

Caesalpinia sappan L. Ethanolic Extract Decrease Intracellular ROS Level and Senescence of 4T1 Breast Cancer Cells

Naufa Hanif¹, Adam Hermawan^{1,2}, Edy Meiyanto^{1,2,*}

¹Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

²Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Highly and uncontrolled cell proliferation on cancer cells may boost ROS level intracellular accumulation up significantly. Sappan heartwood (*Caesalpinia sappan* L.) have been known to have cytotoxicity effect toward several cancer cells. This research was conducted to develop *Caesalpinia sappan* L. heartwood ethanolic extract (CSE) as chemopreventive agent which can be used as cancer therapy, looked from antioxidant and anti-senescence activity toward 4T1 breast cancer cell. CSE was obtained through maceration using ethanol 70% as solvent. Cytotoxicity activity of CSE was done by using MTT assay with IC₅₀ as parameter. This IC₅₀ was used as basic to the next assay, ROS assay by using DCFDA staining flowcytometry to look ROS level intracellular of CSE toward 4T1 cell and senescence assay by using senescence associated β -galactosidase (SA β -gal) assay with % cell senescence as their parameter. CSE toxic to 4T1 cell that proved from IC₅₀ value of 25 μ g/mL. Single treatment of CSE on concentration 12,5; 25; and 37.5 μ g/mL able to suppress ROS level intracellular. If compared with untreated cell, single treatment of CSE on concentration 6 and 12 μ g/mL showed % cell senescence not significantly different. Based on this result, CSE is cytotoxic, has antioxidant and antisenescence activity, so it has great potential to developed as chemoprevention agent on cancer therapy.

Keywords : *Caesalpinia sappan* L., 4T1 breast cancer line, Reactive Oxygen Species (ROS), anti-senescence

INTRODUCTION

Reactive oxygen species (ROS) are continuously produce through normal metabolism process and maintained in balance condition, between ROS production and elimination (Zou, et al., 2017). However, on cancer cell has uncontrolled proliferation which lead to altered ROS expression, which is characterized by the presence of redox homeostasis in the form of increased ROS

production (Costa, et al., 2014; Zhang, et al., 2016) and decreased ROS arrest. Cancer cells require ROS for the initiation, promotion, and progression of cancer cells (Galadari, et al., 2017), but the

Submitted: September 17, 2018

Revised: December 12, 2018

Accepted: December 12, 2018

*Corresponding author: meiyana_e@ugm.ac.id

excessive of ROS can be toxic and damage cancer cells (Redza-Dutordoir and Averill-Bates, 2016), so that it is more susceptible to cell death (Kong and Chandel, 2017) especially senescence (Zhou, *et al.*, 2014). Therefore, a chemoprevention agent is needed which can reduce excess ROS levels in cancer cells.

The development of chemoprevention agents that are able to reduce ROS is a strategic approach in the treatment of cancer through the capture of ROS that can lead to cancer. One of the potential chemoprevention agents to be developed is sappan heartwood (*Caesalpinia sappan* L.). In sappan heartwood contain several chemical compounds such as xanthone, coumarine, chalcones, flavones, homoisoflavonoid and brazilin (Nirmal, *et al.*, 2015). Recent studies reveal that brazilin, the major active compound in sappan heartwood, has antioxidant effect. Based on Uddin, *et al.*, 2015 proved that ROS levels of H₂O₂ can be decreased by pre-treatment with brazilin in a concentration-dependent manner. *Caesalpinia sappan* L. also able to scavenge α,α -diphenyl- β -picrylhydrazyl (DPPH) free radical in a dose dependent manner, and has similar activity with L-ascorbic (as positive control) (Hwang and Shim, 2018). Furthermore, the antioxidant compound is not only able to reduce intracellular ROS levels, but also able to prolong life span. Based on Lee, *et al.*, (2017), brazilin compound was able to extend age of *C. elegans* through antioxidant ability and increase regulation of stress-resistant proteins. Thus, this study was directed to know more and explore the potential effect of sappan heartwood as antioxidant and anti-senescence agent.

