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Screening on Anti-Proliferative Activity of *Psidium Guajava* Leaves Extract towards Selected Cancer Cell Lines

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Abstract: *Psidium guajava* (PG) has been reported to possess an anti-inflammatory, anti-microbial and hypoglycemic activity. However, the information of PG as an anti-cancer is still limited. The purpose of this study is to determine the anti-proliferative activity of organic extracts of PG leaves against the selected cancer cell lines. The three cancer cell lines were cervical cancer (HeLa), breast cancer (MDA-MB-231) and osteosarcoma (MG-63). The PG leaves were extracted using petroleum ether, methanol, and water. For negative control cell, non-malignant cell Madin Darby canine kidney (MDCK) was used where areas cisplatin act as positive control. In this study, methylene blue assay was done to test the anti-proliferative activity of the plant extracts. The anti-proliferative activity was expressed in IC_{50} value. All of the organic extracts of PG showed no anti-proliferative activity on HeLa. However, petroleum ether leaves extract showed the most effective anti-proliferative activity followed by methanol extract and water extract to MDA-MB-231, with IC_{50} of 4.23μg/ml, 18.60 μg/ml and 55.69 μg/ml respectively. Petroleum ether extract also showed similar effects on MG-63 followed by methanol extract and water extract with IC_{50} of 5.42 μg/ml, 23.25 μg/ml and 61.88 μg/ml respectively. The petroleum ether leaves extract also showed the most anti-proliferative activity towards MDCK. As a conclusion, PG extracted with petroleum ether exhibited the most effective anti-proliferative towards MD-MB-231 and MG-63. However, this extract showed cytotoxic effect on non-malignant cell line (MDCK). Thus, the test on other non-malignant cells must be carried out to screen its side effect on normal cells. PG has the potential for anti-cancer agents especially for breast cancer and osteosarcoma. A further investigation on their molecular mechanism is worth in understanding the anti-cancer drug development.

Key words: Psidium guajava, organic extracts, anti-proliferative activity, HeLa cell, cancer cell lines.

1. Introduction

In several years, the increasing study on anti-cancer agent based on traditional used of medicinal plant has become quietly extensive in anti-cancer research. This reason can be support by the recent studies which showed that many plants including their components can be function effectively as tumor suppressor as well as apoptotic inducers in cancer cells. Generally, the tumor suppressing activity arise from the medicinal plants will interfere with cell cycle, thus enhance the immune activity and suppress tumor angiogenesis [1]. Furthermore, there was epidemiological evidence which indicate the association between taking diets rich in fresh fruits can possibly decreased a risk of certain forms of cancer [2] due to the high content of variable bioactive compounds known as phytochemicals [3]. *Psidium guajava* (PG) is one of the most common plants which is important in food industry and have been widely searching for its medicinal properties. PG belongs to the family of Myrtaceae and is a large spreading shrub or a small tree up to 15 m high [4]. It is grown throughout the tropics and subtropics and is known for its edible fruits [5]. This plant is available in the South America, European, Africa and Asia [6]. The common names of PG are guava (English), jambu batu (Malay), mansala (India) and gwaaba (Africa). Phytochemical studies undertaken on different parts of the plants have resulted

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in the isolation and identification of various terpenoids, flavonoids and tannins [5–10]. Different parts of PG have known to pose several medicinal properties such as anti-inflammatory, analgesic and anti-pyretic [11], anti-cough [4], anti-microbial [8, 12], intestinal anti-spasmodic [13], anti-diabetic [14] and anti-oxidant [15]. According to Chen and co-researcher in 2007 also reported that the PG leaves extract contain a soluble polyphenolics including (in mg/g) gallic acid (348), catechin (102), epicatechin (60), rutin (100), quercetin (102), and rutin (100). Leaves and bark decoction of PG has been used widely in India to treat diarrhea, dysentery, vomiting and as well as sore throats. While among the Amazon tribes, the leaves decoction is commonly used in treated mouth sores and bleeding gums [16].

