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Cytotoxicity Activities in Local *Justicia gendarussa* Crude Extracts against Human Cancer Cell Lines

Zahidah Ayob[a], Azman Abd Samad[a], Siti Pauliena Mohd Bohari[a*]

[a]*Department of Biotechnology and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia*

*Corresponding author: pauliena@utm.my

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Graphical abstract

Abstract

The present study was designed to evaluate the cytotoxic activity of *Justicia gendarussa* methanolic leaf extracts from five different locations in Johor and also two standards flavonoids (naringenin and kaempferol) as these two compounds were detected in the chromatogram. In this study, leaf extracts and compounds were tested against various human cancer cell lines (HT-29, HeLa and BxPC-3) by using MTT assay. The results showed that methanolic leaf extracts from Mersing and kaempferol were very toxic against BxPC-3 and HeLa cells with IC_{50} values of 16 µg/ml and 5 µg/ml, respectively. It is suggested that *J. gendarussa* leaf extracts from Mersing have potential cytotoxic activity on human cancer cell lines particularly BxPC-3 cells.

Keywords: Cytotoxicity; *Justicia gendarussa*; MTT assay; HT-29; HeLa; BxPC-3

Abstrak

Kajian ini bertujuan menilai aktiviti sitotoksik ekstrak daun *Justicia gendarussa* menggunakan metanol daripada lima lokasi berbeza di Johor dan dua standard sebatian flavonoid (naringenin dan kaempferol) yang mana kompaun sebatian ini yang dikesan pada kromatogram. Dalam kajian ini, ekstrak daun dan sebatian telah diuji ke atas berbagai sel kanser manusia (HT-29, HeLa dan BxPC-3) dengan menggunakan ujian MTT. Hasil kajian menunjukkan bahawa ekstrak daun dari Mersing dan kaempferol sangat toksik kepada BxPC-3 dan HeLa dengan nilai IC_{50} masing-masing adalah 16 µg/ml dan 5 µg/ml. Penemuan ini menunjuk ekstrak daun *J. gendarussa* dari Mersing berpotensi untuk digunakan dalam rawatan sel kanser manusia khususnya sel BxPC-3.

Kata kunci: Sitotoksik; *Justicia gendarussa*; ujian MTT; HT-29; HeLa; BxPC-3

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1.0 INTRODUCTION

Cancer is the fourth leading death in Malaysia after diseases of the circulatory, respiratory systems and motor vehicle accidents that responsible for 10.3% of medically certified deaths.[1] Cancer causes significant morbidity, mortality and also the major health problem worldwide. The incidence of colon cancer, cervix cancer and pancreas cancer are rising in every country in the world. Although, many clinical anti-cancer drugs today are effective in killing cancer cells, the price still expensive and not affordable for some people. Furthermore, there are also undesirable side effects like excessive hair lose, loss of appetite and nausea.

Medicinal plants have provided a wide variety of phytochemical uses which have been shown to have activity across multiple signalling pathways. Medicinal plants not only disrupt aberrant signalling pathways leading to cancer (proliferation, deregulation of apoptosis, angiogenesis, invasion and metastasis), but also synergize with chemotherapy and radiotherapy. It is not surprising many researchers are constantly turning to natural products for alternative source of medicines.[1]

Justicia gendarussa is also commonly known as Gendarussa is a member of Acanthaceae. Traditionally, *J. gendarussa* extracts have been used to treat ailments such as emetic, antipyretic, amenorrhea, stomach troubles, hemoptysis, cough and asthma.[2-3] Recently, *J. gendarussa* leaves and stem extracts were reported to have antiangiogenic,[4] hepatoprotective activity,[5] anti-fungal,[6] antioxidant,[7-8] anthelmintic,[9] anti microbial,[10] anti-bacterial[11-12] activities. Also, *J. gendarussa* leaf extracts have been used traditionally as a male contraceptive agent by several ethnic groups in central part of Papua, Indonesia and this extracts could inhibit mice spermatozoa penetrate onto mice ovum.[13]

Phytochemical study on the leaves of *J. gendarussa* revealed the alkaloids, amino acids, aromatics amines,[14] flavonoids[14-15] and triterpinoidal saponins[7,14] exist in this plant.

