

The effect of ethyl acetat fraction of *Caesalpinia sappan L.* wood on PC3 cancer cell line : cell viability and migration study

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Abstrak

Latar belakang: Tingginya insidensi kanker di Indonesia, termasuk kanker prostat menimbulkan beban ekonomi kesehatan yang tinggi bagi Indonesia. Pengembangan terapi kanker berbasis sumber daya alam lokal dapat membantu meringankan beban negara. Penelitian ini bertujuan untuk mengetahui potensi aktivitas anti-kanker fraksi ethyl acetat *Caesalpinia sappan L.* terhadap sel line kanker PC3 yang merupakan model *in vitro* kanker prostat.

Metode: Fraksi ethyl acetat kayu secang (*Caesalpinia sappan L.*) diperoleh melalui proses liquid chromatography. Efek fraksi 9 dari Ffraksi ethyl acetat kayu secang terhadap aktivitas anti-proliferasi dan migrasi sel diuji menggunakan desain uji *in vitro*. Hambatan proliferasi sel diukur dengan metode MTT assay, sedangkan aktivitas migrasi sel diukur dengan metode migration assay

Hasil: Fraksi 9 dari fraksi ethyl acetat kayu secang memperlihatkan hambatan proliferasi sel line kanker PC3 dengan IC50:14.99µg/ml. Hasil migration assay menunjukkan pada dosis 10µg/ml fraksi 9 menghambat migrasi sel line kanker PC3, sedangkan pada dosis 100µg/ml sel line kanker PC3 mati.

Kesimpulan: Fraksi 9 dari fraksi ethyl acetat kayu secang menunjukkan aktifitas anti-proliferasi dan anti-migrasi yang kuat terhadap pertumbuhan sel line kanker PC3 secara *in vitro*. (*Health Science Journal of Indonesia 2020;11(2):100-5*)

Kata kunci: ethyl acetat fraction, *Caesalpinia sappan*, prostate cancer, PC3, migrasi sel

Abstract

Background: The high incidence of cancer, including prostate cancer, in Indonesia create a high burden on health economic cost. Development of cancer therapy based on local natural resources may help the country to alleviate the burden. This research aimed to find out the potency of selected compound of ethyl acetate fractions of *Caesalpinia sappan* as anti-cancer by using PC3 cancer cell line as an *in vitro* model of prostate cancer.

Methods: Ethyl acetate fraction of *Caesalpinia sappan L.* heartwood was prepared using a liquid chromatography method. The effect of ethyl acetate fraction 9 on anti-proliferative and cell migration activities was assessed using MTT assay and migration assay.

Results: Fraction-9 of ethyl acetate fraction of *Caesalpinia sappan L.* wood showed inhibition of PC3 cancer cell line proliferation. The IC50 of the fraction was 14.99µg/ml. The migration assay showed inhibition of cell migration on dose 10µg/ml compared to the 0 doses, while most of the cells cultured was dead when treated with 100µg/ml fraction 9.

Conclusion: Ethyl acetate fraction 9 of *Caesalpinia sappan L.* heartwood possibly has anti-cancer properties based on its anti-proliferative and anti-migration activities against PC3 cancer cell line. (*Health Science Journal of Indonesia 2020;11(2):100-5*)

Keyword: ethyl acetate fraction, *Caesalpinia sappan*, prostate cancer, PC3, cell migration

The increasing cancer incidence worldwide put the disease on the high rank of global health challenge. The high mortality rate of the cancer patient and the expensive cost of cancer therapy justify cancer as a catastrophic disease.¹ Global cancer observatory (Globocan) reported 11.361 new prostate cancer cases in 2018 in Indonesia. The incidence rate and mortality rate of prostate cancer are in the third rank among other cancer in the male in Indonesia.²

The application of the screening method on patients with urologic symptoms increases the detection rate of prostate cancer.³ Current therapeutic approaches for prostate cancer in Indonesia include surgery, External Beam Radiation Therapy, and Adjuvant Androgen Deprivation Therapy.⁴ Although advanced cancer therapy has been available nowadays, the cost of cancer therapy hampers many cancer patients from low-income society to seek immediate and appropriate cancer treatment. This condition urges the need to investigate the potential use of local plant medicine as cancer therapy.

