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Cytotoxicity of Ethanol Extract, Polar, Semipolar, and Nonpolar Herb Citolod (Isotoma longiflora (L.) C. Presl.) Cells on MCF-7 Cells

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Abstract

Breast cancer is a major cancer suffered and the most cause of death by women in the world. The existing drugs of anticancer are not selective, disturbing the growth of normal cells. It encourages researchers to explore a chemopreventive agent from the nature materials. This study was conducted to determine the cytotoxic activity of ethanol extract, polar fractions, semipolar, and nonpolar herb chitolod (*Isotoma longiflora* (L) Presl.) on MCF-7 cells. First, herb chitolod was dried, refined, and sieved using mesh number 40 and then macerated using 96% ethanol for 72 hours. Phytochemical screening was conducted with TLC using GF_{254} silica as stationary phase, n-hexane: ethyl acetate (7: 3) as mobile phase, then the result was visualized on UV 254 nm, 366 nm, and visible light. Anisaldehid, dragendroff, sitoborat, and $FeCl_3$ reagents spray were used as visualizer of TLC spot results. Fractination using KCV method, then cytotoxic test using MTT assay. A result obtained, the ethanol extract of herb chitolod positively contains flavonoids, phenolics, alkaloids, terpenoids, saponins, steroids and tannins. IC_{50} value of the ethanol extract, polar fractions, semipolar, and nonpolar are 521,05; 213,29; 499,94; and 239,43 µg/mL. This study showed that the ethanol extract, polar fraction, semipolar, and nonpolar of herb chitolod had moderate cytotoxic activity on MCF-7 cells.

Keywords: Cytotoxic, chitolod, fractions, MTT assay, MCF-7 cells.

INTRODUCTION

Cancer is a group of diseases that cause cells in the body to change and grow out of control (American Cancer Society, 2016). According to the World Health Organization (WHO) 2008, cancer is the fifth largest cause of death in the world, especially in developing countries.

In 2012 the number of deaths caused by cancer reached 8.2 million people. (Indonesian Ministry of Health, 2015). Cancer that generally occurs in women other than skin cancer is breast cancer, the number of sufferers in the United States reaches more than 3.1 million people (American Cancer Society, 2015), while in the world there are around 522,000 women die of breast cancer they suffer with a mortality rate that is vary across the world in 2012 (Anderson, 2014).

Cancer treatment in general can be through surgery (surgery), giving cytotoxic chemicals (chemotherapy), and through radiation (radiotherapy). Treatment by surgery can only be done for cancers that have not yet spread to the blood vessels or lymph nodes. Cancer therapy using cytotoxic agents (chemotherapy) and radiation (radiation) also causes some side effects such as nausea, vomiting, hair loss, and is not selective, so that it can interfere with normal cell growth (Kurnijasanti et al., 2008). Side effects and shortcomings of existing cancer drugs are encouraging researchers to look for cancer drugs that are safer and selective derived from natural ingredients, such as chitolod. Chitolod (*Isotoma longiflora* (L.) C. Presl.) Is a plant that is empirically used as medicine by the people. This plant has efficacy as a drug to treat eye disorders such as cataracts (Amaliah, 2014), minus the eye and treat blindness caused by glaucoma (Wardani & Siska, 2010), asthma,

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syphilis (Koller, 2009), antivirals (Rothan et al. , 2014), and antibacterial (Siregar, 2015). It also has antimicrobial activity in the bacteria *Stapylococcus hominis* (Ismailova, 2008) and *Staphylococcus aureus* (Safitri et al., 2009). Chitolod leaf contains alkaloid compounds, saponins, flavonoids, and polyphenols (Hariana, 2008). Based on the phytochemical test results of ethanol extract of leaves and positive chitolod flowers containing alkaloids, saponins, flavonoids, and tannins (Siregar, 2015). Flavonoids are natural compounds which are known to have anticancer properties. Flavonoid derivatives show antitumor activity and are also candidates for multidrug resistance-reversing agents in cancer chemotherapy. Flavonoids work significantly by the mechanism of inhibiting p-glycoprotein in cancer chemotherapy, increasing the efficacy of anticancer drugs and fighting the action of MDR or multi-drug resistance (Bansal et al., 2009). Previous studies have also shown that the ethyl acetate fraction of chitolod leaves has a moderate ability to inhibit WiDr cancer cells with an IC_{50} value of 191.74 µg / mL (Magfiroh, 2015) and in the ethanol extract of the chitolod herbs it can inhibit the growth of HELa cells with an IC_{50} value of 227 µg / mL (Hapsari et al., 2016).

