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Maprang seed extracts suppressed chemoresistant properties of breast cancer cells survived from ionizing radiation treatment via the regulation of *ABCB1* genes

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ABSTRACT

Background: Minimal Residual Disease (MRD) is a major obstacle for eradication of cancer cells due to therapeutic resistance which results from the expression of multidrug resistant (MDR) proteins. MRD contributes to tumor metastasis and relapse in cancer patients. Therefore, any strategy that can reduce this resistance or eliminate all cancer cells must be one of the main goals for cancer treatment.

Objectives: This study aims to investigate the effect of Maprang Seed Extracts on drug resistance of minimal residual breast cancer cells that have survived radiotherapy.

Materials and methods: The treatment of resistant breast cancer cells MCF-7/IR6 and MCF-7/MPIR6 were established from parental MCF-7 cells having two different conditions for radiation treatment. Chemoresistant phenomenon of MRD was determined by MTT assay. A spectrofluorometric technique was used to determine the cellular drug uptake and the MDR protein-mediated drug efflux. MDR gene expression was confirmed by reverse transcription-polymerase chain reaction (RT-PCR).

Results: MCF-7/IR6 cells have increased resistance to doxorubicin when compared to the parental cells with the descending order of the concentration that inhibiting cell growth by 50% (IC_{50}) as follows: MCF-7/IR6 (IC_{50} =342.95±30.94 nM) > MCF-7/MPIR6 (IC_{50} =282.75±24.64 nM) > MCF-7 (IC_{50} =215.42±23.73 nM), accompanied by a remarkably enhanced expression level of MDR1 genes in MCF-7/IR6 compared with parental MCF-7 cells. For the multidrug protein function, it showed that MCF-7/IR6 cells mediated higher rate of drug efflux out of cells (V_a =0.2308 $nM.s^{-1}$) than MCF-7/MPIR6 (V_a =0.0679 nM.s-1) and the parental MCF-7 cells (V_a =0.0232 $nM.s^{-1}$). MPSEs effectively decreased chemoresistance phenomenon in the MCF-7/IR6 by aberrant MDR gene expression and decreased MDR protein-mediated drug efflux function.

Conclusion: The novel strategy combination of MPSEs and radiotherapy might be one strategy for improving curative effects in breast cancer treatment.

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Introduction

Breast cancer is the most common cancer found in women around the world. Currently, many advanced strategies in breast cancer treatment are developing in many countries to improve the curative effects and to decrease cancer-related morbidity and mortality. Unfortunately, the goal of breast cancer treatment is still not achievable in many patients because of residual cancer cells called Minimal Residual Disease (MRD), defined as the cellular population that can survive after treatment which can lead to tumor relapse and treatment failure.[1] Mounting evidence suggests that the failure of the treatment associated with the use of cancer stem cells represents a more chemoresistant and radioresistant subpopulation within many cancers.[2-4] Recent studies have shown that tumor radioresistance mechanisms are associated with several signaling pathways including the Epithelial-Mesenchymal Transition (EMT), Adenosine Triphosphate Binding Cassette (ABC) transporters, and phosphatidylinositol 3-kinase/protein kinase B.[5-7]

In cancer patients, radioresistance may occur simultaneously with chemoresistance.[8] Furthermore, resistance to conventional anti-cancer therapies remains a major obstacle in achieving successful treatment, highlighting the urgency of improving tools for eliminating minimal residual disease in order to prevent metastasis and to improve the therapeutic effects in breast cancer patients.

