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Pichia-Expressed Recombinant D6 and DARC Negatively Affect Cell Migration and Invasion of Breast Cancer Cells

(Rekombinan D6 dan DARC Hasilan *Pichia* Mempengaruhi Migrasi Sel dan Serangan Sel Kanser Payudara secara Negatif)

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ABSTRACT

Atypical chemokine receptor proteins are termed 'decoy proteins' as their binding to the respective ligands does not lead to a typical signaling pathway but intercepts the action of chemokines. This method of chemokine activity regulation may also function in tumor suppression. D6 and DARC (Duffy Antigen Receptor for Chemokines) have been reported as decoy chemokine receptors in cancer studies. Purified Pichia*-expressed D6 and DARC, produced in-house, were used in cell-based studies to test their biological activities. Cell viability tests showed that recombinant D6 and DARC did not affect cell viability significantly, suggesting that they were not involved in breast cancer cell death. Wound healing assays showed that the presence of recombinant D6 or DARC at 10 µg/mL optimally inhibited the migration of breast cancer cells. ELISA showed an inverse relationship between the recombinant proteins and CCL levels in the treated cells. Migration assay using Boyden chamber demonstrated the function of the recombinant proteins in inhibiting chemotaxis activity of treated cells. Invasion assay showed the ability of the recombinant proteins in inhibiting the invasion property of treated cells. Comparison of single and combinatorial effects of the recombinant proteins showed that the combination of D6 and DARC at a 1:1 ratio (10 µg/mL) is most effective in reducing CCL levels and inhibiting the migration and invasion of treated cells. It was shown that the purified* Pichia*-expressed recombinant D6 and DARC are the negative regulators of breast cancer cell migration and invasion, and the inhibition effects were greater when they were used in combination.*

Keywords: Breast cancer cells; CCL2; cell migration and invasion; D6; DARC

ABSTRAK

Protein reseptor kemokin atipikal disebut 'umpan protein' kerana pengikatannya dengan ligan masing-masing tidak membawa kepada jalan isyarat yang khas tetapi memintas tindakan kemokin. Ia merupakan satu kawalan aktiviti kemokin dan boleh berfungsi dalam penyekatan tumor. D6 dan DARC telah dilaporkan sebagai reseptor kemokin umpan dalam kajian kanser. D6 dan DARC ekspresi Pichia *yang dihasilkan di makmal telah digunakan dalam kajian berdasarkan sel untuk menguji aktiviti biologinya. Ujian daya hidup sel menunjukkan bahawa rekombinan D6 dan DARC tidak mempengaruhi daya maju sel secara signifikan, menunjukkan bahawa mereka tidak terlibat dalam kematian sel barah payudara. Ujian penyembuhan luka menunjukkan bahawa kehadiran D6 atau DARC rekombinan pada 10 µg/mL menghalang penghijrahan sel barah payudara secara optimum. ELISA menunjukkan hubungan terbalik antara protein rekombinan dan tahap CCL pada sel yang dirawat. Ujian migrasi menggunakan ruang Boyden menunjukkan fungsi protein rekombinan dalam menghalang aktiviti kemotaksis sel yang dirawat. Ujian penaklukan menunjukkan kemampuan protein rekombinan dalam merencat sifat penaklukan sel yang dirawat. Membandingkan kesan tunggal dan gabungan protein rekombinan, gabungan D6 dan DARC pada nisbah 1: 1 (10 µg/mL) didapati paling baik dalam mengurangkan tahap CCL dan seterusnya menghalang migrasi dan penaklukan sel yang dirawat. Hasil kajian menunjukkan bahawa rekombinan D6 dan DARC hasilan* Pichia *bukan hanya pengawal negatif migrasi dan penaklukan sel barah payudara tetapi kesan perencatannya lebih besar ketika digunakan dalam gabungan.*

Kata kunci: CCL2; D6; DARC; migrasi dan penaklukan sel; sel payudara

INTRODUCTION

Breast cancer has been one of the most commonly occurring cancers in the world in recent years, with over two million new cases and 600,000 deaths in 2018 alone (Bray et al. 2018). In oncology study, breast cancer research had become one of the most progressively evolving fields. To date, with the advanced understanding of the key molecular features, breast cancer is no longer

considered a single disease but a combination of different subtypes with different biological behaviors and clinical outcomes (Sandhu et al. 2010). Novel molecules and new diagnostic methods are being discovered and developed globally. Recently, the identification of the various signaling pathways implicated in the cellular processes of breast cancer cells has drawn the attention of researchers worldwide. The involvement of growth factors or signaling molecules in breast cancer cell proliferation and invasion has also been reported (Ahmad et al. 2011; Cabioglu et al. 2009).

The expression of chemokine receptors has been found to be restricted and specific in many cancer cells. Besides aiding in cell growth and survival, chemokine receptors also facilitate the characteristic patterns of metastasis (Slettenaar & Wilson 2006). Recently, many studies have reported findings on the atypical action of chemokine receptor proteins. These receptor proteins were termed 'decoy proteins' or 'scavenger proteins' since the binding of these proteins to their respective ligands does not lead to the typical signaling pathway but intercepts the respective pathway and neutralizes the action of chemokines. Hence, they are well-known as 'intercepting receptors', the decoy chemokine receptors that confiscate chemokines without activating the respective signaling pathway (Hansell et al. 2006; Wang et al. 2006; Wu et al. 2008). The binding of chemokines without triggering the G-protein signaling pathway is a way of regulating chemokine activity and may function as a tumor suppressor (Graham 2009). It has emerged as a general strategy in recent years to tune the actions of cytokines and growth factors.

To date, six types of atypical chemokine receptors (ACKR) have been reported and categorized, namely ACKR1 (formerly known as DARC), ACKR2 (also known as D6 or Chemokine-binding protein 2, CCBP2), ACKR3 (Cysteine-X-cysteine chemokine receptor 7, CXCR7 or RDC1), ACKR4 (C-C chemokine receptor 1, CCRL1), ACKR5 (CCRL2), and ACKR6 (Phosphatidylinositol transfer protein 3, PITPNM3 or Nir1) (Lokeshwar et al. 2020). Although chemokine decoy receptors differ from antibodies and small molecule receptor antagonists, they generally have broad specificity of ligands that are recognized by different receptors (Mantovani et al. 2006). For examples, DARC (currently known as ACKR1) binds angiogenic chemokines (Maryam et al. 2020) and was suggested to play a vital role in leukocyte recruitment in inflammatory diseases (Gencer et al. 2019), whereas D6 (ACKR2) is an atypical chemokine receptor with a non-redundant role in controlling inflammation and immunity by scavenging inflammatory chemokines (Saçmacı & Özcan 2020; Vacchini et al. 2020; Wilson et al. 2020). A single use of chemokine decoy receptor proteins, such as D6 or DARC, has been shown to neutralize the action of CCL2 *in vitro* (Wang et al. 2006; Wu et al. 2008). However, a combinatorial effect of D6 and DARC in invasive breast cancer cells and their effects on cell invasion and migration is yet to be explored. Thus, in this study, two types of decoy chemokine receptor proteins, *Pichia*-expressed recombinant D6 and DARC produced in-house, were introduced to the breast cancer cells, namely MDA-MB-231 and MCF-7. The effects of recombinant D6 and DARC on cell migration and invasion were investigated. They were used individually as well as in combination.

Materials and Methods

Cell culture and reagents

Invasive breast cancer cell line MDA-MB-231 (ATCC number: HTB-26) and non-invasive breast cancer cell line MCF-7 (ATCC number: HTB-22) were obtained from the American Type Culture Collection (ATCC; Manassas, USA). Both MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cells were maintained at 37 °C in a humidity-controlled incubator with 5% CO_2.

MTT assay

The MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay was carried out to determine the effects of recombinant D6 and DARC on the viability of MDA-MB-231 and MCF-7 cells, respectively. Both the cells were individually seeded in 96-well plates at a density of 1×10^4 cells per well and cultured at 37 °C in a humidity-controlled incubator with 5% CO_2. After 24 h of incubation, different concentrations of recombinant D6 and DARC (2, 6, 10, 20, 50, and 100 µg/mL) were introduced to the cells. Negative control wells of MDA-MB-231 and MCF-7 cells were treated with 0.5% (v/v) of dimethyl sulfoxide (DMSO). After 24 h of treatment, 20 µL of MTT solution (5 mg/mL in PBS) was added to each of the wells, and the plates were incubated at 37 °C in a CO_2 incubator for 4 h. Following removal of the medium, 100 µL of DMSO was added to each well, and the absorbance intensity was measured at 570 nm (650 nm as the reference or background wavelength) using a microplate reader. All MTT assays were performed in triplicates, and the results were expressed as a percentage of treated viable cells relative to untreated viable cells.

WOUND HEALING ASSAY

Wound healing assay was performed on 100% confluent cells in 24-well plates. Approximately 2×10^5 cells were seeded with fresh DMEM and incubated overnight at 37 °C in a 5% humidified CO_2 incubator. A sterile 10 µL micropipette tip was used to scratch the confluent cell monolayer to create a wound or cell-free gap approximately 1 mm wide. The cells were then rinsed with sterile phosphate buffer saline (PBS) pH 7.2 to remove detached cells and cell debris. Next, the cells were treated with different concentrations (2 µg/mL, 6 µg/mL, and 10 µg/mL) of recombinant D6 or DARC. The plate was finally incubated at 37 °C in a CO_2 incubator and monitored under a phase contrast microscope after 0, 6, 24, and 30 h. Images of cells were captured, and the wound gap of cells was analyzed using Image J 1.51p. Two-way ANOVA was performed to further analyze the data statistically.

