

# The Cellular Activities of the Subfraction of Red Onion Peel Crude Ethanolic Extract in MDA-MB-231 Cells

Sin Pei Choe<sup>1</sup>, Poh Chiew Siah<sup>1</sup>, Shern Kwok Lim<sup>1</sup>, Kok Khiang Peh<sup>2</sup>, Ming Thong Ong<sup>1</sup>, Boon Yin Khoo<sup>1</sup>

<sup>1</sup>H53, Jalan Inovasi, Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 Penang, Malaysia; <sup>2</sup>J01, Lengkongan Teknologi, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

## ABSTRACT

**Aims:** This study aimed to evaluate the cellular activities of a subfraction (F1) of red onion peel crude ethanolic extract in MDA-MB-231.

**Materials and Methods:** The cell cycle profile and apoptosis induction in F1-treated MDA-MB-231 were measured using flow cytometry, and the migratory activity was measured using wound healing assay, Boyden chamber motility assay, and Matrigel chamber invasion assay, whereas other related protein expressions were measured using Western blotting.

**Results:** Our results demonstrated that the treatment of MDA-MB-231 with F1 reduced cell proliferation of the cells via inducing cell cycle arrest at S and G2 phases. F1 also possessed a better cell cycle arrest profile than camptothecin and executed better cell migratory effect in MDA-MB-231, which was likely via inhibition of the Akt signaling pathway in MDA-MB-231. **Conclusion:** F1 may have the potential to be developed as a nutraceutical agent that promotes anticancer effects or to be used in combination with other chemotherapy drugs to reduce the toxicity of drugs for MDA-MB-231-type breast cancer treatment.

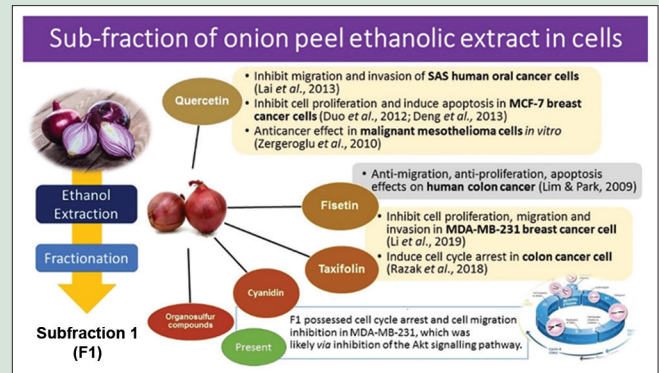
**Key words:** Akt signaling pathway, cell cycle, cell migratory activity, cellular activity, MDA-MB-231, red onion peel

## SUMMARY

This study aimed to evaluate the cellular activities of a subfraction (F1) of red onion peel crude ethanolic extract in MDA-MB-231. The results found that the treatment of MDA-MB-231 with F1 reduced cell proliferation and possessed a better cell cycle arrest profile than camptothecin, as well as executed better cell migratory effect in MDA-MB-231, which was likely via inhibition of the Akt signaling pathway. Therefore, F1 may have the potential to be developed as a nutraceutical agent for MDA-MB-231-type breast cancer treatment.

**Abbreviations Used:** FBS: Fetal bovine serum; CO<sub>2</sub>: Carbon dioxide; DMSO: Dimethyl sulfoxide; PI: Propidium iodide; USM: Universiti Sains

Malaysia; %: Percentage; DPPH: 2,2-diphenyl-1-picrylhydrazyl; AHR: Aryl hydrocarbon receptor.



## Correspondence:

Assoc. Prof. Dr. Khoo Boon Yin,  
H53, Jalan Inovasi, Institute for  
Research in Molecular Medicine  
(INFORMM), Universiti Sains Malaysia,  
11800 Penang, Malaysia.  
E-mail: boonyin@usm.my  
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## INTRODUCTION

Chemotherapy is the use of pharmacologic medication to hinder the development of intrusive cancer disease. Due to its systemic effects, chemotherapy remains the mainstay therapeutics for breast cancer treatment. The use of modern and standard chemotherapy regimens has enabled the application of chemosensitivity tests not only on chemotherapy-eligible patients, but also to those who have failed standard chemotherapy, including patients with highly advanced, prone-to-relapse, or chemoresistant tumors. Although chemotherapy helps to control cancer progression effectively, the toxicity of the chemotherapeutic agents imposed to healthy cells is high and causes a lot of side effects that led to discomfort in cancer patients, for example, vomiting, hair fall, nausea, loss of appetite, lowered resistance toward infection, and so forth.<sup>[1]</sup> Therefore, improving the effectiveness of standard chemotherapy regimens remains ongoing research work. The discovery of more viable therapeutic agents with bioavailable, safe, cost-effective, and minimal side effect properties is required with a closer inspection into several alternative medical treatments to find new solutions for patients by screening potential natural products that contain a high level of bioactive compounds.

In this study, we evaluated the cellular activities of a subfraction of red onion peel crude ethanolic extract (F1) in MDA-MB-231. The study strategies to seek high-quality quercetin-rich natural products that can potentially be developed as supplements for the single-agent treatment or be used in combination with other chemotherapeutic drugs. These natural products or supplements are expected to produce similar anticancer effects, but display less toxic and adverse effects, as well as cause less damage to healthy cells in cancer patients. The cellular activities of F1 were first evaluated using the approaches, as described below. The

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cell cycle profile and apoptosis induction of the cancer cells treated with F1 were evaluated using flow cytometry, whereas the migratory activity of the F1-treated cancer cells was evaluated using wound healing assay, Boyden chamber motility assay, and Matrigel chamber invasion assay. Migratory activity, in this study, refers to cell migration and invasion capacity of the treated cells. Complex migratory activity and process are the metastasis. The migratory activity-related gene protein expression in the treated cancer cells was also evaluated by Western blotting.

