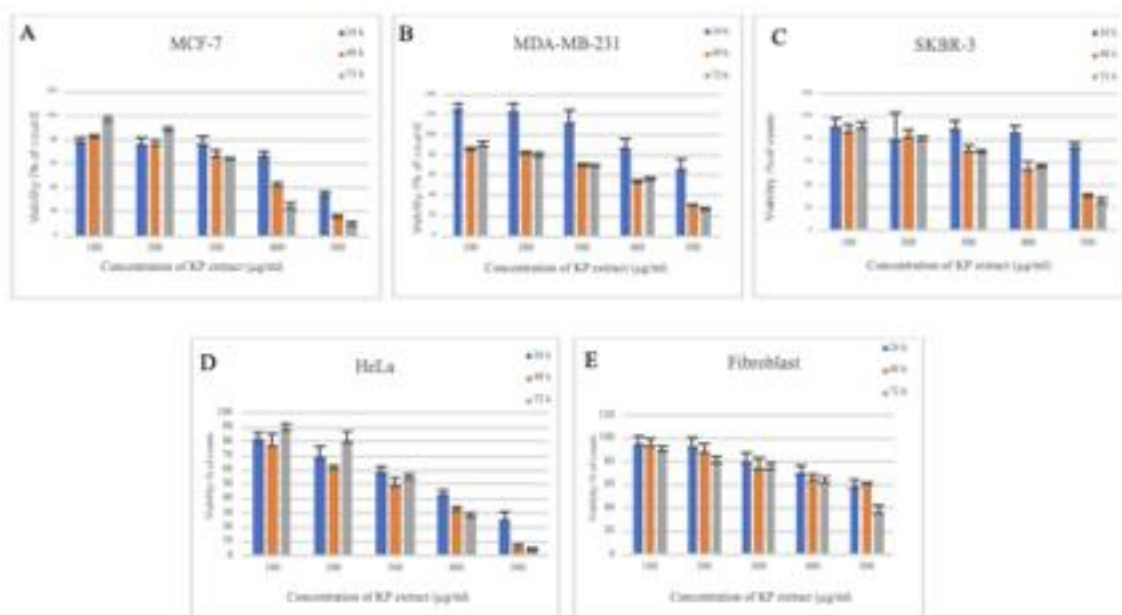

Figure 1 Dose and time of KP extract was responded to the cytotoxicity effect on breast cancer cell line. (A) Showed the percentage of cell viability on MCF-7 cell. (B) Showed the percentage of cell viability on MDA-MB-231. (C) Showed the percentage of cell viability on SKBR-3 cell. (D) showed the percentage of cell viability on HeLa cell. (E) Showed the percentage of cell viability on fibroblast cell. The bar indicated percentage of cell viability on cancer cell treated with KP extract at difference concentration at 24, 72, 48 hours. The results obtained from flow cytometry were expresses three experiments mean and SD.

The effect of ethanolic extract of KP to cell cycle distribution

To investigate the effect of KP extract on cell cycle distribution, MCF-7, HeLa, and fibroblast were treated with KP extract at concentration 470, 330, and 650 µg/ml; respectively for 24 hours. Treated cell lines were stained with PI and analyzed by flow cytometry. In MCF-7 control cells, the percentage of cell in subG0/G1, G0/G1, S and G2/M phase were 6.84%, 55.32%, 19.94%, and 14.79%; respectively. After 24 hours incubation with KP extract, the percentage of cells in sub G0/G1 phase increased to 64.19%, whereas the cells in G0/G1, S and G2/M phase decreased to 9.98%, 3.27%, and 2.6%; respectively. In positive control HeLa cells, the percentages in sub G0/G1, G0/G1, S and G2/M phase were 6.35%, 60.41%, 9.44%, and 7.30% respectively. After 24 hours incubation with KP extract, the percentage of cell in sub G0/G1 phase was increased to 15.01%, whereas the cells in G0/G1, S, and G2/M phase were decreased to 60.17%, 3.24%, 6.46%, and 6.46%; respectively. These results suggested that KP extract had able to induce cell cycle arrest at subG0/G1 of MCF-7 and S-phase of HeLa cell lines (**Figure** 2).

