



THE INVESTIGATION OF CYTOTOXIC EFFECT OF *CINNAMOMUM ZEYLANICUM* EXTRACTS ON HUMAN BREAST CANCER CELL LINE (MCF-7)

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ABSTRACT

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Cinnamon is one of the oldest herbal medicine that has been historically known to possess anticancer property. In this study, the cytotoxicity of the cinnamon extracts towards human breast cancer cell line (MCF-7) was investigated. *Cinnamomum zeylanicum* was extracted using Soxhlet and water extraction methods, producing 7.06% and 2.54% of yield respectively. The extracts were analysed using Gas Chromatography/ Mass Spectrometry (GCMS) and it was found that cinnamaldehyde is the major constituents which contribute more than 70% of the major constituents. The cytotoxicity of *C. zeylanicum* extract against MCF-7 was determined through colorimetric MTT assay at 24 and 48 hours of incubation periods. The IC₅₀ values of Soxhlet extract were 58 µg/ml and 140 µg/ml while the IC₅₀ values of water extract were 9 mg/ml and 4.8 mg/ml, at 24 and 48 hours treatment respectively. In conclusion, *C. zeylanicum* extracts has been confirmed to have cytotoxic effect towards MCF-7 cell line in different concentrations at two different incubation periods.

1. INTRODUCTION

Cancer is one of the major health problems worldwide and it is one of the main causes of human death nowadays. According to American Cancer Society, cancer is the second most common cause of death in US, after heart diseases [1]. The total death rate due to cancer rose significantly in 20th century because of tobacco epidemic. The rate peaked in 1991 at 215 cancer deaths per 100,000 persons. However, the rate reduced by 23%, which equals to 1.7 million cancer deaths, from 1991 to 2012 due to smoking reduction and improvements in early detection and treatment. For the year of 2016, it is expected that about 1,685,210 new cases of cancer will be diagnosed in United States and from that figure it is estimated that 595,690 of cancer deaths will be recorded, which corresponds to approximately 1,600 deaths per day [1,2]. A group researcher added that the most common cancers for men in America are prostate, lung and bronchus, and colorectal cancers, which accounts for 44% of all cases in men [2]. Meanwhile, breast cancer is reported to be the most common type of cancer among women in America which contribute to 29% of cancer cases diagnosed.

Some researcher also reported that breast cancer is the most common type of cancer among women [3]. It is caused by many factors including genetic, familial, hormonal and environmental factors. Unfortunately, it is difficult to reduce the risks of breast cancer because mostly it involves complex hormonal responses, high obesity rates and also elevated levels of blood estrogen among menopausal women. There are many therapies used to remove malignant cells nowadays, commonly used method is chemotherapy. Cisplatin, adriamycin and taxol are some of the various compound that are used in cancer chemotherapy [4].

However, people recently get back to natural resources instead of modern methods as an alternative to treat cancer. In another study highlighted that there are several plants that can be a new source for the production of novel anticancer agents [5]. The plants they have tested are ginger (*Zingiber officinale*), lemon (*Citrus lemon*), grapefruit (*Citrus paradisi*), jasmine (*Jasminum grandiflora*), thyme (*Thymus vulgaris*), chamomile (*Matriaria chamomilla*), rose (*Rosa damascene*) and also cinnamon (*Cinnamomum zeylanicum*).

Cinnamon can be considered as one of the oldest herbal medicine since it has been mentioned in Chinese texts for over 4000 years ago [6]. They cited the traditional uses of cinnamon which are as flavoring agent, insecticidal, antifungal and also as a treatment of dental problems. Nowadays cinnamon is used in pharmaceuticals preparation, perfume production, chemical industry and in food industry. They emphasized that the most important components of cinnamon is cinnamaldehyde, which contributes to the fragrance and various biological properties of cinnamon [6]. Some researcher in their study reported that there are several biological activities that can be related to cinnamaldehyde which are antitumor, antifungal, cytotoxic and anti-mutagenic activities [4]. Besides that, a study also stated that cinnamaldehyde can exhibits antifungal, antipyretic, antioxidant, antimicrobial and larvicidal activities [7]. Additionally, they cited that through anticancer studies, cinnamaldehyde is found to be active against human liver, lung and leukemia cancer cells.