Red onion, which is rich in bioactive compounds and contains numerous pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, anti-hypertensive, and immunoprotective effects, has shown to have anticancer therapeutic values.<sup>[2-5]</sup> The products derived from onion are also shown to carry anticarcinogenic properties and with less toxicity. Numerous epidemiology researches also revealed that dietary intake of onion reduced the risk of developing breast cancer.<sup>[6,7]</sup> It has been demonstrated that quercetin is widely distributed in the outer skin and inner parts of the red onion and the content decreases toward the inner part of the bulb. Although a large number of *in vitro* and *in vivo* studies have been conducted to prove the anticancer property of red onion peel, the cell cycle profile, apoptosis induction, and migratory activity, as well as the related protein expressions, induced by the selected red onion peel extract's subfraction have not been studied in detailed. Further investigations are required to justify the potential of F1 as there is a need to investigate this natural product as new anticancer agents, though the preliminary phytochemical composition, antifungal, antibacterial, and cytotoxic activities had been evaluated in a previous study.<sup>[8]</sup> Indeed, the chemosensitivity of the test compounds that cannot be evaluated by studying patients can be predicted with this strategy.<sup>[9]</sup> The potential substance selection using this strategy may reduce false selection and accelerate the search for more potential anticancer agents from various natural resources to combat deadly human diseases.

## MATERIALS AND METHODS

### Collection, extraction, and fractionation of red onion peel

The red onion peel (*Allium cepa*) was collected from the Cameron Highlands, Malaysia. A sample of the peel was evaluated and deposited at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia (USM) (Herbarium No: USM. Herbarium. 11563). The peel was first dried at 40°C using a conventional oven for 4 days. Next, the peel was powdered using a blender and mixed thoroughly. The dried peel (20 g) was then extracted with absolute ethanol at a 1:10 (w/v) ratio for 48 h. After filtration with filter paper, the dried residue was re-extracted twice using the procedures as mentioned above. The pooled extracts were collected and vacuum dried at 40°C in a rotary evaporator. The dry extract was then transferred to a petri dish and further dried at 40°C in an oven to obtain the powdered form of the peel crude ethanolic extract (E1). This extract was further fractionized into the ethanolic subfractions by dialysis fractionation using SnakeSkin tubing (Pierce Biotechnology, Rockford, IL, USA) with a molecular weight cutoff of 3,500 Daltons against water at 5°C for 48 h. The dialysis water was collected and changed every 24 h. The solutions from the dialysis in the tube and outside the tube were collected as subfraction 1 (F1) and subfraction 2 (F2) of red onion peel crude ethanolic extract, respectively. The crude extract and subfractions were then freeze-dried. F1 was the main focus in this project, whereas E1 and F2 were used to compare the effects with F1 in measuring migration activity.

### Cell culture and stock preparation

The human breast MDA-MB-231 cell line was maintained with Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA, USA)

supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 U/ml of penicillin (Gibco, Carlsbad, CA, USA), and 100 mg/ml of streptomycin (Gibco, Carlsbad, CA, USA). MycoKill (1%; Gibco, Carlsbad, CA, USA) was added to this complete growth medium (contained 10% FBS) to prevent mycoplasma contamination. The growth medium of the cells was changed every 2–3 days, and the cells were incubated at 37°C in a humidified atmosphere of 5% (v/v) carbon dioxide (CO<sub>2</sub>). E1, F1, and F2, as prepared above, as well as camptothecin (97% purity; control drug; Sigma-Aldrich, St. Louis, MO, USA), were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) as 10<sup>-2</sup> M stock. All stocks were then further diluted to the working concentration with growth medium or stored at -20°C.

### Cell cycle profile and apoptosis induction analyses by flow cytometry

The MDA-MB-231 cell line, which was identified to show promising effects after F1 treatment, was used for subsequent experiments. First, MDA-MB-231 (1.0 × 10<sup>5</sup> cells/ml) were plated in T-25 flasks and then incubated in a humidified CO<sub>2</sub> incubator. After 24 h, the cells were synchronized in serum-free medium and treated with F1 at EC<sub>50</sub> value (~50 µg/ml) in assay medium (2% FBS) for 24, 48, and 72 h. After the end of each incubation period, the old-growth medium was collected in 15 ml tubes combined with trypsinized cell suspensions. The cell suspensions were centrifuged at 1,000 rpm for 4 min, and the supernatants were removed using pipettes. The cell pellets were suspended in PBS for cell counting in order to adjust the cell number to 1,000 cells/µl. Ice-cold 70% ethanol was added into the cell suspensions dropwise to fix the cells at 4°C overnight. Thereafter, the cell suspensions were centrifuged at 4,500 rpm for 10 min to remove the ethanol. The cell pellet was washed with 500 µl of PBS, followed by the addition of 500 µl of FxCycle<sup>™</sup> propidium iodide (PI)/RNase staining solution (Thermo Fisher Scientific, Waltham, MA, USA). The cell suspension was incubated in the dark for 30 min. The stained samples were transferred into a new sterile flow tube and kept on ice until subjected to flow cytometer analysis by BD FACSCanto<sup>™</sup> II (BD Biosciences, San Jose, CA, USA), following the parameters recommended by the manufacturer, at Advanced Medical and Dental Institute, USM. Cell cycle distribution was analyzed from a total of 15,000 events with CellQuest Software 3.3 (BD Biosciences, New Jersey, USA). The percentage (%) of cells in G1, S, and G2/M phases was calculated and plotted into the bar chart. The pellets of fixed cells (collected at 72 h of treatment) after removal of PBS were also added with 195 µl of binding buffer (×1) from the Annexin V-FITC Apoptosis Detection Kit (Thermo Fisher Scientific, Massachusetts, USA). After that, 5 µl of Annexin V-FITC was added to the cell suspension. The suspension was incubated in the dark at room temperature for 10 min. The cells were then centrifuged at 400 g for 4 min and washed with 200 µl of binding buffer again prior staining with 10 µl of 20 µg/mL PI. The samples were transferred into new sterile flow tubes and kept on ice until subjected to analysis, as described above. Cell death distribution was analyzed from a total of 15,000 events with CellQuest Software 3.3 (BD Biosciences, New Jersey, USA). The % of cells in viable, early apoptosis, later apoptosis, and necrosis phases was analyzed and calculated.

