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Antimigratory Activity of Brazilin-Containing Fraction from *Caesalpinia sappan* L. on MDAMB-231 Cells

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ABSTRACT

***Caesalpinia sappan* is studied for several biological activities. The aim of this research is to determine the cytotoxic and antimigratory activities of *Caesalpinia sappan* active fraction in combination with cisplatin on human TNBC cells (MDA-MB-231). *Caesalpinia sappan* heartwood was extracted with methanol. Then, several fractions of the methanol extract were obtained by using a liquid-liquid extraction method followed by column chromatography. The cytotoxicity was determined using MTT assay. Synergistic effects were analyzed by calculating the combination index (CI). Migration was examined using wound-healing assay. Levels of MMP2 activity were determined with gelatin zymography assay. The results showed that most of the fractions included in this study exhibited cytotoxic effects against MDA-MB-231 cells, and C fraction demonstrated the highest cytotoxic activity of all fractions. The combination of C-cisplatin revealed a synergistic inhibitory effect on MDA-MB-231 cell growth (CI<1). Furthermore, C fraction, alone and in combination with cisplatin, inhibited migration of MDA-MB-231 and suppressed MMP2 activity. The C fraction isolated from *Caesalpinia sappan* increased the cytotoxic and antimigratory activities of cisplatin on MDA-MB-231 cells. Based on these findings, the potential of *Caesalpinia sappan* to act as a supportive agent in metastatic TNBC treatment with cisplatin warrants further exploration.**

1. Introduction

There are six hallmarks that govern the transformation of normal cells to cancer cells (Hanahan and Weinberg 2011). Metastasis is the latest hallmark of cancer progression that difficult to be overcome. Migration and invasion are the important processes in early step of metastasis event (Brooks *et al.* 2010). Cancer progression usually involve aberration of cell proliferation and cell migration as well as invasion (Kemper *et al.* 2014). Strategy on cancer treatment is not only focused on one target mechanism, but it should be developed on several target mechanism.

Cisplatin (CDDP) is one of the first line therapy in metastatic triple negative breast cancer (Zhang *et al.* 2015). However, low concentration of cisplatin induces epithelial-to-mesenchymal transition (EMT) and followed by increasing instead of inhibiting of cancer metastasis (Latifi *et al.* 2011). Combination therapy with other agent increases the better outcomes.

Several studies revealed the potential effect of *Caesalpinia sappan* L. extract and active fraction for cancer treatment (Nurzijah *et al.* 2012; Tirtanirmala *et al.* 2015; Rachmady *et al.* 2016; Jenie *et al.* 2017). Brazilin and brazilein isolated from *Caesalpinia sappan* L. inhibit cancer cell growth through inducing of apoptosis, cell cycle arrest and inhibiting of migration on cancer cells (Kim *et al.* 2012; Tao L *et al.* 2013; Hsieh *et al.* 2013; Handayani *et al.* 2016; Handayani *et al.* 2017; Jenie *et al.* 2018). Moreover, combination of brazilin or brazilein with chemotherapeutic agent show synergistic effect (Handayani *et al.* 2016; Handayani *et al.* 2017; Jenie *et al.* 2018). Thus, *Caesalpinia sappan* L. can be a promising supportive agent for TNBC breast cancer

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patient treated with chemotherapeutic agent. Therefore, the purpose of this research is to explore the cytotoxic and antimigratory effect of several fractions of *Caesalpinia sappan* L. and its synergistic effect with cisplatin focusing on its proliferation and migration on human triple negative breast cancer (TNBC) cells, which is a highly metastatic cancer cells.