In this study, the cytotoxicity of the cinnamon extract towards cancer cells was investigated, specifically on human breast cancer cell line (MCF-7). MCF-7 cell line was grown in Rosewell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic. The extracts of *Cinnamomum zeylanicum* were obtained using Soxhlet and water extraction methods and analysed using Gas Chromatography/ Mass Spectrometry (GCMS). The cytotoxic effect of the extracts towards the cells was assessed by using MTT assay.

2. METHODS

2.1 Preparation of *Cinnamomum zeylanicum* Extracts

2.1.1 Soxhlet Extraction

One hundred grams of dried *Cinnamomum zeylanicum* bark was crushed and ground completely using homogenizer. The *C. zeylanicum* powder was filled into a thimble and was subjected to Soxhlet extraction at 90 °C for 24 hours using methanol as the solvent. The extract was then collected and concentrated using rotary evaporator at 60 °C until the solvent was dried. The extract was placed in the drier until it dried completely. Then, it was powdered using pestle and mortar and was

stored at room temperature for future use.

2.1.2 Water Extraction

Another 80 g of dried *C. zeylanicum* bark was crushed and ground completely using homogenizer. After that, 400 ml of distilled water was added to the powder and the mixture was stirred on magnetic stirrer at 80 °C for 15 hours. Later, the extract was filtered using cheese cloth and transferred into falcon tube. The filtered extract was then stored in -80 °C freezer until it completely frozen. Then it was subjected to freeze drier for 1 week. Lastly the extract was weighed and stored at room temperature.

2.2 Gas Chromatography/ Mass Spectrometry (GCMS) Analysis

Prior to the analysis, 0.01 g of cinnamon Soxhlet extract and 0.05 g of cinnamon water extract were dissolved in 1 ml methanol separately. Both of the dissolved extracts were filtered using 0.45 µm filter syringe. The volatile composition of the extracts were analysed using Auto System XL Gas Chromatography and TurboMass Gold Mass Spectrometer (Perkin Elmer). The carrier gas used was helium at a flow rate of 1.3 ml/min. The injector and detector were set at the temperature of 250 °C. The identification of compound was based on the comparison of retention time and peak area of authentic standards from sources such as National Institute of Standards and Technology (NIST) libraries and by comparing with previous works reported.

2.3 Maintenance of MCF-7 Cell Lines

2.3.1 Thawing of Cells

Complete growth media was first prepared by mixing 10% FBS and 1% Penicillin-streptomycin into RPMI medium. As the cells were obtained from -80 °C storage, the vial was rubbed against palms until the cells melted. Then, quickly the cell was transferred into falcon tube under the biosafety cabinet. The cells were added drop wisely with 5 ml of growth media before being centrifuged at 1500 rpm at 4 °C for 7 minutes. Supernatant was removed, and another 5 ml of growth media was added and it was again centrifuged to ensure complete removal of DMSO from freezing medium. It is important to completely remove DMSO from the cells as DMSO is detrimental at temperatures above -70 °C. The pellet was resuspended in 5 ml growth media. Then the cells were transferred into T25 flask and were incubated at 37 °C with 5% CO₂. The cells were left undisturbed for a few days to give enough cell-cell contact to multiply.

2.3.2 Changing of medium

The growth medium was changed every 2 days or by depending on the color of the medium. Rapid growth caused the color of medium to change from red to yellowish due to production of acid. Therefore, yellowish medium indicated that the medium had to be refreshed or changed. To change the medium, the flask was first taken out from the incubator and placed under the biosafety cabinet. First, the media was aspirated out. Then 5 ml of complete growth media was added, and the flask was incubated back in the incubator.

2.3.3 Passage of Cells

A confluent flask was taken out from the incubator and placed under the biosafety cabinet. All of the media was aspirated out and 1 ml of 50% trypsin was added into the flask. The flask was then being incubated for 3 minutes until all of the cells were detached. After 3 minutes of incubation, cell detachment was observed under the inverted microscope. The cell suspension was transferred into falcon tube and was centrifuged at 1500 rpm at 4 °C for 7 minutes. The pellet was resuspended in 10 ml growth medium and it was transferred into two new T25 flasks, 5 ml in each flask. The flasks were then being incubated in 5% CO₂ at 37°C.

