Comparison of flavonoid from clove leaf oil cytotoxic activities with doxorubicin and cisplatin on liver cancer cell culture

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Abstract. In the development of new drugs as candidates for cancer therapy agents, cytotoxic testing is needed as an initial screening to determine the effect of a natural substance in inhibiting tumour cell growth. One of the active compounds of flavonoid derivatives that are being studied as anticancer is a compound derived from clove leaf oil. The purpose of this study was to determine the cytotoxic activity of flavonoid compounds from clove leaf oil on liver cancer cell culture and make comparisons with standard drugs for cancer therapy. Examination of cytotoxic activity was carried out on HepG2 cell line culture used MTT method. The absorbance of each well was measured with spectrophotometer at a wavelength of 595 nm. The absorbance results were calculated to create a cytotoxic curve. Based on the cytotoxic curve, IC\textsubscript{50} values of flavonoid compounds, doxorubicin and cisplatin were 50.62\mu g/mL, 20.25\mu g/mL, 15.42\mu g/mL respectively. Those showed that flavonoid compounds from clove leaf oil have strong anti-cancer activity (IC\textsubscript{50}<100\mu g/mL) against liver cancer cells. It was concluded that flavonoid compounds isolated from clove leaf oil were shown to have anticancer activity on liver cancer cell and thus could be used as a new candidate for liver cancer therapy agents.

1. Introduction
Natural ingredients are a source of potential therapeutic agents that have many advantages such as easy to obtain, inexpensive and have minimal side effects with low toxicity [1-3]. Indonesia is known to have many natural ingredients that are trusted by the community to prevent and even treat cancer, one of which is clove plants. Potential active compounds that are often isolated from medicinal plants are flavonoids and isoflavones are derivate from flavonoids [4-7].

In vitro studies show the anticancer activity of ginestein as derivate substance from isoflavon is caused by antioxidant effects and antiproliferative effects through modulation of the expression of genes involved in the cell cycle and apoptosis [8]. Isolation of genistein from soybeans requires large amounts of soybeans and expensive costs so it is not economical, therefore some researchers try to synthesize genistein and its derivatives to get more economical compounds in large quantities. One of the active
compounds of flavonoid derivatives that are being studied as anticancer is a compound derived from clove leaf oil (eugenol) which is widely available in Indonesia [9,10].

Research to find effective treatments for liver cancer is still being developed. Curative approaches such as surgery and transplantation can only be done on a limited basis due to various causes such as lack of donors and considering its effects on liver function. Therapy recommendations are based on the Barcelona Clinic Liver Cancer (BCLC) classification for advanced liver cancer with a palliative approach in the form of systemic chemotherapy. Curative approaches such as resection and transplantation can only be limited to early stage patients. Chemotherapy is still the best choice for patients with advanced stages, however, until now the effectiveness of chemotherapy in cancer patients also remains in debate, and is often considered relatively not effective. This therapeutic option for patients with liver cancer is still very limited which is also one of the causes of prognosis for liver cancer remain poor [7,11-17].

There have been many testimonies of the successful use of flavonoids as empiric anticancer, so that the biochemical potential of flavonoid compounds from clove leaf oil as anticancer, especially for liver cancer systemic chemotherapy is very interesting to be studied as scientific evidence. The ideal model in the search for potential anticancer agents is bioactive compounds that have the potential to kill cancer cells, through the initial stages of isolation of pure compounds, stages of biological screening and pharmacological tests, as well as toxicology and safety testing [18,19].

Cytotoxic test is used as an initial screening to determine the effect of a natural substance in inhibiting tumor cell growth. A compound is considered active if it can inhibit the growth of 50% of the population of tumor cells at a certain concentration. In the development of new anticancer drugs as cancer chemotherapy agents, preclinical evaluation is one of the important things to know the potential of neoplastic activity. This evaluation is not only used for anticancer drugs, but also for other medicines, cosmetics, food additives, pesticides and others. Standardized evaluation to determine whether a material contains biologically hazardous (toxic) material is called a cytotoxicity test [18,20,21]

The conditions that must be done for the cytotoxicity test include the testing system must be able to produce a reproducible dose-response curve with low variability, the response criteria must show a linear relationship with the number of cells and the information obtained from the dose-response curve must be in line with the effects that appear in in vivo. One method commonly used to determine cell viability is the MTT method [18,20,22].

This study aims to examine the cytotoxic activity of flavonoid compounds from clove leaf oil as anticancer against liver cancer cells using HepG2 cell line culture, and compare them with standard drugs for chemotherapy.

2. Methods
The test material used in this study is flavonoid compounds isolated from clove leaf oil compounded (1,2-epoxy-3 (3,4-dimethoxyphenyl) -4H-1-benzopiran-4on) which is namely “Epoxy”, while the standard cancer drugs used were doxorubicin and cisplatin for comparison. The cells used were Hepatocellular carcinoma cells from ATCC, HepG2 cell line. The chemicals used consisted of materials for MTT examination, Phosphate Buffer Saline 1x, Media Culture (DMEM), DMSO, MTT 5 mg / mL PBS (50 mg MTT and 10 mL PBS), 10% SDS in 0.1 N HCl and Aluminum foil.

The equipment used in this study includes glass equipment commonly used in the Organic Chemistry Laboratory. Equipment for MTT examination include 200 micropipets, 1000 μL, small reaction tubes, small tube racks, 96-well plates, Conical tubes, Yellow tips and blue tips and ELISA readers.