In Malaysia, PG leaves are used to treat diarrhea and stomach ache with an astringent solution and to expel the placenta during childbirth [17]. However, there is still limited information regarding the anti-cancer property of PG. In addition, PG has also been tested for its anti-cancer properties especially for osteosarcoma cells [9]. In previous study by Sato and co-worker in 2010 found out that leaves of PG may have anti-cancer activity although it is based on very limited studies. Development of new anti-cancer agents is vital because cancer has been the second major cause of death around the world population which already accounted for 7.6 million of death in 2005. The number will be expected to rise to 9 million in 2015 with further arise of more than 11 million deaths in 2030 [18]. Therefore, more studies in this area are required to establish PG as potential anti-cancer drugs to overcome the rise of deaths from cancer. The objectives of this study were to extract the leaves of PG using different polar solvents to obtain an organic extracts of the plants and to determine the anti-proliferative activity of organic extracts of PG leaves on selected cancer cell lines.

2. Methodology

2.1 Plants Collection and Preparation

The PG leaves were collected from Bukit Bidang, Kelantan, Malaysia. All the plant parts were first washed using tap water and were dried in oven at 50°C. The dried leaves were then blended into powder form.

2.2 Successive Extraction of Plants

An amount of 25 g of leaves powder was subjected to successive extraction using Soxhlet instrument according to [19] with few modifications. The leaves were extracted using in turn with petroleum ether, methanol and water. Extraction was stopped when the solvent became clear. All the extraction products were concentrated through vacuum using Rotoevaporater and let to completely dry in fume hood to eliminate solvent residual. After completely dry, the weights of the extracts were measured. Then, stock solutions of each plant extract were prepared in 10 mg/ml by diluting with DMSO and were kept at 4°C for further uses.

2.3 Cell Culturing

Three types of cancer cell lines were used; cervical cancer cell line (HeLa), breast cancer cell line (MDA-MB-231) and osteosarcoma cell line (MG-63). Non-malignant cell line, Madin-Darby canine kidney cell line (MDCK) was used as a control. Cells from stocks were cultured separately on cell culture flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin). Cells were incubated at 37°C humidified incubator supplemented with 5% (v/v) CO_2. All culturing works were done in Biohazard Safety Cabinet class II with sterile techniques.

2.4 Cell Treatment

Cultured cancer cells with 80–90% confluence were used for plating. The adherent cells were trypsinized to detach cells. 100 µl of cells were seeded into each well of the 96-wells microtiter plates (1-5×10^4 cells per well). The plate was maintained at 37°C in a humid incubator with an air mixture containing 5% (v/v) CO_2

for 24–48 hours until 80–90% confluence. Then, old medium were discarded and 200 µl of new medium were added. Next, the cells were treated with 2 µl of series dilution of plant extracts (Table 1). The cells were also treated with 2 µl of series dilution of cisplatin as positive control and DMSO as negative control. The plate was returned to incubator for 72 hours. All treatments were done in triplicate (n = 3). The same procedure was also done on non-malignant cell lines.

2.5 Methylene Blue Assay

After 72 hours treatment, anti-proliferative activity of plant extracts were studied using methylene blue assay [20]. Firstly, 22.5 µl of 25% glutaraldehyde was added into each well to fix the viable cells to bottom of the well and run off on shaker for 15 minutes. Then, glutaraldehyde and old medium were removed and dead cells were washed away with 100 µl 0.15 M sodium chloride (NaCl) for three times. Subsequently, the viable cells were stained with 100 µl of 0.05% methylene blue dye and run off on shaker for 15 minutes. The balance of methylene blue was then removed and rinse with 0.15 M NaCl for three times. 200 µl of 0.33 M HCl was added and placed on shaker for 15 minutes to get good colour elution. Finally, the absorbance reading (O.D) was read using ELISA reader at 660 nm wavelength. The amount of colour present corresponds directly with the number of cells that survived until assay works finished.

