

Anticancer Activities of the Herbal Recipe Benjakul Extract against Human Breast Cancer Cells by Inducing Apoptosis and Suppressing Migration

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ABSTRACT

Background: Benjakul is a Thai traditional formulation and has been reported for cancer treatment. **Objectives:** In this study aimed to examine the cytotoxic effects of Benjakul extract on suppression of breast cancer cells proliferation and migration. **Materials and Methods:** Cytotoxic effects were determined by sulforhodamine B, colony formation, ROS formation, caspase 3 activity, and mitochondrial function. Migration assay was studied by wound healing and gelatin zymography. **Results:** Piperine level was showed the highest concentration in the Benjakul extract. Further, Benjakul extract exhibited cytotoxicity against breast cancer cells with low IC₅₀ equally effects in two cancer cells and further it suppressed the colony formation. The mechanism of Benjakul to induce cells death through activating ROS formation, stimulating caspase 3 activity, and repressing mitochondrial function. Moreover, the suppression of migration by Benjakul was acted on reduction of MMP-9 expression level in culture medium. **Conclusion:** Our findings demonstrated that Benjakul extract can suppress human cancer cells proliferation along with induction of apoptosis and attenuation of migration by low concentration.

Key words: Benjakul, Breast cancer cells, Growth, Apoptosis, Migration.

Key Message

- Benjakul has a several active compounds and piperine shows the highest levels after detecting with HPLC method.
- Benjakul extract with 95% ethanol can inhibits the human breast MCF-7 and MDA- MB-231 cancer cells by dose- and time-dependent manner and it suppresses the colony formation.
- The mechanism of action of Benjakul relates to stimulate ROS production, activate caspase 3 activity, and stimulate mitochondrial dysfunction.
- Benjakul decreases breast cancer cells migration by reduction of MMP-9 expression.
- This provide the information of Benjakul on treatment of breast cancer in the further.

INTRODUCTION

Cancer is one of important disease that increases the mortality rate in worldwide and the estimation of new cases in 2030 approximately 14 million.^[1] Breast cancer is one of the mostcommon malignant tumors and poses a serious threat to the physical and mental health in women. The data has been reported that breast cancer is showed the high rate in developing country and cancer-related deaths is about 60%.^[2] The several strategies are reported including surgery, radiotherapy, chemotherapy, hormonal therapy and other new targeted therapies; however, breast cancer is still facing to the important issues to improve survival rate, reduce drug-resistance, and suppress metastases.^[3] Therefore, new agents or new traditional medicines are needed to improve chemosensitization and therapeutic

efficacy,^[4] in breast cancer. Many Thai people still use traditional medicine as an alternative for cancer treatment,^[5] Including Benjakul recipe.

Benjakul preparation is a known herbal remedy used for balance the four basic elements in Thai traditional medicine and treatment cancers in folk medicine. This preparation is also used as an adaptogen and for treatment of dyspepsia.^[6] Benjakul preparation comprised five plants:fruit of *Piper retrofractum*, root of *Piper samentosum*, stem of *Piper interruptum*, rhizome of *Zingiber officinale* and root of *Plumbago indica*, in equal proportions.^[7] Benjakul extract has reported many active compounds, including piperine, methyl piperate, myristicin, 6-shogaol, plumbagin, and 6-gingerol.^[8,9]

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It has been reported on biological activities such as anti-allergy, anti-inflammation, hepatic improving, and anticancer activity. Inhibition of cancer indicated, Benjakul suppressed cancer cells growth, migration, and invasion; however, the data are still less information.