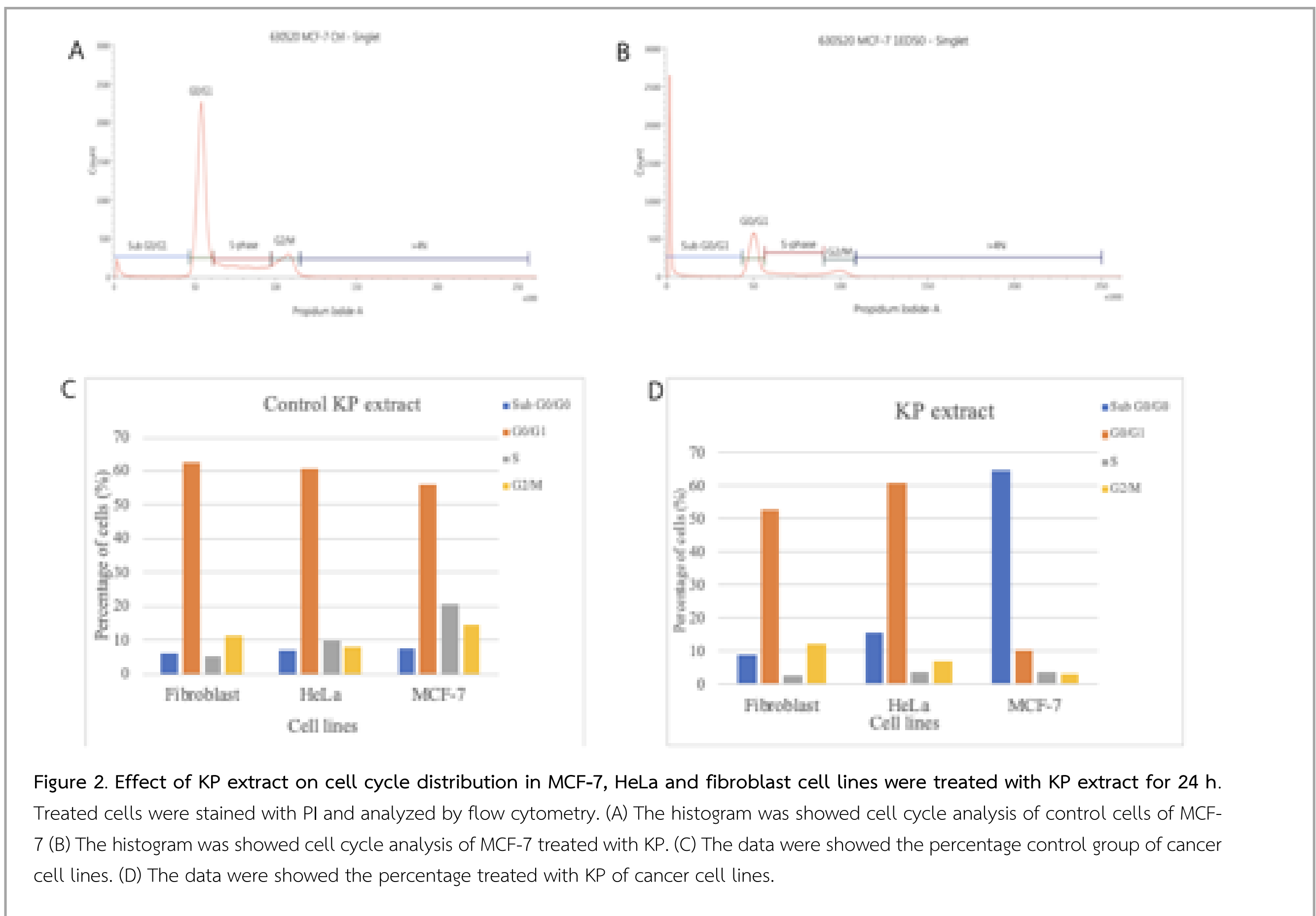

Figure 2. Effect of KP extract on cell cycle distribution in MCF-7, HeLa and fibroblast cell lines were treated with KP extract for 24 h. Treated cells were stained with PI and analyzed by flow cytometry. (A) The histogram was showed cell cycle analysis of control cells of MCF-7 (B) The histogram was showed cell cycle analysis of MCF-7 treated with KP. (C) The data were showed the percentage control group of cancer cell lines. (D) The data were showed the percentage treated with KP of cancer cell lines.