Table 1 Leaves extracts and cisplatin concentration used for treatment of the cells.

Extract concentration (µg/ml)	Final concentration in wells (µg/ml)
10000.00	99.00
5000.00	49.50
2500.00	24.80
1250.00	12.40
625.00	6.19
312.50	3.09
156.30	1.55
78.10	0.77
39.10	0.39

2.6 IC_{50} Determination

Inhibitory Concentration (IC) was defined by IUPAC Compendium of Chemical Terminology as a concentration of a substance that causes a defined inhibition of a given system. IC_{50} is the median concentration that causes 50% inhibition. IC_{50} value of the extract was determined from the plot of viable cells percentage and final concentration of the extract. Viable cells percentage was counted as:

[Mean O.D of treated cells/Mean O.D of control cells] ×100%

2.7 Statistical Analysis

After all the data have been collected, the means ± S.D of the data will be analyzed by statistical analysis using SPSS package for Window (version 18.0.1).

3. Results and Discussion

3.1 Extraction Yields

The successive extraction of PG was started in turn with the less polar solvent, i.e., petroleum ether followed by the more polar solvent, i.e., methanol and water. Petroleum ether was used to extract fatty acid materials from the plants. Methanol and water were used to extract more polar compounds such as alkaloids. Three types of extracts were obtained from the successive extraction method. From 25 g of each dried powder, each extraction produced a different extraction yields (Table 2). Extraction yield is defined as the percentage of final extraction product after extraction:

[Weight after extraction/weight before extraction]×100%

3.2 Anti-proliferative Activity of Plant Extracts on Tested Cancer Cells

The anti-proliferative activity of the plant extracts on cancer cells were expressed in IC_{50} value. IC_{50} is the inhibitory concentration that causes 50% inhibition of the cancer cell population. The extract that has IC_{50} value $\leq$ 20 µg/ml was the one which has a significant anti-proliferative value [21]. The three types of the leaves extracts exhibited a different pattern of

antiproliferative effects (Figs. 1–4). The values for anti-proliferative activity with standard deviation were shown in Table 3. For HeLa cells, all the organic extracts of PG (petroleum ether, methanol and water) showed no anti-proliferative activity (Table 4). For MDA-MB-231 cells, petroleum ether extract of PG showed the most effective anti-proliferative activity followed by methanol extract of PG and water extract of PG with IC_{50} of 4.23 μg/ml, 18.60 μg/ml and 55.69 μg/ml respectively (Table 4). For MG-63 cells, petroleum ether extract of PG showed the most effective anti-proliferative activity followed by methanol extract of PG and water extract of PG with IC_{50} of 5.42 μg/ml, 23.25 μg/ml, and 61.88 μg/ml respectively (Table 4). For control cells, MDCK, petroleum ether extract of PG showed the most effective anti-proliferative activity followed by methanol extract of PG, water extract of PG with IC_{50} of 5.03 μg/ml, 11.55 μg/ml and 15.5 μg/ml, respectively (Table 4). The positive control drug, cisplatin showed no effect on HeLa cells while it showed anti-proliferative activity on MG-63,

Table 2 Percentage of final extraction products.

Types of Extract	Weight after extraction (g)	Weight before extraction (g)	Percentage of final extraction products (%)
P. guajava pet. ether extract	1.8	25.0	7.2
P. guajava methanol extract	5.5	25.0	22.0
P. guajava water extract	7.1	25.0	28.4

Table 3 Mean with standard deviation values for each plant extracts on all tested cell lines.

