In Vitro Cytotoxic and Antiproliferative Effects of Portulaca oleracea Methanol Extract on Breast, Cervical, Colon and Nasopharyngeal Cancerous Cell Lines

(Sitotosik In Vitro dan Kesan Perencatan Proliferatif oleh Portulaca oleracea Pati Metanol Terhadap Sel Kanser Payudara, Serviks, Kolon dan Nasofarinks)

Gek Choo Sarah Tan, Kar Mun Wong, Gui Qi Pearle-Wong, Siau Li Yeo, Swee Keong Yeap, Beow Chin Yiap & Hueh Zan Chong*

ABSTRACT

Portulaca oleracea *is a ubiquitous garden weed that has been traditionally used as antidiabetic and anti-inflammation agent. However, the potential anti-proliferative and cytotoxic effects of* Portulaca oleracea *towards cancerous cells are still unclear. Human hormone dependent breast cancer* MCF-7 *cell, colon cancer* HT-29, *cervical cancer Hela cell and nasopharyngeal cancer* CNE-1 *cell were used in this study.* P. oleracea *was extracted using methanol and the cytotoxicity against various cancerous cell lines was evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (*MTT*) assay. The antiproliferation effect and cell cycle arrest were assessed using BrdU proliferation assay and flow cytometry cell cycle RNase/*PI *analysis, respectively.* Portulaca oleracea *methanol extract was able to reduce viability of all the tested cancerous cell lines. However,* IC_{50} *was only observed in* CNE-1 *cell (92 µg/mL). BrdU incorporation assay indicated anti-proliferation of* Portulaca oleracea *treated* MCF-7 *cells in a dose-dependent manner. A significant increase in the sub G0/G1 cell population phase observed by cell cycle analysis indicates the occurrence of apoptotic events. In conclusion,* Portulaca oleracea *showed anti-proliferative effect on* CNE-1, *HeLa and* HT-29 *and* DNA *fragmentation on* MCF-7 *cells.*

Keywords: BrdU; cell cycle; MCF-7; Portulaca oleracea

ABSTRAK

Portulaca oleracea *adalah sejenis rumpai taman yang digunakan secara tradisi sebagai agen antidiabetik dan anti-keradangan. Walau bagaimanapun, kesan potensi anti-proliferatif dan sitotosik* Portulaca oleracea *terhadap sel-sel kanser masih tidak jelas. Sel kanser payudara yang bergantung kepada hormon* MCF-7, *sel kanser kolon* HT-29, *sel kanser serviks Hela dan sel kanser nasofarinks* CNE-1 *telah digunakan dalam kajian ini.* Portulaca oleracea *telah diekstrak dengan metanol dan kesan sitotosiknya terhadap pelbagai jenis kanser telah dikaji menggunakan pengasaian 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (*MTT*). Kesan anti-proliferatif dan tangkapan kitaran sel telah dinilai melalui pengasaian BrdU dan analisis kitaran sel Rnase/*PI *menggunakan aliran sitometri. Metanol ekstrak* Portulaca oleracea *dapat mengurangkan kebolehhidupan untuk kesemua sel kanser yang diuji. Akan tetapi,* IC_{50} *hanya dapat diperoleh pada sel* CNE-1 *(92 µg/mL). Pengasaian BrdU menunjukkan kesan anti-percambahan oleh* Portulaca oleracea *terhadap sel* MCF-7 *adalah bergantung kepada kepekatan. Peningkatan sel populasi yang ketara pada fasa G0/G1 telah didapati pada analisis kitaran sel menandakan berlakunya apoptosis. Kesimpulannya,* Portulaca oleracea *menunjukkan kesan anti-proliferatif terhadap sel* CNE-1, *Hela dan* HT-29 *dan kesan* DNA *fragmentasi terhadap sel* MCF-7.

Kata kunci: BrdU; kitaran sel; MCF-7; Portulaca oleracea

INTRODUCTION

The application of ethnopharmacology and complimentary alternative medicine in combating current health issues is eminent. Numerous studies have evaluated the biological activities of various phytochemicals produced by plants, particularly the anti-proliferative and cell cycle regulatory effects, in relation to cancer prevention (Cheng et al. 2011; Chu et al. 2002; Zhang et al. 2005). *Portulaca oleracea* or commonly known as Purslane or 'gelang pasir' in Malaysia, falls under the family of Portulacaceae. It is an annual sprawling succulent weed with thick, hairless red stems, obovate leaves and small yellow flowers that widely grows in tropical and subtropical regions (Chan et al. 2000; Dweck 2001; Global Information Hub on Integrated Medicine 2011). *Portulaca oleracea* is a fascinating plant recognised in most cultures for its extensive nutritional benefits. It has been used traditionally as a vegetable for human consumption. On the other hand, traditional medicinal systems of China, India, Europe and Middle Eastern countries have used *P. oleracea* to treat various human ailments such as haemorrhoids, burns and wounds, pain, headache, scurvy, fever and urinary disorder. Therefore, this edible vegetable is dubbed the 'global panacea' (Bosi et al. 2009).

