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Original Article

Piperine Induces Cell Death, Apoptosis and Inhibits Migration in Cholangiocarcinoma Cells

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ABSTRACT

Background/Aim: Piperine (Pip) is an alkaloid that found from natural products and it has been reported to exert anticancer activities. Nevertheless, its anticancer effect has not yet been illustrated in cholangiocarcinoma (CCA) cells. In this work will be explored the Pip effects on two CCA cells and studied the underlying molecular mechanism. **Materials and Methods:** The KKU-100 and KKU-M452 cells proliferation were detected by sulforhodamine B assay, colony formation, and flow cytometric method. Migratory ability was explored via the wound healing and Transwell chamber model. Apoptosis was discovered through double staining of PI and Annexin V-FITC dye. JC-1 dye, and DCF-DA staining by flow cytometry. **Results:** The results revealed that Pip induced CCA cells death by a dose- and time-dependent along with inhibiting colony formation in KKU-100 cells. Following cell cycle arrest, treatment with Pip arrested the cell cycle distribution at G0/G1 phase in KKU-100 and S to G2/M phase in KKU-M452 cells. Furthermore, cell migration revealed that Pip suppressed cell migration in a dose-dependent manner. Apoptosis was significant detected by flow cytometry showed that Pip induced late apoptosis in these two CCA cells. The mechanism was indicated that Pip treatment also decreased mitochondrial membrane potential and increased ROS formation. **Conclusion:** Therefore, Pip may useful for prevention and treatment of CCA.

Keywords: Piperine, Apoptosis, Migration, Cholangiocarcinoma cells, Mitochondrial function, Reactive oxygen species production.

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INTRODUCTION

The active compounds have been extracted from spices, including genus piper, which have been indicated to be a reservoir of efficacy for anticancer agents.[1] Piperine (Pip) is an alkaloid present in *Piper nigrum*, *Piper retrofractum,* and *Piper betle*, which is one of the most common spices used in food and in traditional Indian and Chinese medicine. Pip has been widely used in pharmacological activities, including anti-convulsion,[2] anti-inflammatory and antioxidant,[3] and anticancer effects. The anticancer action of Pip has been reported in many cancer cell types such as breast, colon, ovarian, rectal and prostate cancer.[4] Pip inhibited the cancer cells death via inducing cell cycle arrest at G0/G1 along with downregulating cyclin D1 and cyclin and activating $p21^{Cip1}$ and $p27^{Kip1}$.[5] Induction of apoptosis was observed after treating with Pip, accordingly stimulation of cytochrome c, caspase-3 and caspase-9,[6] finally the apoptosis was occurred. The suppression of angiogenesis was blocked by reduction of Akt phosphorylation and inhibition of matrix metalloproteinases that are likely to promote tumor growth and metastasis.[7] Furthermore, Pip also inhibited drug resistance by suppression of P-glycoprotein-mediated transport, thus increasing the efficacy of anticancer agents.[8] Interestingly, it has been shown to rise the effect of docetaxel against prostate cancer in xenograft animal models.[9] Despite more documents of Pip on many cancer cells; however, it remains to be identified whether Pip effects on cholangiocarcinoma (CCA) cells.

CCA is cancer that originates in epithelium of bile duct, is a rare case of liver cancer; however, it is a serious problem in Thailand. Nowadays, it is the very high incidence and mortality rate in the world.[10] Presently, the surgical resection is greatest effective treatment of CCA[11] and further treatment with anticancer drugs to kill the remaining cancer cells. The efficacy of standard anticancer agents for treating CCA still disappointed, with showing a low survival and cure rate. The alternative treatment with high efficacy is urgently needed for CCA treatment. Presently, the medicinal plants or active compounds have been demonstrated on the anticancer activity to suppress CCA. For example, ethanolic extract of *Piper nigrum* with high levels of Pip caused inhibition of colon and breast cancer cells,[12-13] and

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represents tumor inhibitory effect in mammary adenocarcinoma mouse.[14] However, some data indicated that Pip-free *Piper nigrum* extract strongly inhibited breast cancer and CCA cells with low IC_{50} values.[15] Therefore, we interested to examine the Pip effects on CCA cells in term of proliferation, migration, and apoptosis. Interestingly, the effect of Pip has less information to be reported on CCA cells. This work aimed to explore the inhibitory effects of Pip on two CCA cells, KKU-100 (slowly growth) and KKU-M452 (rapidly growth) along with to elucidate its potential molecular mechanisms *in vitro* study.

