Cytotoxic Assay of Semipolar Fraction Of Ethanolic Extract From Sugar Apple (*Annona Squamosa* L.) Stem Bark on T47D Cells

Cita Hanif Muflihah*, Haryoto Haryoto, Peni Indrayudha
Fakultas Farmasi Universitas Muhammadiyah Surakarta, Jl. A. Yani Tromol Pos I, Pabelan Kartasura Surakarta 57102
*Email: cita.h.muflihah@ums.ac.id

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Abstract

Previous research has shown that some compounds in leaves and seeds of sugar apple have a cytotoxic activity. The aim of this research was to determine the cytotoxicity of semipolar fraction of ethanolic extract from sugar apple stem bark (Annona squamosa L.) on T47D cancer cells. The semipolar fraction of ethanolic extract from sugar apple stem bark was collected by fractionation using Vacuum Liquid Chromatography (VLC) with hexane:ethyl acetic (9:1, 8:2, 7:3, and 6:4) as mobile phase. Cytotoxicity from the fractions of five different concentration namely; 25, 50, 100, 150, and 250, µg/mL was measured by MTT assay. The potency of the cytotoxicity was defined by the ability of the fraction to inhibit the growth of T47D cells indicated by the value of IC_{50}. Qualitative analysis of contained compounds in the fraction was done by Thin Layer Chromatography (TLC) method using silica gel F 254 as a stationary phase and hexane:ethyl acetic (7:3) as a mobile phase. UV 254 and 366 nm lamp also Dragendorff, citroboric, and $FeCl_3$ spray reagents were used to visualize the spots of the secondary metabolites. The result proved that the semipolar fraction of ethanolic extract from sugar apple stem bark showed potential cytotoxicity on T47D cancer cells with IC_{50} value of 70,77 µg/mL. Qualitative analysis showed that the fraction contained flavonoids and alkaloids which is presumably responsible for its cytotoxic activity.
Keywords: Cytotoxic assay, sugar apple (Annona squamosa L.), T47D cells, semipolar fraction.

INTRODUCTION

The prevalence of cancer in Indonesia ranks at the top ten, reigning as the eight in Southeast Asia. It increased from 1,4 per 1000 in 2013 to 1,79 per 1000 citizens in 2018, with 42,1 per 100.000 cases was caused by breast cancer that contributes to the 17 in 100.000 of deaths in woman (Kementrian Kesehatan RI, 2019). The main goal for cancer therapy is to eradicate the cancerous cells by means of killing the cells or removing the cells (Sullivan *et al.*, 2015). This can be achieved by surgery, radiation, chemotherapy, or immunotherapy. However, it is well known that these methods are expensive, and more likely accompanied by aggravating side effects (Kementrian Kesehatan RI, 2018). Our nature contains abundant sources of compounds that have numerous pharmacological activities. Traditional medicine practice has proved that the empirical use of natural source to fight against diseases is comparatively has lesser side effects than modern medicines (Karimi, Majlesi and Rafieian-Kopaei, 2015). Therefore, the exploration of other potential natural sources is important in order to search for another options for cancer therapy that have relatively less detrimental effects to the patients. One of the potential natural sources is sugar apple (*Annona squmosa L.*).

Various parts of sugar apple plant have previously been investigated for their pharmacological effects, especially in their cytotoxic activity. The methanol extract of sugar apple's leaves and seeds were reported to have LC_{50} value of 0.63 µg/mL and 0.10 µg/mL respectively, in Brine Shrimp lethality Test (Pisutthanan *et al*. 2004). Other report showed that the organic and water extracts of sugar apple's seeds could induce the apoptosis of MCF-7 and K-562 cells (Pardhasaradhi *et al*., 2005). In another research it is proved that the crude extract and the ethyl acetate extract of sugar apple leaves possessed cytotoxicity against human

epidermoid carcinoma cell line KB-3-1 and colon cancer cell line HCT-116 (Wang *et al.*, 2014)

Based on the previous findings, it could be concluded that the parts of sugar apple plant have diverse advantages, thus, this research was aimed to evaluate the cytotoxicity of semipolar fraction of ethanol extract from sugar apple's stem bark against T47D cells. We also performed a qualitative test of the fraction in order to predict the secondary metabolites content responsible for its activity using TLC method.