DETERMINATION OF CCL2 EXPRESSION LEVEL WITH ELISA

Approximately 2×10^5 cells were seeded in 24-well plates with fresh DMEM and incubated overnight at 37 °C in a 5% humidified CO_2 incubator. Cells achieving 100% confluence were then treated with different concentrations (2 µg/mL, 6 µg/mL, and 10 µg/mL) of recombinant D6 or DARC. Negative control was cells without any treatment but added only with fresh DMEM. After 24 h of incubation, the cells were rinsed twice with sterile PBS pH 7.2. Then, 100 µL of M-PER reagent was added to each well and incubated at 37 °C in a CO_2 incubator for 10 min. The content of each well was centrifuged at 600 × g for 2 min, and the supernatant was collected for enzyme-linked immunosorbent assay (ELISA). For indirect ELISA, the 96-well plate was coated with the sample and incubated overnight at 4 °C. The plate was washed with PBS pH 7.2 and then blocked with 3% bovine serum albumin solution, with agitation at 300 rpm for an hour at 37 °C. Then, the plate was washed, and the primary antibody anti-CCL2 was added to the sample wells, followed by agitation at 300 rpm for 2 h at 37 °C. After washing, anti-IgG (secondary antibody) was added, and the plate was incubated for 1 h. Then, the plate was washed, TMB (3,3',5,5'-Tertramethylbenzidine) solution added, and the plate incubated for 30 min. The reaction was stopped by adding 100 µL of 1 M H_3PO_4, and the plate was read at 450 nm using a microplate reader.

MIGRATION ASSAY

Boyden chamber system (24 wells, transparent PET membrane of 8 µm pore size; Corning, United States) was used to perform the migration assay. Upon attaining 70% confluency, the cells were trypsinized and resuspended in serum-free DMEM containing different concentrations (2 µg/mL, 6 µg/mL, and 10 µg/mL) of recombinant D6 or DARC or a combination of both in different ratios. The cell suspension was added into each insert at a density of 2×10^5 of cells per well. A 10% FBS-containing medium was then added to the lower chamber that served as a chemoattractant. The cells in the 24-well migration plate were allowed 24 h to migrate across the 8 µm polycarbonate membrane, and then, the cells were fixed in 100% methanol (Fisher Scientific, United States) for 20 min. Methanol was then removed, and the insert was rinsed with PBS pH 7.2 before the membranes and cells were stained with 0.25% crystal violet (Sigma-Aldrich, United States) for 15 min. The inserts were rinsed twice with sterile PBS. The non-migratory cells remaining in the upper chamber were removed using a sterile cotton swab. Inserts were air-dried, observed under a phase contrast microscope, and images were photographed in randomly selected fields of view at 400× magnification. Finally, the bottom parts of the inserts were soaked in glacial acetic acid for 10 min at room temperature with intermittent gentle shaking to lyse the invaded cells and elute the crystal violet dye. The stained glacial acetic acid was then transferred to a 96-well plate, and OD_{570} was read using a microplate reader.

INVASION ASSAY

Corning® BioCoat™ Matrigel® Invasion Chambers (Corning, United States) with 24 wells was used for the invasion assay along with 12 inserts. Prior to use, the invasion plate was rehydrated with serum-free DMEM at 37 °C in a 5% humidified CO_2 incubator for 2 h. For invasion assays, cells were grown and treated as described in the migration assay section. An equal number of cells (1×10^5 cells) in 300 µL serum-free DMEM were loaded into the Matrigel precoated chambers. Then, 600 µL of DMEM supplemented with 10% FBS was placed in the lower compartment of the chamber as the chemoattractant. After allowing 24 h for invasion, cells were fixed, stained with 0.25% crystal violet, and quantified as previously described after the non-invading cells were removed with cotton swabs.

STATISTICAL ANALYSIS

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS Inc., USA) statistical software and GraphPad Prism 7.0 (GraphPad, USA). The statistical significance was analyzed by one-way ANOVA with post hoc Duncan test with only one independent variable in the respective

experiments. On the other hand, two-way ANOVA with Tukey post hoc test was performed to analyze the result of the experiments with two independent variables. Experiments were performed in triplicates, and data were reported as the mean ± standard deviation (SD). Statistical significance was determined at a probability value of <0.05. Means with different letters represent a significant difference (p <0.05). Standard deviation was calculated and presented as an error bar on graphs.

Results

MTT assay

The cytotoxic effect of the purified yeast-expressed recombinant D6 and DARC on MDA-MB-231 and MCF-7 cancer cells was investigated to determine the non-cytotoxic concentration of the recombinant proteins for further experiments. Figure 1(a) showed that after 24 h of treatment with varying concentrations of recombinant D6, ranging from 2 µg/mL to 100 µg/mL, the viability of MDA-MB-231 and MCF-7 cells was not significantly affected compared to the non-treated cells. Similarly, Figure 1(b) illustrated that recombinant DARC did not affect the cell viability of MDA-MB-231 and MCF-7 cells. The viability of both cancer cell lines was observed to remain above 50% even after treatment with 100 µg/mL of recombinant DARC protein.

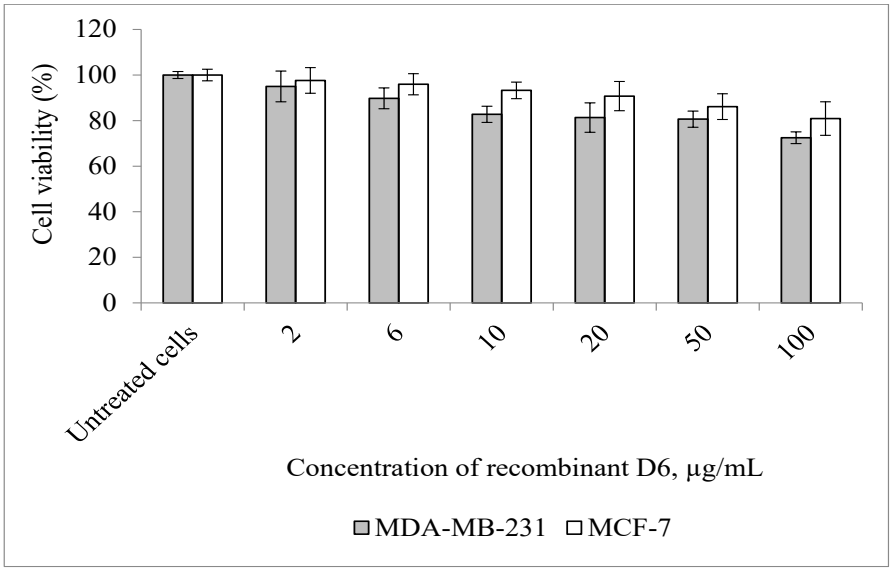


FIGURE 1(a). Effect of purified recombinant D6 on the viability of selected breast cancer cell lines after 24 h of treatment

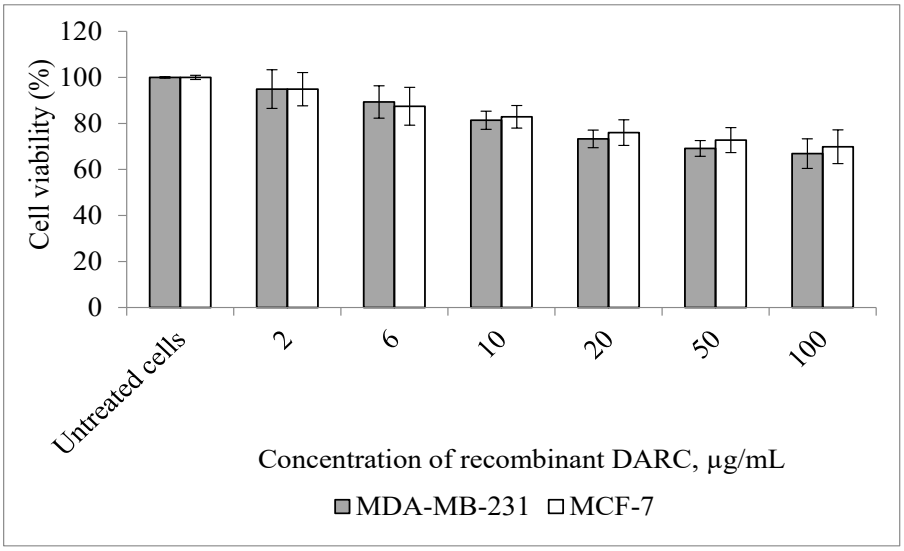


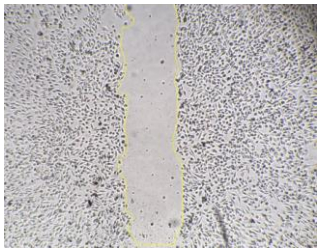
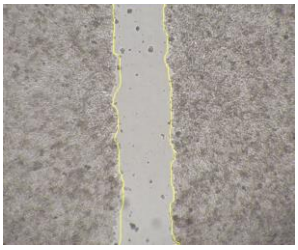
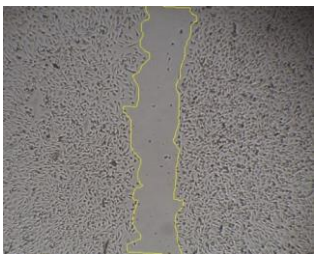
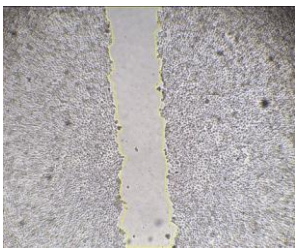
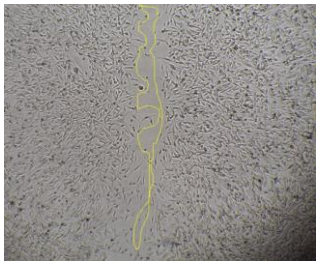
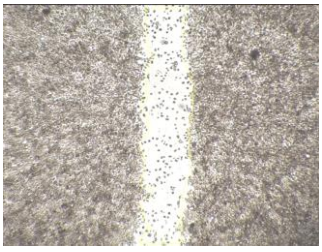
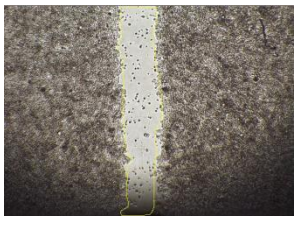
FIGURE 1(b). Effect of purified recombinant DARC on the viability of selected breast cancer cell lines after 24 h of treatment