Cancer stem cells (CSCs) are the small number of cells within a tumor that drive the tumor's growth. These cells are capable of self-renewal and differentiations leading to the heterogeneous nature of cancer cells that comprise the tumor.[9, 10] CSCs are now thought to be contributing to drug and radiation resistance in solid tumors. CSCs express specific markers, cells expressing $CD44^+/CD24^-$ and $CD133^+$ on their surface, are thought to be breast cancer stem cells. CSCs in breast cancer also express high levels of drug resistance transporter proteins including P-glycoprotein (P-gp also known as MDR1), Multidrug Resistance-associated Protein 1 (MRP1), and Breast Cancer Resistance Protein (BCRP),[11, 12] which renders them resistant to drug and radiation therapy. Moreover, several studies have demonstrated that ionizing radiation can enrich cancer stem cells *in vitro* and *in vivo*.[13] Therefore, targeting CSCs may be a promising strategy for improving breast cancer treatment.[14, 15]

Alternative therapy by natural products has attracted much attention due to its considerable chemical and biological activity and beneficial potential effects on human health. The anticancer activity research on natural products demonstrated that it can be used for cancer prevention and treatment in combination with current conventional therapies, including chemotherapy and radiotherapy.[16] Maprang Seed Extracts (MPSEs) is the natural product extract from the Thai fruit *Bouea macrophylla* Griffith. It has demonstrated to exhibit anti-proliferative activity against drug-sensitive as well as drug-resistant leukemic and lung cancer cells.[17] Moreover, we demonstrated that MPSEs effectively sensitize breast cancer cells MCF-7 to radiation, and the combination of MPSEs and irradiation increase numbers of cell death in comparison to irradiation alone.[18] However, the benefits of MPSEs in breast cancer treatment related with chemoresistance have rarely been reported.

In the present study, we demonstrated the effects of MPSEs in terms of how they can overcome treatment resistance, especially chemoresistance, in minimal residual breast cancer cells. We performed comparative analysis of the chemoresistance phenomenon in radiation-survived MCF-7 cells (MCF-7/IR6) and MPSEs pretreatment radiation-survived MCF-7 (MCF-7/MPIR6). Our findings demonstrate that MPSEs can be a sensitizer to chemotherapy and may lead to the identification of a new strategy for improving curative effects in breast cancer treatment.

Materials and methods

Chemicals

Doxorubicin (Dox), pirarubicin (THP), (±)-verapamil, MK-571 and novobiocin were purchased from Sigma-Aldrich (Singapore). Maprang Seed Extracts (97% purity by HPLC) were provided by the MSN Nature Solution Ltd., Thailand.

Establishment of resistant breast cancer cell sub-lines and culture conditions

The two resistant breast cancer cell lines were established from the parental cell known as MCF-7 and were used for two different types of treatment. The first sub-line, MCF-7 was exposed to 6 Gy x-rays (Linear Accelerator 6 MV, Primus; Siemens Healthineers, USA) and the surviving cancer cells (MCF-7/IR6) were collected after two months of exposure. The other sub-line known as MCF-7 was pretreated with 50 µg/mL MPSEs for twenty-four hours before being exposed to 6 Gy x-rays. The surviving cancer cells (MCF-7/MPIR6) were collected after four months of exposure. All cell lines were maintained as a monolayer attachment with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 95% air humidified atmosphere and 5% CO_2 at 37°C.

Chemo-resistant pattern determination by MTT assay

Briefly, 5×10^4 cells were seeded per well into 24-well plates and were cultured for twenty-four hours before being treated with various concentrations of doxorubicin. After forty-eight hours of treatment, 1 mg/mL MTT solution was added and was incubated for four hours to form formazan crystals. These crystals were later dissolved by DMSO. The absorbance was measured at 550 nm. Cell viability (%) = $(OD_1/OD_0)\times100$ was calculated, where OD_1 and OD_0 were the absorbance of treated and untreated cancer cells, respectively. The cytotoxicity curve was plotted and the concentration of doxorubicin required for inhibiting 50% of cell growth (IC_{50}) was determined.[19]

MDR gene expression by RT-PCR (Reverse Transcription -Polymerase Chain Reaction)

The total RNA was extracted with an E.Z.N.A.® Total RNA Kit I (OMEGA bio-tek, USA) and reverse transcription reactions were performed using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (TOYOBO, Japan) following the manufacturer's guidelines. PCR was performed in 10 µL of reaction mixture at 40 cycles for *ABCB1* and 30 cycles for *GAPDH, ABCC1*, and *ABCG2*. PCR products were resolved in 1.5% agarose gel that was stained with redsafe™

nucleic acid staining solution. *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control. The primer sequences are listed below.[20]

