

Apoptosis Induction Activity and Inhibition of MCF-7 Cell Proliferation in Active Isolate of Butterfly Pea Flower (*Clitoria ternatea* L.)

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Abstract

Clitoria ternatea L. is one of the plants whose all parts have functional benefits for the human body. The flower petals are reported to be useful as anticancer. This study aims to determine the activity of inhibiting cell proliferation, and apoptotic induction activity and identify the chemical content of isolates from *Clitoria ternatea* L. Analysis of the results of the MTT assay test ethanol extract of butterfly pea flower has an IC₅₀ value of 17.46 g/mL, CTEE has an IC₅₀ value of 13, 19 g/mL, isolate 1 and isolate 2 of EAFCT had IC₅₀ values of 6.79 and 7.12 g/mL. The results obtained were categorized as potent cytotoxic. Isolation of EAFCT compounds using radial system chromatography then analyzed GC-MS EAFCT isolates containing pyridine hydrochloride (51.42 %) and pentanal (2.79 %) and identified compounds using UV-Vis spectrophotometry and FTIR. The results of the cell proliferation inhibition test used a series of concentrations of IC₅₀, ½ IC₅₀, ¼ IC₅₀ and ⅛ IC₅₀, the CTEE obtained a doubling time of 178.47; 160.86; 151.18; 150.09, EAFCT is 188.53; 164.37; 156.82; 154.79, isolate 1 EAFCT is 212.96; 174.64; 148.53; 145.82 and isolates 2 EAFCT were 190.62; 168.23; 150.24; 148.94. Analysis of the apoptotic potential test results can be concluded from CTEE, EAFCT, isolates 1 and 2 EAFCT have apoptotic potential.

Keywords: Active isolate, *Clitoria ternatea* L., Cytotoxic, Proliferation, Apoptosis

Introduction

Breast cancer is one of the main health problems, especially for women in the world. In 2011 it was reported that more than 508,000 women died of breast cancer [1]. According to GLOBOCAN data (IARC) in 2012 obtained from the Ministry of Health in 2015 it is known that breast cancer is the cancer with the highest percentage of new cases (after controlling for age), which is 43.3 %, and the percentage of deaths (after controlling for age) due to breast cancer by 12.9 % [2].

Therapeutic treatment of cancer now of them is using chemotherapy agents. A chemotherapy agent that is often used in breast cancer therapy is cisplatin. Cisplatin causes side effects including neurotoxicity, nephrotoxicity, and bone marrow suppression. In addition, it was also reported that the use of cisplatin caused resistance. The mechanism of cisplatin resistance occurs through changes in cellular uptake, drug efflux, inhibition of apoptosis, and increased DNA repair. The resistance of cancer cells and the side effects of cisplatin are caused by its use at high doses to produce a more effective treatment [3]. Due to the rapid resistance of cancer cells to existing drugs, the search for new chemotherapeutic agents continues to this day. In addition, the toxicity of the chemotherapy drugs used is a problem that is considered so as not to give unwanted side effects [4].

Several studies have begun to be directed at testing the potential of natural ingredients as chemoprevention agents that have the potential as chemotherapy companion agents. The goal is to increase the sensitivity of cancer cells and reduce the side effects caused by chemotherapy agents. The chemopreventive agent is an agent that can inhibit the development of cancer cells, suppress the growth of abnormal cells into cancer, and reverse the stages of the carcinogenesis process [5]. The chemoprevention agents referred to here generally have the activity of inhibiting tumor growth through cell cycle arrest mechanisms, promoting apoptosis, or inhibiting the expression of proteins that play a role in Multi-Drug Resistance [6]. One approach to finding chemopreventive compounds is through the exploration of natural materials, especially plants [7].

Indonesia is one of the countries with a wealth of flora in the world and has various kinds of plants that have the potential for cancer treatment, one of which is the butterfly pea flower plant. The anti-cancer effect of the extract of butterfly pea flower (*Clitoria ternatea*) has a significant effect on the breast cancer line hormone (MCF-7) [8]. The bioactive components in butterfly pea flower that are thought to have functional benefits are 3 flavonol glycosides, kaempferol 3-O-(200-O-a-rhamnosyl-600-O-malonyl)-b-glucoside, quercetin 3-O-(200-O-a-rhamnosyl- 600-O-malonyl)-b-glucoside, and myricetin 3-O-(200600-di-O-a-rhamnosyl)-b-glucoside along with eleven flavonol glycosides [4,9].

There are at least 4 mechanisms of a component of an active substance against cancer: Antiproliferative activity (prevent or slow the spread of cancer cells, inhibition of angiogenesis (formation of new blood vessels), induction of apoptosis (cancer cells commit suicide), and prevention of metastasis [10]. Researchers previously reported that the cytotoxic activity of the methanolic extract of CT flower was investigated against the MCF-7 breast cancer cell line using the MTT Assay. The cytotoxicity test results of *Clitoria ternatea* showed high cytotoxic activity against MCF-7 values ($IC_{50} = 1.14$ mg/mL). The results of the cytotoxic activity assay for the CT plant correlate with its traditional use as an anticancer agent, thus making it an attractive source for further drug development.

Materials and methods

Research tools

The equipment used in this study are: The tools used for extraction, fractionation, and isolation are glass jars, stirring rods, filter paper, evaporating dish, rotary evaporator, Waterbath, 5 mL vials, 10 mL vials, dropper pipettes, 250 mL separating funnels, portable UV lamp, chromatogram tool set. The tools used for the cytotoxic test are an appendorphic tube, conical tube, 96-well plates, 24-well plates, tissue culture flask, Elisa reader SLT 340 ATC, analytical balance, vortex, CO₂ incubator, refrigerator, hemocytometer, object glass, Cover clip, Inverted microscope, fluorescence microscope, micropipette, digital camera.