2.4 Cytotoxicity Test

2.4.1 Seeding of Cells

Two 96-well plates were seeded for cell treatment with incubation period of 24 hours and 48 hours. For each plate, one confluent flask was taken out from the incubator and placed under the biosafety cabinet. The growth media was pipetted out and PBS was added to wash the cells. One millilitre of trypsin was added and the flask was incubated for 3 minutes until the cells detached. Cell detachment was observed under inverted microscope. As the cells completely detached, 9 ml of complete media was added to the flask, making a total volume of 10 ml. Then the cell

suspension was transferred into a sterile dish. Using a multichannel pipette, 100 µl of cell suspension was added to each well of 96-well flat-bottomed plate. The plate was incubated in 5% CO₂ at 37°C. The seeding density was based on the growth characteristics of the cells and was chosen to avoid 100% confluency of untreated cells [8].

2.4.2 Cell Treatment

2.4.2.1 Treatment with *Cinnamomum zeylanicum* Soxhlet Extract

Firstly, stock solution was prepared by dissolving *C. zeylanicum* Soxhlet extract powder in DMSO to a concentration of 50 mg/ml. From the stock solution, working solution of 1600 µg/ml was prepared by diluting 28.8 µl of stock solution with 871.2 µl of complete media, making the total volume of 900 µl. The media from first well was removed and 200 µl of 1600 µl/mg extract was filled into the well. Then 100 µl from the first well was pipetted into the second well without removing the media, making the extract became double-diluted into 800 µl/ml. The serial dilution was continued to produce working solution of 400, 200, 100, 50, 25, 12.5 and 6.25 µl/ml. The cells were treated in triplicate for each concentration. Untreated cells or wells containing cells but no extract was used as control. Then one plate was incubated for 24 hours and another plate for 48 hours.

2.4.2.2 Treatment with *Cinnamomum zeylanicum* Water Extract

The stock solution of *C. zeylanicum* water extract was prepared at 100 mg/ml by mixing 0.2 g of the extract powder with 2 ml of complete media. It was then mixed thoroughly using vortex and stored at room temperature until use. The concentrations of working solutions used were between 0.39 and 6.25 mg/ml, also prepared in the well by using serial dilution method. All concentrations were tested in triplicate and in two incubation period which were 24 and 48 hours.

2.4.3 MTT Assay

Incubated plates were taken out from the incubator and placed under the biosafety cabinet. The media from each well was aspirated and being replaced with 100 µl of new complete media. Then 20 µl of MTT solution was added to each well and the cells were incubated for 4 hours in the incubator. After 4 hours, the media and MTT solution was removed and 100 µl of DMSO was added to each well. Then the absorbance was read using a 96-well micro plate reader at 570 nm to analyse the viability of cells.

3. RESULT AND DISCUSSION

3.1 Extraction of *Cinnamomum zeylanicum*

Table 1: Total Yield of *C. zeylanicum* Extraction

Type of Extract	Sample weight (g)	Extract weight (g)	Yield (%)
Soxhlet Extract	100	7.0633	7.06
Water Extract	80	2.0287	2.54

Table 1 shows the yield of *C. zeylanicum* extracts obtained from Soxhlet and water extraction method. The total yields were calculated using the formula used by a researcher [9]:

$$\text{Percentage of total yield (\%)} = \frac{\text{Weight of extract powder (g)}}{\text{Weight of ground } C. zeylanicum \text{ (g)}} \times 100\%$$

The Soxhlet extraction was done using methanol as the solvent. The extraction of 100 g of ground *C. zeylanicum* produced 7.06 g of dark brown powder, which equal to 7.06% of total yield. The yield obtained was almost the same as the Soxhlet extraction, which attained 6.84% of yield. The yield obtained by a group researcher was slightly lower possibly due to the extraction period was shorter which were 10 hours, compared to 24 hours of extraction period in this study. Meanwhile for the water extraction of *C. zeylanicum*, 2.03 g of extract was obtained, which equal to 2.54%. This value is quite similar to water extract produced by Syahila (2012) which was 2.6%. Comparing the total yield of Soxhlet extraction and water extraction of *C. zeylanicum*, Soxhlet extraction is observed to produce more extract. This finding proves a statement a group scientist who mentioned that organic solvent such as methanol is more effective than water for extraction.