In addition, naringenin and kaempferol were detected in green callus and *in vitro* leaf extracts of *J. gendarussa* using GC-FID.[16] On the other hand, two flavonoid compounds (naringenin and kaempferol) were successfully detected and quantified in *J. gendarussa* methanolic leaf extracts (wild type) using GC-FID and RP-HPLC (unpublished reports). Naringenin and kaempferol are the natural phytoestrogen that presence in this leaf extracts and exhibit strong anti-proliferation of colon (HT-29), cervix (HeLa) and pancreas (BxPC-3) human cancer cells. The former studies reported naringenin have significant cytotoxicity and suppress apoptosis in mouse leukemia P388 cells[17] and cytotoxic activity on breast cancer cell, MCF-7,[18] HT-29,[19] while kaempferol were reported to inhibit cell proliferation and induce apoptosis in pancreatic cancer cells.[20]

To our knowledge, there is no scientific report pertaining cytotoxic activities on *J. gendarussa* methanolic leaf extracts against human cancer cell lines (HT-29, HeLa and BxPC-3). Therefore, in this study, we report for the first time on crude extracts of *J. gendarussa* against human cancer cell lines. This study was performed to screened the cytotoxic activities of methanolic leaf extracts from five different locations (Mersing, Muar, Pulai, Skudai and Batu Pahat) in Johor and two flavonoids (naringenin and kaempferol) against human cancer cell lines that might have correlation between the remain compounds in crude extracts and flavonoids compounds with the current findings. These data will provide scientific validation in the use of *J. gendarussa* leaves that have potential uses as an anti-cancer.

■2.0 EXPERIMENTAL

2.1 Plant Materials

J. gendarussa plants were collected from five different locations in Johor (Mersing, Muar, Skudai, Batu Pahat, Pulai) and maintained in a greenhouse at Faculty Biosciences and Medical Engineering, University Teknologi Malaysia (UTM). The commercial standards (kaempferol and naringenin) were purchased from Sigma Aldrich Corporation, Malaysia. Mature leaves and commercial standards (kaempferol and naringenin) were used as a starting material. The samples were used after diluted the stock solution with dimethysulfoxide (DMSO) where DMSO at the concentrations lower than 1% had no effect on cell growth.

2.2 Flavonoids Detection of *J. gendarussa* Methanol Leaf Extracts

Detection of flavonoids in leaf extracts were performed using gas chromatography - flame ionization detector (GC-FID).[16,21]

2.3 Preparations of J. gendarussa Methanol Leaf Extracts

The matured leaves of *J. gendarussa* were air dried for 4 weeks. Then, the dried leaves were ground into small particles and approximately, 50 g of small particles of leaves were soaked into 1000 ml of methanol at room temperature for 72 hours in ratio of 1:20 (w/v).[22] The mixtures were filtered through sterile cotton and filtered again using Whatman No. 1 filter paper to obtain the methanol supernatants. The filtered methanol extraction was evaporated at 40 °C under reduced pressure by using rotary evaporator (EYELA N-1000, EYELA, Tokyo, Japan). The dried crude extracts were kept at 4 °C prior to use.

2.4 Culture of Cells

Three human cancer cell lines, HT-29 (colon adenocarcinoma), HeLa (cervix adenocarcinoma), BxPC-3 (epitheloid cervix adenocarcinoma) and one normal cell line CHO (chinese hamster ovary) were obtained from American Type Culture Collection (ATCC) and were a generous gift from Dr Salehhuddin Hamdan (Animal Cell Culture Laboratory, Faculty of Biosciences and Medical Engineering, UTM). HT-29, HeLa, BxPC-3 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% v/v fetal calf serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin as a complete growth medium. Cells were maintained in 25 cm^2 flask at 37 °C with 5 % CO_2. All of the materials were obtained from the (Gibco, Bio-Diagnostics, Petaling Jaya, Selangor, Malaysia).

2.5 MTT Assay

Cytotoxicity assay was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) based on previous study.[23-24] In this assay, cells were harvested when it reached 80 % confluent. Before starting the MTT assay, cells were optimized at a different seeding density that ranging from 2.0×10^3 cell/ml to 1.0×10^6 cell/ml in light to get the suitable seeding number for the experiment. Then, each well of the microtiter plate (96-well) was filled with 100 µl of cells suspension (HT-29 with seeding number; 5×10^4 cell/ml), (HeLa with seeding number; 5×10^4 cell/ml), (BxPC-3 with seeding number; 1×10^5 cell/ml) and (CHO with seeding number; 5×10^4 cell/ml) in RPMI- 1640 complete growth medium. After 24 hour of incubation, the cells were treated (100 µl/well) with the plant extracts in different concentration that ranging from 7.81 to 1000 µg/ml. The total volume of each well is 200 µl with technical replicates. The microtiter plates were further incubated for 72 hours with the plant extracts. After 72 hours of incubation, 20 µl of 5 mg/ml MTT was added to each well and the plates then incubated for 4 hours at 37 °C. Then, the medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by addition of 225 µl of 100mM HCl-isopropanol to each wells. After completing the solubilization of the purple formazan the absorbance was measured using the BioRad microplate reader (BioRad, Tokyo, Japan) at a wavelength of 575 nm. The cytotoxicity was recorded as the drug concentration causing 50 % growth inhibition of cell lines (IC_{50} value) using the formula given below:

$$\% \text{ cell viability} = \frac{\text{Asample (mean)}}{\text{Acontrol (mean)}} \times 100\ \%$$

2.6 Statistical Analysis

All of the samples were run in three replicates. Data obtained were analyzed using SPSS for Window software (SPSS 16.0 for Windows Evaluation Version software, SPSS Inc., USA). The normality of the data was tested using The Shapiro-Wilk test. The data were analyzed using Independence t-test for normal data and Mann-Whitney test for non-normal data.[25] Differences were considered to be significant if the probability $p < 0.05$.

■3.0 RESULTS AND DISCUSSION

In this study, screening of cytotoxicity activities on *J. gendarussa* methanolic leaf extracts from five different locations were evaluated against human cancer cell lines using MTT assay. IC_{50} values defined as the concentrations of the extracts to produce a 50 % reduction in viability of cells.[26] According to [27-29] studies reported that the cytotoxicity was evaluated based on IC_{50} values i.e. IC_{50} values below than 20 µg/ml (cytotoxicity), range between 21 - 40 µg/ml (weak cytotoxicity) and above than 41 µg/ml (not cytotoxicity). Figure 3.1, shows chromatogram that correspond to both flavonoid compounds (naringenin and kaempferol) in leaf extract from Mersing by using GC-FID. Also, *in vitro* leaf extracts and green callus extracts of *J. gendarussa* were detected and quantified the naringenin and kaempferol contents.[16] It shows the presence of flavonoids (naringenin and keampferol) in *J. gendarussa* leaf extracts that could be associated to inhibit the proliferation of HT-29, HeLa and BxPC-3 cell lines.

The *J. gendarussa* leaf extracts from five different locations showed the cytotoxicity in a dose- dependent manner from lowest to highest concentration in HT-29, colon cancer cells. The similar trend of inhibitory cells was recorded in all extracts. Table 3.1 shows IC_{50} values of leaf extracts from Mersing, Muar, Skudai, Batu Pahat and Pulai. The IC_{50} values of leaf extracts from Muar, Skudai and Pulai showed high significantly difference ($p<0.001$) between different concentration of extracts. Leaf extracts from Mersing showed weak cytotoxicity with the approximately IC_{50} values of 21 µg/ml followed by leaf extracts from Batu Pahat (36 µg/ml), Pulai (39 µg/ml), Muar (65 µg/ml) and Skudai (76 µg/ml). The results are summarized in Table 3.2. From the data obtained, leaf extracts from Mersing was found effective inhibit the proliferation of the HT-29 cells and it is believed could be due the presence of more kaempferol contents rather than naringenin as shown in chromatogram (Figure 3.1). The results are summarized in table 3.6 showed the high cytotoxic activity on kaempferol as compared to naringenin against HT-29 cells.

Furthermore, production of flavonoids and various flavonoid compositions could be found in different regions or locations due to genetic variations and different environment conditions.[30] Based on the results, *J. gendarussa* leaf extracts from Mersing, Batu Pahat and Pulai were more cytoxicity contrary on other study in *M. calabura* leaf extracts with IC_{50} value of 46 µg/ml against HT-29 cells.[22]

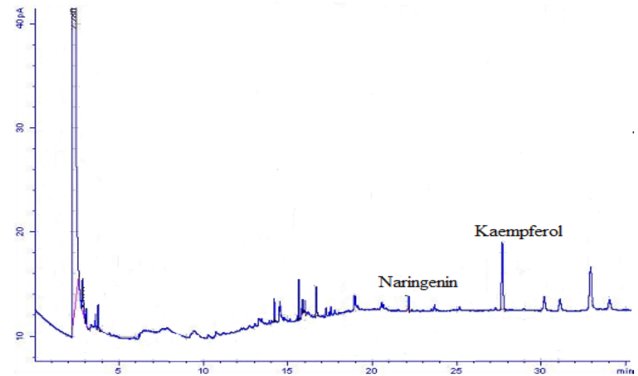

Figure 3.1 Chromatogram of naringenin and kaempferol contents in leaf extract from Mersing using GC-FID

