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Anticancer Potential of Ethanolic Extract *Artocarpus heterophyllus* Lam. Leaves against Human Colon Cancer WiDr Cell Line

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Abstract

Jackfruit (*Artocarpus heterophyllus* Lam.) is a plant contains various compounds that has potential as anticancer drugs. Secondary metabolites of jackfruit leaves are flavonoids, alkaloids, tannins, triterpenoids, and saponins. A typical flavonoid compound group in the *Artocarpus* genus, namely artocarpin, is able to kill cancer cells through apoptosis. Not many have conducted cytotoxicity research and apoptosis induction of ethanolic extracts from jackfruit leaves, especially against WiDr colon cancer cells. Therefore, this study aims to study the cytotoxic effects and apoptosis induction of ethanolic extracts of jackfruit leaves against WiDr colon cancer cells. The research was conducted by cytotoxicity test using the MTT assay. Apoptosis test was done using double staining method with AO/PI dye. The treatment was conducted at various concentrations of ethanolic extract of jackfruit leaves, doxorubicin as positive control, and DMSO as solvent control. The data were analyzed by one way ANOVA ($p \leq 0.05$) and continued using Tukey HSD Post Hoc test. The results showed that the ethanolic extract of jackfruit leaves was not toxic to WiDr cells with an IC_{50} 740.43 µg/mL, but could significantly reduce cell viability at a concentration of 500 µg/mL. The ethanolic extract of jackfruit leaves could also induce apoptotic cell death at a concentration of 500 µg/mL. Based on these results, ethanolic extract of jackfruit leaves has little potential to be developed as an anticancer drug.

Keywords: Apoptosis; cytotoxicity; *Artocarpus heterophyllus* Lam leaves; ethanolic extract; WiDr cells.

INTRODUCTION

Cancer is disease that causes significant deaths in the world. Based on 2022 data, cancer deaths worldwide reached nearly 10 million people. Lung, colorectal, and liver cancer are the three cancers with the highest number of deaths. In 2022, there were 19.255 deaths people from colorectal cancer in Indonesiat (Ferlay *et al*., 2024).

Various cancer therapies that are commonly done until now still have side effects. Treatment for cancer, especially colon cancer, such as surgery can cause urogenital disorders. Chemotherapy has the effect of hair loss, numbness, and increases the chance of infection due to a lack of white blood cells. Radiation therapy can cause reactions of nausea, diarrhea, inflammation, rectal bleeding, bladder dysfunction, and even cause infertility in women (American Cancer Society, 2020). Therefore, it is necessary to develop cancer drugs that can minimize these side effects.

Drugs with natural ingredients can be one of the solutions for the development of cancer treatment. One of the natural ingredients that has the potential to be developed as a natural cancer drug is jackfruit tree (*Artocarpus heterophyllus* Lam.). Several studies have been conducted to study the potential of jackfruit tree organs, among others, *Artocarpus heterophyllus* seeds are known to have cytotoxic activity with an IC_{50} value 35.26 µg/mL against lung cancer cells (A549) (Patel & Patel, 2011).

Water extract of *Artocarpus heterophyllus* flowers has cytotoxic activity on colon cancer cells (Caco-2) with an IC_{50} 29.37 µg/mL (Gupta *et al*., 2020). *A. heterophyllus* wood extract showed cytotoxic activity against colon cancer cells (HCT116) with an IC_{50} 4.23 mg/L (Morrison *et al*., 2021). Methanolic extract of jackfruit leaves showed cytotoxic activity with an IC_{50} 119 µg/mL (Marka *et al*., 2016). Based on those researches, jackfruit leaves may also have the potential to be utilized as an alternative to natural cancer drugs because they contain various secondary metabolites such as anthocyanins, coumarins, anthraquinones, flavonoids, phenolic acids, and terpenoids (Ngbolua *et al*., 2019). Research by Arung *et al*. (2010), showed that artocarpin, one of the flavones contained in A. *heterophyllus*, can cause breast cancer cell death (T47D) through induction of apoptosis.