The effect of ethanol extract of KP to cell apoptosis

To investigate the effect of KP extract to induce apoptosis, MCF-7 and, HeLa cell lines were treated with KP extract at concentration 470, 330, and 650 μg/ml; respectively for 24, and 48 hours. Treated cells were stained with Annexin V-FICT/PI and analyzed by flow cytometry. The data was showed increasing number of early and late apoptotic cells when compared with control group. In treated cells of MCF-7 at 24 hours, the percentage of early apoptosis in N1, N2, N3 were showed 13.98%, 15.45%, and 15.05% respectively. In treated cells of cells MCF-7 at 48 hours, the percentage were showed 24.58%, 38.91%, 31.21%; respectively (Figure 3). At the same time point. The result of early apoptosis in HeLa were showed increasing percentage of cells. In treated cells of HeLa for 24 hours, the data were showed 6.05%, 6.39%, and 4.96%; respectively. In treated cells of HeLa for 48 hours, the percentage were showed 53.94%, 42.14%, and 52.26%; respectively. The percentage of late apoptosis in MCF-7 and HeLa cell lines were showed increasing the number of cell compared with control cells.

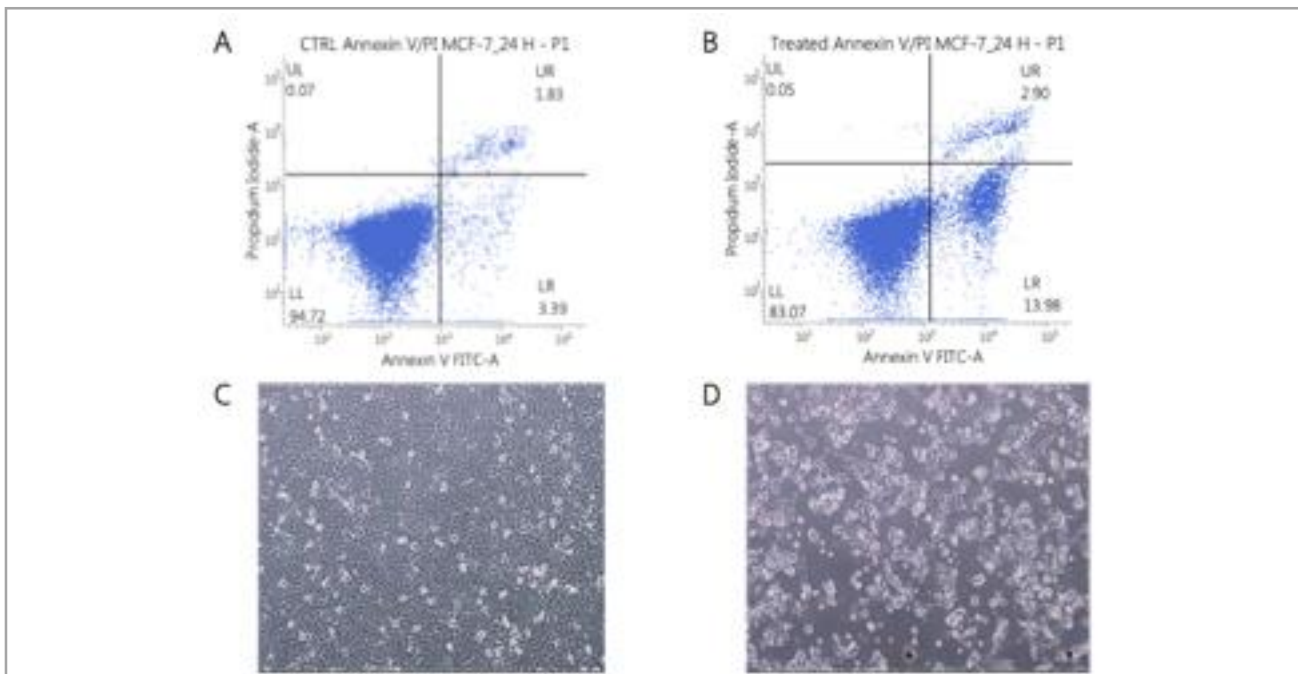

Figure 3 The effect of KP extract on cell apoptosis, MCF-7, HeLa and fibroblast were treated with KP extract for 24, 48 hours and stained with AnnexinV/PI. (A) MCF-7 cells were incubated without KP extract (B) MCF-7 cells were incubated with KP extract 470 μg/ml. The lower right quadrant of histogram was indicated percentage of early apoptotic cells (Annexin V+/PI-) (C) Photo was represented round shape and organized nuclear structure (D) Photo was showed cells floating and irregular cell wall.

DISCUSSION

The data present here demonstrated that the KP extract inhibited proliferation of breast cancer cell lines. The KP extract induced cell cycle distribution in sub G0/G1 and induced cells apoptosis in breast cancer cell lines. Currently, there are several studies that determine anti proliferative effect of extract obtained from herbs on cancer cells. Our results showed that MCF-7 breast cancer cell lines had highly effective response to KP extract. MDA-MB-231 and SK-Br3 were slightly responded when compared with MCF-7 and controlled cells. Our result is consistence with previous studies. They reported that KP extract had cytotoxicity effect to human ovarian cancer (SKOV-3), Human cervical cancer HeLa229, human promyelocytic leukemic (HL60), human promonocytic leukemic (U397), human bile duct (HuCCA-1 and RMCCA-1) and human urinary bladder cancer (T24) (11-15). Several herb extract had been studied in breast cancer cell lines. Our study is the first trial of using the KP extract on breast cancer cell lines. The previous study investigated the anti-proliferation effect of hualiang on MCF-7, MDA-MB-231, SKBR-3 and T47D breast cancer cell lines (16). MCF-7 and MDA-MB-231 had high response to hualiang extract. These finding show that different breast cancer subtype may affect response to herb extract. Further study to elucidate the signaling mechanism of KP extract and the involvement of hormone receptor are need.

