In vitro evaluation of the antiproliferative activity of *Piper retrofractum* on human cholangiocarcinoma cells

Benjaporn BURANRAT*

Mahasarakham University, Faculty of Medicine, Biomedical Sciences Research Unit, Muang District, Mahasarakham 44000, Thailand; benjaporn.b@msu.ac.th

**Abstract**

To explore the effects of *Piper retrofractum* (PR) on CCA cells death, apoptosis, and migration. Two CCA cells, KKU-100 and KKU-M452, were used in this study. Cells were observed the growth by sulforhodamine B (SRB), colony formation, cell cycle arrest, and reactive oxygen species (ROS) formation. Migratory capability was determined by Wound healing and matrigel migration method. From the results indicated that PR significantly suppressed the growth of both CCA cells through reducing cancer cells number, inhibiting colony formation, and arresting cell cycle distribution at G0/G1 phase in KKU-100 cells and S to G2/M phase in KKU-M452 cells. Furthermore, PR strongly increased late apoptosis and necrosis in both cancer cells via increasing ROS formation and decreasing mitochondrial function. In line with this finding, PR extracts suppressed migration in Wound healing and matrigel migration assay, especially in KKU-100 cells. Collectively, the present study suggested PR serve as an anticancer drug for the prevention and treatment of CCA.

**Keywords:** apoptosis; cholangiocarcinoma; mitochondrial dysfunction; *Piper retrofractum*; reactive oxygen species

**Introduction**

Nowadays, many researchers conducted to discover new drug natural resources for cancer therapy, including *Piper retrofractum* (PR). PR is an economically and medicinally important species in the genus *Piper* (Piperaceae) and it is an outstanding pharmacetical species which grows naturally in Indonesia, Malaysia, Vietnam, India, and also in Thailand (Hasan et al., 2016). Several parts of PR have been reported for pharmacological activities including leaves, root, stem bark, and fruit. Especially PR ripe fruit, it demonstrated to possess various bioactivities such as antifungal, insecticidal, antibacterial, antileishmanial, antidiabetic, antiobesity, and anticancer activity. In Thailand, PR is known as a local name as “Dee-Plee” and it has been used as a drug for gastrointestinal system treatment. Moreover, PR has been used in many Thai receipts including ben-ja-kul, prab-chom-poo-tha-weeb, yha-lerd-kham, yha-tri-phi-kad, and yha-phai-pra-lai-kun, for treatment in several diseases. Therefore, the study of PR extract was more interested to evaluate in cancer treatment.
The major compounds that found in PR extract including lignans and alkaloids (Bhandari et al., 1988). Alkaloids are extracted mainly from fruit of PR and it mainly contains piperine and piperlonguminine (He et al., 2016) that more potency against several cancer cells. The data of PR extract in cancer treatment is less information and mechanism of action is still lack information. However, the other plant from genus Piper has been demonstrated against cancer cells including *Piper nigrum*, *Piper betle*, *Piper ribesoides*, *Piper sarmentosum*, *Piper cubeba*, and *Piper porphyrophyllum* including PR. The report from Sriwiriyajan et al., found that PR, *Piper betle*, and *Piper nigrum* extracts have high antigrowth action on human breast cancer cells (MDA-MB-231, MDA-MB-468, and MCF-7) and *Piper nigrum* had more strong effects (Sriwiriyajan et al., 2014). Confirmation with PR extract on breast cancer cells, it showed the low IC₅₀ values at 72 h incubation, 12.27±2.14, 17.10±0.46, and 19.69±0.88, µg/mL in MDA-MB-468, MDA-MB-231, MCF-7, and respectively. Thus, the mechanism of PR extract on cholangiocarcinoma (CCA) is still unknown.

CCA is a bile ducts cancer which is aggressive and poor prognosis in Thailand (Sripa et al., 2011), especially the North-East part. The strategy of CCA therapeutic treatment is limited and has no effective treatment for cure the advance stage of CCA patients (Gatto and Alvaro, 2010). Advances in surgical techniques is the only chance for cure and then treatment with chemotherapy and radiotherapy is needed for prolong the survival rate in this CCA patients. Unfortunately, 5-year survival rate of CCA patients after diagnosis still remain about 10% (van der Gaag et al., 2012; Squadroni et al., 2017). Chemotherapeutic agents are less effective treatment and show many adverse effects through harmful to rapidly normal cells and bone marrow suppression (Barreto et al., 2014). Hence, alternative treatments with less serious side effect for CCA are immediately required.