The effect of natural ingredients on cancer cells depends on various factors. The extraction method and

the choice of solvent type are influential factors in the results of making an extract. Extraction is the first step taken to separate the desired natural ingredients or compounds from certain raw materials (*Zhang et al*., 2018). In the extraction method, solvent selection is very important because the polarity level of each solvent has the ability to dissolve different substances or compounds (Abubakar & Haque, 2020). Based on the law of *like dissolves like*, solvents with polarity values close to the polarity of the solute tend to have better performance (Zhang *et al*., 2018).

Research on jackfruit tree parts and their metabolite compounds against cancer cells has been carried out using various methods and several types of solvents. However, not many have conducted cytotoxicity research and apoptosis induction of ethanolic extracts from jackfruit leaves, especially against WiDr colon cancer cells. Therefore, this study aims to study the cytotoxic effects and apoptosis induction of ethanolic extracts of jackfruit leaves against WiDr colon cancer cells.

MATERIALS AND METHODS

Sample

The leaves of *Artocarpus heterophyllus* Lam. were collected from the Ambarawa region, Semarang Regency, Central Java Province, Indonesia. The plants were identified by taxonomists at the Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada.

Extraction

Jackfruit leaves to be used were selected that are old, intact, fresh, green and then cleaned. Next, leaves were dryed under the sun. The dried leaves were then crushed into powder with a blender. The powder was then sieved with a 40 mesh sieve and weighed on analytical scales as much as 20 grams. Maceration method extraction was carried out by soaking the leaves powder using 70% ethanol solvent for 3x24 hours in a dark bottle. All extracts were put together and evaporated with a rotary evaporator at a temperature of approximately ±50 ^{o}C. Next, evaporation was carried out again by heating in a porcelain dish until it becomes a thick extract.

Cytotoxicity Test (MTT)

Extract testing was conducted on WiDr colon cancer cells obtained from the Laboratory of Parasitology, Faculty of Medicine, Public Health, and Nursing (FK-KMK), UGM. Harvested cells cultured with density1 x 10^4 cells/well in 96-well plate with complete medium RPMI contained, 10% FBS, 2% penicilin-streptomycin, and fungizone (Amphoterizine B) 0.5%. The cells were incubated for 24 hours in an incubator with 5% CO_2 flow and 37^{o}C temperature. After that, the cells treated with various concentrations (7.81; 15.62; 31.25; 62.5; 125; 250; 500; and 1000 µg/mL) of ethanolic extract of jackfruit leaves. Doxorubicin concentrations were 0.062; 0.125; 0.25; 0.5; 1; 2; 4; and 8 µg/mL. All concentration series were made for treatment with 24 and 48 hours incubation. After incubating for 24 and 48 hours, each treatment was dripped with MTT reagent and incubated for 4 hours. The plate was then observed under an inverted microscope, if formazan was clearly formed then 100 µL of 10% SDS was added. Cells were re-incubated in the dark at room temperature overnight. Next, the absorbance was read with an ELISA reader at 595 nm. The absorbance results are used to calculate the percentage of cells viability based on the following calculation formula (Cancer Chemoprevention Research Center (CCRC), 2013).

$$\text{Percentage of live cells} = \frac{(\text{Treatment absorbance} - \text{Media control absorbance}) \times 100\%}{(\text{Solvent control absorbance} - \text{Media control absorbance})}$$

The calculation was also continued by calculating the IC_{50} value.

Apoptosis Test (Double Staining)

WiDr colon cancer cells were cultured with density 5 x 10^4 cells/well on the cover slip in 24-well plate. After that, the cells were allowed to stand for 30 minutes and then added completed medium RPMI and incubated in an incubator CO_2 5% with a temperature of 37^{o}C for 24 hours. After incubation was completed, treatment was conducted with negative control DMSO 0.5%, positive control of doxorubicin 0.125 µg/mL, and ethanolic extract of jackfruit leaves at concentrations of 250 and 500 µg/mL. Next, the cells were re-incubated in an incubator with 5% CO_2 and 37^{o}C temperature for 48 hours. After 24 hours incubation, cover slip with cells on it taken from the plate and then placed on a glass object and stained with AO/PI (acridine orange and propidium iodide). The staining results were observed using a confocal microscope. Green fluorescent cells indicate live cells and red fluorescent cells are dead or apoptotic cells. Apoptotic cells were counted in a population of at least 100 cells per well.