MATERIALS AND METHODS

Materials

The verification of the plant was performed in the Biology Pharmacy Laboratory of Universitas Muhammadiyah Surakarta. The stem bark of sugar apple was collected directly from the local neighborhood plant, dried, and powdered. Various organic solvents were used for extraction such as hexane, ethyl acetate, and ethanol. Dragendorff, sitroboric, and $FeCl_3$ reagents were used to visualize alkaloids, flavonoids, and phenolic compounds. T47D cell line was obtained from CCRC UGM. RPMI media, PBS (phosphate buffer saline), trypsin-EDTA, DMSO (dimethyl sulphoxide), and MTT reagent were used during cytotoxic assay.

Extraction and fractionation

Extraction was performed on powdered samples by maceration using 96% ethanol (ratio 1:7.5) for 24 hours in a dark, closed container followed by filtration. The filtrate was concentrated using rotary evaporator and waterbath. The ethanol extract was then fractioned using VLC method. 25 g extracts were impregnated with silica G60 (30-70 mesh) weigh twice as much as the extract. This mixture was then placed on top of the dense and compact 14 cm column of silica gel GF254. Fractionation was performed in triplicates. Elution was performed fourteen times for every replication, each time using 150 mL solvent with escalated polarity system as follows; three times of hexane: ethyl acetate (9:1); four times of hexane:ethyl acetate (8:2); four times of hexane: ethyl acetate (7:3); three times of hexane: ethyl acetate (6:4); and twice of 96% ethanol. 50 mL of each fraction was collected and each sample was spotted onto a TLC silica plate and developed. This step was done in order to classify all of the fractions into three different categories of nonpolar, semipolar, and polar fractions respectively. Three fractions were then evaporated until thick consistency was obtained. In this experiment, we focused on evaluating the semipolar fraction of the extract.

Qualitative analysis using TLC

Secondary metabolites content in the semipolar fraction was identified using TLC method. The stationary phase used was silica GF254 while the solvent system was hexane:ethyl acetate (7:3). The spots were visualized under UV 254 and 366 nm light prior as well as after sprayed with reagents such as Dragendorff, sitroboric, and FeCl3.

T47D culture media

T47D cells were cultured in RPMI 1640 media. The media was made by mixing RPMI powder, hepes buffer, and sodium bicarbonate buffer of pH 7.4. Complete media was obtained after adding 10% of FBS (fetal bovine serum), 2% of penicillin-streptomycin, and 0.5% of fungizone.

MTT cytotoxic assay

After T47D cells achieved 80% of confluence, the cells were detached using trypsin-EDTA. The cells were then washed, and counted using hemacytometer. The cells were then plated into a 96-well plate with 10^4 cells for each well. The fraction was diluted in DMSO into five different concentrations of 25, 50, 100, 150, and 250, µg/mL and then 100 µL of each concentration was added into the wells. The plate was then incubated for 24 hours. The following day, the cells were washed with PBS and 100 µL of the MTT (3-(4,5-dimethylthiazole-2il)-2,5-diphenyltetrazoliumbromide) in media solution was added into the wells. MTT is the

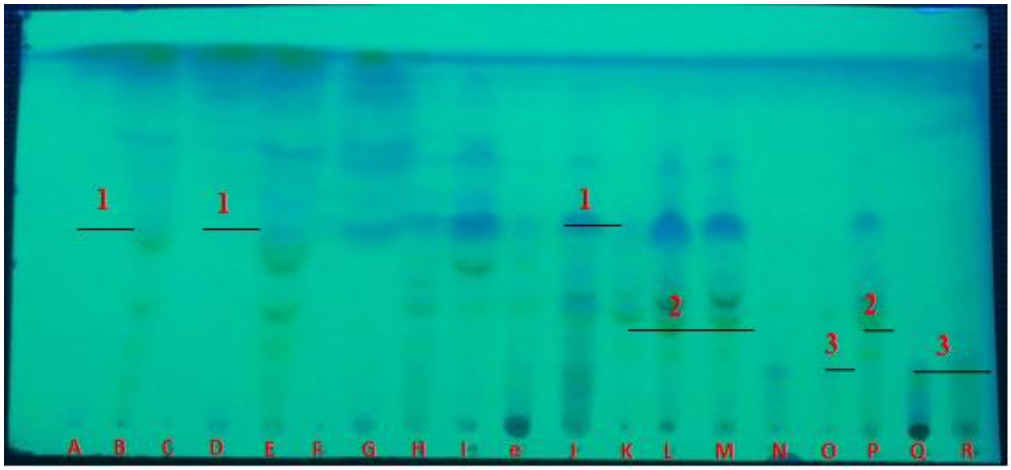

Figure 1. Chromatogram from TLC of eighteen fractions were classified into three groups. (1) Nonpolar fraction, (2) semipolar fraction, and (3) polar fraction.

