Anticancer potential from ethanol extract of *Zanthoxylum acanthopodium* DC. seed to against MCF-7 cell line

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**Abstract.** Andaliman (*Zanthoxylum acanthopodium* DC.) is a wild plant species from North Sumatera. Studies conducted before show that *Z. acanthopodium* seeds contain high antioxidants. Based on International Agency for Research on Cancer (IARC) data, it is known that in 2012 the highest type of cancer in women in the world is breast cancer (38 per 100 000 women). MCF-7 cell line is one of breast cancer model cell. The purpose of this research is to identify types of secondary metabolites which can be found in Andaliman’s seed extract and to test the ability of these compound in stopping the MCF-7 cell line activity. The method used in this research is an experimental method with different concentration and MTT Assay. Secondary metabolites that has been identified are phenol, saponins, tannins, triterpenes, flavonoids, and alkaloids. The result obtained by using MTT Assay with value IC₅₀ and the absorbance of 550 nm was 221.31 mg L⁻¹ which indicate that ethanol extract of Andaliman’s seed has potential as an anticancer and quite actively affect the inhibition of MCF-7 cell line proliferation. Moreover, the pure extract of Andaliman seed may have a strong effect to inhibit MCF-7 cell line proliferation.

**Keywords:** Andaliman, herbal medicine, breast cancer, biological activity, MTT assay, secondary metabolites

1. **Introduction**

Cancer is a disease that can cause death and become a threat in the health sector not only in Indonesia but also in the world since the old time until now. International Agency for Research on Cancer (IARC), assume that breast cancer is a type of cancer with the highest percentage of new cases (43.3 %) and the highest percentage of deaths (12.9 %) in women in the world [1]. In 2013, Basic Health Research data show that the prevalence of breast cancer in Indonesia reaches 0.5 per 1 000 women [2].

The use of various herbal medicine has become a major concern for the community. Besides its safer use, the process to find the active ingredients from plants is relatively easy because it is readily available in nature. Biological activity in plants is caused by the presence of the secondary metabolite compounds which contained there, such as alkaloids, terpenoids, steroids, saponins, flavonoids, polyphenols, and others. Secondary metabolites are compounds produced or synthesized in specific taxonomic cells and groups at certain growth or stress levels.
Andaliman (Zanthoxylum achantopodium DC.) is a wild plant species typical of North Sumatra. The morphology of Z. achantopodium is a shrub or small tree with a height of 5 m. This species has a lot of thorns on the stem. The leaves spread with a length of 5 cm to 20 cm and width 3 cm to 15 cm. The top surface color of the leaves is glints green while the lower surface is light green. The flowers are in the armpits. The fruit can be light green, old red; each fruit of one seed, hard shell, or shiny black color. The shape is round or capsule with a diameter of 2 mm to 3 mm [3]. Z. achantopodium fruit usually uses as a spice of Batak typical cuisine. Studies conducted by [4] show that Z. achantopodium seeds contain high antioxidants. Based on research conducted by [5], an extract or a compound which contain high antioxidant has a potency as anticancer. It is mean that Andaliman has the potential to be an alternative anticancer drug.

The purpose of this experiment was to determine the type of secondary metabolite found in seeds Andaliman and test the ability of secondary metabolites as anticancer for MCF-7 cell line. MCF-7 cell line is a model of breast cancer cells that widely used in research. Cancer treatment, in general, is still dependent on chemotherapy derived from synthetic chemicals. However, the chemical compound may have an effect of resistance, a phenomenon in which cancer cells treated with certain drugs will become resistant to other drugs with similar structures and mechanisms [6]. In addition, synthetic anticancer not only affects the target cells but also affects healthy cells around them. One of the concerns in chemotherapy is the use of bioactive ingredients from the isolation of natural resources because their characteristics are renewable, easily decomposed, and can be removed from the body [7]. Thus, until now the research on chemotherapy drugs using the natural resource like plants is still ongoing and continuously.

