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In-vitro and In-silico Anticancer Activity of Parasitic Tea Plant *Scurrula atropurpurea* (Blume) Danser against Cervical Cancer

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Abstract: Cervical cancer is one of the deadly disease in Indonesia. Recently, the benefits of plants as a herbal medicine have been studied thoroughly, following so many epidemological evidence about plants activities to prevent the progression of some diseases and act as drug for treatment. This study was aimed to identify flavonoid component(s) from *Scurrula atropurpurea* (Blume) Danser (SAD) as anti-cancer agent. In this study, we examined the potency of flavonoid of SAD in-vitro and in-silico for inhibition related to proliferation and induction of apoptosis in HeLa cells. We found that the active compounds from chloroform extracts (flavanon, dihydroflavonol and catechin), ethanolic extract (EGCG, flavonol and flavon) and n-Hexane (flavanon, dihydroflavonol and flavon) of SAD are effectively inhibit the proliferation and induce the apoptosis of HeLa cells. The IC_{50} values of chloroform, ethanolic and n-hexane extracts of SAD were 96,15 µg/ml, 298,81 µg/ml and 498,66 ng/ml, respectively. Computational study show that chloroform and ethanolic extract are the best for increasing expression of p53 protein that have crucial role in induction of apoptosis and inhibit the cell proliferation. It can be concluded that in-vitro and insilico approach from SAD extracts show that the active compound can act as anticancer through the activation of p53, by enhancing the expression of p53. So the active compound is potential for anti-cancer agent in the future.

Key words: *Scurrula atropurpurea* (Blume) Danser, HeLa cells, IC_{50}, apoptosis.

Introduction

Cervical carcinoma is the second cancer type that suffered by woman and the most frequent woman death caused in worldwide. In South East Asia, the case of this disease was in the first place which among 180.000 from 1.726.000 of all cancer cases[1,2]. In Indonesia, this cancer is the fourth death causes after stroke, hypertension, and diabetes mellitus. Recent studies were shown that Human Papilloma Virus (HPV) infection was found in 99,7% cervical cases, and make it as one of the major risk factor in cervical carcinoma progression. Many evidences have showed that infection of HPV type 16 and 18 is high risk factor of cervical cancer progression[3,4].

Recently, the benefits of plants as a medicine have been studied thoroughly, following many epidemological evidences about plants activities to prevent the progression of some disease and act as drug for treatment. Many studies about flavonoid from parasitic plant were succeed in collecting evidences which have many bioactivities as anti-cancer. This study was aimed to isolate and identify flavonoid from parasitic tea plant *Scurrula atropurpurea* (Blume) Danser (SAD). This study was also examined the anticancer activity of n-hexane, chloroform and ethanolic fraction from SAD, respectively in inducing apoptosis and inhibition proliferation against He-La cells.

Material and Methods

Plant Material and Preparation Extract

SAD were collected from Wonosari Lawang Malang, East Java, Indonesia. Morphological identification was done at Purwodadi Botanical Garden, Malang, East Java, Indonesia. Extract source was dry leaves of SAD. Maceration was done overninght using n-hexane, chloroform and etanol as solvent, at room temperature. The separation of extract flavonoid was done by TLC method, and the spots were detected using UV light at wave length 254 nm and 366 nm[5,6]. The layer then scanned by CAMAG winCATS-3 scanner at wavelength 254 nm and 366 nm for flavonoid spectrometry. LCMS was done to ensure the identification of EGCG compounds in SAD extract.

Cell Lines and Cultures

HeLa cells were found from Agency for the Assessment and Application of Technology (ASPT), Serpong Tangerang. Cell lines were maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured as adherent monolayer and maintained at 37°C in a humidified atmosphere of 5% CO2. Cells were harvested after subjecting them to brief trypsinization.

MTT Cells Viability Assay

The Proliferation inhibition activity of n-hexane, chloroform, and ethanolic fraction of SAD against He-La cells was examined by MTT assay. Then, 100 µl He-La cells were cultured in 96 well plate and grown in the incubator for 24 h. Cells were treated by SAD extract at specific concentration and added when cells already reach the 80% confluence. The control used the mixture of medium, DMSO and cells without extract. Each treatment was done three times. Sample and cells were incubated for 24 hours. At the end of the second incubation period, the cell culture were washed by PBS, twice, then 110 µl MTT was added and incubated for 2-4 h, in the incubator CO2 5%, until formazan formed. Stopper solution of 100 µl SDS 10% in 0,1 N HCl was added. Plate were incubated in the dark for 24 h. The absorbance was measured by ELISA reader at wavelength 595 nm. MTT assays data were analyzed by counting percentage of viable cells. The IC_{50} value with SPSS probit.