In this study, we report the anti-cancer activity of one of Indonesian plant medicine, known with local name as secang. The Latin name of Secang is *Caesalpinia sappan* L. The heartwood of *Caesalpinia sappan* has been widely used as Javanese traditional medicine.⁵ In the field of cancer research, attention has been largely spent to study the potential anti-cancer activity of compounds isolated from *Caesalpinia sappan* L. *Caesalpinia sappan* heartwood contains many active compounds with anti-cancer activities including protosappanin B⁶ Brazilein,⁷ Brazilin⁸. Brazilin is the major compound of *Caesalpinia sappan* L. heartwood which gives the red color when dissolved in water.⁹ This study investigated the anti-cancer activity of the ethyl acetate fraction of *Caesalpinia sappan* on PC3 cancer cell line.

METHODS

The experiment was conducted under the Ethical Approval issued by The Health Research Ethics Committee of Faculty of Medicine Universitas Sebelas Maret No. 59/UN27.6/KEPK/2018.

Preparation of ethyl acetate fraction of *Caesalpinia sappan* L.

Caesalpinia sappan heartwood was obtained from an herbal store in Surakarta, Central Java. An

amount of 180 g of *Caesalpinia sappan* heartwood powder was macerated in 1 L of methanol® (Sigma) for 24 hours. The methanolic soluble compound was filtered using Whatman paper. The process was repeated three times. The filtrate of the methanolic soluble compound was then subjected to rotary evaporation to get concentrated crude methanolic extract. The methanolic extract was partitioned using n-hexane® (Sigma): ethyl acetate® (Sigma) (v:v) to separate the non-polar compound from the polar compound. The n-hexane soluble fraction was taken out and filtered. The ethyl acetate soluble fraction was then concentrated and applied to a silica gel column using 1:9 of Chloroform: Ethyl Acetate eluent. The final process yielded 24 separated fractions. All the fractions were subjected to a rotary evaporator to remove ethyl acetate residue. One hundred and twenty (120) mg of fraction-9 with yellowish red color was used in this experiment. The selection of Fraction-9 was based on the physical reference of Brazilin.^{10,11} Further identification of Fraction-9 compound was conducted by using comparative HPLC with Brazilin® (Sigma) as standard. The stock solution used in anti-proliferative and migration assay was made by dissolving Fraction-9 with cell culture grade of *Dimethyl Sulfoxide* (DMSO®) from Sigma. The DMSO concentration on the tested fraction was adjusted not to exceed 0.1% for all of the experiments.

Anti-proliferative assay

Anti-proliferative activity of ethyl acetate fraction of *Caesalpinia sappan* wood was measured using MTT assay. PC3 cancer cell line was used as an *in vitro* model of prostate cancer.¹² RPMI 1640 culture medium supplemented with 10% FBS and 1% Penicillin + Streptomycin was used. A number of 1×10^4 cells were seeded in 96-well microplates and incubated in 37°C and 5%CO₂. After 24 hours the medium was replaced with 100 µl medium containing—serial concentration (100, 50, 25, 12.5, 6.25, 3.125, 1.55, and 0 µg/ml) of the tested fraction. The cells were incubated for the next 24 hours. After 24 hours the culture medium was replaced with 100 µl MTT stock solution (5 mg/ml, Sigma, St. Louis, MO) and incubated for 4 hours on 37°C and 5%CO₂. Sodium Dodecyl Sulphate (SDS) 10% in 0.1 N HCl was added as stop solution after 4 hours incubation, and the plate was wrapped with aluminum foil and kept at room temperature overnight. The absorbance was measured at 570 nm wavelength using ELISA reader (Biorad®) All

the measurements were performed in triplicate. The cell viability was measured by comparing the optical density of the treated (dose 100 – 1.55 $\mu\text{g/ml}$) and the non-treated (dose 0 $\mu\text{g/ml}$) cells as a reference. The data were presented as the percentage of cell viability.