2. Materials and methods

The method used in this research is an experimental method in the laboratory using a completely randomized design (CRD) of one factor. Cytotoxicity tests are carried out in vitro on MCF-7 cells using the MTT-based cytotoxicity assay method with five stages of extract concentration ranging from 0 µL L⁻¹ (control), 1 µL L⁻¹, 10 µL L⁻¹, 100 µL L⁻¹, and 1 000 µL L⁻¹. Each treatment is repeated three times. The measured parameter is the concentration of extract which can inhibit 50 % proliferation of MCF-7 breast cancer cell line (IC₅₀). The next stage is the analysis phase of the percentage inhibition calculation and probit analysis to determine the IC₅₀ value.

The research was conducted in the Laboratory of Taxonomic Department of Biology Faculty of Mathematics and Science Padjadjaran University, Central Laboratory of Padjadjaran University, and the Cell Culture and Cytogenetics Laboratory of Eijkman Education Hospital. The study was conducted from Apr 2018 to June 2018. The ingredients which is used in this experiment were Andaliman, acetic acid, concentrated hydrochloric acid, sulfuric acid, DMSO solvent extract, 70 % ethanol, 80 % ethanol, 96 % ethanol, ferric chloride, fatty test kit, magnesium, sodium hydroxide, reagent Dragendorff, Mayer reagent, and MCF-7 cells. Z. achantopodium seed extract is made by applying the maceration method. First, Andaliman seed is cleaned and dried then weighed as much as 500 g. A total of 3 L of ethanol 70 %, 80 %, and 90 % is added as solvents respectively.

This extraction process is carried out for 5 d at room temperature. The extract is evaporated to remove the ethanol solvent by using an evaporator. Secondary metabolite analysis is performed using several tests, such as hydroquinone phenol test, saponin test, flavonoid test, tannin test, Lieberman Burchard test, Dragendorff reagent, and Mayer reagent. Each extract diluted by adding distilled water to get some concentrations, those are 1 µL L⁻¹, 10 µL L⁻¹, 100 µL L⁻¹, and 1 000 µL L⁻¹. The 0 µL L⁻¹ concentration used as a control because MCF-7 cell line didn't give by Andaliman seed extract. The shape visualization of MCF-7 cell line that has been added Andaliman seed extract will observe by using a light microscope with magnification 100 times. An anticancer activity analysis is performed using a Microculture Tetrazolium Salt (MTT) Assay test followed by calculating the percent of the inhibitor using the following equation [8]:

\[
\text{Percent inhibition} = \left( \frac{\text{absorbance of the sample} - \text{absorbance of the blank}}{\text{absorbance of the control} - \text{absorbance of the blank}} \right) \times 100 \%
\] (1)
The probit analysis is done by taking into account the value in the table. The calculation is continued by calculating the IC\textsubscript{50} value obtained by determining the antilog of the equation.

3. Results and discussion

Based on maceration method using 96 % ethanol, lipid concentration that is contained in this extract is 0.21 % with pH is 7.24. Secondary metabolites is identified by some methods that commonly use. The list of secondary metabolites, that has been identified by the method above can be seen in table 1.

| Secondary metabolites | Test method | Result |
|-----------------------|-------------|--------|
| Phenolic              | Add FeCl\textsubscript{3} 5 % | +      |
| Saponins              | Heated      | +      |
| Flavonoids            | a. Add concentrated HCl + Mg | + |
|                      | b. Add H\textsubscript{2}SO\textsubscript{4} 2N | - |
|                      | c. Add NaOH 10 % | + |
| Tannins               | Add FeCl\textsubscript{3} 3 % | +      |
| Triterpenoids         | Add concentrated H\textsubscript{2}SO\textsubscript{4} + CH\textsubscript{3}COOH | + |
| Steroids              | Anhydrous   | -      |
| Alkaloids             | a. Dragendorff reagent | + |
|                      | b. Mayer reagent | + |

The result column in table 1 shows the result of qualitative that has done. The positive sign means there are secondary metabolites in the extract while the negative sign means there are no secondary metabolites in the extract. Identified secondary metabolites that are phenol, saponins, tannins, triterpenes, and alkaloids. Steroids are not present in the extract. The flavonoids in the extract are flavonoid, flavonols and flavanone which are characterized by positive results when added HCl and Mg while flavonoids from phenol groups characterized by positive results when added 10 % NaOH [6].