Double Staining Apoptosis Assay

The Apoptotic induction activity of n-hexane, chloroform, and ethanolic fraction of SAD against He-La cells was analyzed by double staining method. The number of cells that needed in double staining apoptotic assay were 5 X 10^4 cell/well (5 X 10^4 sel/1000 µl complete medium). 1000 µl suspension were transfered to 24 well-plate, then incubated for 24 h. Cells are ready for treatment when it has been attached at the base of well. Based on the IC_{50}, 1000 µl extract put into the 24 well-plate that already contains the cells. The control used well which contains cells and complete medium and DMSO only. Plate was incubated in dependent time manner (10-24 h). After the incubation period, cells were washed by PBS twice. Treatment of ethidium bromide-acridine orange objects were done and observed by fluorescence microscope.

Biological activity prediction

All active compounds were evaluated using PASS software for evaluating the general biological potential of an organic drug-like molecule. PASS used Structure Actvity Relation (SAR) approach and provides simultaneous predictions of many types of biological activity based on the structure of organic compounds. Thus, PASS can be used to estimate the biological activity profiles for virtual molecules, prior to their chemical synthesis and biological testing[7].

Result and discussion

Identification of Flavonoid in SAD

In early study, it has done the separation of bioactive compounds from SAD by n-hexane solvent, chloroform, and ethanolic. Based on its spectrum pattern, the fraction of n-hexane contains flavonoid compounds including flavanon, dihydroflavonol, and flavon. Meanwhile, the fraction of chloroform contains flavonoid compounds including flavanon, dihydroflavonol, and cathecin. The fraction of ethanolic contains flavonoid compounds including EGCG, flavonol, and flavon. The fraction of ethanolic contains EGCG at concentratrion 763,88 ppm (Figure 1).