WOUND HEALING ASSAY

The effects of recombinant D6 and DARC proteins on cell migration were first evaluated qualitatively by wound healing assay. In wound healing assay, cell migration, or in other words the movement of cells into the wound area, was an indication of wound recovery. The wound gaps were observed after 6 h, 24 h, and 30 h post-treatment with selected recombinant proteins at fixed concentrations (Table 1). The area of the wound measured at indicated time points using ImageJ was compared with the untreated cells (Negative Control). Mean values of triplicates (three measurements on the wound) were presented, and standard deviations were reported as error bars in bar charts. Two-way ANOVA was performed to analyze the results. In this study, the concentration of recombinant proteins and incubation times are the two independent variables. A p value of <0.05 was considered statistically significant.

Figure 2(a) and Figure 2(b) showed the effects of recombinant D6 and DARC proteins on the migration of non-invasive MCF-7 cells, as assessed by wound healing assay. The untreated cells (Negative Control) healed after 30 h of incubation. Wound area for cells treated with 2 µg/mL, 6 µg/mL, and 10 µg/mL of recombinant D6 reduced in a time-dependent manner. Moreover, at every indicated time point (6 h, 24 h, and 30 h post-treatment), the wound gap of the treated cells was observed to reduce with the reduction of recombinant D6 dosage. The lower the dosage, the smaller the wound gap.

TABLE 1. Wound gaps observed at indicated time points after treatment with recombinant proteins

Incubation time (h)	MDA-MB-231 cells	MCF-7 cells
0		
6		
24		
30		

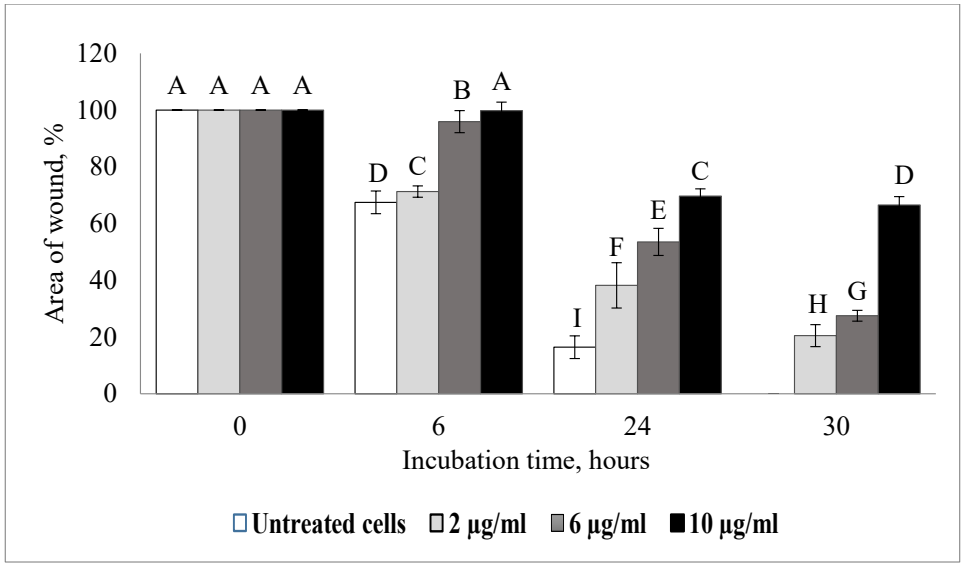


FIGURE 2(a). Effect of recombinant D6 on the migration of MCF-7 cells in wound healing assay

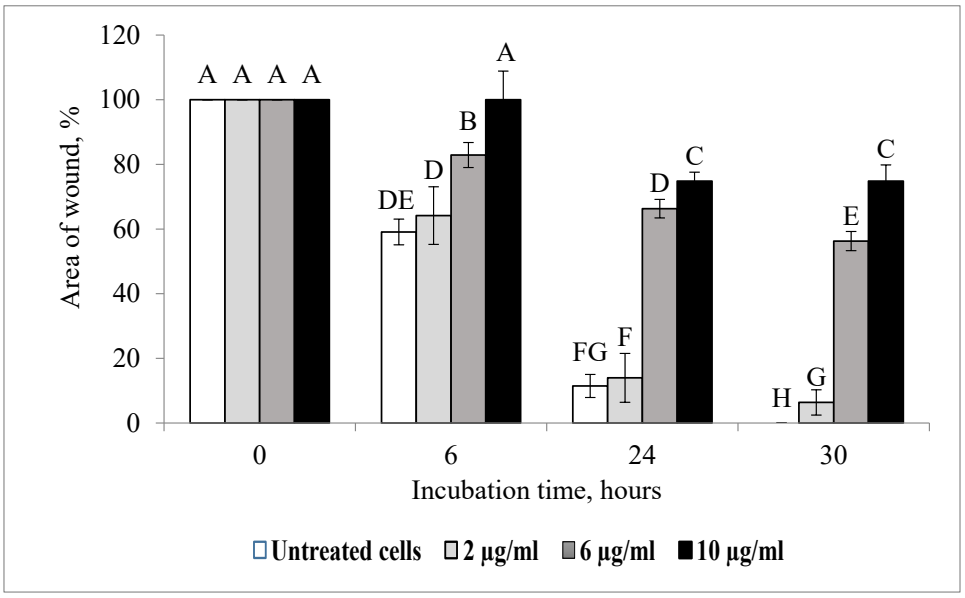


FIGURE 2(b). Effect of recombinant DARC on the migration of MCF-7 cells in wound healing assay

Figure 2(c) and Figure 2(d) showed the results of wound healing assays on MDA-MB-231 cells treated with recombinant D6 and DARC. As observed in Figure 2(c), no wound gap was seen on cells treated with 2 µg/mL and 6 µg/mL of recombinant D6 at 24 h of post-treatment. The result was the same with the Negative Control, the cells which were not treated with recombinant D6. When treated with 10 µg/mL of recombinant D6, the wound gap reduced as incubation time increased from 6 h to 24 h, and no wound gap was observed at 30 h post-treatment. Figure 2(d) reflected the effects of recombinant DARC at various concentrations on cell migration in MDA-MB-231

cells. Non-treated cells showed an aggressive movement of cells, as observed at 6 h, 24 h, and 30 h. The wound area of the non-treated cells was <50% at 6 h compared to 0 h. At 24 h, the wound gap was filled with migrated cells. For treatment with recombinant DARC at concentrations of 2 µg/mL and 6 µg/mL, the rate of migration was reduced compared to non-treated cells. At 6 h post-treatment, wound gaps of treated cells were larger compared to non-treated cells. However, the wound gap was observed to be filled at 24 h post-treatment. For cells treated with 10 µg/mL of recombinant DARC, the wound gap remained above 50% at 6 h post-incubation compared to 0 h. At 24 and 30 h, the wound areas remained visible, but the width of the wound gap was observed to be narrower with increased incubation time.

