Phytochemical and in-vitro Cytotoxicity Study of Ethanol Extract of Fresh and Steamed Cassava Tuber (Manihot Esculenta) Against Hela Cells.

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ABSTRACT

Background
Cervical cancer is one of the most common cancers in women. Although some treatments increase survival rates, they are relatively expensive. Research shows some naturally occurring metabolites in cassava may have anti-cancer properties. This research aims to investigate the phytochemical components of cassava tuber and their cytotoxic abilities.

Methods
Extracts of fresh and steamed cassava tuber (*Manihot esculenta*) were made using ethanol as a solvent. These extracts were then tested to examine their phytochemical constituents using various phytochemical assays and thin-layer chromatography. Additionally, their cytotoxic abilities were also inspected using an MTT assay and the HeLa cell line.

Results
Ethanolic extracts of fresh and steamed *Manihot esculenta* tubers contained 2 phytochemicals. These are alkaloid and triterpenoid. They both also have 'weakly active' cytotoxic activity as the ethanolic extracts of fresh *Manihot esculenta* tuber had an IC_{50} value of 159.56µg/mL and the ethanolic extracts of steamed *Manihot esculenta* tuber had an IC_{50} value of 157.11µg/mL towards HeLa cervical cancer line.

Conclusion
Ethanolic extract of both fresh and steamed *Manihot esculenta* contained phytochemicals that show cytotoxic activity towards the HeLa cervical cancer line.

Keywords: Cassava tuber, cytotoxicity, *Manihot esculenta,* MTT assay, phytochemical

INTRODUCTION

Cancer is a type of disease that consists of alteration in cell signaling and metabolism. This results in the division and survival of abnormal cells.[1] It is the leading cause of death worldwide and decreases life expectancy in many countries. There were an estimated 19.3 million new cases of cancer worldwide, with 10 million deaths due to cancer.[2] In Indonesia, cervical cancer is rank second in the most common cancer and has an incidence of 348,809 cases. Mortality rate of this type of cancer is around 60% of the incidence, at around 207,210 deaths. If no further actions are taken, deaths caused by cervical cancer is expected to rise in the future.[3]

Amongst the types of cancers, cervical cancer is one of the most common carcinomas among women. It ranks fourth after breast cancer, colorectal cancer, and lung cancer. In 2018, there was an increase of 7.8% in the number of patients with cervical cancer, at around 570,000 patients.[4] There is a disparity in incidence rates of cervical cancer between Western and more developed countries and developing countries. Developing countries are disproportionately affected by infectious agents that can cause cervical cancer (such as the human papillomavirus, hepatitis B and C, and H.*pylori*).[5] There are many preventative measures to avert the development of cervical cancer. Important preventative measures include the administration of the HPV vaccine, undergoing screening tests (such as pap smears), and education. Treatment of cervical cancer depends on many key factors such as the staging, if it has metastasized to other regions of the body, its size, and the patient's age and overall health. The treatments include surgery, radiation, chemotherapy, or a combination of them.[6]

Survival rates of cervical cancer depend on their staging. These are 83.5%, 80.6%, 66.0%, and 37.1% for Stage I, Stage II, Stage III, and Stage IV, respectively. These are improved with treatment with the afore-mentioned methods. For example, the survival rate jumps to 95.6% when treated with surgery only.[7] However, treatment can be quite expensive. A study showed that cervical cancer patients who had surgery and those who underwent radiotherapy spent an average of USD 56,250 during the first year after receiving their diagnosis. This is a very substantial amount, especially for those who may not necessarily have the means to fund it.[8] Financial burden in cancer patients is quite common and is experienced in up to half of them, leading to worse treatment compliance and health-related quality of life.[9] Therefore, an alternative method needs to be found to lessen the burden of this disease on families. Additionally, some cancer treatments produce serious adverse effects on the body. An alternative method needs to be found that has minimum side effects on the patient's body. This is where plant-based drugs come in. Currently, they are considered the best alternative due to their minimal side effects, high tolerability to the body, and affordability.[10]

Over the past decades, natural plant products have been a source of discoveries for new drugs. These plant-based drugs have now been a crucial source of cancer treatment.[11] Antioxidants and phytochemicals that are naturally present in plants have shown anti-proliferative and pro-apoptotic abilities. This results in them being recently introduced as anti-cancer adjuvant therapies.[12] Some of examples these phytochemicals are flavonoids, taxanes, and alkaloids, amongst others. One of these vegetables is cassava (*Manihot esculenta*). They contain many phytochemicals such as saponin, tannin, oxalate, phytate, alkaloid, hydrogen cyanide, and trypsin inhibitors.[13] Additionally, cassava also contains linamarin. This secondary metabolite has some anti-cancer properties.[14,15] This is shown in a study where linamarin was extracted from cassava leaves that shows cytotoxic activity and inhibits proliferation on Raji cells. A conclusion can be drawn that cassava leaves contain metabolites that have the potential to develop as anti-cancer agents.[16] Additionally, another study showed that extracts of amygdalin, a phytochemical from cassava, showed antioxidant activity and anti-inflammatory properties. The extracts from its leaves showed anti-cancer properties in cancer-prone Wistar rats.[17] Research on different species of cassava tuber revealed that hexanoic extract of cassava Sao Pedro Petro showed the potential to be an anti-cancer candidate as it had a low IC_{50} value and can prevent the growth of P-388 murine leukemia cancer cells.[18]

