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ORIGINAL ARTICLE

Anticancer Potential of *Piper retrofractum* on Breast Cancer MCF-7 Cells Via Induction of Cell Death and Reduction of Cell Migration

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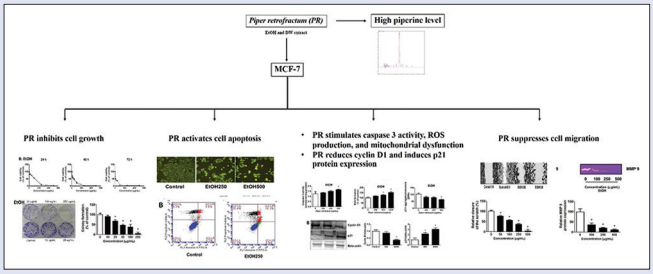
ABSTRACT

Background: *Piper retrofractum* (PR) is traditional medicine in the South-East country. **Objectives:** This study examined the PR effects on anticancer and anti-migratory activity in human breast cancer cells. **Materials and Methods:** Determination of cancer cell death by the sulforhodamine B, colony formation, flow cytometry methods. Mechanism-induced cells death was assessed by intracellular reactive oxygen species generation, caspase 3 activity, mitochondrial function, gene expression, and protein expression. **Results:** The ethanolic extract of PR had higher effects against MCF-7 cells than DW extract expressing the high levels of piperine. The extracts stimulated cell death in MCF-7 cells and induced apoptosis with increasing caspase 3 activity, ROS generation, and decreasing mitochondria function. Moreover, the extract also inhibited gene-related cell proliferation, *cdk2, cdk4,* and *ckd6*, and altered protein-related cell growth, cyclin D1 reduction and p21 induction. Finally, the extract caused inhibition of cells migration by reducing MMP 9 levels. The extracts of PR stimulated MCF-7 cells death in association with increasing apoptosis and inhibiting cancer cells migration. The induction of cancer cell death may be through modulating mitochondrial function. **Conclusion:** These extracts may be a novel strategy to improve the efficacy of chemotherapy to treat MCF-7.

Key words: Apoptosis, breast cancer cells, cell death, cell migration, *Piper retrofractum* (PR)

SUMMARY

- PR extract found potential anti-cancer and anti-migratory activity against MCF-7 cells human breast cancer.



Abbreviations used: AMC: Ac-DEVD-7-amino-4-methylcoumarin; AO/EB: acridine orange/ethidium bromide; CDK: Cell cycle dependent kinase; DMEM: Dulbecco's modified eagle medium; PI: Propidium idodide; PR: *Piper retrofractum*; ROS: Reactive oxygen species; SRB: Sulforhodamine B.

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INTRODUCTION

Presently, herbal remedies or plant medicine have become popular for treating several diseases, due in part to the lower risk of side effects. For several decades, anticancer drug discovery has focused on natural compounds or derivatives.[1,2] *Piper retrofractum* (PR) is one of the most interested in the anticancer effect from the Piperaceae family. PR has been used as herbal medicine in Asia and South-East Asia including Thailand,[3] for long time. Several parts of PR are reported for treating/preventing many diseases such as menstrual pain, inflammation, tuberculosis, depression, respiratory tract infections, pain in the Gastrointestinal tract, arthritic conditions, hepatotoxicity, and especially cancer.[4,5]

The ripe fruit of PR used traditionally in herbal remedies. It has been indicated that the active compounds extracted from PR are piperine, piperlongumine, piperlonguminine, pipermonaline, piperundecalidine, diaeudesmin sylvatin, and sesamin.[4] Most abundant in ripe fruit is piperine, the alkaloid which contributes to their pungency. For cancer studies, PR and piperine have been reported to inhibit cancer cells proliferation such as cervical, prostate, colon ovarian, lung, and breast cancer.[6] Moreover, they inhibit cancer cells proliferation and apoptosis through arresting at G0/G1 phase in the cell cycle with down-regulating cell cycle-dependent kinase (CDK) inhibitors, p21 and p27 expression as well as up-regulating caspase 3 enzyme level and increasing the cleavage of PARP.[7] In osteosarcoma and melanoma, piperine inhibiting at the G2/M phase and G1 phase with inhibiting cyclin B1 levels.[8] Many evidences suggested that PR or piperine caused induction the several cancer cells' death.

For more information, PR extract and piperine caused induction breast cancer cells apoptosis by unbalancing of reactive oxygen species (ROS) homeostasis,[9] as well as melanoma.[10] Furthermore, piperine induces rectal adenocarcinoma and oral squamous carcinoma cell death via modulating the mitochondrial pathway.[11,12] The induction of caspases cascade by piperine is detected after the release of cytochrome c from

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mitochondria to cytosol, then activating caspase 3 and 9, the cleavage of PARP and inactivating of MAPKs pathways such as JNK and p38/MAPK.[13] On the other hand, piperine has been reported to inhibit cancer cells migration by down-regulating of matrix metalloproteinase 9 (MMP 9) expression.[14] However, the PR extract of ripe fruit on the human breast cancer cells is still less information.

Based on the previous studies, we hypothesized that the crude extract from the rip fruit of PR showed anticancer effects by reducing cell growth, increasing apoptosis, and inhibiting metastasis. In this present study, the experiment used the human breast cancer cells MCF-7 to examine whether both PR extracts by DW and EtOH against breast cancer MCF-7 cell proliferation, activate cell apoptosis, and inhibit migration. The measurement of piperine in both parts of PR extract is explored.

MATERIALS AND METHODS

Reagents

Sulforhodamine B, RIPA lysis, crystal violets were obtained from Sigma Chemical (St. Louis, MO). Antibodies for primary against cyclin D1, p21, beta-actin, and secondary were purchased from Cell Signaling Technology (Dancer, MA, and USA). Reagents for cell culture were from Gibco-BRL Life Technologies (Grand Island, New York).

