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ORIGINAL ARTICLE

Anticancer Effects of *Piper nigrum* Extract against Cholangiocarcinoma Cells

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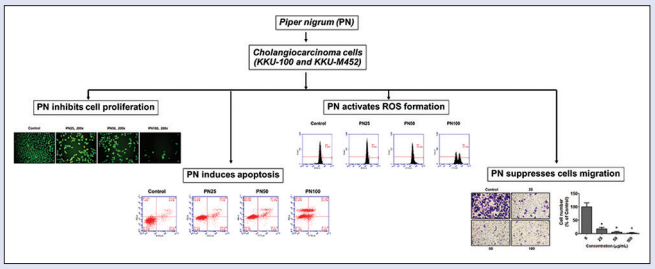
ABSTRACT

Objectives: *Piper nigrum* (PN) is well known for its cytotoxic and pharmacological benefits in several cancer cells. However, there is less documented evidence about its cytotoxic efficacy against cholangiocarcinoma (CCA). The roles of PN extract in two CCA cells, KKU-100, and KKU-M452, were evaluated. **Materials and Methods:** Viability was determined by sulforhodamine B and cell cycle distribution. Apoptotic effects were examined by flow cytometry after staining with Annexin V-FITC and Propidium idodide, JC-1, and Dichlorodihydrofluorescein (DCF-DA) staining. Migration was studied by Wound healing and Matrigel migration assay. **Results:** The results indicated that PN treatment significantly inhibited cells viability and cell replication by dose-and time-dependent both of two CCA cells. Growth was decreased by detecting the cell cycle arrest at G0/G1 phase in KKU-100 cells and S to G2/M phase in KKU-M452 cells. PN extract markedly induced cancer cells apoptosis after treatment for 24 h by loss of mitochondrial membrane potential and increasing of reactive oxygen species production. Furthermore, a significant reduction of migration was observed in both two cells, KKU-100 cells was suppressed the migratory ability more than KKU-M452 cells. **Conclusion:** The data demonstrated that PN extract directly suppresses proliferation and inhibits cells migration in two CCA cells. Finally, PN may be useful for CCA treatment.

Key words: Apoptosis, cell cycle arrest, cholangiocarcinoma, mitochondrila function, *Piper nigrum,* reactive oxygen species

SUMMARY

- *Piper nigrum* extract found potential anticancer and antimigratory activity against cholangiocarcinoma cells.



Abbreviations used: AO/EB: Acridine orange/ethidium bromide; DMEM: Dulbecco's modified eagle medium; PI: Propidium idodide; PN: P*iper nigrum;* ROS: Reactive oxygen species; SRB: Sulforhodamine B.

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INTRODUCTION

Cholangiocarcinoma (CCA) is bile duct cancer that arising from epithelium cells in intrahepatic and extrahepatic organs. It is a rare liver cancer but has been reported a major health problem in the North-East part of Thailand. Currently, CCA has a slightly high incidence and mortality rate in the world.[1] The best effective for CCA treatment is surgical resection in the early stage but not in the late stage, which leads to a good prognosis.[2] At present, only 1 of 4 of CCA patients is suitable for surgical resection, while the most of CCA patients are received chemotherapeutic agents.[3] Unfortunately, chemotherapy is less effective in this tumor through developing with several resistance or tolerance mechanisms.[3] Therefore, it is extremely significant to find a novel management with low side effects and high efficacy for treating CCA.

This circumstance stimulates researchers to discover new effective compounds/agents to defeat cancer. *Piper nigrum* (PN) in Piperaceae family is a herbal medicine and has been reported in several pharmacological activities, including diarrhea, cardiovascular diseases, indigestion, insomnia, cold, fever, and asthma.[4] At present, it has been claimed to have anticancer activity and high efficacy in many cancer cells including breast, colon, and lung cancer.[4] Several active compounds have been obtained from PN such as piperine, pipernonaline, and purpurogallin.[5] Nevertheless, the data have been inadequate study focusing on PN extracts, which displayed several pharmacological actions including antiinflammation, antioxidation, anticancer effects.[4] Therefore, PN effects on anticancer activities were interested.

The data of anticancer activity of PN extract were explored in many cancer cell types. Ethanolic extract of PN activated high levels of ROS formation in intracellular cells, which was the main mechanism to suppress the human breast cancer cells growth by arresting cell cycle distribution in the G1/S phase.[6] Methanolic and dichloromethane crude extracts of PN promoted cancer cell death through activating p53 levels[7] and exhibited less induced normal cells death. Piperine is an active compound of PN and widely studied in many cancer studies; however, the yield of piperine

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only a 2%–4% yield from PN,[8] and it sensitive to heat and easily labile. Previously, PN crude extract with piperine free showed a strong effect on human MCF-7 breast cancer cells with lowering IC_{50} values than piperine. Moreover, PN with piperine free had no acute toxicity during 14 days observation period,[7] and tumor size is significantly decreased. Our previous study found that crude extract of PN caused induction of breast cancer cells death with arresting at G1 phase of cell cycle distribution.[9] The crude extract of PN was more interested.

