Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 24-33
Received: 10-05-2019
Accepted: 12-06-2019

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Cytotoxicity of ethyl acetate extract of Cantigi (*Vaccinium varingiaefolium* (Blume) Miq. young leaves on *Artemia salina* L. larvae, MCF-7, T47D, and vero cell lines

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Abstract
Cantigi (*Vaccinium varingiaefolium* (Blume) Miq.) is endemic plant of Java dominating sub-alpine area of Mount Tangkuban Parahu. Its young leaves are red and will turn green. Previous study reported that ethyl acetate extract of young leaves had antioxidant activity using DPPH method and cytotoxic activity against leukemia L1210 cell lines. This experiment was designed to know whether ethyl acetate (EA) extract of Cantigi leaves had cytotoxic activity against *Artemia salina* L. larvae, breast cancer cell lines T47D and MCF-7, and normal cell line Vero. Dried leaves were extracted with maceration method, firstly, using hexane, and then EA. Obtained EA extract was dried using rotary evaporator. EA extract was examined its cytotoxic activity using Brine Shrimp Lethality Test (BSLT) method, against breast cancer cell lines T47D and MCF-7, and normal cell line Vero using Microculture Tetrazolium Salt (MTT) method. As the positive control, cisplatin was used. This experiment showed that LC_{50} of the extract was 320.83 ppm, IC_{50} against breast cancer cell line T47D was 75.23 ppm, IC_{50} against breast cancer cell line MCF-7 was 88, 89 ppm, IC_{50} against normal cell line Vero was 305 ppm, and IC_{50} of cisplatin against breast cancer cell line T47D was 3.91 ppm. It can be concluded that the EA extract has cytotoxic activity with LC_{50} < 1000 ppm, strong cytotoxic activity against both breast cancer cell lines tested with IC_{50} < 100 ppm, has weak cytotoxic activity against normal cell line Vero with IC_{50} > 300 ppm, and potentially as a candidate for breast cancer therapy.

Keywords: Cantigi, *Vaccinium varingiaefolium* (Blume) Miq, EA extract, Cisplatin, BSLT, T47D

Introduction
Indonesia is a country with a plenty of endemic plants spreading out over the nation. One of plants is Cantigi (a local name) or *Vaccinium varingiaefolium* (Blume) Miq. belonging to Ericaceae family. This endemic plant has a smiliarity with billberry (*V. myrtillus*) and blueberry (*V. corymbosum*) [1]. But, unfortunately, very little studies had been reported.
Mount Tangkuban Parahu (MTP), having a peak at 2,081 meters above sea level (masl) is a volcanic mountain located on Java Island, Indonesia. Its location is about 20 km north of Bandung, the provincial capital of West Java or near 6°40′00″ south latitude and 107°37′00″ east longitude. Plants of MTP varies along the altitudinal gradient of the mountain. There are three parts in this area. First, the area between 1,980 and 2,080 masl as Upper Montane Zone. Second, the area between 1,600 and 1,930 masl was as Mid Montane Slope Zone. Third, the site within the Upper Montane Zone (protected forest) and its surroundings – in the Mid Montane Zone was Vaccinium forest (1,900 masl, 06°45′40″ S & 107°37′07″
E) in an area near the crater dominated by *Vaccinium varingiaefolium.* Cantigi grew well in the area closed to sulphur vents or volcano region, such as MTP [2].
Microscopic and macroscopic studies of Cantigi had been reported. Morphological observation showed that Cantigi had a tap root, circular stem with lenticels on the surface. Cantigi had red young stem with a lot of trichomes, brown old stem, oval leaves with integer margins. Leaves had stone cells, cuticles, idioblasts. Young leaves were red and then turned green (old leaves). The color of flowers were red purple having five sepals, five petals, 10 androecium, and one syncarp gynoecium. Trichomes spread all over flower structures. Its microspore was tetraeder type. The gynoecium sat on the receptacle with a composition of five carpellum with inpherus ovulus. The fruits were changing in color from green, globular, with trichomes to black purple when ripe. It had stone cells with purple cytoplasm and golden brown seeds [1].

The previous study showed that EA extract of young leaves Cantigi tested for phytochemical screening was positive for flavonoid, steroid, tannin, and triterpenoid. It also had antioxidant activity (IC_{50}) of 56, 75 ppm using DPPH method. Moreover, it had cytotoxic activity (IC_{50}) of 12.67 ppm against Leukemia 1210 cell lines using MTT method [3].