ABCB1 Forward primer: 5'- CTGGTTTGATGTGCACGATGTTGG - 3'
Reverseprimer:5'-TGCCAAGACCTCTTCAGCTACTG-3'
ABCC1 Forward primer: 5'- CAGATGACACCTCTCAACAAAACC - 3'
Reverse primer: 5'- GCTAGGGCACACACTAGGGCT - 3'
ABCG2 Forward primer: 5'- CATGTTAGGATTGAAGCCAAAGGC - 3'
Reverse primer:5'-TGTGAGATTGACCAACAGACCTGA-3'
GAPDH Forward primer: 5'- CACCATCTTCCAGGAGCGAGATC - 3'
Reverse primer: 5'- GTGGTGCAGGAGGCATTGCTGA - 3'

Cellular drug uptake and the MDR protein-mediated drug efflux function by spectrofluorometry

The rationale and validation of the experiment setup for monitoring the kinetics of pirarubicin uptake and MDR protein- mediated drug efflux function in cancer cells had been widely described.[21, 22] In order to initiate the monitoring process, 2×10^6 cells were incubated in two mL HEPES-Na^+ buffer pH 7.30 in the presence of 10 mM NaN_3 for thirty minutes. The absence of glucose was necessary in order to off-function MDR proteins since there is no energy. The solution was placed in a 1 cm quartz cuvette that was continuously stirred at 37 °C. Next, 1 µM of pirarubicin was added and the fluorescence intensity at 590 nm (excited at 480 nm) was observed as a function of time by using a spectrofluorometer (Perkin Elmer Luminescence Spectrometer LS 50B). The initial rate of pirarubicin uptake was determined as a decrease in fluorescence intensity due to quenching after intercalation between base pairs of DNA. At steady state, 5 mM glucose was added to be the substrate of glycolysis to permit ATP production that led MDR proteins to re-function which can be observed as an increase in fluorescence intensity. The MDR protein-mediated drug efflux function could be determined as the slope of tangent, dF/dt after glucose addition.

To create condition for inhibition, the experiment was performed in the same manner as described above with notable differences. The difference was the addition of a specific inhibitor for MDR proteins to the cuvette five minutes before the addition of 5 mM of glucose. Verapamil, MK-571, and novobicin were used as a specific inhibitor for P-gp, MRP1, and BCRP, respectively.[23-25]

Statistical analysis

The presentation data is shown as a mean±SD. The difference of means was determined by two sample t-test with the *p* value =0.05, 0.01 and 0.001 for the entire data, using OriginPro 2018 software.

Results

MPSEs decrease chemoresistance of MRD

The chemoresistant pattern of MRD was determined by MTT assay. The cell viability at forty-eight hours after being exposed to doxorubicin (Figure 1a) in MCF-7/IR6 cells was highest, followed by MCF-7/MPIR6, with MCF-7 cell having the lowest viability. The IC_{50} value (Figure 1b) was 215±23.73 (RF=1.0), 342.95±30.94 (RF=1.6), and 282.75±24.64 nM (RF=1.3) for MCF-7, MCF-7/IR6, and MCF-7/MPIR6 cells, respectively. The Resistant Factor (RF) was determined by the IC_{50} of an irradiated cell line divided by IC_{50} of the MCF-7 parental cell line. The results show that MCF-7/IR6 cells were more resistant to doxorubicin than MCF-7/MPIR6 and MCF-7 cells. These findings were statistically significant (p<0.05).