Extensive modern pharmacological studies have attested its wide range of biological effects. It was reported to contain a high antioxidant property (Dkhil et al. 2011; Lim & Quah 2007; Sulaiman et al. 2011), which is mainly attributed to the rich source of omega-3 polyunsaturated fatty acids (Oliveira et al. 2009) and flavonoid compounds; particularly kaempferol, apigenin, myricetin, quercetin, luteolin, carotene and alkaloids (Liu et al. 2000; Xiang et al. 2005; Xu et al. 2006). Studies have also shown that the plant possesses significant analgesic and anti-inflammatory activities when compared with synthetic drugs (Sanja et al. 2009). This claimed support the traditional use of this plant in treating ulcers and inflammations. Moreover, this plant is reported to have hepatoprotective (Ahmida 2010), neuropharmacological (Radhakkrishnan et al. 2001), antihyperglycemia (Gong et al. 2009), antibacterial (Rashed et al. 2003) and even bronchodilatory effects (Boskabady et al. 2004). Nevertheless, the anti-proliferative effect of *Portulaca oleracea* has rarely been reported. However, Chen et al. (2010) reported that water soluble polysaccharides isolated from this plant possesses mild cytotoxic activity against cervical cancer HeLa cell line and the sulphated form of these polysaccharides enhances the anti-tumour effect (Chen et al. 2010). In addition, luteolin which can be found from *P. oleracea* was previously proved to induce cell cycle arrest and apoptosis of colon cancer HT-29 cell via decreased of IGF-II production and downregulated insulin-like growth factor-I receptor signaling (Lim et al. 2007, 2012).

Many of these researches have verified the importance of *P. oleracea*. Furthermore, with its high ethnopharmacological values and the present of cytotoxic polysaccharides and flavonoid especially luteolin, it is a promising plant in the investigation of cancer prevention. The aim of this study was to investigate the cytotoxicity and antiproliferative effect of *Portulaca oleracea* towards human breast, cervical, colon and nasopharyngeal cancer cells.

MATERIALS AND METHODS

PLANT MATERIAL AND EXTRACTION

A commercially available extract of *Portulaca oleracea* (Protusana) was purchased from Frutarom, Switzerland. The capsules containing 60 mg of the powdered herb extract were stored in an air tight container at room temperature for further use. Twenty grams of the powdered herb was extracted thrice by absolute methanol (Fisher Scientific, UK) at room temperature for 72 h. The extracts were then filtered and subjected to evaporation in a rotary evaporator at 40℃. From the initial 20 g of *Portulaca oleracea* powder, a yield of 66.2% of crude methanolic extract was obtained. The residual crude extract was weighed dissolved in DMSO (dimethyl sulfoxide solution) and stored at room temperature.

CANCER CELL LINES

Breast cancer MCF-7, cervical carcinoma cells, HeLa, colon cancer HT-29, nasopharyngeal CNE-1 and normal Chang liver cell were obtained from the Tissue Culture Laboratory, IMU. The entire tested cells were below 20 passages. Change liver cell line was used as a normal cell control to determine the selectivity of the extract against cancerous cell line. The cells were maintained in RPMI 1640 medium supplemented with 10% Foetal Bovine Serum (PAA, Austria) and 5% penicillin/streptomycin (PAA, Austria).

MTT CELL VIABILITY ASSAY

All cell lines were seeded in a flat 96-well plate (Becton-Dickinson, US) at a concentration of 1×10^5 cells/well. The cultured cells were then treated with *P. oleracea* extract at concentrations between 0 and 100 µg/mL for 72 h. After 72 h, 1 µg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma Aldrich, USA) was added to all well and all the plates were further incubated for another 4 h. Lastly, DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm (reference wavelength: 630 nm) (Opsys MR, Dynex Technologies, USA). Anthracycline antibiotic Doxorubicin was used as a positive control. Cell viability was calculated using the following formula:

$$Percentage\ of\ cell\ viability = \frac{Optical\ density\ of\ treated\ cells}{Optical\ density\ of\ control\ cells} \times 100\%.$$

A percentage inhibition curve was plotted against the corresponding concentrations and the half maximal inhibitory concentration (IC_{50}) was determined.