WHO data published in 2017 reported that breast cancer deaths in Indonesia was 21,287 (1.27% of total deaths). The age adjusted death rate is 19.33 of 100,000 of population, and it ranks Indonesia #68 in the world [4]. Studies had identified hormonal, lifestyle, and environmental factors that might increase the risk of breast cancer. But it is not clear why some people who have no risk factors develop cancer, but other people with risk factors never do. Breast cancer is more likely caused by a complex interaction of genetic makeup and environment [5].

There are some treatment options such as chemotherapeutic, but they have resistance and many adverse effects that prevent the usage. For centuries, herbs and plants have been used for medicinal purposes and as food as well. Some different types of herbals retain the immune stimulating and anti-tumor properties. Various active phytochemicals such as carotenoids, flavonoids, ligands, polyphenolics, terpenoids, sulfides, lignans, and plant sterols has been identified in many types of herbs. The phytochemicals have different pharmacological action, such as either to stimulate the protective enzyme like glutathione transferase or to prevent the cell proliferation [6].

This study was aimed to continue the previous study by testing the cytotoxic activity of the EA extract against against Artemia salina L. larvae using Brine Shrimp Lethality Test (BSLT) method, breast cancer cell lines T47D and MCF-7, and normal cell line Vero using Microculture Tetrazolium Salt (MTT) method.

Material and Method

1. Materials

Young red Cantigi leaves were collected from MTP, north Bandung, West Java, Indonesia. Plants were identified at Pusat Penelitian Biologi, Lembaga Ilmu Pengetahuan Indonesia, Cibinong Science Center, Cibinong, Bogor, Indonesia. Firstly, the powder of dried Cantigi leaves was extracted by maceration methods using hexane. Secondly, the residual solid part of the first step was extracted using ethyl acetate (EA). Finally, the EA extract was concentrated and dried by vacuum rotary evaporator.

2. Brine shrimp lethality test (BSLT)

The assay was carried out based on to the principle and protocol previously explained by Meyer, with slight modification. Eggs of *Artemia salina* L. were inserted into a box containing seawater; the box was then placed under a UV lamp. After 48 h the eggs hatched into larvae, and ready for the test [7].

The EA extracts were diluted in 10 mL seawater containing 10 larvae (1% DMSO (v/v)) until concentrations 10, 100, 500, and 1000 ppm were reached. After 24 h, the live and dead shrimp were counted. The mortality rate (%) was obtained by comparing the number of total dead larvae and the total number of larvae. The experiment was conducted in triplicate. The concentrations (dose)-response (% mortality) data were transformed into a straight line using a logit transformation, and the concentration required to kill 50% of the population (LC_{50} values) was derived from the best fit line obtained by linear regression analysis (Probit Analysis).

3. MTT Test

In this study, human breast cancer cell lines of T47D, MCF-7, and normal cell line of Vero were obtained from Labtiab, Badan Pengkajian dan Penerapan Teknologi (BPPT), Puspiptek, Serpong, Banten, Indonesia. Cells of MCF-7 and T47D were maintained as monolayer cultures in RPMI 1640 medium, supplemented with 1% antibiotics (50 IU/ml penicillin and 50 □g/ml streptomycin) and 10% heat inactivated of fetal bovine serum, in a humidified incubator containing 5% CO2 at 37 ºC. Subcultures were obtained by tripsin treatment of confluent cultured. Cells were seeded in 100 µl of medium in 96 microwell plate at a density of 5×103 cells/well, and the plates were placed in a 37 ºC, 5% CO2 incubator. One day later the cell culture was added with 100 µl medium containing a series of EA extract concentrations in DMSO, all done in triplicate. After 24 hours of treatment, washed twice with phosphate buffer saline, then the medium was changed and 100 µl MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide] 0.5 mg/ml was added and maintained at 37 ºC in 5% CO2 incubator for 4 hours to allow MTT to be converted to formazon crystals by reacting it with metabolically active cells. The viable cells were directly proportional to the production of formazan. The reaction was stopped by adding SDS (sodium dodecyl sulfate) 10%, and the cell viability was measured at 570 nm using a plate reader. IC_{50} value of extract was determined based on the equation of linear regression of log concentration vs% cell viability. As the positive control, this study used cisplatin tested against T47D cell line [8].

4. Morphology changes of MCF-7 and T47D Cells treated with EA extracts

Morphology changes of both cell lines were evaluated microscopically after treatment a series of concentrations of AE extract and the positive control (Cisplatin).

5. Selectivity index

The selectivity of EA extract on each breast cancer cell lines was evaluated using the value of Selectivity Index (SI) with a formula as follow:

$$\text{SI value} = \frac{IC_{50} \text{ on Vero cell lines}}{IC_{50} \text{ on breast cancer cell lines}}$$

EA extract is selective if the value is more than 3, and not selective if less than 3 [9].