2. Materials and Methods

2.1. Sample Preparation

Dried heartwood powder of *Caesalpinia sappan* L. was obtained from B2P2TOOT Tawangmangu, Indonesia. Dried powder was extracted in methanol to obtain a methanol-soluble extract. The methanol-soluble extract was diluted in methanol:water (4:1) and then partitioned with hexane. The aqueous layer was fractioned with ethyl acetate and concentrated in a vacuum rotary evaporator to obtain the fraction of ethyl acetate. Subsequent fractions (A-H) were obtained by separation of ethyl acetate fractions using Sephadex G-15 column (Sigma-Aldrich) chromatography (15 x 7 cm) with gradient polarity of the mobile phase ($CHCl_3$:MeOH) and collected using thin-layer chromatography (Jenie *et al.* 2018). A brazilein standard was obtained from the Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada (Laksmiani *et al.* 2015).

2.2. Cell Culture

The MDA-MD-231 cell line was a kind gift of Prof. Hiroshi Itoh, Ph.D. (Nara Institute of Science and Technology, Japan) and was cultured in a non-CO_2 incubator (37°C) in L-15 medium (Sigma) containing 15% Fetal Bovine Serum (Gibco), 1.5% Penicillin-Streptomycin (Gibco), and 0.5% fungizone (Gibco).

2.3. Cytotoxicity Assay

MDA-MD-231 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. Confluent cells were treated with various concentrations of samples. After 24 h of incubation, the culture medium was removed and the cells washed in PBS (Sigma). Then, the cells were incubated with 100 µl culture medium and 10 µl MTT (Sigma) at 5 mg/ml in every well for 4 h. The MTT reaction was stopped with 10% sodium dodecyl sulfate (Merck) in 0.01M HCl (Merck) and incubated overnight. The absorbance was measured in a microplate reader (Bio-Rad) at a wavelength of 595 nm.

2.4. Wound-healing Assay

Cells were seeded in each well of a 24-well plate with 1×10^5 cells and incubated at 37°C in a CO_2 incubator for 24 h. Thereafter, cells were starved by using serum-free medium (containing 0.5% fetal bovine serum) for 24 h. Confluent cell monolayers were wounded, and DMEM culture medium containing ¼ of the IC_{50} of the samples, alone and in combination, was added. Time-lapse images were acquired at 18, 24, and 42 h. The wound closure percentage was analyzed by using Image-J software.

2.5. Gelatin Zymography Assay

The gelatinolytic activity of MMP2 was assayed by gelatin zymography. Cells were seeded in each well of a 6-well plate at a density of 1×10^6 cells and incubated at 37°C in a CO_2 incubator for 24 h. Cells were incubated with ¼ of the IC_{50} of the samples, alone and in combination, in serum-free-medium for 24 h. The supernatants were collected and subjected to gel electrophoresis in a 10% running gel containing 0.1% gelatin. The gels were washed in renaturing solution containing 2.5% Triton X-100 for 30 min, followed by incubation for 20 h at 37°C with incubation buffer. The gels were stained for 30 min in 0.5% Coomassie brilliant blue and then destained with destaining solution (10% v/v methanol and 5% v/v acetic acid) and the protein bands documented.

2.6. Statistical Analysis

The parameters inhibitory concentration (IC_{50}) and combinatory index (CI) were measured as described in previous reports (Mosmann 1983; Reynolds and Maurer 2005). The IC_{50} values of three replications were expressed as mean±standard deviation (SD). The significance of differences between the control (untreated group) and treated groups was analyzed with an unpaired Student's t-test (Microsoft Excel 2013). Differences were considered significant at $p<0.05$.

3. Results

3.1. Cytotoxic Effect of *Caesalpinia sappan* Fractions on MDA-MB-231 Cells

Previous studies revealed that the ethyl acetate fraction of *Caesalpinia sappan* extract inhibits growth in various cancer cells. In this study, several fractions of *Caesalpinia sappan* inhibited MDA-MB-231 cell growth in a dose-dependent manner (Figure 1). The IC_{50} value of the ethyl acetate fraction was 115.4±3.34

