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Indonesian Journal of Pharmaceutical and Clinical Research

Cytotoxic and Apoptotic Activities of *Vernonia amygdalina* Extract in HepG2 Cell Line

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Abstract. Liver cancer is a malignant tumor originating from the development of chronic hepatitis or cirrhosis and is responsible for damage to the shape and function of the liver. The loss of the ability of conventional chemotherapy to inhibit the growth of cancer cells is a major focus in the world. Natural ingredients are the solution to this problem, for example the use of Vernonia amygdalina leaves. Vernonia amygdalina contains flavonoids, cardiac glycosides, and steroids/triterpenoids which are reported to have anticancer properties. This study aims to examined the activity of these plants as anticancer liver through cytotocix and apoptotic activity. Vernonia amygdalina ethanol extract (VAEE), Vernonia amygdalina ethyl acetate extract (VAEAE), and Vernonia amygdalina n-hexane extract (VAHE) were obtained from a multistage maceration process. The extracts were tested for cytotoxic activity by the MTT method on HepG2 cells. VAEAE had the best inhibitory concentration 50 (IC_{50}) value of 19.91 ± 0.24 µg/mL. Apoptosis test was performed using double staining method. Subjective observations were made using a fluorescence microscope. It was seen that Vernonia amygdalina ethyl acetate extract was able to trigger apoptosis in HepG2 cells.

Keyword: *Vernonia amygdalina*, Cytotoxic, Apoptotic, HepG2, Cancer

Abstrak. *Kanker hati merupakan tumor ganas yang berasal dari perkembangan hepatitis kronis atau sirosis dan bertanggung jawab terhadap kerusakan bentuk dan fungsi organ hati. Hilangnya kemampuan kemoterapi konvensional dalam menghambat perkembangan sel kanker menjadi fokus utama dunia kesehatan, Bahan alam menjadi solusi dari masalah tersebut, contohnya penggunaan daun Vernonia amygdalina. Vernonia amygdalina mengandung flavonoid, glikosida jantung, dan steroid/triterpenoid yang dilaporkan berkhasiat sebagai antikanker. Penelitian ini bertujuan untuk menguji aktivitas dari tumbuhan tersebut sebagai antikanker hati secara in vitro. Ekstrak etanol Vernonia amygdalina, ekstrak etil asetat Vernonia amygdalina, dan ekstrak n-heksan Vernonia amygdalina diperoleh dari proses maserasi bertingkat. Ekstrak-ekstrak tersebut diuji aktivitas sitotoksiknya dengan metode MTT pada sel HepG2. Ekstrak etil asetat Vernonia amygdalina memiliki nilai IC50 terbaik yaitu 19,91 ± 0,24 µg/mL. Uji apoptosis dilakukan dengan menggunakan metode double staining. Pengamatan secara subjektif dilakukan menggunakan mikroskop fluoresens. Terlihat bahwa ekstrak etil asetat Vernonia amygdalina mampu memicu terjadinya apoptosis pada sel HepG2.*

Kata Kunci: *Vernonia amygdalina*, *Sitotoksik*, *Apoptosis*, *HepG2*, *Kanker*

Received 10 June 2021 | Revised 31 December 2021 | Accepted 14 October 2022

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1. Introduction

Hepatocellular carcinoma or liver cancer is the most common widespread cancer and causes high mortality rates. However, the main problem of chemotherapy to treat hepatocellular carcinoma is the cancer resistance mechanism [1]. Thus, the discovery of new compound with effective anticancer activity is needed to control liver cancer and apoptosis induction is the desirable effect for successful liver cancer treatment [2]. Natural products have provided a significant contribution to the development of several drugs currently used in cancer chemotherapy. Although many natural products are known to affect the redox state of the cell most studies on these compounds have focused on their antioxidant activity instead of on their pro-oxidant properties [3].