The effect of exposure to ethanol extract of Andaliman seeds on MCF-7 cell line was observed using a light microscope with 100 times magnification. The results of this observation can be seen in figure 1, figure 2, and figure 3. Figure 1 shows the effect of exposure to extracts with a concentration of 1 µL L\textsuperscript{-1}, 10 µL L\textsuperscript{-1}, 100 µL L\textsuperscript{-1}, and 1 000 µL L\textsuperscript{-1}. The MCF-7 cell line is still alive after added by those various concentrations. This is characterized by cells that look epithelioid and polygonal in shape and large cell sizes.

![Figure 1](image_url)

**Figure 1.** Exposure effect of ethanol extract (70 %) Z. Anthopodium seed to MCF-7 cell line (a: 1 µL L\textsuperscript{-1}; b: 10 µL L\textsuperscript{-1}; c: 100 µL L\textsuperscript{-1}; d: 1 000 µL L\textsuperscript{-1}).
Figure 2 shows the effect of exposure to extracts which are made from the maceration method by using 80% ethanol as the solvent. The extract is diluted to be some concentrations such as 1 µL L⁻¹, 10 µL L⁻¹, 100 µL L⁻¹, and 1 000 µL L⁻¹. The MCF-7 cell line is still alive after added by those various concentrations. The characteristic of the alive cell is epithelioid and polygonal with a large size. Cell visualization is looked as same as the control which MCF-7 cell line is not treated with Z. acanthopodium seed extract.

The observation continued with the determination of IC₅₀ values. The IC₅₀ values will show the most effective extract concentration to cause 50% of cell death or give a cytotoxic effect on cells. ELISA Reader with 550 nm wavelength is used to identify the absorbance value of every extract concentration. The result can be seen in table 2.

| µL L⁻¹ | Ethanol extract 70 % | Ethanol extract 80 % | Ethanol extract 96 % | Control |
|--------|----------------------|----------------------|----------------------|---------|
| 1000   | 1.256                | 1.350                | 0.126                | 1.487   |
| 100    | 1.946                | 1.670                | 1.287                |         |
| 10     | 1.534                | 1.352                | 1.385                |         |
| 1      | 1.279                | 1.458                | 1.589                |         |

The absorbance value in table 2 is obtained by calculating the average absorbance value of three repetitions at each ethanol extract concentration of Z. acanthopodium seeds. Then the absorbance value used to determine the amount of percent inhibition. Percentage of inhibition is determined using the equation (1).

The percentage inhibition value of each concentration and ethanol extract of Z. acanthopodium seeds can be seen in table 3. Positive values on percent inhibition indicate that the cell is inhibited. A positive percentage of inhibition can be used for further statistical analysis, namely the determination of the probit value. The IC₅₀ value is determined by the following linear equation [9]:

\[ y = ax - b \]
\[ x = \frac{y - b}{a} \]  
(2)

\[ a = \frac{Y2 - Y1}{X2 - X1} \]  
(3)

\[ b = Y1 - aX1 \text{ or } b = Y2 - aX2 \]  
(4)

With \( y \): probit inhibition 50%  
\( x \): extract concentration that has 50% inhibition (IC₅₀)  
\( a \): probit equation from two different concentration  
\( b \): probit value of percent inhibition  
Y1: probit value of percentage inhibition in concentration 1  
Y2: probit value of percentage inhibition in concentration 2  
X1: log of concentration 1  
X2: log of concentration 2

The IC₅₀ value which obtained is 221.31 µL L⁻¹. This IC₅₀ value is in the concentration range of 1 000 µL L⁻¹ and 100 µL L⁻¹ of 96% ethanol extracts because percent inhibitions at these concentrations are 98.13% and 14.44% respectively. Visualization of IC₅₀ values can be observed in figure 4. The IC₅₀ inhibition percentage is between the vertical axis of 40% and 60% which states the percent inhibition value and between the horizontal axis of 100 µL L⁻¹ and 1 000 µL L⁻¹ which states the extract concentration.
Figure 2. Exposure effect of ethanol extract (80 %) Z. acanthopodium seed to MCF-7 cell line (a: 1 µL L⁻¹; b: 10 µL L⁻¹; c: 100 µL L⁻¹; d: 1 000 µL L⁻¹).