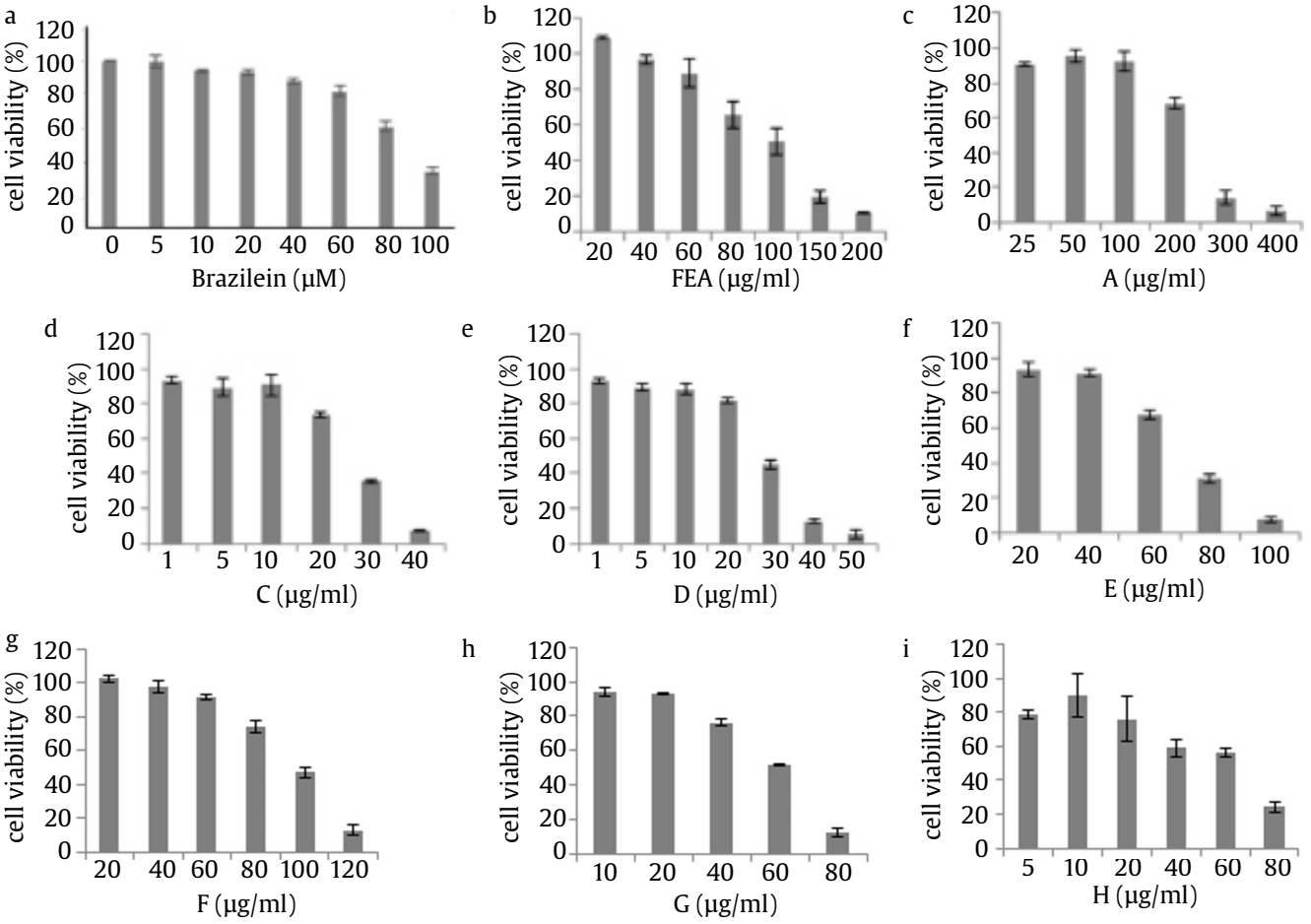

Figure 1. Cytotoxic effect of *Caesalpinia sappan* fractions on MDAMB-231 cells. Cells were treated with various concentrations of samples for 24 h before assessed by MTT assay. Data was collected from three replications