3.2 Gas Chromatography/ Mass Spectrometry (GCMS) Analysis

Since it is invented, gas chromatography has been the method of choice to analyse different substances in a test sample. In identifying the chemical constituents, various combination of GC techniques is used. GC

technique in combination with mass spectrometry is the most powerful technique [10]. Therefore, GCMS was applied in this study to analyse the constituents in *C. zeylanicum* extracts.

Table 2: Major Constituents of *C. zeylanicum* Extracts

No.	Major Chemical Constituent	Retention Time (min)	
		Soxhlet Extract	Water Extract
1.	Cinnamaldehyde	16.05	16.79
2.	Cinnamyl alcohol	16.96	17.70
3.	Eugenol	18.18	18.92
4.	Methoxy cinnamaldehyde	22.59	23.29
5.	Hexadecenal	28.62	28.74
6.	Hexadecenoic acid	31.27	29.23

The major contents of *C. zeylanicum* extract are tabulated in Table 2 shown above. There were 6 major peaks observed in the chromatograph, indicating 6 major chemical constituents detected in the extract. Ordered in increasing retention time, the major constituents are cinnamaldehyde, cinnamyl alcohol, eugenol, methoxy cinnamaldehyde, hexadecenal and hexadecenoic acid. A group scientist explained that depending on its chemical properties, compounds in the sample travel with different velocities and get separated in the column [11]. Consequently, individual compounds reach the detector at different retention time. This list of constituents is coherent with a study by a researcher whom proved that there were 9 major volatile compounds in cinnamon, including aldehydes, alcohols, alkenes, carboxylic acid, ether, ester and ketone [9].

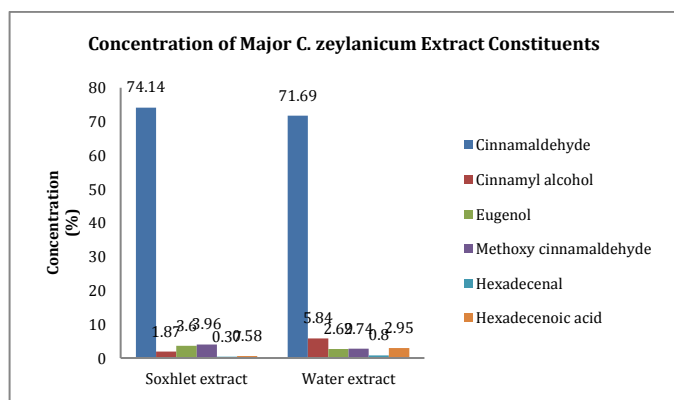


Figure 1: Concentration Major of *C. zeylanicum* Extracts Constituents

Figure 1 represents the concentration of the extract of *C. zeylanicum* based on GCMS analysis. The classification of compound was made based on the percentage of relative peak area since it represents the concentration of chemical constituents in the extracts. It can be clearly seen that cinnamaldehyde was the major components in both Soxhlet and water extract which contributed to 74.14% and 71.69% of total constituents, respectively. This result is parallel with many previous studies [6,7,12-14]. These studies showed that cinnamaldehyde is the major component in *C. zeylanicum* which contribute from 60% to 80% of total components. The percentages of cinnamaldehyde detected might vary due to different extraction method, different solvent, such as hexane, petroleum ether, dichloromethane and ethanol, and different period of extraction were used during the extraction.

3.3 Cell Viability

MTT assay was done to test the cytotoxicity of *C. zeylanicum* Soxhlet and water extract towards human breast cancer cell line MCF-7. MTT is a yellow solution of tetrazolium salt that can be cleaved by living cells into purple coloured formazan [15]. Therefore, more formazan produced indicates higher number of living cells available. In this study, MTT assay was carried out using different concentration of *C. zeylanicum* extracts.