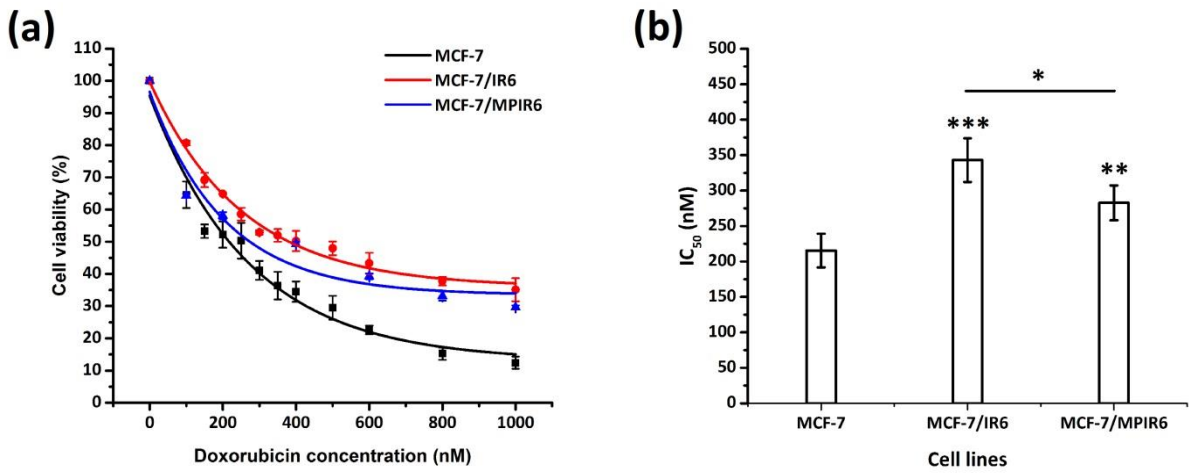


Figure 1. *MPSEs decrease chemoresistance of MRD. (a) Cytotoxicity curve depends on doxorubicin concentration, MCF-7 (black line), MCF-7/IR6 (red line), and MCF-7/MPIR6 (blue line). (b) IC50 value. Data is shown as mean±SD. *p<0.05, **p<0.01 and ***p<0.001.*

MPSEs decrease MDR protein-mediated drug efflux function of MRD

Pirarubicin, a substrate of P-gp, MRP1 and BCRP was used to monitor cellular drug uptake and MDR protein mediated-drug efflux function in MRD breast cancer cells by using spectrofluorometry.[23, 24, 26] The typical pirarubicin uptake and MDR protein-mediated drug efflux is indicated in Figure 2 (a, b, and c). The apparent rate of MDR protein-mediated pirarubicin efflux (V_a) can be determined from the slope of tangent after addition of glucose to the ATP-deprived cells and be calculated by the equation below;

$$V_a = (dF/dt)_t = {}_{glucose} \times (C_T/F_0)$$

Where $(dF/dt)_t = {}_{glucose}$ is the slope of tangent after glucose addition

C_T is the total concentration of pirarubicin (µM)

F_0 is the maximum intensity (a.u.)

As seen in Figure 2c, among the three cell lines, MCF-7/IR6 shows a higher active efflux of pirarubicin than MCF-7/MPIR6 and MCF-7. The efflux rate (V_a) obtained was about 0.0232±0.0001, 0.2308±0.0213 and 0.0678±0.0082 nM. s^{-1} for MCF-7, MCF-7/IR6, and MCF-7/MPIR6 cells, respectively (Figure 2d).

In addition, a specific inhibitor of MDR proteins present in MRD breast cancer (verapamil for P-gp, MK-571 for MRP1, and novobiocin for BCRP) was used to inhibit the MDR protein-mediated drug efflux functioning. MCF-7/IR6 was used as a model to test the inhibition effect of inhibitors because it has the highest potential of drug efflux function. The typical pattern of pirarubicin uptake while using inhibitors is shown in Figure 3a and the decrease in efflux rate was observed as a function of concentration. The efflux rate had indeed decreased. However, when using the two inhibitors (Figure 3b), verapamil and MK-571, there was no noticeable effect for novobiocin. Verapamil and MK-571 can decrease efflux rate about 30% and 20%, respectively at the concentration of 10 µM. MPSEs aberrant multidrug resistance gene expression of MRD