On the other hand, the PN extract on antimigratory ability of cancer is very interested to stop the cancer cell migrate to contribute the secondary tumor. Our previous study indicated that PN crude extract suppressed the breast cancer cells' migratory activity by inhibiting the matrix metalloproteinases (MMP)-9 protein expression and *MMP-2, MMP-9, ICAMP1,* and *VEGFA* mRNA expression.[9] Moreover, the active compounds of PN, such as piperine suppressed the MMP-2 and MMP-9 activity, and further it induced the tissue inhibitors of metalloproteinase 1/2 levels, leading to inhibited cells migration.[10] Less information of PN crude extract was explored on migration in CCA cells; further PN effects on CCA migration are very interested.

While PN effects have been less information in many cancer cell types, however; its underlying molecular mechanisms of action in CCA cells are still less data.

In this study, we interested in the young fruit of PN effects on two CCA cells, KKU-100 (non-migrated cells), and KKU-M452 (migrated cells), in cell growth, apoptosis, and migration with the mechanism(s) responsible.

MATERIALS AND METHODS

Plant and extraction

The procedure of extraction from young fruit of PN was used as the previous study.[9] The specimen number MSUT_7234 and percentage yield of the extracts are 7.56% per dry weight of PN young fruits.

Cell and cell cultures

Two CCA cells (KKU-100 and KKU-M452) were kindly provided by Liver Fluke and CCA Research Center, Khon Kaen University. The cells were grown Dulbecco's modified eagle medium (DMEM) with 1% antibiotics and 10% fetal bovine serum (FBS) and plated in 5% CO_2 incubator at 37°C.

Sulforhodamine B method

Two CCA cells were plated onto 96-well cultured plate (1×10^4 cells/well) for overnight. After that, the cell lines were exposed to PN extract (5, 10, 25, 50, 100, and 250 μg/mL) and then cultured for 24–72 h. Cell viability was measured by Sulforhodamine B (SRB) method, the cells were exposed to 10% trichloroacetic acid, stained with 0.4% SRB, solubilized the with 200 μl of 10 mM Tris base, read the optical density at 540 nm.

Colony formation method

KKU-M452 was not formed the colony formation, and this study we studied only KKU-100 cells. Cells were cultured onto 6-well plate (500 cells/well) for overnight. After that, the cell lines were exposed with PN extract (5, 10, 25, 50, 100, and 250 μg/mL) for 24 h, then cultured for further 15 days. After the end of incubation period, the cells exposed to 100% cold-methanol and stained with 0.5% crystal violet. Colonies were captured, counted, and compared to control group.

Acridine orange and ethidium bromide staining method

Two CCA cells were cultured onto 96-well cultured plate (1×10^4 cells/well) for overnight. After that, the cell lines were exposed to PN extract (25, 50, and 100 μg/mL) for 24 h incubation periods and then the cells were stained with AO/EB solution (final concentration of each dye 1 μg/ml). The cell images were photographed using an inverted fluorescence microscope (CKX53, Olympus).

Cell cycle distribution method

Two CCA cells were plated onto 6-well cultured plate (2.5×10^5 cells/well) for overnight. After that, the cell lines were exposed to PN extract (25, 50, and 100 μg/mL) for 24 h, collected the cells, washed, and incubated with 70% ethanol for overnight at −20°C. Next, the cells were washed with PBS buffer for 3 times, stained with propidium iodide (PI, Cat No. 550825, BD Biosciences, CA, USA) for 30 min at room temperature. Measurement of cell cycle distribution using flow cytometry (BD Biosciences, San Jose, CA, USA) with BD Accuri C6 Plus software and fluorescent signals were displayed as histograms.

Apoptosis by flow cytometric method

Two CCA cells were plated onto 6-well cultured plate (2.5×10^5 cells/well) for overnight. After that, the cell lines were exposed to PN extract (25, 50, and 100 μg/mL) for 24 h, collected the cells, washed, stained with 5 μL Annexin V-FITC for 15 min in dark and added 1.5 μL propidium iodide before measuring apoptosis (Cat No. 558547, BD Biosciences, CA, USA). Live, apoptotic and necrotic cells were measured by flow cytometry (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Reactive oxygen species by flow cytometric method

The reactive oxygen species (ROS) generation assay was performed using DCF-DA fluorescent dye (Cat. no. D6883, Sigma Merck KGaA, Darmstadt, Germany). Two CCA cells were plated onto 6-well plate (2.5×10^5 cells/well) for overnight. After that, the cell lines were exposed to PN extract (25, 50, and 100 μg/mL) for 24 h, collected the cells, washed, and stained with 25 μM DFC-DA for 30 min in dark at 37°C. Subsequently, ROS production was detected by flow cytometry (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Mitochondrial function by flow cytometric method

Two CCA cells were plated onto 6-well cultured plate (2.5×10^5 cells/well) for overnight. After that, the cell lines were exposed to PN extract (25, 50, 100 μg/mL) for 24 h, collected the cells, washed, and stained with JC-1 dye (Cat. No. 1-800-346-9897, Cayman Chemical, Michigan, USA) for 30 min in dark at 37°C. Cells were then analyzed mitochondrial function ($\Delta\Psi m$) with flow cytometry (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Wound healing method

To determine the ability of PN extract to inhibit the migration of cancer cells, scratch assay was used. Two CCA cells were plated onto 24-well cultured plate (2.5×10^5 cells/well) for overnight. After that, the cell lines were made a wound by 0.2 mL pipette tips, washed with PBS buffer, and exposed to PN extract (25, 50, 100 μg/mL) for 48 h. After that, the wound at the time interval of 0 h and 48 h and were compared with the control cells. Images were taken using the Olympus CKX53 inverted microscope (×4 magnifications). The denuded area was compared between treatment and control groups.