Results

1. Brine Shrimp Lethality Test (BSLT)

Table 1: Probit analysis of BSLT method of EA extract resulting LC50 of 320.83 ppm.

Observed and Expected Frequencies

VAR00002	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	10.0	7.0	8.418	-1.418	.84177
500.00	10.0	3.0	6.042	-3.042	.60421
100.00	10.0	4.0	3.723	.277	.37232
10.00	10.0	10.0	3.233	6.767	.32331
1000.00	10.0	10.0	8.418	1.582	.84177
500.00	10.0	4.0	6.042	-2.042	.60421
100.00	10.0	2.0	3.723	-1.723	.37232
10.00	10.0	.0	3.233	-3.233	.32331
1000.00	10.0	10.0	8.418	1.582	.84177
500.00	10.0	7.0	6.042	.958	.60421
100.00	10.0	6.0	3.723	2.277	.37232
10.00	10.0	1.0	3.233	-2.233	.32331

Prob	VAR00002	95% Confidence Limits Lower	Upper
.01	-1256.38636	.	.
.02	-1071.56983	.	.
.03	-954.30966	.	.
.04	-866.09941	.	.
.05	-794.34717	.	.
.06	-733.27474	.	.
.07	-679.72618	.	.
.08	-631.77982	.	.
.09	-588.17453	.	.
.10	-548.03578	.	.
.15	-381.85066	.	.
.20	-249.77199	.	.
.25	-136.46026	.	.
.30	-34.70282	.	.
.35	59.59062	.	.
.40	149.06587	.	.
.45	235.63420	.	.
.50	320.83003	.	.
.55	406.02586	.	.
.60	492.59420	.	.
.65	582.06945	.	.
.70	676.36288	.	.
.75	778.12032	.	.
.80	891.43206	.	.
.85	1023.51073	.	.
.90	1189.69585	.	.
.91	1229.83459	.	.
.92	1273.43989	.	.
.93	1321.38624	.	.
.94	1374.93481	.	.
.95	1436.00723	.	.
.96	1507.75947	.	.
.97	1595.96973	.	.
.98	1713.22990	.	.
.99	1898.04643	.	.

2. MTT Test
2.1 MCF-7 Cell Lines

Table 2: Data of EA extract concentration (ppm) versus average MCF-7 cell viability (A) and data of log EA extract concentration versus average MCF-7 cell inhibition (B).

(A)

Sample	C (ppm)	Average cell viability (%)
EA extract	12.5	83.214
	25	69.298
	50	67.544
	200	39.713
	400	17.185

(B)

Sample	Log C	Average cell inhibition (%)
EA extract	1.097	16.786
	1.398	30.702
	1.699	32.456
	2.301	60.287
	2.602	82.815

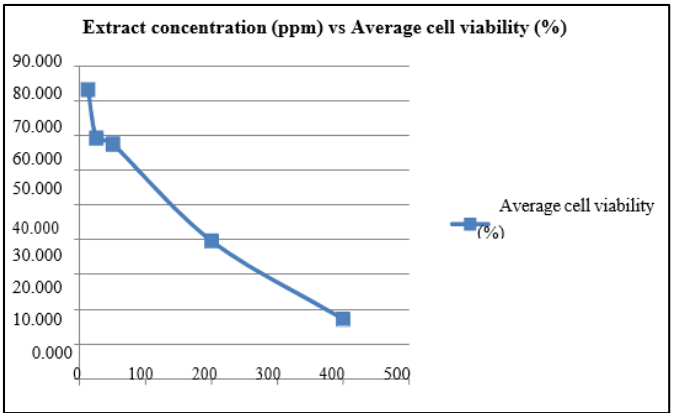


Fig 1: Profile of EA extract concentration (ppm) versus average MCF-7 cell viability (%).

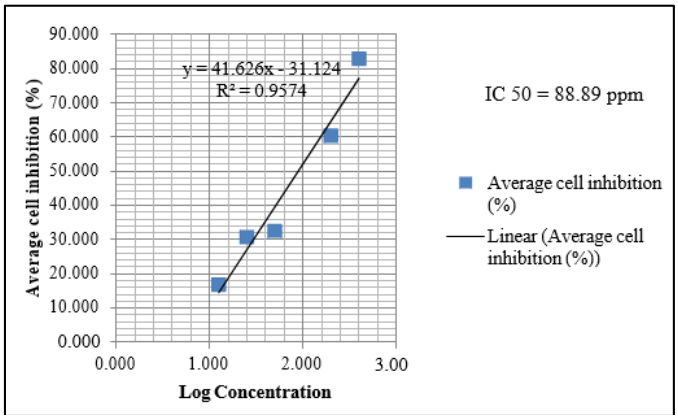


Fig 2: Linear regression profile of EA extract log concentration versus average MCF-7 cell inhibition. The IC_{50} was 88.89 ppm.