Migration assay

The experiment was conducted using a 6 well plate. Following the standard procedure on migration assay¹³, an amount of 7×10^5 PC3 cancer cell line/well was seeded in 6 well plate. RPMI 1640 culture medium supplemented with 10% FBS and 1% Penicillin and Streptomycin were used. The cells culture reached the monolayer, after 24 hours of incubation at 37°C and 5% CO₂. The wound was made by scratching the monolayer cells using a 200 μl pipet tip. After scratching, the monolayer was carefully washed using PBS to remove the cell's debris. The culture medium was replaced with a medium containing 0, 10, and 100 $\mu\text{g/ml}$ of

fraction-9. The picture of the wound was taken at 0 and 18 hours after the scratching by using an inverted microscope. The cell migration was measured by the narrowing of cell free area (the width of the wound area) during the experiment time. The width of the wound area was measured using ImageJ software (NIH, Bethesda, MD). The cell migration was expressed as the reduction of the width of the cell-free area or the proximity of the wound edge.

RESULTS

Identification of selected compound from ethyl acetate fraction (fraction-9)

The HPLC analysis performed using HPLC system (Shimadzu Corp., Tokyo, Japan). The eluent system consisted of an isocratic mode of 100% (v/v) Methanol, running at a flow rate of 0.5 ml/min at a column temperature of 25°C. The spectrometric result of comparative HPLC showed in the Figure1.

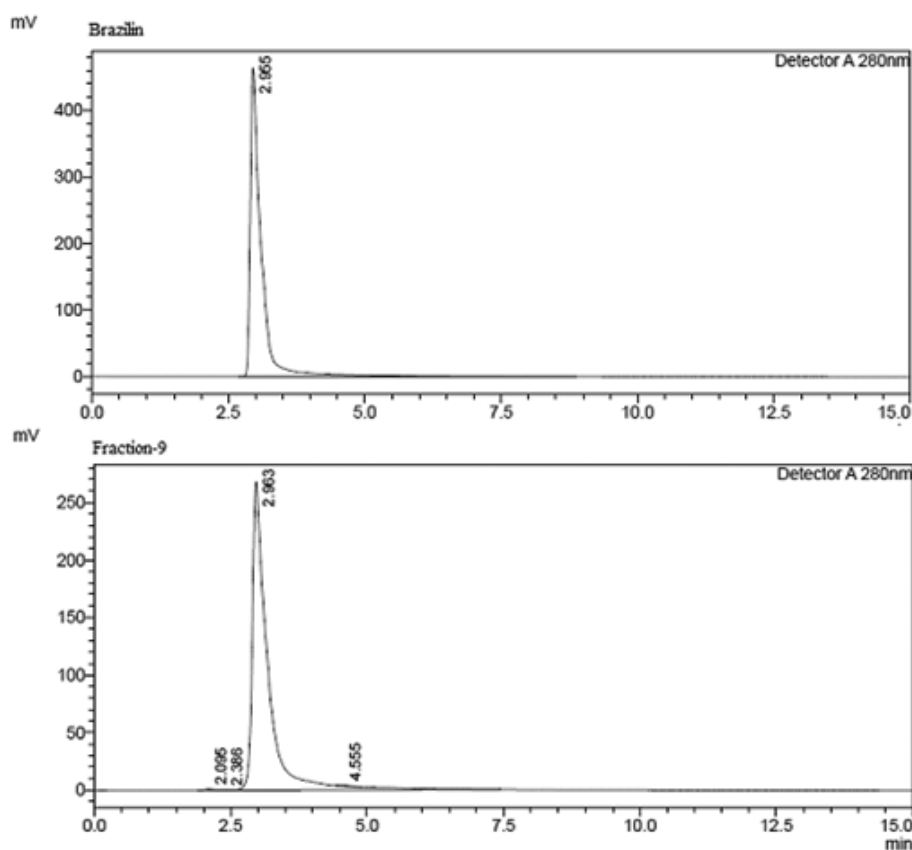


Figure 1. HPLC Chromatograph of Brazilin® and Fraction-9.