Matrigel migration method

To determine the ability of PN extract to inhibit the migration of CCA cells, matrigel migration assay was performed. The cell suspension in 200 µL DMEM-free FBS medium with PN extract (25, 50, 100 µg/mL) was added to the insert well of upper chamber. The lower chamber was added the complete DMEM medium and incubated for 24 h in this condition. At the end of incubation period, cells at the bottom of insert well were stained with 0.5% crystal violet for 1 h and washed several times to wash out excess dye. The migrated cells were photographed using a Olympus CKX53 phase contrast microscope.

Statistical analysis

All data of this study were analyzed using student *t*-test between the control and treatment groups in independent three experiments using GraphPad Prism analysis software (version 5.0, GraphPad Software, San Diego, California, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Piper nigrum extract inhibits cell viability and colony formation

To determine the PN effects on cell growth was analyzed using various concentrations, PN in two types of CCA cells, KKU-100 and KKU-M452, to measure the cytotoxic effects by SRB method. From data showed that PN extract decreased the CCA cells growth of KKU-100 and KKU-M452 cells, and PN effects showed the greatest action at the dose of 250 µg/mL [Figure 1a and b]. Following 72 h exposure to PN extract revealed the low IC_{50} values, KKU-100 cells were 12.76 ± 2.33 µg/mL and KKU-M452 cells were 38.32 µg/mL, respectively. KKU-100 cells were sensitive to PN extract more than KKU-452 cells in SRB method.

To confirm the data of SRB assay, a colony formation assay was examined to measure the cell replication of CCA cells. This experiment we chose only KKU-100 cells because KKU-M452 cells did not form the colonies. Data indicated that long-term incubation with PN extract caused reduction in the colony formation of KKU-100 cells [Figure 1c]. Especially, PN extract promoted the suppression on the number of colonies and, with no colony formation remarked at the high concentration of PN treatment (250 µg/mL). The result indicated that PN extract suppressed the cell growth and colony formation in CCA cells.

Piper nigrum extract reduces the cell number and arrests cell cycle distribution

To determine the PN effects on CCA cell number was examined by AO/EB staining method. The data indicated that cell number was reduced after PN treatment for 24 h by dose-dependent manner. The results showed a large number of KKU-100 and KKU-M452 cells in the untreated control group were stained green color and increased in drug concentration, the number of normal cells was gradually reduced. For KKU-100 cells, number cells, were decreased with 43.06% ± 12.58%, 37.78% ± 6.51%, 12.50% ± 5.12% for 25, 50, and 100 µg/mL, for KKU-M452 with 50.61% ± 8.51%, 43.32% ± 5.57%, and 25.91% ± 10.01%, respectively. For apoptotic induction, PN extract caused