MATERIALS AND METHODS

Cell Cultured and Cytotoxic Assay

KKU-100 and KKU-M452, two CCA cell lines were used in this study and these cancer cells were kindly provided by Faculty of Medicine, Khon Kaen University. CCA cells were grown up in DMEM with 1% antibiotics and 10% FBS. Further, cancer cells were plated in a 5% CO_2 incubator.

Pip effects on cell proliferation were investigated by SRB method. Briefly, the cancer cells were seeded onto a 96-well culture plate (1×10^4 cells/well) for overnight. After incubation with Pip (0-250 μM) for 24-72 hr, the cells in culture plates were added with 0.4% SRB, for 30 min and then dissolved with 10 mM Tris base solution. The absorbance was measured by microplate reader at 540 nm.

Colony Formation Assay

The cell colony was examined by colony formation method. The cancer cells were seeded onto a 6-well plate (500 cells/well) for overnight. The cells were exposed with Pip (0-250 μM) for 24 hr, changed to the new complete medium, cultured for another15 days, methanol was added and 0.5% crystal violet was incubated for 60 min. The colony formation was counted by visual inspection.

Acridine orange/Ethidium bromide (AO/EB) staining assay

Pip effects on cell number was investigated by AO/EB staining method. In brief, the cancer cells were cultured onto a 96-well plate (1×10^4 cells/well) for overnight. The cells were exposed with Pip (0-250 μM) for 24 hr, then the medium was removed, and added with AO/EB dye (1 μg/mL for each dye). Cancer cells were observed immediately by inverted fluorescence microscopy, and images were captured (×20 magnification). The cells number of viable cells per field was counted.

Cell Cycle Distribution

Pip effects on cell distribution was investigated by flow cytometry. In brief, the cancer cells were plated onto a 6-well culture plate (2.5×10^5 cells/well) for overnight. The cells were exposed with Pip (0-250 μM) for 24 hr, fixed with 70% ethanol for overnight and the following components were then added 1 mL propidium iodide (PI) (Cat No. 550825, BD Biosciences, CA, USA). The stained-cells were incubated at 37°C for 30 min and measured cell distribution by flow cytometer (BD Biosciences, CA, USA) using BD Accuri C6 Plus software.

Wound Healing Assay

The Pip effects on cancer migration was investigated by wound healing method. Cancer cells were seeded onto a 24-well plate (2.5×10^5 cells/well) for overnight. Then, the wounds were created by scratching using 0.2 mL sterile pipette tips. The cells were exposed with Pip (0-250 μM) for 24 hr for KKU-M452 cells and for 72 hr for KKU-100 cells, respectively. The wound area was recorded using phase contrast microscopy (Olympus CKX53, USA) at 0, 24 and 72 hr.

Matrigel Migration Assay

The Pip effects on migration was examined by Transwell migration method. Cells were seeded onto the insert well (upper chamber) (2×10^4 cells/well) in serum-free DMEM medium with Pip (0-250 μM) for 24 hr. In the lower chamber, DMEM was containing with 10% FBS. The migrated-cells were added with absolute methanol for 15 min at -20°C and soaked with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA). The migrated-cells were randomly selected fields and counted.

Apoptosis by Flow Cytometry

The Pip effects on apoptosis was measured by flow cytometry. Briefly, the cells were cultured onto 6-well plate (2.5×10^5 cells/well) for overnight and incubated with Pip (0-250 μM) for 24 hr. Afterwards, cells were mixed with 1.5 μl PI and 5 μl Annexin V-FITC solution (Cat No. 58547, BD Biosciences, CA, USA) and apoptotic cells were determined by flow cytometer (BD Biosciences, CA, USA) using BD Accuri C6 Plus software.

Mitochondrial Membrane Potential (MMP) by Flow Cytometry

Pip effects on mitochondrial function was measured by flow cytometry. Briefly, the cells were cultured onto 6-well plate (2.5×10^5 cells/well) for overnight and then exposed to Pip (0-250 μM) for 24 hr. After that, the cancer cells were stained with JC-1 buffer (Cat. No. 551302, BD Biosciences, USA). Mitochondrial function was measured by flow cytometer (BD Biosciences, CA, USA) using BD Accuri C6 Plus software.

