In Vitro Cytotoxicity of Hibiscus sabdariffa Linn Extracts on A549 Lung Cancer Cell Line

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ABSTRACT

Background: Lung cancer is the one of the leading causes of cancer death. However, current treatments for lung cancer are expensive and show negative side effects. Therefore, the study concerning natural anticancer from plants has intensified. Hibiscus sabdariffa Linn are Indonesian herb plants which have been consumed as a drink, are known to have anticancer activity against several cancer cell lines. However, its potential cytotoxic activity on A549 lung cancer cell line is still unclear. **Objective:** This study aimed to identify cytotoxic activity of Hibiscus sabdariffa Linn extracts on A549 lung cancer cell line. **Materials and Method:** Hibiscus sabdariffa Linn flowers from Tangerang, province of Banten, Indonesia, were macerated in three different solvents: ethyl acetate, ethanol, and n-hexane. Afterwards, cytotoxic activity of Hibiscus sabdariffa Linn extracts on A549 lung cancer cell line were evaluated using MTT assay. There were eight variety of concentration of the extracts, the experiment has been done triplicate for each concentration. The anticancer activity is expressed by IC50 value.

Results: Hibiscus sabdariffa Linn extracts in ethanol, ethyl acetate, and n-hexane showed IC50 value of 374.01 µg/mL, 719.28 µg/mL, and 906.57 µg/mL respectively, in which indicated weak cytotoxic activity on A549 lung cancer cell line. **Conclusion:** Ethanol, ethyl acetate, and n-hexane extracts of Hibiscus sabdariffa Linn are potential to be further developed as natural anticancer agents.

Key words: A549 Lung Cancer Cell Line, Cytotoxicity, Hibiscus sabdariffa Linn.

INTRODUCTION

Lung cancer is a disease when the cells in the body, specifically in the lung, begin to proliferate out of control.1 Lung cancer is the leading cause of cancer death globally. In Indonesia, based on WHO and GLOBOCAN data in 2018, lung cancer is a leading cause of cancer death, which has the highest mortality rate in male and the third highest mortality rate in female after breast and cervical cancers.2,3 Primary cancer of the lung divided into two main types: Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). Non-Small Cell Lung Cancer is the most common type of lung cancer, accounting for 80-85% of total lung cancers. There are three subtypes of NSCLC, which are adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. A549 lung cancer cell line which is used in this study is collected from adenocarcinoma lung cancer patients.4 Various factors can increase the risk of lung cancer, including smoking, industrial exposure, genetic polymorphism, overexpression of EGFR protein, and a family history of p53 mutation.5 EGFR protein is related to the activation of Ras and PI3K that leads to cell proliferation.6

The treatment options for lung cancer such as surgery, chemotherapy, and radiotherapy, may produce undesired adverse effects including nausea, vomiting, hair loss, bleeding, skin problems, blood clotting, and required high cost.6,7 Therefore, cancer patients may tend to choose alternative treatment that is considered cheaper and has no side effects, as stated in the study of Naja et al. Naja et al are also stated that the prevalence of alternative medicine users among lung cancer patients is 41%, with the most commonly used modality is dietary supplements, and herbal remedies.8,9 In this regard, the study about potential anticancer from the herbs is widely conducted by many researchers.

In Indonesia, one of the herb plants that are known to have medicinal properties and widely consumed as tea, syrup, pudding, and cake, is Hibiscus sabdariffa Linn.10 Study by Lin et al. reported that the phenolic compound of Hibiscus sabdariffa Linn could mediate activation of P38 MAPK/FASL cascade pathway and p53 signaling which can lead to apoptosis against human gastric carcinoma cells.11 However, the study about cytotoxic activity of Hibiscus sabdariffa Linn on A549 lung cancer cell line is still unclear.