substrate for metabolic enzymes of the cells. MTT would be reduced into purple formazan crystals by the living cells, indicating that the growth was not inhibited by the presence of the fractions. Next, the plate was then incubated again for another four hours. The reduction of tetrazolium salts into formazan by the survived cells was happened in this period. In order to stop the reaction as well as to dilute the formazan crystals formed, 10% of SDS (sodium dodecyl sulphate) in HCL solution was added. Subsequently, the plate was incubated in the closed dark room temperature place overnight. The next day, the absorbance of the solution from each well was measured in 550 nm. The data obtained was used to calculate the percentage of the survived cells using following equation;

$$\text{Surviving cells (\%)} = \frac{(\text{abs. treated groups} - \text{abs. background})}{(\text{abs. control of living cells} - \text{abs. background})} \times 100\%$$

with abs. background means the absorbance of the control media as the background. Then, IC50 value was extrapolated from the linear regression equation obtained by the correlation between log of the concentration of the fractions and the percentage of survived cells. X value represented the log of the concentration, while Y value represented 50% inhibition of the cell growth.

RESULTS AND DISCUSSION

Extraction and fractionation

Extraction with 96% ethanol by maceration resulted in 12.34% yield, as much as 295.55 g concentrated extract of sugar apple's stem bark was obtained at the end of the extraction process. Following the fractionation step, in total, fourteen fractions from the mixture of hexane:ethyl acetate solvent and two fractions of 96% ethanol were collected. Preliminary TLC was performed in order to categorize the polarity of the fractions obtained. The spots were observed under the UV light. The result showed that there were in total of eighteen fractions (A-R) obtained (Figure 1). Eighteen fractions were subsequently spotted onto silica GF254 and developed with hexane:ethyl acetate (8:2). This step was done in order to classify them into three groups of nonpolar, semipolar, and polar fractions based on their chromatogram patterns. As we used hexane:ethyl acetate (8:2) for a mobile phase and silica GF254 as a stationary phase, it is clear that this was a normal phase for a TLC method where the solvent is less polar than the plate. Thus, with *"like dissolve like"* principle, all the nonpolar compounds would be at the end point of the

Table 1. Concentrations of the fraction used during MT assay and their abilities to inhibit T47D cells proliferation indicating by the percentage of surviving cells after the treatments

Concentration (µg/mL)	Log concentration	% of surviving cells
25	1.398	96.301
50	1.699	74.463
100	2.000	37.828
150	2.176	4.893
250	2.398	0.636

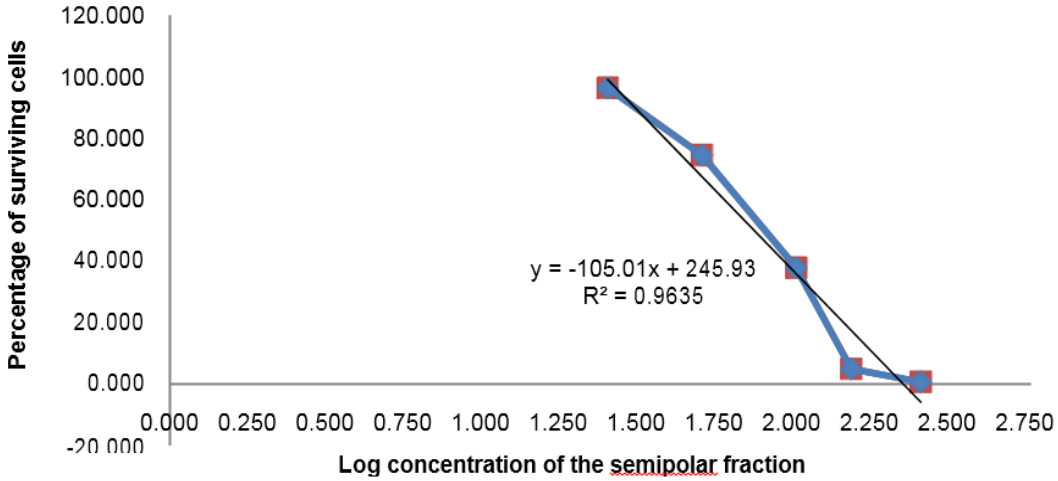

Figure 2. Graphic shows the linear correlation between the percentage of surviving cells and the log of concentrations of the fraction (R=0.963).