Based on the previous studies, we hypothesized that PR ripe fruit extract showed anticancer effects by inhibiting cell growth, inducing apoptosis, and suppressing migration. The purpose of this work was to explore the cytotoxic, apoptotic, and migratory activity of PR fruit extract on two CCA cells including KKU-100 (non-migrated cells) and KKU-M452 (migrated cells) cells by which activation of ROS formation.

**Materials and Methods**

**Plant and extraction**

The ripe fruit of PR were collected from Chiang Mai Province, Thailand, in 2020 and identified by Pornpimon Wongswuan, Assistant professor, Faculty of Medicine, Mahasakham University. The herbarium specimen number of this material has been deposited in the Faculty of Sciences, Mahasarakham University (No. MSUT7235). The PR extract was extracted by 95% ethanol and made as our previous work described (Buranrat, 2022). Briefly, dried ripe fruits (250 g) were macerated with 95% ethanol (7 days), filtered, evaporated and lyophilized to obtain the dry extract. The % yield was 4.14% and the extracts were kept at -20 °C until use.

**Cell cultured and cytotoxicity**

Two CCA cells were used in this work, KKU-100 and KKU-M452, were kindly provided by Faculty of Medicine, Khon Kaen University. To determine the effects of PR extract on cell viability, SRB assay was used. The cell was plated into 96-well culture plates (1x10⁴ cells/well) for overnight and the various concentration of PR extract (0-250 µg/mL) was added to each well for 24, 48, 72 h, respectively. In brief, each well was fixed with 10% trichloroacetic acid, stained with 0.4% SRB solution, and solubilized with 10 mM Tris base, and read the absorbance by spectrophotometer at 540 nm.
Acridine orange/ethidium bromide (AO/EB) double staining
Cell number or morphology was measured using AO/EB assay. Cancer cells were plated in 96-well culture plates (1×10^4 cells/mL) for overnight. Subsequently, the cells were treated with various concentrations of PR extract (0-250 µg/mL) for 24 h and observed the morphology using a fluorescent inverted microscope (200x magnification, CKX53, Olympus, USA). For a staining method, each well was added with 1 µg/mL AO/EB of each dye for 20 min and photographed using a fluorescence microscope.

Colony formation
To determine the effects of PR extract on colony formation, colony formation assay was used. Cancer cells were plated in 6-well culture plates (500 cells/well) for overnight and then incubated with various concentration of PR extract (0-250 µg/mL) for 24 h. Afterwards, cells were cultured for another 15 days, fixed cells with methanol, stained with 0.5% crystal violet, and then counted the colonies.

Cell cycle distribution
To examine the PR extract on cell cycle distribution, flow cytometry was used after staining with propidium iodide (PI) solution. Cancer cells were plated into a 6-well culture plate (2.5×10^5 cells/well) for overnight. Subsequently, cells were incubated with various concentration of PR extract (0-250 µg/mL) for 24 h and were added with 70% ethanol overnight at −20 °C. Cancer cells were washed with PBS buffer for several times, stained with PI solution for 30 min at room temperature in dark. To analyse the proportions of cell cycle, BD Accuri C6 Plus software and fluorescent signals were displayed as histograms.

Reactive oxygen species (ROS) formation
To examine the PR extract on ROS formation, flow cytometry was used after staining with DCF-DA. Cells were seeded (2.5 x 10^5 cells/well) for overnight, and exposed to various concentration of PR extract (0-250 µg/mL) for 24 h. The cells were stained with DCF-DA (25 µM) for 30 min in dark, measured the fluorescence intensity using flow cytometry, BD Accuri C6 Plus software and fluorescent signals were displayed as histograms.