Benjakul was extracted by ethanol caused induction of cancer cells death with low concentration in three cancer types, lung, cervical, and liver cancer cells with 19.80 ± 1.89 , 47.72 ± 2.11 , and 45.58 ± 1.26 $\mu\text{g/mL}$, respectively,^[7] however, no cytotoxic effects in water extracts. Three active compounds were obtained from Benjakul extract were piperine, plumbagin, and 6-gingerol showed as 7.48, 4.18, and 0.54% yield of crude extract, respectively.^[7] The mechanism of Benjakul was reported to inhibit cancer cells growth by increasing the population of sub-G1 phase along with activating the early and late apoptotic cells.^[8] Moreover, apoptosis was induced by Benjakul and its active compound, 6-shogaol and plumbagin, and at the high concentration through increasing caspase 3 activity.^[8] The Benjakul extract on human breast cancer treatment has still less information; however, some data reported that Benjakul demonstrated the great effects on breast cancer cell with low IC_{50} value was 33.20 $\mu\text{g/ml}$,^[10] however, the mechanism of Benjakul on cancer cells death and migration is still unknown in breast cancer.

This work purposed to explore the role of ethanolic extract of Benjakul on the proliferative, apoptotic, and migratory ability of two human breast cancer cells, MCF-7 and MDA-MB-231. The potential mechanisms underlying were explored in the production of ROS levels, caspase 3 activity, and mitochondrial function.

MATERIALS AND METHODS

Benjakul Recipe Preparation and Extraction

Benjakul was composed of five Thai plants including *Piper retrofractum* fruits, *Piper sarmentosum* Roots, *Piper interruptum* stems, *Plumbago indica* roots, and *Zingiber officinale* rhizomes, were obtained from Maha Sarakham Province, Thailand. Proof of identity was made by Applied Thai Traditional Medicine Departments, Faculty of Medicine, Mahasarakham University (specimen no. MSUT-7235, MSUT-7224, MSUT-7287, MSUT-7288, MSUT-7284) and placed at the Faculty of Science, Mahasarakham University, Thailand.

In brief, the five plants with equal proportions were dried, fermented in 95% ethanol, filtered, evaporated, and lyophilized. Percentage yield of the extracts are 4.25% per dry weight of the dry power of each plant.

HPLC method

To examine the pellitorine, 6-gingerol, plumbagin, and piperine contents of Benjakul extract by using HPLC method. Briefly, the extract loaded into C_{18} analytical column by using the mobile phase composed of acetonitrile and deionized water, the injection was performed using a 20 μL loop at 205, 256, 280, and 340 nm for pellitorine, plumbagin, 6-gingerol, and piperine, respectively.

Cell culture and cell proliferation method

Two human breast cancer cell line, MCF-7 and MDA-MB-231 cells, was grown in DMEM medium added with antibiotics (1%), and fetal bovine serum (10%). The cells were cultured under 5% CO_2 in air at 37°C and then subcultured every 2-3 days.

The effects of Benjakul extract on the cancer cells viability was measured by sulforhodamine B (SRB) method. Cells were plated on culture plates (1×10^4 cells/well) overnight and exposed to different concentrations of Benjakul extract (0-100 $\mu\text{g/mL}$) for 0-72 hr. Afterwards, the cancer cells were fixed, stained with 0.4% SRB at room temperature for 30 min. Cells were added 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 Benjakul extract on the colony formation were measured by colony formation method. The cells were plated about 500 cells onto 6-well culture plates for overnight and added the new complete DMEM medium containing various concentration of Benjakul extract (0-100 $\mu\text{g/mL}$) for 24 hr. Next, the cells were exposed to the new DMEM medium and grown for 14 days at 37°C and 5% CO_2 . Afterwards, the each well was added with 100% methanol, stained with 0.5% crystal violet, and measured the colony formation.

Wound healing method

The effects of Benjakul extract on the cell migration were measured by wound healing method. The cells were plated on 24-well culture plate (2.5×10^5 cells/well) for overnight and next day cells were scratched to make a wound by using pipette tip (0.2 mL). Afterwards, cells were exposed to various doses of Benjakul extract (0-50 $\mu\text{g/mL}$) and the images were taken by inverted microscopy at 0 and 72 hr after treatment.

