

Cytotoxic and MMPs inhibitory activities of Sappan Wood (*Caesalpinia sappan L.*): various extracts on 4T1 breast cancer cell line

DOI: 10.22435/hsji.v9i1.483

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Received: February 1, 2018; Revised: April 12, 2018; Accepted: May 9, 2018.

Abstrak

Latar belakang: Kayu secang (*Caesalpinia sappan L.*) merupakan salah satu tanaman potensial dengan berbagai khasiat obat, termasuk antikanker. Gelatinase (MMP-2 dan MMP-9) merupakan matriks metaloproteinase (MMPs) dan memiliki peran penting dalam inisiasi, invasi, dan metastasis kanker. Penelitian ini bertujuan untuk menguji aktivitas sitotoksik dan penghambatan MMP dari ekstrak kayu secang pada sel kanker payudara 4T1.

Metode: Serbuk kayu secang dibagi menjadi 4 bagian, masing-masing diekstraksi dengan pelarut berbeda. Etanol 96%, etanol 70%, dan metanol digunakan untuk metode maserasi, sedangkan air untuk metode infusa. Uji MTT digunakan untuk menentukan efek sitotoksitas, dan uji gelatin zymography untuk mendeteksi aktivitas MMP-2 dan MMP-9. Profil fitokimia ekstrak diamati dengan Kromatografi Lapis Tipis (KLT).

Hasil: Profil fitokimia ekstrak air menunjukkan profil KLT yang berbeda, sementara tiga ekstrak lainnya memiliki profil yang sama. Ekstrak etanol 96% kayu secang memberikan efek sitotoksik paling kuat terhadap 4T1 dengan nilai IC_{50} 13,1 $\mu\text{g/mL}$, diikuti metanol (21,4 $\mu\text{g/mL}$), etanol 70% (22,5 $\mu\text{g/mL}$) dan air (25,5 $\mu\text{g/mL}$). Analisis gelatin zymograph dengan software ImageJ menunjukkan bahwa semua ekstrak kecuali air, menghambat aktivitas gelatinolitik MMP-9

Kesimpulan: Ekstrak kayu secang dengan berbagai pelarut polar memiliki aktivitas sitotoksik dan penghambatan MMP pada sel kanker payudara 4T1 dan berpotensi untuk dikembangkan sebagai anti kanker payudara. (*Health Science Journal of Indonesia 2018;9(1):51-6*)

Kata kunci: *Caesalpinia sappan*, MMP, sitotoksik, 4T1

Abstract

Background: Sappan wood (*Caesalpinia sappan L.*) is one of potential plant with wide variety of medicinal properties, including anticancer. Gelatinases (MMP-2 and MMP-9) are the member of matrix metalloproteinases (MMPs) and having a key role in cancer initiation, invasion, and metastasis. This study evaluated the cytotoxic and MMPs inhibitory activities of sappan wood in various extract on 4T1 breast cancer cell lines.

Methods: Sappan wood powder were divided into 4 parts, each part was extracted with different solvent. Ethanol 96%, ethanol 70%, and methanol were used for maceration methods, while water for infusion method. MTT assay used to identify cytotoxicity effect, and gelatin zymography assay to detect the activity of MMP-2 and MMP-9. Phytochemical profiling of the extract were observed by Thin Layer Chromatography (TLC).

Results: Phytochemical profiling of water extract showed different TLC profile, while three others had similar profile. The results of MTT assay showed that ethanolic 96% extract exhibited the strongest cytotoxic effect against 4T1 with the IC_{50} value 13,1 $\mu\text{g/mL}$, followed by methanolic (21,4 $\mu\text{g/mL}$), ethanolic 70% (22,5 $\mu\text{g/mL}$) and water (25,5 $\mu\text{g/mL}$). The analysis of gelatin zymograph bands using ImageJ software proved that all extracts except water, inhibited gelatinolytic activity of MMP-9.