MATERIALS AND METHODS

Sample preparation

Sappan heartwood was obtained from Medicinal Plant and Traditional Medicine Research and Development Center, Tawangmangu, Central Java, Indonesia. Sappan heartwood was dried in

40°C oven, pulverized and extracted with ethanol 70% (ratio 1:10) by maceration method for twice of 3x12 hours (shaked for 12 hours/day). The macerat was concentrated by rotary evaporator in 60°C. The dried extract of sappan heartwood (CSE) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, Missouri, United States).

Cell line culture

The 4T1 breast cancer cell line culture was obtained from Prof. Masashi Kawaichi (Nara Institute of Science and Technology, NAIST, Japan). The cells were preserved in Dulbecco's Modified Eagles medium (DMEM) high glucose (Sigma) supplemented with 10% FBS (Sigma), HEPES, sodium bicarbonate, 1.5% Penicilin-Streptomycin and 0.5% Fungizone (Gibco, Massachusetts, USA). These cells were cultured with 5% CO₂ in 37°C.

Cell viability assay

A total of 2.3x10³ cells were planted into 96-well plates and incubated for 24 hours, then treated with various concentrations of CSE in culture medium as much as 100 μ L/well and incubated for 24 hours. Then added 100 μ L of culture medium containing MTT 0.5 mg/mL into each well, incubated again for 4 hours at 37°C. The condition of the cell under the inverted microscope was examined, if formazan had been formed, a stopper solution of 100 μ L SDS 10% was added in 0.01 N HCl and the plate was left overnight. The absorbance was measured using ELISA microplate reader at λ 595 nm. The single treatment absorbance data is converted into percent viability and used to calculate IC₅₀.

Intracellular ROS Level with DCFDA Staining Flowcytometry

4T1 cell line was cultured with 5x10⁴ in culture media (DMEM) and incubated for 24 hours at 37°C. Cultured media was removed and washed 1x PBS once. Cells were treated with increasing concentration of CSE (12.5 μ g/mL (1/2

IC₅₀); 25 µg/mL (IC₅₀), and 37.5 µg/mL (1½ IC₅₀) incubated for 24 hours. Then discarded the media, then washed 1x PBS once. Next, 300 µL trypsin is added and leveled for 30 seconds. Then remove trypsin and incubate for 3-5 minutes at 37°C. Then 500 mL of 1x supplemented buffer is added for trypsin inactivation. Moved to the new microtube. Microtube is coated with aluminum foil or use a dark microtube. The next step, stain cells with 25 µM 2',7' -dichlorofluorescein diacetate (DCFDA) by adding 6 µL of 2 mM DCFDA then incubating for 30 minutes in a 37°C CO₂ incubator. Finally, the sample is read using a flowcytometry device at Ex 485 nm/Em 535 nm (Anonymous, 2016).

Senescence Associated β-Galactosidase (SA β-Gal) Assay

The planted 4T1 breast cancer cell of 5x10⁴ cells/well on 6-well plate was incubated for 24 hours in a 37°C CO₂ incubator. After confluent cells, then the media was discarded, washed the cells with 2 mL of 1x PBS twice, then treated using CSE concentration of 6 and 12 µg/mL both single and combination with 250 nM doxorubicin. There are two different period of treatment, for 24 and 48 hours. Next, discarded the media and washed 2 mL of 1x PBS twice. Then, a fixation buffer (1-2 mL) was added, incubated for 10 minutes at room temperature, removed the fixation buffer, washed the cell with 2 mL PBS 1x twice, added X-Gal dye solution (1-2 mL), and incubated at 37°C (non CO₂ incubator). After passing through a series of staining stages with X-gal substrate, cells were observed at 24, 48, 72, 96 and 120 hours in the incubation phase contrast microscope. Finally, counted cells that are greenish blue and displayed as % cells senescence (Eccles and Li, 2012)

Statistical analysis

Data are presented as the mean±SD using SPSS 16.0 application. The significance of difference between each of two different treatments were added to every chart (*).