### Cell movement analyses by Wound healing migration and Boyden chamber motility assays

The wound healing migration assay was performed based on the repopulation of wounded culture. The MDA-MB-231 (1 × 10<sup>5</sup> cells/ml/well) were first seeded with growth medium in 24-well culture plates. The monolayer was then wounded using a 200 µl pipette tip. The wounded monolayer was incubated with growth medium containing 1 µg/ml, 10

$\mu\text{g/ml}$  and an  $\text{EC}_{50}$  value of F1 for 24 h. Photographs on the repopulation of wounded culture were then taken at 0, 12, and 24 h of incubation before the cells started dying. On the other hand, to perform Boyden chamber motility assay, chambers (Corning, New York, NY, USA) were placed in 24-well plates. The MDA-MB-231 ( $2 \times 10^4$  cells/well) were then resuspended in assay medium containing  $\text{EC}_{50}$  value of F1. The cell suspension was carefully transferred into the upper chambers of 24-well plates, whereas lower chambers were filled with growth medium to attract the cell transfer. The plates were incubated at  $37^\circ\text{C}$  in a humidified  $\text{CO}_2$  incubator for 24 h. Next, the cells on the upper surface of the chambers were removed by wiping with a cotton swab, whereas the filter of the chambers was stained with crystal violet stain solution (Sigma-Aldrich, St. Louis, MO, USA). Randomly, the number of treated MDA-MB-231 in five randomly selected microscopic fields ( $\times 200$ ) on the lower surface of the chamber (migrated cells) were counted as % over non-treated MDA-MB-231, which were defined as 100% invaded cells. Both assays were repeated using E1 and F2 to replace F1.

### Cell invasion analysis by Matrigel chamber assay

The Matrigel chamber assay was performed by filling the chambers (BD Biosciences, San Jose, CA, USA) with serum-free medium and was adapted at  $37^\circ\text{C}$  in a humidified  $\text{CO}_2$  incubator. The MDA-MB-231 ( $2 \times 10^4$  cells/well), which were suspended in assay medium containing  $\text{EC}_{50}$  value of F1, were then carefully transferred into the upper chambers; lower chambers were filled with growth medium to attract the cell transfer. The Matrigel matrix of the assay acted as the basement membrane, in which noninvasive cells could not penetrate the matrix. After incubating the Matrigel chambers for 24 h, the cells on the upper surface of the chambers were removed by wiping with a cotton swab and the lower surface of the chambers was stained with crystal violet stain solution (Sigma-Aldrich, St. Louis, MO, USA). The number of treated MDA-MB-231 in five randomly selected microscopic fields ( $\times 200$ ) on the lower surface of the chamber (invaded cells) were counted as % over non-treated MDA-MB-231, which were defined as 100% invaded cells. The assay was repeated using E1 and F2 to replace F1.

### Protein expression by Western blotting

Western blotting was performed by seeding MDA-MB-231 ( $1.0 \times 10^5$  cells/ml) in T-75 flasks and incubated the cells in growth medium in a humidified  $\text{CO}_2$  incubator. After 24 h, the cells were synchronized in serum-free medium and treated with an  $\text{EC}_{50}$  value of F1 in assay medium for 24, 48, and 72 h. After each incubation period, the F1-treated cells were scraped off the dish with a plastic cell scraper and transferred to a centrifuge tube. The cells were then washed twice with ice-cold PBS and lysed with sonication. The protein concentration was determined using a Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples at  $10 \mu\text{g}/\mu\text{l}$  were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and then were electrophoretically transferred onto polyvinylidene difluoride membrane (Roche, AG, Switzerland). The member was then incubated in 5% dry milk powder blocking buffer for 60 min at room temperature with constant shaking. The membrane was then washed and incubated at  $4^\circ\text{C}$  overnight with 1:500 rabbit polyclonal antibodies against phosphorylated Akt1 (Ser473), phosphorylated c-Cbl (Tyr700), phosphorylated Gab1 (Tyr627), phosphorylated Shc (Tyr239/240), or phosphorylated Stat5 (Tyr694) (Santa Cruz, California, CA, USA). After 3 washes with washing buffer (10 min each), the membrane was incubated at room temperature for 1 h with 1:5,000 horseradish peroxidase-conjugated mouse anti-rabbit antibody (Santa Cruz, California, CA, USA). The membrane was then washed again, and the

peroxidase signals were detected using a Western Blotting Analysis System (Thermo Fisher Scientific, Waltham, MA, USA). The blot was exposed to X-ray film (Kodak, Rochester, NY, USA). Human  $\beta$ -actin (control) was used as a control to verify equal sample loading. The protein expression of the molecular target in the target pathway was analyzed by quantifying the target protein band intensity relative to the control.

### Data analysis

All experiments were repeated several times to ensure the repeatability and reproducibility of the results. The data were presented as mean  $\pm$  standard deviation of triplicates in at least two independent experiments. The significant levels of cell cycle arrest, migratory activity, and protein expression level were analyzed using a one-way analysis of variance. A *post hoc* test, Tukey's honestly significant difference, was performed to determine the groups that differ from one another.  $P < 0.05$  was reported as statistically significant.