The peak of fraction-9 at the same retention time indicates that brazilin is the major compound of fraction-9.

1. Anti-proliferative assay

The result of anti-proliferative assay of fraction-9 on PC3 cancer cell line was shown in Figure 2. The fraction-9 was markedly showed a dose-dependent anti-proliferative effect on the cell. The increase of F9 concentration resulting in a decrease in the percentage of the viable cell. The IC50 of the compound was calculated using the following equation ¹⁴.

$$IC50 = \frac{(X2 - X1) \times (50 - Y1)}{(Y2 - Y1)} + X1$$

X1/X2 = the higher/the lower concentration bordering the concentration that reduces the cell growth of 50%

Y1/Y2 = percentage of viable cells at concentration X1/X2

The IC50 of fraction-9 acquired by the calculation was 14.99µg/mL.

2. Migration assay

The migration assay showed that fraction-9 of ethyl acetate fraction extracted from *Caesalpinia sappan* capable of inhibiting the migration of PC3 cancer cell line from the scratch line to the middle part of the wound. Figure 3 and Figure 4 showed that the addition of fraction-9 on PC3 cancer cell line monolayer after scratching inhibits cell migration activity. The migration activity was calculated as a percentage of the closure of the wound edge at 18 hours in respect to those at 0 hours. The percentage of the closure of the wound edge was calculated using the following formula ¹⁵:

$$wound\ closure\ \% = \frac{At0h - At\Delta h}{At0h} \times 100\%$$

A_{t0h} = the area of the wound measured immediately after scratching (t_{0h})

A_{tΔh} = the area of the wound measured Δ hours after the scratch is performed

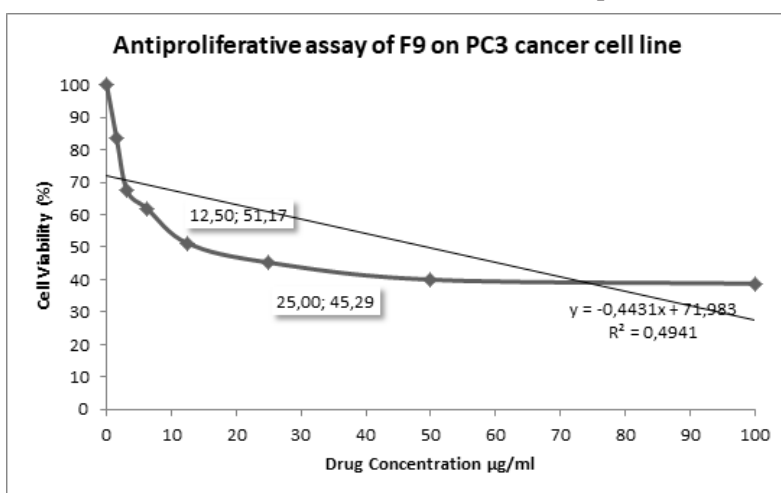


Figure 2. MTT Assay

The graphic showed a dose dependent effect of Fraction-9 on the percentage of PC3 cancer cell line viability. The IC₅₀ of the fraction-9 was 14.99µg/ml.