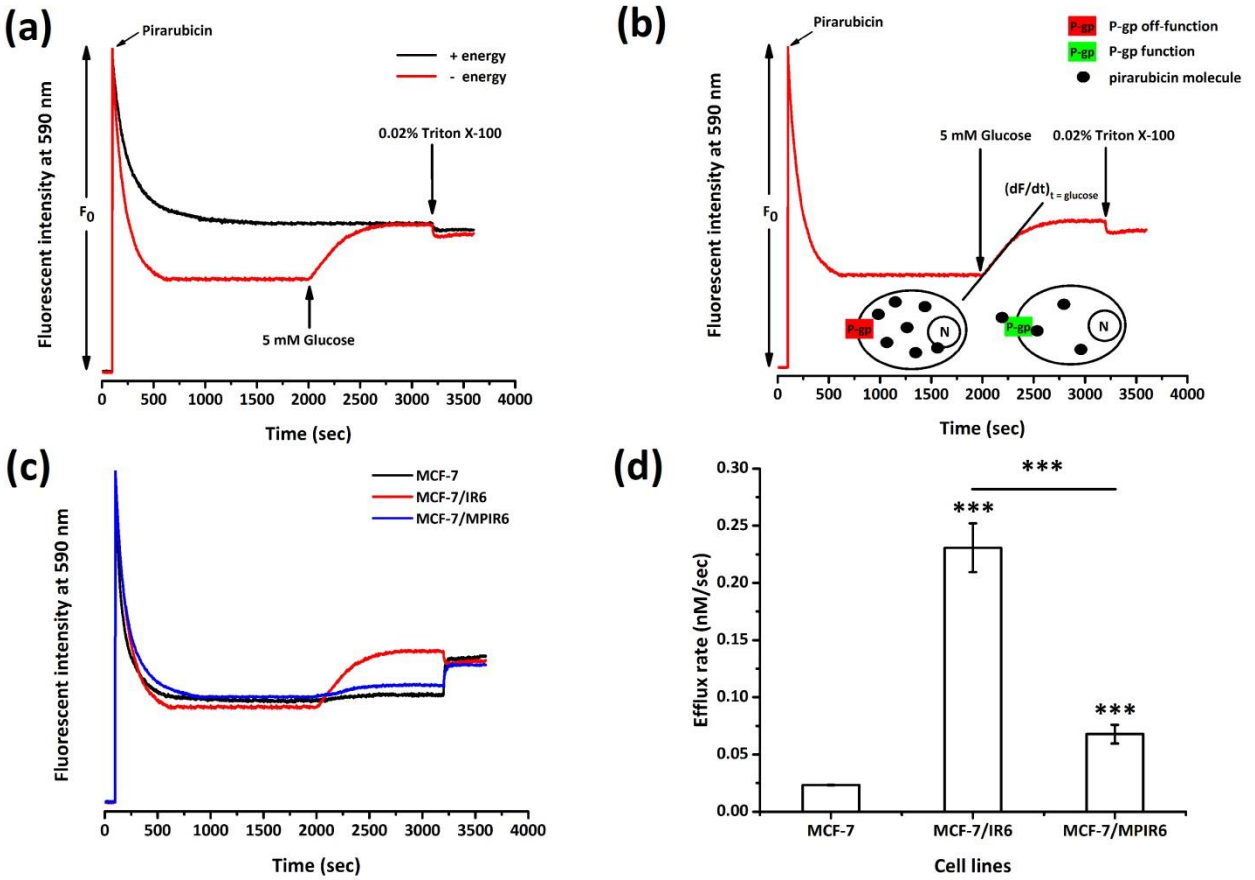


Figure 2. *Typical kinetics pattern of pirarubicin (THP) influx and efflux by resistant breast cancer cell, MCF-7/IR6. (a) Typical pattern of THP uptake by breast cancer cells in a condition with and without energy. F = fluorescent intensity at 590 nm (excited at 480 nm) observed as a function of time. After adding pirarubicin, the maximum intensity (F_{max}) and the C_T (concentration of pirarubicin that add into solution) were yield. F_0 was calculated from F_{max} – background. (b) At steady state, 5 mM glucose was added and the observing of an increasing in fluorescent intensity and slope of tangent, $(dF/dt)t={}_{glucose}$ was monitored. (c) The typical pattern of pirarubicin uptake in breast cancer cell. (d) The MDR protein-mediated drug efflux rate. Data was presented as mean±SD. *p<0.05, **p<0.01, ***p<0.001.*

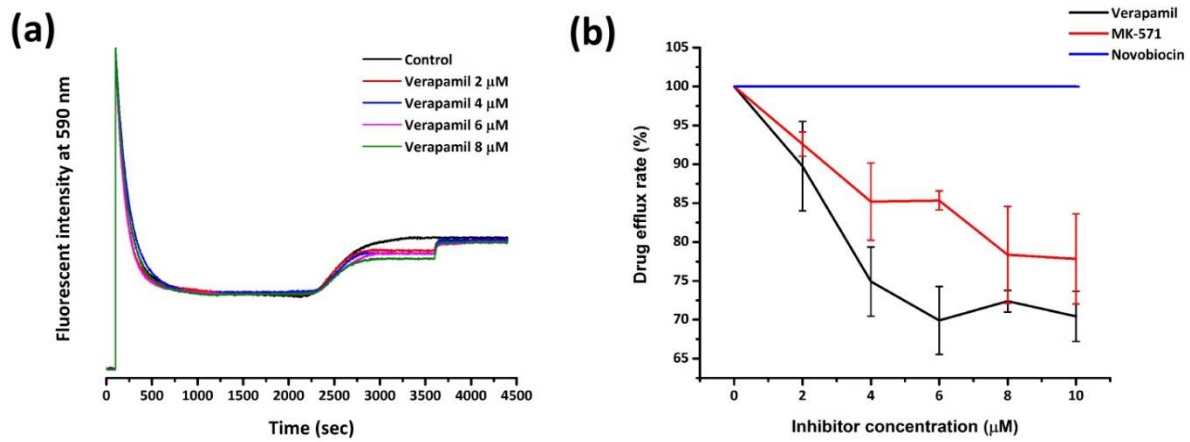


Figure 3. *(a) Typical kinetics of pirarubicin uptake by MCF-7/IR6 cell with verapamil inhibitor. (b) Effect of inhibitors, verapamil (black line), MK-571 (red line), and novobiocin (blue line) on the THP efflux rate of MCF-7/IR6 cell.*