BROMODEOXYURIDINE (BRDU) PROLIFERATION ASSAY

The anti-proliferative effect of *P. oleracea* extract (60 and 80 µg/mL) against CNE-1, HeLa, HT-29 and MCF-7 cells was determined using BrdU proliferation assay (Roche, Germany, Cat. no. 11647229001) according to manufacturer's protocol. Briefly, cells were seeded in a flat 96-well plate at a concentration of 1×10^5 cells/well and treated with 60 and 80 µg/mL of the extract for 48 and 72 h, respectively. The bromodeoxyuridine solution (BrdU) labelling solutions were then added. The absorbance was measured at 540 nm by a microplate reader. The percentage of cells in the S phase was calculated using the following formula:

$$Percentage\ of\ cell\ in\ S\ phase = \frac{Optical\ density\ of\ treated\ cells}{Optical\ density\ of\ control\ cells} \times 100\%.$$

FLOW CYTOMETRY CELL CYCLE ANALYSIS

CNE-1, HeLa, HT-29 and MCF-7 cells were seeded in 6-well plate (Nunc, Denmark) at a concentration of 2×10^5 cells/mL and treated with 60 µg/mL of the *P. oleracea* extract for 36 and 60 h. After the incubation periods, the

cells were harvested and fixed in 80% absolute ethanol and stored at -20ºC for 72 h. Prior to analysis, the cells were stained with staining solution (500 μL of PBS, 0.1% TritonX, 10 mM EDTA, RNAse (50 μg/mL), PI (2 μg/mL)) and were further incubated on ice for 30 min in the dark. The stained cells were then analysed by flow cytometer (FACSCalibur, Becton-Dickinson, USA) at 488 nm. The data from 10000 events was collected and processed using CellQuest Pro software.

STATISTICAL ANALYSIS

All the assays were performed in triplicate. All values were presented as mean ± standard deviation (SD). Statistical comparisons were analysed by one-way analysis of variance (ANOVA) using Dunnett's test for multiple comparisons (SPSS software version 18.0). Differences were considered significant at $p<0.05$.

RESULTS

EFFECTS OF *PORTULACA OLERACEA* EXTRACT ON CELL VIABILITY

Methanolic extract of *Portulaca oleracea* showed an inhibitory effect towards all the tested cancerous cells compared with normal cells after 72 h treatment (Figure 1). However, IC_{50} was only obtained in extract treated CNE-1 cell at 92 μg/mL. When compared to the positive control doxorubicin, IC_{50} of doxorubicin was much lower in all the tested cell lines (CNE-1: 0.25 μg/mL; HeLa: 0.14 μg/mL; HT-29: 1.2 μg/mL; MCF-7: 0.35 μg/mL; Chang Liver: 21 μg/mL).

EFFECTS OF *PORTULACA OLERACEA* EXTRACT ON CELL PROLIFERATION

BrdU proliferation analysis, performed in a time- and dose-dependent manner, showed a significant decrease ($p<0.05$) of BrdU-labelled cells with both 60 and 80 μg/mL of the extract on CNE-1, HeLa and HT29 cells (Figure 2). The results showed better reduction of cells proliferation in CNE-1 and HeLa but not MCF-7 and HT-29 at 48 h as compared with 72 h. *P. oleracea* extract inhibited highest percentage of cell proliferation over CNE-1 cell at 48 and on HT-29 at 72 h as compared with other types of cell.