Final Concentration (μg/ml) of PG Extracts		0	0.39	0.77	1.55	3.09	6.19	12.4	24.8	49.5	99.0
Replicates		n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3
Water	HeLa	100±0	110.47± 13.17	104.18± 11.21	106.32± 8.57	109.85± 12.61	108.86± 20.34	114.15± 27.25	104.24± 18.06	79.64± 10.55	72.11± 2.67
	MDA-MB-231	100±0	108.54± 3.84	94.65± 11.88	90.32± 4.08	91.50± 4.97	93.06± 9.18	98.00± 14.45	74.00± 8.48	54.00± 10.27	19.00± 2.46
	MG-63	100±0	99.61± 9.51	100.65± 6.28	97.92± 7.77	96.25± 11.23	94.08± 3.93	92.31± 1.60	87.71± 12.10	57.99± 0.31	19.70± 2.08
	MDCK	100±0	103.40± 2.49	104.05± 3.97	104.81± 6.62	104.88± 5.38	105.75± 6.55	98.00± 5.31	74.00± 0.13	54.00± 2.02	19.00± 1.77
Methanol	HeLa	100±0	109.92± 15.36	104.65± 11.58	106.68± 4.33	113.21± 24.31	107.08± 21.51	103.03± 4.33	92.77± 24.25	76.16± 26.91	56.97± 14.31
	MDA-MB-231	100±0	96.54± 1.70	90.37± 5.21	91.26± 6.30	84.06± 2.66	76.75± 3.19	63.24± 3.92	39.57± 8.75	16.27± 0.69	15.70± 1.04
	MG-63	100±0	92.79± 4.72	93.82± 3.93	95.50± 16.24	88.04± 20.84	91.68± 22.43	91.88± 10.09	45.23± 7.34	33.21± 2.40	35.33± 3.00
	MDCK	100±0	102.63± 2.27	103.28± 5.03	102.60± 6.48	100.16± 4.49	87.11± 10.65	48.62± 3.34	12.25± 2.28	10.36± 0.48	9.35± 0.48
Pet-Ether	HeLa	100±0	109.15± 7.85	113.86± 14.11	101.58± 9.97	86.82± 14.12	71.92± 18.23	72.13± 20.34	72.18± 17.69	55.21± 21.57	56.54± 14.91
	MDA-MB-231	100±0	87.78± 8.71	104.79± 7.81	91.32± 3.49	87.66± 1.84	44.29± 3.10	24.21± 3.11	12.19± 0.85	13.58± 0.89	18.59± 1.40
	MG-63	100±0	113.57± 9.09	96.78± 1.22	95.40± 4.84	93.15± 10.02	34.05± 0.84	23.63± 1.53	20.32± 1.16	21.30± 1.81	25.06± 3.59
	MDCK	100±0	106.13± 7.08	101.80± 6.14	108.46± 11.48	74.80± 12.07	33.46± 2.10	21.44± 3.44	5.10± 0.20	5.11± 0.70	5.98± 0.79
Cisplatin	HeLa	100±0	104.55± 14.96	108.09± 6.38	103.50± 9.71	100.83± 14.28	102.60± 10.63	94.80± 9.21	93.46± 15.71	90.96± 18.25	65.71± 22.32
	MDA-MB-231	100±0	96.36± 1.73	90.19± 5.17	90.96± 6.29	83.69± 2.67	76.33± 3.17	62.83± 3.86	39.11± 8.80	15.66± 0.67	15.09± 0.93
	MG-63	100±0	92.62± 9.41	99.17± 7.24	74.14± 16.15	53.08± 9.71	39.41± 10.53	21.29± 2.01	21.67± 2.15	23.88± 5.28	24.19± 1.92
	MDCK	100±0	105.86± 7.55	103.05± 6.61	88.41± 1.76	81.06± 2.27	70.96± 9.08	58.01± 4.53	32.16± 12.62	12.74± 10.45	9.90± 0.47