µg/ml toward MDA-MB-231 cells. Brazilein is one of the major compounds in *Caesalpinia sappan* L. As additional data, the IC_{50} values of cisplatin and the brazilein standard on MDA-MB-231 cells were 112.5±1.06 µM and 89.3±3.65 µM, respectively. We further separated the ethyl acetate fraction collected by using column chromatography based on polarity to obtain fractions A through H. The nonpolar A and B fractions appeared as the same spot on the thin-layer chromatography profile, indicating identical compounds (data not shown). The A fraction showed the largest IC_{50} value: 221.8±10.18 µg/ml. The semi-polar fractions C, D, E, and F decreased MDA-MB-231 cell viability, with IC_{50} values of 24.2±0.19 µg/ml, 27.0±0.43 µg/ml, 67.0±1.44 µg/ml, and 103.5±2.96 µg/ml, respectively. The polar fractions G and H decreased MDA-MB-231 cell viability, with IC_{50} values of 55±1.18 µg/ml and 54.9±1.42 µg/ml, respectively. The semi-polar C fraction had the highest cytotoxic activity of all fractions (Figure 2). Previous study revealed that the active C fraction of *Caesalpinia sappan* L contained almost a single flavonoid compound, brazilin. Combinations of two or more drugs is one possible way to overcome the side effects and offer a greater chance of a cure. This study investigated whether the active C fraction from *Caesalpinia sappan* L. had a synergistic cytotoxic effect in combination with cisplatin on MDA-MB-231 cells. In addition, we investigated the synergistic effect of brazilein and cisplatin together. We evaluated the synergistic effect of the combinations by using the CI. The results showed that the combination of ½ IC_{50} of C-cisplatin and brazilein with cisplatin inhibited cell viability by 61% and 60%, respectively (Figure 3a and b). Furthermore, combinations of $^1/_{10}$, $^1/_8$, ¼, and ½ IC_{50} of C-cisplatin and brazilein with cisplatin inhibited MDA-MB-231 cell growth, with CI values less than 1 (Figure 3c).