EFFECTS OF *PORTULACA OLERACEA* EXTRACT ON CELL CYCLE DISTRIBUTION

In correlation to the cell proliferation assay which demonstrated a decreased proportion of proliferating cells after *P. oleracea* treatment, we next studied the effects of *P. oleracea* on cell cycle progression based on their DNA ploidy of all the tested cancerous cells using RNase/PI staining prior to the flow cytometric analysis. At both 36 and 60 h of treatment, a significant increase ($p<0.05$) in the proportion of CNE-1 and HeLa cells at the S phase was seen as compared with their respective untreated control suggesting retardation in progression of cells through the S phase (Figure 3 & Table 1). For HT-29, after 36 and 60 h treatment, significant decrease in the S and G2/M phases couple with accumulation of cells in G_1 phase were observed. This effect suggests that the reduction in cell viability and proliferation on HT-29 was caused by cytostatic G_1 phase arrest without induction of apoptosis. Unlike other types of cells, significant increase of cell population at hypo-diploid population of subG$_0$/G$_1$
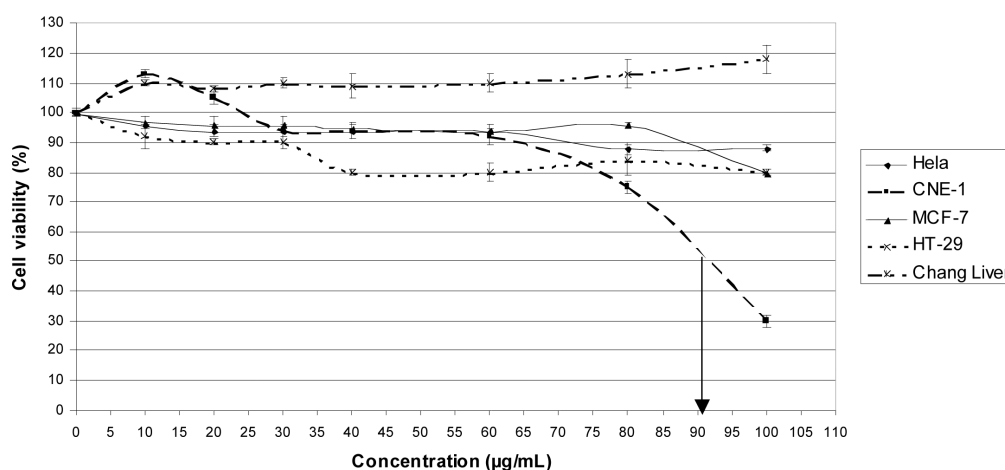

FIGURE 1. Effects of *P. oleracea* methanol extract in CNE-1, HeLa, HT-29, MCF-7 and Chang Liver cells viability. After seeding of cells in 96 well plates overnight, *P. oleracea* methanol extract was added to the final concentrations shown in the figure. Effect of *P. oleracea* methanol extract after 72 h against the viability of treated cells was evaluated through mitochondrial activity using MTT assay. Each value represents the means ±S.E.M. triplicate in three independent experiments. The differences between the untreated control group and treated group were determined by one-way ANOVA (*$p{\leq}0.05$).
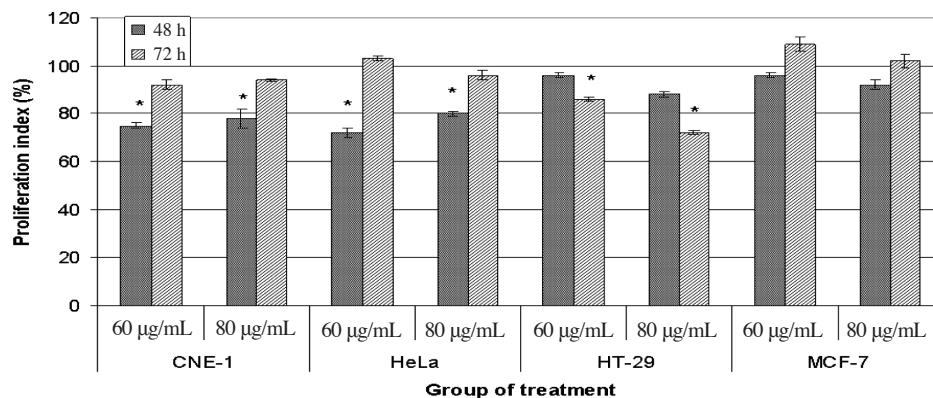
FIGURE 2. Effects of *P. oleracea* methanol extract in CNE-1, HeLa, HT-29, MCF-7 and Chang Liver cells DNA synthesis. After seeding the cell in 96 well plates for overnight, *P. oleracea* methanol extract were added to the final concentrations shown in the figure. Effect of *P. oleracea* methanol extract after 48 and 72 h against the DNA synthesis of treated cells was evaluated through BrdU proliferation assay. Each value represents the means ±S.E.M. triplicate in three independent experiments. The differences between the untreated control group and treated group were determined by one-way ANOVA ($*p \leq 0.05$).

phase was observed in MCF-7 cell treated with *P. oleracea* extract for both 36 and 60 h. This result indicated that the extract was able to induce DNA fragmentation which is the hallmark for apoptosis.

DISCUSSION

Recently, experts in cancer prevention, detection and treatment have reviewed the need of more research in chemoprevention (Follen et al. 2003). Thus, relevant approaches particularly food-based entities remain essential in reducing the risk of cancer. *P. oleracea,* an ubiquitous garden weed has shown to provide a rich plant source of nutritional benefits. With that, the potential anti-proliferative activities of standardized *P. oleracea* methanol extract towards various types of cancerous cells were explored. These were determined through MTT assay, BrdU incorporation assay and flow cytometry RNASe/PI staining to quantify on the cell cycle progression.