For Soxhlet extract, the concentrations tested were 1600, 800, 400, 200, 100, 50, 25, 12.5 and 6.25 µg/ml. Meanwhile for water extract of *C. zeylanicum*, the extract concentrations used were between 0.39 and 6.25 mg/ml. On every test, cells with no extract added were prepared as the control. Two different incubation periods were used which were 24 hours and 48 hours. The amount of formazan produced was quantified by using microplate reader and the percentage of cell viability was calculated. The data was presented as charts of cell viability against extract concentration. The percentage of viability was calculated using the formula by a scientist:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control}} \times 100\%$$

In analysing the viability percentage of cells tested with *C. zeylanicum* Soxhlet extract, the absorbance readings from the two highest concentrations of extracts, 1600 and 800 µg/ml, were omitted. This is due to high percentage of DMSO in the extract which was 3.2% and 1.8% respectively. Based on personal communication with Dr Wastuti, the maximum percentage of DMSO that cells can tolerate is only 1% since DMSO is hazardous and possesses cytotoxic property. High percentage DMSO can kill the cells thus resulting in low absorbance reading. This could have masked the cytotoxic effect of *C. zeylanicum* extract towards MCF-7.

3.3.1 MCF-7 Cell Viability in *C. zeylanicum* Soxhlet Extract

Table 3 shows the concentration and time dependent effect of *C. zeylanicum* Soxhlet extract on MCF-7 cell line. The data was presented in the form of bar chart in Figure 2. It can be clearly seen that generally, the cell viability decreased with the increasing of extract concentration in both period of incubation. The cell viability was highest in lowest concentration of extract and vice versa. Focusing on cell viability at 24 hours incubation, the cell viability was at the highest, which was 81.66 ± 5.5%, at the lowest extract concentration, 6.25 µg/ml. The percentage of viable cell decrease steadily to 57.29 ± 7.5% as extract concentration was increased up to 50 µg/ml but it dropped significantly to 21.73 ± 3.47% in 100 µg/ml of extract. The viability of MCF-7 was lowest in 400 µg/ml of *C. zeylanicum* extract with viability percentage of 13.09 ± 1.3%.

Table 3: Cell Viability in *C. zeylanicum* Soxhlet Extract ± SD

Soxhlet Extract Concentration (µg/ml)	Cell Viability (%)	
	24 Hours	48 Hours
0 (control)	100 ± 0.1	100 ± 0.04
6.25	81.66 ± 5.5	80.88 ± 8.0
12.5	76.22 ± 10.7	78.98 ± 11.8
25	71.81 ± 10.8	76.28 ± 7.1
50	57.29 ± 7.5	77.69 ± 4.4
100	21.73 ± 3.5	71.28 ± 0.7
200	23.49 ± 3.4	35.38 ± 4.7
400	13.09 ± 1.3	14.50 ± 0.7

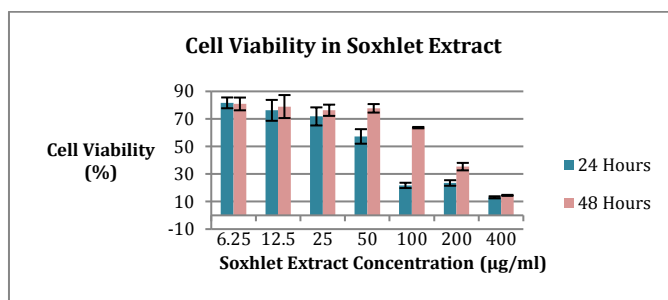


Figure 2: Percentage of MCF-7 Cell Viability in *C. zeylanicum* Soxhlet Extract

For the cells that were incubated with Soxhlet extract for 48 hours, the lowest concentration of 6.25 µg/ml gave the least cytotoxic effect as the viable cell was at the highest percentage, 80.88 ± 8.0%. Not much difference in cell viability was observed when the cell was incubated in 12.5, 25 and 50 µg/ml of *C. zeylanicum* extract, which were 78.98 ± 11.8%, 76.28 ± 7.1% and 77.69 ± 4.4% respectively. Meanwhile in the highest concentration of extract, 400 µg/ml, the percentage of viable was at the lowest, 14.50 ± 0.7%.