These facts inspired us to identify cytotoxic activity of Hibiscus sabdariffa Linn extract on A549 lung cancer cell line. This study is conducted using the experimental study design. We explore the cytotoxic activity of Hibiscus sabdariffa Linn collected from Tangerang, province of Banten, Indonesia. The first step is collecting Hibiscus sabdariffa Linn sample, drying, and grinding them become dry powder. Then, the powder were extracted and macerated in ethanol, ethyl acetate, and n-hexane, respectively. Furthermore, the cytotoxic activity of Hibiscus...
The plates consisting of cancer cells, the extract, and the reagent of MTT were incubated for 4 hours. After that, it will be examined with the microscope, if the purple sediment of formazan has formed, in amount of 100 μl of DMSO is added to dissolve the purple sediment. The absorbance of the mixture is measured at 595 nm using an ELISA reader. Then, the calculation of percentage inhibition of cancer cells is carried out by the formula: 14

\[ \% \text{Inhibition} = \left( \frac{\text{absorbance of negative control} - \text{absorbance of treatment}}{\text{absorbance of negative control}} \right) \times 100\% \]

The parameter which represented the concentration of the extract which can inhibit 50% of the growth of cancer cells is IC_{50} value. The calculation of IC_{50} value is obtained using the linear equation between log concentration of the extract and percentage inhibition of A549 lung cancer cell line, or using this formula below, with a is the gradient of the line, and b is a constant. 14

\[ \text{IC}_{50} = 10^{(50-b)/a} \]

RESULTS

Cytotoxic activity of ethanol, ethyl acetate, and n-hexane extract hibiscus sabdariffa Linn

Figure 2 shows the relationship between the percentage inhibition of lung cancer cells with the concentration of ethanol extract Hibiscus sabdariffa Linn. As shown in Figure 2, none of the concentrations achieved a 50% inhibition of the cancer cell activity. The IC_{50} value of ethanol extract of Hibiscus sabdariffa Linn is calculated from the linear equation: y = 14.895x + 7.4465 and the formula IC_{50} = 10^{(50-b)/a}, with a = 7.4465 and b = 14.895 to give IC_{50} value of ethanol extract Hibiscus sabdariffa Linn is 374.01 μg/mL.

Figure 3 shows the relationship between the percentage inhibition of lung cancer cells with the concentration of ethyl acetate extract Hibiscus sabdariffa Linn. Based on the graph, none of the concentrations achieved a 50% inhibition of the cancer cell activity. IC_{50} value of ethyl acetate extract of Hibiscus sabdariffa Linn is calculated from the linear equation y = 18.896x + 1.3827, or with formula IC_{50} = 10^{(50-b)/a}, with a = 1.3827 and b = 18.896 to generate IC_{50} value of ethyl acetate extract of Hibiscus sabdariffa Linn is 719.28 μg/mL.

Figure 4 shows the relationship between the percentage inhibition of lung cancer cells with the concentration of n-hexane extract Hibiscus sabdariffa Linn. The IC_{50} value of n-hexane extract of Hibiscus sabdariffa Linn is 906.57 μg/mL, which is calculated from the linear equation y = 16.071x + 2.4714 and the formula IC_{50} = 10^{(50-b)/a}, with a = 2.4714 and b = 16.071.

IC_{50} value of ethanol, ethyl acetate, and n-hexane extracts of Hibiscus sabdariffa Linn are lower than the positive control, cisplatin (IC_{50} value of 3.60 μg/mL). Further, cytotoxic activity of ethanol, ethyl acetate, and n-hexane extract of Hibiscus sabdariffa Linn has the greatest cytotoxic activity on A549 lung cancer cell line among the others, as it has the lowest IC_{50}. Furthermore, cytotoxic activity of ethanol, ethyl acetate, and n-hexane extract of Hibiscus sabdariffa Linn are lower than the positive control, cisplatin (IC_{50} value of 3.60 μg/mL).