MTT assay that is commonly used to screen for the cell proliferation, viability and cytotoxic effects was done to determine the cell viability by assessing healthy cells (Radhakkrishnan et al. 2001). Under the current experimental conditions, no IC_{50} was obtained within the range of the tested concentration (0 – 100 µg/mL) on all the cell lines except for CNE-1. However, there was a significant decline in cell viability at concentrations higher than 40 µg/mL (Figure 1). The reduction of cell viability as compared with untreated control cell may be contributed by apoptosis or antiproliferation. On the other hand, the extract showed selective inhibition on all the cancerous cell as compared with Chang liver cells, which indicates the non toxic effect of the plant and is safe for daily consumption. BrdU assay is a method to quantify cell proliferation in which BrdU, a thymidine analogue, incorporates during DNA synthesis and gives a direct measurement of cells

undergoing proliferation. The extract showed a significant inhibition of DNA synthesis on CNE-1, HeLa and HT-29 cells but not on MCF-7. This effect was further evaluated by quantifying the cell cycle progression of all tested cancerous cell lines using flow cytometric analysis.

From our results, accumulation of cells at S phase was observed in CNE-1 and HeLa cells treated with the *P. oleracea* extract (Figure 3 & Table 1). This result indicated that the extract was able to reduce DNA synthesis that was detected in BrdU assay and arrest both CNE-1 and HeLa cell under S phase of the cell cycle. Various plant-based phytochemicals have been reported to arrest cancer cells in the S phase, in agreement to this current finding (Srivastava et al. 2009; Zhang et al. 2011). This similarity may be contributed by comparable phytochemicals found among the plants with *P. oleracea*. Those phytochemicals present in *P. oleracea* included omega-3 fatty acids, β-carotene, flavonoids and alkaloids (Liu et al. 2000; Xiang et al. 2005; Xu et al. 2006). Some of these compounds are known to cause cell cycle arrest in cancer cells from other reports (Androutsopoulos et al. 2010; Casagrande & Darbon 2001; Zhang et al. 2009). For example, Chen et al. (2010) demonstrated a significant increase of cells in the S phase which led to the S phase arrest and an apoptotic phenomenon, when HeLa cells were treated with *P. oleracea* sulphated-polysaccharides. Besides, alkaloids extract from *Scutellaria barbata* were found to arrest cells at S phase of the cell cycle. The extract was also found to induce apoptosis leading to cell death (Wang et al. 2011).