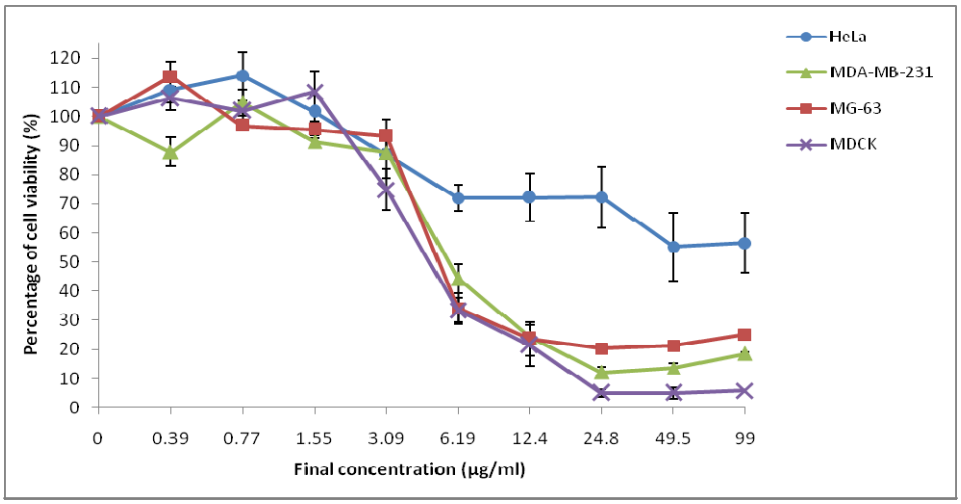

Fig. 1 Anti-proliferative activity of PG petroleum ether extract on HeLa, MDA-MB-231, MG-63 and MDCK cell lines at different final concentrations of 0.39–99 µg/ml for 72 hours. Each point showed the percentage of viable cells. n = 3, $P < 0.05$.

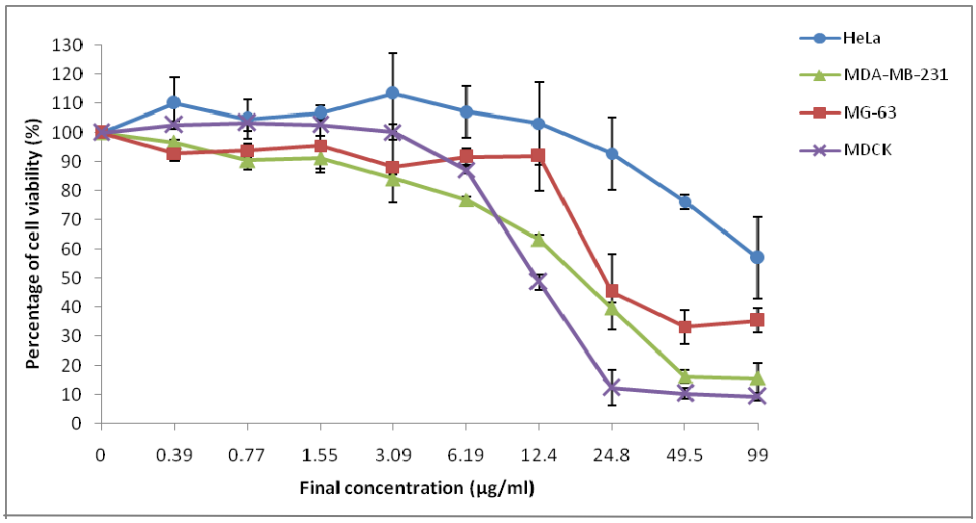

Fig. 2 Anti-proliferative activity of PG methanol extract on HeLa, MDA-MB-231, MG-63 and MDCK cell lines at different final concentrations of 0.39–99 µg/ml for 72 hours. Each point showed the percentage of viable cells. n = 3, $P < 0.05$.