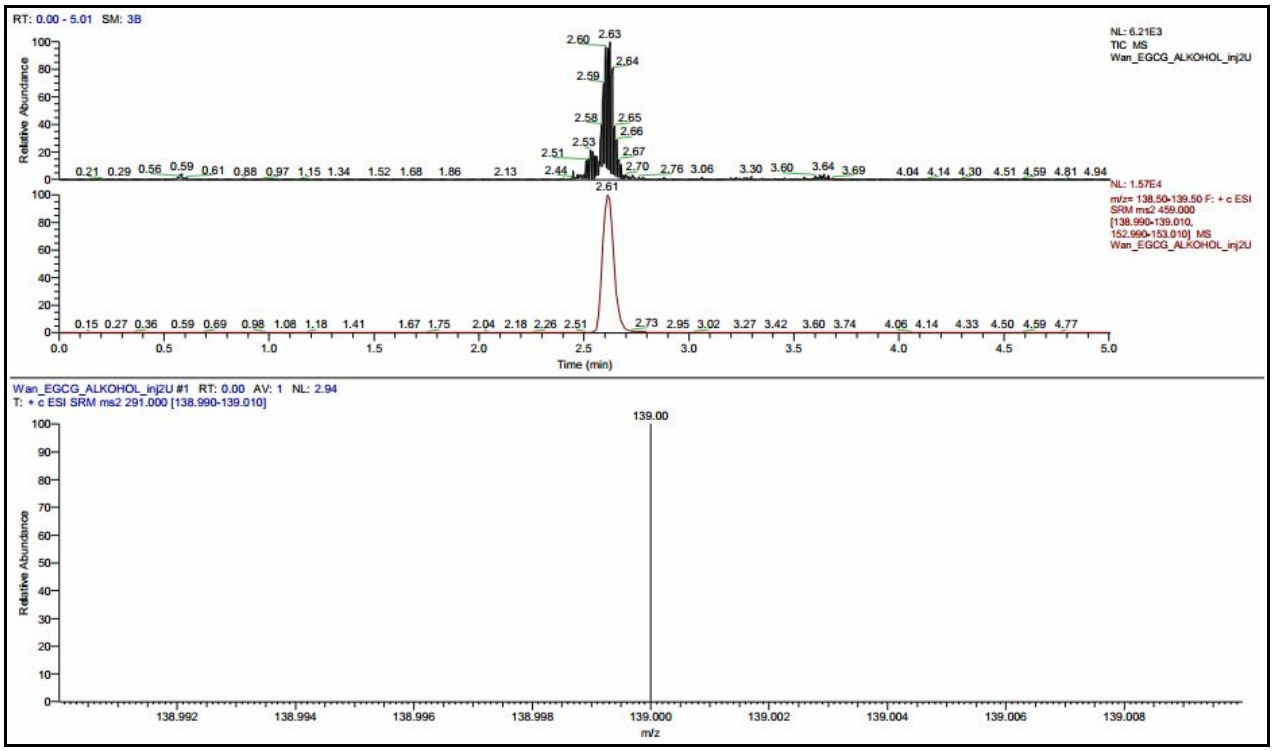

Figure 1. LCMS results on the fraction of ethanolic from SAD-Lawang East Java showed EGCG at concentration 763,88 ppm.

SAD extract inhibits the proliferation of HeLa cells

MTT assay showed that treatment with SAD extract from n-hexane, chloroform, and ethanolic resulted as an inhibitor of proliferation in dose dependent manner. Proliferation and cell viability were decreased alongside with the dose of SAD extracts (Figure 2).

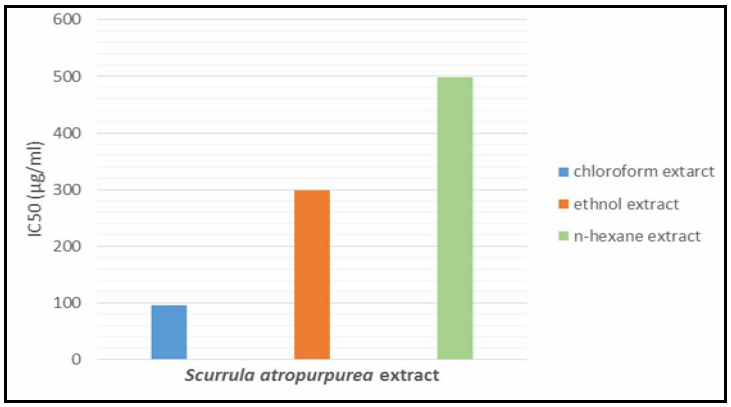

Figure 2. IC_{50} value of chloroform, ethanolic and n-hexane fraction from SAD extract. Chlorofrom fraction of SAD extract has the lowest IC50 value (96,15 µg/ml) , the content of this fraction are flavanon, dihydroflavonol and catechin.

This results were measured based on the He-la Cells viability (%). The cells viability were decrease alongside with the increased of extract dose. The IC_{50} value were 96.15, 298.81, and 498.66 µg/ml for chloroform, ethanolic, and n-hexane extract fraction, respectively. From all extracts fraction, chloroform extract showed the most potential inhibitor for proliferation activity against He-La Cell. The chloroform fraction extract had the lowest IC_{50}, 96.15 µg/ml (Figure 2).

SAD extract induce apoptosis of HeLa cells

Apoptosis Assay show that apoptic He-La cells was induced bt SAD extract (Figure 3). Red orange cells population increased significantly, compared to control culture. The red orange cells after extract treatment were cells on the apoptosis condition. In control culture, the number of apoptotic cells were 1,01%. Domination of green cell population shown that apoptotic mechanism was in low frequent. In n-hexane extract treatment the green colour of He-La cells were decreased significantly. The apoptotic cells population were 14,92%. In chloroform extract treatment the population of green cell also decreased significantly. The apototic cells population were 99,97%. In ethanolic extract treatment, the population of green cell was also decreased significantly. The apoptotic cells population were 66,79%. From all extract fraction, chloroform extract showed the most potential as apoptosis induction toward He-La cells.