Cassava is a staple food in many countries. It is included in many people's diets.[19] It is very accessible in Indonesia as well. Steaming cassava tuber is a popular way to enjoy the crop. There is little to no research detailing the phytochemical components and cytotoxicity effects of steamed and fresh cassava tuber. Ethanol was used as the solvent because it has been used in multiple studies and is a universal solvent for phytochemical analysis.[20] Both fresh and steamed cassava were used to compare their bioactivity. A study states that steaming retains vitamins and phytochemicals in vegetables better than boiling and therefore, it is important to promote this cooking method.[21] Therefore, this cooking method needs to be further analyzed. An investigation on how this cooking style can affect cassava should be done. This study aims to investigate the phytochemical constituents of fresh and steamed cassava tuber (*Manihot esculenta*) alongside its cytotoxic effect on a cervical cancer line (HeLa cells).

METHODS

Study Design

The research will be conducted in the format of an experimental research design to evaluate the cytotoxic activity of the fresh and steamed tuber of *Manihot esculenta* towards

cervical cancer cells (HeLa). The tuber will be prepared in two ways: one will be fresh and the other will be steamed. Afterwards, their cytotoxic activities will be compared. The entirety of the research will be conducted in the laboratory of the Department of Medical Chemistry Faculty of Medicine Universitas Indonesia.

Preparation of ethanol extract of fresh and steamed cassava tuber

Firstly, some of the cassava tubers ((*Manihot esculenta)*) were steamed in 30 minutes. Then, both the fresh and steamed cassava tuber were dried. 25 g of each cassava tuber was soaked in 100 mL of ethanol for 48 h. The cassava tubers underwent a multi-stage maceration process. The multi-stage maceration entails the cassava tuber to be prepared (either fresh or steamed), cut into small pieces, and dried. Then, the cassava was put into a blender so that it can become even smaller. The cassava tubers were poured into a flask of ethanol to make the extract. To obtain the filtrate, a filter paper was used. Afterwards, filtration and evaporation of the filtrate was carried out using a rotary evaporator. The extract was weighed and the next steps of the research (such as for phytochemical testing and for further analysis) was carried out. There were a number of concentrations used such as 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, and 1.625 µg/mL. The purpose of this concentration series is to find which is the appropriate concentration to use. Each test will be repeated thrice. There will be 48 samples will be used in this experiment.

Phytochemical Tests

Phytochemical tests will be conducted to determine if the cassava tuber contains saponin, flavonoid, tannin, glycoside, triterpenoid, steroid, and alkaloid.

1. Alkaloid
   1 ml of the extract is first evaporated. 5 mL of HCl 2N is then added. The extract is divided into two tubes. One will be used as a blank (tube 1) and the other will be given 3 drops of Mayer's solution (tube 2). A positive alkaloid test will result in a yellow precipitate in tube 2.
2. Flavonoid
   1 ml of the extract is first dissolved in hot water. Then, 0.5 mL of concentrated HCl is added in the test tube. Afterwards, 0.5 gram of magnesium powder is added along with 1 mL amyl alcohol and let the mixture sit. A positive flavonoid test will be indicated if the color changes to either red, orange, or green.
3. Glycoside
   1 ml of the extract is dissolved in hot water and then evaporated. 5 mL of anhydrous acetic acid is added along with 10 mL of concentrated H_2SO_4. A positive glycoside test will result in a blue or green ring.
4. Saponin
   1 ml of the extract is dissolved in hot water and mixed for 10 seconds and let sit for 10 minutes. If a stable foam is formed, 1 drop of HCl 2N is added to the mixture. The foam will stay if it is a positive saponin test.
5. Steroid and Triterpenoid
   1 ml of the extract is dissolved in hot alcohol and then evaporated. Then, 0.5 mL of anhydrous acetic acid and $CHCl_3$ is added. Afterwards, H_2SO_4 is added in through the wall of the test tube. A positive triterpenoid test will produce a violet or chocolate ring. A positive steroid test will produce a blue-green ring.
6. Tannin
   1 ml of the extract is first dissolved in hot water. Then, 1 mL of $FeCl_3$ is added. A positive tannin test will produce a dark blue or black-green color change.