Wound healing
To determine the cancer cells migration, wound healing assay was used. Cells were seeded (2.5x10^5 cell/well) for overnight and the wound was made by a 0.2 mL pipette tip. The cells were incubated with various concentration of PR extract (0-250 µg/mL) for 48 h. Images of the wound surfaces were recorded at 0 and 48 h (40x magnification, CKX53, Olympus, USA) and then measured the denuded area.

Matrigel migration
To determine the cancer cells migration, a matrigel migration assay was used. Cancer cells were plated (2x10^4 cell/well) into the upper chamber of Transwell insert in a 24-well culture plate in serum-free DMEM medium with various concentration of PR extract (0-250 µg/mL) and the lower chambers was added with complete DMEM medium. After 24 h incubation period, the migrated cells were fixed with ice-cold methanol, stained with 0.25% crystal violet. Migrated cells were captured by phase-contrast microscope (200x magnification, CKX53, Olympus, USA).

Statistical analysis
All results are showed as the mean±SE. from three independent experiments and used GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) for testing statistical analysis. P<0.05 was statistically significant difference.
Results

PR effects on cell growth

The cytotoxic effects of PR indicated that PR extract inhibited CCA cell number at 24 h incubation periods by dose-dependent manner (Figure 1A and B) correlated with reduction of cell viability by dose- and time-dependent manner in two CCA cells. Compared with the incubation time point, a significant difference was identified in IC\(_{50}\) values of 72.16±8.04, 49.72±4.86, and 33.32±1.19 µg/mL for 24, 48, 72 h in KKU-100 cells and 245.33±23.25, 54.95±16.74, and 39.64±3.10 µg/mL for 24, 48, 72 h in KKU-M452 cells, respectively (Table 1).

![Figure 1](image_url)

**Table 1.** The IC\(_{50}\) values and Emax of PR extract on CCA cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Incubation times (h)</th>
<th>IC(_{50}) (µg/mL)</th>
<th>Emax</th>
</tr>
</thead>
<tbody>
<tr>
<td>KKU-100</td>
<td>24</td>
<td>72.16±8.04</td>
<td>86.38±1.01</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>49.72±4.86*</td>
<td>86.87±0.64</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>33.32±1.19*</td>
<td>87.69±1.09</td>
</tr>
<tr>
<td>KKU-M452</td>
<td>24</td>
<td>245.33±23.25</td>
<td>81.11±1.17</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>54.95±16.74*</td>
<td>85.44±0.86</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>39.64±3.10*</td>
<td>87.16±0.95</td>
</tr>
</tbody>
</table>
Cancer cells replication was observed by colony formation assay and the PR extract suppressed the formation of colonies on KKU-100 cells with IC₅₀ values was 70.03±3.93 µg/mL. KKU-M452 cells could not form the colony like as KKU-100 cells (Figure 2A). The suppression of PR on CCA cells growth was confirmed by cell cycle distribution. For KKU-100 cells, the percentage of cells in G0/G1 phase was increased after treating with PR extract at 24 h by dose-dependent and KKU-M452 cells was increased in S to G2/M phase (Figure 2B and C). Two CCA cells were induced cancer cells growth inhibition by PR through arresting the cell cycle distribution.

**Figure 2.** PR effects on colony formation and cell cycle distribution in CCA cells. (A) KKU-100 cells were incubated with PR extract (0-250 µg/mL) for 24 h, further cultured the cancer cells for 14 days, and counted the colonies. (B-C) KKU-100 and KKU-M452 were incubated with various concentration of PR extract for 24 h and measured cell cycle distribution after staining with PI solution. Mean±SE. n=3. *p<0.05

**PR effects on cancer cells apoptosis**

The percentage of apoptosis from flow cytometry after staining Annexin V-FITC and PI found that the cell number of late apoptotic and necrotic cells in the PR treatments were significantly increased when compared with untreated control groups (Figure 3A and B). Especially in KKU-100 cells, PR extract
significantly decreased the percentage of viable cells along with increasing late apoptotic and necrotic cells. Finally, PR extract decreased the number of viable cells and also induced necrotic cells in both CCA cells.