Research material

The materials used in this study were CT flower powder (*Clitoria ternatea* L.), chemicals for extraction, phytochemicals, and fractionation, including acetone, ethyl acetate, methanol, 96 % ethanol, n-hexane, and distilled water. The material used in the cytotoxic test is MCF-7 cancer cells. The culture medium consisted of Gibco Life Technologies RPMI 1640 with phenol red and 2 mM glutamine, 100 U/mL, Doxorubicin, DMSO; MTT (3-(4, 5-dimethyl thiazolyl-2)-2,5-diphenyl-tetrazolium bromide); sodium dodecyl sulfate (SDS, Sigma), Ethidium bromide, Acridine.

Making of CT flower extract

The plant used is a true butterfly pea flower obtained from Klaten, Central Java, Indonesia. Butterfly pea flower extract was made using the maceration method by weighing 300 g of dry *Simplicia* powder and then extraction using a ratio of 1:10. Maceration was carried out with 96 % ethanol solvent of 2,250 mL each in a container protected from light for 2 days while occasionally stirred every 12 h. Then the obtained macerate is filtered and a remaceration process is carried out with the remaining ethanol solvent until the color of the ethanol solvent is clear which indicates that the solvent is no longer able to attract the compounds contained in the *simplicia*. The obtained macerate was separated using filter paper and the remaceration process was carried out with the same solvent. The obtained macerate was collected and then evaporated the filtrate of the butterfly pea flower extract using a rotatory evaporator at a temperature of 78 °C and residue with a water bath at a temperature of < 65 °C to obtain a thick ethanol extract [21].

Preparation of ethyl acetate and methanol fraction

Extract fractionation was carried out with several modifications. Fractionation was carried out by suspending the thick extract using methanol as a solvent. Partitioning was then carried out using ethyl acetate. The ratio between methanol and ethyl acetate used is 1:1. Finally, thicken the results of the fractionation with a water bath at a temperature of 60 °C so that a thick extract of each fraction is obtained [22].

Radial system chromatography

Purification using radial system chromatography begins with activating the chromatographic plate in the form of silica gel by placing the plate in an oven at 50 °C for 30 min. The silica plate formula used with a plate thickness of 2.0 mm requires 55 g of silica, the volume of silica used is added by 10 mL so that the

total required is 120 mL, and the sample used is 200 mg. Prior to the elution of the sample, the plate must be eluted with n-hexane as the eluent until the first drop comes out. After the first drop comes out, close the eluent tap and wait 15 min before adding the sample. The sample fraction used was 200 mg with the addition of 40 mL of eluent for each elution. The sample is dissolved in acetone, let the silica plate dry. After the plate is dry, it is carefully put in. Then the eluent tap was opened and elution with n-hexane was started. Catch the liquid that comes out according to the visible stains with the help of a portable UV. The results of the reservoir were evaporated with a vacuum rotary evaporator and carried out on TLC. Fraction results with the same R_f value are accommodated in the same vial. [23].

GC-MS analysis

Sample preparation of 20 mg of isolate in 1 mL of ethyl acetate pro-analysis was taken using a filter syringe (0.5 µL). GC-MS analysis: GC-MS analysis was carried out with the system Column Oven Temperature set at 50.0 °C, Injection Temperature 260.0 °C, split injection model, pressure 28.3 kPa with a total flow of 73.8 mL/min, column flow 0.70 mL/min, a linear velocity of 30.4 cm/s and a purge flow of 3.0 mL/min. The mass detector is set with a start time of 2.5.00 min and an end time of 30.00 min. The spectrum of the unknown component is compared with the spectrum of the known component stored in the NIST and WELLY libraries [25].

Identification of Isolation by UV-Vis spectrophotometry

The isolated isolate was dissolved in a solvent, then put into a cuvette that had been rinsed with the sample solution. Furthermore, the absorbance of the sample solution was measured at a wavelength of 200 - 800 nm [24].

Identification of isolation by FTIR spectrophotometry

Identification of isolates by IR spectrophotometry was carried out by dissolving the sample and then dripping it into an infrared spectrophotometer and measuring its absorbance at a frequency of 4,000 - 400 cm⁻¹ [24].

Cytotoxic testing using MTT assay

Cells were grown in 96 wells microplate so that the density was 1X10⁴ cells/well and incubated for 24 h. Test solutions of various concentrations were added with DMSO cosolvent and incubated at 37 °C in a 5 % CO₂ incubator for 24 h. Cells were washed with PBS. In each well was added 100 L of culture media and 10 L of MTT 5 mg/mL, Incubated for 4 - 6 h. The MTT reaction was stopped with a reagent stopper (10 % SDS in 0.01 N HCL), and the wrapped plate was left overnight. Absorption was read with a microplate reader at a wavelength of 595 nm [17].

Cell proliferation inhibition test (doubling time)

MCF-7 cells were planted in 96 well microplates to obtain a density of 5×10⁴ cells/well and incubated for 48 h. Extracts with various levels were added to the wells and sampling was performed at 24, 48 and 72 h. Cell proliferation was observed by the MTT method as was done in the cytotoxic test [25].

Apoptotic potential test

The cover slip is inserted into the well (24-well plate). A total of 5×10⁴ cells/well were grown on a coverslip by transferring 500 µL of MCF-7 cell suspension. Cells were incubated for 24 h. 24 well plates were taken from the incubator, all culture media were removed and each was filled with 500 L of PBS. The PBS is then removed and the sample is inserted into the well. After incubation, the coverslip was placed on the object glass. 10 µL of ethidium bromide-acridine reagent was dripped onto the coverslip and then observed with a fluorescent microscope [26].

Statistical analysis

Cytotoxic activity was expressed by IC₅₀ (concentration leading to the death of 50 % of the cell population) which was analyzed with GraphPad Prism 8. Absorbance data obtained from the proliferation test of each sample and cell proliferation kinetics were converted into percent living cells and analyzed by the ANOVA test followed by Tukey's test using SPSS 25 to determine the significant difference between the control group and the treatment group. Observations of cell apoptosis were carried out under a fluorescence microscope with a magnification of 10 X by observing changes in cell morphology which were marked by visible colors, namely orange to indicate dead cells and green to indicate living cells.