Reactive Oxygen Species (ROS) Formation Assay by Flow Cytometry

Pip effects on ROS formation was determined by flow cytometry. Briefly, the cells were cultured onto 6-well plate (2.5×10^5 cells/well) for overnight and incubated with Pip (0-250 μM) for 24 hr. The cells were added with DCF-DA solution (25 μM) for

30 min at 37°C. ROS formation was measured by flow cytometer (BD Biosciences, CA, USA) using BD Accuri C6 Plus software.

Statistical Analysis

Data from three independent experiments are presented as the mean±SE. The difference between treatment and control groups were tested using Student's *t-test* and statistical significant difference must less than 0.05.

RESULTS

The Effects of Pip on Cell Viability and Colony Formation

Results demonstrated the Pip effect on CCA cells, KKU-100 and KKU-M452 cells. At 24 hr, Pip suppressed the cell viability in both of two types of CCA cells and Pip effects showed higher levels of anticancer actions in KKU-M452 cells more than KKU-100 cells (Figure 1A and C). The IC_{50} values of KKU-100 cells were 539.47±131.66 µM, 103.93±16.99 µM, 37.50±3.42 µM, and IC_{50} values of KKU-M452 cells were 149.07±34.52 µM, 67.58±8.83 µM, 45.46±13.13 µM for 24, 48, 72 hr incubation periods, respectively (Table 1). Consequently, with colony formation assay, the result indicated that clonogenic survival of KKU-100 cells were decreased following incubation with Pip by dose-dependent manner (Figure 1B). We used only KKU-100 cells in colony formation assay because KKU-M452 did not form the colony formation.

The effects of Pip on cell number and cell cycle distribution

The results from AO/EB staining assay demonstrated that viable cells were observed to have large number in control group and Pip treatment decreased the cell number by dose-dependent

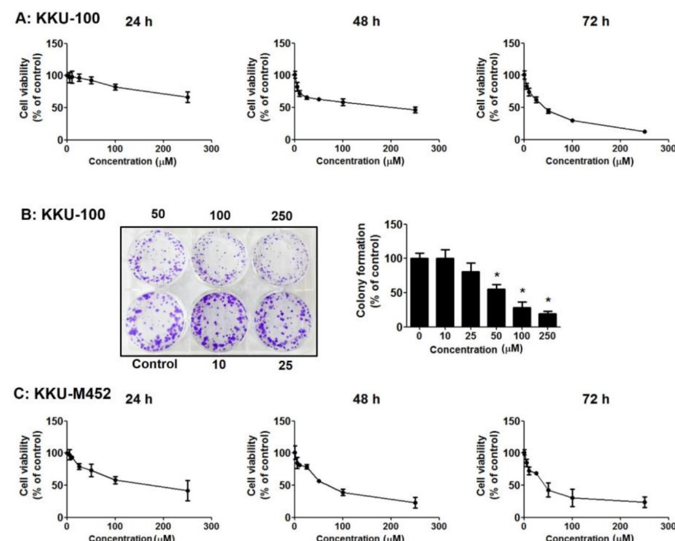


Figure 1: The Pip effects on KKU-100 (A) and KKU-M452 (C) cells viability and colony formation (B) (n=3, Mean±SE., *p<0.05).

Table 1: IC_{50} values and E_{max} of Pip on two CCA cells.

Cell types	Incubation times (h)	IC_{50} (µM)	E_{max}
KKU-100	24	539.47±131.66	58.37±9.05
	48	103.93±16.99*	76.90±4.66*
	72	37.50±3.42*	76.08±8.22*
KKU-M452	24	149.07±34.52	33.46±4.73
	48	67.58±8.83*	53.79±4.52*
	72	45.46±13.13*	87.40±1.41*

* p<0.05 Vs. 24 hr incubation period.