Thin Layer Chromatography Analysis

A thin layer chromatography (TLC) is used to separate the different components found in each extract. The extract, TLC plate, and elution on the eluent bottle is prepared. The TLC plate will act as the stationary phase with cutting, washing, and activation whilst the elution on the eluent bottle is used for the mobile phase. The extract is placed in different spots in a TLC plate starting at 1 cm distance from the tip of the plate. The TLC plate is lowered into the chromatography tank filled with the aforementioned elution (n-hexane:ethyl acetate=3:1). The metabolites will travel up the TLC plate. After it is dried and put in an iodine vapor spot viewer, it is then observed with bare eyes and with UV light (at 366 nm). The Rf value can be found by dividing the distance traveled by the sample by the distance traveled by the solvent.

Cytotoxic Test

An MTT assay is performed as a way to test cytotoxicity capabilities. It is done after a period of incubation of more than 24 hours. The first step is to note down the number of cells in a 100 µL culture medium and then it is incubated. Then, the extract is diluted and poured into a well with HeLa cells. 100 µL of doxorubicin is added to one well to act as a positive control. Afterwards, 100 µL of MTT reagent with a concentration of 5 mg/mL is added to each well. The cells are then

incubated for 3- 4 hours at a temperature of 37°C. Observe the formazan crystals form under an inverted microscope. Afterwards, to cease all activity in the well, 100 µL of 10% sodium dodecyl sulfate (SDS) in 0.1 N of HCl is added. The plate is incubated for 24 hours and the optical density (OD) score is read through a wavelength of 540 nm. This OD will be used to determine the percentage of inhibition using the formula below.

$$\%\ \textbf{inhibition} = \frac{\textbf{OD control} - \textbf{OD concentration}\ \textbf{x 100\%}}{\textbf{OD control}}$$

OD control = optical density of blank
OD concentration = optical density of sample at certain concentration

Statistics Analysis

Data analysis will be conducted with a normality test, a comparative hypothesis testing of more than two groups, and a post hoc test if the result is significant enough on the previous tests. The normality test that will be used is the Shapiro-Wilk test as the number of samples are less than 50. A homogeneity test will be performed if the data is normally distributed. If the data is not normally distributed, the data will be transformed and the test is performed again. Next, a one-way ANOVA test will be performed as a comparative hypothesis test if the data is normally distributed. If not, the Kruskal-Wallis test will be used.

Posthoc test will be performed only if the data is significant enough after the one-way ANOVA or Kruskal-Wallis test is conducted. The Bonferonni test (if the data is normally distributed with the same variants), Tamhane test (if the data is normally distributed with the different variants), or Mann-Whitney test (if data is not normally distributed) will be performed as the posthoc test.

Data processing will be performed in SPSS and Microsoft Excel. The IC_{50} value depict at which value does the extract exhibit cytotoxic properties to 50% of the cells. To find the appropriate IC_{50} value, linear regression analysis will be used for each extract. This is done by plotting the extract concentrations against growth inhibition and trying to fit with on a straight line, in which the IC_{50} value will be calculated on. This can be demonstrated using some equations:

$$Y = aX + b$$

$$IC_{50} = \frac{(0.5 - b)}{a}$$

Research Ethics

The experiment was not conducted on humans or animals. Only cancer line cells (in-vitro) were used. We have already proposed an ethical review to the research module and have gotten ethical approval with the number No.1275/UN2.F1/ETIK/PPM.00.02/2020.

RESULTS

Phytochemical Analysis

After the *Manihot esculenta* has been through the processed and have gone through multilevel maceration, they are both submerged in ethanol extract and are labelled accordingly: F for ethanol extract of fresh *Manihot esculenta,* S for ethanol extract of steamed *Manihot esculenta*. The phytochemicals that were tested are saponin, flavonoid, tannin, glycoside, alkaloid and triterpenoid. The results of the phytochemical analysis is shown in Table 1. For both F and S, all phytochemicals tested was negative, with the exception of alkaloid and triterpenoid.

Table 1. Results of the phytochemical analysis.

Cassava Tuber Preparation	Metabolites					
	Alkaloid	Flavonoid	Glycoside	Saponin	Tannin	Steroid and Triterpenoid
Fresh Cassava Tuber	+	-	-	-	-	Triterpenoid

Thin Layer Chromatography (TLC)

A thin layer chromatography (TLC) was done for both extracts. The results were shown in Fig 1. It should be noted that Ag indicates gallic acid which tests for the presence of phenol and q denotes quercetin which tests for the presence of flavonoid. After, the retention factors (Rf) value was calculated. This is shown Table 2.

Table 2. Rf values of the TLC analysis.

Cassava tuber preparation	Rf value	Phytochemical Components
Fresh cassava tuber	0.975 (1), 0.925 (2), 0.400 (3)	3
Streamed cassava tuber	0.425 (1)	1

Figure 1. Results of the TLC analysis. 0 – mixed, all phytochemicals were used.