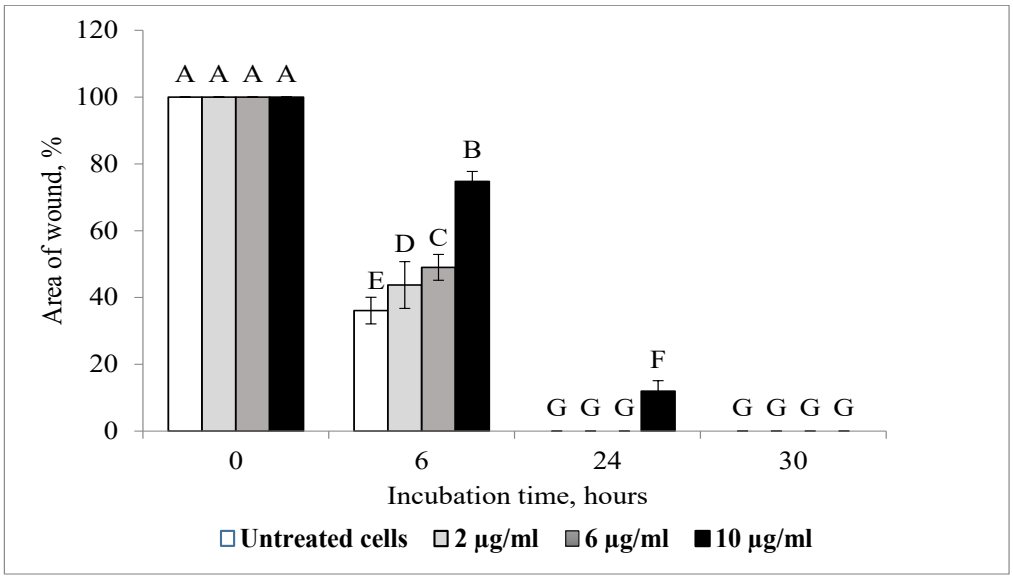


FIGURE 2(c). Effect of recombinant D6 on the migration of MDA-MB-231 cells in wound healing assay

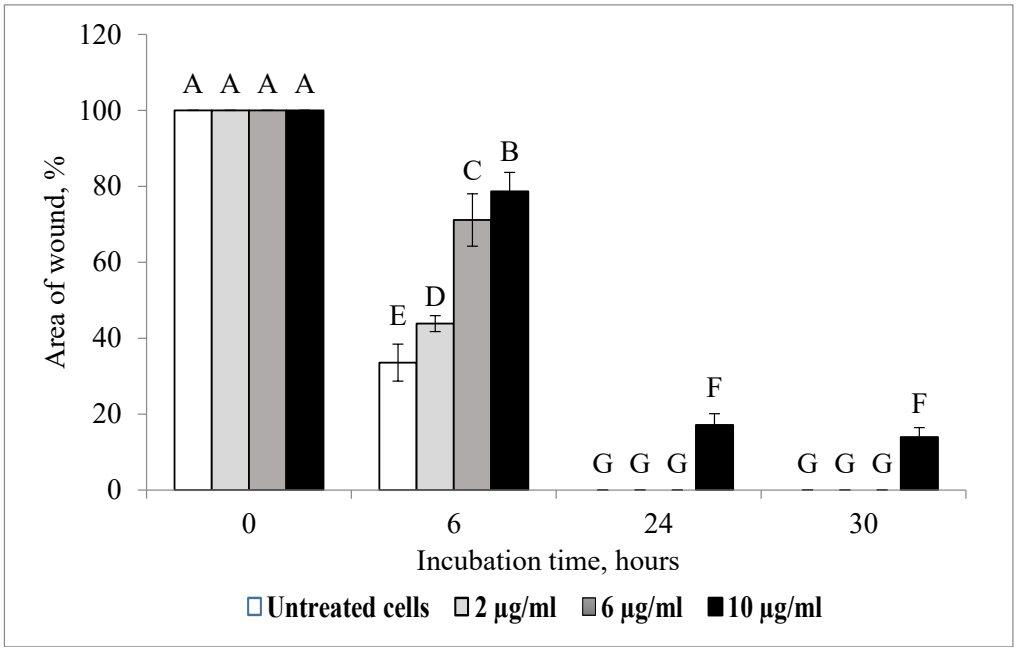


FIGURE 2(d). Effect of recombinant DARC on the migration of MDA-MB-231 cells in wound healing assay

After studying the individual effects of recombinant D6 and DARC on cell migration, the combinatory effect of recombinant D6 and DARC was evaluated by wound healing assay on MDA-MB-231 cells with MCF-7 cells as a background control. Recombinant D6 and DARC were combined at three different ratios, as illustrated in Figure 2(e). At 24 h post-treatment, non-treated MDA-MB-231 cells healed, while non-treated MCF-7 cells were observed to have a wound area as small as 16.10% compared to 0 h. Among the three different ratios of combinations, the wound gap of MDA-MB-231 treated with recombinant D6 and DARC at the ratio of 1:1 was observed to be the largest (16.99% compared to 0 h), followed by ratio of 2:1 and 1:2. However, every treatment group of MCF-7 cells was observed to have wound areas above 50% after 24 h post-treatment. Results showed that the combination of recombinant D6 and DARC at a ratio of 1:1 was the best among the three tested combination ratios.

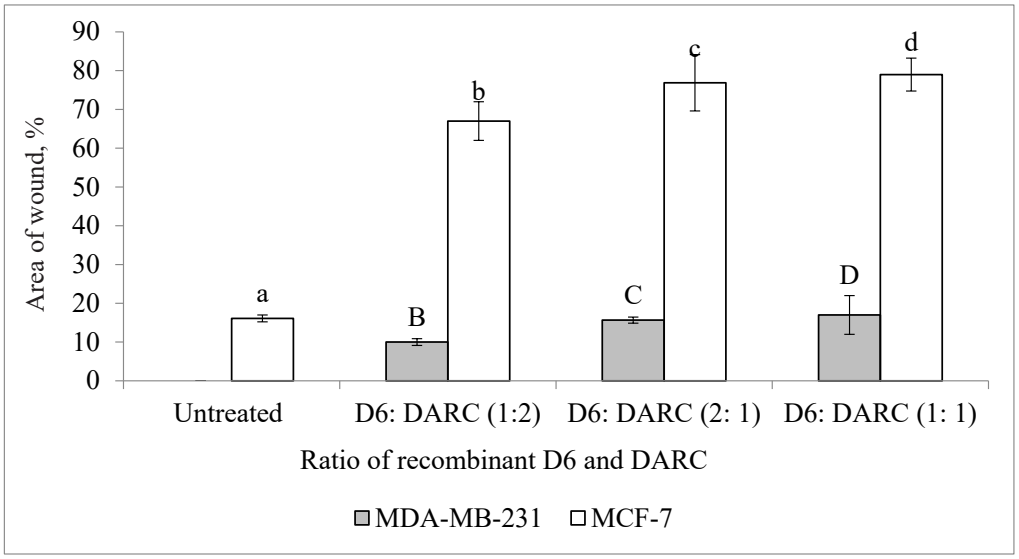


FIGURE 2(e). Effect of combination of recombinant D6 and DARC on the migration of MDA-MB-231 and MCF-7 cells in wound healing assay

DETERMINATION OF CCL2 EXPRESSION LEVEL WITH ELISA

In addition to wound healing assay, determination of CCL2 level was performed through ELISA to further justify the effects of recombinant D6 and DARC on cell migration. Figure 3(a) showed that the amount of CCL2 detected from MDA-MB-231 cells and MCF-7 cells decreased with the increased dosage of recombinant D6. The lowest CCL2 level was detected from cells (MDA-MB-231 and MCF-7) treated with 10 µg/mL of recombinant D6. Approximately 48.96% of CCL2 was obtained from MDA-MB-231 cells and 76.16% from MCF-7 cells after 24 h of treatment.

As shown in Figure 3(b), cells treated with recombinant DARC showed similar trends as the cells treated with recombinant D6. As the dosage of recombinant DARC increased, the amount of CCL2 obtained from MDA-MB-231 or MCF-7 cells decreased. Cells treated with 10 µg/mL of recombinant DARC expressed the lowest CCL2 level of 57.67% in MDA-MB-231 and 71.38% in MCF-7. In Figure 3(c), among the three different combination ratios of recombinant D6 and DARC, the lowest level of CCL2 was detected in MDA-MB-231 cells (55.99%) treated at the ratio of 1:1, followed by ratio 2:1 and 1:2. However, for MCF-7 cells, the lowest CCL2 level was detected for ratio 1:2 (40.76%).

MIGRATION ASSAYS

Migration assay was conducted using the Boyden chamber to quantitatively justify the effects of recombinant D6 and DARC on cell migration. A phase contrast microscope was used to capture images of the

cells that have migrated across the membrane barrier of the Boyden chamber at the studied time points. The migration ability of treated and non-treated cells was determined through absorbance measurement by a microplate reader. Figure 4(a) demonstrated the cell migration of treated and untreated cells after 24 h of treatment. Images of non-treated cells showed plenty of cells at the bottom of the Boyden chamber as untreated cells exhibited an aggressive phenotype and metastasized across the membrane barrier. On the other hand, treated cells were observed to show reduced migration ability after 24 h of treatment, and lesser cells were seen at the bottom of the Boyden chamber under the phase contrast microscope.

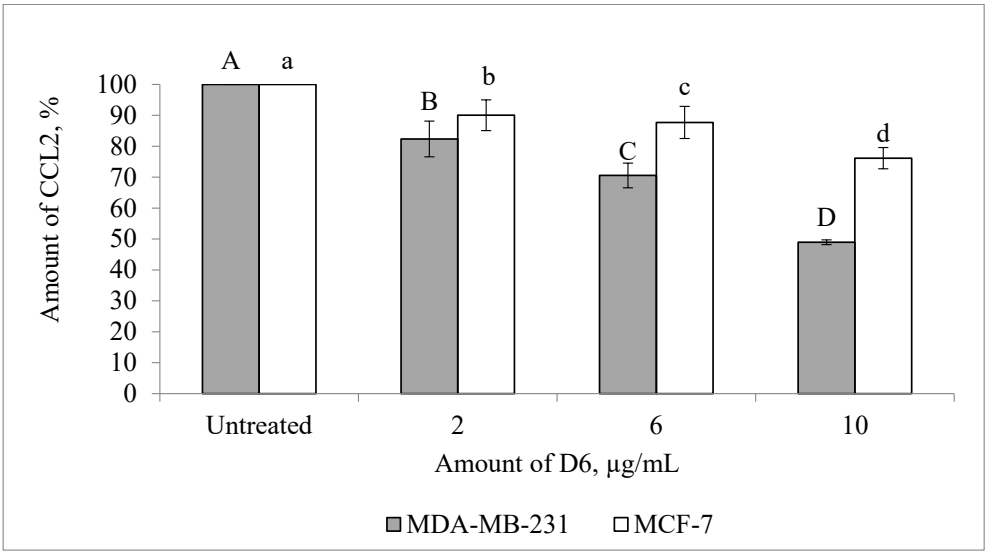


FIGURE 3(a). Effect of recombinant D6 on CCL2 expression level in MDA-MB-231 and MCF-7 cells