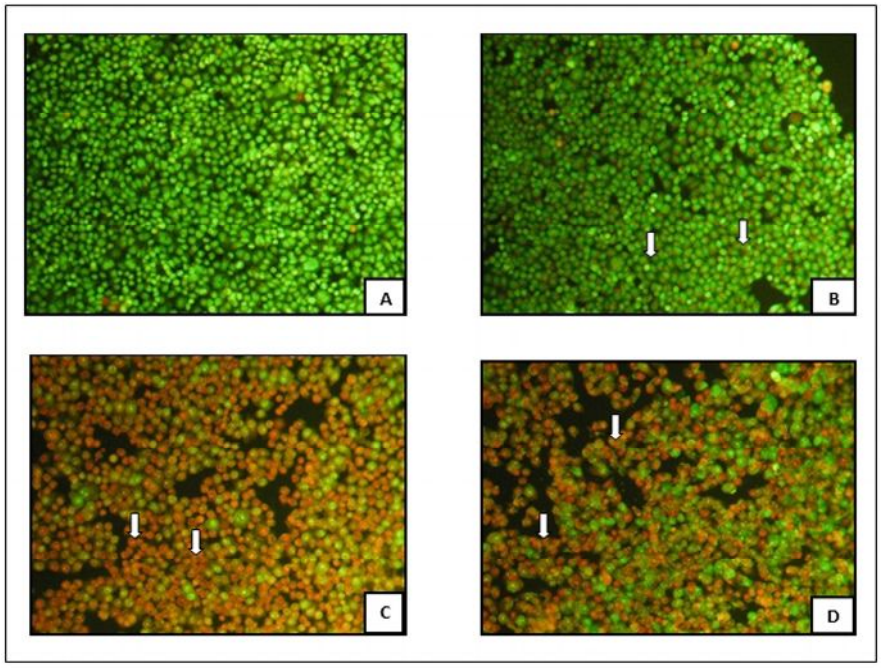

Figure. 3. Apoptosis assay on HeLa cell (A) Control, (B) treatment of 250 µg/ml n hexan-fraction, (C) Treatment of 50 µg/ml cloroform-fraction, (D) Treatment of 150 µg/ml ethanolic-fraction. Apoptotic cell show by white arrow on the picture.

Apoptosis Assay show that apoptic He-La cells was induced bu SAD extract. Apoptotic cells were indicated by red-orange color. The treatment show that red-orange cells population increased significantly, compared to control culture. In control culture, the number of apoptotic cells were 1,01%. Domination of green cell population shown that apoptotic mechanism was in low frequent. In n-hexane extract treatment the green colour of He-La cells were decreased significantly. The apoptotic cells population were 14,92%. In chloroform extract treatment the population of green cell also decreased significanty. The apototic cells population were 99,97%. In ethanolic extract treatment, the population of green cell was also decreased significantly. The apoptotic cells population were 66,79%. From all extract fraction, chloroform extract showed the most potential as apoptosis induction toward He-La cells.

Biological Activity Prediction

Active compound is the set of different types of biological activity that reflect the results of the compound's interaction with various biological entities. The computational study of biological activity can explain the mechanism of action. This research showed that comparison of Probability activity (Pa) from flavanone, dihydroflavonol, flavone, catechin, flavonol, and EGCG. Based on the group of extraction, we found that the combined compound from chloroform extract have high biological activity for anticancer agent. We choose three kind of category that are, apoptosis agonist, Tp53 expression enhancer and proliferative disease treatment (Figure 4).

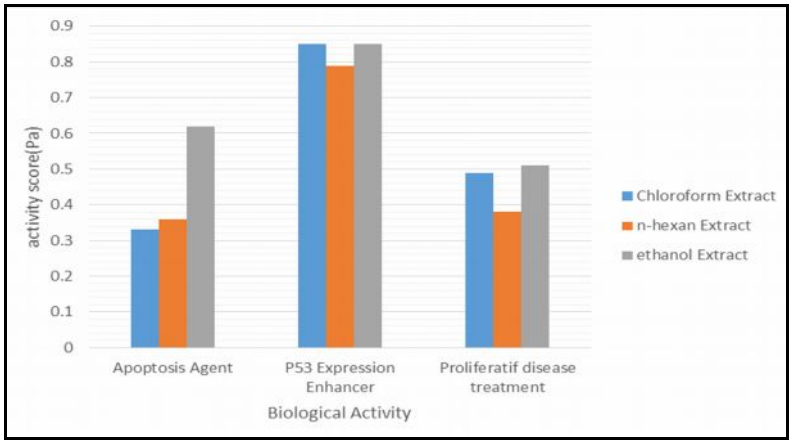

Figure 4. Prediction of biological activity of SAD extract. Chloroform, n-hexan and ethanolic extract have 3 activities that related to anti-cancer activity that are; Apoptosis agent, P53 Expression enhancer and Proliferative disease treatment. The highest activity of SAD extract is P53 expression enhancer. The predicted activity has coefficient Pa > 0.7.