METHOD

Sample Preparation. A total of 339.08 grams of chitolod herbs which had been pollinated and sifted with mesh number 40 were put into the maceration vessel. Samples were macerated with solvent 2.5 L 96% ethanol, allowed to stand for 72 hours at room temperature, while occasionally stirring, then filtered (maceration was carried out 3 times). The maserate obtained was evaporated with a rotary evaporator until a thick blackish-brown mass was formed. Then proceed with the compound test.

Compound Content Test. The compound content test is carried out using TLC of the spray reagent and the test tube. TLC test using the mobile phase n-hexane: ethyl acetate in a ratio of 7: 3. The compound that has been eluted is then sprayed with anisaldehyde- H_2SO_4 reagent, dragendorff, sitoborate, and $FeCl_3$ to show the spots and as an affirmation of what compounds are contained in the chitolod herbl extract. Anisaldehyde-H_2SO_4 spray reagents are used to detect the presence of terpenoid compounds and saponins, dragendroff for alkaloids, cytoborates for flavonoids, and $FeCl_3$ for phenolic compounds. Next, the thick ethanol extract was fractionated by the KCV method.

Fractionation. Fractionation is done using the vacuum liquid chromatography (KCV) method. The stationary phases used are silica G_{60} and silica GF_{254} as well as N-hexane: ethyl acetate (9: 1; 8: 2; 7: 3; 6: 4; and 5: 5) and ethanol used as the mobile phase with volume respectively as much as 150 mL (Haryoto et al., 2015). The thick extract was weighed as much as 20 g which had been dissolved with methanol and mixed with silica G60 (30-70 mesh). The sample is placed at the top of the KCV column and eluted with a mobile phase, then fractionated. The results of the fractions are collected and eluted using TLC and separated based on the nature of polarity (polar, semipolar, and nonpolar) with a mobile phase of n-hexane: ethyl acetate 7: 3 ratio. Polar, semipolar, and non-polar fractions were thickened with a rotary evaporator, then a cytotoxic test was performed on MCF-7 cells.

Cytotoxic Activity Test. Ethanol extras, 10 mg polar, semipolar and nonpolar fractions were weighed and dissolved in 100 µL of DMSO solvent, then added DMEM culture media ad 10 mL. Substrate solution is made with a dilution process, so that a concentration of 31.25 is obtained; 62.5; 125; 250; and 500 µg / mL. Samples were distributed into each well as much as 100 µL, sequentially from low to high concentrations, then incubated in a CO2 incubator for 48 hours. At the end of the incubation, the culture media containing the sample was removed, then 100 µL of MTT solution was added to each well and incubated for 2 hours in a CO2 incubator at 37°C.

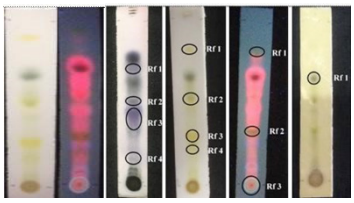
Living cells will react with MTT to form purple crystals. All processes must be carried out under sterile conditions and in Laminar Air Flow.

RESULTS AND DISCUSSION

The process of extracting compounds using 96% ethanol, because it is dissolve polar compounds, such as flavonoids (Nirwana et al., 2015). Chitolod herbl pollination needs to be done before starting the maceration process. The purpose of the pollination process is so that the material submerged in the solvent has a wide surface, so that the solvent is able to reach the compounds contained in the cell or inter-cell space more optimally (Saifudin, 2014). Pollination of chitolod herbs that have been dried in the sun for 3-5 days is done using a blender. Pollination results should not be too fine, so as not to cause the solution to become turbid and not to form a dispersion in the process of dissolution and diffusion (Saifudin, 2014)