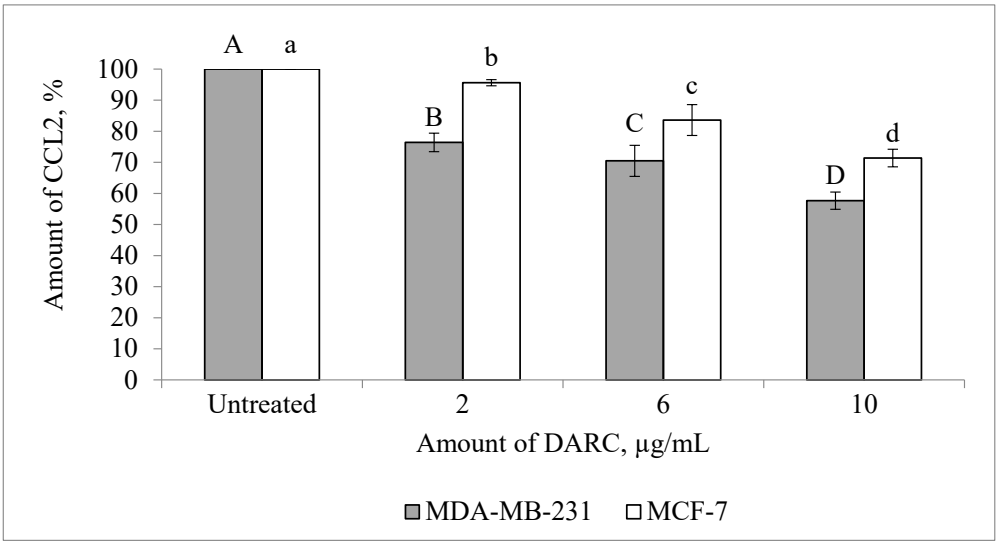
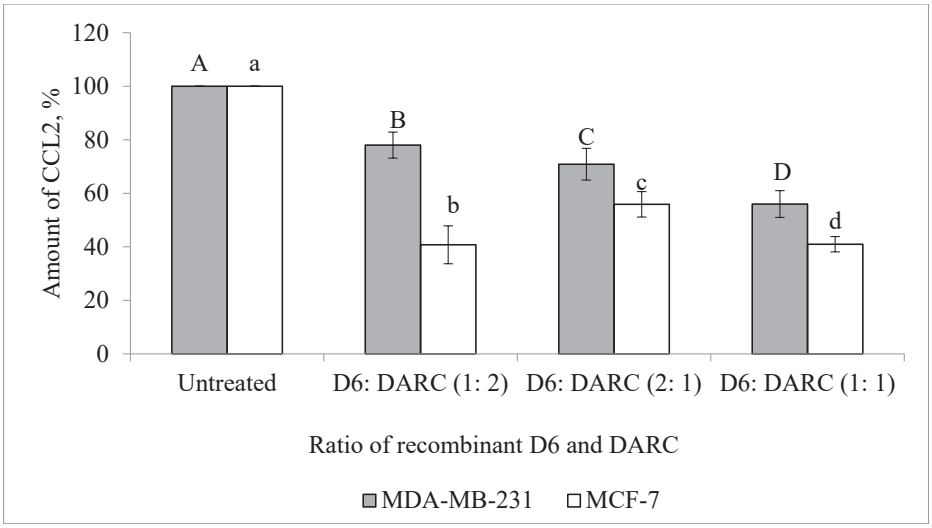


FIGURE 3(b). Effect of recombinant DARC on CCL2 expression level in MDA-MB-231 and MCF-7 cells



The experiments were carried out in triplicates and means with a significant difference are represented with different letters (Duncan, $p < 0.05$). Capital letters indicate the means for the amount of CCL2 from MDA-MB-231, whereas small letters indicate the means for the amount of CCL2 from MCF-7. Error bars indicate the standard deviation values of the triplicatesInvading cells are indicated by arrows

FIGURE 3(c). Effect of combination of recombinant D6 and DARC on CCL2 expression level in MDA-MB-231 and MCF-7 cells

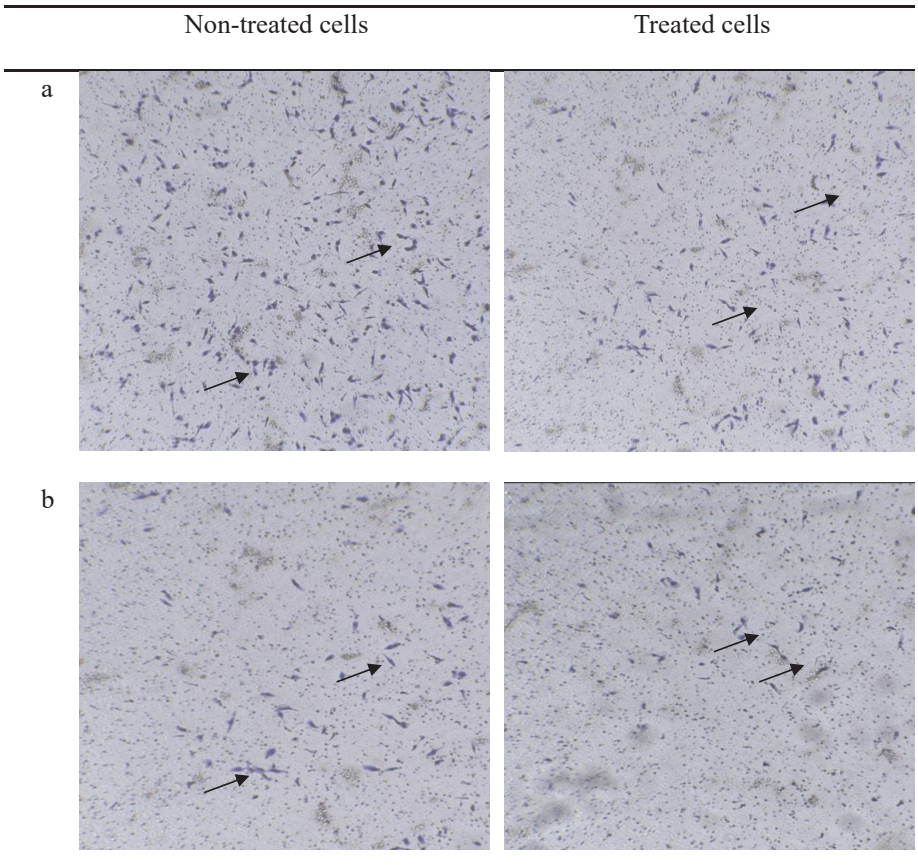


FIGURE 4(a). Cell migration images of (a) MDA-MB-231 & (b) MCF-7 under phase contrast microscope (400×magnification). Invading cells are indicated by arrows

The migration abilities of MDA-MB-231 and MCF-7 cells were shown to be affected by treatment with recombinant D6. Figure 4(b) showed that the number of migrated cells was negatively correlated with the concentration of recombinant D6. As the dosage of recombinant D6 increased in the cell medium, the migration ability of cells reduced. The least number of migrated cells was observed after treatment with 10 µg/mL of recombinant D6 (MDA-MB-231 cells: 65.97%; MCF-7 cells: 39.88%). Similar results were obtained after the treatment with recombinant DARC. Figure 4(c) depicted that the higher the concentration of recombinant DARC introduced to the cell medium, the lower the number of migrated cells detected at the bottom of the Boyden chamber. The lowest number of migrated cells was detected after treatment with 10 µg/mL of recombinant DARC, with around 60.77% for MDA-MB-231 and 29.09% for MCF-7. Figure 4(d) showed that the combination of recombinant D6 and DARC at the ratio of 1:1 showed the highest reduction effect on the migration of MDA-MB-231 and MCF-7 cells. The number of migrated cells treated with recombinant D6 and DARC at the ratio of 1:1 was significantly lower than untreated cells (Duncan, $p < 0.05$).