## RESULTS

### Cell cycle profile and apoptosis induction of F1 and camptothecin in MDA-MB-231

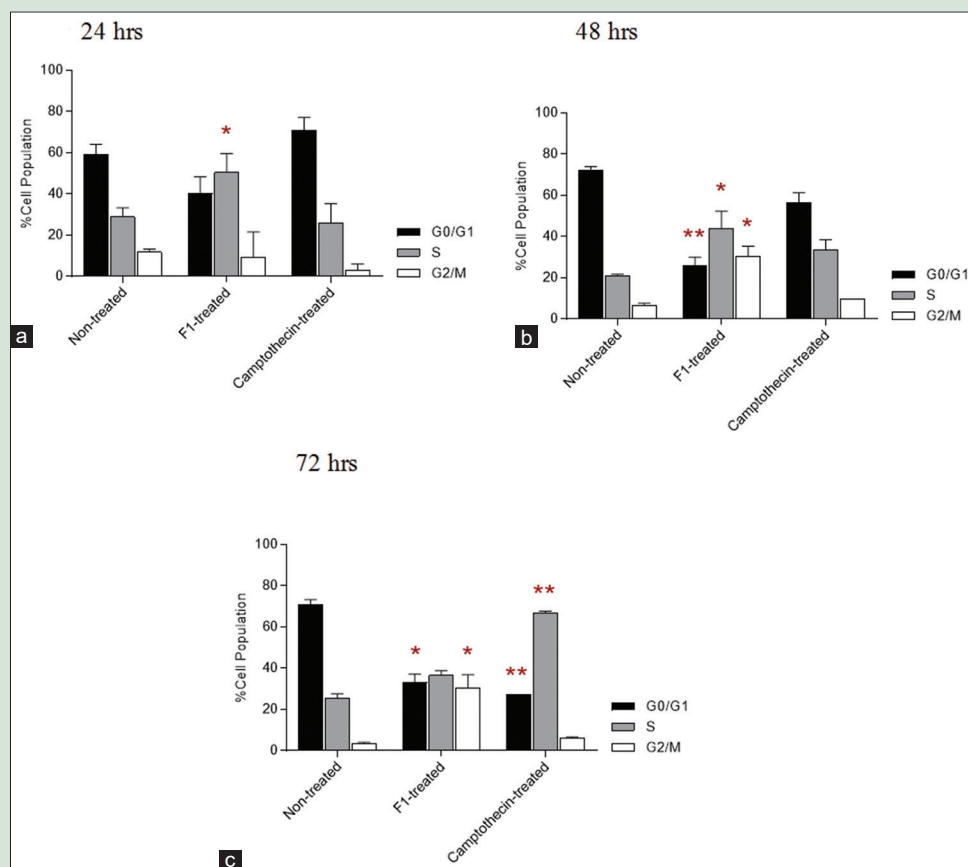
The cell cycle profile of MDA-MB-231 upon treatment with  $50 \mu\text{g/mL}$  of F1,  $1.0 \mu\text{g/mL}$  of camptothecin, and 0.5% of DMSO (control) for 24, 48, and 72 h was analyzed using flow cytometry post staining with PI. Approximately  $50 \mu\text{g/mL}$  of F1 was used because this concentration produces similar chemosensitivity as camptothecin in MDA-MB-231 at 24 h of treatment in our previous study (data not shown). Flow cytometric results showed that the cell population of F1-treated MDA-MB-231 at S phase increased significantly to 50.5% ( $P < 0.05$ ) at 24 h of treatment [Figure 1a]. The increase in S phase cell population of F1-treated MDA-MB-231 to 43.9% ( $P < 0.05$ ) at 48 h of treatment was accompanied by a decrease of the cell population at G0/G1 phase to 25.7% ( $P < 0.01$ ) and with a significant effect on the cell population at G2/M phase to 30.4% ( $P < 0.05$ ) when compared to respective controls [Figure 1b]. Conversely, the distribution of cell cycle profile in MDA-MB-231 treated with F1 for 72 h showed an only significant difference in the cell populations at G0/G1 phase (33.1%,  $P < 0.05$ ) and G2/M phase (30.4%,  $P < 0.05$ ) [Figure 1c]. The increase in S phase cell population was also found in camptothecin-treated MDA-MB-231 to 66.9% ( $P < 0.01$ ) at 72 h of treatment that was accompanied by a decrease of the cell population at G0/G1 phase to 27.1% ( $P < 0.01$ ) but with no significant effect on the cell population at G2/M phase. This phenomenon evinced that F1 induced cell cycle arrest at S and G2/M phases in MDA-MB-231, whereas only cell cycle arrest at S phase was induced in camptothecin-treated MDA-MB-231. Cell viability reduction was also found to 49.7% ( $P < 0.001$ ) in camptothecin-treated MDA-MB-231 along with the detection of apoptosis induction, where camptothecin was found to induce a series of apoptotic effect in the treated MDA-MB-231 at 72 h of treatment: early apoptosis (18.6%,

**Table 1:** Cell distribution profile of F1- and camptothecin-treated MDA-MB-231 for 72 h

	Viable cells	Early apoptosis	Late apoptosis	Necrosis
Control	95.9 $\pm$ 1.50	0.2 $\pm$ 0.05	0.3 $\pm$ 0.10	3.6 $\pm$ 1.70
F1	90.5 $\pm$ 2.65	0.4 $\pm$ 0.25	2.4 $\pm$ 1.90	6.4 $\pm$ 0.05
Camptothecin	49.7 $\pm$ 2.65***	18.6 $\pm$ 17.2*	6.0 $\pm$ 2.05	25.8 $\pm$ 16.6**

Each data set represents the percentage (%) of mean $\pm$ SD of two independent experiments with triplicate reading each ( $n=2$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  as compared to DMSO-treated MDA-MB-231 (control).