Conclusion: The results of the study suggest that various extract of the sappan wood have been found to posses cytotoxic and MMPs inhibitory activities. (*Health Science Journal of Indonesia 2018;9(1):51-6*)

Keywords: *Caesalpinia sappan*, MMP's, cytotoxic, 4T1

Caesalpinia sappan L., commonly known as sappan wood or secang in Indonesia, is a plant of Leguminosae family, which has been used widely as traditional medicine in Asia. Chemical investigation resulted that brazilin is the major compound of sappan wood. Brazilin is easily oxidized producing brazilin by air and light. Both brazilin and brazilin responsible for various biological activities of sappan wood, including anticancer.¹

Breast cancer was the most common type of female cancer among diagnoses. The incidence rates of breast cancer is still remain much higher in the world, and continuing to emerge as a major health issue for women in more developed countries such as Asia, Africa and South America. During 2012, close to a quarter (24%) of all breast cancers were diagnosed within the Asia-Pacific region (approximately 404,000 cases at a rate of 30 per 100,000), with the greatest number of those occurring in China (46%), Japan (14%), and Indonesia (12%). It is predicted that the incidence rates of breast cancer in this region will continue to increase.²

Based on the biological molecular breast cancer subtype, more women in the Asia-Pacific region have estrogen receptor (ER)-negative and progesterone receptor (PR)-negative tumors than in America and the European Union.³ This subtype is associated with lower survival rates and earlier onset than ER-PR positive tumors.⁴ The 4T1, a highly metastatic human breast cancer cell line with negative ER-PR, are derived from a spontaneously arising BALB/c mammary tumor breast cancer cells.⁵⁻⁷ It shows breast cancer malignancy 8, and having capability to metastasize to distant organ such as bone, lung, liver, spleen, and brain.^{9,10}

Metastasis is a multistep process by which a subset or individual cancer cells disseminate from a primary tumor to distant secondary organs or tissues. Matrix metalloproteinases (MMPs) have been regarded as major critical molecules assisting tumor cells during metastasis, especially on extra cellular matrix degradation and cancer cell invasion.¹¹ The MMP-2 and MMP-9 are subtype of MMPs with frequently elevated in human cancer, which correlates with advanced tumor stage, increased metastasis, and poor prognosis.¹²

Various medicinal plants' phytochemical constituents have been shown to exert anticancer effects via metastasis. Sappan wood (*Caesalpinia sappan L.*) has potential capacity as promising chemoprevention agent by its cytotoxic and anti MMPs activities.^{13,14}

The most efficient solvent used for sappan wood extraction haven't been explored yet. This study aimed to evaluate cytotoxic effect and MMPs inhibitory activities of sappan wood various extract (water, methanolic, ethanolic 70%, and ethanolic 96%) on 4T1 murine breast cancer cell lines. The 4T1 cells showed characteristic of highly metastatic breast cancer cells, and usually used as appropriate model in metastatic studies.

METHODS

Extract preparation. The heartwood of secang powder were obtained from Medicinal Plant and Traditional Medicine Research and Development Centre (B2P2TO-OT), Tawangmangu, Central Java, Indonesia. The powder was divided by four, then each powder was macerated using ethanol 96%, ethanol 70%, and methanol for 3x24 hours, then filtrated, and evaporated to get dried extract. The water extract was done using hot infusion method for 15 minutes, then filtrated, and evaporated in oven 40°C to obtain dried extract. Each extract was assayed for their cytotoxic activity and MMPs inhibitory effect.

Thin Layer Chromatography. Extract was dissolved in DMSO, spotted 5 µL on plate TLC 60F254, then developing in TLC system with chloroform:ethyl acetate:methanol (5:1:1) with 5 drops of acetic acid. TLC plate was then observed under visible light, UV 366nm, and 254nm. The organic solvents used for TLC method was pro analysis grade (p.a, Merck)

Cell culture and condition. The 4T1 cell lines were obtained from Cancer Chemoprevention Research Centre, Faculty of Pharmacy, Gadjah Mada University Yogyakarta. The cell line was kindly given by Prof. Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. They were grown in B2P2TO-OT molecular biology laboratory, using tissue culture dish (Iwaki) with Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma), and 100 µg/mL penicillin-streptomycin (Sigma). Cells were maintained in incubator at 37°C with 5% CO₂.

Cytotoxic evaluation. Cytotoxic activity was determined by MTT-assay. DMSO (Sigma) was used as co-solvent. Cells were detached from the bottom of tissue cultured dish using 0,05% trypsin-EDTA (GIBCO) and counted with haemocytometer. The cells were seeded into 96-well culture plates at a density of 6000 cells/well, and incubated for 24 hours.