Plant extraction

The ripe fruit of PR was collected from Chiang Mai Province, Thailand, in 2019 and identified by Pornpimon Wongsuwan, Assistant Professor, Faculty of Medicine, Mahasarakham University. The herbarium specimen number of this material has been deposited in the Faculty of Sciences, Mahasarakham University (No. MSUT_7235). The PR extract was extracted by distilled water (DW) and ethanol (EtOH) and made as our previous work described.[15] Briefly, dried ripe fruits (250 g) were boiled with water or macerated with 95% ethanol (7 days), filtered, evaporated, and lyophilized to obtain the dry extract. The % yield was 9.24% and 5.26% for DW and EtOH extract and the extracts were kept at -20°C until use.

HPLC method

To examine the piperine contents of both PR extracts by using the HPLC method as described previously.[15] Briefly, PR extract was loaded into C18 analytical column by using the mobile phase composed of acetonitrile: DW: Acetic acid (60.0:39.5:0.5), the injection was performed using a 20 µL loop at 340 nm.

Cell culture and cell proliferation method

MCF-7 cells, human breast cancer cell line, was cultured in Dulbecco's modified eagle medium (DMEM) added with sodium bicarbonate ($NaHCO_3$), penicillin (100 U/mL), streptomycin (100 µg/mL), and fetal bovine serum (10%) and incubated under 5% CO_2 in air at 37°C. Cancer cells were subcultured every 2-3 days after confluent using trypsin-EDTA (0.25%).

The effects of PR extract on the viability of the MCF-7 cells was measured by the SRB method.[15] Cells were plated on culture plates (1×10^4 cells/well) for 24 hr and then cells were exposed to various concentrations of PR extract both of DW and EtOH (0-500 µg/mL) for 0-72 hr. Afterward, the cancer cells were fixed, stained with 0.4% SRB for 30 min. The cells were solubilized with 10 mM Tris base solution. After that, the optical density (O.D.) was examined by spectrophotometer (540 nm).

Colony formation method

The effects of PR extracts on colony formation were measured by the colony formation method.[15] The cells were plated about 500 cells into 6-well plates overnight and the next day cells were added to the new DMEM medium containing various concentrations of PR extracts (0-250 µ g/mL) for 24 hr. Next, cancer cells were exposed to the new DMEM medium and grown for 14 days at 37°C and 5% CO_2. Afterward, the cells were then fixed, stained with 0.5% crystal violet, and counted the colony formation.

Wound healing method

The effects of PR extract on the cell migration were measured by the wound healing method.[15] The cells were plated on a 24-well culture plate (2.5×10^5 cells/well) overnight and the next day cells were scratched to make a wound by using a pipette tip (0.2 mL). Afterwards, the cells were washed with PBS buffer to remove any detached cells, the wound was photographed as a baseline for 0 hr. The cells were exposed to various doses of PR extract and a series of images of the scratched wound were taken 48 hr after treatment. The distance of the wound was calculated by dividing the area by the length of the scratch.

Gelatin zymography method

The effects of PR extract on the MMP 9 expression in the culture medium were measured by the gelatin zymography method.[15] The cells were plated on a 24-well culture plate (2.5×10^5 cells/well) overnight, next day cancer cells were exposed to PR extract for 48 hr, collected the DMEM medium, centrifuged, and measured the protein concentration. The conditioned DMEM medium (20 µg) was mixed with a 2x non-reducing sample buffer and then subjected to electrophoresis on 10% polyacrylamide gels containing gelatin (1 mg/ml). Afterward, the gel was washed third time with 2.5% Triton X-100 and then exposed to developing buffer overnight at 37°C. After that, the gel was stained with 0.25% Coomassie Blue R-250 and de-stained in the de-staining buffer. The band was detected as clear bands against the blue background of Coomassie BlueR-250 and the band intensity was determined.

Acridine orange/ethidium bromide (AO/EB) method

The effects of PR extract on apoptosis were measured by AO/EB method.[16] The cells were plated on a 96-well culture plate (1×10^4 cells/well) for 24 hr, then cells were exposed to PR extract for 24 hr, washed, added to the AO/EB solution (1 µg/mL each) for 15 min at room temperature. Thereafter, cells apoptosis was determined and captured by inverted microscopy (*10x* magnification).

Apoptotic method by flow cytometry method

The effects of PR extract on apoptosis were confirmed by the flow cytometry method.[17] The cells were plated on a 6-well culture plate (2.5×10^5 cells/well) for 24 hr, then cancer cells were exposed to PR extract for 24 hr, washed with cold PBS buffer for three times, stained with 5 µL propidium iodide (PI) and 5 µL Annexin V FITC for 15 min on ice, and then resuspended in assay buffer, as described by the manufacturer. Cells were analyzed using a BD Accuri™ C6 Plus flow cytometer with BD Accuri™ C6 Plus software FCS 3.1 (Becton Dickinson, San Jose, CA).

Caspase 3 activity method

The effects of PR extract on caspase 3 activity were examined by the kits.[15] Cells were plated on a 6-well culture plate (2.5×10^5 cells/well)

overnight, next day cells were exposed to PR extract for 24 hr, collected the cells pellets, lysed by RIPA buffer, and measured the protein concentration. Measurement of caspase 3 activity was performed by using a substrate, Ac-DEVD-7-amino-4-methylcoumarin (AMC), and AMC as a standard. The fluorescent signals were set to 360 (excitation wavelengths) and 460 nm (emission wavelengths), respectively.

Reactive oxygen species formation method

The effects of PR extract on ROS formation were measured by a DHE-fluorescent probe.[15] The cells were plated on a white 96-well culture plate (1 x 10^4 cells/well) for 24 hr, the cells were exposed to PR extract plus DHE probe for 90 min, and then measured the fluorescent signal using a fluorescent plate reader and the wavelengths were set to 518 (excitation) and 605 (emission) nm, respectively.