Data Analysis

Cytotoxicity and apoptosis data were analyzed by one way ANOVA. If there is a significant difference, then the analysis continued using Tukey HSD post hoc test. The test results are significant if the p value $\leq$ 0.05. (Simanurak *et al*., 2023).

RESULT AND DISCUSSION

In this study, jackfruit leaves were extracted by maceration method using 70% ethanol. The extract was tested for its cytotoxic effect on WiDr colon cancer cells by MTT assay. In this test, the treated cells were

incubated for 24 and 48 hours. The cytotoxic level of the extract was measured by calculating the percentage of cell viability. Cell viability is determined based on purple formazan crystals. Soluble crystals will produce a color intensity that is directly proportional to the number of living cells (Buranaamnuay, 2021) (Figure 1.)

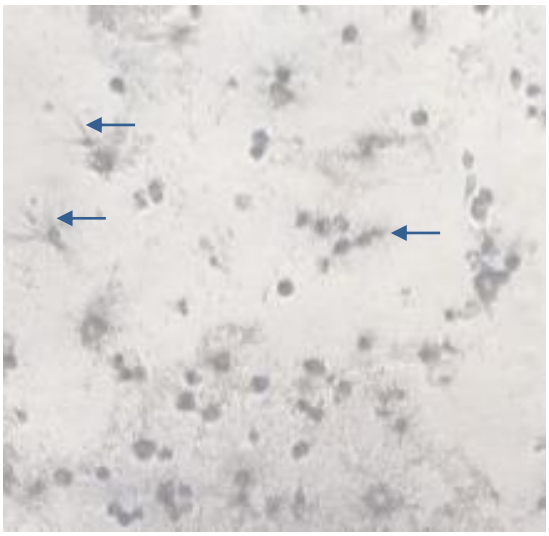

Figure 1. Formazan crystals formed in the MTT assay.

The percentage of cell viability after 24 hours incubation showed that 0.5% DMSO control was not toxic to WiDr cancer cells with a viability of 100 ± 6.56%. Doxorubicin treatment showed cell viability of 85.08 ± 3.10% at the lowest concentration of 0.062 µg/mL, while at the highest concentration of 8 µg/mL the viability was 61.82 ± 4.53% (Figure 2). In the treatment of ethanolic extract of jackfruit leaves (EEJL), cell viability was more than 100% in all concentration groups. The lowest concentration of 7.81 µg/mL had a cell viability of 105.08 ± 3.62% and the highest concentration of 1000 µg/mL had a viability of 136.36 ± 3.22% (Figure 2.). In the EEJL treatment group, data analysis showed that there were several concentrations that were not significantly different and some were significantly different, but in general there were no significant differences between concentrations.

In the EEJL treatment group, data analysis showed that there were several concentrations that were not significantly different ($p > 0.05$), such as concentrations of 31.25 and 250 µg/mL. Concentrations of 15.62 and 125 µg/Ml also showed no significant difference µg/mL. However, other concentrations showed significant differences between concentrations ($p \leq 0.05$). Based on the viability results, the IC_{50} for doxorubicin and EEJL could not be determined. These results indicate that EEJL is unable to exert cytotoxic effects on WiDr cancer cells within the 24 hours incubation period. Therefore, the same study was conducted with a longer incubation period of 48 hours.