Cells were then treated with 20, 40, 60, 80, and 120 $\mu\text{g}/\text{mL}$ of each extracts for 24 hours. Cells with only culture medium without treatment were used as negative controls. Each treatment was done in triplicate. The cells was observed under inverted microscope (Olympus). Treated cells were added with MTT solution and incubated for 3-4 hours to form formazan crystals. This reaction was stopped using SDS 10% in HCl 0,01N. The intensity of formazan solution was measured by enzyme-linked immunosorbent assay (ELISA reader, Biorad) at wavelength 595nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. Data are expressed as the mean percentage of viable cells as compared to the respective control cultures treated with medium. The curve of dose-respon were made and analysed using Excel linier regression to obtain IC_{50} . The percentage growth inhibition was calculated using following formula:

$$\% \text{ cell inhibition} = 100 - [(A_t - A_b) / (A_c - A_b)] \times 100$$

Where, A_t = absorbance value of test compound, A_b = Absorbance value of blank and A_c = Absorbance value of control.

Gelatin Zymography. A number of 500.000 cells/well were cultured at 6 well plate for 24 hours, and then treated with 25 $\mu\text{g}/\text{mL}$ of each extract. Cultured media were collected after 18 hours of treatment, then centrifugated at 10000 rpm, 4°C for 2 minutes. The supernatant mixed with non-reducing sample buffer, and electrophoresed in a polyacrylamide gel containing 0,1% (w/v) gelatin. The gel was washed at room temperature for 30 min with 2,5% Triton X-100 solution, and subsequently incubated at 37°C for 16 h in developing buffer (0,8766 g NaCl, 0,147 g CaCl_2 , 0,05g NaN_3 , and 0,61 g Tris-base in 100 mL ddH₂O). The gel was stained for 30 min with 0,25% (w/v) coomassie brilliant blue in 40% (v/v) methanol, and 7% (v/v) acetic acid and scanned on Canon image scanner. Proteolysis was imaged as a white zone in a dark blue field. Densitometric analysis was performed using imageJ software.

Ethical clearance

Regarding to this research of in vitro study, ethical clearance is no need to be issued.

RESULTS

TLC analysis showed the primary observed spot of methanolic and both ethanolic extract corresponding to brazilin with the R_f 0,6 as shown on Figure 1. This suggestion was based on Hangoluan, 2011 study on brazilin isolation, with similar solvent system

chloroform : methanol (5:1) obtained R_f value 0,54.¹⁵ The water extract had different TLC profile, with extremely slight spot of brazilin, and showed specific spot at R_f 0,2.

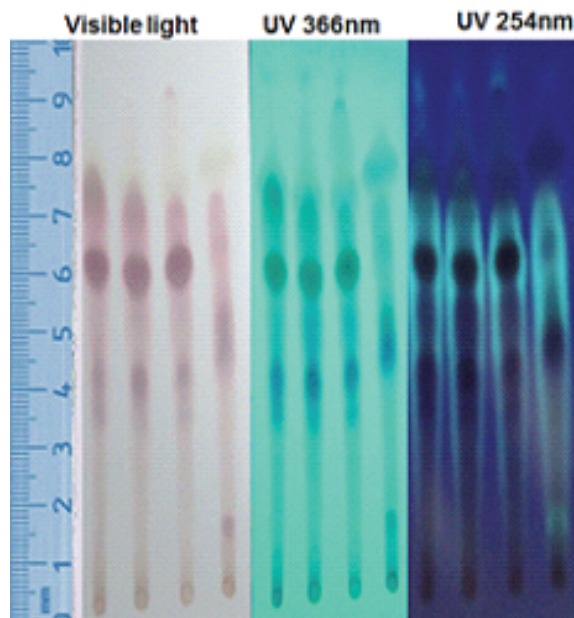


Figure 1. TLC profile of sappan wood extract. From left to right: ethanolic 96%, ethanolic 70%, methanolic and water extract

The evaluation of the morphological changes and cytotoxic activity by MTT assay in 4T1 cells treated with the extracts represented in Figure 2 and 3. The 4T1 cells viability were performed in triplicate, and represented as mean \pm SD and the IC_{50} value was calculated using linier regression. All extracts exhibited strongly cytotoxic activity on 4T1 cells. The ethanolic 96% extract possessed the strongest activity with the IC_{50} value 13,1 $\mu\text{g}/\text{mL}$, followed by methanolic (21,4 $\mu\text{g}/\text{mL}$), ethanolic 70% (22,5 $\mu\text{g}/\text{mL}$) and water (25,5 $\mu\text{g}/\text{mL}$).