It is well known that ABC transporters play an important role in drug efflux function by utilizing the ATP energy. The ABC transporters which help cancer cells resist against chemotherapeutic drugs are P-gp, MRP1, and BCRP which are encoded as *ABCB1*, *ABCC1*, and *ABCG2* genes, respectively. In this study, we found that two types of MRD (MCF-7/IR6 and MCF-7/MPIR6) were different in MDR gene expression patterns (Figure 4a). We determined that ionizing radiation at 6 Gy can diminish the mRNA expression of *ABCC1* and *ABCG2*, but also found that it modulated increasing expressions of *ABCB1* mRNA when compared with its parental cell (MCF-7). Additionally, the MDR mRNA expression pattern of MCF-7/MPIR6 was similar to that of MCF-7.

The relative MDR gene expression value is shown in Figure 4b. For *ABCB1* gene, this value was 1.00, 1.18±0.043, and 1.10±0.003 in MCF-7, MCF-7/IR6, and MCF-7/MPIR6, respectively. In addition, this value was 1.00 and 0.98±0.05 in MCF-7 and MCF-7/MPIR6, respectively for *ABCC1* genes. Finally, for *ABCG2* gene, this value was 1.00 and 1.02±0.07 in MCF-7 and MCF-7/MPIR6, respectively.