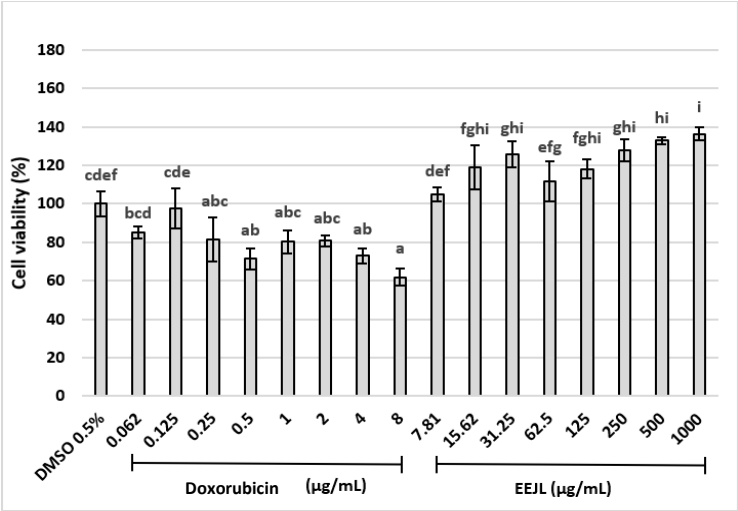

Figure 2. Ethanolic extract of jackfruit leaves (EEJL) for 24 hours did not decrease WiDr cell's viability.

In the treatment with an incubation time of 48 hours, the 0.5% DMSO control showed cell viability of 100 ± 4.42% which means it is not toxic to WiDr cells (Figure 3.). This result shows that DMSO 0.5% in 24 or 48 hours incubation time proved to be nontoxic. These results were consistent with previous research, the DMSO concentration range of 0.1-0.6% does not cause toxic effects on cells (Nguyen *et al*., 2020). In the treatment of doxorubicin, the lowest concentration of 0.062 µg/mL resulted in a viability percentage of 62.83 ± 0.85% while the highest concentration of 8 µg/mL viability was 28.32 ± 5.57% (Figure 3). Doxorubicin treatment for 48 hours resulted in an IC_{50} 0.147 µg/mL. This result is quite different from previous research, doxorubicin produced

an IC_{50} 3.49 µg/mL and was toxic to WiDr cells in only 24 hours (Fathani & Miladiyah, 2021). Based on the US National Cancer Institute (NCI) criteria, the IC_{50} of doxorubicin is classified as high because $IC_{50} \leq 20$ µg/mL (Fathani & Miladiyah, 2021). In the treatment of ethanolic extract of jackfruit leaves with the lowest concentration of 7.81 µg/mL had a cell viability of 132.07 ± 14.91% (Figure 5.). The concentration group of 15.62 µg/mL has the highest viability of 136.94 ± 17.99%, while the concentration of 500 µg/mL has the lowest cell viability of 43.31 ± 3.94% (Figure 3.). EEJL treatment for 48 hours can kill WiDr cancer cells more than 50% at a the concentration of 500 µg/mL so that the IC_{50} can be determined at 740.43 µg/mL (Figure 5). This IC_{50} is classified as nontoxic based on the US National Cancer Institute (NCI) standard because $IC_{50} > 501$ µg/mL. Even though IC_{50} EEJL is classified as non-toxic, at the concentration of 500 µg/mL there was a decrease in cell viability to less than 50%.

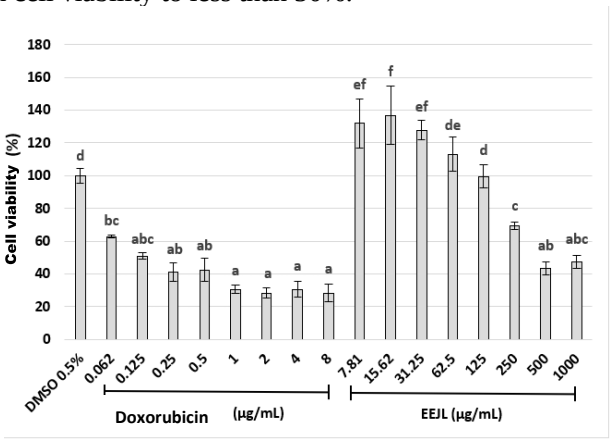

Figure 3. Ethanolic extract of jackfruit leaves (EEJL) for 48 hours decrease WiDr cell's viability.

