Ethyl Acetate Fraction Of Secang As Anti Cervical Cancer By Inducing p53 and Caspase 9

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Abstract. Secang (Caesalpinia sappan L.) contains potentially anti-cancer active compounds, namely brazilin and brazilein which are soluble in ethyl acetate. This study is conducted to evaluate the anticancer activity of secang wood in cervical cancer cells (HeLa) in vitro and molecular mechanisms that selectively mediate their cytotoxic effects on several cancer cells through molecular docking or in silico. The anticancer potential of the ethyl acetate fraction of secang wood through its cytotoxic activity on HeLa cells was carried out with MTT assay and calculated the IC50 value. The molecular mechanism is carried out in silico with the target protein p53 and caspase 9 using the autodock 4.2 program. The docking process includes protein and active compounds preparation, validation of molecular docking methods and docking brazilein as the active substance with the target protein. The docking score is measured by binding energy value between brazilein and the target protein. The fraction of secang wood was cytotoxic in HeLa cells with IC50 values of 48.71 μg/mL. The docking results showed that brazilein had an affinity for the target proteins p53 and caspase 9 with binding energies of -8.24 and -6.71 kcal / mol, respectively.

1. Introduction
In Indonesia, approximately 15,000 cases of cervical cancer occur every year. That makes cervical cancer referred to the most cause of death disease in women in Indonesia. WHO sets Indonesia as the country with the highest number of cervical cancer patients in the world due to its cases number [1]. Cervical cancer is classified as carcinoma, which is a cancer that occurs in squamous cells in the uterine lining of the woman's uterine epithelial tissue. This cancer is caused by infection with HPV (Human Papilloma Virus) [2]. Oncogenous viruses often exploit infected cell working systems, to carry out the life and replication of the virus itself. As part of the process, the virus will affect cell control mechanisms that will cause abnormal cell growth, genetic changes, even malignant transformation. Human papilloma virus 18 encode the formation of 2 oncogens, namely E6 and E7 which interact with the regulator of cell cycle. E6 protein bind to p53 and accelerate p53 degradation [3]. Thus, when p53 is degraded, nothing inhibition process mechanism of cell proliferation and cervical cancer develop and become malignant [4].

Cancer treatment using chemotherapy has many side effects, especially in normal cells. So that research is developed on compounds derived from nature as anticancer agents to increase the sensitivity of cancer cells and reduce side effects due to chemotherapy agents. Many plants contain potential compounds as co-chemotherapy agents [5] [6]. One of which is secang wood (Caesalpinia sappan L.). Secang wood contains brazilian, brazilen, 4-O-methylsappanol, protosappanin A, and caesalpin J homoisofoflavonoids [7]. Which have cytotoxic activity in some cells such as Hep G2 (liver)
and A549 (lung) [8] [9]. Brazilein increases the apoptosis mechanism in liver cancer cells [10]. Brazilein and brazilein are known to be soluble in ethyl acetate solvents, so that in this study the development of ethyl acetate fraction of secang wood (Caesalpinia sappan L.) as an active anticancer through cytotoxic assay on cervical cancer cells (HeLa) and molecular evaluation to determine the molecular mechanism of the anticancer potential possessed by ethyl acetate fraction of secang wood through in silico on the target proteins p53 and caspase 9. These three target proteins play a role in the apoptosis process.

2. Materials and Methods

2.1 Fractination of secang wood
Secang (Caesalpinia sappan L.) heartwood was derived from Center for Research and Development of Medicinal Plants and Traditional Medicine. The dried heartwood of secang was macerated with methanol (Merck) at a volume ratio of 1:10 (for 48 hours). The methanolic extract was then evaporated with reduced pressure by rotary evaporator. The extract was partitioned with n-hexan and ethyl acetate (Sigma Aldrich co. USA.) by liquid liquid extraction (LLE). The ethyl acetate fraction was evaporated using rotary evaporator to get the yield of ethyl acetate fraction [11].

2.2 Cell culture
HeLa cells belong to Cancer Chemoprevention Research Center (CCRC), Pharmacy Universitas Gadjah Mada, obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. HeLa cell lines were cultured in DMEM-low glucose on incubator at 37˚C and 5% CO₂, supplemented with 10% (v/v) FBS, 1.5% (v/v) penisilin-streptomisin, and 0.5 % (v/v) fungizone.