Mitochondrial membrane potential method

The effects of PR extract on mitochondrial functions were examined by JC-1 fluorescent probe.[18] Cells were plated on a white 96-well culture plate (1 x 10^4 cells/well) for 24 hr, the cells were exposed to PR extract plus DHE probe for 90 min, and then measured the fluorescent signal using a fluorescent plate reader and the wavelengths were set to 485 (excitation) and 535 (emission) nm, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) method

The effects of PR extract on gene expression were examined by RT-PCR.[18] Cells were plated on a white 6-well culture plate (2.5 x 10^5 cells/well) for 24 hr, the cells were exposed to PR extract for 24 hr and then extracted the total RNA using trizol® solution (Invitrogen, Grand Island, NY). The total RNA (1 µg) was reversed to single-stranded cDNA using iScript reverse transcriptase (Bio-Rad, Hercules, CA). The primers for PCR were shown as in Table 1.

The RT-PCR was carried out using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Inc., CA, USA), and thermal cycling was performed using CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, Inc., CA, USA) under the following conditions: denaturation at 95°C for 3 min and amplification by cycling 40 times at 95°C for 15 s and 60°C for 31 s.

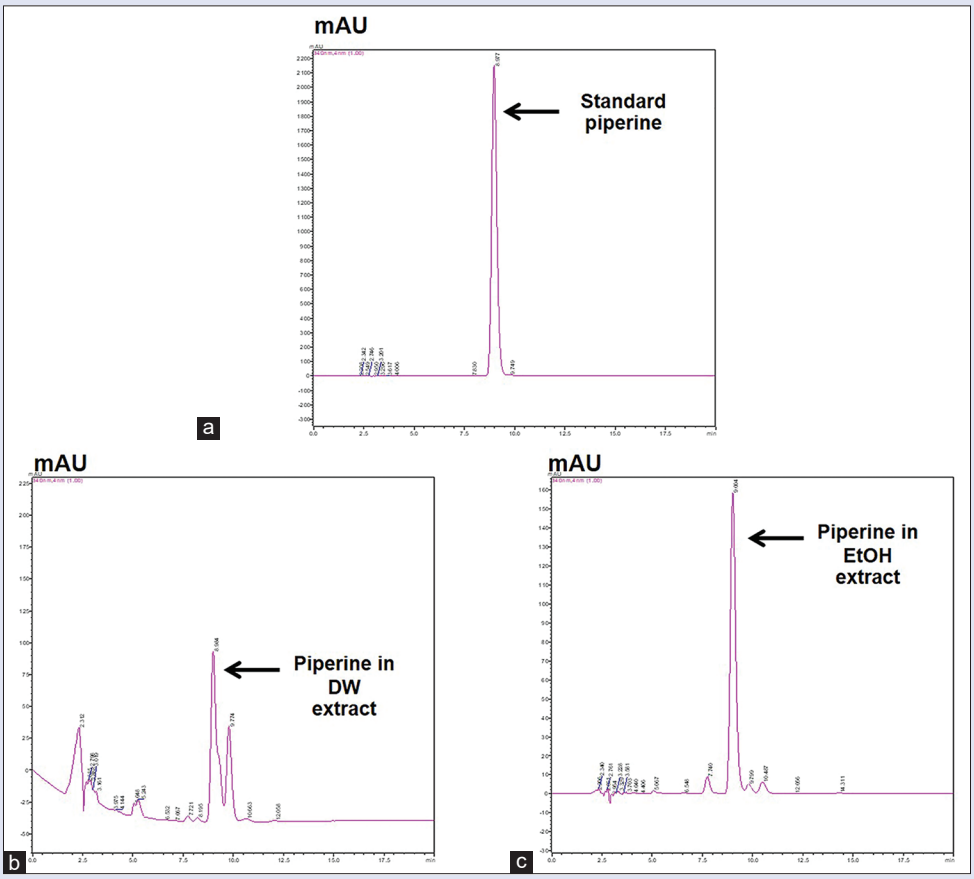

Figure 1: Determination of piperine in PR extracts by HPLC method

Table 1: Primer sequences for targeted genes

Gene symbols	Primer sequences	
	Forward	Reverse
Cdk2	5′-CCA-GGA-GTT-ACT-TCT-ATG-CCT-GA-3′	5′-TTC-ATC-CAG-GGG-AGG-TAC-AAC-3′
Cdk4	5′-ATG-GCT-ACC-TCT-CGA-TAT-GAG-C-3′	5′-CAT-TGG-GGA-CTC-TCA-CAC-TCT-3′
Cdk6	5′-GCT-GAC-CAG-CAG-TAC-GAA-TG-3′	5′-GCA-CAC-ATC-AAA-CAA-CCT-GAC-C-3′
ACTB	5′-GTG-ACG-TTG-ACA-TCC-GTA-AAG-A-3′	5′-GCC-GGA-CTC-ATC-GTA-CTC-C-3′

Western blotting method

The effects of PR extract on protein expression were measured by Western blotting.[18] The cells were plated on a 6-well culture plate (2.5 x 10^5 cells/well) for 24 hr, the cells were exposed to PR extract for 24 hr, lysed the cancer cells, and then measured the protein concentration. The protein (20 µg) was subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Thereafter, the protein was transferred to polyvinylidene fluoride (PVDF) membrane, blocked with 2.5% albumin, and exposed to primary antibodies against cyclin D1 (1:1000), p21 (1:1000), and β-actin (1:2500) overnight. After that, the membrane was exposed to a secondary antibody for 2 hr (1:5000) at room temperature. The specific protein bands were subsequently examined by Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., MA, and USA).

Statistical analysis

The data were analyzed and compared between PR extract and control groups by using the Prism 5 program (GraphPad Software, San Diego, CA, USA) and expressed as mean ± SEM.

Results were considered to be statistically significant at a value of $P < 0.05$.

RESULTS

Piperin concentration in PR extract

Piperine is an active compound in the ripe fruit of PR and it has been reported that piperine caused the induction of cancer cells death and apoptosis. The two extractions of PR from DW and EtOH extract showed the higher piperine concentration in EtOH extract than DW extract [Figure 1a-c] approximately 17.02 ± 3.56 µg/mL and 1.55 ± 0.56 µg/mL, respectively. For the further experiment, this work needs to find the potency between DW and EtOH extract on MCF-7 cells death and cell migration.

