Anticancer and Cytotoxic Potentials of *Vernonia amygdalina* Delile on WiDr Cell Lines

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Abstract

The development of cancer treatment with natural ingredients has gained attention. This is because many of the active compounds present in nature are capable of inhibiting the growth of cancer cells. One of the natural ingredients which has antitumour is *Vernonia amygdalina* Delile, known in Indonesia as African leaves. Colorectal cancer (CRC) is the incidence of cancer in parts of the large intestine that is prevalent in the aging population. This research investigated the effect of *Vernonia amygdalina*, through cytotoxic activity on human colon cancer cells (WiDr). The simplicia of the African leaves was extracted separately using 96% ethanol solvent by the maceration method. The African leaves ethanol extract was then analyzed for toxicity test using 3-(4,5-Dimethylthiazol-2-Yl)-diphenyl tetrazolium bromide-2,5 (MTT) in WiDr cell lines. The findings showed that African leaves exerted cytotoxic activity with an IC_{50} value of 26.92µg/ml which suggested the activity of African leaves extract has cytotoxic impact and to be moderately active on the WiDr cell lines. These data indicate the anti-colorectal cancer activity of *Vernonia amygdalina*.

Key words: African Leaves, Cytotoxic activity, Anticancer, WiDr Cell Lines

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1. Introductions

Cancer is an unregulated process of cell development followed by cell invasion and spreading into other regions of the body in surrounding tissues (metastasis). Cancer cells are distinguished by their continual growth, which results in an imbalance between live and dead cells. Cancer is the world's second biggest cause of mortality, after heart disease. Colorectal cancer is a kind of cancer that affects the epithelial cells of the colon or rectum (Kamaruddin, 2020; Sargowo et al., 2007).

According to the American Cancer Society, colorectal cancer is one of the most prevalent malignancies in the world, and ranks third most commonly diagnosed in the United States and fourth in Asia. According to the World Health Organization data, colorectal cancer in Asian countries is quickly growing. In 2008, it was projected that there were 1.6 million new cancer diagnoses and 1.1 million cancer deaths in Southeast Asia (Rahmadania et al., 2016). According to the Indonesian Health Profile 2008, colorectal cancer ranks ninth out of ten main cancers suffered by inpatients in all hospitals in Indonesia (1,810 cases). Colorectal cancer is one of the most frequent types of cancer in Indonesia, with 1.8/100,000 incidences, according to Dharmais Cancer Hospital statistics. (Rahmadania et al., 2016).

Cancer can be treated through surgery, radiation, and chemotherapy in general. There are numerous limitations to existing chemotherapy drugs, including resistance, adverse effects and poor effectiveness. As a result, there is an inefficient therapy, so it is necessary to develop more effective and efficient chemo-preventive molecules from plant source. Chemo-preventive agents capable of inhibiting cancer cell formation (Rauf et al., 2021), of suppressing aberrant cancer growth and of reversing the phases of the carcinogenesis process. Furthermore, chemo-preventive medicines can lower the risk of cancer by preventing carcinogens from initiating pre-neoplastic lesions. The investigation of natural materials, especially plants, will be a method to identifying chemical preventative substances. (Haryanti & Widiyastuti, 2017).

The use of chemicals found in natural substances can be used to cure cancer. African leaves (*Vernonia amygdalina* Del) is one of the natural substances active in cancer chemoprevention. This plant is well-known and frequently utilized by the general people to prevent and treat a variety of illnesses, including heart disease, lower cholesterol, lower blood sugar, digestive problems, and weight reduction. There are numerous nutrients and chemical substances may be found in African leaves. Protein, fiber, carbs, calcium, and ascorbic acid are all included in the nutritional content. Flavonoids, alkaloids, saponins, steroids, and tannins are among the chemical substances found in African leaves (Hadi, 2017).

Based on previous research, the IC_{50} values for the n-hexane, ethyl acetate, and methanol fractions were 2.8920 ±0.1998 µg/mL, 4.3052 ± 0.2776 µg/m, 2.6711 ± 0.1660 µg/mL, respectively, and were strongly toxic to *Artemia salina* Leach (Rosnani, Abd. Halim Umar, Hamdayani, L.A., 2018). The IC_{50} values of the ethanol extract from African leaves were determined in a cytotoxic examination of cancer cells as an initial data for the provision of information on African leaf plants' potential as alternative anticancer medicinal medicines, based on the preceding description. The present study is to determine the antitumour activity of *Vernonia amygdalina* Del in WiDr cell lines.