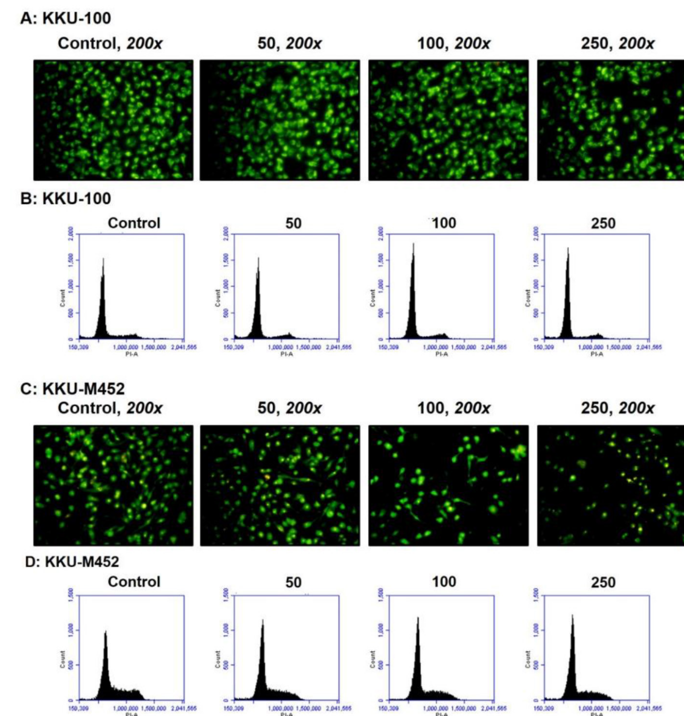


Figure 2: The Pip effects on cell numbers (A and C) and cell cycle distribution (B and D). Cell number and cell cycle distribution of Pip in CCA cells.

manner in both two CCA cells (Figure 2A and C). Especially, 250 µM of Pip treatment significantly showed the induction of cells number of nuclear condensation and apoptotic body formation. Moreover, cell cycle distribution was conducted and the results demonstrated that Pip treatment significantly increased the percentage of G0/G1 phase in KKU-100 cells and increased in S to G2/M phase in KKU-M452 cells (Figure 2B and D).

The effects of Pip on cell migration

The results indicated that Pip significantly suppressed the cell migration assay after making a wound by 0.2 mL pipette tips by dose-dependent manner. At the dose of 250 µM of Pip had the higher levels of anti-migratory activities more than the other concentrations; the percentage of inhibition were 41.37±2.13% in KKU-100 cells and 47.23±4.18% in KKU-M452 cells (Figure 3A and B).

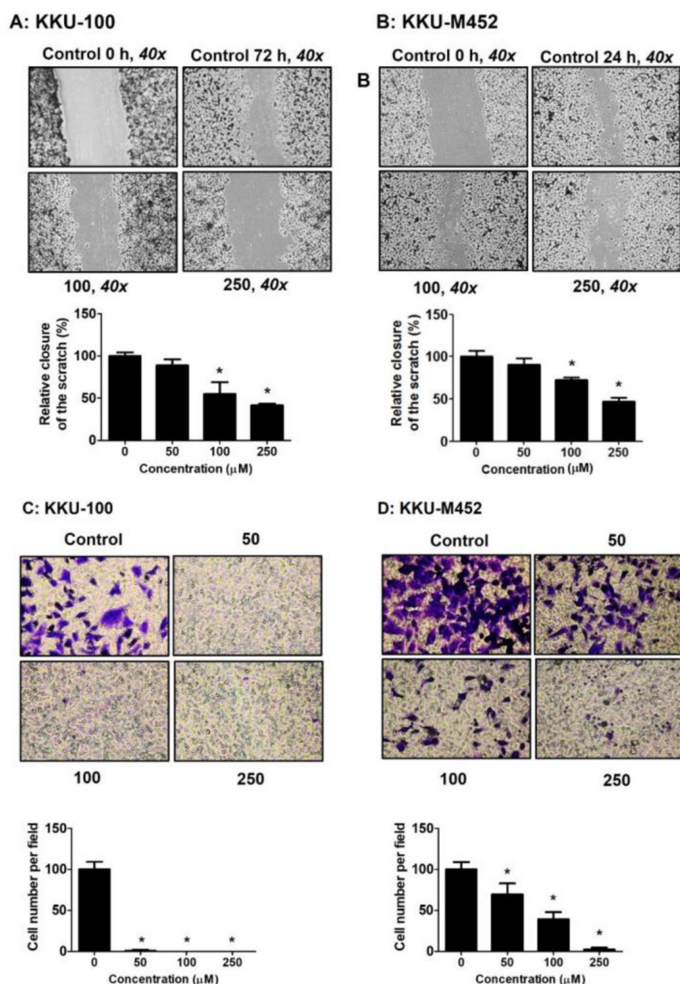
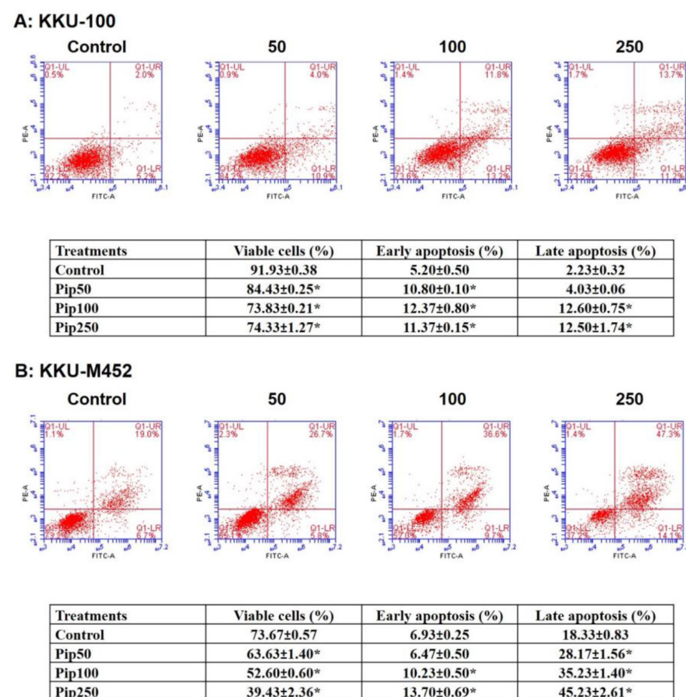