Table 3.1 Cytotoxicity study on *J. gendarussa* methanolic leaf extracts from five different locations against HT-29 cells

Concentration (µg/ml)	7.81	15.63	31.25	62.5	125	250	500	1000
Leaf extracts from Mersing	86.37± 9.78	* 75.13± 9.00	* 9.67± 1.19	*** 3.97± 0.46	*** 2.44± 0.77	*** 2.24± 1.58	*** 1.78± 0.40	*** 1.71± 0.36
Leaf extracts from Muar	*** 81.75± 0.29	*** 77.66± 3.16	*** 73.56± 0.97	** 51.92± 4.12	*** 17.76± 1.23	*** 3.86± 0.85	*** 2.10± 0.26	*** 1.74± 0.21
Leaf extracts from Skudai	*** 73.87± 2.52	*** 71.24± 2.10	** 68.04± 2.35	*** 56.83± 1.96	*** 19.72± 0.42	*** 3.69± 0.36	*** 2.15± 0.33	*** 2.49± 0.03
Leaf extracts from Batu Pahat	* 67.43± 8.53	** 61.04± 6.81	* 51.46± 7.57	* 47.49± 7.71	*** 14.44± 3.16	*** 3.02± 0.70	*** 2.99± 0.84	*** 2.87± 0.31
Leaf extracts from Pulai	*** 73.52± 3.31	** 69.51± 4.39	*** 57.12± 4.62	*** 31.28± 3.25	*** 4.69± 1.35	*** 3.64± 1.10	*** 3.62± 0.29	*** 3.38± 0.11

Values are mean±SD for 3 replicates *,p<0.05, **,p<0.01,***,p<0.001 compared with control

Table 3.2 Comparison of IC_{50} values the various extracts of *J. gendarussa* leaf extracts in human cancer cell lines

Extracts of *J. gendarussa*	**IC_{50} values (µg/ml)**		
	HT-29	**HeLa**	**BxPC-3**
Mersing	21	22	16
Muar	65	88	157
Skudai	76	39	105
Batu Pahat	36	146	410
Pulai	39	205	264

The morphological changes of the cells were observed under Nikon fluorescence microscope (Nikon, Tokyo, Japan) (10× magnification) after 72 hours treatment. The IC_{50} leaf extracts from various locations treated in HT-29 cancer cell lines revealed morphology changes (Figure 3.2: B,C,D,E,F) as compared to non-treated cells (Figure 3.2:A). Treated cells

showed a more prominent growth inhibition and shrinkage of the cells. On the contrary, untreated cells remained confluent throughout the incubation period (Figure 3.2:A).

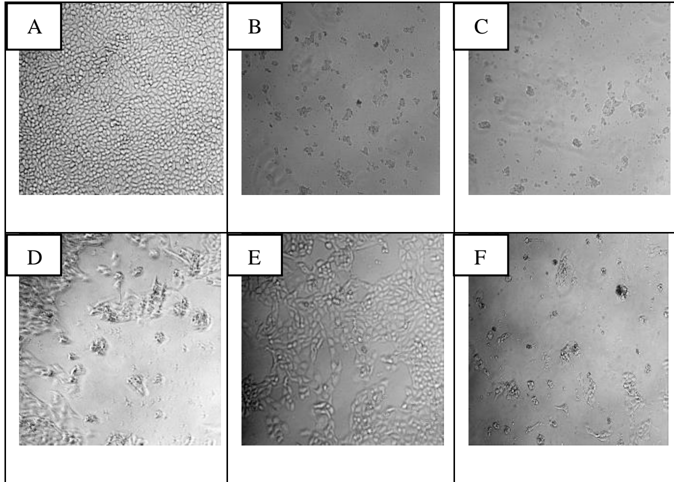

Figure 3.2 Morphology of IC_{50} in *J. gendarussa* leaf extracts from five different locations against HT-29 cancer cell lines after MTT assay (10× magnification). (A) HT-29 cells without any treatment, control; (B) leaf extract from Mersing, 31.25 µg/ml; (C) leaf extract from Muar, 125 µg/ml; (D) leaf extract from Skudai, 125 µg/ml; (E) leaf extract from Batu Pahat, 62.5 µg/ml; (F) leaf extract from Pulai, 62.5 µg/ml