Vernonia amygdalina (VA) leaves has been reported to have anticancer activity. VA was tested by in vitro and in vivo among other against nacofaring cancer, skin cancer cell [4], prostate cancer cell [5], breast cancer cell [6], and pancreatic cancer cell [7]. The anticancer activity of VA is thought to be due to the content of secondary metabolites, such as polyphenols, steroids/triterpenoids, and cardiac glycosides [8]. Based on this, VA has the potential to be developed as an anticancer agent, so it is necessary to test the activity of VA in inhibiting the development of liver cancer cells.

Testing of VA as an anticancer agent will begin with a cytotoxic test. This test was conducted to determine the IC_{50} value as a description of the toxic nature of the agent against cancer cells. Then IC_{50} will be used as a reference for other activity tests, such as apoptosis [9]. Apoptosis is a controlled program of cell death. This program occurs naturally to select and exclude cells that are considered abnormal [10]. Apoptosis also has a role in monitoringchanges in cancer cells and becomes the first line of defense to fight mutations by cleaning abnormal DNA cells which can be malignant. Thus, apoptosis is part of the immune system which controls normal cell population in the body [11]. The apoptotic-stimulating activity of anticancer agents is very important to know [12]. This study will test whether VA can induce apoptosis in HepG2 cells.

2. Material and Methods

2.1 Materials

All chemicals and reagents were obtained from certified suppliers and were of the highest analytical standard. The liver cancer cell (HepG2) was obtained from Laboratory of Parasitology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

2.2 Sampel Preparation

VA extracts were prepared using multilevel maceration method. Ethanol, ethyl acetate, and n-hexane have been using as a solvent for extraction process. The process begins with soaking 200 g of VA simplex in 2 L of n-hexane solvent (1 x 24 h) and then filtered using filter paper. This

process is repeated 3 times, and all filtrate is put into a glass container. For the macerate reextraction was carried out using ethyl acetate solvent with a same action and this workflow is done also for ethanol solvent. All filtrate was evaporated to give a VA extract [13].

2.3 Cytotoxic Test

Cytotoxic activity of VAEE, VAEAE, and VAHE was carried out using MTT method. The extracts with the test concentration were added to the well plate. Then the well plate containing HepG2 cells and extracts were prepared by adding MTT reagent. After incubation Viable cells reacted with MTT to produce purple formazan crystals. The sample was determined by microplate reader λ 595 nm to get the absorbance value. Absorbance value of the sample is converted to %viability using a common formula [14].

2.4 Apoptosis Stimulating Activity

The concentration of the sample in this test using the IC_{50} value. The apoptosis test was carried out using the double staining method. HepG2 cells were planted on a well plate with a cover slip at the bottom. The extract was added to the well plate and incubated for 24 hours. After incubation the cover slip was taken and acridine orange - ethidium bromide was added. The process of cell apoptosis was observed under a fluorescence microscope [15].

2.5 Statistical Analsysi

The data were presented as mean ± SD and all data were anelyzed with probit regression analysis using SPSS 22.

3. Results and Discussion

Cytotoxic test is a preliminary test that can be done to determine the ability of the sample as an anticancer. As a first step in the development of cancer drugs, of course, this test is very important [16]. The sample will be incubated with the cancer cells in the hope that they will kill the cells. With the addition of MTT reagent the number of live cells can be determined [17]. In this study, each of the VA extracts was incubated with HepG2 cells at concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31,125 µg/mL. Then doxorubicin was used as a comparison with concentrations of 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL. The result of cytotoxic test VA extracts in HepG2 cell can be seen in Table 1.

IC_{50} value was obtained from regression plot between %viability cell and concentration sample. SPSS version 22 was used to helping data analysis process. Based on Table 1, VAEAE have a more cytotoxic activity than another sample. The IC_{50} value of VAEAE was 19,91 ± 0,24 µg/mL, while another sample like VAEE was 190,99 ± 0,63 µg/mL and 1029,80 ± 27,95 µg/mL to VAHE. VAEAE have a good activity alhtough compared with doxorubicin. It is