Results and discussion

Extract fraction

CTEE was obtained by maceration from 300 g of powdered CT flower with 96 % ethanol as solvent. Then it was fractionated using ethyl acetate solvent, the obtained fraction was collected and evaporated to obtain its weight, and the yield of CT flower extraction was 32.57 %. The fractionation method used is the fractionation method with liquid-liquid extraction based on the difference in solubility or partition coefficient of the compound between 2 immiscible solvents. The results of the methanol fraction obtained a yield of 28.16 % and the ethyl acetate fraction obtained a yield of 23 %. This is in line with the research of Wicaksono *et al.*, 2021 which showed that there were differences in yield results due to the polarity of the solvent to attract compounds in the CT flower. The yields are produced in succession from the highest, namely in polar solvents, semi-polar and non-polar. the results of fractionation can affect the level of the polarity of the solvent used and the solubility properties of the sample used [27].

MTT assay cytotoxicity test results

The MTT test [3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyltetrazolium bromide] was used to determine cell viability in each observation indicated by the IC₅₀ value that could inhibit cell growth. The results obtained can be seen in **Table 1**.

Table 1 The concentration of inhibition IC₅₀.

Sample	\bar{X} IC ₅₀ (µg/mL) ± SD
CTEE	17.46 ± 1.05
MFCT	207.88 ± 2.78
EAFCT	13.19 ± 1.16
Isolate 1 EAFCT	6.79 ± 0.14
Isolate 2 EAFCT	7.12 ± 0.39
Doxorubicin	1.72 ± 0.67

The difference in the results obtained that CTEE and EAFCT are more active than MFCT, this indicates that the compounds contained in CTEE and EAFCT are more active than the results of fractionation using polar solvents, namely MFCT. Based on the results obtained, testing of compound isolation, inhibition of cell proliferation, and potential for apoptosis were continued in EAFCT. As additional data, the results of the EAFCT fractionated isolation tested for cytotoxicity obtained isolates 1 and 2 with a range of IC₅₀ values that were not much different, namely 6.79 and 7.12 g/mL [28].

The results of the isolation of the ethyl acetate fraction of CT flowers. Isolation of compounds using chromatographic method that is radial chromatogram. Radial system chromatography has the same principles as classical chromatography because the flow of the mobile phase is accelerated by centrifugal force. The chromatogram separation method uses an eluent with increasing polarity [28].

Based on the results of the isolation of the eluted chromatogram using ethyl acetate; n-hexane as an eluent starting from the ratio (0.5:9.5) then continued (1:9) and (2:8) with an increase in polarity of 5 % by 50 mL. The entry of the sample is followed by elution and produces component bands in the form of concentric circles. At the edge of the plate, the tape will be rotated outward by the centrifugal force. The results of the isolation obtained 11 eluate reservoirs, the eluate reservoir was accommodated based on the color band visible under UV light and then monitored by thin layer chromatography. Thin layer chromatography testing process using ethyl acetate; n-hexane as mobile phase with a ratio (1:9). The spots that appear parallel are accommodated as one, namely in vials 3 - 5 an Rf value of 0.3 is obtained, and in vials 6 - 9 an Rf value of 0.2.

GC-MS analysis results

The results of the analysis using GC-MS will obtain 2 data, namely chromatograms derived from the results of gas chromatography (GC) analysis and mass spectra from the results of mass spectroscopic (MS) analysis. The chromatogram of the analysis by gas chromatography showed 4 peaks of the compound as shown in **Figure 1** and **Table 2**.

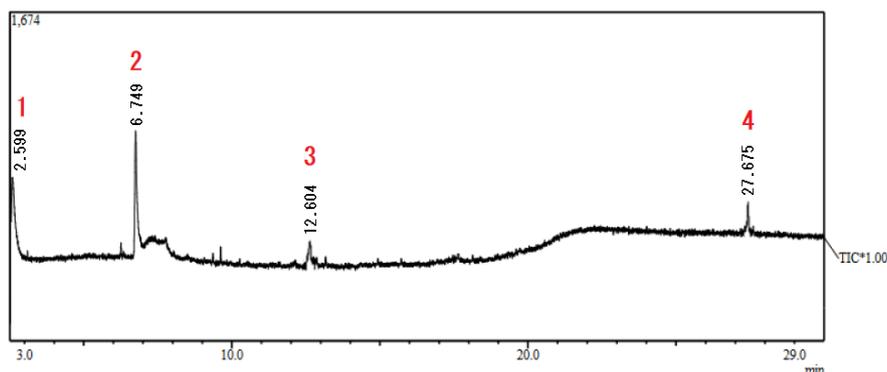


Figure 1 EAFCT isolate chromatogram.

Table 2 Alleged compound isolate EAFCT.

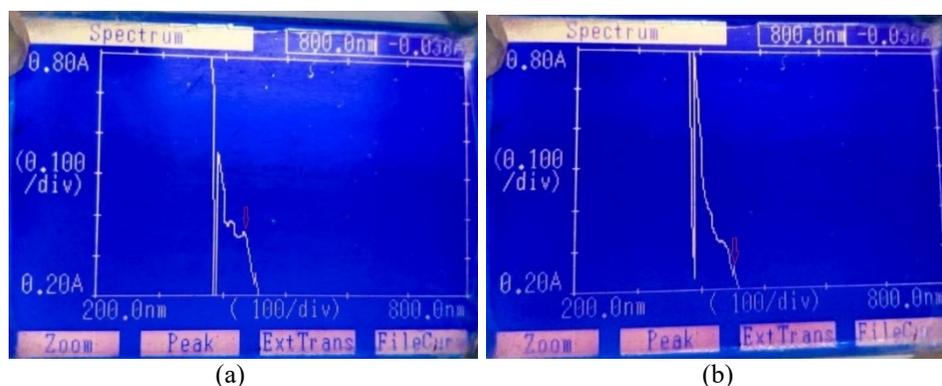
Peak	Retention time (minutes)	Peak (% Area)	Similarity (%)	Compound suspect	Molecular formula	Molecular weight
1	2.599	51.42	87	<i>Perhydropyridazine hydrochloride</i>	$C_5H_6C_1N$	115.56
2	6.749	40.25	92	<i>2-Propylhexanal</i>	$C_9H_{18}O$	142.24
3	12.604	2.79	57	<i>Pentanal (CAS) n-Valeraldehyd</i>	$C_5H_{10}O$	86.13
4	27.675	2.28	55	<i>8-(1-oxo-2-propenyl)-1,4-dioxaspiro [4.5] DCC</i>	$C_{16}H_{18}O_4$	274.31

Based on the analysis of the peak, the highest % area appears at the first peak, with a retention time of 2.599 min, which is probably a *Pyridine hydrochloride* compound with the molecular formula $C_5H_6C_1N$ that appears at the peak of the molecular ion 115.56. This is based on the similarity index to fragmentation, which is 87 % with the highest area being 51.42 %.