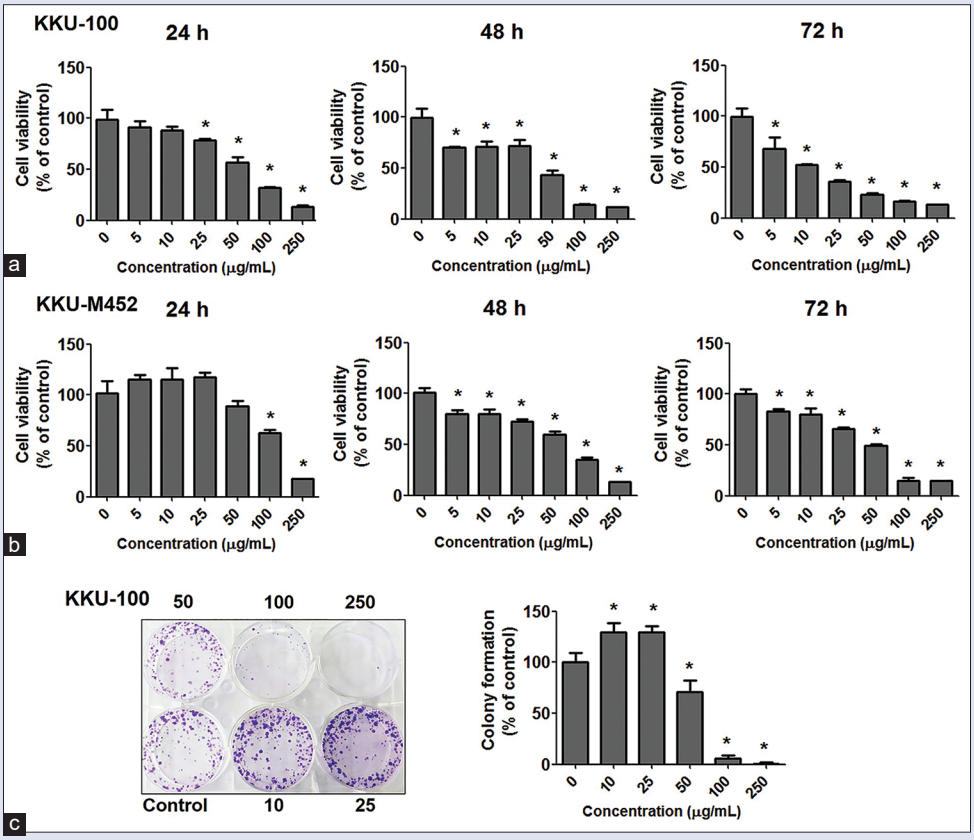

Figure 1: PN extract on colony formation and cells proliferation. (a-b) Cholangiocarcinoma cells were incubated with PN extract (0–250 µg/mL) for 24–72 h, and measured the cells viability by sulforhodamine B method. (c) KKU-100 cells were incubated with PN extract (0–250 µg/mL) for 24 h, cultured further for 15 days and counted the colonies. *$P < 0.05$ versus control group