SD: Standard deviation



**Figure 1:** Cell cycle profile of F1- and camptothecin-treated MDA-MB-231 for (a) 24 h, (b) 48 h, and (c) 72 h. Control: Dimethyl sulfoxide-treated MDA-MB-231. Each data set represents the mean  $\pm$  standard deviation of two independent experiments with triplicate reading each ( $n = 2$ ). The significant differences were analyzed versus control by one-way analysis of variance and Dunnett's multiple comparisons test that indicated \* $P < 0.05$  and \*\* $P < 0.01$

$P < 0.05$ ) and necrosis (25.8%,  $P < 0.01$ ) [Table 1]. However, no apoptosis was induced in the F1-treated MDA-MB-231. Only samples collected from 72 h of treatment were measured because only this treatment time point showed significant effects.

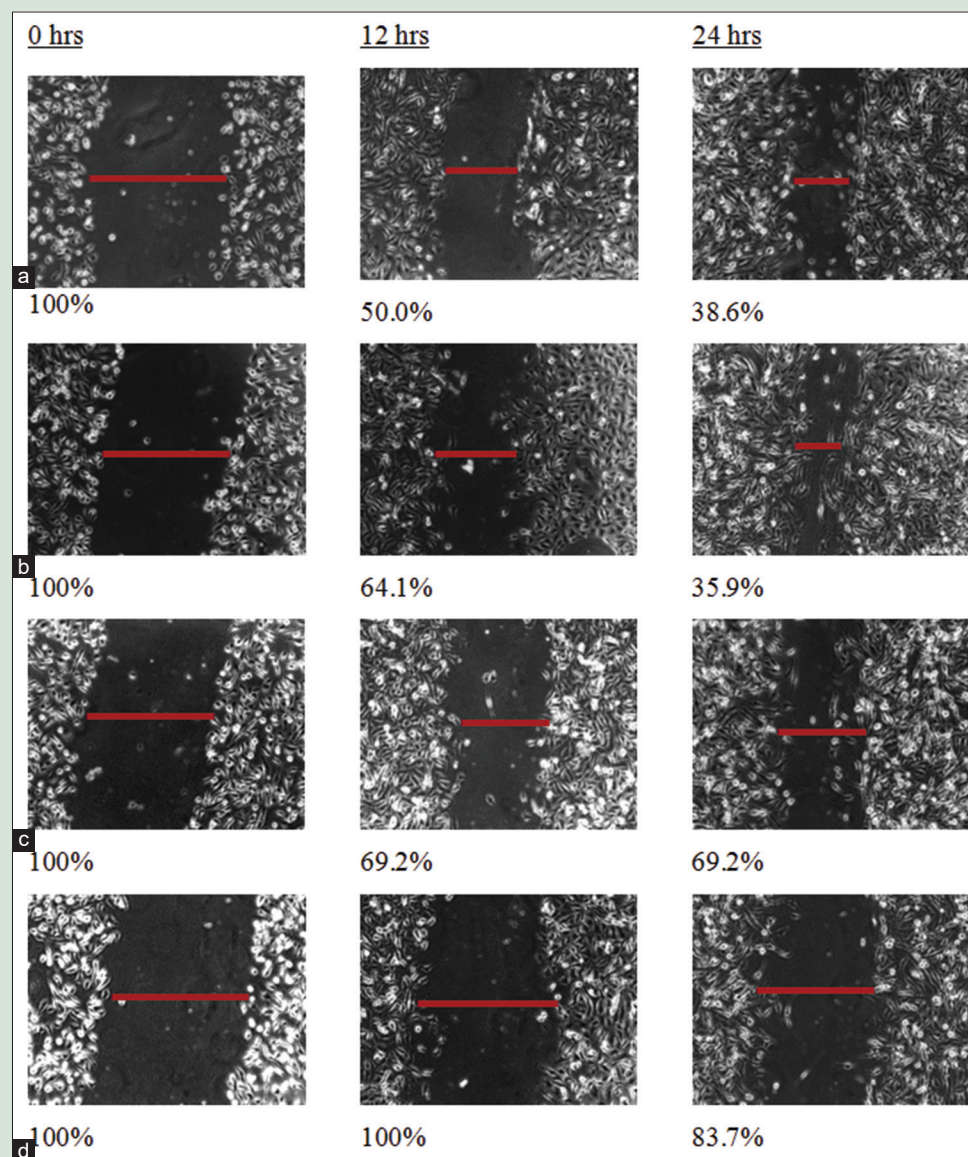
### Cell migratory activity and invasion potential of the F1-treated MDA-MB-231

