Arcangelisia flava leaves ethanolic extract is better to be used as cancer co-chemotherapeutic agent rather than single use

Endah Puspitasari*, Dian Agung Pangaribowo, Alifanti Balinda Pramatasari, Nandan Gilang Cempaka, Muhammad Bayu Sanjaya

Faculty of Pharmacy University of Jember
Jl. Kalimantan 37 Jember, East Java, Indonesia

*coresponding author e-mail: e.puspitasari@unej.ac.id

ABSTRAK

Penelitian dan pengembangan agen kemoprevensi kanker terus dilakukan oleh peneliti, baik yang berasal dari bahan alam maupun hasil sintesis kimia. Hal ini tidak terlepas dari meningkatnya angka kejadian kanker di dunia. Penderita kanker baru mengalami peningkatan dari 12,7 juta kasus pada tahun 2008 menjadi 14,1 juta kasus baru pada tahun 2012. Angka kematian akibat kanker juga meningkat dari 7,6 juta kasus pada tahun 2008 menjadi 8,2 juta kasus pada tahun 2012. Diperkirakan, dalam jangka waktu lima tahun ke depan, terdapat sekitar 32,6 juta penderita kanker baru


Oleh karena itu, A. flava memiliki potensi untuk dikembangkan sebagai agen kemoprevensi kanker. Hasil penelitian tahun pertama menunjukkan bahwa ekstrak etanol A. flava termasuk dalam kategori sitotoksik sedang terhadap sel MCF-7, HeLa, dan WiDr, namun tidak toksik terhadap sel normal (sel Vero). Ekstrak etanol A. flava sangat selektif terhadap sel MCF-7 dan WiDr, namun kurang selektif terhadap sel HeLa. Hasil uji apoptosis menunjukkan bahwa sel mengalami kematian dengan mekanisme nekrosis, meskipun perlu dikonfirmasi kembali. Hasil uji in silico menunjukkan bahwa berberin memiliki afinitas paling stabil terhadap reseptor kinase dibandingkan dengan palmatin dan jatrorrhizin, artinya berberin berperan lebih banyak dibandingkan dua senyawa alkaloid dalam aktivitas sitotoksik dari ekstrak
etanol A. flava. Meskipun demikian, tidak tertutup kemungkinan adanya peran dari senyawa lain selain alkaloid.

Sedangkan penelitian tahun kedua adalah uji aktivitas kemoprevensi ekstrak etanol A. flava dalam penggunaan kombinasi dengan agen kemoterapi (ko-
kemoterapi) kanker pada berbagai sel kanker (sel kanker payudara, sel kanker leher rahim, sel kanker kolon) in vitro meliputi uji sitotoksitas dan uji apoptosis. Hasil pengujian menunjukkan bahwa ekstrak etanol A. flavă lebih baik digunakan dalam kombinasi dengan doxorubicin dibandingkan penggunaan tunggal. Sebab, kombinasi kedua mampu menginduksi apoptosis lebih baik, sedangkan penggunaan tunggal akan memacu nekrosis, kematian sel kanker yang tidak diinginkan. Penelitian lebih lanjut diperlukan untuk menelusuri mekanisme molekuler yang memperantarainya.

**Kata kunci:** Arcangelisia flavă, ko-kemoterapi, doxorubicin, sitotoksitas kombinasi, apoptosis

**Abstract**

Previous studies showed that Arcangelisia flavă ethanolic extract (EEAfL) is cytotoxic against cervical, breast, and colon cancer cell lines. The cytotoxic activity on breast and colon cancer cell line was selective, but not likely on cervical cancer cell line. The cytotoxic activity was apparently contributed to the induction of necrosis, rather than apoptosis. This study was determined whether EEAfL is better to be used in combination with cancer chemotherapeutic agent, doxorubicin. The cytotoxic activity was done in single and combinatorial assay on EEAfL and doxorubicin using MTT method. The apoptosis assay was done using flow cytometry annexin V-FITC method. The results showed that EEAfL in combination with doxorubicin induced apoptosis more than necrosis cells, suggesting that EEAfL is better to be used in combination with doxorubicin (as cancer co-chemotherapeutic agent) rather in single use.

**Keywords:** Arcangelisia flavă ethanolic extract (EEAfL), doxorubicin, cancer co-chemotherapeutic agent, cytotoxic, apoptosis

**INTRODUCTION**

Arcangelisia flavă is a potential candidate to be developed as cancer chemoprevention agent. A. flavă was proven to exhibit antioxidant and cytotoxic activity against MCF-7 breast cancer cell line. These ability were suggested to be related to its alkaloid content: berberine, palmatine, and jatrorrhizine (Keawpradub et al., 2005). Although this plant is stated as a rarely found species (Koran Jakarta, 2012), we could find it abundantly in Meru Betiri National Park, Jember.