MTT Assay

The cytotoxicity of *M. esculenta* was assessed against HeLa human cervical cancer cell line through MTT assays. A graph with log of concentration as the x-axis against inhibtion was made. Then, linear regression was used using the equation "y=ax+b". In order to find the IC_{50}, y was substituted with 50 to find the concentration where 50% of the cells were inhibited. Because the MTT assay of each extract was done thrice, the results will be averaged and this will be used in the linear regression analysis. A positive control is also used to compare with the results, in order to ensure its validity to produce an expected results. In this case, the positive control that is used is doxorubicin.

Figure 2 shows the linear regression analysis for F. The final linear regression equation is y=49.768x-59.635 (R^2=0.9248). Table 3 shows the data for the F.

Table 3. Data of ethanolic extract of fresh *M. esculenta.*

Concentration (ug/mL)	Log Concentration	Repetition			Average	% Inhibition	IC_{50} (ug/mL)
		1	2	3			
31.25	1.4944850022	0.615	0.688	0.602	0.635	22.02210	
62.50	1.7958800170	0.464	0.511	0.825	0.600	26.32010	
125.00	2.0969100130	0.454	0.582	0.541	0.526	35.44822	159.55909
250.00	2.3979400900	0.296	0.317	0.377	0.330	59.47605	
500.00	2.6989700040	0.149	0.167	0.164	0.160	80.35203	

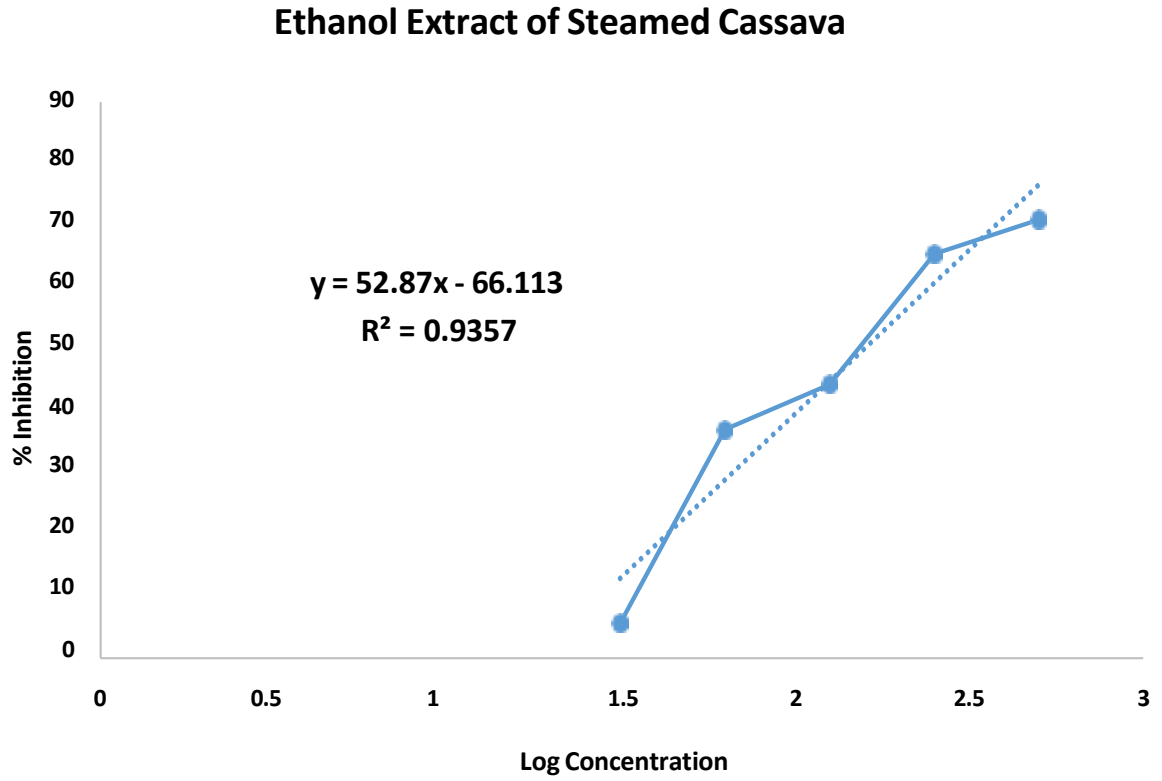

Figure 2. Linear regression analysis of F.

Figure 3 shows the linear regression analysis for S. The final linear regression equation is y=52.87x-66.113 (R^2=0.9357). Table 4 shows the data for the S.

Table 4. Data of ethanolic extract of steamed *Manihot esculenta*.