Based on previous research GC-MS analysis (Gas Chromatogram-Mass spectrometry) showed that the 2 active components in the water extract of CT flower, namely mome inositol (38.7 %) and pentanal (14.3 %) the results obtained were not much different where the EAFCT isolate at peak 3 which is with a retention time of 12.604 min which is probably a Pentanal compound with the molecular formula $C_5H_{10}O$ which appears at the peak of 86.13 molecular ions. This is based on the similarity index to fragmentation, which is 57 % with an area of 2.79 %.

Identification results of isolated compounds using a uv-vis spectrophotometer

Ultraviolet spectrophotometry results from CTEE, EAFCT, Isolate 1 EAFCT, and Isolate 2 EAFCT can be seen in **Figure 2**.



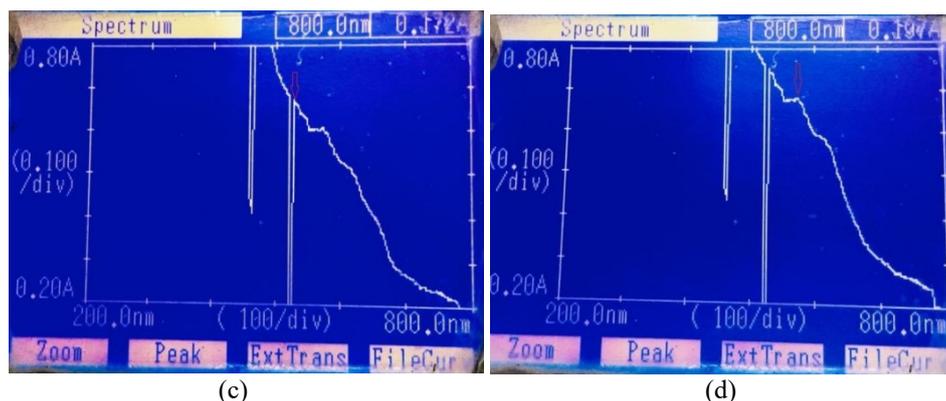


Figure 2 UV-Vis spectrophotometer peak appearance results (a) CTEE, (b) EAFCT, (c) Isolate 1 EAFCT, and (d) Isolate 2 EAFCT.

Ultraviolet spectrophotometry results from CTEE, EAFCT, Isolate 1 EAFCT, and Isolate 2 EAFCT. The CTEE has one peak at a wavelength of 574.0 nm with an absorbance of 0.683. The FEABT has 1 peak also at a wavelength of 574.0 with an absorbance of 0.607. In the sample isolate 1, EAFCT had 5 peaks, namely 657.0, 608.0, 541.0, 432.0, 352.0 nm with absorbances of 0.078, 0.016, 0.042, 0.360, 1.387, respectively. In sample isolate 2, EAFCT had 3 peaks, namely 657.0, 607.0 and 541.0 nm with absorbances of 0.060, 0.011 and 0.037 respectively. Pentanal compound (aldehyde) is an alkane derivative compound that is a saturated aliphatic hydrocarbon compound. Alkane derivatives are compounds that are considered to be derived from alkanes, where one or more of the hydrogen atoms are replaced by certain atoms or groups of atoms. This replacement group is referred to as a functional group. These hydrogen atoms absorb UV light at wavelengths > 270 nm [30].

Identification results of isolated compounds using FTIR (fourier-transform infrared spectroscopy)

CTEE (Clitoria ternatea ethanol extract)

The results of the CTEE analysis using infrared spectrophotometry gave an absorption with a wave number of $3,570.42\text{ cm}^{-1}$ with a transmittance of 78.27 % expressed as an OH group (Hydrogen bonded). At a wave number of $3,145.16\text{ cm}^{-1}$ with a transmittance of 53.61 %, it is expressed as a CH group (Aromatic rings). At wave numbers $2,986.03$ and $1,997.75\text{ cm}^{-1}$ with the transmittance of 49.31 and 80.25 % are expressed as CH (Alkane) groups. At a wave number of $1,737.49\text{ cm}^{-1}$ with a transmittance of 34.12 %, it is expressed as a C = O (Aldehyde) group. At a wave number of $1,447.70\text{ cm}^{-1}$ with a transmittance of 82.97 %, it is expressed as a CH (Alkane) group. At a wave number of $1,373.24\text{ cm}^{-1}$ with a transmittance of 59.92 %, it is expressed as a group of NO₂ (Nitro Compounds). At $1,236.98\text{ cm}^{-1}$ with a transmittance of 32.12 %, it is expressed as a CN group (Amines, Amides). At numbers $1,097.66$ and $1,044.91\text{ cm}^{-1}$ with transmittances of 85.25 and 47.10 % were expressed as CO groups (Ether, Esther). At wave numbers 938.41 and 847.21 cm^{-1} with transmittances of 88.19 and 86.96 % are expressed as CH (Alkene) groups. The results obtained can be seen in **Figure 3**.