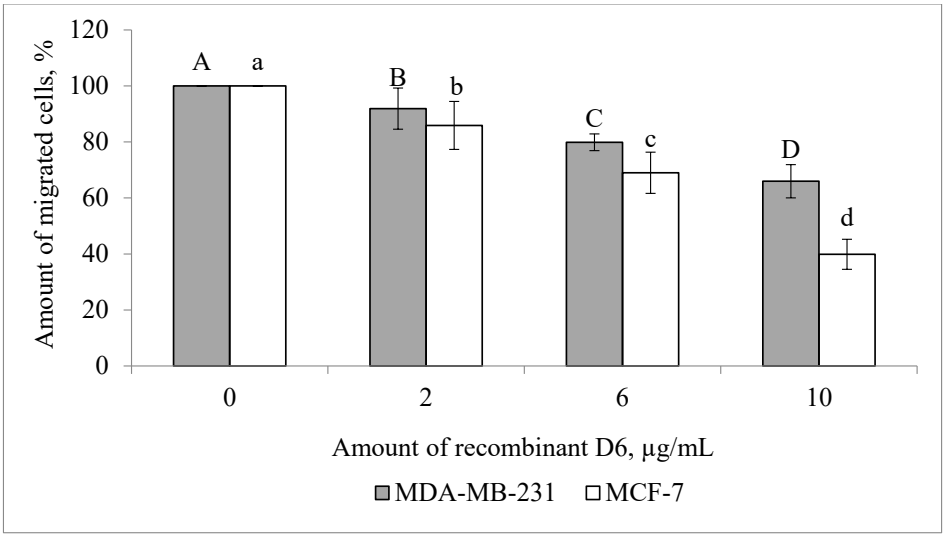


FIGURE 4(b). Effect of recombinant D6 on the migration of MDA-MB-231 and MCF-7 cells via Boyden chamber assay

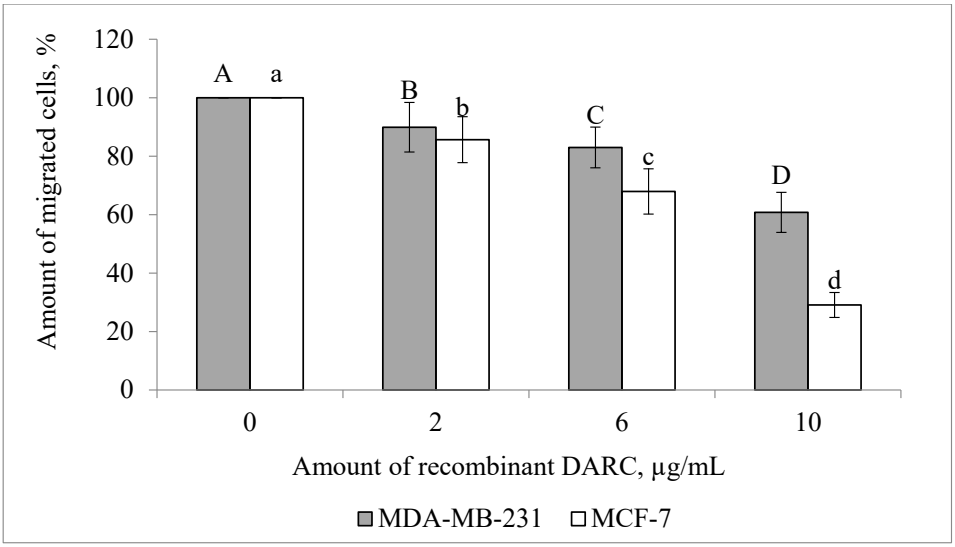
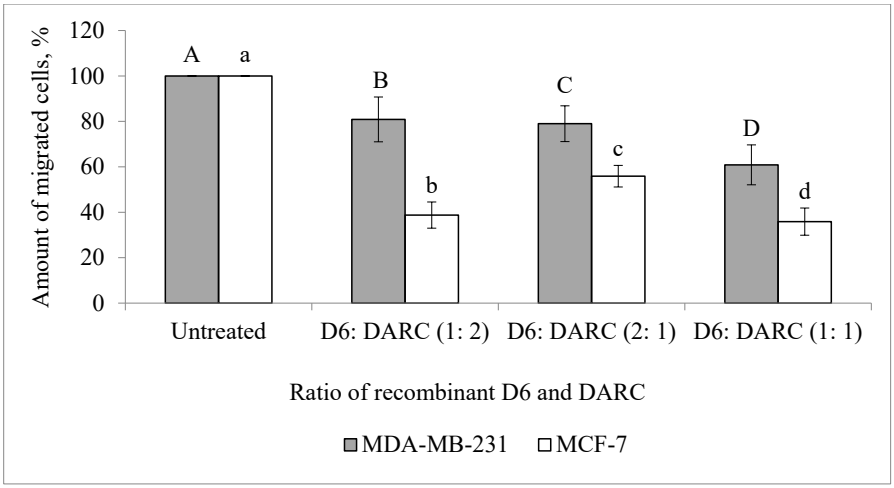


FIGURE 4(c). Effect of recombinant DARC on the migration of MDA-MB-231 and MCF-7 cells via Boyden chamber assay

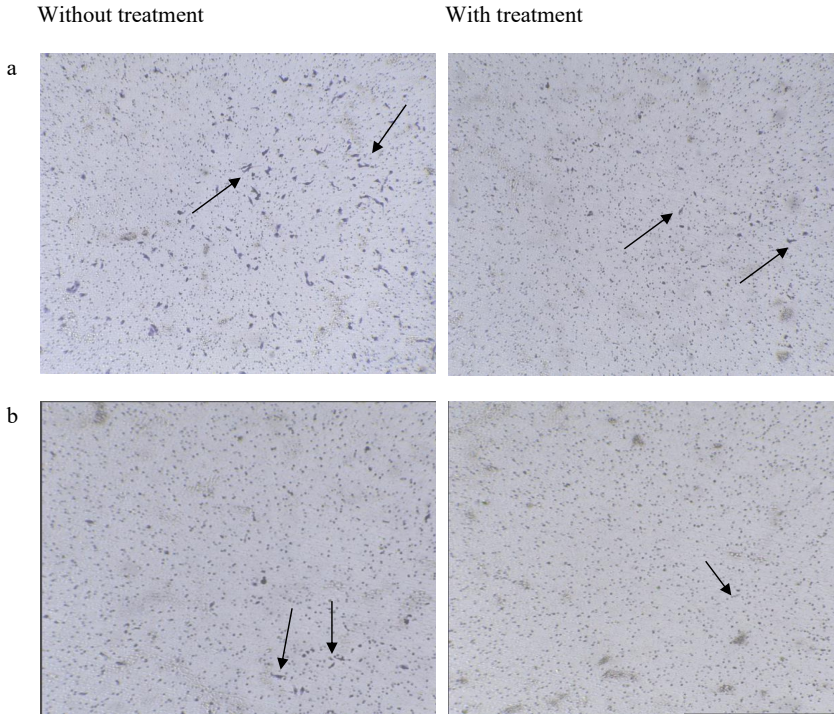


The experiments were carried out in triplicates and means with a significant difference are represented with different letters (Duncan, $p < 0.05$). Capital letters indicate the means for the number of migrated cells from MDA-MB-231, whereas small letters indicate the means for the number of migrated cells from MCF-7. Error bars indicate the standard deviation values of the triplicates

FIGURE 4(d). Effect of combination of recombinant D6 and DARC on the migration of MDA-MB-231 and MCF-7 cells via Boyden chamber assay

INVASION ASSAYS

The cell invasion and metastasis were evaluated using the Matrigel invasion assay. Figure 5(a) illustrated the cell invasion images of treated and untreated cells after 24 h of treatment. Phase contrast microscopy images of untreated cells showed plenty of cells at the bottom of the Boyden chamber, while treated cells showed reduced invasion ability after 24 h of treatment, with a lesser number of cells observed at the bottom of the Boyden chamber. Treatment with recombinant D6 or DARC showed a negative impact on the invading ability of the cancer cells compared to the non-treated cells. The



Invading cells are indicated by arrows

FIGURE 5(a). Cell invasion images images of (a) MDA-MB-231 & (b) MCF-7 under phase contrast microscope (400×magnification). Invading cells are indicated by arrows

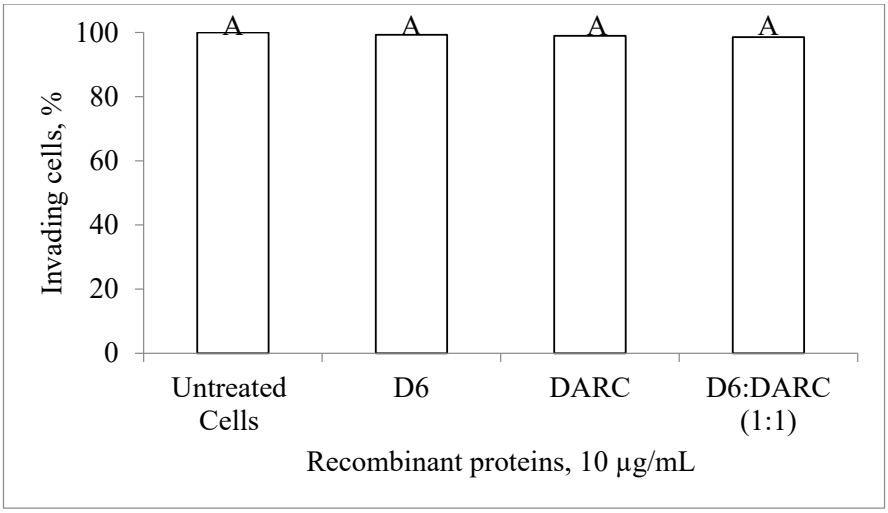
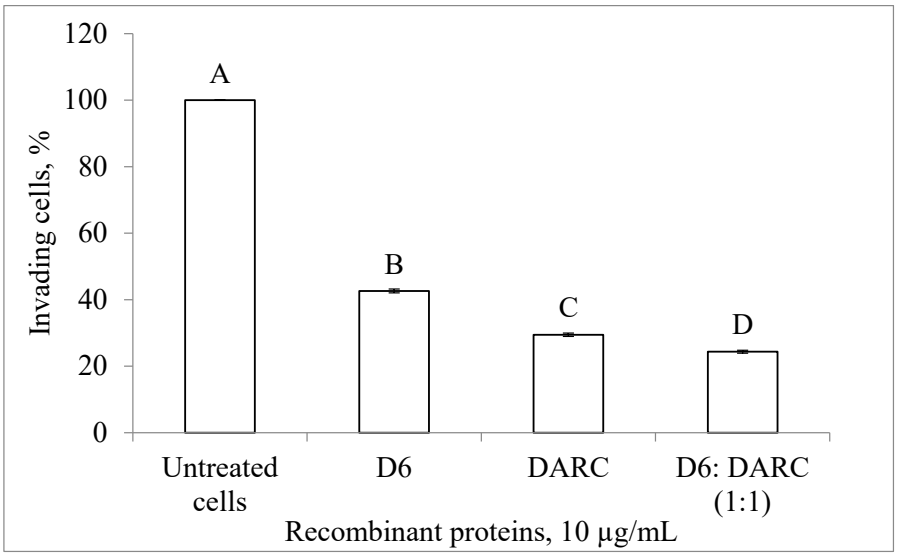


FIGURE 5(b). Effects of recombinant proteins on the invasiveness of MCF-7



The experiment was carried out in triplicates, and the means that are significantly different are represented with different letters (Duncan, $p < 0.05$). Capital letters indicate the means for the number of invading cells from MDA-MB-231, whereas small letters indicate the means for the number of invading cells from MCF-7. Error bars indicate the standard deviation values of the triplicates.