Concentration (ug/mL)	Log Concentration	Repetition			Average	% Inhibition	IC50 (ug/mL)
		1	2	3			
31.25	1.494850022	0.864	0.822	0.498	0.728	5.699482	
62.50	1.795880017	0.402	0.376	0.679	0.486	37.08981	
125.00	2.096910013	0.323	0.407	0.558	0.429	44.38687	157.10800
250.00	2.397940090	0.300	0.301	0.199	0.267	65.45769	
500.00	2.698970004	0.206	0.224	0.240	0.223	71.07081	

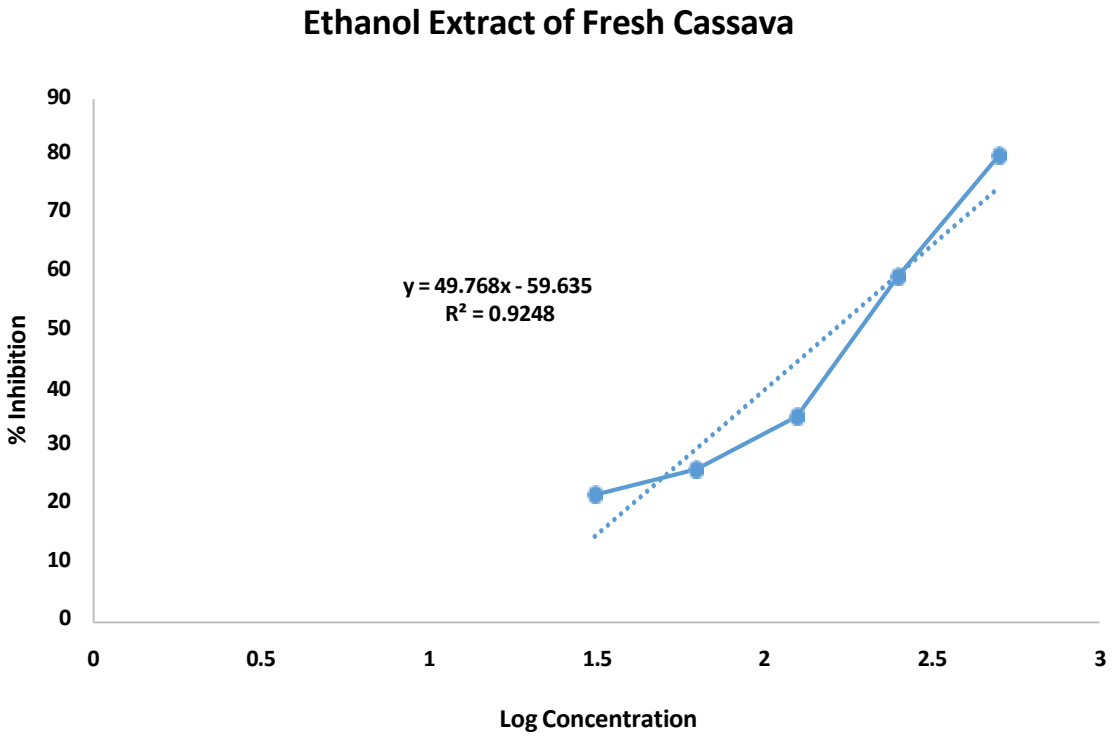

Figure 3. Linear regression analysis of S.

Figure 4 shows the linear regression analysis for doxorubicin. The final linear regression equation is y=37.057x+20.177 (R^2 = 0.9476). Table 5 shows the data for the positive control, doxorubicin.

Table 5. Data of ethanolic extract of the positive control, doxorubicin.

Concentration (ug/mL)	Log Concentration	Repetition			Average	% Inhibitio n	IC50 (ug/mL)
		1	2	3			
1.56	0.1938200260	0.823	0.746	0.494	0.688	23.25149	
3.13	0.4948500220	0.510	0.712	0.427	0.550	38.65327	
6.25	0.7958800170	0.458	0.653	0.313	0.475	47.02381	
12.50	1.0969100130	0.252	0.256	0.254	0.254	71.65179	6.37951
50.00	1.6989700040	0.130	0.149	0.119	0.133	85.19345	
100.00	2.0000000000	0.111	0.104	0.107	0.107	88.02083	