Table 3.3 represent HeLa cancer cells viability on *J. gendarussa* leaf extracts from Mersing, Muar, Pulai, Skudai and Batu Pahat. IC_{50} values of leaf extracts from Mersing, Muar, Skudai and Batu Pahat showed high significantly difference ($p<0.001$) between different concentrations of extracts. Leaf extracts from Mersing showed weak cytotoxicity with IC_{50} values of approximately 22 µg/ml followed by leaves extracts from Skudai (39 µg/ml), Muar (88 µg/ml), Batu Pahat (146 µg/ml) and Pulai (205 µg/ml) (Table 3.2). The cell viability of HeLa cancer cells were decreased in dose- dependent manner and the trend of inhibitory cells are same in all extracts. From the results, leaf extract from Mersing was effective against HeLa cells as compared to other extracts and this activity could be associated with the presence of more kaempferol contents as compared to naringenin contents in the extracts (Figure 3.1).

The IC_{50} methanolic leaf extracts from various locations treated in HeLa cancer cell lines revealed morphology changes (Figure 3.3: B,C,D,E,F) as compared to non-treated cells (Figure 3.3: A). Treated cells show a more prominent growth inhibition and shrinkage of the cells. On the contrary, untreated cells remained confluent throughout the incubation period (Figure 3.3:A).

The effect of *J. gendarussa* leaf extracts from Mersing, Muar, Pulai, Skudai and Batu Pahat on the cytotoxicity potential of BxPC-3 cancer cell lines are demonstrated in Table 3.4. The IC_{50} values showed high significantly difference ($p<0.001$) in leaf extracts from Skudai and Pulai, followed by leaf extract from Mersing, Muar and Batu Pahat showed moderate significant difference ($p<0.01$) between different concentration of extracts. The similar trend inhibition of cells and the cell viability of the cells were decreased in dose- dependent manner in all extracts. The result from cytotoxicity assay on BxPC-3 cancer cell lines are summarized in table 3.2, leaf extracts from Mersing showed cytotoxicity with IC_{50} values of approximately 16 µg/ml and other extracts showed not cytotoxicity against BxPC-3.[27-29] The leaf extract from Mersing showed cytotoxicity and it is believed from the chromatogram indicates the presence of more kaempferol contents rather than naringenin contents that could be associated the proliferation of the cells rather than other leaf extracts (Figure 3.1).

Table 3.3 Cytotoxicity study on *J. gendarussa* methanolic leafextracts from five different locations against HeLa cells

Concentration (µg/ml)	7.81	15.63	31.25	62.5	125	250	500	1000
Leaf extracts from Mersing	82.82±3.33 ***	64.55±2.73 **	38.13±2.86 ***	4.64±0.26 *	1.53±0.21 ***	1.21±0.26 ***	1.54±0.30 ***	4.02±0.39 *
Leaf extracts from Muar	82.18±5.46 *	69.10±6.79 ***	64.51±3.43 **	56.66±2.86 ***	44.34±1.90 ***	32.78±1.90 ***	1.22±0.89 ***	0.89±0.29 ***
Leaf extracts from Skudai	70.12±2.78 **	57.44±3.08 ***	53.06±1.59 ***	43.28±2.45 ***	38.88±2.91 ***	32.49±4.24 ***	4.14±0.95 ***	5.30±0.37 ***
Leaf extracts from Batu Pahat	85.04±3.11 *	78.27±4.92 **	65.48±4.77 ***	62.32±4.09 **	52.95±3.47 ***	41.16±5.37 **	28.74±7.28 **	2.59±1.15 ***
Leaf extracts from Pulai	76.21±7.76 **	63.63±7.27 ***	63.49±1.94 ***	60.39±4.96 ***	57.33±4.63 **	47.16±5.00 ***	32.03±4.51 ***	2.33±0.58 ***

Values are mean±SD for 3 replicates *,p<0.05, **,p<0.01,***,p<0.001 compared with control