PR effects on cell viability

To explore the cytotoxic effect of PR extract on MCF-7 cells by using SRB and colony formation method. After cells were treated with two

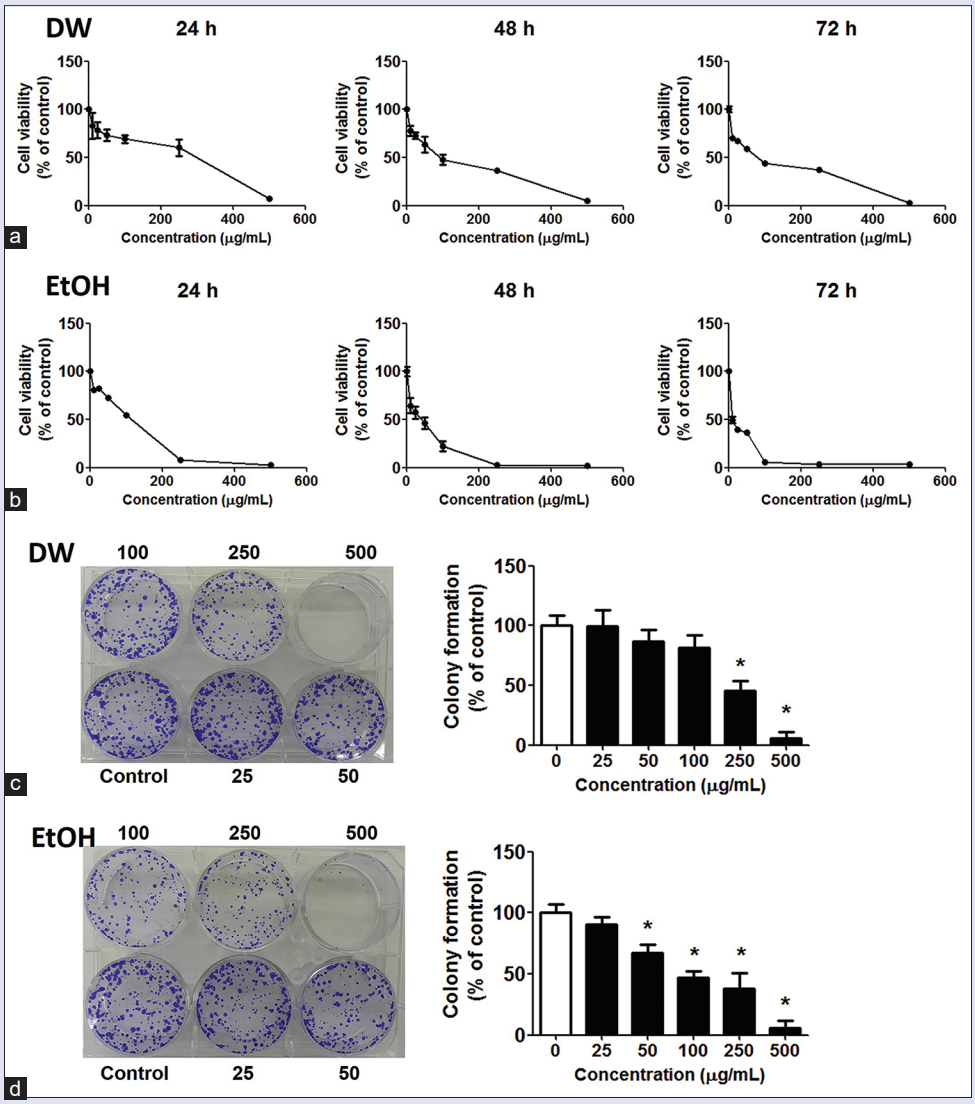

Figure 2: The effects of PR extract on cell viability and colony formation. For SRB method, the cancer cells were treated with PR extract for 0-72 hr (0-500 µg/mL), and measured the cells viability by SRB assay (a-b). For colony formation method, the cells were treated with PR extract for 24 h, changed to the new medium and cultured for another 14 days, stained with crystal violet, and then counted (c and d). *$p < 0.05$ vs. control groups

PR extractions for 0-72 hr and viability of all cultured cells showed a dose- and time-response manner [Figure 2a,b]. MCF-7 cells were more sensitive to EtOH extract than DW extract, with IC_{50} values for EtOH of 177.87 ± 56.07 µg/mL, 83.81 ± 15.63 µg/mL, 67.40 ± 2.97 µg/mL and IC_{50} values for DW of 83.46 ± 3.31 µg/mL, 29.72 ± 72 µg/mL, 14. 55 ± 1.03 µg/mL for 24, 48, and 72 hr, respectively.

The effects of PR extract on MCF-7 cells replication was determined by using colony formation. The colony formation of MCF-7 cells was inhibited by both PR extract and showed the sensitivity with EtOH extract than DW extract with IC_{50} values of 49.84 ± 4.51 µg/mL and 97.08 ± 16.34 µg/mL, respectively [Figure 2c,d]. This suggests that PR extract has the effect to stimulate breast cancer cells death and repress the cancer cells' regrowth by dose-response manner. Moreover, EtOH extract has a higher potency than DW extract with correlating the piperine levels in the extract.

PR effects on cell apoptosis

To explore an apoptotic effect of PR extracts on MCF-7 cells and then AO/EB staining and flow cytometry methods were used. From the results obtained that both PR extracts caused the induction of cells apoptosis. EtOH extract caused a significant reduction of viable cell counts more than DW extract and accelerated apoptosis at the dose of 250-500 µg/mL [Figure 3a]. Furthermore, the data from flow cytometry showed that two PR extractions induced cancer cells to early apoptotic cells with higher than untreated control groups at a dose of 250 µg/mL [Figure 3b].

EtOH showed a higher activity than DW extract. Moreover, EtOH of PR extract caused significant induction of the caspase 3 activity at the dose of 250 µg/mL [Figure 4a], stimulation of ROS formation at the dose of 500-1000 µg/mL [Figure 4b], and reduction of mitochondrial membrane potential at the dose of 500 µg/mL [Figure 4c]. These data indicated that EtOH extract had a greater activity than DW extract.