2.2 Cisplatin

Table 3: Data of cisplatin concentration (ppm) versus average T47D cell viability (A) and data of log cisplatin concentration versus average T47D cell inhibition (B)

(A)

Sample	C (ppm)	Average cell viability (%)
Cisplatin	0.75	97.609
	1.5	85.795
	3	69.866
	6	34.951
	9	14.627
	12	13.537

(B)

Sample	Log C	Average cell inhibition (%)
Cisplatin	-0.125	2.391
	0.176	14.205
	0.477	30.134
	0.778	65.049
	0.954	85.373
	1.079	86.463

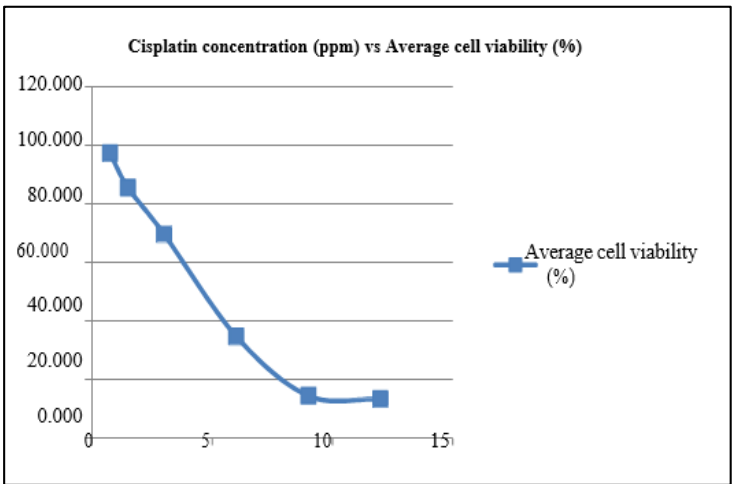


Fig 3: Profile of cisplatin concentration (ppm) versus average T47D cell viability (%).

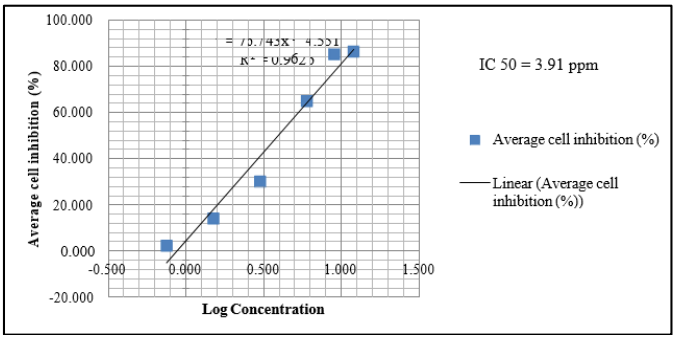


Fig 4: Linear regression profile of Cisplatin log concentration versus average T47D cell inhibition. The IC_{50} was 3.91 ppm

2.3 Vero Cell Lines

Table 4: Data of EA extract concentration (ppm) versus average Vero cell viability (A) and data of log cisplatin concentration versus average Vero cell inhibition (B)

(A)

Sample	C (ppm)	Average cell viability (%)
EA extract	12,5	76.440
	25	70.942
	100	64.660
	200	50.131
	400	47.513

(B)

Sample	Log C	Average cell inhibition (%)
EA extract	1.097	23.560
	1.398	29.058
	2.000	35.340
	2.301	49.869
	2.602	52.487

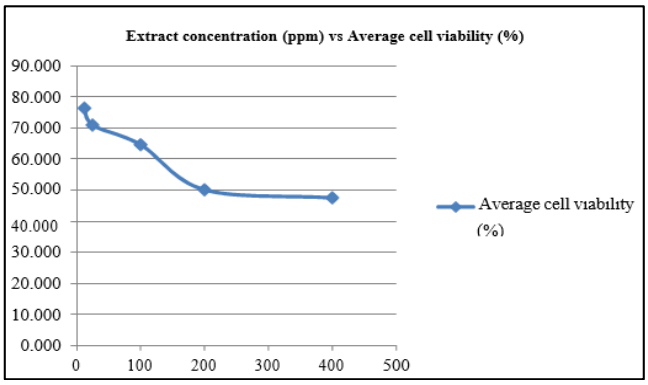


Fig 5: Profile of EA extract concentration (ppm) versus average Vero cell viability (%).