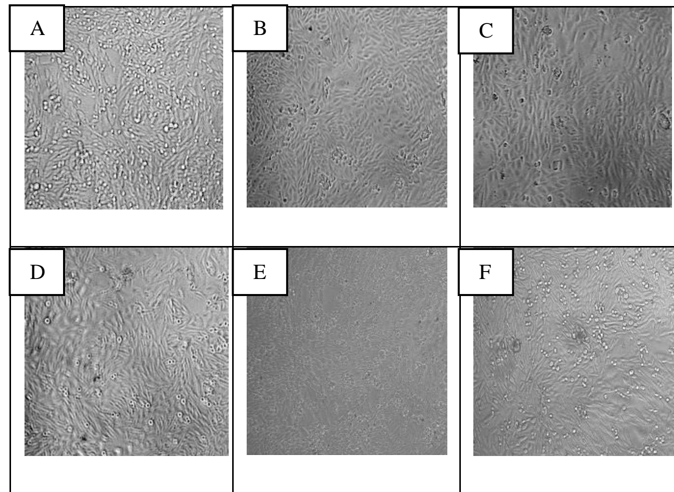

Figure 3.3 Morphology of IC_{50} in *J. gendarussa* leaf extracts from five different locations against HeLa cancer cell lines after MTT assay (10× magnification). (A) HeLa cells without any treatment, control; (B) leaf extract from Mersing, 31.25 µg/ml; (C) leaf extract from Muar, 125 µg/ml; (D) leaf extract from Skudai, 62.5 µg/ml; (E) leaf extract from Batu Pahat, 125 µg/ml; (F) leaf extract from Pulai, 250 µg/ml

Table 3.4 Cytotoxicity study on *J. gendarussa* methanolic leaf extracts from five different locations against BxPC-3 cells

Concentration (µg/ml)	7.81	15.63	31.25	62.5	125	250	500	1000
Leaf extracts from Mersing	* 69.44±4.25	** 45.79±5.25	** 43.16±3.95	*** 25.76±4.54	*** 5.28±0.19	*** 4.42±0.82	*** 3.94±0.87	*** 15.21±2.39
Leaf extracts from Muar	*** 65.88±0.69	** 59.02±13.05	* 57.47±9.92	* 55.85±11.82	* 54.68±11.71	** 36.52±8.12	** 9.55±7.00	*** 6.03±2.21
Leaf extracts from Skudai	*** 74.79±1.13	*** 70.69±0.41	*** 60.45±3.89	*** 54.48±0.33	*** 49.91±1.41	*** 48.52±1.23	* 47.83±14.79	*** 6.48±0.43
Leaf extracts from Batu Pahat	* 90.85±5.19	87.36±5.55	* 87.16±1.97	* 82.92±2.35	*** 75.51±1.98	*** 70.10±1.63	** 41.62±7.33	*** 4.52±0.20
Leaf extracts from Pulai	*** 79.24±2.76	73.29±17.6	* 69.03±6.04	*** 64.42±5.55	*** 58.24±6.41	** 52.49±3.65	*** 14.37±1.05	*** 6.49±0.36

Values are mean±SD for 3 replicates *,p<0.05, **,p<0.01,***,p<0.001 compared with control

The IC_{50} methanolic leaf extracts from various locations treated in BxPC-3 cancer cell lines revealed morphology changes (Figure 3.4: B,C,D,E,F) as compared to non-treated cells (Figure 3.4:A). Treated cells show a more prominent growth inhibition and shrinkage of the cells. On the contrary, untreated cells remained confluent throughout the incubation period (Figure 3.4:A).

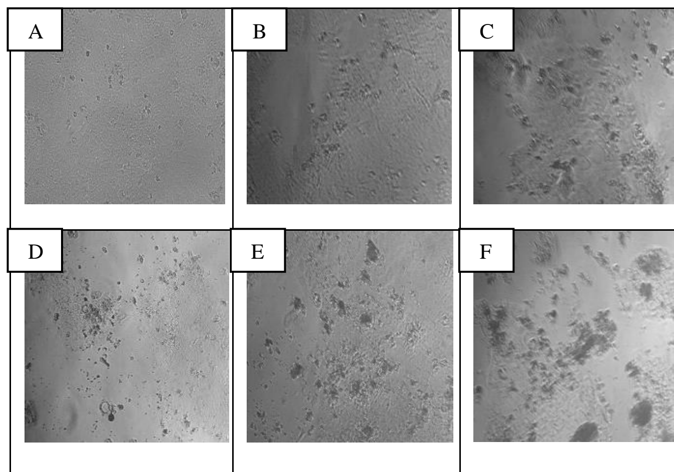

Figure 3.4 Morphology of IC_{50} in *J. gendarussa* leaf extracts from five different locations against BxPC-3 cancer cell lines after MTT assay (10× magnification). (A) BxPC-3 cells without any treatment, control; (B) leaf extract from Mersing, 15.63µg/ml; (C) leaf extract from Muar, 250µg/ml; (D) leaf extract from Skudai, 125µg/ml; (E) leaf extract from Batu Pahat, 500µg/ml; (F) leaf extract from Pulai, 500µg/ml