elution, while semipolar compounds were in the middle and the polar compounds would be retained near the starting marks of the first spots (Figure 1, indicated with number 1, 2, and 3 respectively). Three fractions obtained were then evaporated and concentrated. By at the end of this step, 4.6 gram of the semipolar fraction with the yield of 1.56% was achieved

MTT cytotoxic assay of the semipolar fraction against T47D cells

The highest concentration of 250 µg/mL exhibited the most potent cytotoxic activity causing almost 99.32% of cell death, while the lowest concentration of 25 µg/mL could only cause death on 3.64% of T47D cells population. The percentage of surviving cells receiving treatments of the fraction in every concentration can be seen in Table 1.

The cytotoxic potency of the semipolar fraction of the ethanol extract from sugar apple's stem bark against T47D cells is illustrated in Figure 2. The ability of the fraction in killing the T47D cells is indicated by the decrease of the percentage of the surviving cells along with the increase of the fraction's concentration. From the abovementioned graph (Figure 2), the IC_{50} was then calculated by substituting the Y value with 50 in the equation. The IC50 value obtained of the semipolar fraction of the ethanol extract from sugar apple's stem bark against T47D cells was 70.77 µg/mL. According to Rajabalian (2007), an extract is considered to have weak cytotoxicity if its IC_{50} value turns to be >100 µg/mL. Thus, based on this stipulation, our finding showed that the semipolar fraction of the ethanol

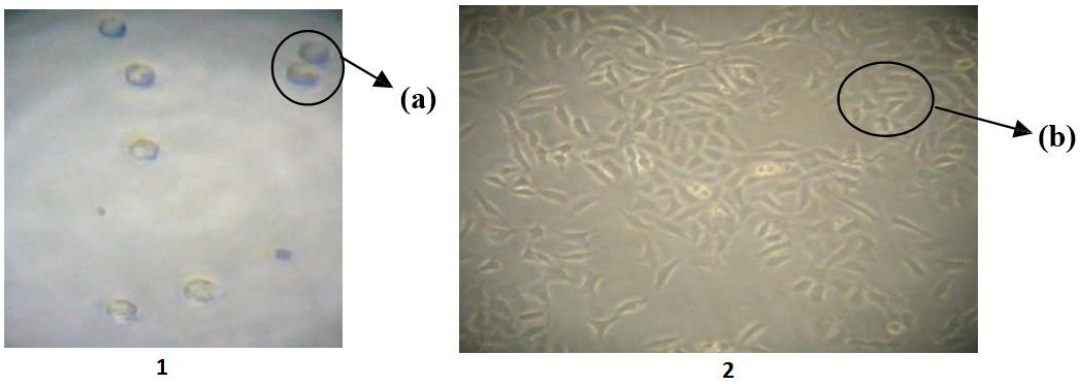

Figure 3. The treatment effect of the fraction on the morphology of T47D cells. (1) Cells were treated with 250 µg/mL, while (2) were treated with 25 µg/mL of the fraction. (a) Dead cells (b) live cells

extract from sugar apple's stem bark have relatively fair cytotoxicity against T47D cells.

This IC_{50} value was then compared with another fractions, which are the nonpolar and polar fractions. The semipolar fraction of the ethanol extract from sugar apple's stem bark has higher cytotoxicity than the nonpolar and the polar fractions with the IC_{50} value of 237.317 (Rahayu, 2012) and 74.665 µg/mL (Rachmayanti, 2012) respectively. However, the semipolar fraction was less effective in inhibiting the growth of T47D cells compared with the ethanol extract from sugar apple's stem bark itself with the IC_{50} value of 43.93 µg/mL (Umma, 2012). Similar cytotoxicity experiments were done using the leaves and seeds of sugar apple on T47D cells. The polar fractions of ethanol extract from sugar apple's leaves and seeds had the IC50 values of 110.30 and 23.34 µg/mL respectively (Meiningrum, 2012; Rimbawan, 2012). Therefore, this data is in line with the previous findings indicating that the seeds of sugar apple has the most satisfying cytotoxic activity on cancerous cells (Pisutthanan *et al*. 2004; Pardhasaradhi *et al*., 2005).