Table 3. The value of IC₅₀.

| Extract          | µL L⁻¹ | Percent inhibition | Probit | IC₅₀       |
|------------------|--------|--------------------|--------|-----------|
| Ethanol extract   |        |                    |        |           |
| 70 %              | 1000   | 16.65              | 4.05   |           |
|                  | 10     | -33.09             | -      |           |
|                  | 1      | 15.04              | 3.96   |           |
| Ethanol extract   |        |                    |        |           |
| 80 %              | 1000   | 9.88               | 3.72   |           |
|                  | 10     | -13.19             | -      |           |
|                  | 1      | 2.11               | 2.95   |           |
| Ethanol extract   |        |                    |        |           |
| 96 %              | 1000   | 98.13              | 7.05   | 221.31 µL L⁻¹ |
|                  | 10     | 14.44              | 3.92   |           |
|                  | 1      | -7.35              | -      |           |

Based on research conducted, the type of extract greatly affects the result of anticancer trials. Condition and type of extract are determined based on the process performed, such as extraction method, solvent type, a difference of solvent concentration, length difference of wave absorbance, and sample condition. Extraction is a separation process of the mixture by using the appropriate type of solvent. The type of extraction can be heated extraction, such as reflux and soxhlet as well as cold extraction such as maceration and percolation. The maceration method is used in this research to avoid the occurrence of damage to secondary metabolite compounds to be extracted from Z. acanthopodium seeds [10].

A solvent used in dissolving a compound must have the same polarity property with the compound that will be extraction. This method is an application of like dissolved like principle [11]. A solvent can be classified into three types, namely polar, semi-polar and non-polar. Ethanol is used in this study because this solvent is a polar solvent that can dissolve flavonoid, tannin, terpenoid, and alkaloid [12].

The difference of solvent type and concentration are used as a modifications from [13] research to know about the effectiveness of the extract containing a secondary metabolite. Different ethanol concentrations that used are 70 %, 80 %, and 96 %. The difference concentration will affect the number of secondary metabolites contained in the extract [14]. From this research, it is discovered that the effective amount of secondary metabolites contained in ethanol with 96 % extraction has greater percentage of an obstacle compared to the other as well as its IC₅₀ value. The extract produced from this extraction is a crude extraction which consist of 0.21 % fat content. The condition of the extract is also influenced by the freshness of the sample used.
Figure 3. Exposure effect of ethanol extract (96 %) Z. acanthopodium seed to MCF-7 cell line (a: 0 µL L⁻¹ (control); b: 1 µL L⁻¹; c: 10 µL L⁻¹; d: 100 µL L⁻¹; e: 1 000 µL L⁻¹).

The IC₅₀ value from each plant species extracted will result in variations. Other factors that affect this difference are extraction methods, the type of extraction solvent used, and the tested cell [15]. The value of IC₅₀ obtained using MTT assay with the absorbance of 550 nm is 221.31 µL L⁻¹. This value indicates that Z. acanthopodium seeds ethanol extract has a potency as an anticancer because the IC₅₀ value is below 1 000 µL L⁻¹ concentration [16]. This potential is shown by the ability of the extract to inhibit the cell proliferation process of MCF-7 cell line which can be seen in the visualization of figure 3.

According to [17], the Z. acanthopodium extract with IC₅₀ value indicates that the extract can be quite active when applied as anticancer. This is because the value of IC₅₀ is in the range 100 µg mL⁻¹ to 500 µg mL⁻¹. Meanwhile, based on the classification of anticancer compounds by the National Cancer Institute (NCI), Z. acanthopodium seeds extract with IC₅₀ value do not have a strong or moderate effect as anticancer. This happens because the extract produced from the extraction process is a crude extract. The cytotoxic compound as anticancer will increase if the IC₅₀ value is smaller.

Figure 4. The graph of percent inhibition of ethanol extract (96 %) from Z. acanthopodium seed to against MCF-7 cell line.

4. Conclusion

Secondary metabolites that has been found in ethanol extract are phenols, saponins, flavonoids, tannins, triterpenoids, and alkaloids. Different types of solvents produce different contents of the
extract and different effects on MCF-7 cell lines proliferation. Ethanol extract of Andaliman seeds has the potential as an anticancer because it has an active effect to inhibit MCF-7 cell line proliferation and has strong effectiveness in pure extracts.

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