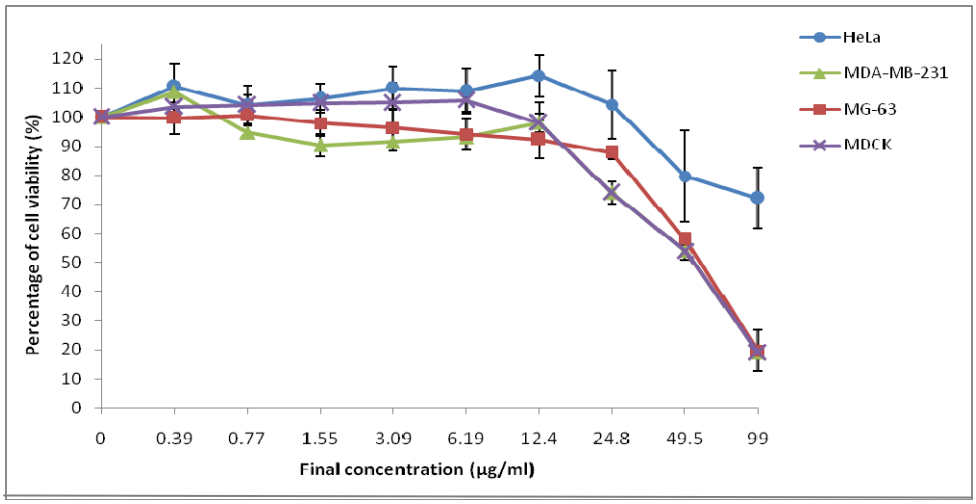

Fig. 3 Anti-proliferative activity of PG water extract on HeLa, MDA-MB-231, MG-63 and MDCK cell lines at different final concentrations of 0.39–99 µg/ml for 72 hours. Each point showed the percentage of viable cells. n = 3, $P < 0.05$.

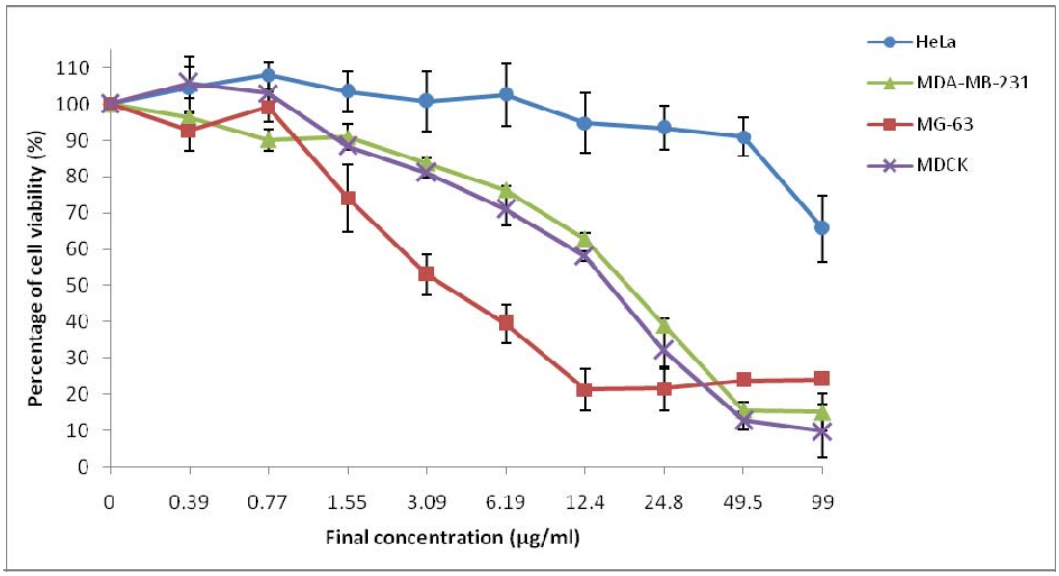

Fig. 4 Anti-proliferative activity of cisplatin on HeLa, MDA-MB-231, MG-63 and MDCK cell lines at different final concentrations of 0.39-99 µg/ml for 72 hours. Each point showed the percentage of viable cells. n = 3, $P < 0.05$.

Table 4 IC_{50} value of each plant extract on all cell lines tested.