As shown in Figure 2, the migratory activity of MDA-MB-231 across the wounds in non-treated MDA-MB-231 (control) was observed as early as 12 h (50%) and 24 h (38.6%) of treatment. Similar migratory activity of MDA-MB-231 across the wounds was also observed in MDA-MB-231 treated with 1.0  $\mu\text{g}/\text{mL}$  of F1 at 12 h (64.1%) and 24 h (35.9%) of treatment. However, the gap between wounded cells, which was indicated by the red line, was still prominent in MDA-MB-231 treated with 10  $\mu\text{g}/\text{mL}$  of F1 for 12 h (69.2%) and 24 h (69.2%) of treatment. A more potent inhibitory effect on MDA-MB-231 migratory activity was seen, when 50  $\mu\text{g}/\text{mL}$  of F1 was used for treatment, indicating that the cell migratory activity of MDA-MB-231 was significantly inhibited by  $\sim 50$   $\mu\text{g}/\text{mL}$  of F1 ( $\text{EC}_{50}$  value) as early as 12 h (100%) and 24 h (83.7%) of treatment. The Boyden chamber motility assay was carried out to validate the migratory activity of F1-treated MDA-MB-231 further. Figure 3 shows that the migrated MDA-MB-231 treated with the  $\text{EC}_{50}$  values of E1 and F1 for 24 h was significantly inhibited at  $5.6 \pm 2.03\%$  ( $P < 0.05$ ) and  $4.9 \pm 0.30\%$  ( $P < 0.05$ ), respectively. The results also showed that only E1 and F1 significantly inhibited the migration of MDA-MB-231 at the concentration of the  $\text{EC}_{50}$  value and

the cell migration inhibitory effect of F1 was approximately the same as E1. The study further examined the invasive potential of F1-treated MDA-MB-231 using the Matrigel chamber invasion assay. All extracts and fractions significantly reduced the invasiveness of MDA-MB-231 at the  $\text{EC}_{50}$  value for 24 h of treatment [Figure 4]. The % of invaded cells was reduced from 100% to  $11.65 \pm 6.45\%$  ( $P < 0.05$ ) for E1 treatment,  $9.9 \pm 1.3\%$  ( $P < 0.05$ ) for F1 treatment, and  $20.45 \pm 1.45\%$  ( $P < 0.05$ ) for F2 treatment in MDA-MB-231 post 24 h of treatment. The analysis showed that the most potent cell invasion inhibitory effect was found with F1.

### Expression of phosphorylated Akt (Ser473) protein in the F1-treated MDA-MB-231

The MDA-MB-231 treated with 10  $\mu\text{g}/\text{ml}$  and 50  $\mu\text{g}/\text{ml}$  of F1 for 72 h showed different levels of phosphorylated Akt (Ser473) protein expression [Figure 5]. The expression level of phosphorylated Akt protein in F1-treated MDA-MB-231 was  $3.02 \pm 1.44$ -fold ( $P < 0.01$ ) for 10  $\mu\text{g}/\text{ml}$  treatment and  $1.61 \pm 0.12$ -fold ( $P < 0.05$ ) for 50  $\mu\text{g}/\text{ml}$  treatment, compared with that in control cells (1.0-fold) at 24 h of treatment by Western blotting, indicating an increase in the protein expression level of phosphorylated Akt in F1-treated MDA-MB-231. This phenomenon may be correlated with the migratory activity of MDA-MB-231 treated with F1 for 24 h, suggesting that F1 might exert anticancer activity via regulation of the Akt signaling pathway. On the other hand, phosphorylated c-Cbl (Tyr700), phosphorylated Gab1 (Tyr627), phosphorylated Shc (Tyr239/240), and



**Figure 2:** Wound healing assay for determination of migration activity of MDA-MB-231 treated with different concentrations of F1 for 0 h (control), 12 h, and 24 h. (a) No treatment. (b) Treatment with 1.0 µg/mL of F1. (c) Treatment with 10 µg/mL of F1. (d) Treatment with EC<sub>50</sub> value of F1. The red line indicates the percentage (%) of the wound area

phosphorylated Stat5 (Tyr694) were not detected post-F1 treatment in MDA-MB-231 (data not shown).

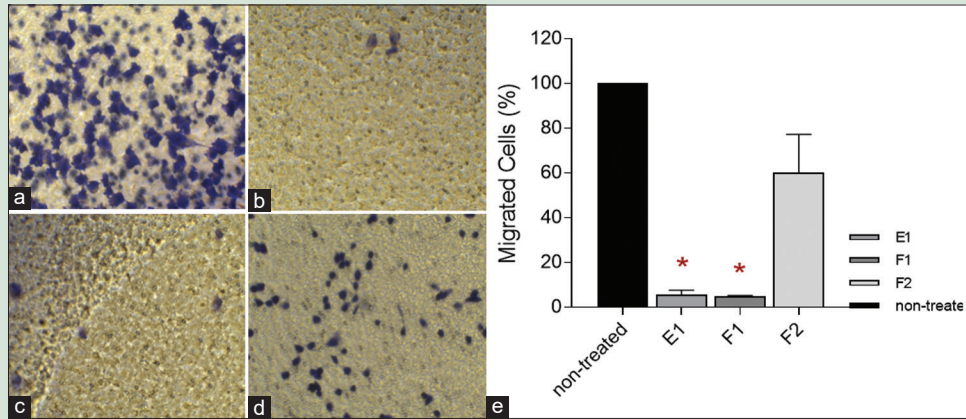
## DISCUSSION

Our study demonstrates that F1 may exhibit a better *in vitro* anticancer effect than camptothecin in MDA-MB-231. F1 also possessed a better cell cycle arrest profile than camptothecin and other migratory activity inhibitions in MDA-MB-231. F1 might exert its anticancer activity via regulation of the Akt signaling pathway in MDA-MB-231.