2.3 Cytotoxicity and Cell viability assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
The potency of ethyl acetate fraction of secang on HeLa cells was evaluated by using MTT cytotoxicity assay. HeLa cells with a concentration of 1x10⁴ cells / wells were planted in a 96 well plate and incubated for 24 hours. Test solution (ethyl acetate fraction of secang) and media of 100 μl each were added to the well then incubated for 24 hours. Cells were washed with 100 μl PBS, then added 100 μl of culture medium containing MTT 5 mg / ml into each well and then incubated again for 4 hours at 37 ° C. Living cells will convert MTT to purple formazan. After 4 hours of media containing MTT removed, sodium dodesylsulfate stopper solution is added, then the plate is left overnight. The plate is shaken on the shaker for 10 minutes then absorbance will calculate using an ELISA reader at λ 595 nm. Absorption absorbance data is converted into percent viability and used to calculate IC₅₀.

The data result in the form of absorbance of each well is then converted into percent cell viability. Equation (1)[13] was used to calculate the percentage of HeLa cells viability

\[ \text{% Viable cells} = \frac{\text{Treated cells abs} - \text{medium control abs}}{\text{Cells control abs} - \text{medium control abs}} \times 100\% \]  

The chart of the concentration of the cytotoxic assay and cell viability compound is presented as an average ± SE of 3 experiments. Data in the form of cell viability were then analyzed with Microsoft Excel 2007 program to obtain linearity regression Y=BX+A and correlation coefficient (r) between concentration and percentage of viability cells and to calculate IC₅₀ values. The r value of the calculation results is compared with r table (p <0.01)[14]. IC₅₀ is a concentration which causes 50% inhibition of cell proliferation so that its cytotoxicity potential is known.
2.4 Molecular docking
Molecular docking techniques can be used to predict the affinity of an active compound to the target protein that its activity can be predicted. Molecular docking was applied to determine the potency and mechanism of brazilein as an active compound in ethyl acetate fraction of secang as an anticancer agent in cervical cancer by in silico through p53 and caspase 9 target proteins.

2.4.1 Preparation of p53 and caspase 9 target proteins. The process of preparation of p53 and caspase 9 by using Chimera 1.10.1 software. The protein will separate from its ligand (native ligand).

2.4.2 Validation method of molecular docking. Validation of the molecular docking method is done by redocking the native ligand back on the target proteins that native ligand have been removed from the proteins using the Autodock 4.2 program. The results of the analysis described which the compound with conformation had the lowest binding energy to bind to the target protein. If the root mean square distances (RMSD) ≤ 3 Å, the protocol is received and docking of the active compound on the target proteins can be done.

2.4.3 Optimization of 3D brazilein. The 3-dimensional structure of brazilein compounds is optimized using the HyperChem 8 program. Optimization is performed on the 3-dimensional structure of brazilein compound complete with hydrogen atoms, semi-empirical AM1 computation methods, and calculations with single points and optimization geometry.

2.4.4 Molecular docking of brazilein and target proteins. Docking was conducted using the Autodock 4.2 program between brazilein from the optimization results and the target proteins that its native ligand has been removed. The results of the analysis will show the conformation of the lowest binding energy to bind to the target protein as score docking.

2.4.5 Data analysis. The results of molecular docking are score docking. Score docking in the form of binding energy score. The value of the binding energy shows the strength of the bond between the compound and the target protein. Lower of the binding energy value means stronger and more stable of the bond. Lower of binding energy score reflect stronger interaction that occurs between brazilein and the target proteins.

3. Result

3.1 Cytotoxicity assay of ethyl acetate fraction of secang
The activity assay of ethyl acetate fraction of secang on heLa cells was carried out by in vitro using MTT cytotoxicity assay through the measuring parameter was IC\(_{50}\). The ethyl acetate fraction of secang has an IC\(_{50}\) value of 48.71 μg / mL. The cytotoxicity assay result is described according to figure 1. In figure 2 seen morphologically, higher concentration of ethyl acetate fraction of secang cause more cells undergoing changes in shape. Condensation occurs, fragmented, membrane blebbing and shrinkage of cells. This indicates that ethyl acetate fraction has a potency to be developed into a natural anticancer agent or later it can be developed into a co-chemotherapy agent as a companion to chemotherapy agents.
3.2 Brazilein from ethyl acetate fraction of secang increase apoptosis process by inducing p53 and caspase 9.