RESULTS

Cytotoxic Activity of Single CSE on 4T1 Breast Cancer Cells

Before we know more the potential effect of CSE in inhibiting Intracellular ROS level and cell senescence, we must know the cytotoxicity of CSE first. The result showed that viability of 4T1 cell was decreased in dose dependent manner. we obtained the IC₅₀ value was 25 µg/mL. These result was exhibited the cytotoxic effect as seen in morphological figure (Figure 1B). Therefore, the result indicated that CSE was cytotoxic to 4T1 breast cancer cells and has the potential effect as chemoprevention agent. These result was collateral with the Haryanti, *et al.*, (2017)'s research which stated that CSE has an IC₅₀ value of 20 µg/mL in cell 4T1. The difference in IC₅₀ values between this study and the previous one is not significantly different. Then, the IC₅₀ value was used as standard concentration of next assay (DCFDA flowcytometry assay and SA β-Gal assay).

Antioxidant Activity of CSE on 4T1 Cells

To find out more about antioxidant activity of sappan heartwood, we conducted the DCFDA staining flowcytometry to look Intracellular ROS level of CSE-treated cells. In this assay, we used both H₂O₂ 200 µM and doxorubicin 6 nM as positive control, and single treatment of CSE concentration of 12.5 µg/mL (½ IC₅₀); 25 µg/mL (IC₅₀); and 37.5 (1½ IC₅₀) µg/mL. The three CSE concentrations of 12.5, 25 and 37.5 µg/mL showed a quite different with untreated cell, where all three were able to decrease significantly (Figure 2).

Anti-Senescence Activity of CSE on 4T1 Cell

The decreased intracellular ROS level after treated with CSE concentration of 12.5, 25 and 37.5 µg/mL, indicating its ability to extend the life span of an organism (Ristow and Schmeisser, 2011). So, to reveal the potential effect of CSE in inhibiting senescence in 4T1 breast cancer cells, a

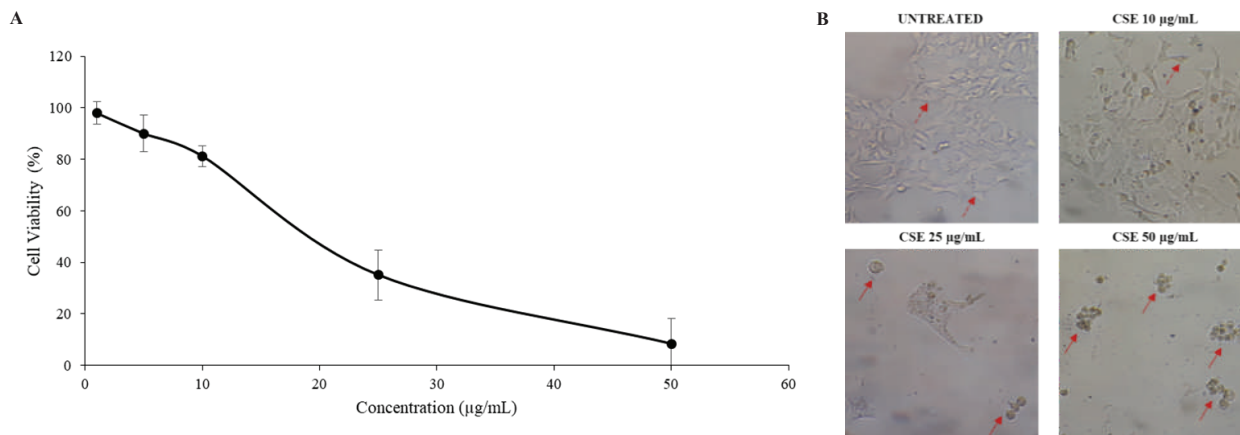


Figure 1. Cytotoxic effects of a single CSE on 4T1 breast cancer cells. **A:** Profile of linear regression curve between cell viability (%) with given CSE concentration (µg/mL), presented from mean±SD of 4 measurements (n=4), IC₅₀ value of 25 µg/mL. **B:** Appearance of cell morphology observed under the inverted microscope from cell control (untreated), CSE 10, 25 and 50 µg/mL. Signs indicate healthy cells, while signs indicate cells that have died after CSE treatment of 25 and 50 µg/mL.

senescence associated β-galactosidase (SA β-Gal) assay was conducted. In this assay, doxorubicin is used as a positive control because it is known that anthracycline compounds are capable of inducing senescence (Piegari, *et al.*, 2013). There

are two concentrations of CSE used in this assay, 6 µg/mL (¼ IC₅₀) and 12 µg/mL (½ IC₅₀). Both concentrations were used so that senescent cells can be observed before cell death. Besides that, in this assay we used two different periods of treatment, 24