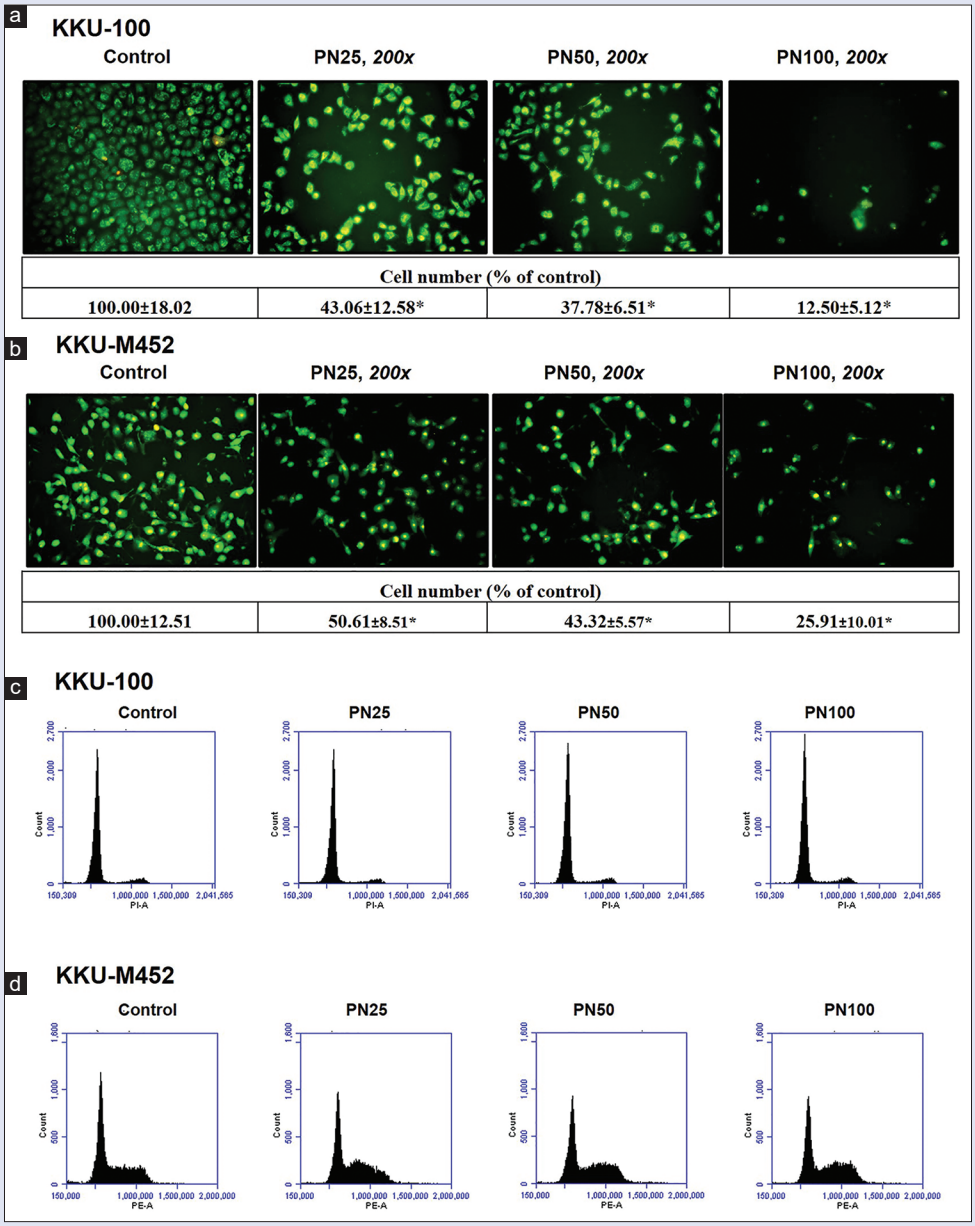

Figure 2: *Piper nigrum* extract on cell number and cell cycle distribution. (a and b) cholangiocarcinoma cells were incubated with *Piper nigrum* extract (0–100 µg/mL) for 24 h, stained with AO/EB (1 µg/mL of each dye) and photographed by fluorescent inverted microscopy. (c and d) cholangiocarcinoma cells were incubated with *Piper nigrum* extract (0–100 µg/mL) for 24 h and measured the cell cycle distribution by flow cytometry. *$P < 0.05$ versus control group

induction of apoptotic both in two cells and found the spindle shaped with a whole nucleus and clear demarcation [Figure 2a and b].

To determine the PN effects on cell cycle arrest was determined by flow cytometric method. As shown in Figure 2c and d, following treatment with PN at 0, 25, 50, and 100 µg/mL concentrations, significant arrested at G0/G1 phase was observed in KKU-100 cells, especially at 100 µg/mL PN. While the percentage of S and a G2-M phase was increased in KKU-M452 at the dose of when compared with untreated group.

Piper nigrum extract induces apoptosis

To examine the percentage of apoptotic rates stimulated by PN extract, flow cytometry was used. Data demonstrated that PN extract decreased the cells number and induced late apoptosis in both KKU-100 and KKU-M452 cells [Figure 3a and b] by dose-dependent manner. Treatment with PN extract led to a decrease the cell number from 89.6% ± 2.6%, 71.2% ± 2.1%, 47.5% ± 4.2%, and 23.6% ± 2.1% for 0, 25, 50, and 100 µg/mL in KKU-100 cells. The cells number in KKU-M452 was showed likely with KKU-100 by decreasing the cells number from 84.7% ± 3.3%, 84.9% ± 4.9%, 62.4% ± 4.8%, and 26.4% ± 3.9%, respectively. Moreover, PN extract led to an increase the percentage of the late apoptotic rate by dose-dependent manner, KKU-100 with 4.6% ± 0.6%, 7.9% ± 1.5%, 12.8% ± 2.1%, and 22.9% ± 2.9% and KKU-M452 with 6.1% ± 1.9%, 6.8% ± 0.5%, 16.8% ± 3.1%, and 21.1% ± 2.9% for 0, 25, 50, and 100 µg/mL, respectively. Thus, PN extract induced apoptosis in CCA cells.