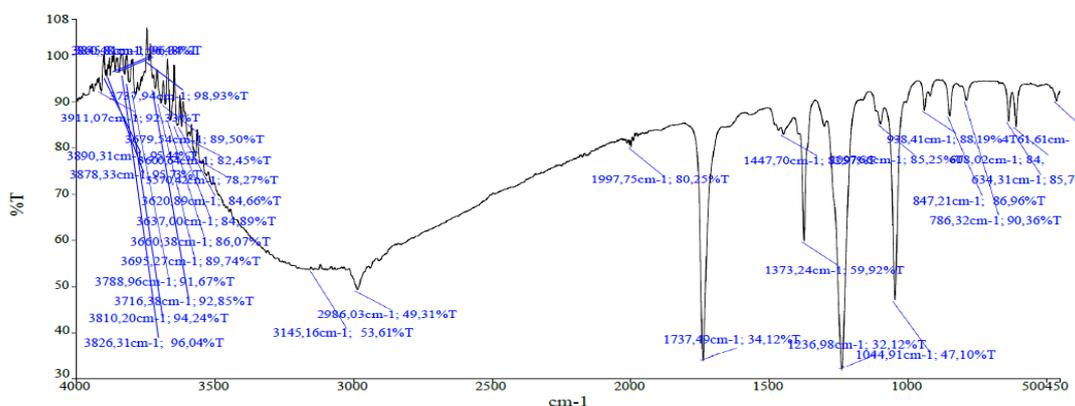


Figure 3 Result of FTIR CTEE.

EAFCT (ethyl acetate fraction of *Clitoria ternatea*)

The results of the EAFCT analysis using infrared spectrophotometry gave an absorption with a wave number of $3,631.12\text{ cm}^{-1}$ with a transmittance of 89.61 % expressed as an OH group (Alcohol). At wave numbers $3,620.60$ and $3,274.50\text{ cm}^{-1}$ with a transmittance of 90.27 and 63.42 % are expressed as OH (Hydrogen bonded) groups. At wave numbers $2,941.03$ and $2,830.93\text{ cm}^{-1}$ with a transmittance of 39.88 and 39.00 % expressed as CH (Alkane) groups. At a wave number of $1,706.96\text{ cm}^{-1}$ with a transmittance of 54.98 %, it is expressed as a C = H (Aldehyde) group. At wave numbers $1,420.53$ and $1,364.19\text{ cm}^{-1}$ with a transmittance of 75.51 and 68.17 % expressed as CH (Alkane) groups. At a wave number of $1,228.74\text{ cm}^{-1}$ with a transmittance of 74.77 %, it is expressed as a CN group (Amines, Amides). At $1,094.48\text{ cm}^{-1}$ and $1,023$. The results obtained can be seen in **Figure 4**.

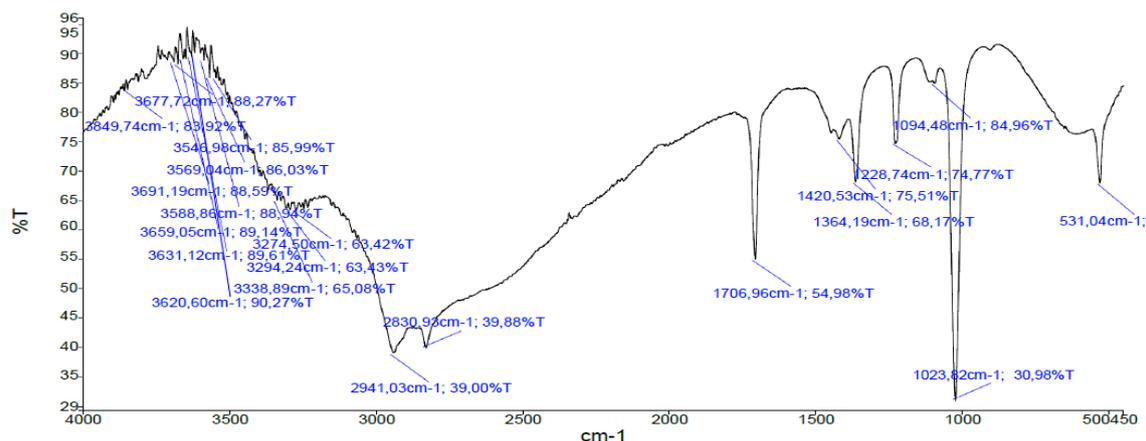


Figure 4 Results of FTIR EAFCT.

Isolate 1 EAFCT (ethyl acetate fraction of *Clitoria ternatea*)

The results of the analysis of Isolate 1 EAFCT using infrared spectrophotometry gave an absorption with a wave number of $3,012.42\text{ cm}^{-1}$ with a transmittance of 108.69 % expressed as a CH group (Aromatic rings). At wave numbers $2,860.03$ and $2,420.33\text{ cm}^{-1}$ with a transmittance of 105.06 and 95.14 % are expressed as CH groups (Alkanes). At a wave number of $1,706.38\text{ cm}^{-1}$ with a transmittance of 59.97 %, it is expressed as a C = O (Aldehyde) group. At a wave number of $1,579.72\text{ cm}^{-1}$ with a transmittance of 99.85 %, it is expressed as a C = C group (Aromatic rings). At a wave number of $1,422.22\text{ cm}^{-1}$ with a transmittance of 95.99 %, it is expressed as a CH (Alkane) group. At a wave number of $1,362.32\text{ cm}^{-1}$ with a transmittance of 74.09 %, it is expressed as a group of NO_2 (Nitro compounds). At the number $1,223.10\text{ cm}^{-1}$ with a transmittance of 76.46 % expressed as a CN group (Amines, Amides). At wave numbers $1,092.61$ and $1,036.36\text{ cm}^{-1}$ transmittances of 97.65 and 95.55 % are expressed as CO groups (Ether, Esther). At a wave number of 908.19 cm^{-1} with a transmittance of 103.24 %, it is expressed as a CH (Alkane) group. The results of the analysis on Isolate 1 are shown in **Figure 5**.