2.1. Liver cancer cell culture HepG2 cell line
HepG2 cell line liver cancer cells were cultured in Medium (DMEM / F12) containing 10% Fetal Bovine Albumin. Previously, cell trypination was carried out using 0.05% - EDTA 0.53mM Trypsin, then the growth medium was added to become cell suspense. Then the cell is calculated using a hemocytometer and then planted with a cell density of 25,000 cells / mL. Then the cell was incubated and the growth medium was replaced every 2 days when the medium color showed changes in pH. After repetition at
certain hours, a curve between cell concentration and cell growth hours can be made, so that the growth curve of HepG2 cell line can be determined.

2.2. Cytotoxic activity test on HepG2 cell line MTT method (3-4-5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide)

The principle of the MTT method is the reduction of MTT tetrazolium yellow salt (3- (4,5-dimethylthiazol-2-il) -2,5-diphenyl tetrazolium bromide) by the reductase system. Tetrazolium succinate which is included in the respiratory chain in mitochondria, living cells form purple formazan crystals and are not water-soluble. The addition of a stopper reagent (detergentic) will dissolve this colored crystal which is then measured by absorbing the ELISA reader. The intensity of the purple color formed is proportional to the number of living cells. So if the intensity of purple gets bigger, then the number of living cells is increasing.

Cytotoxic tests in this study were carried out using the MTT method (3-4-5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide). A series of Epoxy compound concentrations were made using 4 growth medium solvents. Three types of control were made, namely: tumor cell control (100 µL HepG2 + 100 µL media cells), media control (200 µL media), and sample control (100 µL soursop leaf extract + 100 µL media). A total of 100 µL Epoxy compounds from each concentration were included in the microplate wells which contained 2x10^4 HepG2 cancer cells (100 µL). The microplate was incubated for 24 hours in a CO2 incubator. Add 10 µL of MTT to each microplate well and incubate again for 4 hours in a CO2 incubator. The MTT reaction was stopped by adding 10% sodium dodecyl sulfate (SDS), then the microplate was again incubated for 12 hours in a dark room at room temperature. After incubation, each well was measured with a microplate reader spectrophotometer at a wavelength of 595 nm. IC_{50} calculations was used Probit analysis with SPSS software.

3. Result and discussion

Based on the cytotoxic curve in HepG2 cell culture, the IC_{50} value of flavonoid compounds of clove leaf oil was 50.62µg/mL. This showed that the flavonoid compound has strong anticancer activity against the liver cancer.

To evaluate the strength of the cytotoxic activity, the comparison was made with the IC_{50} standard drug doxorubicin and cisplatin. Based on the cytotoxic curve in HepG2 cell culture, IC_{50} doxorubicin values obtained were 20.25µg/mL, cisplatin values were obtained at 15.42µg/mL.

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Cytotoxic test is used as an initial screening to determine the effect of a natural substance in inhibiting tumor cell growth. A compound is considered active if it can inhibit the growth of 50% of the population of tumor cells at a certain concentration. The conditions that must be found in the cytotoxicity test system include the testing system must be able to produce a reproducible dose-response curve with low variability, the response criteria must show a linear relationship with the number of cells and the information obtained from the dose-response curve must be in line with the appear. One common method used to assess the cytotoxic activity is the MTT method. The compound is considered to have the anticancer activity if it can inhibit of the growth of 50% tumor cells population at concentrations below 200µg/mL (IC_{50}: 200µg/mL) [18-21].

In this study the results of cytotoxic tests on flavonoid compounds of clove leaf oil (Epoxy) on HepG2 cell line liver cancer cells culture by MTT method produced IC_{50} values of 50.62µg/mL. With the IC_{50} value, it can be concluded that the flavonoid compound from clove leaf oil was a strong compound with a strong copper potential, so that it can be used in tests subsequently in a clinical evaluation to examine anticancer potential and analyze its mechanism of action by using the IC_{50}
reference value. This IC\textsubscript{50} value is stronger than the IC\textsubscript{50} value of compounds isolated from green tea by 57.53102μg/mL and other studies with IC\textsubscript{50} values of 65.7μg/mL in human breast cancer cells HS579T.

Comparison of IC\textsubscript{50} flavonoid compounds (Epoxy) with doxorubicin and cisplatin were made of the IC\textsubscript{50} value of the oil flavonoid compound, it was dislodged by 50.62μg/mL compared to the doxorubicin IC\textsubscript{50} value of 24.25μg/mL and IC\textsubscript{50} cisplatin of 15.42μg/mL. Graph of IC\textsubscript{50} comparison of flavonoid compounds of clove leaf oil, doxorubicin and cisplatin can be seen in Figure 1.

![Figure 1. IC\textsubscript{50} of epoxy compounds, doxorubicin and cisplatin.](image)

The results of this comparison do not affect Epoxy compounds as potent anti-cancer candidates, especially in terms of their selectivity because doxorubicin and cisplatin have a very low IC\textsubscript{50} to normal cells so that the administration of both drugs causes side effects such as normal cell damage.

4. Conclusion
Flavonoid compounds from clove leaf oil (Epoxy) are a potent natural product that have strong cytotoxic activity against liver cancer cells, thus can be used as candidates for new liver cancer chemotherapy agents.

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