3.3.2 MCF-7 Cell viability in *C. zeylanicum* water extract

Table 4: Cell Viability in *C. zeylanicum* Water Extract ± SD

Water Extract Concentration (mg/ml)	Cell Viability (%)	
	24 Hours	48 Hours
0.39	91.75 ± 26.50	91.39 ± 0.21
0.78	124.86 ± 18.96	83.84 ± 5.58
1.56	94.64 ± 27.73	83.92 ± 3.27
3.13	75.55 ± 20.80	83.15 ± 11.07
6.25	59.43 ± 16.72	17.13 ± 0.79

Table 4 shows the concentration and time dependent effect of *C. zeylanicum* water extract on MCF-7 cell line while Figure 3 depicts the data in bar chart. From the result obtained, it is observed that the growth of the cell line was inhibited in a concentration-dependent manner for 24 and 48-hour exposure to water extract of *C. zeylanicum* (0.39-6.25 mg/ml). Generally, the cell viability decreases as the extract concentration increase. For each test, the initial cell viability percentage was approximately 91%. The extract showed significant cytotoxicity on both incubation times at 6.25 mg/ml. Viability of MCF-7 was inhibited 35% and 81% after 24 and 48 hours of incubation with 6.25 mg/ml of extract, respectively.

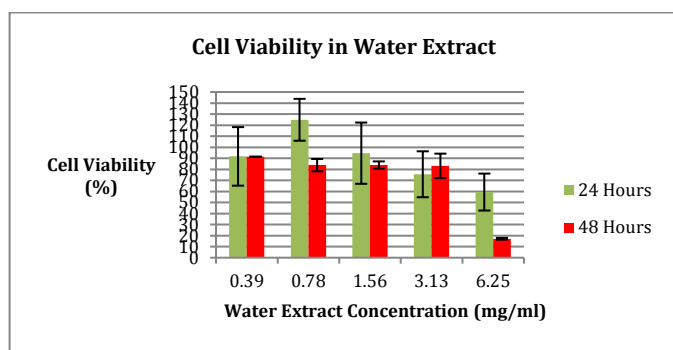


Figure 3: Percentage of MCF-7 Cell Viability in *C. zeylanicum* Water Extract

3.4 Half Inhibitory Concentration (IC50)

A study by a researcher stated that MTT assay is a common method to measure drug sensitivity in established cell lines [16]. The drug sensitivity can be identified from the concentration of drug needed to achieve 50% of growth inhibition as compared to the growth of untreated control. This concentration is called 50% inhibitory concentration, IC50. In other word, IC50 is the value of concentration when the cell viability is at 50%. The value can be determined by extrapolating the graph at 50% cell viability.

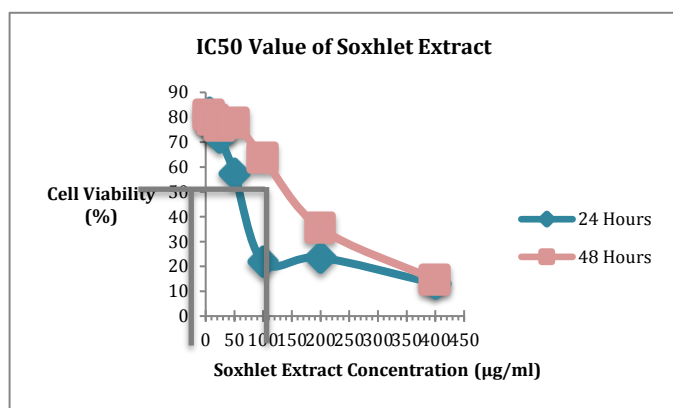


Figure 4: IC50 of Soxhlet Extract

Figure 4 illustrates the half inhibitory concentration (IC50) values of *C. zeylanicum* Soxhlet extract towards human breast cancer cell line MCF-7 at both 24 hours and 48 hours of incubation. Based on Figure 4, it is observed that the IC50 at 24 hours of incubation was lower than 48 hours. The IC50 of *C. zeylanicum* Soxhlet extract towards MCF-7 at 24 hours of incubation period was about 58 µg/ml. Meanwhile when