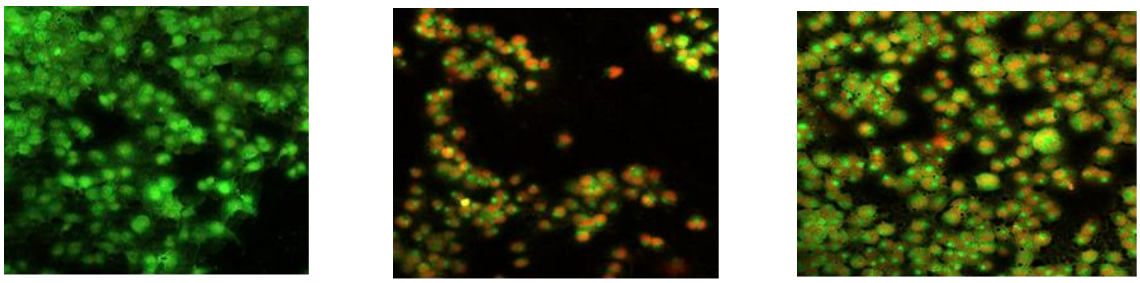
known that doxorubicin is an effective chemotherapeutic agent in inhibiting the growth of cancer cells. Experiencing doxorubicin has developed resistance to HepG2 cancer cells [18].

Table 1. IC_{50} **(**µg/mL) of VA extracts and doxorubicin in HepG2 cells

Groups	**IC_{50} (µg/mL) Value**
VAEE	190,99 ± 0,63
VAEAE	19,91 ± 0,24
VAHE	1029,80 ± 27,95
Doxorubicin	25,39 ± 0,57

Cancer cell death in this test is indicated by the color change that occurs [19]. Dead cells will not be able to reduce MTT compounds to formazan crystals, so the purple color will decrease causing the absorbance to decrease when measured using a microplate reader [20]. The IC_{50} value of VAEAE can be categoried strong as anticancer [21]. Because of that, VAEAE will be tested as apoptotic inducer agent in it's IC_{50} concentration and doxorubicin was also used as a comparison agent.

Apoptotic activity was tested using double stainning method with acridine orange–ethadium bromide as a reagent. VAEAE and doxorubicin have been incubated with HepG2 cell on well plate. The IC_{50} value was used as concentration test. Observation of sample activity was carried out using fluorescence microscope. The result of apoptotic activity can be seen in Figure 1.



(a) **(b)** **(c)**

Figure 1. Observation of apoptosis in fluorescence microscope with 40x magnification and 3 x zoom out. (a); Control Cell; (b): Doxorubicin in 1 IC_{50}; (c): VAEAE in 1 IC_{50}

The process of apoptosis is a complex one involving the expression of pro-apoptotic proteins such as Bax and Caspase-3, as well as anti-apoptotic proteins such as Bcl-2 [22]. Based on figure 1, can be seen HepG2 cell morphology after treatment using VAEAE and doxorubicin. These results have been identified subjectively by looking at the color changes that occur in HepG2 cells. Green cells indicate viable cells, while red cells represent cells that have undergone apoptosis. On the control cells, green fluorescent can be seen because it only absorbs acridine orange. Ethidium bromide cannot enter to the cell control because cell integrity is still

good [23]. The cells with treatment 1 IC_{50} was spurred apoptosis, marked with a red colour formed. Red fluorescent in the cells was showed the cells loss of membrane permeability and leaded ethidium bromide can enter the cells. It was as an indicator that VAEAE and doxorubicin can leading cell deadth [23].

4. Conclusion

Based on the results of the study, doxorubicin decreased its cytotoxic activity against HepG2 cells, because VAEAE has better cytotoxic activity than doxorubicin. This explains that there was resistance of HepG2 cancer cells to doxorubicin. VAEAE can be considered as an anticancer agent of the liver, because in addition to having cytotoxic activity on HepG2 cells, VAEAE is also capable of causing apoptosis. Of course, further testing is needed on the mechanism of its activity.

Acknowledgment

This research was funding by Ministry of Research Technology and Higher Education through "Hibah Penelitian Tesis Magister 2018".

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