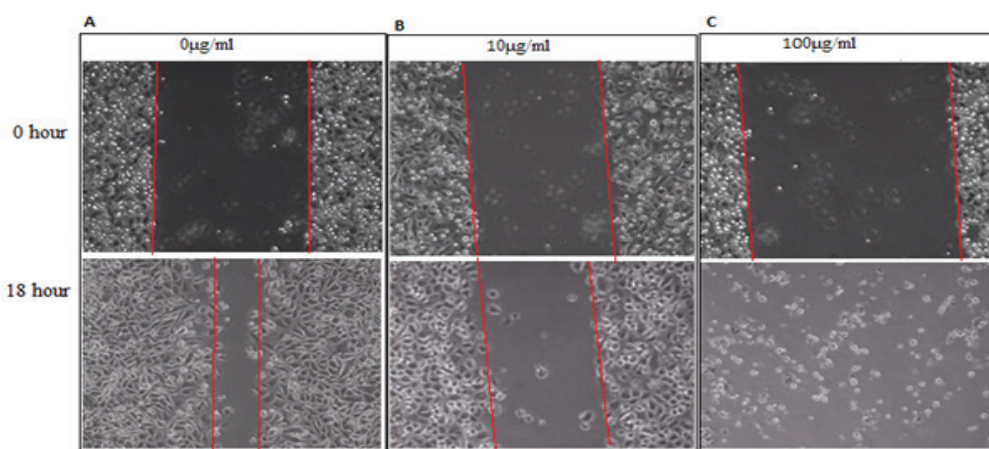


Figure 3 The effect of Fraction 9 on PC3 cancer cell line migration. A. The cell free area of PC3 cancer cell line monolayer without addition of Fraction 9 (0µg/mL) at 0 and 18 hours after scratching. B. The cell free area of PC3 cancer cell line monolayer treated with 10µg/mL of Fraction 9 at 0 and 18 hours after scratching. C. The cell free area of PC3 cancer cell line monolayer treated with 100µg/mL of Fraction 9 at 0 hour after scratching (upper) and at 18 hours after scratching most of the cells died (lower).

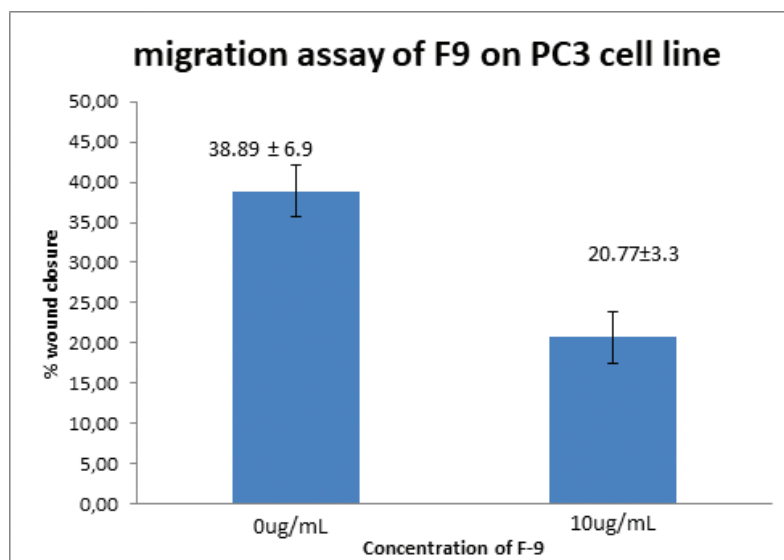


Figure 4. The effect of Fraction 9 of Ethyl acetate fraction of *Caesalpinia sappan* L. heartwood on PC3 cancer cell line migration. Cell migration was presented as a percentage of wound closure. Treatment of PC3 cancer cell line monolayer after scratching decreases the percentage of wound closure as compared to the control.

Figure 3 showed the highest percentage of cell migration on the untreated cells (0µg/ml). The cell-free area was wider in cells treated with 10µg/ml of fraction-9 than those in the untreated cells. The migration activity presented as the percentage of wound closure were 38.89% ± 6.9 20.7% and 20.77% ± 3.3% for untreated cell and 10µg/ml Fraction-9 treated cell respectively.

DISCUSSIONS

Brazilin is considered the major compound of *Caesalpinia sappan*. heartwood.¹⁰ The data resulted from the comparative HPLC of fraction-9 with Brazilin® as standard compound indicated that Fraction-9 consisted mainly of brazilin. As can be shown in the chromatography of HPLC, many other compounds in small proportion were still mixed in fraction-9. Although fraction-9 was not a purified compound, it showed strong cytotoxic activity toward the growth of the PC3 cancer cell line. The IC50 of fraction-9 in this study was 14.99µg/mL.