Table 3.5 demonstrated that the ability of kaempferol and naringenin to inhibit the proliferation of HT-29, HeLa and BxPC-3 cancer cell lines in this study. The results are as summarized in table 3.6, kaempferol showed highly cytotoxicity with IC_{50} values of approximately 5 µg/ml (HeLa) and 6 µg/ml (HT-29). This followed by naringenin with IC_{50} values of approximately 15 µg/ml (HeLa) and 19 µg/ml (HT-29) cancer cell lines. The IC_{50} values for kaempferol shows high significantly difference ($p<0.001$) between difference concentrations in all compounds whereas naringenin shows high significantly difference ($p<0.001$) in HT-29 and BxPC-3 while weak significantly difference ($P<0.05$) in HeLa cancer cell lines between difference concentrations in all compounds (Table 3.5).

From the chromatogram (Figure 3.1) shows both compounds were detected in leaf extracts from Mersing and assessed in this study to determine the effectiveness against human cancer cell lines where both compounds might be contributed the proliferation of cells tested in leaf extracts. It is suggested, the combination of kaempferol and naringenin in leaf extracts that could be associated in proliferation of the cells. Previous study also reported that combination of naringenin and kaempferol showed toxicity with IC_{50} values of 43.73 µg/ml as compared to naringenin and kaempferol alone against HeLa.[31] However, in this study, kaempferol and naringenin alone showed high cytoxicity with IC_{50} values of approximately 5 µg/ml and 15 µg/ml, respectively against HeLa cells. The cell of HT-29, HeLa and BxPC-3 were shrinkage decreased in dose-dependent manner and the similar trends of inhibitory of cells in all compounds. On the other hand, kaempferol showed high toxicity rather than naringenin could be due to kaempferol is strong estrogenic activity as compared to naringenin. It is suggested kaempferol have benefits potential effect in preventing estrogen imbalance diseases such as breast cancer, osteoporosis, cardiovascular,[32] hypertension, headache, abdominal pains and rheumatism.[33] However, naringenin also reported induced inhibitory effects on human cancer cell lines (MDA-MB-231 and Caco-2).[17]

Table 3.5 Cytotoxicity study on kaempferol and naringenin against HT-29, HeLa and BxPC-3 cells lines

Cells	Concentration (µg/ml)	3.91	7.81	15.63	31.25	62.5	125	250	500
HT-29	Kaempferol	** 61.19±5.90	*** 30.09±1.86	*** 15.59±0.95	*** 3.20±0.55	*** 2.67±0.43	*** 2.62±1.38	*** 4.35±1.33	*** 4.09±0.35
	Naringenin	*** 70.17±5.48	** 67.06±2.63	* 56.19±1.37	*** 34.41±7.82	*** 17.04±5.08	*** 1.60±0.31	*** 1.51±1.26	*** 1.33±0.76
HeLa	Kaempferol	*** 51.22±0.67	*** 25.15±0.69	*** 13.28±2.59	*** 6.49±0.66	*** 5.71±0.86	*** 5.80±0.86	*** 4.90±1.12	*** 6.92±0.60
	Naringenin	* 69.73±8.40	*** 60.26±7.12	* 48.58±5.99	*** 27.94±2.66	*** 15.01±2.55	*** 4.08±1.31	*** 3.37±0.16	*** 4.22±0.96
BxPC-3	Kaempferol	** 69.55±3.76	** 67.13±10.39	*** 66.21±5.65	*** 31.54±5.90	*** 27.74±0.78	* 27.09±1.58	*** 30.91±1.69	** 24.51±6.45
	Naringenin	** 76.10±8.98	** 75.69±6.58	* 67.96±11.57	* 62.97±11.32	*** 46.82±0.54	*** 8.24±1.31	*** 6.37±1.12	*** 5.06±0.88

Values are mean±SD for 3 replicates *,p<0.05, **,p<0.01,***,p<0.001 compared with control

Table 3.6 Comparison of IC_{50} values the compounds (kaempferol and naringenin) in human cancer cell lines

Compounds	IC_{50} values (µg/ml)		
	HT-29	HeLa	BxPC-3
Kaempferol	6	5	23
Naringenin	19	15	57

The IC_{50} kaempferol and naringenin in HT-29 (Figure 3.5: B,C), HeLa (Figure 3.5: E,F) and BxPC-3 (Figure 3.5: H,I) cancer cell lines revealed morphology changes as compared to non-treated cells (Figure 3.5:A,D,G). Treated cells showed a more prominent growth inhibition and shrinkage of the cells. On the contrary, untreated cells remained confluent throughout the incubation period.