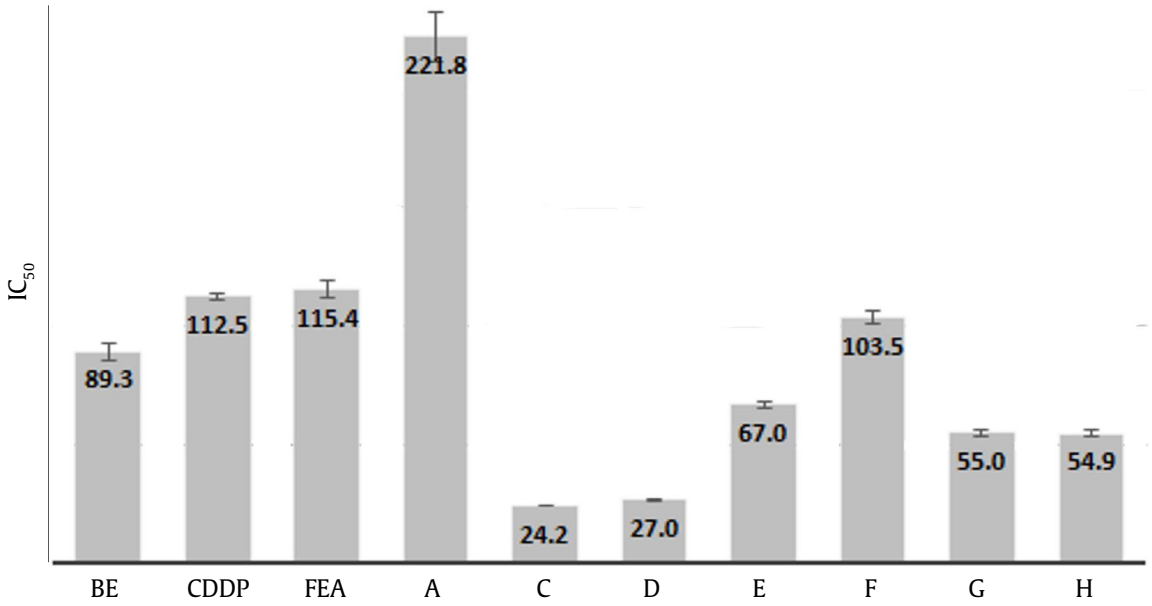

Figure 2. The IC_{50} value of *Caesalpinia sappan* fractions (µg/ml) on MDAMB-231 cells. Cisplatin (CDDP) and brazilein (Be) were in µM