The anti-proliferative activity of fraction-9 selected from the Ethyl acetate fraction of *Caesalpinia sappan* heartwood was markedly different from the anti-proliferative activity of methanolic extract of *Caesalpinia sappan* reported in a previous study, where the IC50 of methanolic extract of *Caesalpinia sappan* was 48µg/ml on MCF-7 cell line. Fraction-9 also exhibits a higher cytotoxic activity compared

to the cytotoxic activity of ethyl acetate fraction reported on HeLa, MCF-7/Moc, MCF-7/Her2, and T47D cancer cells, where the range of IC50 were 40 – 65µg/mL.¹⁷⁻¹⁹

Investigation on anti-cancer activity of purified brazilin showed a potential anti-cancer activity of brazilin. Kim *et al.* reported high cytotoxic activity of brazilin on MM cells (U266 and MM1s), leukemia cell line (K562) with the range of IC50 10-80µM.⁸ Ren *et al.* also reported the inhibition ratio of brazilin purified from sappan wood on the bladder cancer cell line (T24). The study reported the 50% inhibition ratio of brazilin on the T24 cell line was 25.81µg/mL.²⁰ Compare to the anti-proliferative activity of purified Brazilin, the result of this study showed a promising potency to inhibit cancer cell proliferation. However, the cytotoxic dosage of fraction 9 on PC3 cancer cell line was higher than the dose of Doxorubicine and Docetaxel reported by Tsakalozou *et al.* where the IC50 of Doxorubicine and Docetaxel on PC3 cancer cell line were 0.598nM and 0.469nM respectively.²¹

The anti-migratory activity of fraction 9 was evaluated on PC3 cancer cell line in this study. The cell migration activity was examined using wound-healing assay. This method measure cell migration activity as the rate of wound closure. The result of this study showed that fraction-9 inhibits wound closure on dosage 10µg/mL. PC3 cancer cell line treatment with 10µg/L of fraction-9 resulting in 22.77% of wound closure, while the wound closure of untreated PC3 cancer cell line was 38.89%. This

anti-migratory effect was comparable with other reported studies on purified Brazilin, where treatment with 12.5 μ M of Brazilin inhibits up to 16% of MCF/Her2 cell migration.²²

In conclusion, ethyl acetate fraction 9 of *Caesalpinia sappan* L wood possibly has anti-cancer properties based on its anti-proliferative and anti-migration activities against PC3 cancer cell line. Further study to optimize the compound, dosage, and anti-cancer mechanism of *Caesalpinia sappan* L. is required.