In silico evaluation with molecular docking using the autodock method was carried out to determine the molecular mechanism that mediates cytotoxic activity in HeLa cells. The target proteins evaluated were p53, and caspase 9 which the target proteins responsible for apoptosis. In HeLa cells had characteristics, namely the presence of mutations in p53 and interference with the apoptosis process [15]. Before molecular docking was carried out, validation was necessary to ensure that the molecular docking stages using autodock provided appropriate and valid results. Validation could be seen from the RMSD value which is <3 Å (table 1). Molecular docking result between brazilein and protein target were showed on table II dan III.

| Protein | RSMD (Å) | Binding Energy (kcal/moL) | Hydrogen Bond               |
|---------|----------|---------------------------|-----------------------------|
| p53     | 2.26     | -8.24                     | SER152; GLU124              |
| Cappase 9 | 2.44     | -3.62                     | THR181; ARG 355             |
Table II. Binding energy of brazilein with p53 and caspase 3 using molecular docking

| No. | Target Proteins | Ligand   | Binding Energy (kcal/mol) | Hydrogen Bond | (Ligand-Protein) Group |
|-----|-----------------|----------|---------------------------|---------------|------------------------|
| 1   | p53 (2J8Z)      | Native ligand | -6.29 | ARG 42 | HH11 |
|     |                 |          | ALA 149                  | HN            |                        |
|     |                 |          | LYS 178                  | HZ2           |                        |
|     |                 |          | ASN 321                  | HD21          |                        |
|     |                 |          | ASN 323                  | HD21          |                        |
|     |                 | Brazilein | -8.24 | SER 152 | HG |
|     |                 |          | GLU 124                  | OE2           |                        |
| 2   | Caspase 9 (1JXQ)| Native ligand | -3.62 | ARG355 | HN-O2 |
|     |                 |          | ARG355                   | HE-O4         |                        |
|     |                 | Brazilein | -6.71 | THR181 | HN-O |
|     |                 |          | ARG355                   | HN-O          |                        |

4. Discussion

IC\textsubscript{50} value is the concentration needed by a compound to inhibit growth and proliferation of cancer cell by 50% of the cell population. Lower IC\textsubscript{50} value means more potent of compound has anticancer activity. In this study the ethyl acetate fraction of secang had an IC\textsubscript{50} value of 48.71 μg/mL (figure 1) and the cytotoxic activity of the fraction showed a positive correlation. Higher concentration of ethyl acetate fraction of secang cause greater the percentage of cell death with \( r = 0.918 \) (\( r \) table = 0.874) [14]. Correlation (\( r \) test) value is higher than \( r \) table showed concentration of secang treatment on HeLa cells related to percentage of cell death and many cells to be morphological changes (figure 2). According to figure 1 and 2, ethyl acetate fraction of secang had a potent activity to cervical cancer that HeLa as cell line model. This fraction has IC\textsubscript{50} values below 100 μg / mL. A compound to be potent as an anti-proliferative if it has IC\textsubscript{50} values below 100 μg / mL [16].

Molecular mechanism of brazilein in secang had been further observed in silico using autodock 4.2 program to evaluate molecular capability of it that mediates anticancer activity. RMSD (Root Mean Square Deviation) is a measurement of two poses by comparing the atomic position between an experimental structure with a predicted structure or a deviation between a ligand conformation that is tethered to a protein compared to a site that has a native ligand bind to a protein. Getting greater RMSD value means greater deviation which indicates the position of the ligand predicton father away to native confirmation caused greater prediction error of ligand interaction with protein [17]. The results of the validation of the molecular docking method show that the RMSD value is ≤ 3 Å which demonstrated that the method used has met the requirements of its use [17]. The affinity of the compound can be known by comparing the energy value of the compound bond with native ligand on the target protein [18]. The value of binding energy obtained from the target protein docking with native ligand and brazilein was then compared to determine the potency of brazilein as an anticancer agent through the mechanism of inducing p53 and caspase 9. Table 1 and table 2 showed that brazilein compounds in secang have lower binding energy values than native ligand on p53 and caspase 9. Brazilein had lower binding energy than native ligand with the target protein exhibited stronger bind formed between compound and the target proteins [18]. The affinity of compound for the target protein that is directly proportional to the strength and stability of the bond. Comparison of binding energy values in Table 2 shows that brazilein has greater potency as an anticancer agent compared to its native ligand with the mechanism of inducing p53 and caspase 9 in silico by molecular docking method so that the apoptosis process is accelerated and kills cancer cells.
Table III. Interaction between brazilein and target proteins (p53 and caspase 9) with amino acid residue in target proteins