In this research, chitolod herbl powder was screened using mesh number 40. The purpose of sifting was to obtain a homogeneous powder. Powder with mesh number 40 has a broad surface and more for contact with solvents, so that the solvents of compounds by solvents are also more numerous. In addition, powder with mesh 40 can produce more yields when compared to mesh number 20 (the powder is too coarse) and whole powder (Maulida and Guntarti, 2015). The process of thickening the extract is attempted by using the lowest possible temperature, recommended at temperatures less than 60oC. Too high a temperature can cause degradation of compounds contained in the extract (Saifudin, 2014). The yield of chitolod herbl ethanol extract obtained from the maceration that has been done is 10.86%.

Compound Content Test. TLC aims to find out the compounds contained in chitolod herbl extracts based on the nature of their personality (Saifudin, 2014). In experiments with various n-hexane: ethyl acetate mobile phase comparisons (9: 1, 8: 2, 7: 3, 6: 4, and 5: 5), the best separation results and the most spotting (the dotted mark on the image) that is in a ratio of 7: 3. Compound content testing was carried out by TLC spray reagents and test tubes. Spray reagents used are anisaldehyde-H2SO4, dragendoff, sitoborate, and FeCl3. Anisaldehyde H_2SO_4 spray reagent to detect the presence of terpenoids and saponins, dragendoff to detect the presence of alkaloids, cytoborate to detect the presence of flavonoids, and to detect $FeCl_3$ in the presence of phenolic.



(a) (b) (c) (d) (e) (f)

Figure 1. Chromatograms of the test results of the chitolod herbl extract compound with spray reagent TLC. Before being given spray reagent on visible light (a), before being given spray reagent at UV 366 nm (b), after being given anisaldehyde H_2SO_4 spray reagent on visible light (c), after being given dragendroff spray reagent on visible light (d), after being given reagents cytoborate spray at UV 366 nm (e), after being given $FeCl_3$ spray reagents in visible light (f).

Figure 1 above is the result of the detection of chitolod herbl ethanol extract compounds using the spray reagent TLC method. On the addition of anisaldehyde- H_2SO_4 spray reagent a visual

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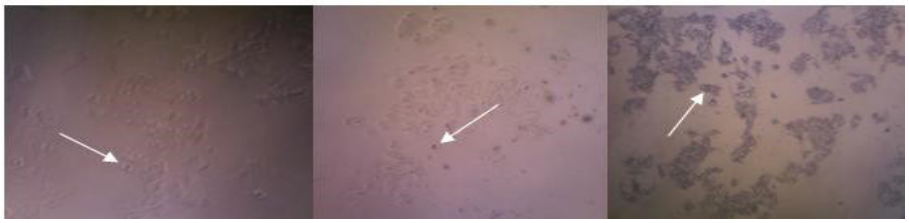
violet blue color appeared on the visible light which showed that positively contained terpenoids and saponins, while on the addition of the dragendroff spray reagent a brownish orange color appeared on the visible light indicating the presence of alkaloids. On the addition of cytoborate spray reagent a greenish-yellow color appears on UV light 366 nm which indicates the presence of flavonoid compounds, while the addition of the $FeCl_3$ spray reagent appears blue gray in visible light indicating the presence of phenolic compounds (Saifudin, 2014).

Table 1. Test results of compound content from ethanol extract of chitolod herbs

Spray reagents	Rf	Spotting color	Compound detection
Anisaldehyde-H_2SO_4 (in visible light)	0.74 0.54 0.44 0.22	Violet Blue	Terpenoids and Saponins
Dragendroff (in visible light)	0.96 0.56 0.32 0.22	Brownish orange	Alkaloids
Cytoborate (in 366 nm UV light)	0.78 0.34 0.12	Greenish yellow	Flavonoids
$FeCl_3$ (in visible light)	0.64	Blue gray	Phenolic

The results of the compound test using the TLC method with spray reagents, showed that 96% ethanol extract of positive chitolod herbs contained terpenoids, saponins, alkaloids, flavonoids, and phenolics, as shown in Table 1. Fractionation is done by the VLC (Vacuum Liquid Chromatography) method. Fractionation is a separation effort carried out after getting an active fraction or active extract. The purpose of fractionation is to separate compounds derived from extracts based on their polarity. Fulfillment of adsorbents is an important process before fractionation. The purpose of fulfillment is to increase the effectiveness of separation (Saifudin, 2014). Elution of the mobile phase using a gradient system that starts from the nonpolar solvent first (n-hexane: ethyl acetate 9: 1) then gradually leads to a combination of polar solvents (n-hexane: ethyl acetate 5: 5) (Saifudin, 2014).