Figure 3: The Pip effects on Wound healing (A and B) and Matrigel migration (C and D) (n=3, Mean±SE., *p<0.05).

The Transwell assay revealed that Pip inhibited the KKU-100 cells migrated through the lower chamber almost 100% compared to the control cells, whereas Pip decreased KKU-M452 migration by dose-dependent manner. These observations suggest that Pip treatment decreased CCA cell migration, especially in KKU-100 cells.

The effects of Pip on apoptosis, mitochondrial function, and ROS formation

In order to further determine the occurrence of apoptosis, cancer cells were measured apoptosis by flow cytometry, mitochondrial function, and ROS formation. Pip treatment reduced the viable cells along with increasing early and late apoptotic rate (Figure 4A and B). The following treatment with 0, 50, 100 and 250 μM Pip for 24 hr, the cells number of late apoptosis was increased from 2.23±0.32, 4.03±0.06, 12.60±0.75, 12.50±1.74% in KKU-100 cells and 18.33±0.83, 28.17±1.56, 35.23±1.40, 45.23±2.61% in KKU-M452 cells respectively. These results indicated that Pip significantly stimulated CCA cells apoptosis by dose-dependent manner at 24 hr incubation.



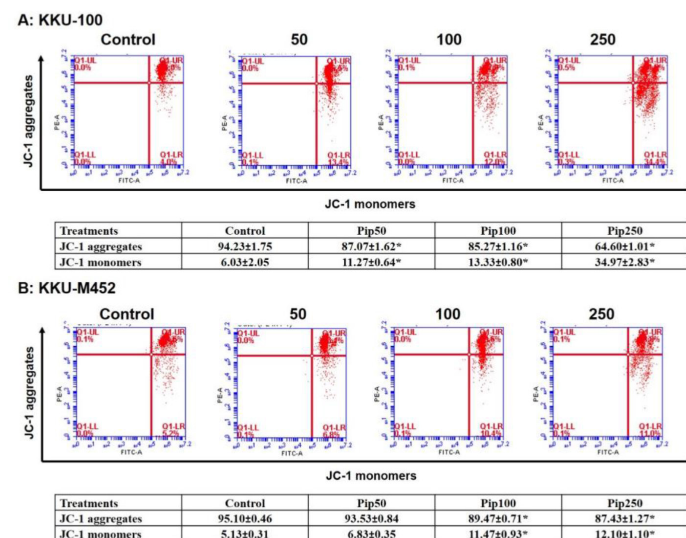
A: KKU-100

Treatments	Viable cells (%)	Early apoptosis (%)	Late apoptosis (%)
Control	91.93±0.38	5.20±0.50	2.23±0.32
Pip50	84.43±0.25*	10.80±0.10*	4.03±0.06
Pip100	73.83±0.21*	12.37±0.80*	12.60±0.75*
Pip250	74.33±1.27*	11.37±0.15*	12.50±1.74*

B: KKU-M452

Treatments	Viable cells (%)	Early apoptosis (%)	Late apoptosis (%)
Control	73.67±0.57	6.93±0.25	18.33±0.83
Pip50	63.63±1.40*	6.47±0.50	28.17±1.56*
Pip100	52.60±0.60*	10.23±0.50*	35.23±1.40*
Pip250	39.43±2.36*	13.70±0.69*	45.23±2.61*

Figure 4: The Pip effects on cell apoptosis (A and B) (n=3, Mean±SE., *p<0.05).