Gelatin zymography method

The effects of Benjakul extract on the matrix metalloproteinase-9 (MMP-9) expression were measured by gelatin zymography method. Cells were plated on 24-well culture plate (2.5×10^5 cells/well) for overnight, next day cancer cells were exposed to Benjakul extract for 72 hr (0-50 $\mu\text{g/mL}$), 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). Afterwards, gels were washed three times with 2.5% Triton X-100 and then exposed to developing buffer for overnight at 37°C. The gels were exposed to 0.5% Coomassie BlueR-250 and detained in destaining buffer. The band was detected as clear bands against the blue background of Coomassie BlueR-250 and the band intensity was determined.

Caspase 3 activity method

The effects of Benjakul extract on caspase 3 activity were examined by the kits. Cells were plated on 6-well culture plate (2.5×10^5 cells/well) for overnight, next day cells were incubated with Benjakul extract (0-50 $\mu\text{g/mL}$) for 24 hr, collected the cells pellets, lysed by RIPA buffer, and measured the protein concentration. 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 Benjakul extract on ROS formation were measured by DHE-fluorescent probe. The cells were plated on white 96-well culture plate (1×10^4 cells/well) overnight, incubated with Benjakul extract (0-50 $\mu\text{g/mL}$) 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 Benjakul extract on mitochondrial functions were examined by JC-1 fluorescent probe. Cells were plated on white 96-well culture plate (1×10^4 cells/well) overnight, incubated with Benjakul extract (0-50 $\mu\text{g/mL}$) plus JC-1 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.

Statistical Analysis

The data were analyzed and compared between Benjakul extract and control groups by using the Prism 5 program (GraphPad Software, San Diego, CA, USA) and expressed as mean±standard error. Results were considered to be statistically significant at a value of $p < 0.05$.

RESULTS

Active compounds in Benjakul recipe extract

Pellitorine, 6-gingerol, plumbagin, and piperine is the most abundant compounds in Benjakul extract, and it is the active anticancer compound. To examine the four compounds in the Benjakul extract, we used the HPLC method. The data revealed the presence of pellitorine, 6-gingerol, plumbagin, and piperine in the Benjakul extract, as shown in Figure 1, to be 22.90±5.71, 22.51±4.76, 6.45±1.82, and 60.82±9.21 µg/mL of Benjakul/dry weight extract. Piperine is the most abundant in Benjakul extract than the other compounds and it suggests to be used as biomarkers for standardization of this preparation.

Benjakul effects on cell death

To explore the cytotoxic effect of Benjakul extract on two breast cancer by using SRB and colony formation method. After cells were treated with Benjakul extract for 0-72 hr and viability of all cultured cells showed a dose- and time-response manner (Figure 2A-B). Both of human breast cancer cells were more sensitive to Benjakul extract, with IC_{50} values for MCF-7 cells of 38.35±5.89, 16.36±1.22, 14.54±1.39 µg/mL and IC_{50} values for MDA-MB-231 of 29.28±1.93, 26.51±1.88, 21.16±2.10 µg/mL for 24, 48, and 72 hr, respectively. Next, the effects of Benjakul extract on cells replication was determined by using colony formation. The colony formation of two breast cancer cells were inhibited by Benjakul extract and MCF-7 cells showed the sensitive to Benjakul extract than MDA-MB-231 cells with IC_{50} values of 5.94±0.73 and 13.16±2.72 µg/mL, respectively (Figure 2C and D).

Benjakul effects on ROS formation, caspase 3 activity, and mitochondrial function

To explore ROS formation of Benjakul extracts on breast cancer cells by using DHE- fluorescent probe. From the results obtained that Benjakul extracts caused the induction of ROS formation only at 50 µg/mL Benjakul extract in MCF-7 cells (Figure 3A and B) and in MDA- MB-231 cells did not detect.