| No | Target proteins | Interaction between brazilein and amino acid residu of target proteins |
|----|----------------|---------------------------------------------------------------------|
| 1  | p53            |                                                                    |
| 2  | Caspase 9      |                                                                    |

5. Conclusion
Ethyl acetate fraction of secang had a potent cytotoxic activity as cervical anticancer agent with HeLa cell lines as a model through inducing p53 dan caspase 9. This fraction promising to be developed into an anticancer agent or co-chemotherapeutic agent.

6. Acknowledgement
Ethyl acetate fraction of secang had a potent cytotoxic activity as cervical anticancer agent with HeLa cell lines as a model through inducing p53 dan caspase 9. This fraction promising to be developed into an anticancer agent or co-chemotherapeutic agent.
References

[1] Ministry of Health Republic of Indonesia 2013 Riset Kesehatan Dasar (RISKESDAS). Jakarta : Badan Litbang Kemenkes RI.

[2] Castellsagué X 2008 Gynecologic Oncology. 110: S4–S7.

[3] Georgieva, S., V. Iordanov, and S. Sergieva 2009 J Buon. 14(3): p. 391-8.

[4] Kines, R.C. and Cole A 2009 Proc Natl Acad Sci USA. 106(48): p. 20458-63

[5] Greenwell, M. and Rahman, P.K.S.M 2015 Int J Pharm Sci Res. 6(10): 4103-4112.

[6] Ho JW, LYK, Pong C 2002 Anticancer Agents 2: 209-214

[7] Chun Fu, Xin-an Huang, Zhen, yun Lai, Ying-jie Hu , Hong, jiao Liu, and Xiao,ling Cai 2008 Molecules 13: 1923-1930.

[8] Yen,C., Kyoko Nakagawa-Goto, Tsong-Long Hwang, Pei-Chi Wu, Susan L.Morris-Natschke, Wan-Chun Lai, Kenneth F. Bastow, Fang-Rong Changb, Yang-ChangWu, and Kuo-Hsiung Lee 2010 Bioorg Med Chem Lett. 20(3): 1037–1039.

[9] Ren L, Yang X, Wang G, Zhang H, Zhao L, Mi Z 2011 World Academy of Science, Engineering and Technology 60: 215-219

[10] Zhong , B. Wu, Y. J. Pan, S.Zheng 2009 Neoplasma 56 (5).

[11] Laksmiani, N.P.L., Meiyanto, E. and Susidarti, R.A 2017 Int J Pharm Pharm Sci. 12, 124-130.

[12] Mosmann T 1983 Journal of immunological methods 65(1): 55-63.

[13] Meiyanto E, Fitriasari A, Hermawan A, Junedi S, Susidarti RA 2011 Orient Pharm Exp Med 11:183-190.

[14] Alhusin, S 2002 SPSS 10 for Windows, Edisi 1, J & J Learning, Yogyakarta.

[15] Buitrago-pérez Á, Garaulet G, Vázquez-carballo A, Paramio JM 2009 Current genomics 10: 26-34.

[16] Teng WY, Yu LH, Chien CS, Ray LH 2005 J Chin Chem Soc 52:1253-5.

[17] Jain, A. J., dan A. Nicholls 2008 J. Comput. Aided Mol. Vol.22: 133-139.

[18] Laksmiani, N. P. L., N. L. P. V. Paramita, and I. M. A. G. Wirasuta 2016 International Journal of Pharmacy and Pharmaceutical Sciences 8(8): 177-181.