In our study, the effect of KP extract to cell cycle distribution was determined by PI staining. Our result showed that KP extract could induce cell cycle arrest at subG0/G1 of MCF-7 breast cancer cell lines. The previous studies have shown that KP extract could suppressed growth and survival signaling pathway. Potikanond et al. reported KP extract significantly to suppressed phosphorylation of PI3K, AKT, and ERK1/2. The PI3K/AKT, and ERK1/2 signaling pathway are role important on process of cell survival, cell proliferation, and cell growth. PI3K/AKT pathway can activated growth factor via their ligand (14). This finding is consistent with Paramee et al, they were studied the effect of KP on SKOV-3 and showed KP extract can suppress phosphorylation of ERK1/2 and AKT (15). In addition, cyclin and cyclin dependent kinase (CDKs) are involve regulation process of cell cycle division. Therefore, further study to elucidate the signaling mechanism of KP extract to cell cycle distribution are need.

We determine the effect of KP extract on breast cancer cell lines. The data showed increase the percentage of early apoptosis. Our result is consistence with previous studies. KP extract induce apoptosis of SKOV-3, HeLa229, HL-60, U397, HuCCA-1 and RMCCA-1, and T24 in a time and dose dependent manner (10-15). The previous evidence demonstrated that KP extract suppressed activation of caspase-3, caspase-7, and caspase-9. This finding is consistent

with Paramee et al. KP extract showed reduction of full length structure of caspase-3, caspase -7, and caspase-9. The apoptotic pathway includes 2 pathway, extrinsic and intrinsic pathway, the caspase-3, capase-7 and caspase-9 are involve with apoptotic intrinsic pathway when the mitochondrial outer membrane permeability (MOMP) is increasing permeability (17-20). This finding is consistence with Banjerdpongchai et al. (2008, 2009) KP ethanol extract involved activation of caspase-3 via a mitochondria-mediated pathway (10,11).

Conclusions

This study presents the effect of KP was able to anti-proliferation on breast cancer cell lines including induction cell cycle distribution at sub G0/G1 and induction apoptosis on MCF-7 breast cancer cell lines. These observations convince us to believe the KP extract can be used as potential agent in breast cancer.

Reference

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.