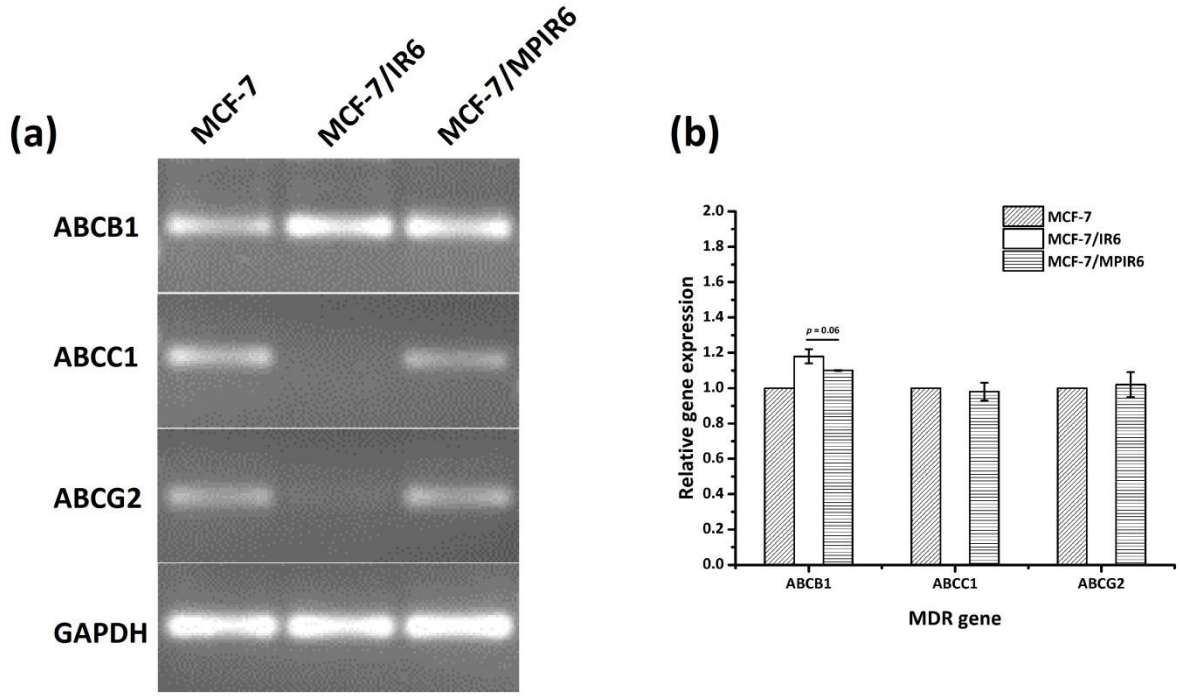


Figure 4. *ABCB1, ABCC1 and ABCG2 mRNA expression by RT-PCR. (a) MDR gene expression pattern of MCF-7, MCF-7/IR6 and MCF-7/MPIR6 cells. (b) Relative MDR gene expression.*

Discussion

Tumor metastasis and recurrence following primary treatment with conventional therapies may develop from the treatment resistant preexisting CSCs such as minimal residual disease or from cancer cells that acquire stemness capacities due to chemo/radiotherapy induced changes in the tumor and microenvironment.[27, 28] In this study, we reported that breast cancer cells MCF-7 that survived ionizing radiation treatment (MCF-7/IR6) have a chemoresistant phenotype by expressing high levels of MDR1 (*ABCB1*), but not MRP1 (*ABCC1*) or BCRP (*ABCG2*). Overexpression of MDR1 was associated with the high activity of P-gp pump and poor response to doxorubicin that is found in MCF-7/IR6 when compared to its parental cells MCF-7. We found that pretreatment of MCF-7 with MPSEs before irradiation has a reversal effect on chemoresistance phenomenon of survived cells (MCF-7/MPIR6). The results detected by RT-PCR indicated that MCF-7/MPIR6 cells decreased the P-gp mRNA (*ABCB1*) expression yet still expresses MRP1 and BCRP mRNA the same way as its MCF-7 parental (Figure 4a). It should be noted that MPSEs can suppress chemoresistance phenomenon of MCF-7/IR6 cells.