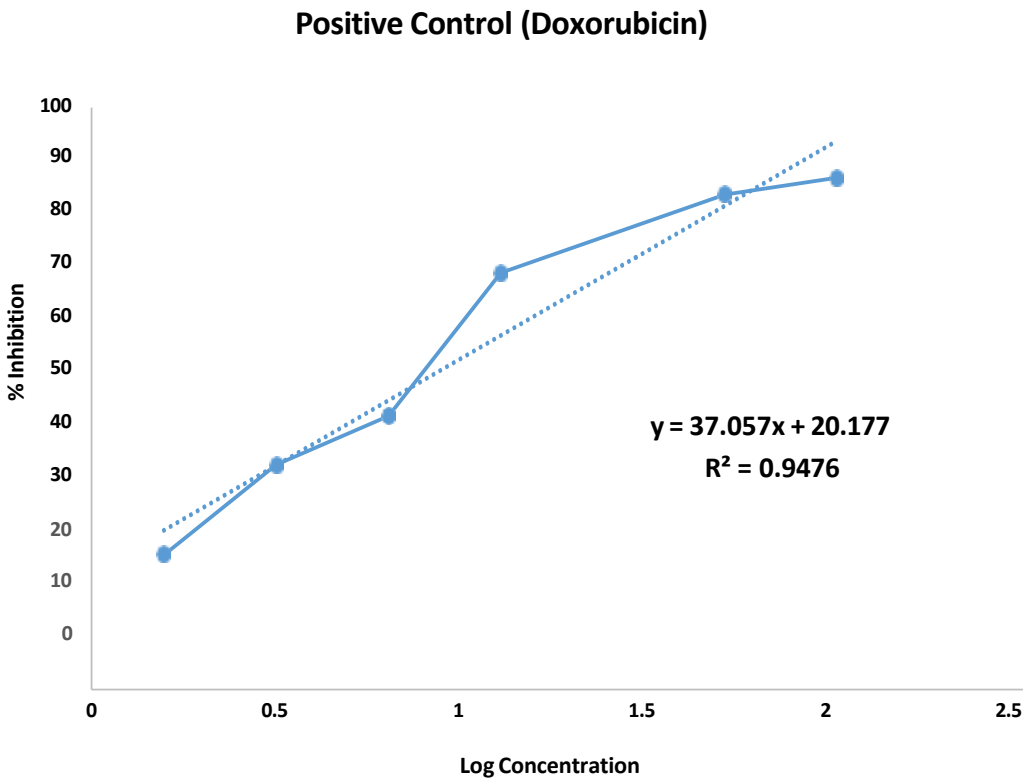

Figure 4. Linear regression analysis of Doxorubicin.

Using these linear regression lines, the IC_{50} can be found for both extracts and the positive control (doxorubicin). This can be found in table 6. The results show that both preparations of cassava (*Manihot esculenta*) were both very similar, at 159.56µg/mL for F and 157.11µg/mL for S. Nevertheless, out of the two, F had the higher IC_{50} than S. Additionally, IC_{50} of the positive control (doxorubicin) is 6.38µg/mL.

Table 6. Results of IC50 for F, S and doxorubicin.

Cassava Tuber Preparation	IC_{50} Value (ng/mL)
Fresh Cassava Tuber	159.56
Steamed Cassava	157.11
Doxorubicin	6.38

Statistical Analysis

In order to perform the parametric test of ANOVA, the data needs to be considered normal. Therefore, Sahpiro-Wilk normality test is used. It is considered normal if the p is above 0.05 (p>0.05). Figure 5 and 6 shows the results of the normality test for doxorubicin, F and S. All normality tests indicate the data is used. Therefore, ANOVA test can be used. The results of the ANOVA test can be seen in Figure 7. The results of the ANOVA shows that there is no statistically significant difference between the ethanolic extracts of fresh *M. esculenta* and the ethanolic extracts of steamed *M. esculenta* (p>0.05).

Tests of Normality[a,c,d,e,f,g]

	Extract	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
PIConc1	Doxorubicin	.299	3	.	.914	3	.431
PIConc2	Doxorubicin	.273	3	.	.945	3	.548
PiConc3	Doxorubicin	.206	3	.	.993	3	.838
PIConc4	Doxorubicin	.175	3	.	1.000	3	1.000
PIConc5	Doxorubicin	.236	3	.	.977	3	.708
PIConc6	Doxorubicin	.204	3	.	.993	3	.843

Figure 5. Normality test of % inhibition of doxorubicin

Tests of Normality

	Extract	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
PIConc1	Ethanol Fresh C	.334	3	.	.860	3	.269
	Ethanol Steamed C	.330	3	.	.867	3	.286
PIConc2	Ethanol Fresh C	.193	3	.	.997	3	.892
	Ethanol Steamed C	.367	3	.	.793	3	.099
PiConc3	Ethanol Fresh C	.259	3	.	.959	3	.609
	Ethanol Steamed C	.227	3	.	.983	3	.749
PIConc4	Ethanol Fresh C	.288	3	.	.928	3	.482
	Ethanol Steamed C	.381	3	.	.760	3	.022
PIConc5	Ethanol Fresh C	.328	3	.	.871	3	.298
	Ethanol Steamed C	.269	3	.	.949	3	.567

Figure 6. Normality test of % inhibition of F and S.

ANOVA

IC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	124.980	1	124.980	.060	.819
Within Groups	8378.963	4	2094.741		
Total	8503.943	5			

Figure 12. Results of the ANOVA test.