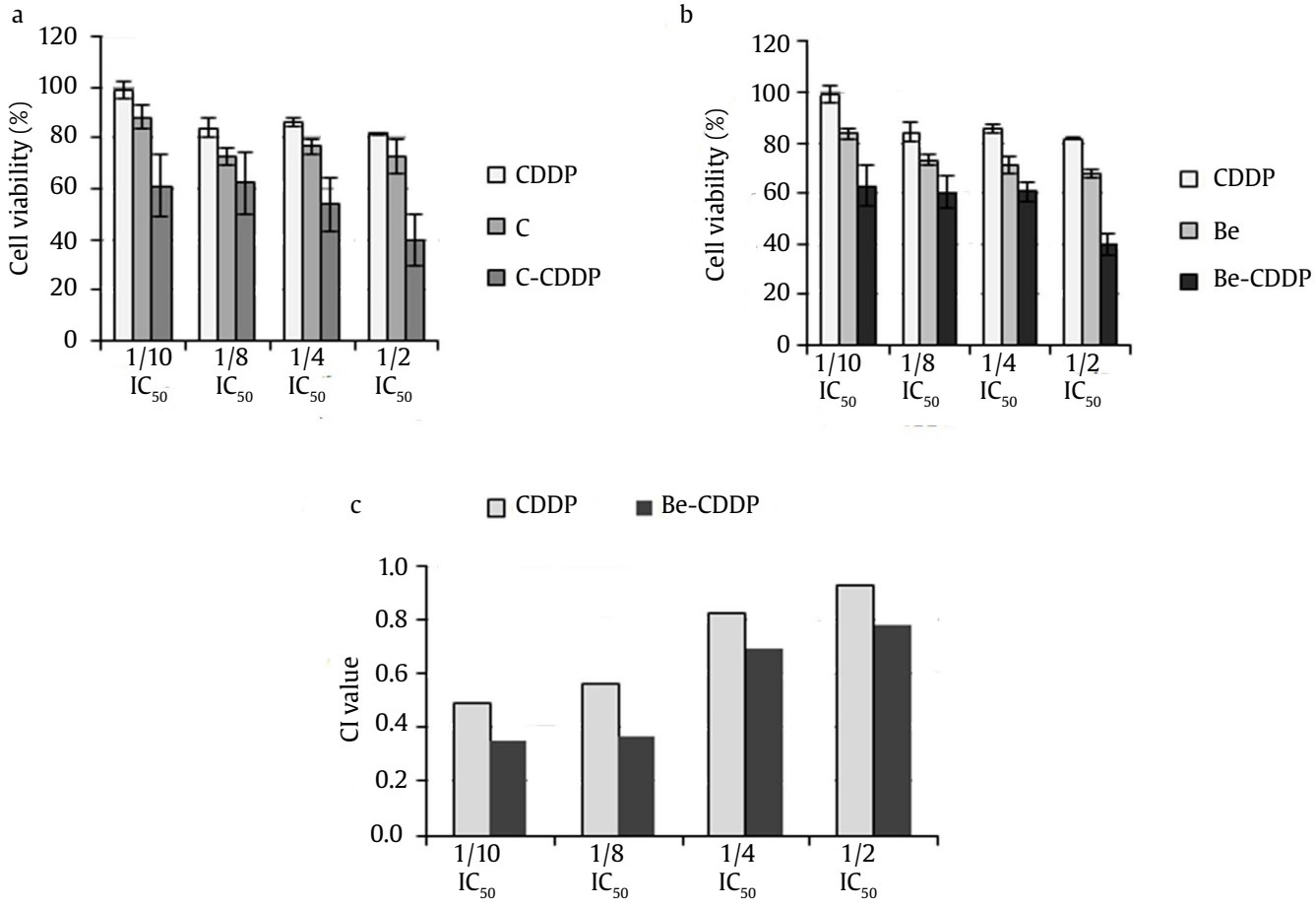

Figure 3. Cytotoxic effect of combination of C-cisplatin (C-CDDP) and brazilein-cisplatin (Be-CDDP) on MDAMB-231 cells. Data was collected from three replications. (a) C-cisplatin (1/10-1/4 IC_{50}), (b) Brazilein-cisplatin (1/10-1/4 IC_{50}), (c) the CI value of combination of C-cisplatin and brazilein-cisplatin

3.2. Wound Closured Inhibition of *Caesalpinia sappan* Active Fraction on MDAMB-231 Cells

Metastatic cancer cells have the ability to migrate from the primary site to other parts of the body. A low concentration of cisplatin was reported to increase, instead of inhibit, metastasis of cancer cells. This study found that treatment with ¼ IC_{50} of cisplatin alone did not affect wound closured in MDA-MB-cells (Figure 4a and b). On the other hand, treatment with ¼ IC_{50} of fraction C alone and ¼ IC_{50} of brazilein alone inhibited wound closured in MDA-MB-231 cells by up to 55% and 56%, respectively, after 18 h of incubation, followed 51% and 54%, respectively, after 24 h of incubation. Furthermore, this phenomenon persisted in combination with cisplatin (Figure 4a and b). The C fraction and brazilein, alone and in