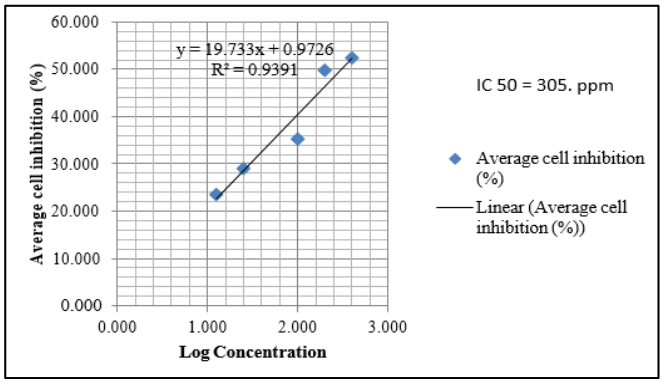


Fig 6: Profile of EA extract concentration (ppm) versus average Vero cell inhibition (%).

2.4 T47D Cell Lines

Table 5: Data of EA extract concentration (ppm) versus average T47D cell viability (A) and data of log EA extract concentration versus average T47D cell inhibition (B)

(A)

Sample	C (ppm)	Average cell viability (%)
EA extract	12.5	68.636
	25	63.256
	50	58.966
	200	41.772
	400	25.316

(B)

Sample	Log C	Average cell inhibition (%)
EA extract	1.097	31.364
	1.398	36.744
	1.699	41.034
	2.301	58.228
	2.602	74.684

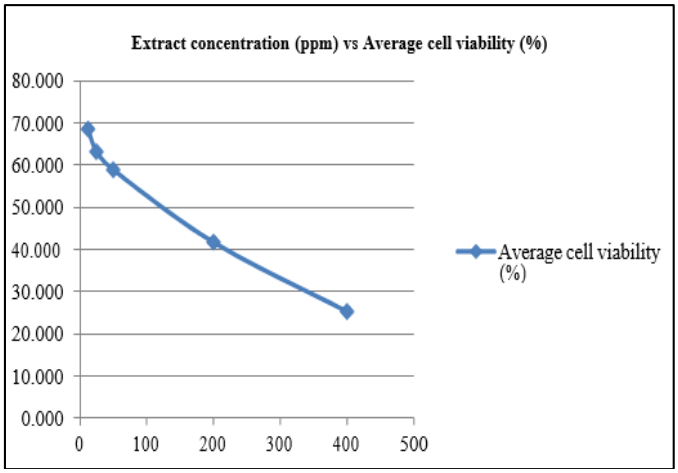


Fig 7: Profile of EA extract concentration (ppm) versus average T47D cell viability (%)

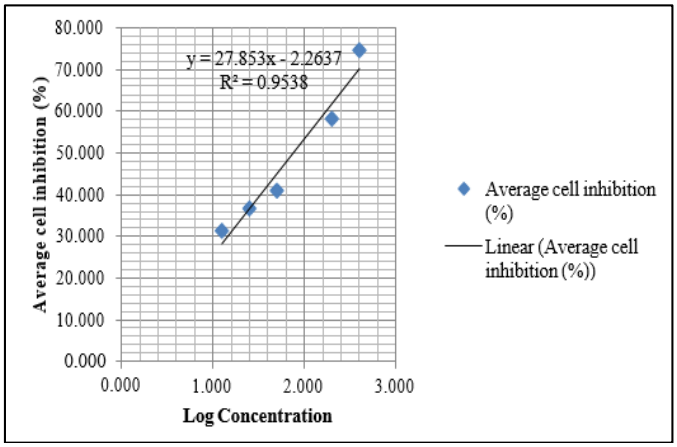
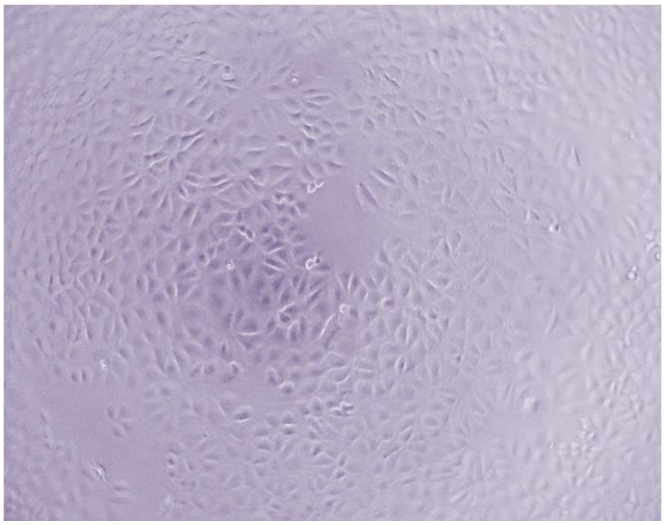