**Figure 3.** PR effects on apoptosis in CCA cells. (A) KKU-100 and (B) KKU-M452 cells were incubated with PR extract (0-250 µg/mL) for 24 h and measured apoptosis after staining with Annexin V-FITC and PI solution. Mean±SE. n=3. *p<0.05

**PR effects on ROS formation**

The results demonstrated that ROS accumulation were increased following PR treatment, especially in KKU-100 cells (Figure 4A and B). PR extract at the dose of 0, 50, 100, and 250 µg/mL were significant induced ROS formation in KKU-100 cells from 0.83±0.35%, 2.68±0.29%, 16.13±1.50%, and 17.23±1.33%, respectively. On the other hand, PR extract slightly activated ROS formation in KKU-M452 cells; however, the significant did not alter from the control group.
Figure 4. PR effects on ROS formation in CCA cells. (A-B) Cancer cells were incubated with PR extract (0-250 µg/mL) for 24 h and measured ROS formation by flow cytometry after staining with 25 M DCF-DA. Mean±SE. n=3. *p<0.05

PR effects on mitochondrial function

The results demonstrated that mitochondrial function (mitochondrial membrane potential, MMP) was decreased following PR treatment, especially in KKU-100 cells (Figure 5A and B). PR extract at the dose of 50, 100, and 250 µg/mL were significant reduced mitochondrial function by detecting with JC-1 monomers (Unhealthy-cells) was increased along with JC-1 aggregates (Healthy-cells) was decreased and the significant was indicated at high dose (250 µg/mL). KKU-100 cells, JC-1 aggregates were showed approximately from 96.63±0.67%, 93.23±0.91%, 80.47±1.25%, and 77.60±1.57% for 0, 50, 100, and 250 µg/mL, respectively and KKU-M452 cells were 93.63±0.51%, 92.83±0.55%, 92.93±0.86%, and 82.73±2.20%. PR extract decreased mitochondrial function in both of CCA cells, especially in KKU-100 cells (non-migrated cells/slow growth cells).

PR effects on cell migration

The data indicated that the migratory activities of CCA cells were markedly inhibited following their exposure to PR (Figure 6A and B). In Wound healing assay found that PR extract significantly inhibited cell migration at the doses of 50, 100, and 250 µg/mL in KKU-100 cells and 100 and 250 µg/mL in KKU-M452 cells. In Matrigel migration assays, PR extract strongly suppressed the migrated cells to the lower bottom of insert well in both of CCA cells, especially in KKU-100 cells. No cells were detected in PR treatment at the doses of 50, 100, and 250 µg/mL in KKU-100 cells and 100, and 250 µg/mL in KKU-M452 cells (Figure 7A and B). These results suggested that PR inhibited the migration in CCA cells.
Figure 5. PR effects on mitochondrial function in CCA cells. (A-B) Cancer cells were incubated with PR extract (0-250 µg/mL) for 24 h and measured mitochondrial function by flow cytometry after staining with JC-1 dye. Mean±SE. n=3. *p<0.05

Figure 6. PR effects on migration by wound healing in CCA cells. (A-B) Cancer cells were made a wound by 0.2 mL pipette tips and incubated PR extract (0-250 µg/mL) for 24 h. The cancer cells migration was measured the denuded area at 0 and 48 h after photographing with 4x magnification by inverted microscopy. Mean±SE. n=3. *p<0.05
Discussion

Presently, many reports demonstrated that the extracts from genus *Piper* has been exhibited anticancer effects including PR. The effects of PR fruit have been reported in several pharmacological actions such as anticancer effect; however, the PR effects on CCA cells are still unknown. Based on the results obtained, we found that PR exhibited growth inhibition by time- and dose-dependent manner in both of two CCA cells, KKU-100 and KKU-M452, and showed the strong effects in KKU-100 cells (non-migrated cells and slow growth cells). The anticancer effect of PR extract found that the extract arrested the cell cycle distribution at G0/G1 phase in KKU-100 cells and S to G2/M phase in KKU-M452 cells. Moreover, PR induced CCA cells apoptosis, especially in KKU-100 cells along with induction of ROS formation and decreased mitochondrial function. Furthermore, PR extract also inhibited cell migration by dose-dependent manner. PR fruit extract could be useful for anticancer agents in CCA treatment.