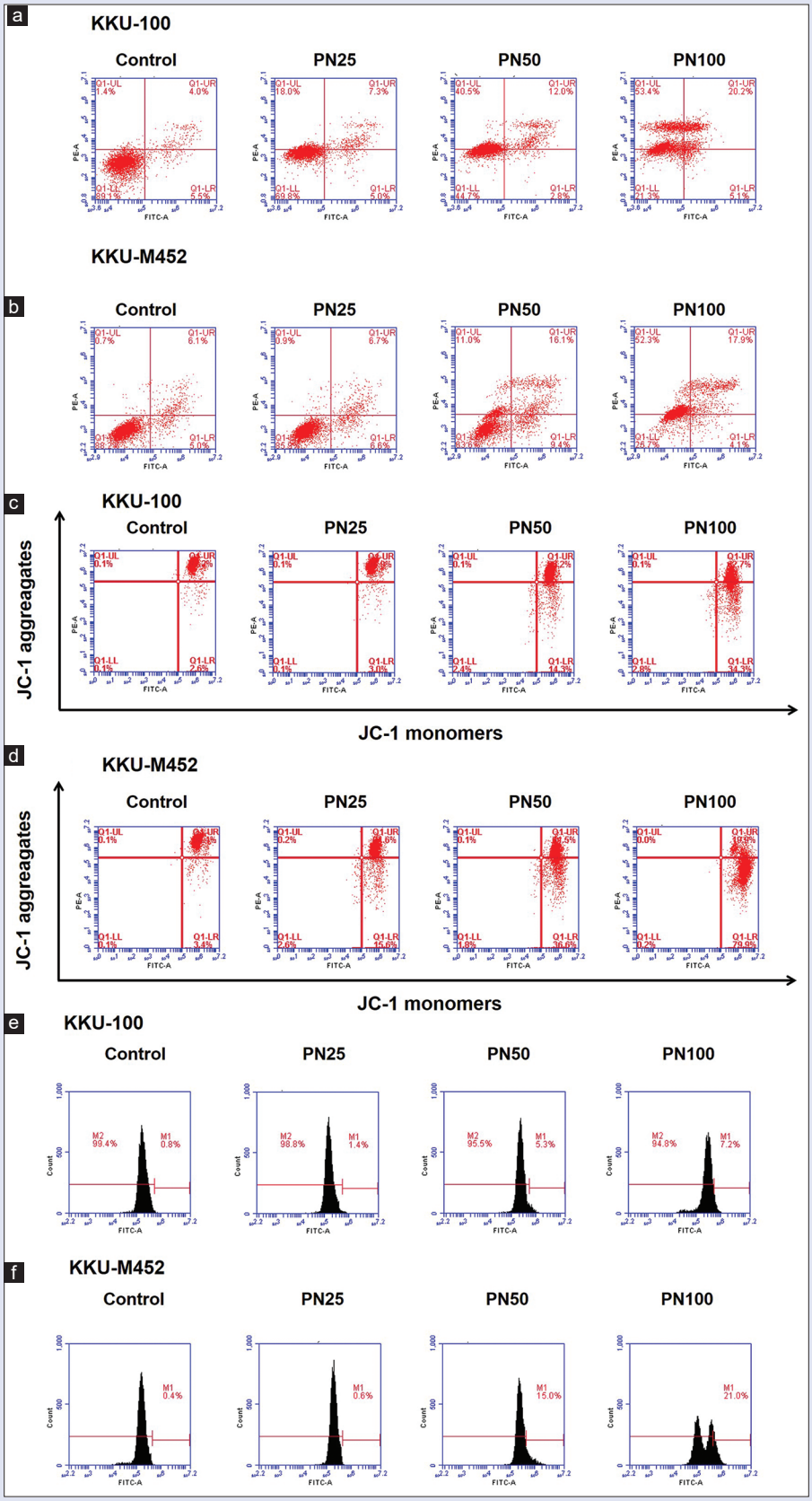

Figure 3: *Piper nigrum* extract on cell apoptosis, mitochondrial function, and reactive oxygen species formation. (a and b) cholangiocarcinoma cells were plated, treated with *Piper nigrum* extract (0–100 μg/mL) for 24 h, then stained with propidium idodide and Annexin V-FITC and measured the apoptosis by flow cytometry. (c and d) cholangiocarcinoma cells were plated, treated with *Piper nigrum* extract (0–100 μg/mL) for 24 h, stained with JC-1 fluorescent dye and measured the mitochondrial membrane potential by flow cytometry. (e and f) cholangiocarcinoma cells were plated, treated with *Piper nigrum* extract (0-100 μg/mL) for 24 h, stained with DCF-DA fluorescent dye and measured the Reactive oxygen species formation by flow cytometry