DISCUSSION

Extraction and Maceration of Hibiscus sabdariffa Linn Samples

The purpose of making three different extracts of Hibiscus sabdariffa Linn is to extract various secondary metabolites in different polarity because the secondary metabolites can be dissolved in the same polarity of the solvent. Therefore, different solvents with different polarity can affect the secondary metabolite containing in the extract. Based on a
**Ethanol Extract of **Hibiscus sabdariffa**

\[ y = 14.895x + 7.4465 \]
\[ R^2 = 0.916 \]

**Figure 2:** The relationship between ethanol extract of **Hibiscus sabdariffa** and percentage inhibition of A549 lung cancer cell lines.

**Ethyl Acetate Extract of **Hibiscus sabdariffa**

\[ y = 18.896x + 1.3827 \]
\[ R^2 = 0.966 \]

**Figure 3:** The relationship between ethyl acetate extract of **Hibiscus sabdariffa** and percentage inhibition on A549 lung cancer cell lines.

**n-Hexane Extract of **Hibiscus sabdariffa**

\[ y = 16.071x + 2.4714 \]
\[ R^2 = 0.9698 \]

**Figure 4:** The relationship between n-hexane extract of **Hibiscus sabdariffa** and percentage inhibition on A549 lung cancer cell lines.

**Table 1:** IC\textsubscript{50} values of Ethanol, Ethyl Acetate, and n-Hexane Extract of **Hibiscus sabdariffa** on A549 Lung Cancer Cell Line.

<table>
<thead>
<tr>
<th>Hibiscus sabdariffa Linn Extracts</th>
<th>IC\textsubscript{50} Values (µg / mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>374.01 ± 109.29</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>719.28 ± 55.19</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>906.57 ± 130.61</td>
</tr>
</tbody>
</table>

*IC\textsubscript{50} is the concentration of the extract which can inhibits 50% activity of cancer cells, expressed in mean value±SD. *SD: Standard deviation.
study by Widyawati et al., ethanol is a polar solvent that can effectively dissolve flavonoids, glycoside, alkaloid, saponin, terpenoid, sterol, phenol, tannin, and anthocyanin. Ethyl acetate is a semipolar solvent that can effectively dissolve flavonoid, alkaloid, and sterol. N-hexane is a nonpolar solvent that can effectively dissolve lignin, wax, sterol, terpenoid, and lipid. Alkaloid can induce apoptosis activity of the lung cancer cells. Condurangogenin A, a derivative compound of glycoside, has cytotoxic activity to the lung cancer cells by increasing activation of p21, p53, and caspase-3 pathway; decreasing activation of the cyclin-dependent kinase, cyclin D1, and protein Bax expression; and arrest cell cycle in G0/G1 phase. Steroid and triterpenoid in Spathodea campanulata have cytotoxic activity on lung cancer in the rat by inhibiting the malondialdehyde enzyme. Therefore, it can be determined at which polarity has the most active cytotoxic activity due to its secondary metabolite.

Cytotoxic Activity of Ethanol, Ethyl Acetate, and n-Hexane Extracts of Hibiscus sabdariffa Linn

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay method was used to analyze cytotoxic activity of ethanol, ethyl acetate, and n-hexane extracts of Hibiscus sabdariffa Linn. This method has been commonly used to identify cytotoxic activity of the extract on different concentrations. The basic principle of the method is the mitochondrial activity in every living cells, so the greater the activity, the greater the amount of the living cells. In mitochondria, there is a cellular reductase enzyme that can convert the tetrazolium compound in MTT reagent into the purple formazan crystal. The effect of cytotoxic activity of the extract is evident when the original yellow color of the tetrazolium compound is not changed to purple, indicates low mitochondrial activity. The lower the mitochondrial activity, the lower the amount of the living cells. The absorption of the sample is read using the ELISA reader in 595 nm. The MTT assay uses IC50 value as a parameter for the interpretation of cytotoxic activity. IC50 is the amount of extract concentration that can inhibit 50% activity of the cancer cells. The relationship between cytotoxic activity and the IC50 value is inversely proportional, therefore the lower the IC50 value indicates the higher cytotoxic activity.