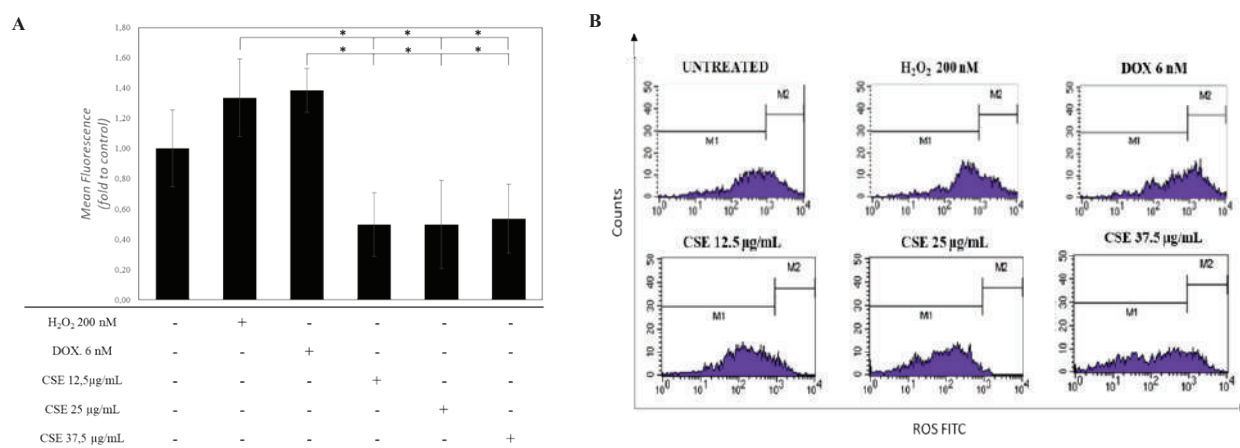


Figure 2. Measurement of intracellular ROS levels was carried out using DCFDA staining flow cytometry method in 4T1 breast cancer cell to see the effect of single CSE-treated. **A:** In 4T1 cells, H₂O₂ 200 µM and doxorubicin (dox) 6 nM as a positive control on ROS test which can be increased ROS expression. Single CSE-treated concentration of 12.5, 25 and 37.5 µg/mL. **B:** A bar chart of the profile of intracellular ROS levels by single CSE against untreated cell/cell control in 4T1 cell. In 4T1 breast cancer cells, a single CSE-treated on concentration of 12.5, 25 and 37.5 µg/mL can decrease intracellular ROS levels significantly (*p<0.05).

and 48 hours. These different periods were used to see the effectiveness of the time needed for the CSE to prevent senescence.

Meanwhile, the counting of senescence cells were carried out by calculating the percentage of colored cells from 100 cell populations. There were almost similar in morphological appearance of 24 hours-treated (Figure 3A) and 48 hours-treated (Figure 3B). Both of them showed the stained cell (undergo senescence cells) of doxorubicin-treated were much more than untreated and single CSE concentration of 6 and 12 µg/mL. However, different things were shown in the treatment of combination between CSE (6 and 12 µg/mL and doxorubicin 250 nM. The majority of cells experience cell

death shown by round cell morphologically (Figure 3A). This indicates that the combination treatment directly triggers cell death and does not go through senescence first.

DISCUSSION

In this study 4T1 cells were used as triple negative breast cancer cell modeling. In addition, 4T1 cells were characterized into highly metastatic cell (Abe, *et al.*, 2016). Based on the research that had been conducted by Piskounova, *et al.*, (2015), the manifestation of high levels of metastatic in cells is an increase intracellular ROS production. In this study, we confirmed that sappan heartwood has