Discussion

This study is demonstrated that SAD extract had potency as a cytotoxicity agent against He-La cells. Data from previous study, showed that flavonoid in the SAD extract could inhibit proliferation in cancer cell. The inhibition was found correlated with apoptosis induction mechanism. Collected data from previous studies were supported that flavonoid could act as an inhibitor in proliferation and apoptotic inducer[8-12]. Those data were confirmed by IC_{50} value from the extract that were used in the study. We predicted that the flavonoid content in the extract from all fraction was the source of the cytotoxic effect on He-La cells in dose dependent manner. Extract chloroform fraction was the strongest of cytotoxic effect (IC_{50}=96,15 µg/ml), followed by etanol (IC_{50}= 298,81 µg/ml) and n-hexane (IC_{50}=489,66 µg/ml). The results from the efficiency of treatment, which was shown from the lowest dose to the strongest effect, stated that SAD extract with chloroform fraction is the most potential inhibitor proliferation agent against He-La Cells.

This study was also indicated that the cytotoxic ability of SAD extract could be shown through apoptotic pathway in dose dependent manner. The percentage of viable cells were decreased significantly in the culture which treated with SAD extract when compared to the control. From this study, we offered that three extracts fraction of SAD (ethanol, n-hexane, and chloroform) had potency to induce apoptotic in dose dependent manner.

He-la Cells were expressed two types of oncogene, which are E6 and E7. E6 will bind to the phosphorylated p53, while E7 will bind with pRb. pRb and p53 are known as tumor suppresor genes, and correlated with cervical carcinoma development. On normal condition, p53 would be upregulated when DNA was damage. DNA damage would induce p53 activation, which then would stopped the cell cycle on G1 or went to the apoptotic pathway. In this period, cells will repair or do the apoptotic pathway, when the repairing could not be done. HPV protein E6 would made a complex with E6-AP and p53, and after it is completed, p53 will be ubiquitinated and degradated by proteolytic enzyme. E7 protein will bind to retinoblastoma (pRb). Phosphorilation of pRb, which was done simultaneously by cyclin/cdk complex, will inhibit repressor activity of pRb. E7 will bind to phosphorylated pRb, and disturbing the binds between pRb complex and transcription

factor E2F. E2F will be released from complexes, and then the cell will continue to S phase, and also the degradation of pRb. The result of inhibition of pRb and p53, which caused by HPV, were able to cause uncontrolled cell cycle, no DNA repair, and inhibition of apoptotic cells.

This study also showed that chloroform extract fraction was the most potential proliferation inhibitor and apoptotic inductor against He-La cells and computational study. We assumed that those potency have strong correlation with the differences of flavonoid compound in the extract. We investigated the potential of individual compound through Activity Relationship (SAR) approach using PASS server[7]. The result show that probability activity(Pa) value. The range of value is 0 up to 1. Higher score of probability activity, more accurate the prediction. The standard threshold of Pa is 0.7. If the score is above 0.7 so this computational result will have the same potential when observing in-vitro[13]. The minimum standard of reliable score is 0.3 for some compounds that predicted have biological activity.mechanism of compound show that Flavonol is the most potential to be apoptosis agent. Dihydroflavanol is the best for inducing expression of p53 protein and EGCG is the first choice for proliferative disease treatment. We compare the potency based on group of extraction, chloroform and ethanolic extract are potential because it has high score in p53 protein expression enhancer and poliferative disease treatment. Chloroform and ethanolic extract are the best for increasing expression of p53 protein that have crucial role in induction of apoptosis and inhibit the cell proliferation.

In previous result, active compound from flavonoid, including silymarin, genistein, quercetin, daidzein, luteolin, kaempferol, apigenin, dan epigallocatech and 3-gallate, were known to have effect towards G1/S and G2/M checkpoints cycle cell in cancer cell culture. Many evidences from studies were stated that flavopiridol could induce cell cycle arrest in G1 or G2/M phase, which caused by inhibition of CDKs action. Those previous studies are similar with the result from recent studies that flavonoid from n-hexane, chloroform and ethanol extract had potential as inhibitor of CDKs activity, so it could inhibit proliferation and induce apoptotic. Extract of SAD also show the potency for increasing Bax protein expression and decresing Bcl-2 protein expression, activating caspase 9 and caspase 3 and increasing cytochrome-C expression in HeLa cells[14-16].

Conclusion

SAD extract contain high flavonoid compounds, it can induce apoptosis on HeLa cell and inhibit the cancer cell proliferation. Furthermore, Computational study of SAD extracts show that the active compound can act as anticancer through the activation of p53, by enhancing the expression of p53. Chloroform and ethanolic extract of SAD are potential for anticancer agent in the future and it was validated by in-silico and in-vitro study.

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