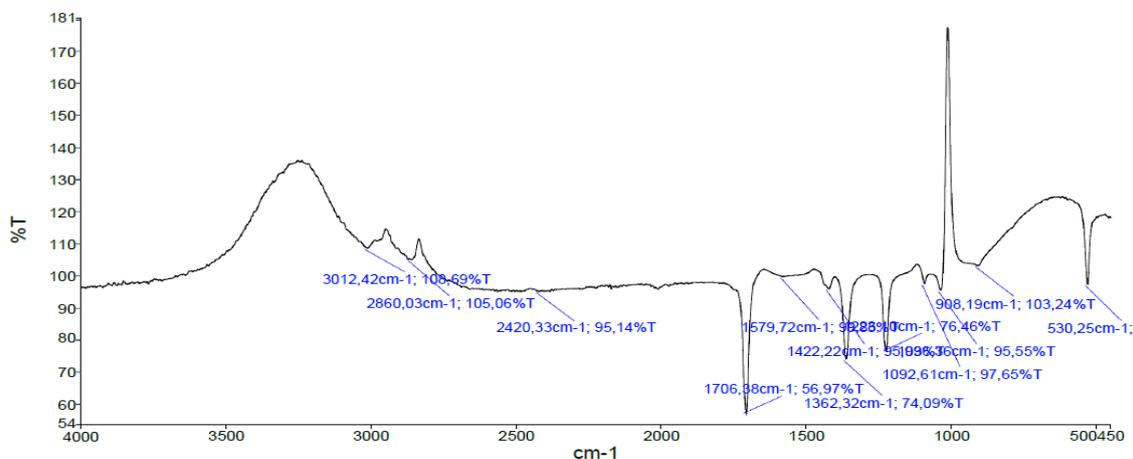


Figure 5 Results of FTIR isolate 1 EAFCT.

Isolate 2 EAFCT (ethyl acetate fraction of *Clitoria ternatea*)

The results of the analysis of Isolate 2 EAFCT using infrared spectrophotometry gave an absorption with a wave number of $3,319.63\text{ cm}^{-1}$ with a transmittance of 56.89 % expressed as an OH group (Hydrogen bonded). At wave numbers 2,943.44 and $2,831.89\text{ cm}^{-1}$ with a transmittance of 68.07 and 69.78 % expressed as CH (Alkane) groups. At wave numbers 1,997.40 and $1,449.13\text{ cm}^{-1}$ with a transmittance of 92.84 and 82.55 % were expressed as CH (Alkane) groups. At wave numbers 1,114.20 and $1,021.48\text{ cm}^{-1}$ with a transmittance of 87.22 and 26.02 %, it is expressed as a CO group (Ether, Esther).

Based on the results of the infrared spectrum of the compound Isolate 1, it has a similar spectrum to pentanal compounds, namely the presence of an aldehyde group at a wave number of $1,706.38\text{ cm}^{-1}$. Isolate 2 is seen in **Figure 6**.

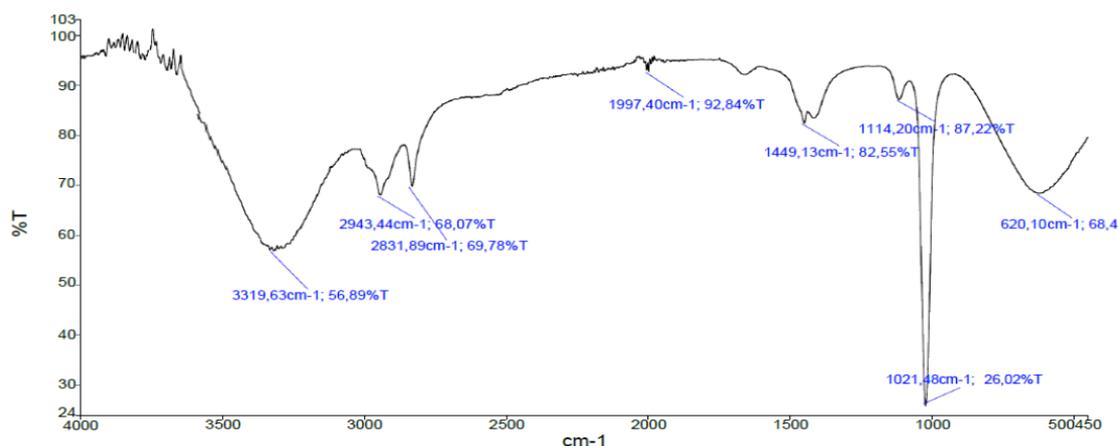


Figure 6 Results of FTIR isolate 2 EAFCT.

Antiproliferation test results (doubling time)

Based on the cell proliferation inhibition test, the concentration of doxorubicin and samples of CTEE, EAFCT, Isolate 1 EAFCT, and Isolate 2 EAFCT were used referring to the values of IC_{50} , $\frac{1}{2} IC_{50}$, $\frac{1}{4} IC_{50}$ and $\frac{1}{8} IC_{50}$ obtained from previous studies. This concentration is chosen because the morphology of cells can be observed, and at that level, it is estimated that not too many cells will die. When used at levels far above the IC_{50} value, it was feared that too many cells died before 72 h of incubation, so cell proliferation observations could not be carried out and growth profiles were not visible [31]. The results of testing the proliferative activity of MCF-7 cells can be seen in **Table 3**.

Table 3 Results of observation of cell proliferation activity.