CCA is one of an aggressive tumor with very poor prognosis and less effectiveness to chemotherapeutic drugs (Wu and Chu, 2019). The researchers have been tried to explore new effective agents for treating and preventing of CCA (Wu and Chu, 2019). Natural compounds and traditional medicines are one of the major sources of anticancer drug including PR ripe fruit. Presently, we demonstrated the effects of PR extract against CCA proliferation and apoptosis. The IC$_{50}$ values to suppress the CCA cells viability was low concentration in KKU-100 cells more than KKU-M452 cells. At 72 h incubation period, the IC$_{50}$ values were 33.32±1.19 and 39.64±3.10 µg/mL, respectively. Confirmation with previous report from human breast cancer cells, PR extract inhibited cancer cell viability at similar range with CCA cells. PR extract has been reported the 4 active
compounds including piperine, methyl piperate, sylvatine, and piperlonguminine and piperine is widely used to study cytotoxic effects (Amad et al., 2017). PR extract was examined the cytotoxic action on lung cancer cells, SCLC-H22 and NCI-H187, compared with human gingival fibroblast cells, HGF, and the results indicated that lung cancer cells sensitive to PR extract than normal fibroblast cells (Amad et al., 2017). PR extract is less adverse effects on the normal cells.

Lack information of PR crude extract on apoptosis; however, piperine is one of most active compounds from PR extract, caused induction of apoptosis in several mechanism of actions and cancer cells. Piperine induced apoptosis on cervical cancer cells through induction of ROS generation, activation mitochondrial dysfunction, and stimulation of caspase 3 activity (Jafri et al., 2019), like as rectal cancer cells. On the other hand, it induced protein-related apoptosis including BAX, cleaved poly (ADP-ribose) polymerase, cleaved caspase-9, phospho-c-Jun N-terminal kinase and phospho-p38 in melanoma cells (Yoo et al., 2019) and apoptosis is occurred. In this study, we demonstrated that PR crude extract caused activation of apoptosis in CCA cells via ROS production and mitochondrial dysfunction as previous data. Finally, PR extract activated cancer cells apoptosis in CCA cells.

Metastasis is the main step of cancer with very poor prognosis and clinical outcome. This study examined the PR extract on cells migration was explored by wound healing and Matrigel migration assay. The data indicated that PR extract showed the powerful to suppress the cell migration by dose-dependent manner, especially in KKU-100 cells. Matrix metalloproteinases (MMPs) is the main step for cancer metastasis, invasion, and migration (Toth et al., 2012). Interestingly, an overexpression of MMP 2 and MMP 9 levels are found in cancer cells migration (Shi et al., 2012). Like as anticancer effects, lack of information of PR extract on cancer cells migration; however, piperine and its active compounds of PR extract still have been reported antimigratory activity with low doses. Piperine significantly reduced the breast cancer cells migration by suppressing MMP 9 and MMP 13 levels, and it significantly suppressed the lung metastasis in vivo study (Lai et al., 2012). In conclusion, our data showed that PR extract attenuated CCA cell migration, led to reduced tumor progression and migration.

Conclusions

The standard treatment for CCA is still very poor response. In this study indicated that the anticancer effect of PR extract has more potency defeated CCA cells through inducing cells death, stimulating apoptosis, and inhibiting migration. PR shows anticancer effects via induction of cell cycle arrest at G0/G1 phase for KKU-100 cells and S to G2/M phase for KKU-M452 cells. Further, PR extract activated the apoptosis signaling pathway through stimulation of ROS production and reduction of mitochondrial function. Finally, PR extract caused reduction of cancer cells migration. In conclusion, these results demonstrated that PR may serve as a potential antitumor agent by its multifunctional effects in CCA.

Authors’ Contributions

The author read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.
Acknowledgements

This research project was finally supported by Mahasarakham University (MSU) and the research project presentation for Dr. Benjaporn Buranrat was supported by Mahasarakham University Development Fund and Faculty of Medicine. Thanks for Dr. Adrian R. Plant for MSU language editing.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References


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