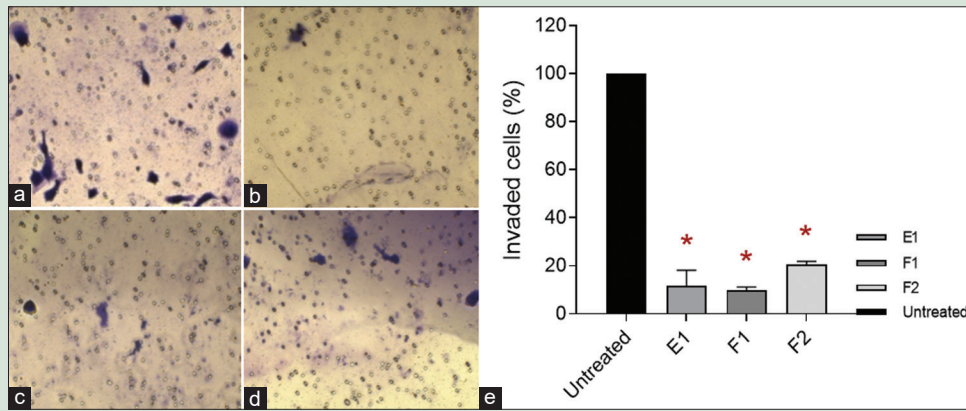
The ethanolic extract of red onion peel (food waste) has been demonstrated to have a growth inhibitory effect on human cancer cell lines, where the ethanolic extraction increased the total phenolic and flavonoid contents in the onion peel extracts and showed greater 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging and antioxidant activities than those by hot water and subcritical water extractions, as determined by the ferric thiocyanate assay.<sup>[10]</sup>

Therefore, F1 may be a potential new solution that can be used as a supplement or cost-effective chemotherapy agent, to reduce the toxicity of currently used chemotherapy drugs, particularly for cancer patients from low-income families. MDA-MB-231 was the focus because the cancer cells were highly aggressive, invasive, and poorly differentiated.<sup>[11]</sup> The cancer cells lack cellular targets compared with another estrogen receptor (ER)-positive breast cancer, which can be effectively inhibited by targeting the estrogen receptor with antiestrogen agents, for example, tamoxifen. The proliferation of ER-negative breast cancer cells, for example, MDA-MB-231, is not affected by estrogen, negating the use of antiestrogen therapies. Hence, the search for potential anticancer agents to treat ER-negative breast cancer effectively is all the more urgent and important now,<sup>[12]</sup> prompting us to investigate the effects of F1 on MDA-MB-231 in the cell cycle and other regulatory cell distributions.

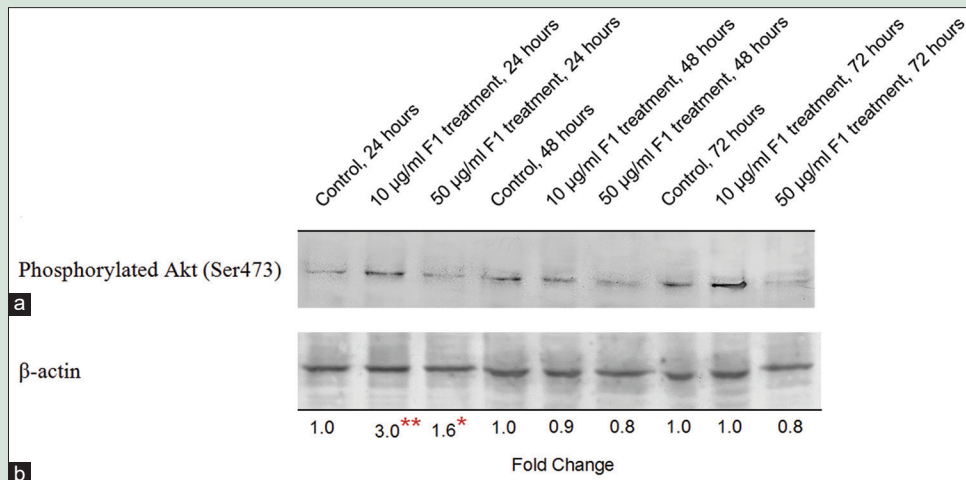
F1 showed an ideal proliferation reduction of MDA-MB-231 in a dose-dependent manner by affecting the cell cycle at S and G2/M



**Figure 3:** Boyden chamber motility assay for determination of migration activity of MDA-MB-231 treated with EC<sub>50</sub> value of E1, F1, and F2 for 24 h. (a) Non-treated MDA-MB-231 (control). (b) Treatment with E1. (c) Treatment with F1. (d) Treatment with F2. The data are expressed as the means ± standard deviation of three independent experiments with triplicate reading each (n = 3). \*P < 0.05 compared with the control. (e) Statistical analysis of the treated MDA-MB-231 cells for 24 h.



**Figure 4:** Matrigel invasion assay for determination of invasion activity of MDA-MB-231 treated with EC<sub>50</sub> of E1, F1, and F2 for 24 h. (a) Non-treated MDA-MB-231 (control). (b) Treatment with E1. (c) Treatment with F1. (d) Treatment with F2. The data are expressed as the means ± standard deviation of three independent experiments with triplicate reading each (n = 3). \*P < 0.05 compared with the control. (e) Statistical analysis of the treated MDA-MB-231 cells for 24 h.



**Figure 5:** Protein expression of (a) phosphorylated Akt (Ser473) and (b) β-actin in MDA-MB-231 treated with F1 for 24 h, 48 h, and 72 h. The determination was performed using Western blotting. 1: Control for 24 h. 2: Treatment with 10 µg/ml F1 for 24 h. 3: Treatment with 50 µg/ml F1 for 24 h. 4: Control for 48 h. 5: Treatment with 10 µg/ml of F1 for 48 h. 6: Treatment with 50 µg/ml of F1 for 48 h. 7: Control for 72 h. 8: Treatment with 10 µg/ml of F1 for 72 h. 9: Treatment with 50 µg/ml of F1 for 72 h. The data were expressed as the means ± standard deviation (n = 3). \*P < 0.05 and \*\*P < 0.01 compared with the control