The results of the cytotoxic test showed that incubation time affected the viability of WiDr cells. Treatment with 24 hours incubation did not reduce cell viability, while 48 hours incubation significantly reduced cell viability to less than 50%. In a study conducted by Rachmawati *et al*. (2012), the incubation time factor in anticancer drug testing affects the expression time and function of tumor suppressor genes. In this study, it is also possible that EEJL and doxorubicin did not work optimally within 24 hours because genes related to cancer cell death, such as tumor suppressor genes, have not worked or have not even been expressed yet. In addition, the dose or concentration also affects the viability of WiDr cells. Our results, especially after 48 hours of incubation, shows that cell viability tends to decrease as the concentration increases. Incubation time and dose affect the results of this study because both factors affect the contribution of the receptor fraction in drug action. The drug will work stronger along with the number of receptor fractions that bind (Rachmawati *et al*., 2012).

Based on the results of the cytotoxicity test, an apoptosis test was carried out to determine the death rate of WiDr cancer cells. The test was carried out by double staining method using AO/PI dye (acridine orange and propidium iodide) and incubated for 48 hours. AO dye works by passing through the plasma membrane of living cells then inserting into DNA and RNA which emit green fluorescence. PI dye emit red fluorescence in dead cells by working through the integrity of the cell membrane that has been damaged or disrupted and then penetrating the cell nucleus (Shahruzaman et al., 2019). The results of the apoptosis test (Figure 3.) showed that in 0.5% DMSO control, all cells have intense green fluorescence which means the cells are still alive. In the control of doxorubicin 0.125 µg/mL, some cells have red fluorescence, meaning that the cells have died or experienced apoptosis. In addition, there are also some green and slightly orange colored cells that indicate cells experiencing early apoptosis. In the treatment of ethanolic extract of jackfruit leaves at a concentration of 250 µg/mL, there were some cancer cells that were still alive and began to experience apoptosis in the early stages. In the ethanolic extract of jackfruit leaves with the concentration of 500 µg/mL, most of the cancer cells experienced death or apoptosis (Figure 4). Living cells have an intact morphological structure of the nucleus. Morphologically, cells that die and experience apoptosis are indicated by rounded cells, bubbling membranes, and apoptotic bodies (Figure 4). Calculation of the percentage of apoptosis cells showed that the 500 µg/mL EEJL concentration has the highest number of apoptosis cells at 93.69 ± 10.93% (Figure 5.). From the apoptosis test, the ethanolic extract of jackfruit leaves has the ability to induce apoptosis of WiDr cancer cells. The results of this apoptosis test also have results that are quite in accordance with the cytotoxic test.

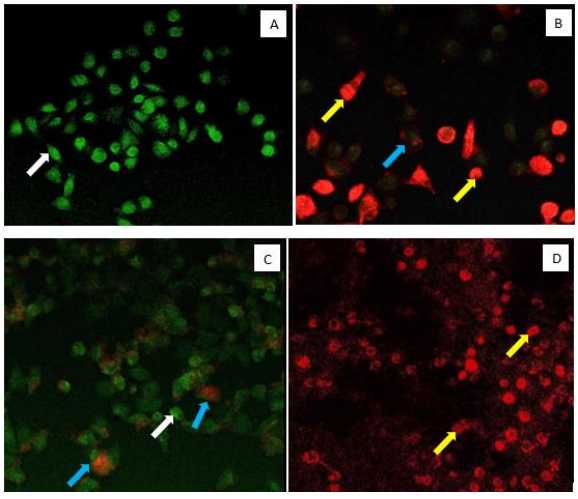

Figure 4. Ethanolic extract of jackfruit leaves can induce apoptosis in WiDr cancer cells. A. DMSO 0.5%; B. Doxorubicin 0.125 µg/mL; C. Jackfruit leaf ethanolic extract 250 µg/mL; and D. Ethanolic extract of jackfruit leaves 500 µg/mL. 10x10 magnification. White arrow indicates live cells. Blue arrow indicates cells undergoing early apoptosis. Yellow arrow indicates dead cells or late apoptosis.