In this experiment, DMSO was used as a solvent to dilute the samples. The highest concentration of the fraction during MTT assay was 250 µg/mL with 0.5% of DMSO. According to the previous finding, DMSO with the concentrations of 0,5% should not interfere with the cells viability (Chen and Thibeault, 2013) which also in line with our data. The morphology of T47D cells receiving the highest and lowest concentration of the fraction can be seen in Figure 3. Based on the results, it can be concluded that the semipolar fraction of the ethanol extract from sugar apple stem bark has potential cytotoxic activity against T47D cellsQualitative analysis of secondary metabolites content of the semipolar fraction of the ethanol extract from sugar apple stem bark

The analysis was done using TLC method focusing on the detection of the alkaloids, polyphenols, and flavonoids content in the fraction visualized by the spray reagents of Dragendorff, FeCl3, and sitroboric, respectively. The result of the TLC can be seen in Figure 4. The presence of alkaloids can be detected with Dragendorff reagent as a brown-red fluorescence under UV light of 366 nm (Wagner *et al*, 1995). The alkaloids group in the semipolar fraction was found to be on RF of 0,53 (Figure 4, picture 2).

Next, we also attempted to detect the flavonoids group in the fraction. Flavonoids would react with boric acid and citric acid in the sitroboric reagent forming bonds that will be seen as yellow-greenish fluorescence under UV light of 366 nm (Markham, 1982). Our samples showed two positive results with

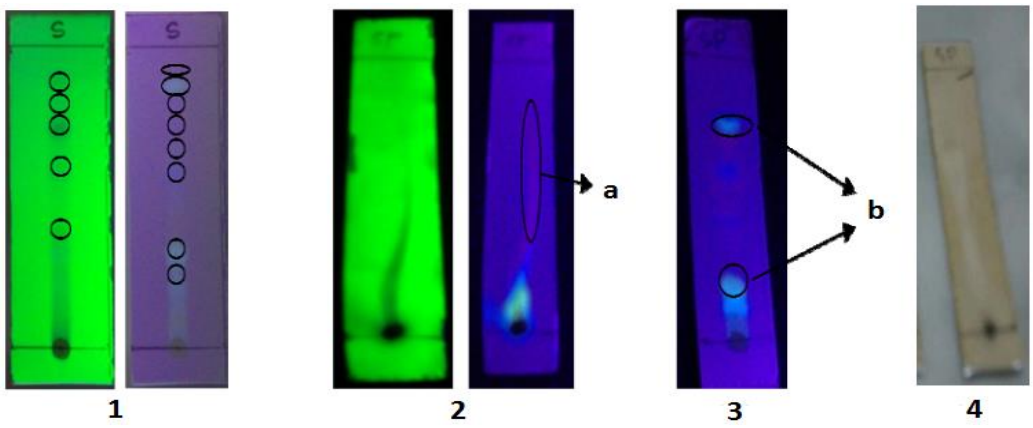

Figure 4. Detection of alkaloids, flavonoids, and polyphenols content using normal phase TLC method with hexane:ethyl acetate (7:3).
(1) The spots visualized under UV 254 nm (left) and 366 nm (right). (2) The plate was sprayed with Dragendorff reagent for alkaloids detection then observed under UV 254 nm (left) and 366 nm (right). (a) alkaloids were indicated with brownish red fluorescence under UV 366 nm. (3) The plate was sprayed with sitroboric reagent for flavonoids detection and observed under UV 366 nm, (b) yellow greenish fluorescence indicated the presence of flavonoids. (4) The plate was sprayed with FeCl3 reagent for polyphenols detection and then observed under visible light. Dark blue-grey spots indicating polyphenols content in the fraction.