Sample	Concentration (ppm)	(%) Cell viability		
		24 h	48 h	72 h
CTEE	17.46	43.68	59.14	69.13
	8.73	50.11	63.20	81.39
	4.36	55.37	75.75	89.12
	2.18	56.42	75.83	91.12
EAFCT	13.19	53.65	66.58	80.95
	6.59	67.82	75.75	97.85
	3.29	69.54	75.62	100.14
	1.64	71.31	76.04	102.32
Isolate 1 EAFCT	6.79	65.17	72.15	86.39
	3.39	69.43	77.65	97.50
	1.69	77.79	80.86	110.31
	0.84	77.63	83.69	113.66

Sample	Concentration (ppm)	(%) Cell viability		
		24 h	48 h	72 h
Isolate 2 EAFCT	7.12	63.56	74.14	88,12
	3.56	66.33	74.77	97.06
	1.78	70,70	77.14	105.93
	0.89	76.13	81.41	111.96
Doxorubicin	1.72	22.25	9.46	5.37
	0.86	24.69	9.67	5.70
	0.43	26.19	11.36	5.82
	0.21	28.35	11.28	5.96

The results of the proliferation inhibition test using the MTT method were seen based on the % (percentage) of cell life (viability) for each concentration series obtained from the absorbance value of the treatment at each incubation time. Based on the results obtained, it was shown that the percentage of cells obtained by the treatment of the test compounds namely CTEE, EAFCT, Isolate 1 and Isolate 2, and doxorubicin as a comparison of positive control showed that the positive control of doxorubicin until the end of incubation did not increase cell growth. This is probably due to cell death. This death is thought to occur through the mechanism of cell cycle arrest, causing the ability of cell proliferation to decrease [31]. The results of this cell proliferation inhibition test in the form of a bar graph can be seen in **Figure 7**.

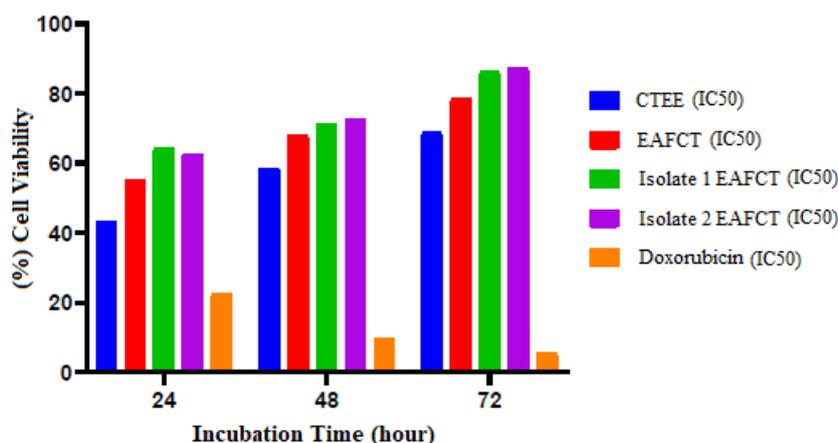


Figure 7 % Cell viability vs incubation time of CTEE, EAFCT, Isolate 1 EAFCT, Isolate 2 EAFCT, and Doxorubicin.

Based on the bar graph, it can be seen that in the CTEE, EAFCT, isolate 1 FEABT and isolate 2 EAFCT with each IC₅₀ concentration, along with the increase in incubation time, % cell viability also increased. This shows that the IC₅₀ concentration of each cell cycle sample is still running. This event may occur because the influence of the active compounds contained is reduced. These active compounds are metabolized by cells so that over time the concentration decreases. This reversible effect is more accurately described as cell cycle delay (delay in cell cycle inhibition) so to the state of arrest, it is necessary to add a test solution (dose-dependent). Based on the results of One Way ANOVA data analysis from the test sample with positive control doxorubicin obtained a value ($p < 0, 05$) with meaning that there is a significant difference in the results of CTEE, EAFCT, Isolate 1 EAFCT, Isolate 2 EAFCT with doxorubicin. The results of doubling time vs concentration can be seen in **Figure 8**.

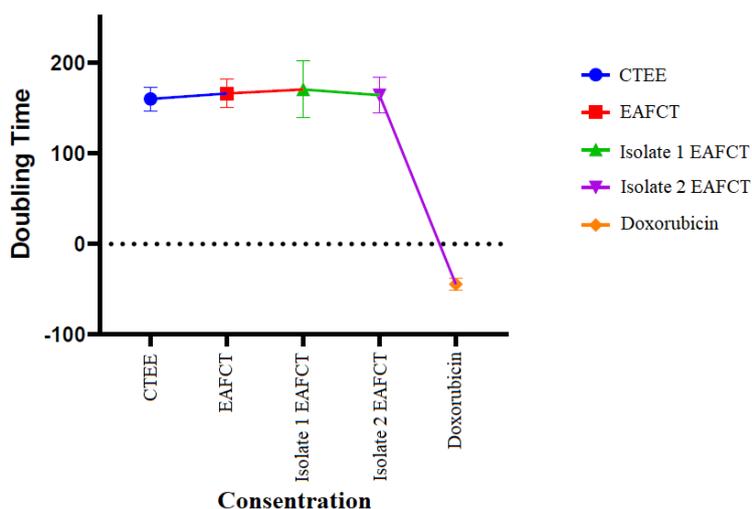


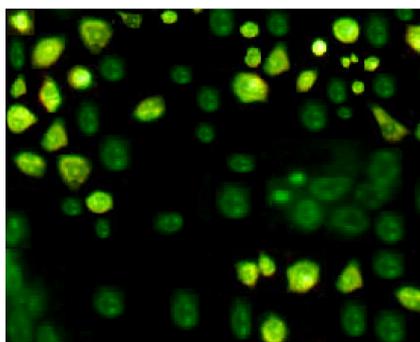
Figure 8 Results of doubling time vs concentration of CTEE, EAFCT, Isolate 1 EAFCT, Isolate 2 EAFCT, and Doxorubicin.

Based on the results of doubling time vs concentration, it can be seen that the CTEE sample obtained the doubling time values of IC_{50} , $\frac{1}{2} IC_{50}$, $\frac{1}{4} IC_{50}$, and $\frac{1}{8} IC_{50}$ concentrations, namely 178.47; 160.86; 151.18; 150.09. The EAFCT sample is 188.53; 164.37; 156.82; 154.79. In EAFCT Isolate Sample 1, namely 212.96; 174.64; 148.53; 145.82. The EAFCT isolate 2 samples were 190.62; 168.23; 150.24; 148.94.