		IC_{50} values (µg/ml)			
Plants	Extracts	HeLa	MDA-MB-231	MG-63	MDCK
P. guajava	Pet. ether	-	4.23	5.42	5.03
P. guajava	Methanol	-	18.60	23.25	11.55
P. guajava	Water	-	55.69	61.88	15.50

MDA-MB-231 and MDCK cells with IC_{50} of 3.87 µg/ml, 18.6 µg/ml and 15.5 µg/ml (Table 5).

Many studies have been done on PG based on their constituents and potential bioactives related to anti-cancer found in fruit, leaves and bark [17]. However, there are limited studies on anti-proliferative activity from PG leaves extract. According to Sato *et al* [17], only seven studies were related to the anti-proliferative activity on cancer cell line by using PG leaves extract. In this study, PG leaves extract of petroleum ether, methanol and water were used to test the anti-proliferative activity on three types of cancer cell lines that were human cervical cancer (HeLa), breast cancer (MDA-MB-231) and osteosarcoma cancer (MG-63) cell lines. All of the three types of PG extract did not showed any anti-proliferative activity on HeLa cells. Based on the three extracts, petroleum ether extract exhibited the most effective anti-proliferative activity towards MDA-MB-231 and MG-63 cell lines followed by methanol extract. Nevertheless, these extract have a cytotoxic effect on non-malignant cell Madine Darby canine kidney (MDCK). It is mean the anti-cancer effect of petroleum ether and methanol extract of PG was accompanied by cytotoxic effect. So, the test for the other non-malignant cells must be carried out to screen its side effect on normal cells. The finding by Ampasavate et al. [22] showed that ethanolic extracts from PG leaves have no cytotoxicity effects to four leukemic cell lines, erythroid (K562), promyeloid (HL60), monocytic (U937) and lymphoblastic (Molt4), and the human peripheral blood mononuclear cells (PBMCs). PBMC is other normal human cell type that also used in the cytotoxicity test to assess the effects of anti-proliferative activity on normal cells. Moreover,

Table 5 IC_{50} value of cisplatin on all cell lines tested.

Positive	IC_{50} values (µg/ml)			
control drug	HeLa	MDA-MB-231	MG-63	MDCK
Cisplatin	-	18.60	3.87	15.50

study by Manosroi et al [23] reported that PG leaf oil showed the highest anti-proliferative activity in human mouth epidermal carcinoma (KB) cells with IC_{50} value of 0.0379 mg/ml which is 4.37 times more potent than vincristine. In other study, an aqueous extract of PG leaves was showed to inhibit the cancer cell line, brain-derived metastatic prostate (DU-145) in a dose-dependent manner to 36.1 percent (48 hours) and 3.59 percent (72 hours) of incubation [24]. In the study by Kaileh et al [25] also reported that the methanolic extract of PG has been found to be effective against murine fibrosarcoma (L929sA) and benign human breast cancer (MCF7), but the extract did not have any anti-proliferative activity on metastatic human breast cancer (MDA-MB-231). Anti-cancer effect was also displayed by methanol extract of PG on mice-induced cancer inoculated with B16 melanoma cells [26]. In addition, aqueous extract of PG have exhibited potent anti-proliferative activity towards KB with IC_{50} value of 0.055 mg/ml and for HeLa cells, the antiproliferative activity of the aqueous extract was significantly lower at an IC_{50} value of 0.051 mg/ml [27].

4. Conclusion

This study demonstrates the PG as a promising anti-cancer agent with petroleum ether extract showed the most anti-proliferative activity on human breast cancer (MDA-MBA-231) and osteosarcoma cancer (MG-63) cells compared to the other extracts. Since the anti-proliferative activity of petroleum ether extract is accompanied with cytotoxic effect, an assessment of their cytotoxic effect must be carried out to ensure the PG is safe for human consumption. A further investigation on their molecular mechanism is can be worth in understanding the anti-cancer drug development.

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