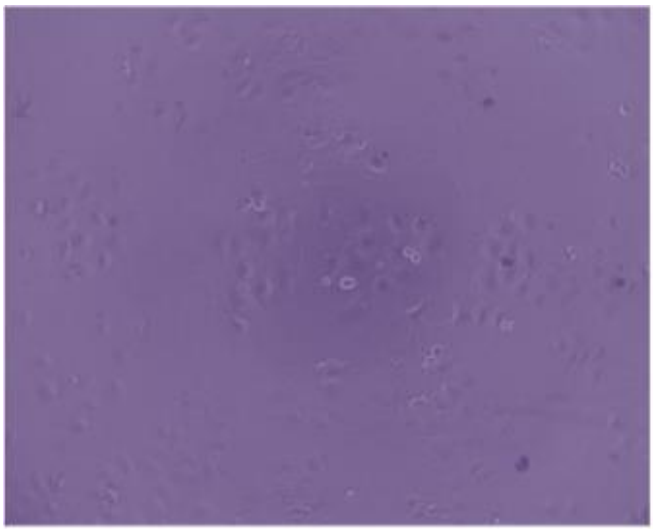
Fig 8: Profile of EA extract concentration (ppm) versus average T47D cell inhibition (%)

3. Morphology Changes of MCF-7 and T47D Cell Lines
3.1 MCF-7 Cell Lines



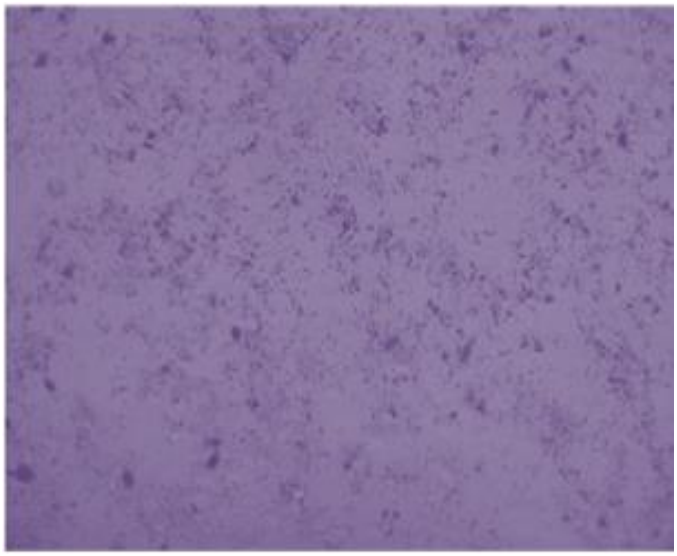
(1) Control cells of MCF-7.

(2) MCF-7 cells treated with EA extract (12.5 ppm)

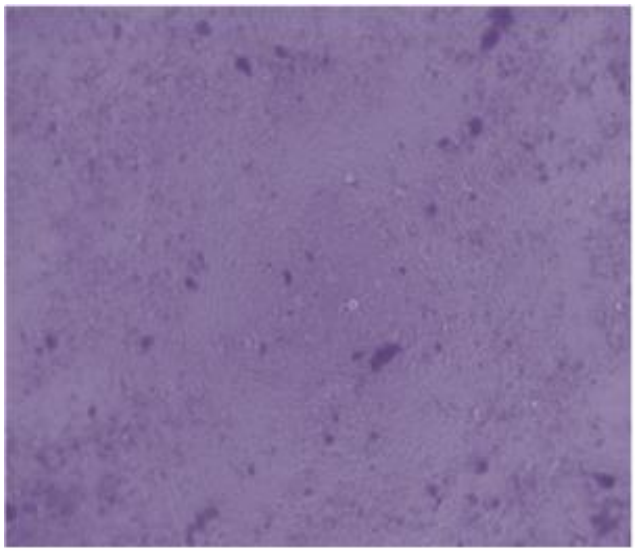


(3) MCF-7 cells treated with EA extract (25 ppm)

(4) MCF-7 cells treated with EA extract (50 ppm)