Cytotoxic Activity Test. Cytotoxic tests were performed with MTT assay. DMSO is used as a solvent because it is a solvent capable of dissolving polar and nonpolar compounds (BPOM RI, 2010). In this cytotoxic test, the DMSO level contained in the sample is 1%. DMSO with levels less than 3%, are not toxic or kill on cancer cells (Purwaningsih et al., 2014), so they can be used as solvent control. The addition of MTT reagents serves to determine cancer cells that are still alive, through the reaction of reductase enzymes that form purple formazene crystals.



(a) (b) (c)

Figure 2. Control of MCF-7 cells (a), MCF-7 cells die in polar fractions with a concentration of 500 µg / mL (b), and formazent crystals (c)

Figure 2 shows the morphology of live MCF-7 cells (before treatment), after treatment, and formazent crystals formed during cytotoxic testing. In the morphology of MCF-7 living cells are irregularly shaped around a membrane with a clear color. Meanwhile, after being treated morphologically from cancer cells change, the cell nucleus becomes black and the cell shape shrinks. This indicates that the cancer cells have died due to treatment. Formazent crystal formation comes from the enzymatic reaction between MTT reagents and enzymes found in living cells. This reaction changes the color of the living cells to purple, so that the results of the reading by ELISA reader, if more and more formazen crystals are formed, the number of cancer cells that are still alive is also increasing.

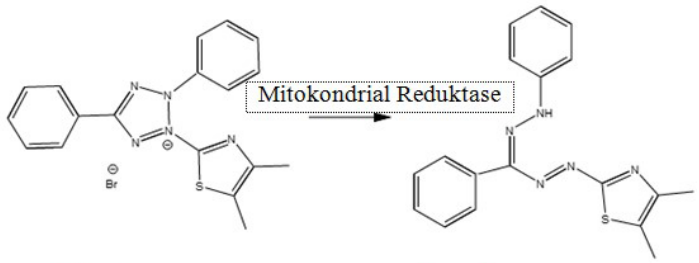


Figure 6. Formazen (purple) crystal formation reaction of MTT reagent (3- (4,5-dimethyltiazol-2il) -2,5-diphenyltetrazolium bromide)

Table 2. Cytotoxic of ethanol extract, polar, semipolar, and nonpolar fractions of the chitolod herbs

Samples	IC_{50} Value	Evidence
Ethanol extract	521,054 µg/mL	moderate cytotoxic activity
Polar fraction	213,294 µg/mL	
Semipolar fraction	499,947 µg/mL	
Nonpolar fraction	239,431 µg/mL	

A compound is categorized as a potent, moderate and non-potent cytotoxic have an IC_{50} value <100 ;100-1000 µg / mL, and > 1000 µg / mL (Prayong et al. ., 2008). The IC_{50} value in ethanol extract was greater than the polar and nonpolar fractions, so that the cytotoxic activity was also weaker than the polar and nonpolar fractions. This is estimated because in ethanol extract, the

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compound content is still complex and diverse, both polar, semipolar and polar compounds, so that their cytotoxic effects will affect each other on cancer cells (Ismiyati et al., 2015; Djajanegara and Wahyudi, 2009).

CONCLUSION

Ethanol extract, polar, semipolar, and nonpolar herbls of the chitolod herb (*Isotoma longiflora* (L.) C. Presl.) have cytotoxic activity with moderate ability on MCF-7 cells with IC_{50} values respectively 521,05; 213,29; 499,94; and 239,43 µg / mL. The compounds contained in the ethanol extract of the chitolod herb (*Isotoma longiflora* (L.) C. Presl.) namely flavonoids, phenolics, alkaloids, terpenoids, saponins, steroids, and tannins.

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