FIGURE 5(c). Effects of recombinant proteins on the invasiveness of MDA-MB-231

impact of treatment was further justified by absorbance measurements. Invading assay conducted with MCF-7 (Figure 5(b)) and MDA-MB-231 (Figure 5(c)) cells showed no significant difference among cells treated either with single recombinant protein or combination between two different types of recombinant proteins when compared to the untreated one.

DISCUSSION

The bioactivity and functions of purified *Pichia*-expressed recombinant D6 and DARC proteins were evaluated using *in vitro* cell-based assays to determine their effects on breast cancer cell migration and invasion. The wound healing assay performed to determine the role of recombinant D6 and DARC in cancer cell migration is an economical method. Apart from that, indirect ELISA was conducted to analyze the level of CCL2 in treated cells. The Boyden chamber-based assays were carried out to further investigate the influences of these recombinant proteins in cancer cell migration and invasion.

Prior to the migration and invasion studies, yeast-expressed recombinant D6 and DARC were tested on MDA-MB-231 and MCF-7 cells to determine their toxicity on the cells. The MTT analysis showed that the viability of MDA-MB-231 and MCF-7 cells was not significantly hampered by the presence of these recombinant proteins. The viability of these two breast cancer cell lines displayed a slightly decreasing trend with the increase in the concentration of the recombinant proteins. However, the reduction in viability was not significant compared to untreated cells, as it remained above 50% even after the addition of 100 µg/mL of recombinant proteins to the cells. The findings suggested that these recombinant decoy chemokine receptor proteins might not be involved in cancer cell death but act as silent receptors to inactivate the respective signaling pathways in breast cancer cells.

In cancer cells, there are numerous pathways leading to chemokine-induced cell migration and invasion, namely mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (P13K), focal adhesion kinase (FAK), and Rho family of GTPases. However, the general mechanism of cancer cell migration and invasion is almost similar (Chen et al. 2020; Raman et al. 2011; Tan et al. 2006; Zhao et al. 2008). In experimental cell biology, migration is defined as the directed movement of cells on a substrate. It can occur on two-dimensional surfaces without any obstructive fiber network. On the other hand, invasion is defined as the movement of cells through a three-dimensional matrix, which is followed by the restructuring of the three-dimensional environment. During invasion, cells change their shape and interact with the extracellular matrix to travel through the matrix (Kramer et al. 2013). In previous studies, the transfer of cancer cells to specific organs by chemokines chemotaxis properties was observed (Ben-Baruch 2008; Rezaeeyan et al. 2018).

Wound healing or '*in vitro* scratch assay' is popular in cell migration study as it is simple, versatile, technically non-demanding, and cost effective for the quantification of the alterations in cell migratory capacity due to experimental manipulations. This method allows the user to determine the migration ability of whole-cell masses. Besides the measurement of wound closure distance with time and comparing the readings to the control, it allows the user to observe specific migration changes or any impaired migratory phenotype that was previously unknown. It is a complex cellular and biochemical process needed for the restoration of the structurally damaged tissues. In the wound healing process, dynamic interactions and crosstalk between various cell types, interaction with extracellular matrix molecules, and the regulated production of soluble mediators and cytokines are involved (Grada et al. 2017). There are three phases in regulating wound healing, namely inflammation, proliferation, and remodeling (Zaja-Milatovic & Richmond 2008). Chemokines play a vital role in the wound healing process and were found to be involved in all three phases of the process (Raman et al. 2011). CCL2 is one of the chemokines that has been reported to improve the wound healing process by enhancing cell migration in studies conducted *in-vitro* with MCF-7 and MDA-MB-231 cells (Fang et al. 2012). CCL2 was also reported to promote wound healing after being induced by erythroid differentiation regulator 1 via MAP kinases *in vitro* and *in vivo* (Lee et al. 2020). It is one of the pro-inflammatory CC chemokines found in invasive and non-invasive breast cancer cells, and it was correlated with cancer cell migration and invasion (Wu et al. 2008). The controlled and specific expression of chemokine receptors plays a vital role in facilitating the characteristic patterns of cell migration and invasion, besides aiding in cell growth and cell survival (Slettenaar & Wilson 2006). The binding of chemokine receptor to its ligand induces signal transduction, direct chemotaxis, and subsequently, promotes invasive responses in cancer cells. However, many recent studies have reported that the atypical action of chemokine receptors, in which the binding of these proteins to their respective ligands does not lead to a typical signaling pathway, neutralizes the action of chemokines and intercepts the respective pathways. The absence of the well-conserved DRYLAIVHA motif (DKYLEIVHA in ACKR2) has been assumed to explain the inability of atypical chemokine receptors in inducing downstream receptor signaling after ligand binding (Sjöberg et al. 2019). The D6 and DARC decoy chemokine receptors had been reported to inhibit the action of CCL2 (Galzi et al. 2010).

Concurring with the findings by Wang et al. (2006) and Wu et al. (2008) on the ability of D6 and DARC to inhibit the action of CCL2, our study showed that

treating the cells with recombinant D6 and DARC reduced the effects of CCL2 in the cells, and thus, affected the recovery of wound or cell migration. The MCF-7 non-invasive breast cancer cells as background control were found to recover at 30 h of incubation after scratching. However, the introduction of recombinant D6 and DARC inhibited the migration of these cells, and a treatment dosage of 10 μg/mL appeared to reduce the migration of cells extensively. The wound gaps treated with 10 μg/mL of recombinant proteins D6 and DARC remained more than 60% open at 30 h post-treatment compared to 0 h. Besides comparing treated MDA-MB-231 cells with the non-treated one, the introduction of 10 μg/mL recombinant D6 and DARC was also found to significantly influence wound healing compared to lower concentrations. Untreated MDA-MB-231 cells were found to have a more aggressive migration in which complete closure of wound gap was observed at 24 h post-treatment compared to the untreated MCF-7 cells, which closed at 30 h post-treatment. D6 or ACKR2 was reported to bind with only CC pro-inflammatory chemokines. The lack of a DRYLAIV motif made D6 a decoy chemokine receptor; thus, the binding of D6 with its ligand will neither elicit signal transduction nor activate a cascade of signaling pathway (Stone et al. 2017). In fact, D6 acts as a scavenger protein and rapidly internalizes and degrades its ligands. Thus, the addition of purified recombinant D6 will compete with the signaling chemokine receptors to bind pro-inflammatory CC chemokines that facilitate breast cancer cell migration and invasion. Upon binding, the CC chemokines were internalized and degraded, which explains the reduction in the level of CCL2 in the treated cells in ELISA. The decrease in CCL2 level and other pro-inflammatory CC chemokines in the cells resulted in the reduction in the number of migrating and invading cells. Wu et al. (2008) had demonstrated that the overexpression of D6 in human breast cancer cells inhibited the proliferation and invasion of cells. This inhibition was associated with a reduction in chemokines, vessel density, and tumor-associated macrophage (TAM) infiltration. D6 inhibited tumor growth, metastasis, angiogenesis, and infiltration of TAMs by reducing the expression of chemokines.

On the other hand, the lack of a DRY motif made DARC another member of the decoy chemokine receptors. The binding of DARC with its ligand silences the action of the respective chemokine (Hansell et al. 2011). Similar to D6, the introduction of purified recombinant DARC silences the actions of CCL2 in cancer cells upon ligand-receptor binding, which explains the reduction of CCL2 level in ELISA. Nevertheless, different from D6, DARC binds both pro-inflammatory CC and CXC chemokines. Comparing the two recombinant proteins, recombinant DARC was observed to be more effective in inhibiting cell migration than recombinant D6 in MDA-MB-231 cells. The wound gap was observed to close completely at 30 h post-treatment with recombinant D6 but remained visible with recombinant DARC. The ability of DARC to bind and silence more than one category of pro-inflammatory chemokines in cancer cells well explains the differences observed between cells treated with D6 and those treated with DARC. As more pro-inflammatory chemokines were silenced, both chemotaxis and cell invasion were effectively downregulated. This explains why cells treated with DARC showed a bigger wound area, lower CCL2 level, and lower migrating and invading cell numbers in cell-based assays compared to cells treated with D6. Wang et al. (2006) showed that low DARC protein expression is associated with estrogen-receptor (ER) status, lymph node metastases (LNM), tumor multivessel density (MVD), distant metastasis, and reduced survival in human breast cancer samples. DARC overexpression inhibited tumorigenesis and metastasis, possibly by interfering with tumor angiogenesis. As previously mentioned, D6 binds CC chemokines and DARC binds CC and CXC chemokines. Hence, the combinatory use of D6 and DARC increased the competitive level of ligand binding among signaling receptor proteins and recombinant proteins to their respective ligands in the cancer cells' microenvironment. In the study with recombinant D6 and DARC combination, wound healing results showed that the combination of the two recombinant proteins at a ratio of 1:1 significantly affected wound recovery compared to the use of a single recombinant protein. Yu et al. (2015) reported a strong association between the co-expression of both DARC and D6 and relapse-free survival (RFS) in breast cancer patients. Their findings also implied that the chemokine decoy receptors might affect disease progression by influencing the tumor micro-environment but not the cancer cells directly.