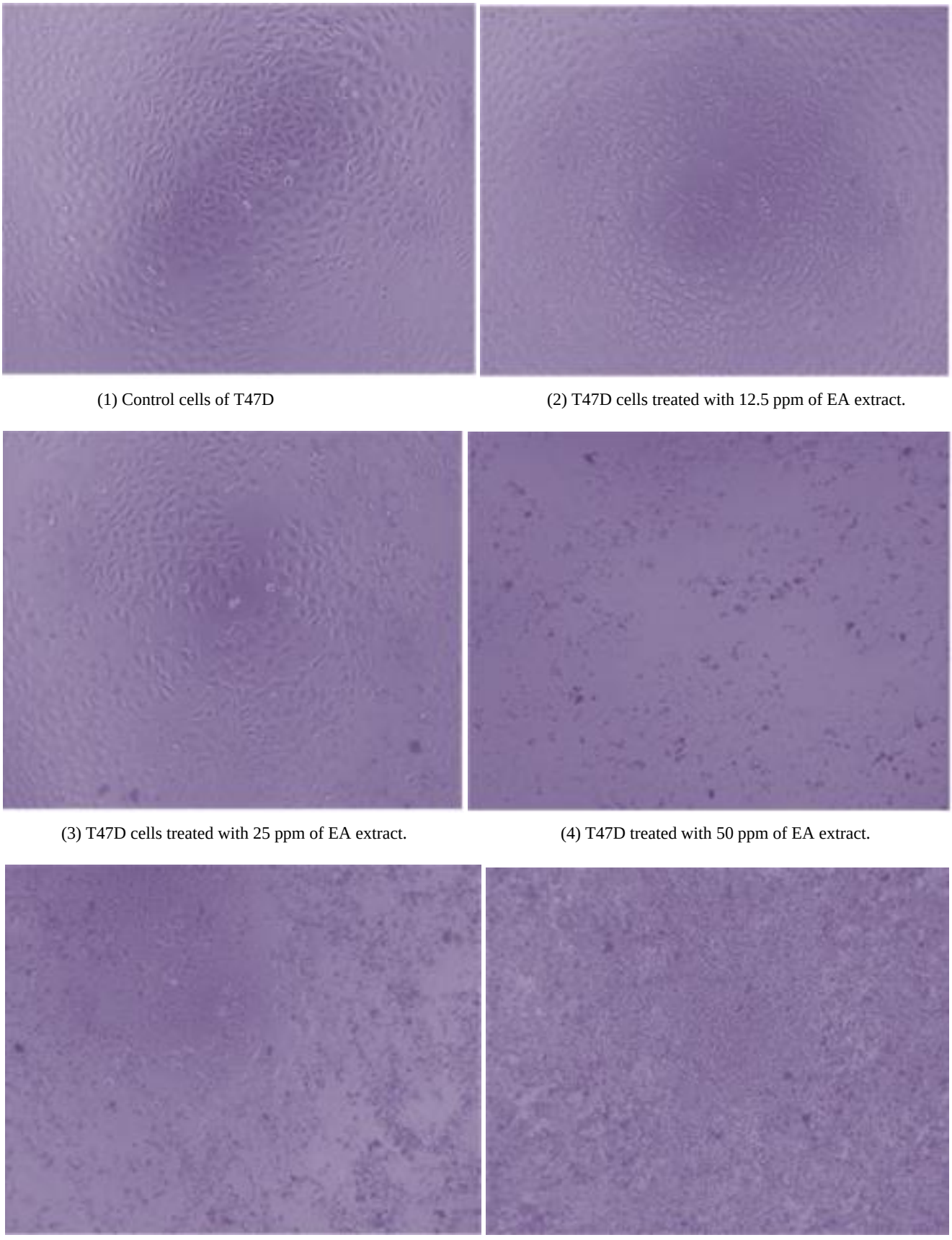
(5) MCF-7 cells treated with EA extract (200 ppm)



(6) MCF-7 cells treated with EA extract (400 ppm)

Fig 9: Morphological changes of MCF-7 cells treated with a series of EA extract concentrations.

3.2 T47D Cell Lines



(1) Control cells of T47D

(2) T47D cells treated with 12.5 ppm of EA extract.

(3) T47D cells treated with 25 ppm of EA extract.

(4) T47D treated with 50 ppm of EA extract.

(5) T47D cells treated with 200 ppm of EA extract.

(6) T47D cells treated with 400 ppm of EA extract

Fig 10: Morphological changes of T47D cells treated with a series of EA extract concentrations

3.3 Cisplatin as Positive Control

(1) T47D cells treated with 0.75 ppm of Cisplatin. (2) T47D cells treated with 1.5 ppm of Cisplatin.