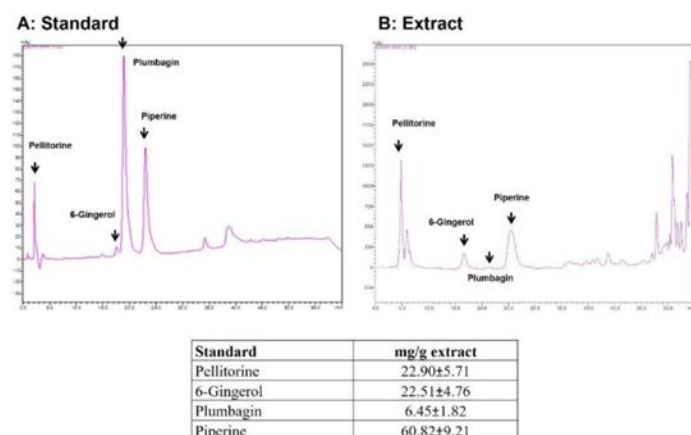


Figure 1: High-performance liquid chromatography chromatogram of active compounds in Benjakul extracts, (A) Standard (B) Benjakul extract.

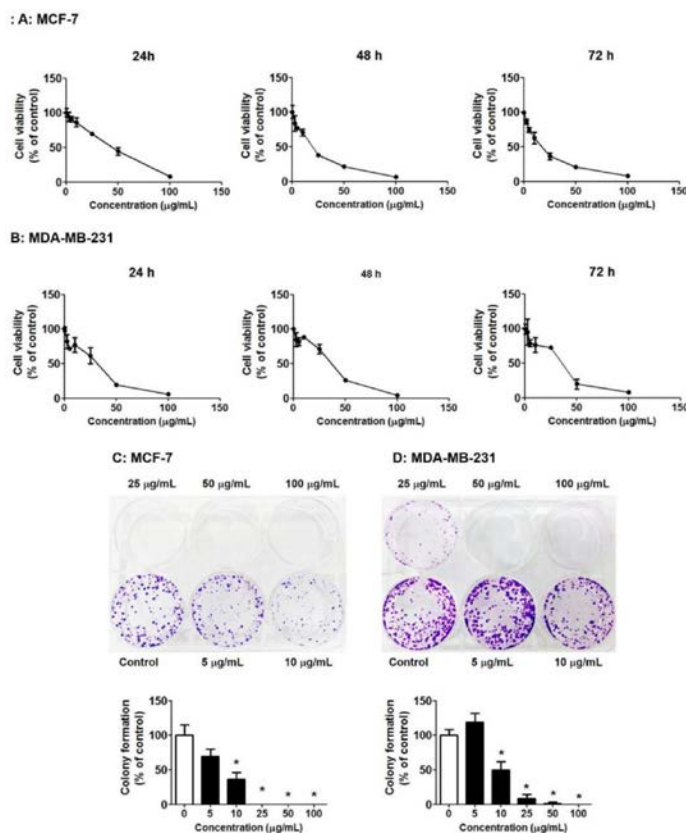


Figure 2: Effects of Benjakul extract on viability and colony formation of breast cancer cells (A and B). MCF-7 and MDA-MB-231 cells were treated with 0–100 µg/mL Benjakul extract for 24–72 hr and then analyzed cancer cells death by SRB. Cells were treated with 0–100 µg/mL Benjakul extract for 24 hr and then added the new complete medium every 2 days for 2 weeks and analyzed colony formation. Data represent mean ± standard error of the mean of three independent experiments. * $P < 0.05$ versus control.

However, Benjakul extract caused induction of apoptosis via caspase 3 activity was detected by dose-dependent manner both in two breast cancer cells. At high dose of Benjakul extract (50 µg/mL) significantly induced caspase 3 activity approximately four times than the untreated control groups both in two cancer cells (Figure 3C and D).

Moreover, Benjakul extract activated cancer cell death and apoptosis by decreasing mitochondrial membrane potential or mitochondrial function at the dose of 50 µg/mL (Figure 3E and F). These data indicated that Benjakul extract had the great activity to induce human breast cancer cell apoptosis both in two breast cancer cells.