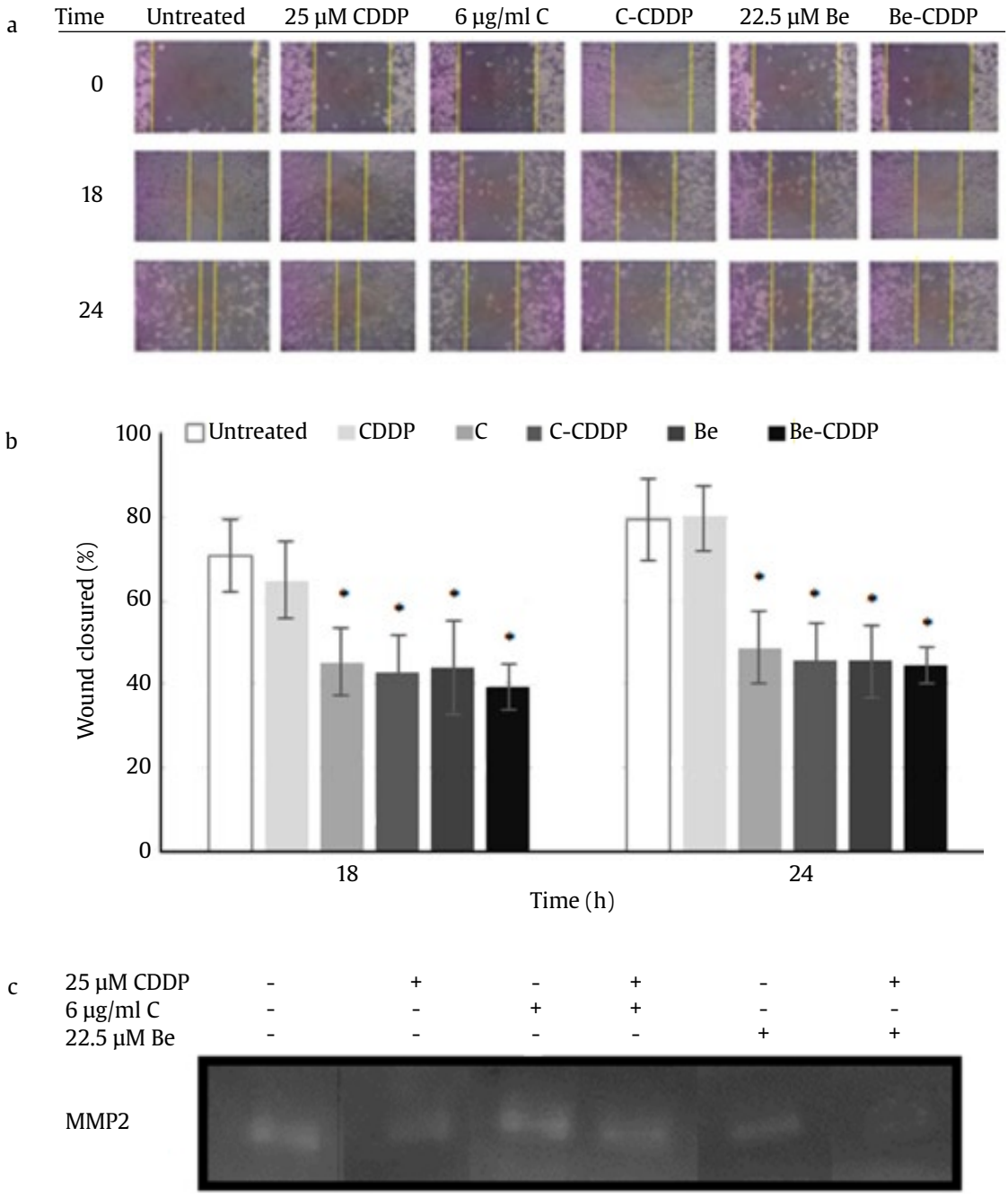

Figure 4. Inhibition of migration of C and brazilein (Be) single and combination with cisplatin (CDDP) on MDAMB-231 cells. (a) wound closured, (b) relative wound closured (18 and 24 h), (c) the levels of MMP2 protein. (P<0.5 by Student's t-test)

combination with cisplatin, inhibited migration of MDA-MB-231 cells.

3.3. Activity of *Caesalpinia sappan* Active Fraction on Alteration of MMP2 Protein Level

The high expression of proteinases such as MMPs in the microenvironment of cancer cells plays a critical role in tumor invasion and metastasis. Thus, this study investigated the effect of fraction C, brazilein, and their combination with cisplatin on alteration of MMP2 protein expression in MDA-MB-231 cells according to gelatinolytic activity using gelatin zymography. The results indicated that ¼ IC_{50} of C and brazilein in combination with ¼ IC_{50} of cisplatin decreased MMP2 protein levels in MDA-MB-231 cells (Figure 4c).