(4) T47D cells treated with 6 ppm of Cisplatin (3) T47D cells treated with 3 ppm of Cisplatin

(5) T47D treated with 9 ppm of Cisplatin.

Fig 11: Morphological changes of T47D cells treated with a series of Cisplatin concentrations

4. Selectivity Index (SI)

Table 6: IC_{50} of breast cancer cell lines and the SI values

Cell lines	IC_{50} (ppm)	SI	Remark
T47D	75.23	4.05	Selective
MCF-7	88.89	3.43	Selective
Vero	305	-	-

Discussion

As a preliminary test, the cytotoxic activity of the EA extract was evaluated firstly using a brine shrimp lethality test (BSLT) method. The EA extract exhibited moderate toxic activity with LC_{50} value of 320.83 ppm (moderate toxicity (LC_{50} value > 100–1000 ppm, high toxic activity (LC_{50} > 30–100 ppm), and very high toxic activity (LC_{50} < 30 ppm). The BSLT method is a fast, cheap, and simple method for predicting the toxicity level of the EA extract. This method is not specific to antitumor activity, however there is a positive correlation between BSLT toxicity and cytotoxicity against some cell lines [10]. Therefore, in this study, the EA extract were followed up to be evaluated for its potential as a candidate of anti breat cancer. The result of BSLT is presentated in Table 1.

The cytotoxic activity of the EA extract was evaluated against a normal cell line Vero, and against breast cancer cell lines MCF-7 and T47D. The IC_{50} values and selectivity index (SI) of the study are presented in the Table 2-6 and Figure 1-8 above. Cell lines are frequently used as *in vitro* tools to mimic specific types of *in vivo* system. For example, MCF-7 and T47D have been widely used in breast cancer researches. MCF-7 is a commonly used breast cancer cell line. It has been propagated for long time by many groups. It proves to be a suitable model cell line for breast cancer investigations around the world. Until now, it has produced a lot of data of practical knowledge for patient care than any other breast cancer cell line. It is ER-positive and progesterone receptor (PR)-positive, also belongs to the luminal A molecular subtype. It is a cell line which is poorly-aggressive and noninvasive cell line, and usually considered to have low metastatic potential [11].

A study reported that T47D and MCF-7 cell lines showed distinct molecular characteristics. The MCF-7 cell line was less susceptible to progesterone than the T47D cell line. MCF-7 cell line did not respond to progesterone in the presence of estrogen Bindings of ERα, PR, and P300 were substantially different between the T47D and MCF-7 cell lines. It concluded that the importance of selecting appropriate cell lines for breast cancer studies. T47D cell lines was suggested as an ideal experimental model to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. Based on the IC_{50} of the cell lines of this study, it could be concluded that T47D cell lines was more susceptible to the EA extract than MCF-7 cell lines, and supported the above statement [12].

Generally, the values of cytotoxicity activities of extracts or chemicals (IC_{50}) against the cell lines could be divided into strong (<100 ppm), moderate (101-200 ppm), and weak (>200 ppm) activities. The EA extracts of Cantigi young leaves showed a strong inhibition against the MCF-7 and T47D cell lines, and weak inhibition against the normal Vero cell lines. It meant that the EA extract could be safe and could be a candidate of anti breast cancer [13]. The morphology changes study above also showed that the EA extract had ability to inactivate MCF-7 and T47D cells by changing the cells from a leaf-like form to a round form (Figure 9-11). It could be predicted that the lysis of the cells because of antiproliferative and apoptotic action. [14-16] In addition, the selectivity index (SI) of the EA extract to Vero, T47D, and MCF7 were more than 3. The greater the SI value, the safer the extract or compound [10]

Conclusions

This study confirmed the strong cytotoxic potential of the EA extract of young leaves of Cantigi against breast cancer cells (IC_{50} < 100 ppm). The effect was more pronounced in T47D cells instead of MCF-7 cells. High concentration of EA extract induced very low Vero cells mortality, indicating the selectivity of EA extract on cancerous cells rather than normal cells (SI > 3). As the preliminary test, the cytotoxic activity of the EA extract was evaluated using a brine shrimp lethality test (BSLT) method, exhibiting moderate toxic activity (LC_{50} value > 100–1000 ppm). The BSLT method is a fast, cheap, and simple method for predicting the toxicity level of the EA extract. This method is not specific to antitumor activity, however there is a positive correlation between BSLT toxicity and cytotoxicity against some cell lines. The morphology changes also confirmed that the EA extract had ability to inactivate MCF-7 and T47D cells by changing the cells from a leaf-like form to a round form. Finally, the data confirmed that the EA extract could be safe and could be a candidate of anti breast cancer.

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