2. Materials and methods

The sample of African Leaves which is grown in Bolaang Mongondow district, a central Pinolosian district Province of North Sulawesi. Maceration was performed by soaking in ethanol as solvent in simplicia as much as 1 kilogram of African leaves. Simplicia was placed into the maceration container and then inserted into sufficient solvent for the wetting procedure. The residual solvent was inserted until all simplicia had fully dipped, and thereafter it may stand for 3 days in a place shielded from sunshine. With the same procedure, the pulp was suppressed 4 times. The evaporation results were transferred to a porcelain cup, which was placed in a water bath to evaporate the residual solvent and obtain a thick extract (Widowati, 2017).

Phytochemical screening

Identification of alkaloids, steroids/triterpenoids, flavonoids, saponins, and tannins was performed by the method of Harbone (1987).

The African leave extract was heated, agitated, and filtered after being combined with 5 ml of chloroform and 5 ml of ammonia. Each filtrate received 5 drops of 2 N sulfuric acid, which was shaken and allowed to stand. The top of each filtrate was taken and tested with Mayer and Dragendorff reagents. The presence of alkaloids is indicated by formation of an orange, brown, and white precipitate.

The presence of steroid compounds in plant extract indicated by a color shift from purple to blue or green. Which, 1 ml of African leaf extract was combined with 3 ml of chloroform, 2 ml of concentrated sulfuric acid, and 2 ml of anhydrous acetic acid. While, triterpenoid compounds shows the development of a brownish hue on the surface.

A combination of 3 ml of ethanol (70%) with 1 ml African leave extract, was shaken, heated and shaken again and then filtered. Added filtrate 0.1 g of magnesium powder and 2 drops of hydrochloric acid. The presence of flavonoids is shown by the development of a red hue on the ethanol layer.

African leave extract was placed in a test tube, then 10 ml of hot water was added, cooled, and rapidly shaken for 10 seconds. This extract includes saponins if a foam produced from 1-10 centimeters in height not less than 10 minutes does not exhaust by adding 1 drop of hydrochloric acid.

For Tannins, a total of 1 ml of the extract was boiled with 20 ml of water then filtered. The obtained filtrate was mixed with 2 t0 3 drops of 1% $FeCl_3$, and tannin compounds were shown to be green in brown or in dark blue.

Extract Test Solution

The extract test solution was created by dissolving 10 mg of extract in 1 ml of DMSO and vortex at 1500 rpm until dissolved to obtain a solution with a concentration of 10,000 µg/ml, which was then pipetted 100 µl and the volume was adjusted up to 1 ml. The African leave extract were prepared in five concentration series (7.8125 µg/ml; 15.625 µg/ml; 31.25 µg/ml; 62.5 µg/ml; and 125 µg/ml), added RPMI media to each tubes and graded dilution was performed.

Cancer Cell Culture (WiDr) Media

RPMI solid media was added to 800 ml sterile aqua in a container, mixed until smooth, then added 2 grams of $NaHCO_3$ for every liters of media made, then stirred repeated. The mixture volume was adjusted up to 1 liter with sterile

aqua and homogenized them. The filtered media was collected in bottles and refrigerated at 40°C.

The WiDr cell suspension was placed in a conical tube, then 4 mL of RPMI media was added. Centrifuged it at 500 rpm for 5 minutes. Discarded its supernatant, and added 4 ml of RPMI 1640 complete media to the precipitate. An inverted microscope was used to monitor the cell conditions after cultivation in three culture bottles and 5 ml of RPMI media was added to each culture bottle. The culture bottles were put in a CO_2 incubator at 37 °C, after 24 hours replaced the media, and cultivated them again until they were confluent and adequate in number for the research.

Take out WiDr cells from CO_2 incubator, then assessed viability of them, if they were confluent, then were harvested. Removed media by using a micropipette, added 1-2 mL of trypsin EDTA 0.25 % to the culture bottle and incubated in a CO_2 incubator for 5 minutes before adding 7 mL of RPMI media for inactivating trypsin and observing the cells under a microscope. The obtained cell volume was transferred to a conical tube with 10 ml adding media. To achieve a cell concentration of 10^4 cells per well, we counted cell suspensions that be taken and medium volume that be supplied.

Cytotoxic Test

Prepared a microplate with 30 wells for extract sample test and 3 wells for negative control. Fill the WiDr cell suspension to each test and control samples were triple replicated, and incubated them in a CO_2 incubator at 37^{o}C for 24 hours. After incubation, discarded the microplate media by flipping the microplate upside down.

Diluted solution of the African leave extract (7.8125 µg/ml; 15.625 µg/ml; 31.25 µg/ml; 62.5 µg/ml; and 125 µg/ml) were put into each wells containing WiDr cells (100 µl respectively), and 100 µl of media sole into negative control wells, then incubated to the CO_2 incubator at 37^{o}C for 24 hours. After incubation, discarded microplate media, and add 100 µl each of 0.5 mg/ml MTT reagent (Microtetrazolium) solution into the test extract wells and negative control. Wrapped the microplate with aluminum foil, and incubated it into CO_2 incubator at 37^{o}C for 4 hours. Final procedure, add 100 µL of DMSO to the each wells for 10 minutes before measuring absorbance using an ELISA reader at a wavelength of 595 nm.