PR effects on gene- and protein-related cell proliferation

The effects of PR extract on gene and protein expression of cell proliferation were further evaluated. The three genes-related cell proliferation including *cdk2, cdk4, cdk6*, were examined by RT-PCR. The data indicated that both PR extracts significantly inhibited *cdk2, cdk4, cdk6* gene expression levels [Figure 5a]. Moreover, PR extracts inhibited the protein-related cell growth, cyclin D1, stimulated protein-inhibited cell cycle, p21 protein [Figure 5b], and finally, PR extract caused inhibited MCF-7 cell growth.

PR effects on cell migration

The PR extract on MCF-7 cells migration was evaluated by wound healing assay. Results showed that the dose of 250 µg/mL of DW extract significantly suppressed MCF-7 cell migration [Figure 6a]. Similarly, exposure to EtOH extract also significantly inhibited the migration at the lower dose than DW extract, 50-500 µg/mL [Figure 6a]. Additionally, MMP 9 expression correlated with cancer cell migration and then these data indicated that both PR extracts significantly suppressed the MMP 9 levels in the DMEM culture medium after incubated with the extract for 48 hr [Figure 6b]. Both PR extracts had the greater activity to inhibit MCF-7 migration.

DISCUSSION

Human breast cancer is cancer with rapid growth and significant metastatic potential.[19] The development of a novel effective agent targeting antiproliferation and antimetastasis could be valuable to improve current treatments in breast cancer patients.[19] These data revealed that both PR extracts had the levels of piperine, the active compound of PR extract that caused induction of many cancer cells types such as Dalton's lymphoma ascites, Ehrlich ascites carcinoma, breast, prostate, colon, and lung cancer cells.[6,20,21] less information on

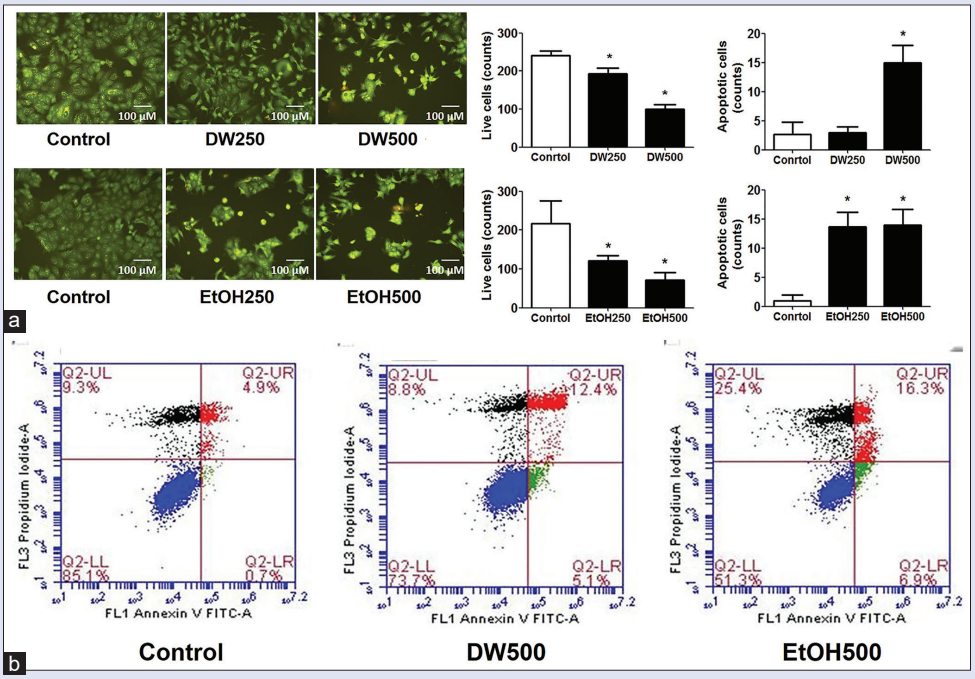

Figure 3: The effects of PR extract on cell apoptosis. For AO/EB method, the cancer cells were treated with PR extract for 24 hr (0-500 µg/mL), stained with AO/EB solution for 15 min, and captured by inverted microscopy (*10x* magnification, (a) For apoptotic by flow cytometry method, cells were treated with PR extract for 24 hr, stained with PI and annexin V FITC for 20 min, measured cell apoptosis by flow cytometry (b) *$p < 0.05$ vs. control groups