Unlike CNE-1 and HeLa cell, cell cycle analysis of HT-29 suggests that *P. oleracea* may exert its anti-proliferative effects in HT-29 cells by arresting the cell at G1 phase. Cell cycle progression of *P. oleracea* treated HT-29 was observed with significant G0/G1 arrest at 72 h and BrdU proliferation assay was observed
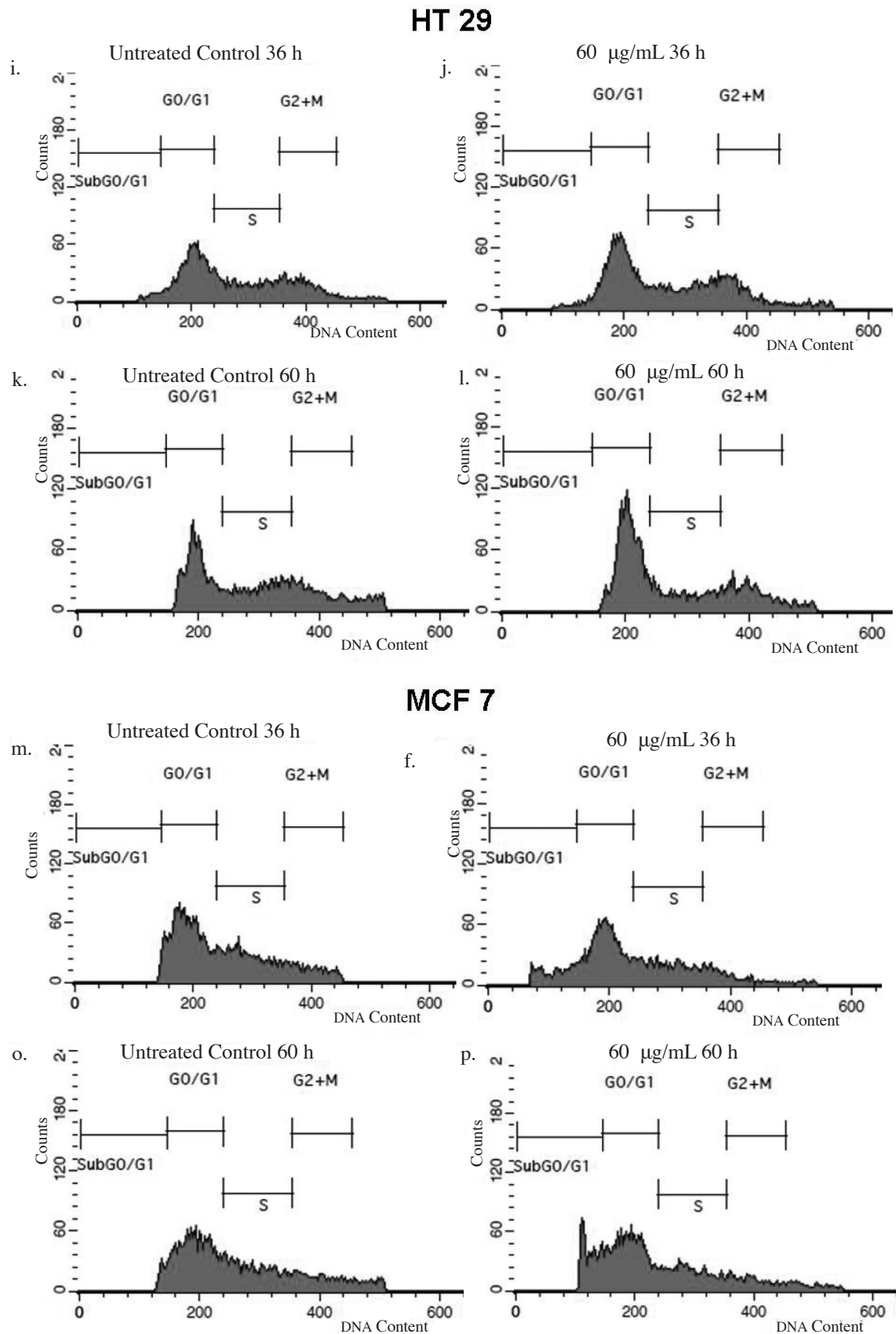
CNE-1
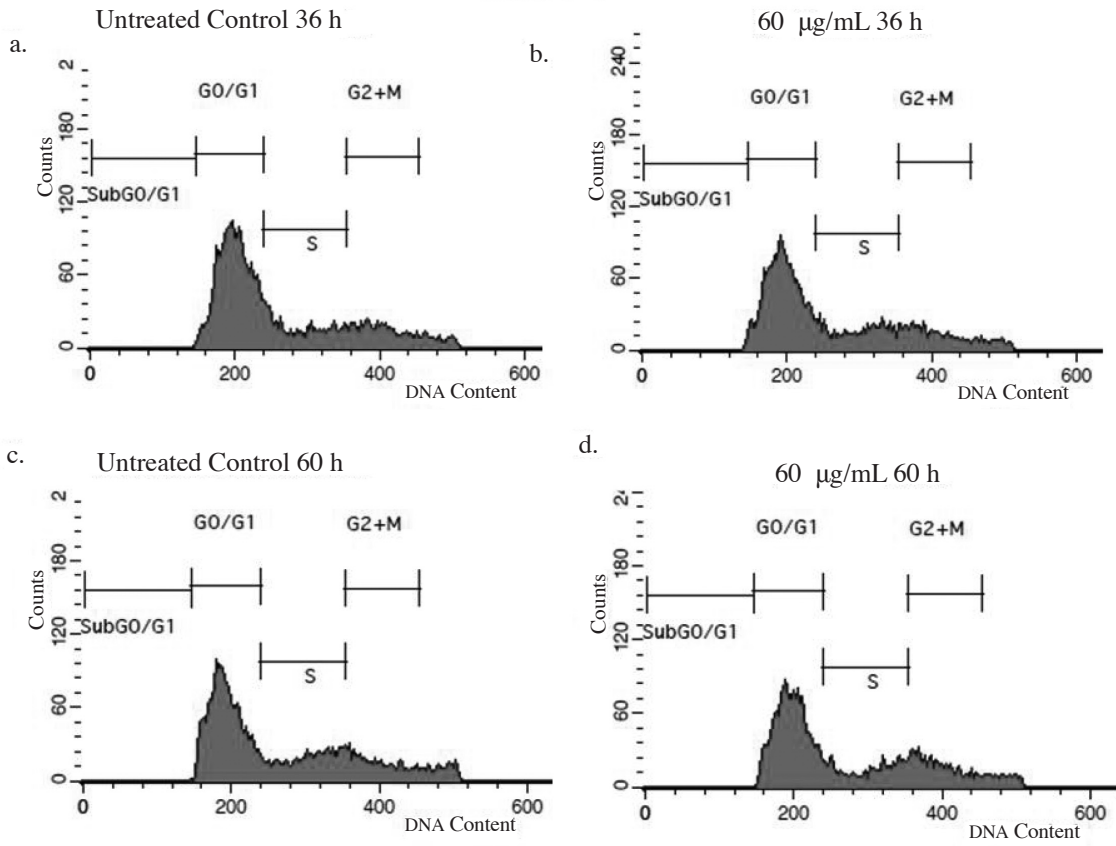
HT 29
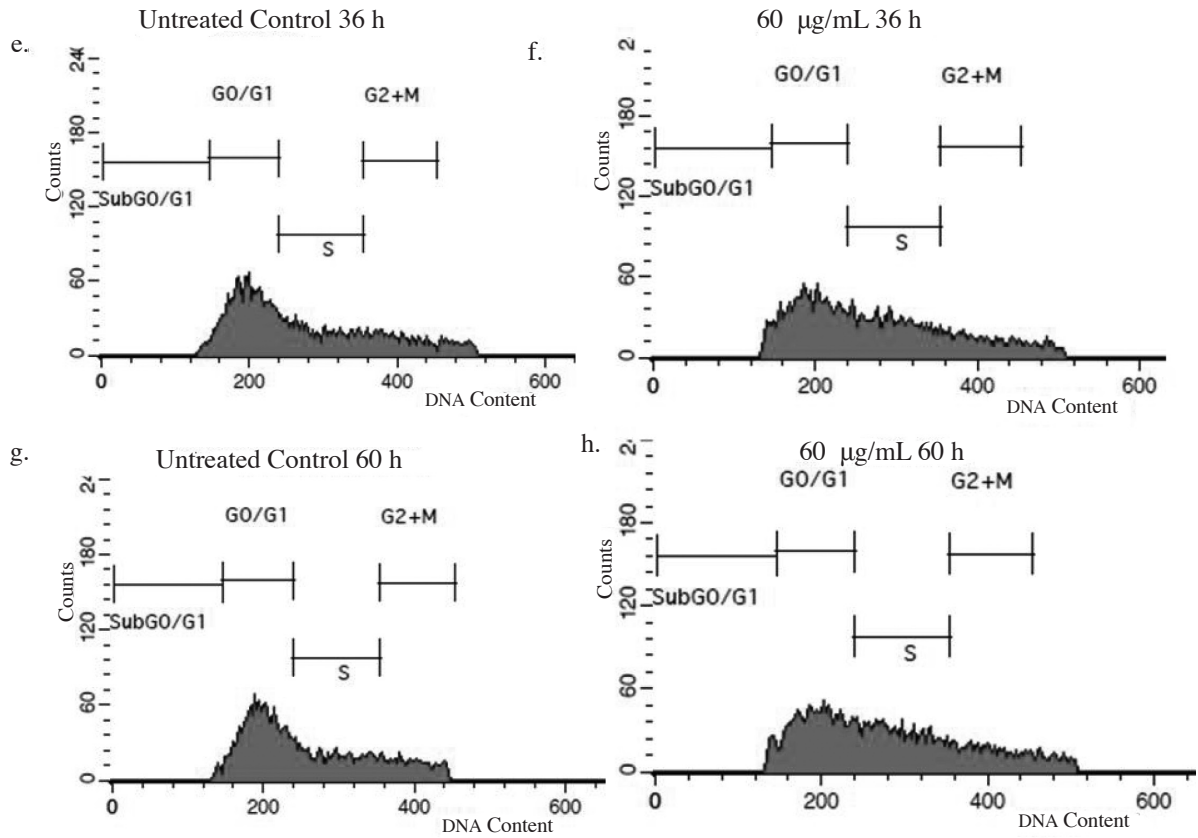
FIGURE 3. Cell cycle histogram for the effects of *P. oleracea* methanol extract (60 μg/mL) on cell cycle distribution of CNE-1 (a-d), HeLa (e-h), HT-29 (i-l) and MCF-7 (m-p) cells after 36 and 60 h were determined by RNase/PI cell cycle flow cytometry. G_0/G_1, G_2/M and S indicate the cell phase and sub- G_0/G_1 DNA content refers to the proportion of apoptotic cells undergoing DNA fragmentation. The detail percentage of the cell cycle progress was summarized in Table 1