4. Discussion

Caesalpinia sappan L. is the main ingredient in the traditional Indonesian herbal drink wedang secang, it is interesting to show the supportive benefits of this plant to the quality of life of cancer patients treated with a chemotherapeutic agent. The active C fraction of *Caesalpinia sappan* L contained only one flavonoid compound, brazilin (Jenie *et al.* 2018). This flavonoid content differs from those of other plants in the genus Caesalpiniaceae. Brazilin and brazilein are not present in the flavonoid HPLC fingerprint of *Caesalpinia crista* (Chethana *et al.* 2018). The IC_{50} value of the C fraction on MDA-MB-231 cells was 24.22±0.19 µg/ml (Figure 2), and that of brazilin was 84.6±0.67 µm. On the other hand, the IC_{50} value of brazilein was 89.32±3.65 µM (Figure 2). The cytotoxic activity of the C fraction (brazilin) was slightly different from that of brazilein against MDA-MB-231 cells (Figure 1a and d). Even though cisplatin (CDDP) is one of the first-line therapies for metastatic TNBC (Zhang *et al.* 2015), it has some negative side effects (Florea *et al.* 2011). Thus, combinations of two or more drugs is one possible way to overcome the side effects and offer a greater chance of a cure (Bozic *et al.* 2013) Furthermore, the combination of C-cisplatin and brazilein-cisplatin showed a synergistic effect, with a CI value less than 1 (Figure 3c). Handayani *et al.* (2017) also revealed that the IC^{50} value of brazilein on 4T1 murine TNBC cells was 50 µM. In cells of different origins, different concentrations of the drug are generally needed to achieve similar effects. However, the synergistic effects of the combination of brazilein and cisplatin on 4T1 cells (Handayani *et al.* 2016) and MDA-MB-231 cells were similar. Brazilein was the oxidized form from brazilin (Nirmal *et al.* 2015), and both compounds are major components of *Caesalpinia sappan* L. heartwood extract. Thus, it is interesting to reveal the cytotoxic effect of both compounds, alone and in combination with cisplatin, on MDA-MD 231 human TNBC cells.

Since the MDA-MB-231 cell line is a human cell line model for metastatic TNBC, this cell line would be suitable for antimetastatic studies. A previous study reported that brazilein (Be) isolated from *Caesalpinia sappan* L. inhibits migration of MDA-MB-231 (Hsieh *et al.* 2013). Our study revealed that ¼ IC_{50} of the C fraction (brazilin) or brazilein combined with CDDP inhibits migration of MDA-MB-231 cells compared with CDDP alone. A flavanone liquiritigenin combined with CDDP induces antimigration and anti-invasion effects on melanoma cells through downregulation of the MMP 2/9 and PI3K/AKT signaling pathway (Shi *et al.* 2015). Formononetin, an isoflavone from *Astragalus membranaceus*, inhibits cancer cell migration by decreasing MMP2/9 and pERK protein expression (Zhang *et al.* 2018). Thus, MMPs are important protein targets for most flavonoid compounds to suppress cancer cell migration.

The MMP2 is one of Matrix metalloproteinases (MMPs) that plays important roles in metastasis via extra cellular matrix degradation (Hua *et al.* 2011). This study describes that the C fraction alone and in combination with cisplatin suppressed the MMP2 activity of MDA-MB-231 cells. Brazilin and brazilein were reported to suppress MMP2 and MMP9 via the HER2 pathway (Jenie *et al.* 2018). However, MDA-MB-231 cells are a TNBC, thus, these cells do not overexpress HER2 protein. Another studies reported that brazilein inhibits MMP9 and MMP2 activity and expression in TNBC cells (Hsieh *et al.* 2013; Handayani *et al.* 2016). Brazilein inhibits MMP2 on MDA-MB 231 by suppressing NF-κB activation through inhibition of IKK (Hsieh *et al.* 2013). NF-κB is a signal transducer that regulates the expression of genes such as MMP2 and MMP9 (Hsieh *et al.* 2013; Shi *et al.* 2015). Several pathways have been studied to show the mechanism of NF-κB activation, related or unrelated to HER2 protein (Serasanambati and Chilakapati 2016). Based on our findings using a TNBC cell line, we suggested that the inactivation of NF-κB by the C fraction (brazilin) in MDA-MB-231 was not related to the HER2 pathway. Nevertheless, further studies are needed to confirm the mechanisms involved in the cytotoxic and antimigratory effects of the C fraction of *Caesalpinia*

sappan L. and its combination with cisplatin on human TNBC cells.

5. Conclusion

The C fraction of *Caesalpinia sappan* L. supported the activity of cisplatin by increasing the cytotoxic and antimigratory activity of cisplatin on triple negative breast cancer, MDA-MB-231 cells.

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