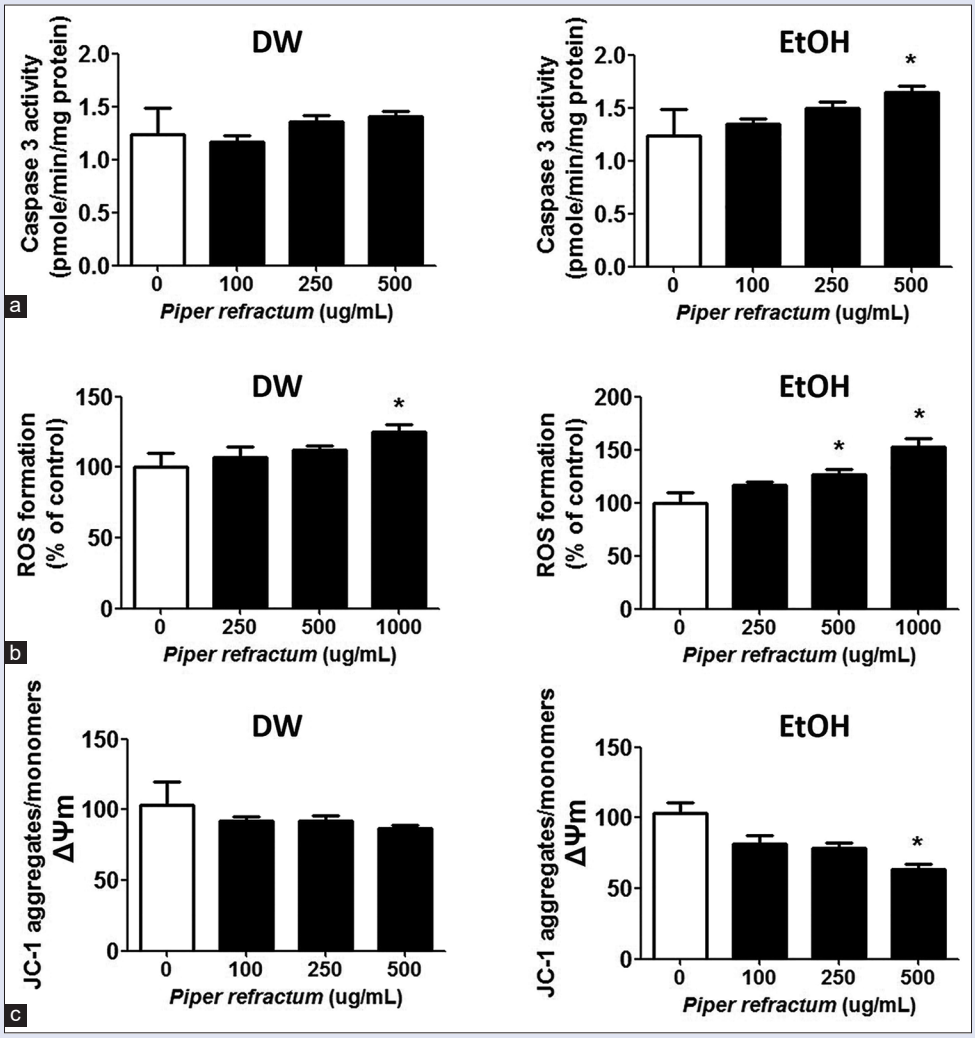

Figure 4: The effects of PR extract on cell caspase 3 activity, ROS formation, and mitochondrial membrane potential (MMP). For caspase 3 activity, the cancer cells were treated with PR extract (0-500 μg/mL) and measured the caspase 3 activity by Caspase 3 assay kit (a) For ROS formation, cells were treated with PR extract (0-1000 μg/mL) plus DHE-fluorescent probe for 90 min, and then measured ROS formation by spectrophotometer (b) For JC-1 method, cells were treated with PR extract (0-500 μg/mL) for 24 h, stained with JC-1 for 30 min, and then measured the fluorescent intensity (c) *$p < 0.05$ vs. control groups

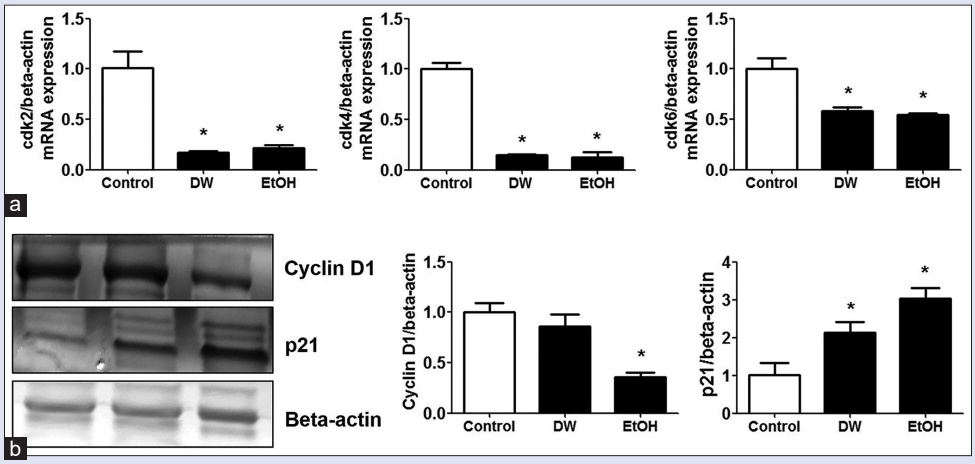

Figure 5: The effects of PR extract on gene and protein-related cell proliferation. For RT-PCR method, the cancer cells were treated with PR extract (250 μg/mL) for 24 hr, and then measured gene expression by RT-PCR (a) For Western blotting method, after cells were treated with PR extract (250 μg/mL) for 24 hr and protein expression by Western blotting (b) *$p < 0.05$ vs. control groups