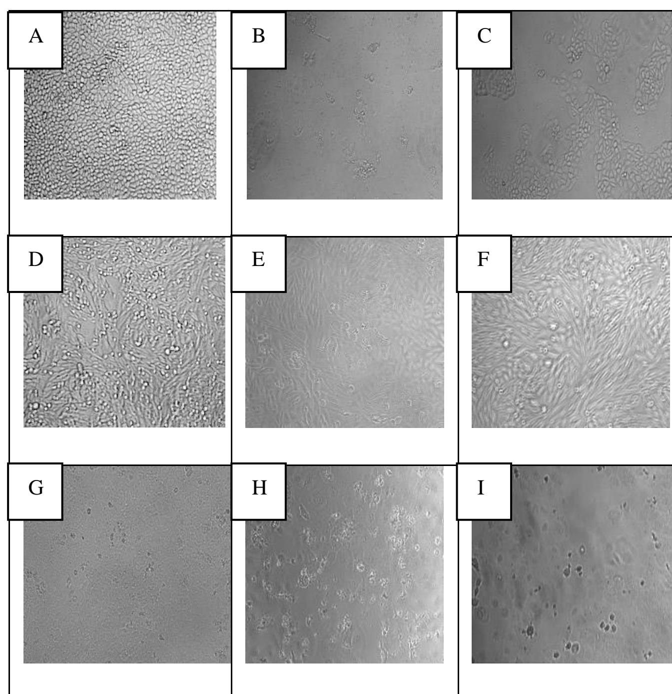

Figure 3.5 Morphology of IC_{50} in kaempferol and naringenin and against HT-29, HeLa and BxPC-3 cancer cell lines after MTT assay (10× magnification). (A) HT-29 cells without any treatment, control; (B) HT-29 cells treated with kaempferol, 7.81 µg/ml; (C) HT-29 cells treated with naringenin, 31.25 µg/ml; (D) HeLa cells without any treatment, control; (E) HeLa cells treated with kaempferol,7.81 µg/ml; (F) HeLa cells treated with naringenin, 15.63 µg/ml; (G) BxPC-3 cells without any treatment, control; (H) BxPC-3 cells treated with kaempferol, 31.25 µg/ml; (I) BxPC-3 cells treated with naringenin, 62.5 µg/ml

Table 3.7 shows the cytotoxicity study on *J. gendarussa* leaf extracts from Mersing, Muar, Pulai, Skudai and Batu Pahat and also flavonoids (kaempferol and naringenin) against CHO normal cell lines. CHO cell lines are positive control used to compare the cytotoxic activity on HT-29, HeLa and BxPC-3 human cancer cell lines. The comparisons of both compounds were performed based on IC_{50} between human cancer and normal cell lines.[27-29] From this finding, leaf extract from Mersing was weak cytotoxicity against CHO cells but not cytotoxicity in others leaf extracts. However, IC_{50} values of leaf extracts in Mersing against human cancer cells highly cytotoxicity as compared in CHO normal cells. It is suggested, the cytotoxic activity on leaf extracts from Mersing could be associated presence high contents of flavonoid compounds. Therefore, the active compounds particularly flavonoid contents in *J. gendarussa* leaf extracts might be contributed the cytotoxicity activity against HT-29, HeLa and BxPC-3 cancer cells lines.

Table 3.7 Comparison of IC_{50} values the compounds and various extracts of *J. gendarussa* leaf extracts in CHO normal cell lines

	IC_{50} values (µg/ml)
Compounds	
Kaempferol	14
Naringenin	21
Extract of *J. gendarussa*	
Mersing	28
Muar	108
Skudai	88
Batu Pahat	190
Pulai	305

■4.0 CONCLUSION

In conclusion, various leaf extracts and flavonoid compounds were evaluated and exhibited a dose- dependent manner on cytotoxicity effects against human cancer cell lines. In this study, leaf extracts from Mersing demonstrated high cytotoxic against BxPC-3 pancreas cell lines. It is suggested, leaf extracts from Mersing containing active compounds particularly flavonoids that exhibit potential inhibitors of growth and act as an anti-cancer plant particularly in BxPC-3 cells. Further investigation to those researches are interested on *J. gendarussa* plant should review on positive standard drugs such as tamoxifen that could provide additional insight of this extracts uses as chemotherapeutic agents. Furthermore, this study justifies the *J. gendarussa* leaf extracts as a folklore medicinal uses and validates its potential uses as anti-cancer.

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