DISCUSSION

Phytochemical Components of *M. esculenta*

There are various phytochemicals that were tested which are saponin, tannin, flavonoid, glycoside, alkaloid, steroids and triterpenoid. These phytochemicals are beneficial for the human body. Saponin have been described to have some pharmacological activity with its ability to modulate the immune system through its interaction with cytokines, and its cytostatic and cytotoxic effects on various malignant tumour cells, including HeLa human epithelial cervical cancer line, MCF-7 human breast cancer line, and HT-29 human colon cancer cell line.[22] Studies show that tannins exhibit anti-inflammatory, anti-fibrotic, antioxidant, and anti- cancer properties.[23,24] Additionally, it also has activity against pathogenic bacteria, parasites, and viruses.[24] Flavonoids has some anticancer properties. They are able to regulate ROS by scavenging enzyme activities, stopping the cell cycle, bring about apoptosis and autophagy, and restrain proliferation of cancer cells and decrease its invasiveness. They have a dual-action property in ROS homeostasis. They are able to act as antioxidants in normal conditions and are able to act as pro-oxidants in cancer cells. This allows them to be able to trigger apoptotic pathways and downregulate pro- inflammatory signalling pathways.[25] Glycoside exhibit strong anticancer abilities as they supress cell proliferation and activate cell death by autophagy or apoptosis.[26] Alkaloids have a supressing effect on the topoisomerase enzyme, thereby stalling DNA replication and cell death. They are also able to restrain oncogenesis.[27] Triterpenoids have a wide range of function that are related with its anticancer property. They are able to supress nuclear factor-κB (NF-κB) activation and signal transduction, angiogenesis, apoptosis, cell proliferation, mitochondrial dysfunction, and control of MDR genes and proteins.[28]

The results the phytochemical assays of both extracts (F and S) can be seen in table 7. Ethanolic extract of fresh *Manihot esculenta* (F) shows that there are 2 phytochemicals present. These are alkaloid and triterpenoid. This is the same case for the ethanolic extract of steamed *Manihot esculenta* (S). It also has 2 phytochemicals present which are alkaloid and triterpenoid. One research shows that raw *Manihot esculenta* contains alkaloids, flavonoids, and tannins.[29] Another research shows that the levels of the phytochemicals depend on the age of the tuber. It is stated that some phytochemicals, namely alkaloids and saponins, increase with age, whilst others, specifically flavonoids and tannin, reduce with age.[30] Additionally, a different study shows that the phytochemical constituent of *Manihot esculenta* are phenolic compounds, flavonoids, and alkaloids.[31] There has been no literature that looks into the phytochemical constituents of steamed *Manihot esculenta*. There are some similarity from the aforementioned studies and in this research, mainly where alkaloid is found in the ethanolic extract of fresh/raw *Manihot esculenta*. However, the studies did not mention any presence of triterpenoid. The difference in phytochemical constituents might be because the specific species looked in the aforementioned research might be different to this research. Furthermore, many factors (such as soil quality, and other environmental factors) might affect the levels of phytochemicals present in the tuber. Additionally, as suggested in a study, the levels of phytochemicals might be different due to the age of the *Manihot esculenta.*

The act of steaming vegetables can change the phytochemicals constituents. This is due to two main mechanisms which are thermal degradation and matrix softening effect. Thermal degradation reduces the concentration of the phytochemicals but the matrix softening effect increases the extractability of phytochemicals, therefore, increases its concentration. However, it should be noted that there are some factors can greatly affect the phytochemical concentration during cooking, such as the cooking parameters, structure of the food, the specific chemical nature of the compound itself. One study claims that steaming is a good technique to ensure a better preservation/extraction yield of phenols and glucosinates than other cooking methods. Additionally, because the food is not in direct contact with the cooking material itself, there is minimal leaching of the soluble compound. This way, thermal degradation is limited.[32] A study on tomatoes suggested that steaming at a higher temperature and a longer time improved phenolic and flavonoid content.[33] This suggests that time and temperature has an effect on the phytochemical constituents. However, a study showed that the phenolic content of steamed *Portulaca* decreased when compared to its raw counterpart.[34] Another study on potatoes showed that there is a decrease in total phenolic and phenolic acid activity when steamed. However, compared to other cooking methods, steaming is one of the best way to retain more phytochemical.[35]

Colchicine, an alkaloid that can be extracted from *Colchcum autumnale*, demonstrate cytotoxic activity. It is able to bind to ends of microtubules and stop

microtubule polymerization. Colchine is able to do this by interacting with soluble tubule to create tubuline- colchcine (TC) complex. This results in the combination of microtubular ends and will disrupt the tubulin lattice dynamics. Additionally, it is a well-known anti-mitotic agent and is able to prevent mitotic cells from entering metaphase. Moreover, colchicine can prevent cancel cell movement and metastatic potential, cell blebbing using the Rho/Rho effector kinase/myosin light chain kinase pathway, restrict the entering of ATP to mitochondria, and release caspase protease and cytochrome- *c* which results in apoptosis of the cells.[27] Triterpenoids and its derivatives have shown anti-cancer activities. A study reported that some triterpenoids are able to decrease the expression of specificity protein (Sp) transcription factor and many pro-oncogenic Sp-regulated genes in various cancer cell lines. They are able to induce ROS which can decrease expression of Sp transcription factors. Additinally, they can also activate or deactivate nuclear receptors and G-protein coupled receptor.[36]