A: KKU-100

Treatments	Control	Pip50	Pip100	Pip250
JC-1 aggregates	94.23±1.75	87.07±1.62*	85.27±1.16*	64.60±1.01*
JC-1 monomers	6.03±2.05	11.27±0.64*	13.33±0.80*	34.97±2.83*

B: KKU-M452

Treatments	Control	Pip50	Pip100	Pip250
JC-1 aggregates	95.10±0.46	93.53±0.84	89.47±0.71*	87.43±1.27*
JC-1 monomers	5.13±0.31	6.83±0.35	11.47±0.93*	12.10±1.10*

Figure 5: The Pip effects on mitochondrial function (A and B) (n=3, Mean±SE., *p<0.05).

Mitochondrial function is an important step in apoptotic pathway and loss of mitochondrial function is essential process apoptosis. Data indicated that Pip treatment stimulated a significant decrease in the mitochondrial function level with increasing concentrations of Pip in two CCA cells (Figure 5A and B). Mitochondrial dysfunction was slightly low in CCA untreated control cells.

Further, to explore the mechanism Pip on cell apoptosis and mitochondrial dysfunction, ROS formation was observed.

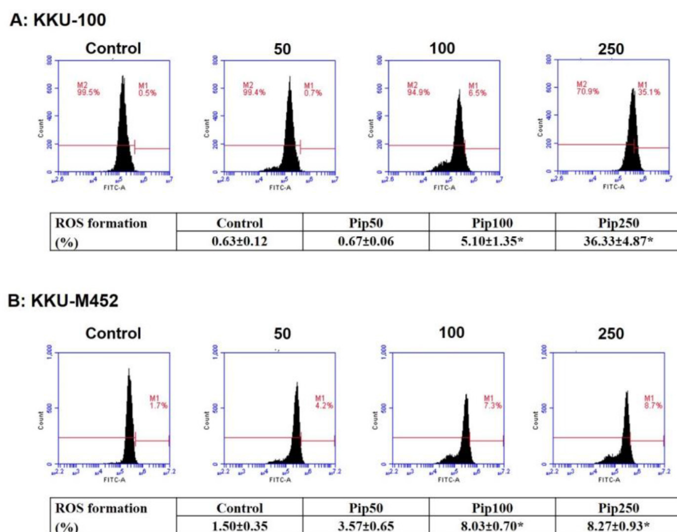


Figure 6: The Pip effects on ROS formation (A and B) (*n*=3, Mean±SE., **p*<0.05).

Intracellular ROS production was detected using a DCF-DA probe by flow cytometric analysis in CCA cells. Following treatment with Pip for 24 hr, Pip significantly accelerated ROS formation in both two CCA cells (Figure 6A and B). The ROS levels was induction from 0.63±0.12, 0.67±0.06, 5.10±1.35, and 36.33±4.87% in KKU-100 cells and 1.50±0.35, 3.57±0.65, 8.03±0.70, and 8.27±0.93% in KKU-M452 cells. Pip induced ROS production in CCA cells and related to induction of CCA cells death.

DISCUSSION

Pip has been reported presently to exhibit anticancer actions, such as anti-growth, anti-migratory, and apoptotic effects. Pip suppresses proliferation and stimulates apoptosis in several cancer cell lines, however, Pip effects on human CCA cells are still lack information. This work, Pip suppressed the proliferation and reduced the cancer cells number in both of two CCA cells, KKU-100 (slowly growth) and KKU-M452 (rapidly growth), through arresting in G0/G1 phase and S to G2/M phase cell cycle, respectively. It significantly suppressed CCA cells migration by detecting with wound healing and Transwell assay. Furthermore, Pip induced both of two CCA cells late apoptosis by which dose-dependent manner, especially KKU-100 cells. These effects are associated with reducing mitochondrial function and ROS formation. It can be concluded that Pip has a great effect to induce cells death and apoptosis in both of two CCA cells.