Benjakul effects on cell migration

The Benjakul extract on breast cancer cells migration was evaluated by wound healing assay and gelatin zymography assay. The data indicated that at the dose of 10-50 µg/mL of Benjakul extract significantly suppressed cancer cells migration (Figure 4A and B) with significant start at the dose of 10 to 50 µg/mL. Additionally, MMP-9 expression was inhibited the migration by Benjakul extract both in two cancer cells and then these data indicated that Benjakul extract significantly suppressed the MMP-9 levels after exposing with these extracts for 72 hr.

DISCUSSION

In this study, Benjakul extract demonstrated stronger cytotoxic effects against all two types of human breast cancer cells, MCF-7

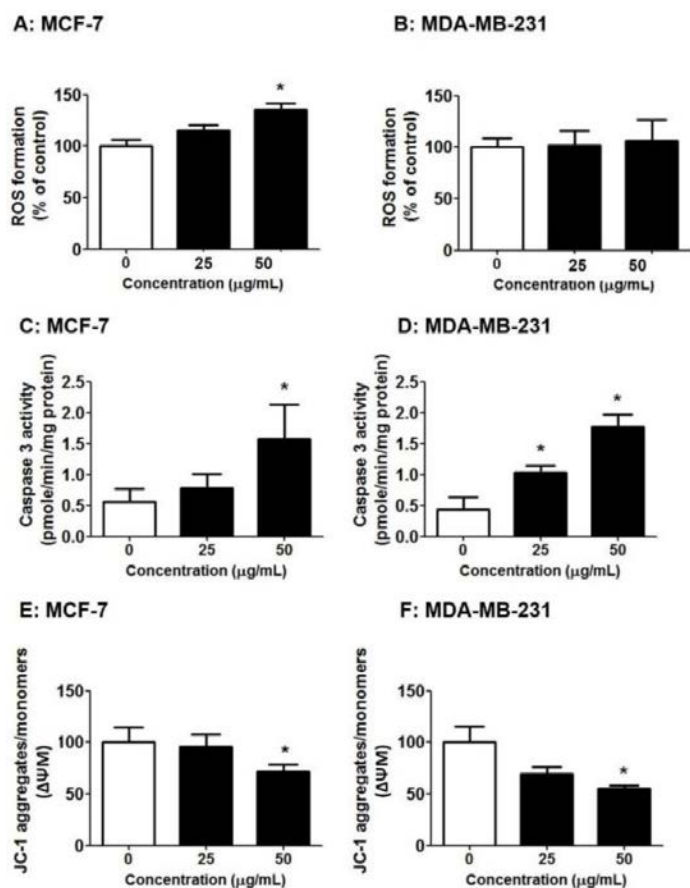


Figure 3: Effects of Benjakul extract on ROS formation, caspase-3 activity and mitochondrial function of breast cancer cells (A-F). After cancer cells were treated with 0–50 µg/mL Benjakul extract with DHE- probe for 90 min in dark and analyzed ROS formation by measured fluorescent intensity. Cells were treated with 0–50 µg/mL Benjakul extract for 24 hr and measured caspase-3 activity by the kits. Cells were incubated with 0–50 µg/mL Benjakul extract for 24 hr and measured mitochondrial function by fluorescence spectrophotometer. Data represent mean ± standard error of the mean of three independent experiments. * $P < 0.05$ versus control.

and MDA-MB-231, with low IC_{50} values. The active compounds of extract were observed in four compounds with pellitorine, 6-gingerol, plumbagin, and piperine and piperine is the highest level than the other compounds. For more information, Benjakul caused suppression of colony formation as inhibited cell viability by dose-dependent manner. Further, Benjakul induced apoptosis by induction of the ROS formation, stimulation of caspase 3 activity, and reduction of mitochondrial function. Finally, these extracts caused suppression of migration via decreasing MMP-9 levels in culture medium. Benjakul extract had the greatest activity to against two types of human breast cancer cells and it may useful to treat the human breast cancer in the further.