Meanwhile, ELISA results showed a remarkable reduction of CCL2 level in treated invasive and non-invasive breast cancer cells, indicating a negative association between recombinant D6 or DARC with their ligands. The CCL2 level of the treated cells was found to decrease with an increase in recombinant D6 or DARC dosage. A drastic reduction in CCL2 level was observed in the cells treated with 10 μg/mL of recombinant D6 or DARC. The combination of recombinant D6 and DARC at a ratio of 1:1 effectively reduced CCL2 level in cancer cells and was more promising compared to treatment with a single recombinant protein. The combination of

D6 and DARC allowed the binding of more than one category of chemokine. Both pro-inflammatory CC and CXC chemokines can be 'captured' by the combination of recombinant decoy proteins. As recombinant D6 and DARC complemented each other in binding with their ligands, more pro-inflammatory chemokines were degraded or silenced. This caused an effective downregulation in signal transduction in cancer cells, and subsequently, reduced the number of migrating and invading cells. The quantitative ELISA results complemented the results from wound healing assays, which qualitatively suggested the negative effects of recombinant D6 or DARC on the migration of breast cancer cells. The role of these recombinant proteins in inhibiting cancer cell mobility was shown to be associated with the downregulation of CCL2 level in the cells. Other than CCL2, other chemokines are reported to be involved in cancer cells' migration and invasion, such as CCL5 and interleukin 6. Among pro-inflammatory CC and CXC chemokines, CXCL1, CXCL2, and CXCL3 were reported to be stimulated by CXCR2 receptor to stimulate the growth of melanoma cells (Dhawan & Richmond 2002). In breast cancer cells, other than CCL2, CXCL12 was another popular chemokine, which is reportedly involved in breast cancer cell metastasis. Upregulation of CXCR4, the receptor of CXCL12, was found to enhance breast cancer cells migration and invasion, which subsequently promoted lymph node metastasis (Allinen et al. 2004; Cabioglu et al. 2005). Fibroblast-derived CXCL14 was found to be involved in epithelial-to-mesenchymal transition (EMT) and metastasis of breast cancer cells. ACKR2 (D6) was identified as a critical mediator of CXCL14-induced signaling, although no direct interaction between CXCL14 and ACKR2 could be found (Sjöberg et al. 2019). Wang et al. (2006) investigated the tumorigenesis effects of DARC on MDA-MB-231 and MDA-MB-435HM breast cancer cells, which have a high capacity of spontaneous pulmonary metastasis, by transfecting these cells with a DARC expression vector using lipofectamine. Results showed that DARC overexpression led to tumorigenesis and/or metastasis inhibition, which was associated with reduced CCL2 levels. It was also associated with the expression of matrix metallopeptidase 9 (MMP-9) and tumor multivessel density (MVD) in xenograft tumors. The results showed that DARC overexpression inhibited tumorigenesis and metastasis of breast cancer, possibly via inhibition of angiogenic chemokines and subsequent sequestration of tumor neovascularity.

Malignant cancer cells invade and metastasize to nearby cells and tissues. Given the impact of yeast-expressed recombinant proteins D6 and DARC on cell migration (via wound healing assays) and CCL2 level in MDA-MB-231 and MCF-7 cells, further investigations were carried out to study the effects of these recombinant proteins on cell migration and invasion via Boyden chamber assay. Basically, the setup of transwell invasion assay is the same as transwell migration assay; the only difference is the coating of extracellular matrix (ECM) on the porous membrane of the insert. The purpose of coating ECM is to block the cells from migrating through the porous membrane to the lower chamber. Only cells which possess invasion ability will be able to invade ECM and migrate through the porous membrane. In this study, the transwell chamber, which was coated with Matrigel, was used to study breast cancer cell invasion. Matrigel is a type of gelatinous protein mixture that has been broadly used to simulate the ECM *in vitro* (Valle Oseguera & Spencer 2017). Matrigel transwells mimic extracellular matrix and enable the user to study the invasion of cancer cells. Membrane pore size is crucial for the active transmigration of cells in a transwell assay. Pore size should be smaller than the cell diameter to prevent unspecific dropping of cells from the upper chamber to the lower chamber. In this study, insert with a membrane pore size of 8 μm was used as it is suitable for MDA-MB-231 and MCF-7 cells. The Boyden chamber helps in analyzing the ability of single cells to directionally respond to the available chemoattractant. The growth medium in the upper chamber was serum-free, while the growth medium in the lower chamber contained FBS as the chemoattractant. The presence of chemoattractant in the lower chamber induced the migration of cells from the upper chamber towards the lower chamber. Cells migrate in the vertical direction through membrane pores and get attached to the bottom of the insert. Transwell migration assay differs from the qualitative wound healing assay and allows the migrated cells to be analyzed quantitatively. This makes the analysis more reliable, and it can be used to complement the results of wound healing assays. Besides, the availability of different cell culture inserts and sizes, as well as the ease of experimental setup, make transwell assay relatively useful.

Cell migration quantified in transwell assays showed that the combination of recombinant D6 and DARC at a ratio of 1:1 (10 μg/mL) significantly affected the mobility of MDA-MB-231 as well as MCF-7 cells compared to cells treated with a single type of recombinant protein (recombinant D6 or DARC). These observations suggested that D6 and DARC complement each other in inhibiting the migration of cells. This finding is similar to the results obtained from wound healing assay and is in agreement with ELISA results where CCL2 levels in the treated

cells were quantified. Invasion assay using Boyden chamber pre-coated with Matrigel demonstrated that the yeast-expressed recombinant D6 and DARC have negative effects on the invasion ability of breast cancer cells. Similar to the findings from wound healing assays, ELISA (CCL2 levels), and migration assays using Boyden chamber, invasion assays showed that the combination of recombinant D6: DARC at the ratio of 1: 1 (10 µg/mL) had the most significant impact on the invasion and metastasis ability of MDA-MB-231 and MCF-7 cells. Different from wound healing assay, instead of merely reporting about the negative effects of recombinant proteins on cell mobility, transwell invasion assay showed cell chemotaxis, or in other words, the directional movement of cells towards a chemoattractant (Justus et al. 2014). Thus, the current findings also showed that *Pichia*-expressed recombinant D6 and DARC decreased the chemotaxis ability of breast cancer cells. Quantitative results obtained from invasion assays justified and further confirmed the effects of *Pichia*-expressed recombinant D6 and DARC in inhibiting breast cancer cell migration and invasion. Invasion assay performed on MCF-7 cells showed no significant difference in invading cell numbers between treated and untreated cells. MCF-7 is a non-invasive breast cancer cell line, and the cells remained at the upper chamber of the transwell because of their inability to invade. In other words, the cells were blocked by the Matrigel and could not pass through the porous membrane of the insert.

Taken together, findings from all the assays conducted in this study suggested that purified *Pichia*-expressed recombinant D6 and DARC are bioactive and functional. The role of these recombinant proteins in decreasing CCL2 level and inhibiting the migration and invasion of breast cancer cells was well observed in the present study. The primary chemokine decoy receptors, D6 and DARC, confer their inhibitory effects on breast cancer cell proliferation and invasion mostly via the sequestration of pro-malignant chemokines (Yang et al. 2013; Yu et al. 2015). Other than being expressed in cancer cells, these chemokine decoy receptors were also present in blood and/or lymphatic endothelial cells and erythrocytes in the circulation. Hematogenous and lymphatic distribution are the two routes for breast cancer metastasis, and thus, chemokine decoy receptors also serve as a systemic barrier against such metastasis (Yu et al. 2015).

Conclusion

In summary, the use of purified *Pichia*-expressed recombinant D6 and DARC was observed to be capable of affecting cell migration, reducing CCL2 level, and inhibiting chemotaxis and invasion of breast cancer cells, as shown by wound healing assay, ELISA, and Boyden chamber-based migration and Matrigel invasion assay. The effects were found to be concentration-dependent. Since there was no previous study on the combinatorial effects of the two decoy proteins, our study proved to be interesting and vital in throwing some light on this aspect. The results showed that the combination of two different types of purified recombinant proteins had extensive negative effects on CCL2 level, cancer cell migration, and invasion compared to single proteins tested at the same concentration. This is the first *in vitro* study to report about the combinatory use of two different types of recombinant decoy chemokine receptor proteins in downregulating breast cancer cell migration and invasion. This study provides useful information about the effects of D6 and DARC on tumorigenesis and the metastatic potential of breast cancer cells and may lead to novel therapeutic strategies against breast cancer in the future.

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