TABLE 1. Effects of *P. oleracea* methanol extract (60 μg/mL) on cell cycle distribution of CNE-1, HeLa, HT-29 and MCF-7 cells after 36 and 60 h were determined by RNase/PI cell cycle flow cytometry. G_0/G_1, G_2/M and S indicate the cell phase, and sub- G_0/G_1 DNA content refers to the proportion of apoptotic cells undergoing DNA fragmentation. Each value represents the means ±S.E.M. triplicate in three independent experiments. The differences between the untreated control group and treated group were determined by one-way ANOVA (*$p \leq 0.05$)

Cell	Time (h)	Treatment	$SubG_0/G_1$	G_0/G_1	S	G_2/M
CNE-1	36	Untreated control	0.01±0.01	65.75±1.46	18.94±0.42	15.30±0.34
			0.27±0.01	62.87±1.79*	21.13±0.60*	15.73±0.45
	60	60 μg/mL	0.21±0.01	58.57±2.10	16.46±0.59	24.76±0.89
			0.93±0.02	60.07±1.52	19.68±0.50*	19.32±0.49*
HeLa	36	Untreated control	1.12±0.04	53.05±1.96	28.82±1.07	17.01±0.63
			2.91±0.09	44.70±1.33*	36.05±1.08*	16.34±0.49
	60	60 μg/mL	4.20±0.13	43.35±1.34	34.82±1.07	17.63±0.54
			2.80±0.13	45.85±2.01	38.73±0.01*	12.61±1.70*
HT-29	36	Untreated control	2.12±1.04	46.23±0.95	28.86±0.59	22.79±0.47
			2.30±0.05	48.13±1.12	29.74±0.69	19.83±0.46*
	60	60 μg/mL	0.43±0.02	45.07±1.91	32.22±1.37	22.28±0.95
			0.93±0.12	56.10±1.10*	20.97±0.41*	22.00±0.43
MCF-7	36	Untreated control	0.31±0.10	54.37±2.03	32.18±1.02	13.14±1.01
			12.37±0.14*	50.07±2.56*	27.42±2.30*	10.14±0.11*
	60	60 μg/mL	3.97±0.13	51.44±1.64	29.39±0.94	15.21±0.48
			17.87±0.45*	48.36±1.22*	23.34±0.59*	10.43±0.26*

higher inhibition in 72 h. This G1 arrest after 72 h may be contributed by the present of flavanoids especially luteolin (Xu et al. 2006). Due to its hydrophilic nature, the intestine is often exposed to superior concentrations of dietary polyphenols compared to other body tissues, making it a potential chemopreventive site (Manach et al. 2004). Luteolin, one of the flavonoids found in *P. oleracea*¸ induced a G1 arrest in the cell cycle progression of HT-29 cells by reducing cyclin D1 and phosphorylated retinoblastoma protein levels, as well as decreasing CDK2 and CDK4 activity (Lim et al. 2007, 2012).

Since DNA fragmentation is the hallmark for apoptosis, mild reduction of the MCF-7 viability as seen in the MTT assay indicates induction of apoptosis by the extract as shown in the cell cycle analysis (Figure 3 & Table 1). This apoptotic event may be contributed by the presence of β-carotene, omega-3 fatty acids, coumarins, flavonoids, monoterpene glycosides, anthraquinone glycosides and alkaloids in *P. oleracea* that have been previously proved to exhibit the induction of apoptosis in some previous researches (Li et al. 2009; Xiang et al. 2005; Xin et al. 2008). In addition, polysaccharides that were found to be rich in *P. oleracea* (Mangalan et al. 1989) have been demonstrated to mediate apoptotic phenomenon towards MCF-7 cells via dysregulating the Bcl-2 and Bax proteins in MCF-7 cells (Yang et al. 2006).

CONCLUSION

In conclusion, our data indicated that the *Portulaca oleracea* extract specifically reduced viability of various cancerous cell lines either through G0/G1 or S phase arrest (on CNE-1, HeLa and HT-29 cells) or via induction of sub-G0/G1 DNA fragmention (on MCF-7). However, the mechanism of the action is still unclear. Thus, further investigations including isolation of individual active flavonoid and elucidation of the molecular mechanisms involved are needed to fully understand the active ingredient and potential of *P. oleracea* as a chemopreventive food.

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Gek Choo Sarah Tan, Kar Mun Wong, Gui Qi Pearle-Wong, Siau Li Yeo & Beow Chin Yiap
Department of Pharmacy
School of Pharmacy and Health Sciences
International Medical University
57000 Kuala Lumpur
Malaysia

Swee Keong Yeap
Institute of Bioscience
University Putra Malaysia
43400 Serdang, Selangor D.E.
Malaysia

Hueh Zan Chong*
Department of Nutrition and Dietetics
School of Pharmacy and Health Sciences
International Medical University
57000 Kuala Lumpur
Malaysia

*Corresponding author; email: meganjournal@gmail.com