incubated for 48 hours, the IC50 value was 140 µg/ml. This means that *C. zeylanicum* Soxhlet extract worked better when being incubated for 24 hours. However, these values were higher than what have been reported in previous studies. A group of scientists stated that the IC50 of *C. zeylanicum* extract on MCF-7 was 14.98 µg/ml at 48 hours of incubation while other researcher cited that the IC50 was 3.75 µg/ml at 76 hours and in other studies showed that the IC50 was less than 20 µg/ml [17-19]. So, it can be said that the *C. zeylanicum* extract obtained in this study does have cytotoxic effect towards MCF-7 but it appeared to be less effective compared to those in previous study as higher extract concentration was needed to decrease the cell viability to 50% of total viable cell [20-28].

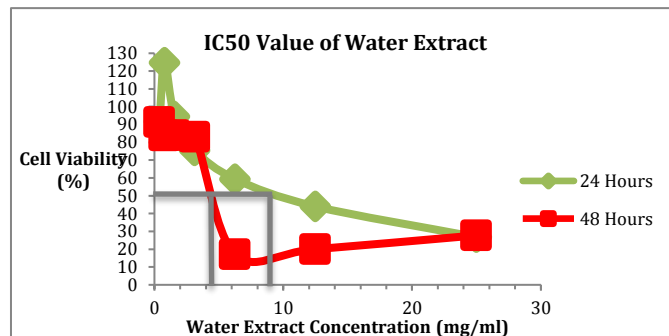


Figure 5: IC50 of Water Extract

The IC50 values of *C. zeylanicum* water extract towards MCF-7 cell line at 24 and 48 hour treatments are figured out from Figure 5. Unlike the treatment with Soxhlet extract, the cell treatment with *C. zeylanicum* water extract showed smaller IC50 value at 48 hours of incubation period as compared to 24 hours of incubation period [29-35]. The IC50 value at 48 hours treatment was 4.8 mg/ml while at 24 hours treatment was 9mg/ml. This means that *C. zeylanicum* water extract worked better at 48 hours treatment. Besides that, the IC50 values of Soxhlet extract were comparatively larger than the IC50 values of *C. zeylanicum* Soxhlet extract. So it can be said that *C. zeylanicum* Soxhlet extract is more cytotoxic towards MCF-7 cell line than *C. zeylanicum* water extract [36]. One reason that can probably be related the different cytotoxic property of *C. zeylanicum* extract in this study compared to previous study is the different amount of cinnamaldehyde content in the extract [37-40]. Since a group of scientists has obtained higher yield of extract which was 21.09%, at least three times higher than the yield in this study (7.06% and 2.54%), it is assumed that the cinnamaldehyde content in their extract was higher [17]. Cinnamaldehyde is the major content of cinnamon and has been shown in many researches to have anticancer and anti-proliferative property [41,42]. According to a study, cinnamaldehyde was found to be active against various human cancer cells such as liver, lung and leukemia cancer cells [7]. A group scientist also has stated that cinnamaldehyde can induce apoptosis to cancer cells through a few mechanisms such as generation of reactive oxygen species and loss of mitochondrial membrane potential [4]. While according to other study, cinnamaldehyde has been synthesized and tested as angiogenesis inhibitors and thus inhibit the growth of tumour [6, 43]. Therefore, it is predicted that higher cinnamaldehyde content can lower the cell viability more, thus lower the IC50 value [44,45].

4. CONCLUSION

This study focused on the investigation of cytotoxic activity of *Cinamomum zeylanicum* extracts against human breast cancer cell line (MCF-7). *C. zeylanicum* was successfully extracted using Soxhlet and water extraction methods and the major content was analysed using GCMS analysis. By means of MTT assay, *C. zeylanicum* extracts has been confirmed to have cytotoxic effect towards MCF-7 cell line in different concentrations at two different incubation periods, 24 hours and 48 hours. Therefore, these results call for further investigations of the mechanism in which *C. zeylanicum* extracts works against cancer cells in vitro and also in vivo, also the high dose or long-term use of the extracts.

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