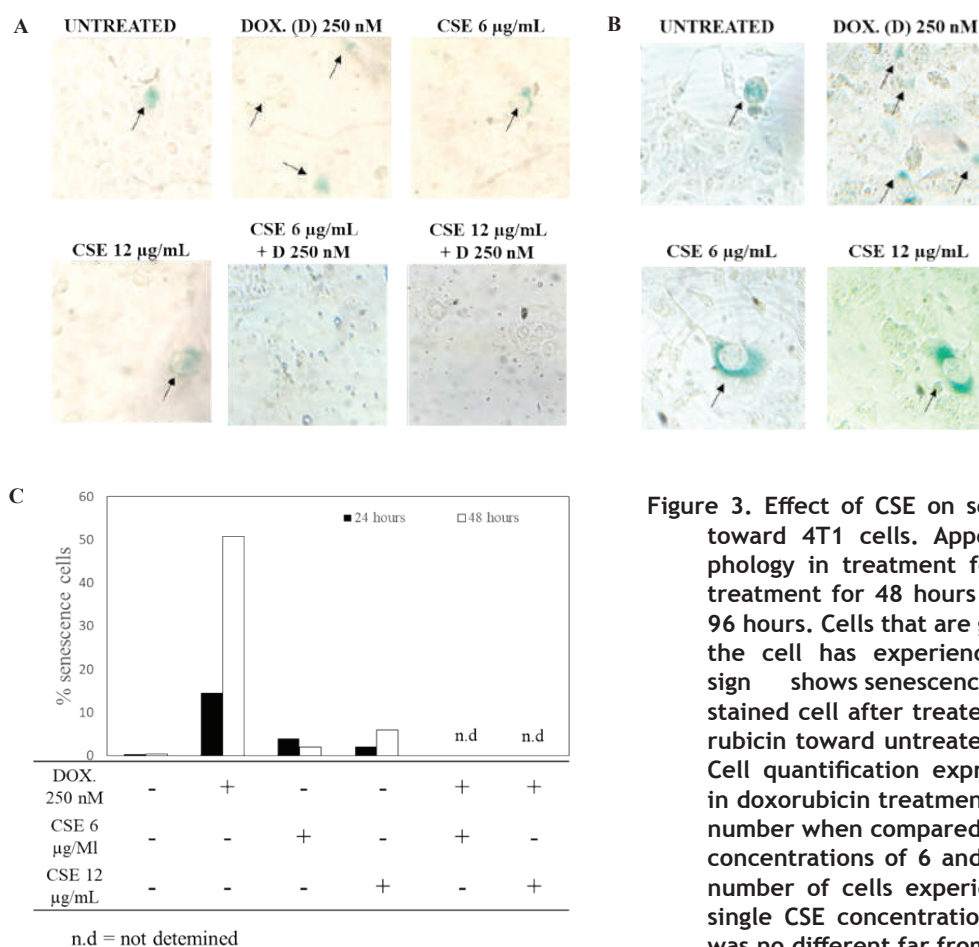


Figure 3. Effect of CSE on senescence expression toward 4T1 cells. Appearance of cell morphology in treatment for 24 hours (A), and treatment for 48 hours (B), after stained for 96 hours. Cells that are greenish blue indicate the cell has experienced senescence. The sign shows senescence cells. Bar diagram of stained cell after treated with CSE and doxorubicin toward untreated cell in 4T1 cell (C). Cell quantification expressed by senescence in doxorubicin treatment showed a very large number when compared to untreated and CSE concentrations of 6 and 12 µg/mL, while the number of cells experiencing senescence at single CSE concentration of 6 and 12 µg/mL was no different far from untreated.

strong cytotoxicity effect on 4T1 breast cancer cell (IC_{50} value on 25 $\mu\text{g/mL}$). The cytotoxicity effect of sappan heartwood extract on 4T1 cancer cell had been tested before by Haryanti, *et al.*, (2017) with IC_{50} value on 20 $\mu\text{g/mL}$. Because of the result of CSE cytotoxicity, further investigation is needed on the CSE cytotoxicity pathway.

Previously, sappan heartwood has been investigated in 4T1 breast cancer cell through cell cycle pathways and apoptosis. Research that had been conducted by Haryanti (2017) stated that the combination of sappan heartwood and awar-awar extract with doxorubicin concentration of 10:1.7:0.23 $\mu\text{g/mL}$ in 4T1 cells were able to inhibit the cell cycle in the G2/M phase and induce apoptosis, decrease phosphorylated I κ B α protein expression, inhibits migration and expression of MMP9 in 4T1 cells and decreases Rac-1 protein expression (Haryanti, 2017). So one of the other pathways that could be explored is cancer cell metabolism primarily on the ROS pathway.