From the results obtained, Pip significantly inhibited the growth of two CCA cells in a dose- and time-dependent manner, with the slow-proliferative cells KKU-100 was more sensitive than rapid-proliferative cells KKU-M452 cells. Consequently, the colony formation confirmed the antigrowth effect of Pip, it was inhibited the colony formation by dose-dependent manner. Similarly, Pip on the antiproliferative activity in colon cancer cells and rectal cancer cells,[16] was confirmed by cell cycle distribution of PI-stained cells cultured. Pip indicated an inhibitory effect on distribution of cell cycle, which was related with arresting of G0/G1 phase in KKU-100 cells and S to G2/M phase in KKU-M452 cells. Likely as Pip effects on rectal cancer cells, it was induced the percentage of cells in G0/G1 phase along with decrease of cells in G2/M phase.[17] Induction of cells in G0/G1 phase suggests a probable inhibitory effect of Pip associated with decreasing protein-related cell growth, such as cyclin-dependent kinases (CDK), CDK4 and CDK6.[18] Moreover, Pip caused a significant increase of p21^{Cip1} in prostate cancer cells, which might account for why Pip caused a significant cell cycle arrest.[19] This is the important data of Pip with anticancer activity on cell cycle distribution, which differ depending on each cancer cell types that are being determined.

Pip reduced CCA cells migration was showed by two experiments including wound healing and Transwell assay. In this study, Pip inhibited CCA cells migration like as prostate cancer cells with regulating via reducing p-Akt, MMP 9 and p-mTOR expression levels,[20] consistent with decreasing the MMP9 and MMP13 in human breast cancer cells.[21] On the other hand, Pip inhibited PDGF-BB that induced migration,[22] that important in the atherosclerosis and restenosis pathogenesis. Pip caused reduced CCA cell migration in slow-growth cells more than rapidly-growth cells; however, the Pip effects on CCA cells migration was still less information, in the further we will deeply explore the mechanism of action on it.

It is a consensus in literature that Pip can be used as an anticancer agent, since it activates apoptosis in various cancer cell types. CCA cells were induced apoptosis in *in vitro* study after exposing with Pip treatment, especially for late apoptosis level. Apoptosis stimulation suggests the activation of caspase in Pip-treated rectal cancer cells,[23] which could be reliable with modulations in mitochondrial dysfunction following treatment Pip in of colon and melanoma cells.[24] Furthermore, induction of ROS levels in intracellular, which is activate of oxidative stress that lead to stimulate cancer cells apoptosis. Like as our work, Pip caused induction of apoptosis by decreasing mitochondrial function and increasing ROS formation levels. Pip mechanism induced apoptosis was correlated with ROS formation, led to induce mitochondrial damage along with protein-related apoptosis induction including Bax, cytochrome c, caspase 9 and caspase 3.[25] Interestingly, Pip is also known to augment the bioavailability of some drugs by inhibiting drug metabolism or by increasing absorption. Pip with 5-fluorouracil (5-FU) augmented the anticancer activity of 5-FU and decreased the side effects.[26] Finally, Pip has the high efficacy of anticancer drugs on CCA cells.

CONCLUSION

Pip can distinctly inhibit the proliferation and colony formation of CCA cells. Furthermore, Pip-treated cells showed induction of cell cycle distribution at G0/G1 phase for KKU-100 and S to

G2/M phase for KKU-M452 cells, respectively. Pip decreased cell migration by dose-dependent manner, especially in KKU-100 cells. On the other hand, it induced cancer cells apoptosis via decreased mitochondrial membrane potential and induced ROS formation. The over formation of ROS levels may be important in inhibiting the growth of CCA cells following Pip treatment. Therefore, the natural product, Pip may be a promising novel drug for the treatment of CCA. Future studies will use Pip in combination with anticancer drugs in CCA cells to investigate the probable helpfulness of Pip as an adjunct to anticancer drugs treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AO/EB: Acridine orange/Ethidium bromide; **CCA:** Cholangiocarcinoma cells; **MMP:** Mitochondrial membrane potential; **Pip:** Piperine; **PI:** Propidium iodide; **ROS:** Reactive oxygen species.

SUMMARY

Pip is an alkaloid that found from natural products and it has been reported to exert anticancer activities. Pip inhibits CCA cells proliferation, induces apoptosis, and suppresses migration with arresting cell cycle distribution at distribution at G0/G1 phase in KKU-100 and S to G2/M phase in KKU-M452 cells. Moreover, the apoptosis was demonstrated via decreasing mitochondrial function and increasing ROS formation. Pip against CCA cells and may be useful for CCA treatment.

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