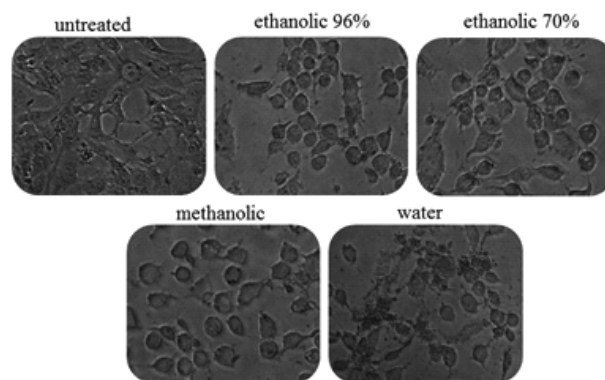


Figure 2. Morphological changes of 4T1 cells caused by each extract 40 $\mu\text{g}/\text{mL}$ for 24 hours under inverted microscope, magnification 200x.

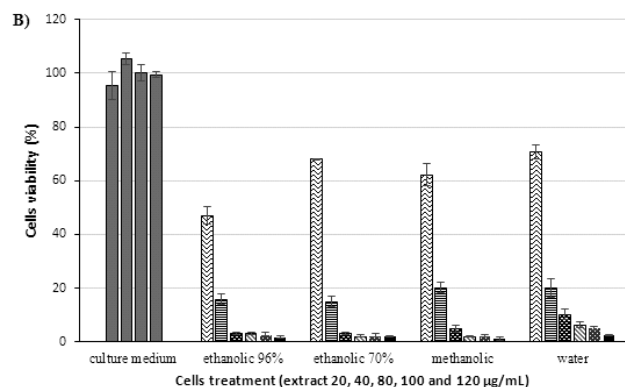


Figure 3. The MTT assay of 4T1 cells treated with the extract 20, 40, 80, 100, and 120 µg/mL.

The investigation of inhibitory effects on MMPs activity was done using gelatin zymography and analyzed with imageJ software, as shown in Figure 4. Gelatin zymography showed that 4T1 cells secreted MMPs, and by its molecular mass we concluded that it was active MMP-9 (92 kDa) and MMP-2 (62 kDa). It was proved that all extracts except water, inhibited gelatinolytic activity of MMP-9. The ethanolic 96% and 70% extract showed similar inhibitory effect, both was able to decrease gelatinolytic activity of MMP-9 by ± 12 fold lower than cell control, whereas methanolic ± 5 fold lower. Meanwhile, all extracts had no significant inhibitory effect on the activity of MMP-2.

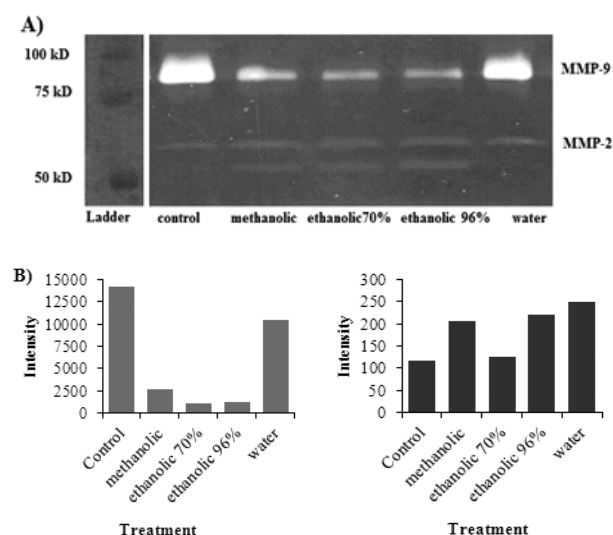


Figure 4. Gelatin zymograph of 4T1 cells treated with sappan wood extract. A) representative gelatin zymogram illustrating MMP-9 and MMP-2 in 4T1 cells. B) ImageJ software analysis of MMP-9 (left graphic) and MMP-2 (right graphic)