In this study indicated that Benjakul significantly inhibit growth and colony formation in two types of breast cancer cells with low IC_{50} values and MCF-7 cells were sensitive to Benjakul than MDA-MB-231 cells. Benjakul is the in Thailand, Benjakul is used for adaptogen before changing seasonal weather or receiving the anticancer drugs,^[11] however, all plants or active compounds in the Benjakul recipe has been reported the anticancer activity and in presently,

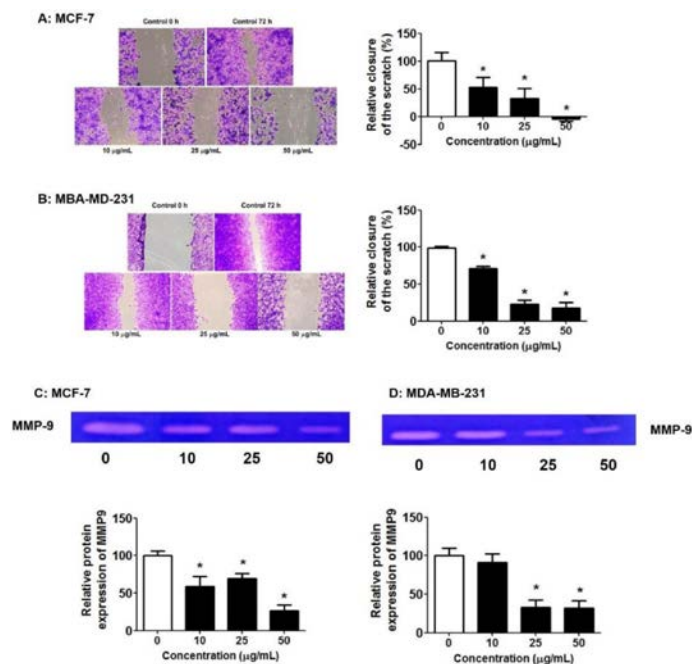


Figure 4: Effects of Benjakul extract on cell migration of breast cancer cells (A-D). Cells were treated with 0–50 µg/mL for 72 hr and examined the wound healing assay. Cells were treated with 0–50 µg/mL for 72 hr and examined the gelatin zymography for detecting MMP-9 level. Data represent mean ± standard error of the mean of three independent experiments. * $P < 0.05$ versus control.

the Thai researchers try to used or used Benjakul in cancer patients. Hence, the basic research in *in vitro* study is more important in many cancer cell types including breast cancer before using in patients. Our work demonstrated that Benjakul inhibited two types of human breast cancer cells including MCF-7 and MDA-MB-231, with low IC_{50} value, 14.54 ± 1.39 µg/mL in MCF-7 cells and 21.16 ± 2.10 µg/mL in MDA-MB-231 cell, like as the previous report showed that IC_{50} value was approximately 33.20 ± 0.80 µg/mL.^[10] The effects of Benjakul on breast cancer cells is still less data, especially in the mechanism of action. For the other cancer cells, Benjakul extract and its active compound, plumbagin, and 6-shogaol, can induce cell cycle arrest at G2/M phase and activate caspase-dependent apoptosis,^[8] that it the mechanism of action of Benjakul on lung cancer cells. Interestingly, Benjakul extract need to explore the mechanism in breast cancer cells.