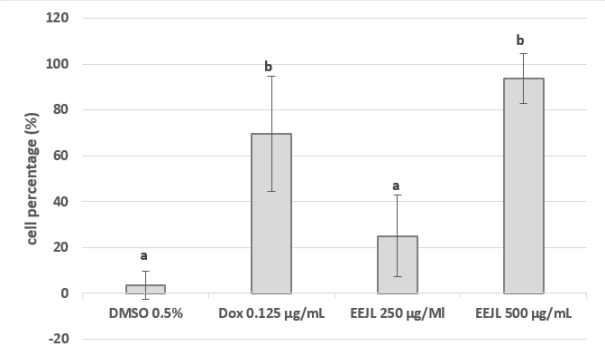

Figure 5. Ethanolic extract of jackfruit leaves can cause apoptotic death of WiDr cancer cells.

Doxorubicin is an anticancer drug used as a positive control in this study. According to the results of the study, doxorubicin can be toxic and induce apoptosis in WiDr cancer cells. This is because doxorubicin is able to induce apoptosis by trapping the topoisomerase enzyme in DNA damage so that it activates the transcription factor of cellular tumor antigen p53 (TP53). p53 (TP53) that is activated can control the expression of proapoptotic genes and inhibitors of antiapoptotic proteins (Kciuk *et al*., 2023). Doxorubicin can also induce apoptosis through increased levels of reactive oxygen species (ROS) that activate ATM-CHK2-TP53 signaling independently of DNA (Kciuk *et al*., 2023).

The ethanolic extract of jackfruit leaves can reduce cell viability and induce apoptosis in this study, possibly due to the effects of compounds contained in jackfruit leaves. This study used 70% ethanol solvent with maceration method to extract compounds in jackfruit leaves. Previous research, jackfruit leaves extraction produced compounds namely, flavonoids, alkaloids, tannins, triterpenoids, and saponins from the extraction process with 70% ethanol solvent (Nilakandhi *et al*., 2023). A typical flavonoid compound group in *A. heterophyllus* is artocarpin. Artocarpin can cause cytotoxic effects by causing cell death through caspase activation, Poly (ADP-ribose) polymerase (PARP) cleavage, and ROS formation (Daud *et al*., 2020). Artocarpin is one of the compounds that may play a role in inducing apoptosis of WiDr cells. According to research conducted by Arung *et al*. (2010), artocarpin isolated from *A. heterophyllus* wood can induce apoptosis accompanied by morphological changes and cell nuclei in T47D cancer cells by increasing caspase 3 and 8.

Factors such as solvent selection and extraction method can influence the results of this study. Ethanol solvent was used because it is able to dissolve almost all compounds and is relatively non-toxic (Mierza *et al*., 2022). The maceration method was chosen because it is classified as cold extraction so that it does not use excessive heating which results in damage to the compound (Mierza *et al*., 2022; Wardhani *et al*., 2023). The test results of jackfruit leaves extract are still not optimal with an IC_{50} 740.43 µg/mL which is classified as nontoxic. Nevertheless, the ethanol extract of jackfruit leaves still has potential to be studied further as an anticancer drug. This potential can be increased by choosing solvents and methods that can maximize the results and quality of jackfruit leaves compound content.

CONCLUSION

Based on this study, WiDr cancer cells treated with ethanolic extract of jackfruit leaves cannot reduce cell viability during 24 hour incubation. However, the extract can reduce cell viability at 48 hours incubation. The IC_{50} value obtained is not classified as toxic but there is a significant decrease in cell viability. Ethanolic extracts can also cause apoptotic death. Therefore, extracts from jackfruit leaves have little potential as anticancer drugs but can be further investigated by maximizing on the selection of solvents and extraction methods.

Competing Interests: The authors declare no conflict of interest in the manuscript

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