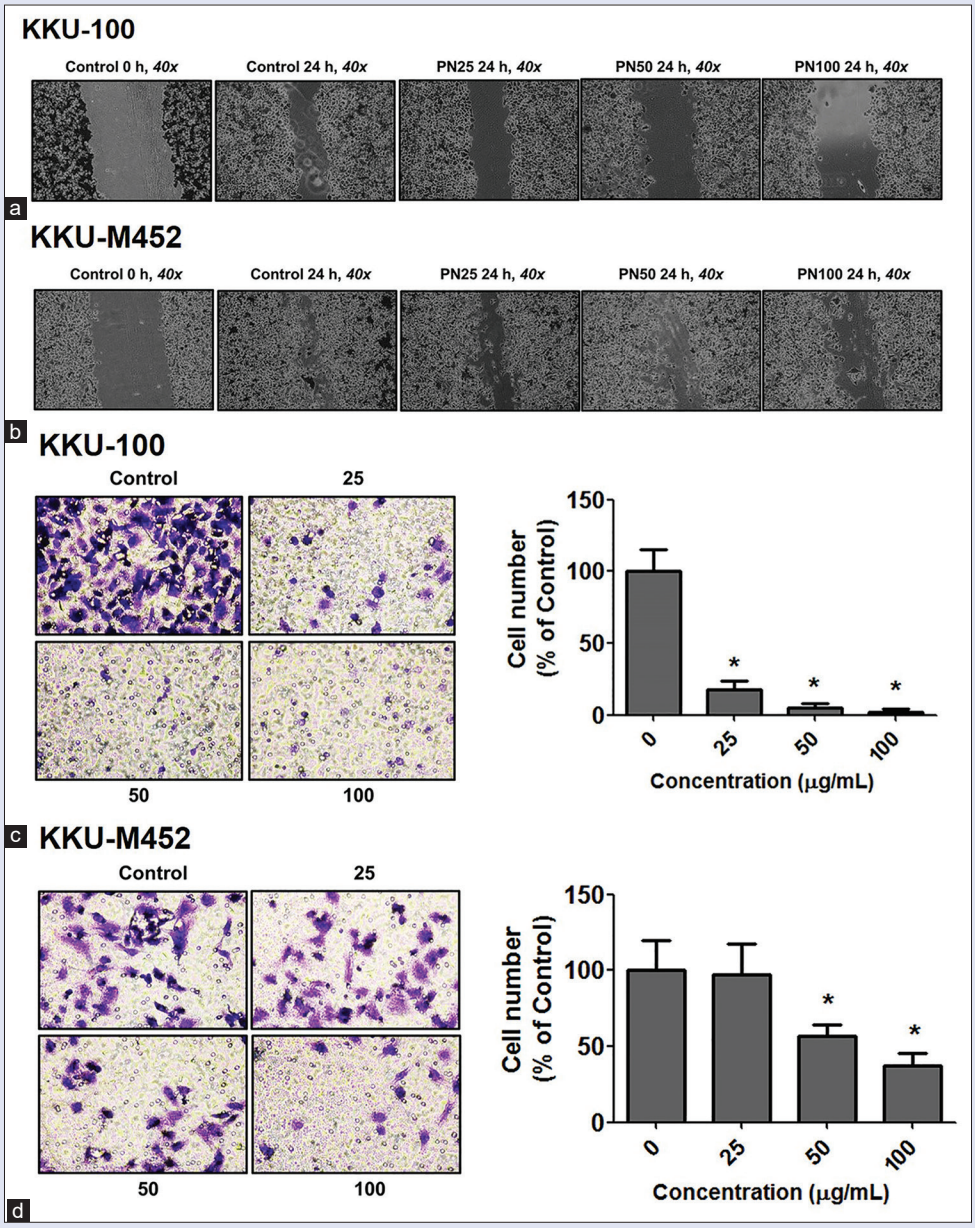

Figure 4: PN extract on cell migration. (a and b) cholangiocarcinoma cells were plated, made a wound by 0.2 mL tips, treated with PN extract (0–100 μg/mL) for 24 h, then stained with 0.5% crystal violet and captured by inverted microscopy (×4 magnification). (c and d) cholangiocarcinoma cells were plated on the upper chamber of insert well and treated with PN extract (0–100 μg/mL) for 24 h. The cells in the lower chamber were stained with 0.5% crystal violet, captured by inverted microscopy (×20 magnification), and then counted. *P < 0.05 versus control group

Piper nigrum extract decreases mitochondrial function and increases reactive oxygen species formation

To determine the mechanism of action of PN extract on cancer cells apoptosis was related with mitochondrial function, ΔΨm and the formation of ROS levels were explored. The data from flow cytometry after staining Annexin V-FITC and PI found that the cell number of late apoptotic cells in the PN extract was significantly increased when compared with untreated control groups [Figure 3a and b]. PN extract caused induced the loss of mitochondrial function when compared with control groups was observed in both KKU-100 and KKU-M452 cells [Figure 3c and d]. The loss of ΔΨm was observed in the reduction of JC-1 aggregates (healthy-cells) and induction of JC-1 monomers (unhealthy-cells), JC-1 aggregates was decreased from 95.2% ± 2.1%, 95.3% ± 1.4%, 82.2% ± 6.6%, 58.5% ± 4.1% for 0, 25, 50, and 100 μg/mL in KKU-100 cells and 96.3% ± 0.9%, 81.8% ± 2.4%, 60.1% ± 3.9%, and 12.7% ± 5.6% in KKU-M452 cells, respectively. Further, the JC-1 monomers were significantly induced in PN treatment from 2.0% ± 0.6%, 3.6% ± 0.5%, 12.8% ± 4.2%, and 38.6% ± 0.6% in KKU-100 cells and 3.4% ± 1.1%, 18.3% ± 2.4%, 37.6% ± 2.3%, and 81.9% ± 3.2% in KKU-M452, respectively.

ROS data indicated that the stimulation of intracellular ROS formation in the CCA cells was markedly induced by PN extract by dose-dependent manner at 24 h incubation peroid [Figure 3e and f]. The induction of ROS formation in the two CCA cells was significantly at the dose of 50 and

100 μg/mL PN extract. By the induction of PN concentrations, ROS level was increased from 0.8% ± 0.3%, 1.7% ± 0.3%, 5.9% ± 1.2%, and 8.2% ± 0.9% for 0, 25, 50, and 100 μg/mL in KKU-100 cells and 0.4% ± 0.1%, 0.8% ± 0.1%, 16.3% ± 3.2, and 22.0% ± 2.1% in KKU-M452 cells, respectively. The data demonstrated that mitochondria dysfunction and ROS formation may serve an important role in PN-induced apoptosis in CCA cells.

Piper nigrum extract suppresses cell migration

Based on the results obtained, PN extract showed a reduction of cell growth and stimulation of apoptosis; further the PN effects on attenuation of migration were more interested. Wound healing method demonstrated that PN extract significant decreased the CCA cells migration by dose-dependent manner, especially at 50 and 100 μg/mL in KKU-100 cells and 100 μg/mL in KKU-M452 cells [Figure 4a and b].

Next, the data from Transwell migration assay were indicated that the numbers of CCA cells after treatment with PN extract showed the reduction of migrated cells on the lower surfaces of the Transwell membranes at 25, 50, and 100 μg/mL in KKU-100 cells and 50 and 100 μg/mL in KKU-M452 cells [Figure 4c and d]. PN extract significantly inhibited CCA cell migratory ability *in vitro*.