Our previous studies showed that A. flavă leaves increase the immune system in doxorubicin-treated rats (Puspitasari et al., 2014b) with no signs of toxicity based on sub chronic toxicity assay (Puspitasari et al., 2014a). The A. flavă leaves ethanolic extract (EEAfL) had been proven to have cytotoxic activity on HeLa, MCF-7, and WiDr cancer cell lines with the IC₅₀ value of 467 ± 70; 136 ± 17; and 213 ± 79 μg/ml, respectively. The activity was selective on MCF-7 and WiDr, but not likely on HeLa cell line (Puspitasari et
The cytotoxic activity on those cells was likely due to the induction of necrosis, rather than apoptosis at the IC$_{50}$ (Puspitasari et al., 2016).

This study was conducted to determine whether EEAfL is better to be used in combination with cancer chemotherapeutic agent, doxorubicin. The cytotoxic activity was done in single and combinatorial assay on EEAfL and doxorubicin using MTT method. The apoptosis assay was done using flowcytometry annexin V-FITC method.

**MATERIALS AND METHODS**

**Plant Materials and Extraction**

The leaves were obtained from Meru Betiri National Park, Jember, Indonesia without any selection on age, only for their health and freshness. The ethanol extract was prepared based on Puspitasari et al. (2015). The leaves were washed and air-dried, then they were grounded and sieved. The ethanolic extract were prepared using 100 g of leaves powder. The ground-dried leaves was extracted with ethanol 96%. The extraction was repeated three times. The ethanol extract was evaporated under reduced pressure (Heidolph, Laborota) resulting EEAfL. EEAfL was then suspended in DMSO never exceed than 1% for flowcytometry annexin V-FITC apoptosis assay.

**Cytotoxicity assay**

The cytotoxicity assay was done using MTT method against HeLa cervical cancer cell line, T47D breast cancer cell line, and WiDr colon cancer cell line. All of the cancer cell lines were the collection of Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University and were grown in RPMI 1640 supplemented with 10% of fetal bovine serum, 1% of penicillin-streptomycin, and 0.5% fungizone.

The cells were seeded at 1$x10^4$ cells/well in 96 well plate and incubated for 24H in 37°C 5 % CO$_2$ for the adaptation. A series of concentration of EEAfL and doxorubicin was given to the cell line. After 24H incubation, the cells were washed with PBS and MTT (0.5 mg/ml) was added. The incubation was continued for 4 hours. Then the stopper reagent (10 % SDS dalam 0.1 N HCl) was added and the absorbance was read at 595 nm. The cell viability was calculated as Formula 1. The IC$_{50}$ was determined by probit analysis based on the plot of concentration vs cell viability (Doyle and Griffiths, 2000).

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\text{Cell viability} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100 \%
\]  

[1]
Co-chemotherapeutic assay was done using combinatorial use of EEAfL and doxorubicin. The concentration used was approx. half, one fourth, and one eight the IC<sub>50</sub> of each EEAfL and doxorubicin on each cancer cell line. The cell viability was calculated based on Formula 1. The combination able inhibit approx. 50% of the cell line was then used for the apoptosis assay.

**Flowcytometry Annexin V-FITC Apoptosis Assay**

The apoptosis assay were done using flowcytometry annexin V-FITC method. Briefly, the cell line (7x10<sup>5</sup> cells/well for HeLa and WiDr, 4x10<sup>5</sup> cells/well for T47D) were seeded in 6 well plate and incubated for 24H in 37°C 5% CO<sub>2</sub> for the adaptation. EEAfL and doxorubicin was then given in single (approx. IC<sub>50</sub>) and combinatorial use (combination that inhibits approx.. 50% of the cells). The apoptosis assay was done for 24H. At the end of the incubation time, the cell line was harvested using trispsin and washed using cold PBS twice. The cell line, then, was centrifuged 3,000 rpm for 2 minutes and added 100 μl binding buffer containing 450 ng annexin V-FITC and 5 μl propidium iodide-PE. The cell line was incubated further for 15 min at a dark place in room temperature before analyzed with flowcytometer (FACSCalibur Becton Dickinson) at λ 528 nm for FITC and 550 nm for PE.