2 Imsamran W, Chaiwerawattana A, Wiangnon S, Pongnikorn D, Suwanrungrung K, Sangrajrang S, et al. Cancer in Thailand: Vol. VIII, 2010–2012. 2015.

3 Dai X, Li T, Bai Z, Yang Y, Liu X, Zhan J, et al. Breast cancer intrinsic subtype classification, clinical use and future trends. American journal of cancer research. 2015;5(10):2929-43.

4 Rujjanawate C, Kanjanapothi D, Amornlerdpison D, Pojanagaroon S. Anti-gastric ulcer effect of Kaempferia parviflora. Journal of ethnopharmacology. 2005;102(1):120-2.

5 Sudwan P, Saenphet K, Saenphet S, Suwansirikul S. Effect of Kaempferia parviflora Wall. ex. Baker on sexual activity of male rats and its toxicity. The Southeast Asian journal of tropical medicine and public health. 2006;37 Suppl 3:210-5.

6 Tewtrakul S, Subhadhirasakul S, Kummee S. Anti-allergic activity of compounds from Kaempferia parviflora. Journal of ethnopharmacology. 2008;116(1):191-3.

7 Tewtrakul S, Subhadhirasakul S, Karalai C, Ponglimanont C, Cheenpracha S. Anti-inflammatory effects of compounds from Kaempferia parviflora and Boesenbergia pandurata. Food Chemistry. 2009;115(2):534-8.

8 Malakul W, Ingkaninan K, Sawasdee P, Woodman OL. The ethanolic extract of Kaempferia parviflora reduces ischaemic injury in rat isolated hearts. Journal of ethnopharmacology. 2011;137(1):184-91.

9 Kobayashi H, Suzuki R, Sato K, Ogami T, Tomozawa H, Tsubata M, et al. Effect of Kaempferia parviflora extract on knee osteoarthritis. Journal of natural medicines. 2018;72(1):136-44.

10 Banjerdpongchai R, Suwannachot K, Rattanapanone V, Sripanidkulchai BJAPJCP. Ethanolic rhizome extract from Kaempferia parviflora Wall. ex. Baker induces apoptosis in HL-60 cells. 2008;9(4):595-600.

11 Banjerdpongchai R, Chanwikruy Y, Rattanapanone V, Sripanidkulchai BJAPJCP. Induction of apoptosis in the human Leukemic U937 cell line by Kaempferia parviflora Wall. ex. Baker extract and effects of paclitaxel and camptothecin. 2009;10(6):1137-40.

12 Leardkamolkarn V, Tiamyuyen S, Sripanidkulchai BJAPJCP. Pharmacological activity of Kaempferia parviflora extract against human bile duct cancer cell lines. 2009;10(4):695-8.

13 Ninomiya K, Matsumoto T, Chaipech S, Miyake S, Katsuyama Y, Tsuboyama A, et al. Simultaneous quantitative analysis of 12 methoxyflavones with melanogenesis inhibitory activity from the rhizomes of Kaempferia parviflora. 2016;70(2):179-89.

14 Potikanond S, Sookkhee S, Na Takuathung M, Mungkornasawakul P, Wikan N, Smith DR, et al. Kaempferia parviflora extract exhibits anti-cancer activity against HeLa cervical cancer cells. 2017;8:630.

15 Paramee S, Sookkhee S, Sakonwasun C, Takuathung MN, Mungkornasawakul P, Nimlamool W, et al. Anti-cancer effects of Kaempferia parviflora on ovarian cancer SKOV3 cells. 2018;18(1):178.

16 Kang JX, Liu J, Wang J, He C, Li FP. The extract of huanglian, a medicinal herb, induces cell growth arrest and apoptosis by upregulation of interferon-beta and TNF-alpha in human breast cancer cells. Carcinogenesis. 2005;26(11):1934-9.

17 Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology. 2007;35(4):495-516.

18 Wong RS. Apoptosis in cancer: from pathogenesis to treatment. Journal of experimental & clinical cancer research : CR. 2011;30:87.

19 Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, et al. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell proliferation. 2012;45(6):487-98.

20 Jin Z, El-Deiry WS. Overview of cell death signaling pathways. Cancer biology & therapy. 2005;4(2):139-63.