phases but not toxic to the test cells. The cell cycle is a major regulatory mechanism of cell growth.<sup>[13]</sup> Regulation of the cell cycle is crucial for the development of healthy cells. Nevertheless, cancerous cells exhibit uncontrolled cell proliferation and evasion of apoptosis resulting from the dysfunctions of checkpoint and the destruction of cell cycle.<sup>[14]</sup> The uncontrolled cell growth and apoptosis resistance are the major defects in cancer cells; thus, the discovery of effective compounds targeting cell cycle mechanism and apoptotic machinery could be effective against uncontrolled cell proliferation in neoplasia. F1 is able to obstruct cell cycle progression in MDA-MB-231 that is due to the presence of different compound compositions in F1, for example, quercetin.<sup>[15,16]</sup> The composition gives rise to the different molecular mechanisms of cell cycle regulation induced by F1, as compared to other pure compounds. As for the apoptosis analysis, the results showed that F1 did not induce apoptosis in MDA-MB-231, suggesting that the inhibition of cell proliferation by F1 in MDA-MB-231 was through different mechanisms. The F1 also showed remarkable inhibition of MDA-MB-231 migratory activity in a dose-dependent manner. Metastatic cells, for example, MDA-MB-231, are highly invasive with exceptional migratory ability,<sup>[17]</sup> whereby 90% of cancer death is due to metastatic progression. Migration is needed for cancerous cells to migrate from the primary location to the secondary site.<sup>[18,19]</sup> Disruption of migration would interrupt the metastatic cascade and the translocation of cells from one location to a distant location.<sup>[20]</sup> The use of natural products as anti-breast cancer agents has been widely studied in many drug discovery.<sup>[21]</sup> However, the search for effective therapies from natural products to reduce metastatic progression remains lacking. Inhibition of the migratory activity of MDA-MB-231 by F1 likely occurs via the inhibition of cell fibronectin adhesion. Fibronectin is one of the components of ECM that regulates cancer cell migration and adhesion.<sup>[22]</sup> Other components of the ECM, including laminin and type IV collagen. The Matrigel invasion assay was performed to investigate the inhibition of cell migration and cell-fibronectin adhesion by F1 in MDA-MB-231 that mimics the actual extracellular component in the human body. The regulation of cell fibronectin adhesion activity of F1 in MDA-MB-231 also occurs likely via regulation of the Akt signaling pathway, which is consistent with the study that reported the Akt/ERK signaling pathway as the molecular target to regulate the invasion of cancer cells.<sup>[23-25]</sup>

Red onion provides a great amount of antioxidant phytochemicals, which are well known to exhibit various biological activities and might be potent agents to interrupt the metastatic processes of cancer cells. The anticancer activity of F1 tested in this study is most likely due to the phytochemical characteristic of the subfraction. Phytochemicals, for example, flavonols, which are primarily detected as glycosides of quercetin and kaempferol in onions, have been identified as major contributors to the antioxidative activities and health benefits of onions, as well as to exert antimetastatic activity in the brain, oral, lung, breast, and gastric cancers.<sup>[26,27]</sup> Flavonols are often found to be concentrated in the skin/peel of onions that give the yellow, brown, or red color to the onions.<sup>[28]</sup> The dry peel of the red onion that is always discarded as waste contains large amounts of quercetin. Quercetin is widely distributed in the outer skin and inner parts of the bulb.<sup>[27,29]</sup> Quercetin in onion peel was the most effective DPPH radical scavenger, and it also reacted faster than other flavonols, especially rutin and kaempferol.<sup>[30]</sup> Therefore, the inhibition of metastasis in breast cancer may be achieved using onion-derived natural products. Consistently, the results of DPPH-free radical-scavenging activity from a previous study demonstrated that the onion peel extract acted as strong hydrogen donors.<sup>[10]</sup> The outer peel of the red onion that displays a high yield in phenol and total flavonoid contents has been demonstrated to contain higher antioxidant activity than yellow and white onions.<sup>[2]</sup> The peel was

also reported to have a liver protective effect, immune enhancement potential, anti-infection, anti-stress, anticancer, and other pharmacological properties.<sup>[31,32]</sup> It is interesting to know that the red onion peel has been used all worldwide as a colorant, flavor agent, and various types of food. However, available information on the anticancer activity of the red onion peel remains scanty.

Aryl hydrocarbon receptor (AHR), which is responsible for activating CYP genes transcription, is one of the proteins to affect cell cycle regulation.<sup>[33-35]</sup> Several findings had shown that dietary flavonoids play a role as AHR ligand either with antagonist or agonist activity to inhibit cancer cell growth.<sup>[36,37]</sup> In addition, flavonoid may also undergo CYP1-mediated oxidative metabolism to become antiproliferative products.<sup>[36]</sup> Research also showed the antiproliferative and cytostatic effect of a flavonoid lipid molecule, namely eupatorin, in breast cancer cells that are due to the involvement of CYP1-mediated metabolism.<sup>[38]</sup> The studies showed cell cycle arrest at G2/M phase induced by eupatorin could be reversed when co-incubated with CYP1 inhibitor of acacetin in MDA-MB-468 cells. Another finding proved that metabolites produced from isoflavones daidzein and genistein via CYP1A1, CYP1A2, and CYP1B1 metabolism activities induced antiproliferative response in MCF-7 cells.<sup>[39]</sup>

## CONCLUSION

The subfraction F1 of red onion peel crude ethanolic extract may be developed as a supplement or used in combination with other drugs as a new agent to treat MDA-MB-231-type breast cancer. This finding is based on our current evaluation that is quantitatively measured using *in vitro* assays. The strategy used in this study may also help to accelerate the prediction of the cytotoxic activity and other *in vitro* effects of natural products, which can be used in new alternative regimens that display less toxic and fewer adverse effects to cancer patients while also causing less damage to healthy cells in cancer patients.

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## Conflicts of interest

There are no conflicts of interest.

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