Figures 2-4 show the relationship between the percentage inhibition of lung cancer cells with the concentration of ethanol, ethyl acetate, and n-hexane extract Hibiscus sabdariffa Linn. The results indicate that the log concentration of the extracts is directly proportional to the percentage inhibition of the cancer cell activity. Therefore, we can conclude that the ability of the extract to inhibit the growth of lung cancer cell is concentration-dependent.

Based on the study from Atjanasuppat et al, the anticancer activity can be classified into four groups according to the IC50 value: IC50<20 μg/mL is classified active; IC50: 20–100 μg/mL is classified moderately active; IC50: 100–1000 μg/mL is classified weakly active; and IC50 >1000 μg/mL is classified inactive. As shown in Table 1, IC50 value of ethanol, ethyl acetate, and n-hexane extracts are included in 100-1000 μg/mL range, which means that all of Hibiscus sabdariffa Linn extracts are weakly active in inhibiting A549 lung cancer cells activity. Meanwhile, the positive control, cisplatin, is very active in inhibiting A549 lung cancer cell activity. The most active Hibiscus sabdariffa Linn extract is ethanol extract, as it has the lowest IC50 among the three extracts. Therefore, Hibiscus sabdariffa Linn extracts is potential to be further developed as a natural anticancer agent.

According to previous research, the study about cytotoxic activity of Hibiscus sabdariffa Linn extract on lung cancer is still limited. But, the studies on anticancer activity of Hibiscus sabdariffa Linn against other cancer cells has conducted by many researchers. A study by Amran et al. reported that anticancer activity of methanol extract of Hibiscus sabdariffa Linn on breast cancer cell MCF-7 is weakly active, with IC50 value of 112.1 μg/mL. Furthermore, a study by Chin et al. which evaluated anticancer activity of Hibiscus sabdariffa Linn on prostate cancer cells using western blot discovered that Hibiscus sabdariffa Linn extract inhibits P38 expression which involved in migration pathway and metastasis of prostate cancer cells. P38 is known to be involved in the pathogenesis of lung cancer. Another study by Cheng et al. reported that the acetone extract of another species of Hibiscus, Hibiscus syriacus, has an active anticancer activity to A549 lung cancer cells with IC50 value of 9.4 μg/mL, by modulation of p53 pathway and apoptosis induced factor.

CONCLUSION

The ethanol, ethyl acetate, and n-hexane Hibiscus sabdariffa Linn extracts showed anticancer activity which can be further developed as a source of natural anti-lung cancer from plants. Further studies are needed to evaluate the specific anticancer mechanism of Hibiscus sabdariffa Linn extracts on A549 lung cancer cell line.

CONFLICTS OF INTEREST

The authors declare no conflict of interest in this study.

ACKNOWLEDGMENT

We express our gratitude to Faculty of Medicine and Directorate of Research and Public Service, University of Indonesia for the research grant of PITTA B (Publikasi Internasional Terindeks Scopus Untuk Tugas Akhir Mahasiswa B) for the 2019 fiscal year.

ABBREVIATIONS

g: gram; mL: milliliter; nm: nanometer; CO2: Carbon dioxide; FKUI: Fakultas Kedokteran Universitas Indonesia; IC50: Inhibition Concentration 50%; MTT:3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); NSCLC: Non-Small Cell Lung Cancer; PBS: Phosphate-Buffered Saline; P38K: Phosphoinositide 3-kinase; %: Percentage; μg: microgram; μl: microliter; μg/mL: microgram/milliliter.

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Fithrotunnisa, et al.: In Vitro Cytotoxicity of Hibiscus sabdariffa Linn Extracts on A549 Lung Cancer Cell Line

Pharmacognosy Journal, Vol 12, Issue 1, Jan-Feb, 2020


GRAPHICAL ABSTRACT

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SUMMARY

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