Based on the mechanism on cell death, Benjakul extract acted on the stimulation of ROS formation in only MCF-7 cells; however, the extract induces cancer cells apoptosis through increasing the caspase 3 activity and reducing the mitochondrial function in both two cancer cell lines. Previously, the early and late apoptosis was detected in lung cancer cells after receiving Benjakul extract. ROS are harmful substances which indefinitely damages cells, it's activated mitochondrial dysfunction and led to cancer cells apoptosis.^[12] Intrinsic pathway of apoptosis was involved the permeability of the mitochondrial outer membrane and generates the membrane pores that lead to release apoptosis signaling pathway, such as caspase cascade, thus leading apoptosis and cancer cells death. It is value pointing that the production of high levels of ROS precedes dysfunction of mitochondria, condensation of nuclear, and formation of apoptotic body.^[13] *Piper nigrum* and *Piper retrofractum* are contained in Benjakul recipe that have been indicated the production of ROS formation and induction of apoptosis.^[14,15] The results appropriately

support the traditional use of Benjakul; it is not enough. The further investigation of its extract and components in the treatment of cancer is needed to be explored and larger population studies would be needed to recommend it as a safe and effective treatment in general.

The metastasis of cancer cells is a basic mechanism of cancer cells to migrate to distant organs.^[16] Truly, an herbal medicine with high capability to inhibit the metastasis could be a potential candidate for preventing and treating cancer. This is showing the report of Benjakul extract on the cancer cells migration and these studies indicated that Benjakul extracts suppressed the Basal activity of human breast cancer cells migration through inhibiting MMP-9 expression. Likely, our previous report demonstrated that *Piper nigrum* inhibited breast cancer cells migration via inhibition of MMP-9 protein expression with reduction of *MMP-2*, *MMP-9*, *ICAM1*, and *VEGFA* gene expression. Our results indicate that the Benjakul extract may probably exert this migratory effect via inhibition of MMP-9 expression.

CONCLUSION

Benjakul extracts exhibited the potency of anticancer activity on human breast cancer cells, MCF-7 and MDA-MB-231 cells, through inducing cancer cells apoptosis with stimulating ROS formation, activating caspase 3 activity, and reducing mitochondrial function. In addition, the Benjakul extract that contains pellitorine, 6-gingerol, plumbagin, and piperine, piperine is showed the high level in these extracts and could serve as a promising anticancer agent for human breast cancer development. Moreover, Benjakul extract can suppress the cancer cells migration with decreasing MMP-9 expression levels. Finally, Benjakul herbal recipe could be provided a new natural compound to inhibit breast cancer cells growth and migration.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

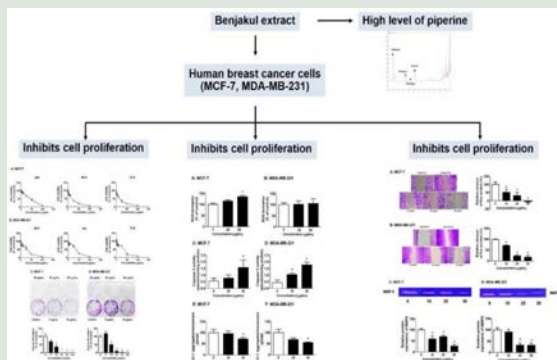
ABBREVIATIONS

AMC: Ac-DEVD-7-amino-4-methylcoumarin; **DMEM:** Dulbecco's Modified Eagle Medium; **SRB:** Sulforhodamine B; **OD:** Optical density; **ROS:** Reactive oxygen species.

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GRAPHICAL ABSTRACT



SUMMARY

The Ayurvedic medication play an important role in the traditional healing system in Thailand. Benjakul is a known herbal remedy used for balance the four basic elements in Thai traditional medicine. Piperine is the highest levels of active compounds in Benjakul extract. Further, Benjakul against human breast cancer cells both in reduction of proliferation and colony formation. The mechanism of Benjakul has more effects on cell apoptosis by detecting ROS formation, caspase 3 activity, and mitochondrial dysfunction. The migratory inhibition was observed in the reduction of wound healing assay and suppression of MMP-9 expression. This provide the information of Benjakul on treatment of breast cancer in the further.

ABOUT AUTHORS



Dr. Benjaporn Buranrat, is a Lecturer, Faculty of Medicine, Mahasarakham University. She is passionate researcher on *in vitro* plant screening studied against cancer.

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