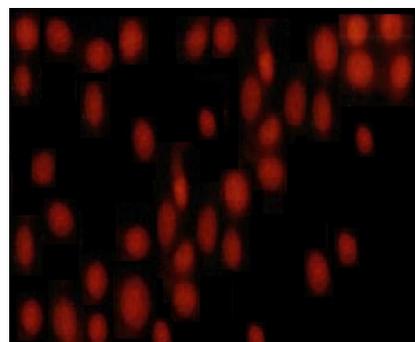
Based on the results obtained, it is not much different from the research of Neda *et al.*, (2013), namely the proliferation test on MCF-7 cells of the water extract of CT flower obtained 175.35 g/mL. The correlation of the doubling time value with the higher concentration, the higher the doubling time value, and vice versa, the lower the concentration, the lower the doubling time value. This is related to the ability to inhibit cell proliferation, which can be related to the mechanism of cell cycle arrest, namely the presence of DNA or RNA damage that will trigger the activation of the p53 gene so that the cell cycle will be temporarily stopped for the DNA or RNA repair process. If the damage is severe enough and cannot be repaired, the cells will undergo apoptosis or a natural and programmed cell death mechanism [32].

Apoptotic test results (double staining)

Observation of the induction of apoptosis was carried out to determine the cause of cell death, both apoptosis, and necrosis. Cell apoptosis can be detected by acridine orange-ethidium bromide staining. This method is based on differences in DNA fluorescence in living and dead cells due to the binding of acridine orange-ethidium bromide. Orange acridine will penetrate all parts of the cell and the nucleus will appear green. Meanwhile, ethidium-bromide can only calibrate with cells whose membranes have been damaged and the nucleus will turn red [33]. The results of the apoptotic induction test using the Double Staining method which is seen qualitatively are shown in **Figure 9**.



(a)



(b)

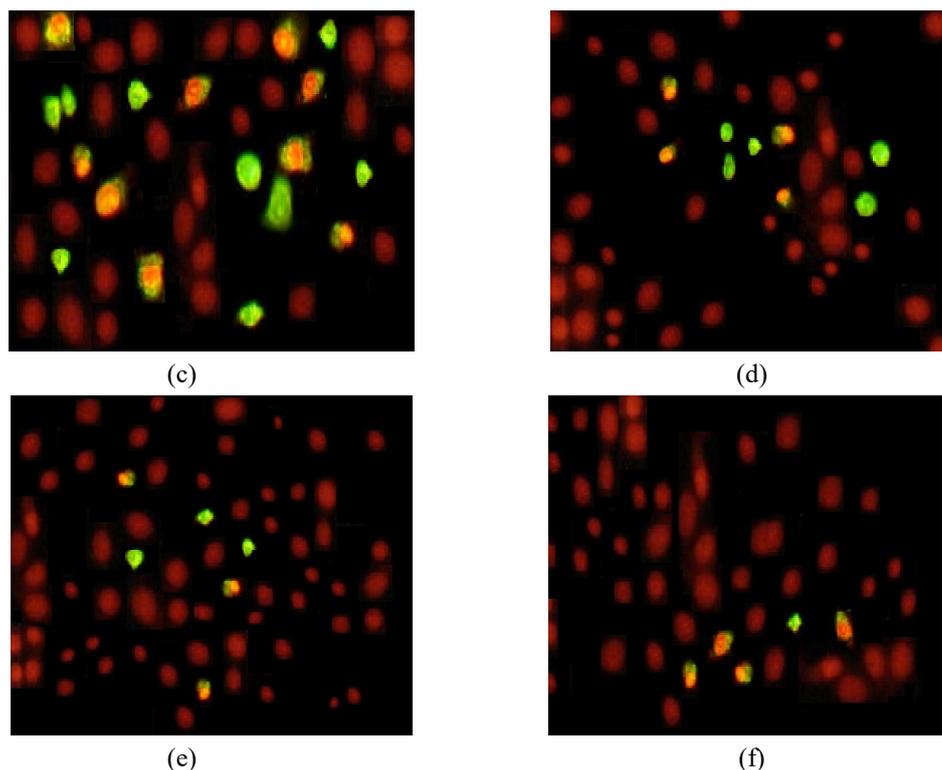


Figure 9 Results of testing potential for apoptosis (a). Control Cells, (b) Doxorubicin Positive Control, (c) CTEE, (d)EAFCT, (e) Isolate 1EAFCT, (f) Isolate 2EAFCT.

The apoptosis test was carried out by giving CTEE, EAFCT, isolate 1 and isolate 2 EAFCT treatment using IC_{50} concentration. From the results of apoptosis, it can be seen that all control cells that were not treated with a bright green fluorescent test sample indicated that the cells were alive. Cells treated with the test sample showed that several cells fluoresce which indicates the cell is dead. Based on the results of DNA staining, cells treated with CTEE, EAFCT, Isolate 1, and isolate 2 EAFCT showed a non-uniform color, i.e. green mixed with orange, indicating that the cell had membrane blebbing which indicated cell apoptosis [34].

The process of apoptosis in the intrinsic pathway involves mitochondria. Mitochondrial stress that induces apoptosis can be caused by chemical compounds or loss of growth factors that cause mitochondrial disruption and the release of cytochrome-C which is a proapoptotic factor from the mitochondrial intermembrane. Cytochrome-C binds to a cytoplasmic protein called Apaf-1 so that it will activate an apoptotic initiator, caspase 9 in the cytoplasm. Furthermore, caspase 9 will activate procaspase 3 into caspase 3 which is an effector caspase that carries out apoptosis [36].

Conclusions

Based on the results of the study, several conclusions were obtained, namely:

1) EAFCT 1 and 2 isolates had a cytotoxic effect on MCF-7 cells by showing IC_{50} values of 6.79 and 7.12 $\mu\text{g/mL}$ and were able to inhibit the growth rate of MCF-7 cells with doubling time values of 212.96 and 190.62 h.

2) EAFCT 1 and 2 isolates had apoptotic induction activity against MCF-7 cells indicated by the presence of orange fluorescent cells which indicated that the cells were dead.

3) EAFCT isolate contains Pyridine hydrochloride and pentanal compounds which have the potential as anticancer compounds.

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