DISCUSSIONS

The ethanolic and methanolic extract showed similar TLC profile which correlate to brazilin as the main compound. Brazilin and brazilein is semi polarity molecule, so their content in the water extract was extremely smaller, as shown on TLC profile. Brazilin can be isolated from ethanolic or methanolic extract and found in ethyl acetate fraction. It can be easily oxidized producing brazilein.¹ Extraction procedures is first necessary step in the analysis of bioactive compound from medicinal plant. Based on traditional uses, medicinal plant is usually prepared with boiling water. Water infusion is known as the simplest method to extract phytochemical compound from plants. Plant extracts usually contain a combination of different phytochemical compounds with various polarities. Based on that fact, the separation, identification, and characterization method still remain a big challenges for the invention of new bioactive compound.¹⁶

Based on MTT assay, all sappan wood extract showed strong cytotoxic activity on 4T1 cells. The water extract still revealed strong cytotoxic activity (IC_{50} 25,5 µg/mL), although it was the lowest effect compared to the other extracts. The cells treated with the extract showed morphological changes compared to untreated cells. The cells developed to be rounded and shrinkage, as the typical features of apoptosis (programmed cell death). Similar with this study, Hung et al. 2014 evaluated the cytotoxic activity of sappan wood ethanolic, methanolic and water extracts. The methanolic extract exhibited the most potent cytotoxic activity against HeLa, HL-60, MCF-7, HepG2, and KB cell lines; followed by ethanolic extract. In accordance with our results, the water extract also showed the weakest activity against all cell line. Based on the DNA fragmentation and biochemical assay, Hung et al reported the methanolic extract inhibited HeLa cells growth through apoptosis induction and mediated by caspase-3 activation. Caspase-3 activation is needed for some typical characteristic of apoptosis and is absolutely necessary for apoptotic chromatin condensation, DNA fragmentation, and finally is one of the determinants in cancer growth and development.¹⁷

Cancer research on microenvironment, especially the extracellular matrix is arising as a key points bringing great impact on cancer cells progression. MMPs are shown in almost all human cancers. MMPs affect on cancer environment by rebuilding new blood vessels (angiogenesis), and promoting tumor growth and metastasis. Therefore, MMPs expression is tight

corelated to cancer aggressiveness, carcinogenesis stage, and patient prognosis. The inhibition of MMPs activity should play an important role in cancer therapy, because MMPs trigger the degradation of extracellular matrix and induce tumor invasion.¹⁸ Gelatin zymography showed that 4T1 cells secreted MMP-9 and MMP-2, and it was proved that the extracts except water, inhibited gelatinolytic activity of MMP-9 but had no significant inhibitory effect on the activity of MMP-2. The MMP-2 and MMP-9 are highly expressed in aggressive breast tumors and are associated with poor clinical outcome. The transcription of MMPs is reported to be regulated by NF- κ B.¹⁹ Interleukin-4 (IL-4) Researchs shows that brazilin and brazilein were the main compound of sappan wood. Both brazilin and brazilein have the ability to inhibit MMPs activities.¹

Hsieh, et al., 2013, reported that brazilein could inhibit the MMP-2 expression, and deliberated the effect of anti-migration and anti-invasion on MDA-MB-231 cells.²⁰ Another research showed that brazilin demonstrated the inhibition of TPA-induced MMP-9 expression and cell invasion mediated by the suppression of the NF- κ B pathway in MCF-7 cells.²¹ Hsieh et al. 2013 suggest that brazilein could suppress both PI3K/Akt and p38 MAPK signaling pathways thus inhibited NF- κ B activation of MDA-MB-231 cells.²⁰ A large number of human cancers express constitutive NF- κ B activity; which promotes cells growth, avoids apoptosis, withdraws angiogenesis, and induces metastasis.²² Brazilin is a potent NF- κ B inhibitor that selectively disrupts the formation of the upstream IL-1R signalling complex.²³

In conclusion, the results in the present study suggest that various extract of the sappan wood with polar solvent have been found to possess cytotoxic and MMP-9 inhibitory activities and may potentially be explored as anti breast cancer. The ethanol 96% would be the best solvent of sappan wood extraction for further research as new anticancer agent.

Acknowledgments

We would like to thank to the Director of Medicinal Plant and Traditional Medicine Research and Development Centre, National Institute of Health Research and Development Ministry of Health of Indonesia for all the funding and facilitation so that this research could be done.

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