On the DCFDA staining assay, we could look the antioxidant effect of CSE. The ability of CSE to reduce intracellular ROS levels can be seen from the structure of the brazilin and brazilein. Both of these compounds have phenolic hydroxyl. Phenolic compounds are known to have antioxidant activity (Hassanzadeh, *et al.*, 2014) These results lead to antioxidant effects of CSE on 4T1 breast cancer cells. Not only its ability to reduce ROS levels in cells, sappan heartwood and brazilin are also known to be able to induce the expression of the glutathione peroxidase 7 (GPX7) enzyme. Research conducted by Hwang and Shim (2018) shows that *Caesalpinia sappan* L. (CSL) extract has an effect on DPPH free radical capture in dose dependent manner. When compared with L-ascorbic acid (positive control), CSL extract also showed similar antioxidant activity. In addition, CSL extract is also able to reduce the formation of H_2O_2 significantly (Hwang and Shim, 2018). One of the confirmation steps regarding these results is to see effect of CSE on the occurrence of senescence cells in 4T1 cells.

However, research on the potential effect of sappan heartwood in inhibiting the occurrence of senescence in cancer cells has been carried out by Lee, *et al.*, (2017) showing the treatment of brazilin concentrations of 50 and 100 μM able to prolong the life of *Caenorhabditis elegans* (*C. elegans*), when compared to control were 11.3% and 17.9% respectively. Brazilin is able to prolong life time of *C. elegans* both under normal conditions and when the levels of stress inside it increase, through the ability of antioxidants and increase the regulation of resistant stress proteins. In addition, the previous research conducted by Putri, *et al.*, (2018) explained that ethanolic extracts of sappan heartwood were able to prevent senescence in mesenchymal stem cells. This is indicated by a decrease in the intensity of the greenish stained cells at CSE concentration of 10 $\mu\text{g/mL}$ combined with 5 μM doxorubicin concentration. The combination of CSE concentration of 10 $\mu\text{g/mL}$ was able to reduce % senescence cells from a single treatment of doxorubicin at a concentration of 5 μM by 76% to 38%. The potential shown in previous studies leads to further suggestions for the SA β -Gal test by adding a combination treatment of CSE samples with doxorubicin. The concentration used is based on IC_{50} values obtained from the cytotoxic test of a combination of both. Therefore, it is necessary to do a cytotoxic test combination between CSE and doxorubicin so that it can be know at what concentration of CSE and doxorubicin combination can have a synergistic effect and have a high enough viability (60-70%) so that it can be seen in the potential for inhibiting the occurrence of senescence cells.

CONCLUSION

In summary, we can conclude that CSE has cytotoxic effect to cancer cells. It means that CSE has potential to be developed as chemoprevention agent. Furthermore, CSE also able to scavenge free radicals seen from its ability to reduce intracellular

ROS levels and inhibit the occurrence of senescent cells. From this result, we can see the cytotoxicity of CSE is not through the ROS or senescence pathway. So on the next research it is needed to looking for another pathway that may induce cytotoxic on cancer cells.

ACKNOWLEDGEMENT

We express our appreciation and gratitude to Ministry of Research, Technology, and Higher Education of the Republic of Indonesia that has been funding this research via PKM 2017.