Data was obtained in the form of living cells, apoptotic cells, and likely undergo necrotic cells distibution. The analysis was done based on annexin V and propidium iodide fluorescence intensity. Annexin V would identified the flip out cell membrane at the apoptotic cells (phosphathydilserine directs to the outside of the cell). While propidium iodide would detect the exposed DNA either due to the cell membrane breakage during the the apoptotic bodies formation as well as necrosis.

**RESULTS AND DISCUSSION**

The EEAfL obtained was 12.46 g from 100 g ground-dried leaves, resulting the yield of 12.46%. The extract was cytotoxic against HeLa, T47D, and WiDr cancer cell lines (Fig. 1), while the cytotoxicity of doxorubicin was, of course, much more higher than that of EEAfL (Fig. 2).
Fig 1. The IC$_{50}$ value of EEAfL on HeLa, T47D, and WiDr for 24H. The data was presented as mean ± SD of independent triplicate.

Fig 2. The IC$_{50}$ value of doxorubicin on HeLa, T47D, and WiDr for 24H. The data was presented as mean ± SD of independent triplicate.

The combinatorial cytotoxicity assay was done using the concentration under the IC$_{50}$ of EEAfL and doxorubicin. The results were shown in Fig. 3-5. The combination of EEAfL and doxorubicin able to inhibit approx. 50% of the cell viability was then used further for apoptosis assay.
Fig. 3. Combinatorial cytotoxicity assay of EEAfL and doxorubicin on HeLa cell line (24H incubation). Data was shown as mean of three independent replication

Fig. 4. Combinatorial cytotoxicity assay of EEAfL and doxorubicin on T47D (24H incubation). Data was shown as mean of three independent replication
Fig. 5. Combinatorial cytotoxicity assay of EEafL and doxorubicin on WiDr (24H incubation). Data was shown as mean of three independent replication

The apoptosis assay using flowcytometry annexin V-FITC was shown in Fig. 6-8. The flowcytometry annexin V-FITC apoptosis assay showed that the method was valid, since more than 90% of the cell control was alive. EEafL induced more necrotic cells rather than apoptotic cells when it was used singly on all of the cancer cell lines. While when it was used in combination with doxorubicin, it induced more apoptotic cells rather than necrotic cells. Suggesting that EEafL is better to be used in combination with cancer chemotherapeutic agent (co-chemotherapy).

Necrosis is a cell death process that is avoided in cancer therapy. It would cause inflammation at the cancer microenvironment. The pro-inflammatory agent released would further generate malignant growth of cancer itself, resulting worse prognosis for the patients (Hanahan and Weinberg, 2011).
Fig. 6. The flowcytometry annexin V-FITC apoptosis assay of HeLa cell line treated with EEAfL and doxorubicin in single and combinatorial use at 24H incubation. EEAfL in combination with doxorubicin induced more apoptosis rather than single use (data was presented as mean ± SD , n =2)

The necrosis activated by the EEAfL in single use was suggested due to the high concentration used. Though IC$_{50}$ was supposed to induced apoptosis than that of the IC$_{75}$ (Mahassni and Al-Reemi, 2013), of course it would correlate to the compounds content in EEAfL. EEAfL contains alkaloids, flavonoids, terpenoids, and saponin (Maryani et al., 2013). Alkaloids may cause apoptosis as well as necrosis on cancer cell line (Lamchouri et al. 2013; Song et al., 2006), while most flavonoids are inducing apoptosis rather than necrosis (Kuno et al., 2012). The effect might be less harmful if the concentration used is lower, e.g. the using of EEAfL in combination with cancer chemotherapeutic agent (co-chemotherapy).
Fig. 7. The flowcytometry annexin V-FITC apoptosis assay of T47D cell line treated with EEAfL and doxorubicin in single and combinatorial use at 24H incubation. EEAfL in combination with doxorubicin induced more apoptosis rather than single use (data was presented as mean ± SD , n =2)

Combinatorial use of EEAfL and doxorubicin at concentration under the IC\textsubscript{50} was proven to induce more apoptotic cells than necrotic cells. Co-chemotherapy is intended to increase the effectivity as well as to reduce the side effect that might happen when we use higher concentration. The effectivity of EEAfL in combination with doxorubicin is proven with the apoptosis induction rather than necrosis. While the side effect reduction is the use of doxorubicin under the IC\textsubscript{50} would reduce the side effects due to doxorubicin unselectivity, i.e. reduction on immune system, hair loss, as well as other unkonvenience symptoms.
CONCLUSION

We can conclude that EEAfL is better to be used in combination with doxorubicin (as cancer co-chemotherapeutic agent) rather in single use.

Further research on combinatorial use of EEAfL and cancer chemotherapeutic agent is needed to study the mechanism of actions.

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