Cytotoxicity test results were used to measure levels that induced 50 percent cell proliferation inhibition (IC_{50}) with probite analysis. The percentage of cell death after treatment at each stage is determined using the following formula:

$$\% \text{ of cell death} = \frac{\Sigma \text{ living cells in the control} - \Sigma \text{ living cells in the sample}}{\Sigma \text{ living cells in the control}} \text{ X } 100\%$$

IC_{50} is the concentration that causes the death of 50% of the cell population, so that its potential cytotoxicity can be known (Ismaryani et al., 2018).

3. Results and discussion

Cytotoxic activity of African leave extract on WiDr cancer cells in vitro was tested using the MTT technique (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) as shown by the parameter IC_{50} in this study. A phytochemical screening was performed to evaluate the content of secondary metabolites in the African leave samples. According to the findings of phytochemical screening tests performed on African leaves, the ethanol extract included flavonoid, saponin, tannin, and steroid components as indicated in the table below:

Table 1. Phytochemical screening of *Vernonia amygdalina* leaves extract

Compounds Reagents		African leave extract	Result
Alkaloid	Mayer	No precipitated	-
	Dragendrof	No precipitated	-
	Wagner	No precipitated	-
Flavonoid		Red orange	+
Saponin		Foam	+
Tannin		Dark blue	+
Steroid		Red ring	+

Information:
+ = Contains compounds (changes in color)
- = Does not contain compounds

Flavonoid compounds are widely recognized to be one of secondary metabolite compounds with anticancer action. Flavonoid compounds able to cause apoptosis based on previous studies. Flavonoids induce apoptosis via inhibiting DNA topoisomerase I/II activity, modulating signaling pathways, decreasing expression of Bcl-2 and Bcl-XL genes, increasing expression of Bax and Bak genes, and inhibiting endonuclease activity. Quercetin, a flavonoid molecule, has been shown to promote apoptosis in Caco-2,HT-29, and HL-60 leukemia cancer cells by stimulating cytochrome c release of from mitochondria. (Amit K. Taraphdar, 2001).

Tannin compounds have anticancer properties as well. Tannins' anticancer mechanism is similar to the antioxidant activity, through an apoptotic pathway of cancer cells stimulation. According this concept, the mechanism of cell apoptosis caused by DNA fragmentation. The release of the DNA chain by reactive oxygen molecules such as hydroxy radicals initiates the fragmentation process. Another function of tannin inhibits cancer growth by reducing the activation of protein kinases, therefore inhibiting signal transduction routes from membranes to nucleus cells.

Saponins are also known to have anticancer properties by preventing the synthesis of overexpressed Bcl-2, generating overexpressed caspase-3 protein, boosting p53 expression, and inducing G1 cell cycle arrest. Cytotoxic test against cancer cells is a popular fundamental test on the anticancer medicines and chemo-preventive compounds (Fitria, M., Armandari, I , Septhea, D.B, Ikawati, A.H.M , Meiyanto, 2011).

One of the procedures utilized in the cytotoxic test is MTT assay test. In this technique, the tetrazolium salt of the MTT reagent is broken down into formazan crystals by the succinate tetrazolium reductase system, which is active in live cells (Asril Burhan, Akbar Awaluddin, Zulham, Burhanuddin Taebe, 2019). An ELISA reader may be used to measure the absorbance of this formazan crystal. In this study, an ELISA reader at a wavelength of 595 nm was used to determine the absorbance value of formazan produced. An increase in purple color indicates an increase in living cells, if the resultant color is light (yellow), however, this indicates more dead cells (Table 2).

Table 2. Cytotoxic on WiDr cancer cells using an African leave ethanolic extract (*Vernonia amygdalina*)

Sample	Concentration (µg/ml)	% of cell death	Concentration Log	Probit	IC_{50} (µg/ml)
African leaf ethanol extract	7.8125	31.0572	0.89279003	4.5	26.92 µg/mL
	15.625	40.3243	1.193820026	4.75	
	31.25	53.2824	1.494850022	5.08	
	62.5	62.1276	1.795880017	5.31	
	125	75.5405	2.096910013	5.71	

The results of the cytotoxic test for African leave extract and fraction are shown in Table 2. The ethanol leaf extraction of *Vernonia amygdalina* was considered to have anticancer activity against WiDr cells with an IC_{50} value of 26.92µg/ml.

4. Conclusion

In conclusion, this study showed that the African leaves ethanol extract has a cytotoxic impact and to be moderately active (IC_{50} = 26.92µg/ml) in WiDr cell lines.

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