REFERENCES

- Abe, H., Wada, H., Baghdadi, M., Nakanishi, S., Usui, Y., Tsuchikawa, T., et al., 2016, Identification of A Highly Immunogenic Mouse Breast Cancer Sub Cell Line, 4T1-S, *Hum. Cell*, **29**(2), 58-66.
- Anonymous, 2016, *Cellular Reactive Oxygen Species Detection Assay Kit (Red Fluorescence) (ab186027)*, <http://www.abcam.com/cellular-reactive-oxygen-species-detection-assay-kit-red-fluorescence-ab186027.html>, accessed on June 7, 2018.
- Costa, A., Scholer-Dahirel, A. and Mechta-Grigoriou, F., 2014, The Role of Reactive Oxygen Species and Metabolism on Cancer Cells and Their Microenvironment, *Semin. Cancer Biol.*, **25**, 23-32.
- Eccles, M. and Li, C., 2012, Senescence Associated B-galactosidase Staining, *BIO-Protoc.*, **2**(16), e247. doi: 10.21769/BioProtoc.247.
- Galadari, S., Rahman, A., Pallichankandy, S. and Thayyullathil, F., 2017, Reactive Oxygen Species and Cancer Paradox: To Promote or To Suppress? *Free Radic. Biol. Med.*, **104**, 144-164.
- Haryanti, S., 2017, *Potensi Antikanker Kombinasi Ekstrak Tanaman Dengan Doxorubicin Melalui Penghambatan Proliferasi dan Metastasis pada Sel Kanker Payudara Secara In Vitro*, Dissertation, Fakultas Farmasi Universitas Gadjah Mada, Yogyakarta.
- Haryanti, S., Murwanti, R., Putri, H., Ilmawati, G.P.N., Pramono, S. and Meiyanto, E., 2017, Different 4T1 Cells Migration under Caesalpinia sappan L. and Ficus septica Burm.f Ethanolic Extracts, *Indones. J. Cancer Chemoprevent.*, **8**(1), 21-26.
- Hwang, H.S. and Shim, J.H., 2018, Brazilin and Caesalpinia sappan L. Extract Protect Epidermal Keratinocytes from Oxidative Stress by Inducing the Expression of GPX7, *Chin. J. Nat. Med.*, **16**(3), 203-209.
- Kong, H. and Chandel, N.S., 2017, Regulation of Redox Balance in Cancer and T cells, *J. Biol. Chem.*, **293**(20), 7499-7507.
- Lee, E.B., Xing, M.M. and Kim, D.K., 2017, Lifespan-extending and Stress Resistance Properties of Brazilin from Caesalpinia sappan in Caenorhabditis Elegans, *Arch. Pharm. Res.*, **40**(7), 825-835.
- Nirmal, N.P., Rajput, M.S., Prasad, R.G.S.V. and Ahmad, M., 2015, Brazilin from Caesalpinia sappan Heartwood and Its Pharmacological Activities: A Review, *Asian Pac. J. Trop. Med.*, **8**(6), 421-430.
- Piegari, E., Angelis, A., Cappetta, D., Russo, R., Esposito, G., Costantino, S., et al., 2013, Doxorubicin Induces Senescence and Impairs Function of Human Cardiac Progenitor cells, *Basic Res. Cardiol.*, **108**, 334. doi: 10.1007/s00395-013-0334-4.
- Putri, A.M., Putri, N.B., Rachmady, R., Dilalah, I., Murwanti, R. and Meiyanto, E., 2018, Secang Heartwood Ethanolic Extract (Caesalpinia sappan L.) Inhibits Mesenchymal Stem Cells Senescence, *Indones. J. Cancer Chemoprevent.*, **8**(3), 126-134.
- Redza-Dutordoir, M. and Averill-Bates, D.A., 2016, Activation of Apoptosis Signalling Pathways by Reactive Oxygen Species, *Biochim. Biophys. Acta*, **1863**(12), 2977-2992.
- Ristow, M. and Schmeisser, S., 2011, Extending Life Span by Increasing Oxidative Stress, *Free Radic. Biol. Med.*, **51**(2), 327-336.
- Uddin, G.M., Kim, C.Y., Chung, D., Kim, K.A. and Jung, S.H., 2015, One-step Isolation of Sappanol

- and Brazilin from *Caesalpinia sappan* and Their Effects on Oxidative Stress-induced Retinal Death, *BMB Rep.*, **48**(5), 289-294.
- Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., *et al.*, 2016, ROS and ROS-Mediated Cellular Signaling, *Oxid. Med. Cell. Longev*, **2016**, 4350965. doi: 10.1155/2016/4350965.
- Zhou, D., Shao, L. and Spitz, D.R., 2014, Reactive Oxygen Species in Normal and Tumor Stem Cells, *Adv. Cancer Res.*, **122**, 1-67.
- Zou, Z., Chang, H., Li, H. and Wang, S., 2017, Induction of Reactive Oxygen Species: An Emerging Approach for Cancer Therapy, *Apoptosis*, **22**(11), 1321-1335.