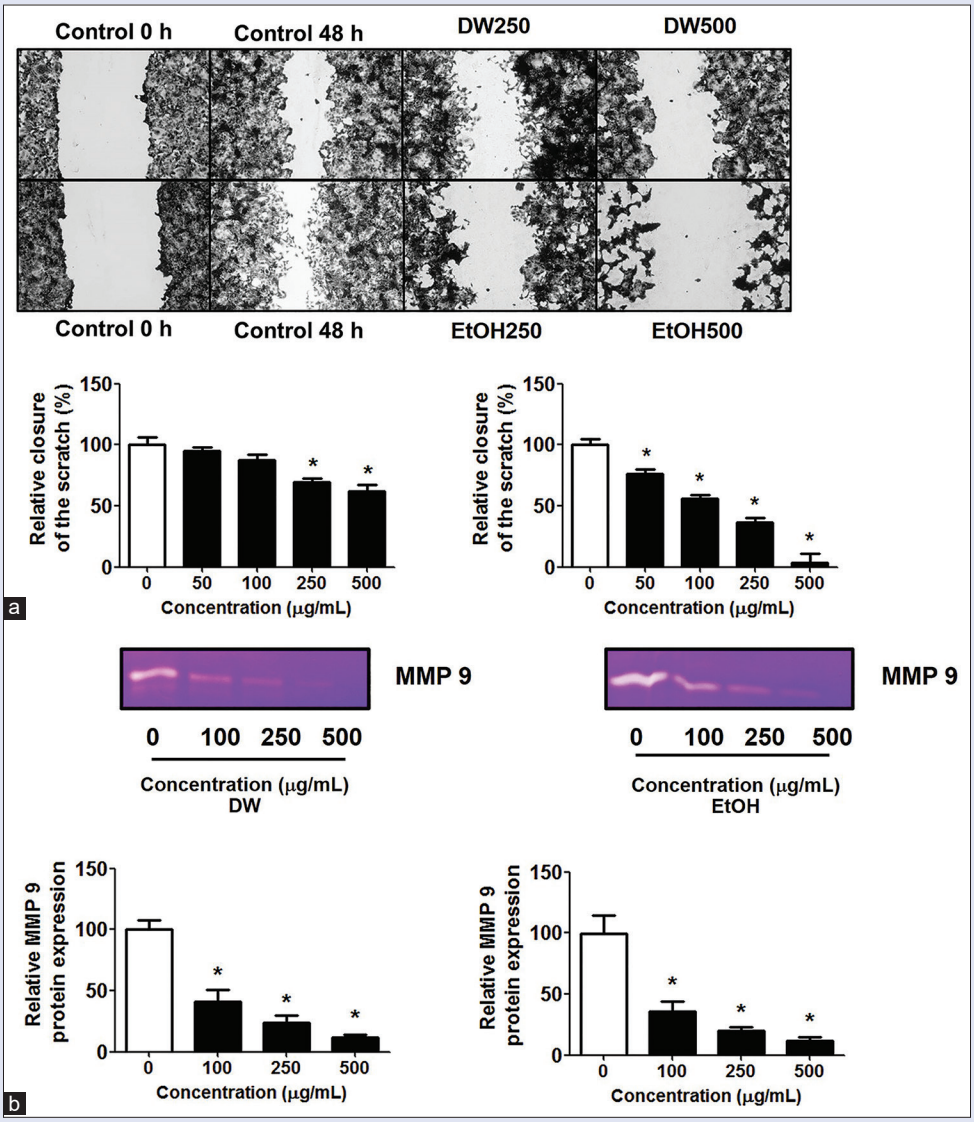

Figure 6: The effects of PR extract on cell migration. For wound healing method, the cancer cells were treated with PR extract (0-500 μg/mL) for 48 hr and then calculated the distance between the edges of wound with control group (a) For gelatin zymography method, cells were treated with PR extract (0-500 μg/mL) for 48 hr, collected the cultured medium, and measured the MMPs expression by gelatin zymography (b) *$p < 0.05$ vs. control groups

PR extract on human cancer; however, many reports have been indicated that the active compounds from PR such as piperine and piperlongumine suppressed the many cancer cells growth.[21,22] Especially, piperine found the higher levels in Piper species; Furthermore, it induced several cancer cells death by arresting the cell cycle at the G1 phase and relating with the cdk inhibitor-1 (p21/WAF1) activation and cyclin D1 protein suppression.[10] In the same situation, the piperine interrupts the ROS homeostasis that caused induction of the damage of DNA, lipids and proteins,[10] and then leads to cancer cells death. The other active compound is piperlongumine, it has been reported that piperlongumine inhibits prostate cancer cells' growth and is associated with the reduction of cdk such as cdk2, cdk4, cyclin D1, and cyclin E, which are related to G1 phase accumulation.[23] Like this work, PR extracts found the inhibition of breast cancer cells growth with decreasing *cdk2, cdk4, cdk6* gene expression and then reducing cyclin D1 and increasing p21 as well. Finally, the PR extract and its active compound were stimulated MCF-7 cell's death by several mechanisms.

Consistency with the effects of PR extracts on *cdk* gene expression levels; furthermore, both of the extracts had the high potency to inhibit cyclin D1 and stimulate p21 protein levels. Sriwiriyajan *et al.*,[24] reported that PR extract induced breast cancer cells such as MDA-MB-231, MDA-MB-468, MCF-7, MCF-12A, with very low IC_{50} values with 17.10 ± 0.46, 12.27 ± 2.14, 19.69 ± 0.88, 32.41 ± 5.94 μg/mL for 72 hr, respectively. The step of apoptosis is typically accomplished via two major pathways: intrinsic and extrinsic pathways in mitochondrial function.[25] In these same circumstances, it was detected that the alkaloid from PR extracts disturb the homeostasis of ROS levels that, in order, cause DNA damages,[10] with stimulated G1 phase arrest through activating the checkpoint kinase-1. On the other hand, some data found that the MDA-MB468 and MDA-MB-231, were exposed to piperine, G1 phase arrest was occurred through inactivating the G1 phase by modulating cyclin D3, cdk4, growth factors (E2F-1). The data indicated that piperine or PR extracts with higher piperine concentration may be suppressed the cancer cells proliferation and supported the chemopreventive action.

In addition, PR extracts inhibited the breast cancer cell migration with wound healing study by suppressing MMP 9 expression levels in the culture medium as well. Therefore, the extract may be a promising agent for treating human breast cancer by targeting antigrowth- and metastasis-associated events. Piper species such as *Piper nigrum* has been reported that inhibited breast cancer cells migration with very low concentration. The last step of cancer cells migration was triggered by a proteolytic enzymes such as MMPs, a family of Ca^{2+}-, and Zn^{2+}-dependent endopeptidases.[26] Piperine positively decreased the expression level and/or stimulation of MMPs and also indirectly hampers tumor spreading. As well, piperine suppressed lung metastasis of murine breast cancer model (4T1) by reduction of expression levels of MMP 9 and MMP 13.[9] Subsequently, migration was inhibited by piperine and/or PR extract and all these molecular mechanisms down-regulated MMP 9 expression.

CONCLUSION

This finding revealed that both PR extracts elicit an anti-growth and anti-metastatic effect against breast cancer MCF-7 cells. Down-regulating caspase 3, inducing ROS generation, and interfering mitochondrial function contributes in part to its effect as well as reducing proliferating-gene (*cdk2, cdk4, cdk6*), proliferating-protein (cyclin D1), and increasing proliferating-protein inhibitor (p21) protein levels. Thus, this compound may be a potential candidate for treating metastatic breast cancer by inhibiting MMP 9 expression. Further study is required to obtain more pharmacological data and to verify the effects of *in vivo* study.