Maprang seed extracts (MPSEs) from the Thai fruit *Bouea macrophylla* Griffith exhibits anti-proliferative activity against drug-sensitive as well as drug-resistant leukemic and lung cancer cells. Previous studies suggest that ethanolic extracted MPSEs have more effect on drug-resistant leukemic (K562/adr) and lung (GLC4/adr) cancer cells, although the cells selected exhibited an overexpression of P-gp and MRP1.[17] However, it is unclear whether MPSEs can actually affect drug-resistant cancer cells. In addition, several studies have shown the reversal effects of polyphenol in drug-resistant cell lines, which is a polyphenol suppressed P-gp expression that functions as the main reason for the reversal effect.[29, 30]

We investigated the MDR protein-mediated drug efflux function and found that MCF-7/IR6 has the highest potential drug efflux function, while MCF-7/MPIR6 has a lower rate of extrude drug. In addition, we have investigated the expression level of MDR genes (*ABCB1*, *ABCC1* and *ABCG2*) to confirm MDR proteins function. The MDR protein mediated efflux pump was identified as ATP-binding cassette (ABC) transporters. Among them P-gp is the most important ABC transporter. Notably, another three eminent ABC transporters that were attributed to anticancer drug uptake and efflux such as MRP1, MRP3, and BCRP do not correlate as closely as P-gp with chemoresistance phenotype.[31] We demonstrated that radiation diminished *ABCC1* and *ABCG2*, but increased *ABCB1* expression in MCF-7/IR6 cells. Also, MCF-7/MPIR6 that had completely expressed three types of MDR genes including *ABCB1*, *ABCC1*, and *ABCG2* had lower regulation of *ABCB1* than MCF-7/IR6 coincident with the efficiency of drug efflux function that was significantly lower than that of MCF-7/IR6. Interestingly, we found that the MCF-7/MPIR6 cells that survived from irradiation in the presence of MPSEs show the same genetic profile as parental cells MCF-7 (Figure 4a).

To further confirm multidrug resistance protein function, we used specific inhibitors to test their activity in MCF-7/IR6 cells. Verapamil, a specific inhibitor of P-gp proteins, significantly decreased the drug efflux function of MCF-7/IR6 cells that showed an overexpression of *ABCB1* genes which encoded the expression of P-gp proteins and plays a key role in chemoresistance. Surprisingly, we found that in the presence of specific inhibitors of MRP1 protein (MK-571), MCF-7/IR6 also exhibit decreased drug efflux function. According to the mRNA expression profile we did not observe MRP1 mRNA expression in MCF-7/IR6 cells. It should be noted that ABC transporters such as P-gp, MRP1 and BCRP co-expressed in tumors show a broad and overlapped specificity for substrates and MDR modulators.[32] However, further studies are needed to confirm this underlying mechanism.

Our findings demonstrate that MPSEs could have ability to overcome chemoresistance phenomenon in cancer cells that had survived irradiation treatment. We found that pretreatment with MPSEs before irradiation treatment altered the resistance features of survived-cancer cells after normal treatment and that these phenomena were accompanied by a reversal in P-gp mRNA expression. Furthermore, our results also suggest that breast cancer cells that survived ionizing radiation treatment have a chemoresistant- and radioresistant phenotype.

Conclusion

Maprang Seed Extracts (MPSEs), the natural product extract from the Thai fruit *Bouea macrophylla* Griffith, can suppress chemoresistance properties of breast minimal residual diseases through prevention of ionizing radiation induced upregulation of *ABCB1* gene expression and protein function. Therefore, MPSEs might be used in combination with conventional therapy as a novel strategy for improving the curative effects in breast cancer treatment.

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Conflicts of interest statement

The authors declare no conflict of interest.

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