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REFERENCES

1. Moelock NF. Indonesia national health policy in the transition of disease burden and health insurance coverage. *Med J Indones.* 2017;26(1):3–6. Available from: <http://dx.doi.org/10.13181/mji.v26i1.1975>.
2. International Agency for Research on Cancer. Indonesia Source GLOBOCAN 2018. Available from: <https://gco.iarc.fr/today/home>.
3. Mochtar CA, Atmoko W, Umbas R, Hamid ARAH. Prostate cancer detection rate in Indonesian men. *Asian J Surg.* 2018; 41:163-9.
4. Umbas R, Safriadi F, Mochtar CA, Djatisoesanto W, Hamid ARAH. Urologic cancer in Indonesia. *Jpn J Clin Oncol.* 2015 June 25:708–12.
5. Mulyani H, Widyastuti S, Ekowati V. Tumbuhan herbal sebagai jamu pengobatan tradisional terhadap penyakit. *J Penelit Hum.* 2016 Oktober 21:73–91. Indonesian.
6. Yang X, Ren L, Zhang S, Zhao L, Wang J. Antitumor effects of purified Protosappanin B extracted from *Lignum sappan*. *Integr Cancer Ther.* 2016;15:87–95.
7. Tirtanirmala P, Novarina A, Utomo RY, Sugiyanto RN, Jenie RI, Meiyanto E. Cytotoxic and Apoptotic-inducing effect of fraction containing Brazilin from *Caesalpinia sappan* L. and Cisplatin on T47D cell lines. *IJCC.* 2015;6:89–96.
8. Kim B, Kim S, Jeong S, Sohn EJ, Jung JH, Lee MH, et al. Brazilin induces apoptosis and G2/M arrest via inactivation of histone deacetylase in multiple myeloma U266 cells. *J Agric Food Chem.* 2012;60:9882–9.
9. Fardhyanti DS, Riski RD. *Jurnal Bahan Alam Terbarukan.* 2015;4(1):6–13. Indonesian.
10. Nirmal NP, Rajput MS, Prasad RGSV, Ahmad M. Brazilin from *Caesalpinia sappan* heartwood and its pharmacological activities: a review. *Asian Pac J Trop Med.* 2015;8(6):421–30.
11. Batubara I, Mitsunaga T, Ohashi H. Screening antiacne potency of Indonesian medicinal plants : antibacterial, lipase inhibition, and antioxidant activities. *J Wood Sci.* 2009;55:230–5.
12. Cunningham D, You Z. In vitro and in vivo model systems used in prostate cancer research. *J Biol Methods.* 2015;2(1):1–28.
13. Justus CR, Leffler N, Ruiz-echevarria M, Yang LV. In vitro cell migration and invasion assays. *J Vis Exp.* 2014; 88:1–8.
14. Mathieu V, Neve N De, Mercier M Le, Dewelle J, Gaussin J-F, Dehoux M, et al. Combining Bevacizumab with Temozolomide increases the antitumor efficacy of Temozolomide in a human glioblastoma orthotopic xenograft model. *Neoplasia.* 2008;10:1383–92.
15. Grada A, Otero-vinas M, Prieto-castrillo F, Obagi Z, Falanga V. Research techniques made simple: analysis of collective cell migration using the wound healing assay. *J Invest Dermatol* 2018;137:e11–6.
16. Suyatmi S, Azzumar F, Pesik RN, Indarto D. Potential anticancer activity of *Caesalpinia sappan* Linn., in silico and in vitro studies. In: The 1st International Conference on Health, Technology, and Life science (ICO-HELICS) Surakarta. *KnE Life Sciences;* 2019. p. 96–102.
17. Husnaa U, Putu N, Laksmiani L, Susidarti RA, Meiyanto E. Ethyl acetate fraction of *Caesalpinia sappan* L. enhances cisplatin's cytotoxicity on HeLa cells via G1 and S arrest through p53 expression. *IJCC.* 2017;8:51–60.
18. Jenie RI, Handayani S, Susidarti RA, Udin Z, Meiyanto E. Cytotoxic and Antimetastasis effect of Ethyl acetate fraction from *Caesalpinia sappan* L. on MCF-7 / HER2 cells. *IJCC.* 2017;8:42–50.
19. Utomo RY, Novarina A, Tirtanirmala P, Kastian RF, Jenie RI. Enhancement of Cytotoxicity and Apoptosis induction of Doxorubicin by Brazilin containing fraction of Secang (*Caesalpinia sappan* L.) on T47D cells. *IJCC.* 2018;9:32–40.
20. Ren L, Yang X, Wang G, Zhang H, Zhao L, Mi Z. Inhibition effect of Brazilin to human bladder. *Int Sch Sci Res Innov.* 2011;5:657–61.
21. Tsakalozou E, Eckman AM, Bae Y. Combination effects of Docetaxel and Doxorubicin in hormone-refractory prostate cancer cells. *Biochemistry Research International.* 2012 Juli: 1-10. Article ID 832059, <https://doi.org/10.1155/2012/832059>.
22. Jenie RI, Handayani S, Susidarti RA, Udin LZ, Meiyanto E. The Cytotoxic and Antimigratory activity of Brazilin-Doxorubicin on MCF7/Her2 cells. *Adv Pharm Bull.* 2018;8:507–16.