Highlight

1. This study showed that PR extract significantly inhibits human breast cancer cells proliferation, induces cancer cells apoptosis and suppresses cells migration with a low dose of PR extract.
2. The mechanism of PR extract generated ROS formation and decreased mitochondrial function in intracellular MCF-7 cells.
3. These results suggested that PR is a potential therapeutic agent against breast cancer.

Author's contributions

Benjaporn Buranrat designed, conducted the experiments, analyzed data, and wrote the manuscript.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Habib MR, Karim MR. Antitumour evaluation of di-(2-ethylhexyl) phthalate (DEHP) isolated from Calotropis gigantea L. flower. Acta Pharm 2012;62:607-15.
2. Patel MS, Antala BV, Dowerah E, Senthilkumar R, Lahkar M. Antitumor activity of Pogostemon benghalensis Linn. on ehrlich ascites carcinoma tumor bearing mice. J Cancer Res Ther 2014;10:1071-5.
3. He H, Guo WW, Xu RR, Chen XQ, Zhang N, Wu X, *et al.* Alkaloids from piper longum protect dopaminergic neurons against inflammation-mediated damage induced by intranigral injection of lipopolysaccharide. BMC Complement Altern Med 2016;16:412.
4. Kumar S, Kamboj J, Suman SS, Sharma S. Overview for various aspects of the health benefits of Piper longum linn. fruit. J Acupunct Meridian Stud 2011;4:134-40.
5. Singh YN. Kava: An overview. J Ethnopharmacol 1992;37:13-45.
6. Zadorozhna M, Tataranni T, Mangieri D. Piperine: Role in prevention and progression of cancer. Mol Biol Rep 2019;46:5617-29.
7. Ouyang DY, Zeng LH, Pan H, Xu LH, Wang Y, Liu KP, *et al.* Piperine inhibits the proliferation of human prostate cancer cells via induction of cell cycle arrest and autophagy. Food Chem Toxicol 2013;60:424-30.
8. Zhang J, Zhu X, Li H, Li B, Sun L, Xie T, *et al.* Piperine inhibits proliferation of human osteosarcoma cells via G2/M phase arrest and metastasis by suppressing MMP-2/–9 expression. Int Immunopharmacol 2015;24:50-8.
9. Lai LH, Fu QH, Liu Y, Jiang K, Guo QM, Chen QY, *et al.* Piperine suppresses tumor growth and metastasis *in vitro* and *in vivo* in a 4T1 murine breast cancer model. Acta Pharmacol Sin 2012;33:523-30.
10. Fofaria NM, Kim SH, Srivastava SK. Piperine causes G1 phase cell cycle arrest and apoptosis in melanoma cells through checkpoint kinase-1 activation. PLoS One 2014;9:e94298. doi: 10.1371/journal.pone.0094298.
11. Siddiqui S, Ahamad MS, Jafri A, Afzal M, Arshad M. Piperine triggers apoptosis of human oral squamous carcinoma through cell cycle arrest and mitochondrial oxidative stress. Nutr Cancer 2017;69:791-9.
12. Yaffe PB, Doucette CD, Walsh M, Hoskin DW. Piperine impairs cell cycle progression and causes reactive oxygen species-dependent apoptosis in rectal cancer cells. Exp Mol Pathol 2013;94:109-14.
13. Si L, Yang R, Lin R, Yang S. Piperine functions as a tumor suppressor for human ovarian tumor growth via activation of JNK/p38 MAPK-mediated intrinsic apoptotic pathway. Biosci Rep 2018;38:BSR20180503. doi: 10.1042/BSR20180503.
14. Zeng Y, Yang Y. Piperine depresses the migration progression via downregulating the Akt/mTOR/MMP9 signaling pathway in DU145 cells. Mol Med Rep 2018;17:6363-70.
15. Buranrat B, Boontha S. Effect of *Piper nigrum* ethanolic extract on human breast cancer cell growth and cell migration. Pharmacogn Mag 2019;15:538-46.
16. Buranrat B, Senggunprai L, Prawan A, Kukongviriyapan V. Simvastatin and atorvastatin as inhibitors of proliferation and inducers of apoptosis in human cholangiocarcinoma cells. Life Sci 2016;153:41-9.
17. Buranrat B, Prawan A, Senggunprai L, Kukongviriyapan V. Inhibition of growth and migration of cholangiocarcinoma cells by pamidronate. Exp Ther Med 2019;18:3977-83.
18. Buranrat B, Bootha S. Antiproliferative and antimigratory activities of bisphosphonates in human breast cancer cell line MCF-7. Oncol Lett 2019;18:1246-58.
19. Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, *et al.* A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PLoS One 2009;4:e6562. doi: 10.1371/journal.pone.0006562.
20. Anuradha V, Srinivas PV, Rao JM. Isolation and synthesis of isodihydropiperlonguminine. Nat Prod Res 2004;18:247-51.
21. Pradeep CR, Kuttan G. Effect of piperine on the inhibition of lung metastasis induced B16F-10 melanoma cells in mice. Clin Exp Metastasis 2002;19:703-8.
22. Thongsom S, Suginta W, Lee KJ, Choe H, Talabnin C. Piperlongumine induces G2/M phase arrest and apoptosis in cholangiocarcinoma cells through the ROS-JNK-ERK signaling pathway. Apoptosis 2017;22:1473-84.
23. Lee W, Kim KY, Yu SN, Kim SH, Chun SS, Ji JH, *et al.* Pipernonaline from Piper longum Linn. induces ROS-mediated apoptosis in human prostate cancer PC-3 cells. Biochem Biophys Res Commun 2013;430:406-12.
24. Sriwiriyajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. Cytotoxicity screening of plants of genus piper in breast cancer cell lines. Trop J Pharm Res 2014;13:921-8.
25. Tanaka T. Role of apoptosis in the chemoprevention of cancer. J Exp Clin Med 2013;5:89-91.
26. Kajanne R, Miettinen P, Mehlem A, Leivonen SK, Birrer M, Foschi M, *et al.* EGF-R regulates MMP function in fibroblasts through MAPK and AP-1 pathways. J Cell Physiol 2007;212:489-97.