DISCUSSION

PN is one of the most popular traditional medicines that have been reported many bioactive compounds with pharmacological actions. Interestingly, it has been reported to defeat many cancer cells types and against cancer in animal models;[4] however, the PN effects on CCA are still unclear. Thereby, the effects of ethanolic PN extract on two CCA cells (KKU-100, non-migrated and KKU-M452, migrated cells) were interested to compare the action and mechanism. Our data indicated that PN extract inhibited CCA cells growth by dose- and time-dependent manner with low IC_{50} values and also suppressed colony formation. Moreover, it suppressed the cells number through stopping the cell cycle at G0/G1 phase in KKU-100 cells and S to G2/M phase in KKU-M452 cells. Next, PN extract significantly induced apoptosis by reducing mitochondrial function and increasing ROS formation levels. Finally, PN extract significantly attenuated migration in both two cancer cells, especially KKU-100 cells. Hence, PN may be a gorgeous plant medicine for prevention and treatment for CCA.

The present study, we try to use the young fruit of PN as we eat in daily life to demonstrate as chemoprevention or cancer treatment in CCA. PN inhibited cell growth in many cancer cell types, such as colon, lung, and breast.[4] PN extract suppressed breast cancer cell growth through inducing intracellular ROS overproduction,[11] which induced oxidative stress damage in DNA and then stimulated cell cycle arrest and caused cancer cells apoptosis. As our results indicated that PN extract induced CCA cells death by arresting cell cycle distribution. The different effects of PN extract on two CCA were indicated in the different of cell distribution, KKU-100 was stopped at G0/G1 phase, and KKU-M452 was arrested at S to G2/M phase. It suppressed the cancer cells growth with increasing protein-related cell death, Bax and p53 expression, and reducing protein-related cell growth, Bcl-xL, and cyclin A expression.[11] As our previous study showed that PN crude extract had high levels of piperine, and this active compound may cause induction of MCF-7 cells death.[9] Piperine is one of active compound in PN extract that reported in inhibition of cancer cells growth and induction of apoptosis. The active compound, piperine, induced cancer cells death was mediated by induction of ROS formation in intracellular cells.[12] Moreover, piperine treatment resulted inhibition of the proliferation by arresting cell cycle distribution at G0/G1 along with downregulating cyclin D1 and cyclin A and p21Cip1 and p27Kip1.[13] Both of PN crude extract and active compound, piperine, had high potency to against cancer cells by interfering cell cycle, inducing protein-related cell death, and inhibiting protein-related cell growth.

From our results obtained, apoptosis is one of programmed cell death that plays the major role in anticancer effects natural products. Apoptosis is a process that related with multiple pathway including mitochondrial dysfunction, ROS activation, caspase stimulation, cleaved-PARP elevation, and chromatin condensation.[14,15] This study, our results showed that the apoptotic properties of PN extract significantly increased the late apoptotic and necrotic levels in both CCA cells. Further, the mechanism of apoptosis was observed by increasing the mitochondrial dysfunction, including increased of permeability transition pore opening, induced mitochondrial dysfunction, and activated caspases activity.[16] This study found that treatment of CCA cells with PN extract showed in a decrease of $\Delta\Psi m$ in a dose-dependent manner at 24 h incubation period. Likely, Mdivi-1 sensitized CCA cells to cisplatin effects in association with increased the mitochondrial dysfunction.[17] Moreover, the excessive generation of ROS plays an import role in oxidative stress, DNA damage, and cell death through apoptosis.[18] Our result demonstrated that PN extract significantly induced ROS formation after 24 h incubation when compared with control group. Taken together, these data certified that PN extract induced apoptosis by activating ROS formation and suppressing mitochondrial function in CCA cells.

As a specific cell migration pathway, the metastasis of cancer cells is a basic mechanism of cancer cells to migrate to distant organs and contribute to the second tumor.[19] Correctly, a herbal medicine with the ability to prevent metastasis could be a potential candidate for preventing and treating cancer. In this study indicated that PN inhibited CCA cell migration was detected by wound healing and matrigel migration assay. Previously, the crude PN extract caused reduction of migration by decreasing MMP-9 protein expression correlate with inhibiting *MMP-2, MMP-9, VEGFA*, and *ICAMP1* mRNA expression.[9] Piperine is an active compound of PN extract and has more efficacies on many cancer cell types. Piperine significantly decreased the MMP-9 and MMP-13 expression and led to inhibit breast cell migration.[20] On the other hand, piperlongumine as one of active compounds of PN indicated that it markedly suppressed the human glioma cells migration; however, it did affect in normal astrocytes with wound healing method.[21] As the results obtained, PN extract and active compound had the potent activity to inhibit cancer cells migration including CCA cells.

CONCLUSION

Based from the results obtained, PN treatment significantly suppresses cell proliferation, induces apoptosis, and inhibits CCA cells migration. PN extract significantly decreased cell growth by arresting cell cycle distribution at G0/G1 phase in KKU-100 cells and S to G2/M phase in KKU-M452 cells. Moreover, the results demonstrated that PN induced the apoptotic properties of CCA cells by reducing the mitochondrial function and activating ROS formation in intracellular cells. Further, PN treatment had more efficacies on antimigratory capability in CCA cells. The present data suggest that PN could be applied in the treatment of CCA cells.

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Conflicts of interest

There are no conflicts of interest.

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