Thin Layer Chromatography (TLC) of *Manihot esculenta*

As shown in table 2 and figure 6, there are 3 phytochemicals present in the ethanolic extract of fresh *Manihot esculenta,* whilst there is only 1 phytochemical present in the ethanolic extract of steamed *Manihot esculenta* when TLC is done. There is some disparity between the TLC and the phytochemical assays. In the TLC of ethanolic extract of fresh *Manihot esculenta*, the first and second spot, denoted by 1 and 2, might be alkaloid and triterpenoid as the Rf value is similar to the spots in the 0 or mixed column. The third spot might be due to a phytochemical present that was not tested during the phytochemical assay tests. The missing phytochemical that was present in the phytochemical assay but not in the TLC might be due to it being in very low amount and, therefore, it couldn't be detected. In the TLC examination, gallic acid tests for phenol whilst quercetin tests for flavonoid. The Rf value of gallic acid and quercetin is

0.125 and 0.3 respectively. There are no spots in both the ethanol extract of fresh and steamed cassava tuber (*Manihot esculenta*) that have the same Rf value. Therefore, based on the TLC and the Rf value of these standards, the ethanol extract of both fresh and steamed cassava tuber (*Manihot esculenta*) do not contain any phenolic and flavonoid.

Cytotoxic Evaluation of fresh and steamed *Manihot esculenta*

To evaluate the cytotoxicity of the extracts, an MTT assay was done against HeLa cervical cancer cell line. An MTT assay uses the basic principle of mitochondrial activity in every living cell. The greater the mitochondrial activity, the greater the amount of living cells. In a mitochondrium, a cellular reductase enzyme is present that can change the tetrazolium compound in the MTT reagent into a purple formazan crystal. Cytotoxicity activity is when there is low mitochondrial activity, therefore this means that it is when tetrazolium compound has not changed to purple. A low mitochondrial activity indicates a low amount of living cells. After the MTT assay method is done, the absorption of the sample is read in 595 nm. The final purpose was to obtain an IC_{50} value, the exact concentration that can inhibit 50% of the activity of cancer cells. The IC_{50} values can be seen in table 7. The IC_{50} is very similar in both extracts with the ethanolic extract of fresh *Manihot esculenta* having an IC_{50} of 159.56µg/mL, whilst the ethanolic extract of steamed *Manihot esculenta* has an IC_{50} of 157.11µg/mL. On the other hand, the positive control, doxorubicin, has a very low IC_{50} which is 6.38µg/mL. Doxorubicin is a chemotherapeutic drug derived from *Streptomyces peucetius* bacterium. The IC_{50} values are evaluated based on a study from Atjanasuppat *et al.* This accepted classification can be seen in table 7.[37]

Table 7. Classification of cytotoxity based on IC_{50}.[37]

IC_{50} (µg/mL)	Classification
<20	Active
20-100	Moderately Active
100-1000	Weakly Active
>1000	Inactive

When using the classification in the table 7, both of the ethanol extracts of fresh and steamed *Manihot esculenta* are in the 'weakly active' category. On the other hand, doxorubicin has a 'active' classification. This suggests that doxorubicin is still a more effective compound as its classification is higher than the extracts.

There are limited research on the cytotoxicity activity of *Manihot esculenta* on HeLa cancer line. However, there is some research about this shrub on other types of cancer. A study reported that ethanolic extract of different cultivars of *Manihot esculenta* yielded an IC_{50} of 58.497µg/mL, 90.311µg/mL, and 61.218µg/mL against P-388 murine leukemia cancer cells.[18] All of the results are in the 'moderately active' category based on table 7. The IC_{50} is higher in this research, suggesting lower cytotoxicity level, which

could be due to different phytochemical constituents as well as different levels of it in the *Manihot esculenta* being used in this research. As mentioned above, specific phytochemicals have different mechanisms to show their cytotoxic activity. Therefore, the specific phytochemical constituents that may be present in this research may have different mechanism of cytotoxic activity that could not be detected using the MTT assay, showing a low IC_{50} value. Normality tests were conducted and it showed that the data is normal. The ANOVA test showed that there is no statistically significant difference both of the the ethanolic extracts of fresh *Manihot esculenta* and the ethanolic extracts of steamed *Manihot esculenta*.

Limitation of Study

There was a limited amount of *Manihot esculenta*, therefore there was a limited amount of extract that could be used. Other cancer lines were not used, hence comparisons cannot be made. Additionally, only one solvent (ethanol) was used and comparisons amongst solvents could not be made.

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