Table 2. TLC result of the secondary metabolites content of the semipolar fraction of ethanol extract from sugar apple stem bark

Detection	Rf	Secondary metabolite groups	Results
UV 254 nm	0.37; 0.59; 0.74; 0.81; and 0.89	-	Five spots detected
UV 366 nm	0.21; 0.32; 0.56; 0.67; 0.70; 0.74; 0.88; and 0.92.	-	Eight spots detected
Dragendorff	0.53	Alkaloid	Positive
Sitroboric	0.19 and 0.76	Flavonoid	Positive
$FeCl_3$	-	Tannins and Polyphenols	Negative

this test on RF of 0.19 and 0.76 after heating the plate on 105^0C for five minutes (Figure 4, picture number 3). Last, polyphenols content was identified using $FeCl_3$ reagent. $FeCl_3$ reagent can be to detect polyphenols and tannins where positive are indicated by grey, dark green to bluish spots under the visible light as the result of the reaction between the Fe^{3+} and the hydroxyl groups. Interestingly, even though polyphenols are abundant in plants but we could not find the dark spots throughout the elution area indicating the negative result. However, there was black-greyish spot at the start line, the lower part of the plate (Figure 4, number 4). Therefore, we assume that our mobile phase was not able to separate the sample. This may happen since the solvent used was relatively less polar than the polyphenols, so the polyphenols group would have stronger bond with the stationary phase, and thus would be retained at the lower part of the plate. Based on that assumption, this result is possibly false negative, further improvement of the system is needed in order to successfully identify the polyphenols group. The complete result from the TLC step can be referred in Table 2.

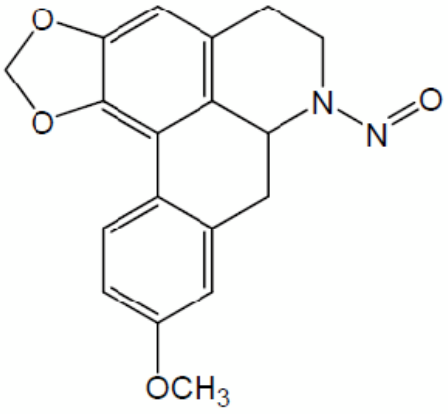

Figure 5. N-nitrosoxylopine compound found in the ethanol extract of sugar apple stem bark

Based on the previous research, the compounds responsible for the cytotoxic activity of sugar apple stem bark was acetogenins (Villo, 2008). Acetogenins are potential anticancer agents isolated from *Annonaceae* family, including sugar apple (*Annona squamosa* L). Acetogenin is an amphiphilic molecule consists of polar group binds with two lipophilic chains connected with the lactone ring (Villo, 2008), which makes acetogenins are highly possible to be extracted with semipolar solvent, especially in this experiment. Unfortunately, this research was not able to confirm the presence of acetogenins in the fraction, thus, the compounds responsible for the cytotoxic activity is still unidentifiable.

Alkaloids have been widely investigated as one of potential agents that possess significant cytotoxic activity and have been used in a cancer therapy (Mondal *et al.*, 2019). Alkaloids in *Catharantus roseus* were shown to have the ability to inhibit metaphase step in the leukemic P-1534 cell cycle in rats leading them to death (Pardhasaradhi, 2004). Another research proved that the alkaloids isolated from *Cananga odorata*, sampangine, were able to increase the formation of ROS (*Reactive Oxygen Species)* inside the cells, thus inducing their apoptosis (Wink, 2007). One of the alkaloids contained in the sugar apple stem bark called annonaine, was claimed to have various pharmacological activities (Pandey *et al*., 2011). In addition, Johns *et al*., (2011) isolated three aporphine alkaloids of the ethanol extract from sugar apple stem bark which showed effective antimalarial activity. Between these three alkaloids, there was one compound namely N-Nitrosoxylopine (Figure 5) which possessed cytotoxicity against CHO (Chinese Hamster Ovarian) cells. The mechanism of its cytotoxic activity was not clearly elucidated; however, it was reported that the nitroso group was possibly account for the activity (Figure 5). Therefore, to conclude, the semipolar fraction of the ethanol extract from sugar apple stem bark has potential cytotoxicity against T47D cells and based on the literature, it is likely that the alkaloids contained in the fraction played role in its cytotoxic activity. Considering its potential as the new option for cancer therapy, it is necessary to further isolate the major compound responsible for its cytotoxic activity and to elucidate the mechanism of action in its cytotoxicity of this fraction.

CONCLUSION

This research proved that the semipolar fraction of the ethanol extract from sugar apple stem bark is toxic towards T47D cells with the IC50 value of 70.77 µg/mL. On top of that, this fraction was indicated to have

alkaloids and flavonoids content which is possibly to be responsible for its pharmacological activity, including its cytotoxicity.

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