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Phenolics-saponins rich fraction of defatted kenaf seed meal exhibits cytotoxicity towards cancer cell lines

Latifah Saiful Yazan[1,2*], Napsiah Abd Rahman[1,2], Kim Wei Chan[1], Wan Nor Hafiza Wan Abd Ghani[1,2], Yin Sim Tor[1,2], Jhi Biau Foo[1,2]

[1] *Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia (UPM), 43400, Serdang, Selangor, Malaysia*

[2] *Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), 43400, Serdang, Selangor, Malaysia*

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ABSTRACT

Objectives: To determine the cytotoxicity of crude ethanolic extract, *n*-butanol fraction and aqueous fraction on selected cancer cell lines, and to observe the morphological changes of the cancer cells treated with *n*-butanol fraction.

Methods: The cytotoxic effect of *n*-butanol fraction, crude ethanolic extract and aqueous fraction on breast cancer (MCF-7 and MDA-MB-231), colon cancer (HT29), lung cancer (A549), cervical cancer (HeLa) and normal mouse fibroblast (3T3) cell lines was determined using MTT assay. The morphological changes of the treated cells were observed under an inverted light microscope.

Results: *n*-Butanol fraction was the most cytotoxic towards HT29 and MCF-7 cells in a dose-dependent manner compared to crude ethanolic extract and aqueous fraction ($P < 0.05$). The IC_{50} of *n*-butanol fraction for HT29 and MCF-7 was (780.00 ± 28.28) and (895.00 ± 7.07) μg/mL, respectively. Cell shrinkage, membrane blebbing and formation of apoptotic bodies were noted following treatment of HT29 cells with *n*-butanol fraction.

Conclusions: In conclusion, *n*-butanol fraction was more cytotoxic than crude ethanolic extract and aqueous fraction towards the selected cancerous cell lines and induced apoptosis in HT29 cells.

1. Introduction

Kenaf (*Hibiscus cannabinus* L.) belongs to the Malvaceae (Mallow) family, which is locally known as “Ambadhi” in Marathi or “Ambashta” in Sanskrit [1]. Kenaf is an annual plant that grows to 3–4 m in height within 4–5 months and can be harvested 2–3 times a year [2]. Traditionally, the kenaf flower is used to treat biliousness with acidity, the seed to promote weight gain, the leaf to relieve cough and the stem to treat anemia [1]. Malaysia has taken the initiative to cultivate kenaf plant in order to replace tobacco plantation. The kenaf fiber is commercially used in industrial products such as bio-composite materials, absorbents and fiber board. In general practice, kenaf seeds are usually disposed as waste material after the plants have been harvested [3]. Although kenaf is mainly used for fiber, the seed (by-product) also has the potency to be turned into value-added products [4]. Previous study has shown that kenaf seed oil possessed substantially high antioxidant activity [5]. In addition, the kenaf seed oil extracted by supercritical carbon dioxide fluid extraction exhibited anticancer properties towards colon cancer in rats [6], and ovarian cancer cell line [7]. Large scale extraction of oil from kenaf seeds produces huge amount (80% of the seed) of

*Corresponding author: Latifah Saiful Yazan, Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia (UPM), 43400, Serdang, Selangor, Malaysia.

Tel: +60 03 89472303, +60 03 89472308

E-mail: latifahsy@upm.edu.my

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defatted kenaf seed meal (DKSM), which contains considerable amounts of health beneficial bioactives such as phenolic compounds and saponins [8]. DKSM has been compared with some edible flours (rice flour, sweet potato flour and wheat flour), and it showed the highest content of phenolic compound and antioxidant properties [3]. The fractionation of DKSM crude ethanolic extract by *n*-butanol successfully elevated the level of phenolics and saponins as well as antioxidant activity of the extract. Most of the active compounds are extractable in solvent system of intermediate polarity, which justifies higher recovery in *n*-butanol fraction [8]. Based on the strong antioxidant activities, it is postulated that *n*-butanol fraction might also possess better anticancer properties in comparison to crude ethanolic extract and aqueous fraction. This study aimed to investigate the cytotoxic effects of DKSM crude ethanolic extract as well as its derived *n*-butanol and aqueous fraction against the breast cancer-hormone dependent (MCF-7), breast cancer-hormone independent (MDA-MB-231), colon cancer (HT29), cervical cancer (HeLa), lung cancer (A549) and normal mouse fibroblast (3T3) cell lines.

2. Materials and methods

2.1. Materials

2.1.1. Cell lines

Breast cancer-hormone dependent (MCF-7), breast cancer-hormone independent (MDA-MB-231), colon cancer (HT29), cervical cancer (HeLa), lung cancer (A549) and normal mouse fibroblast (3T3) cell lines were purchased from the American Type and Culture Collection, USA.

2.1.2. Reagents

The tissue culture medium (RPMI-1640) was purchased from Nacalai TesqueInc (Kyoto, Japan). Penicillin/streptomycin antibiotic and trypsin-ethylene diamine tetraacetic acid were purchased from PAA Laboratories (Pasching, Austria). Mycoplex™ fetal bovine serum was purchased from Commerce Ave (California, USA). Trypan blue solution, MTT powder and propidium iodide were purchased from Sigma Chemicals (St. Louis, USA).

2.2. Methods

2.2.1. Preparation of crude ethanolic extract, aqueous fraction and n-butanol fraction from DKSM

The crude ethanolic extract, aqueous fraction and *n*-butanol fraction of DKSM extracted from kenaf seeds were kindly provided by Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia [8]. Kenaf seeds were purchased from Malaysian Kenaf and Tobacco Board, Kelantan, Malaysia. Firstly, the seeds were pulverized and homogenized with *n*-hexane to extract out the oil. The residue which is DKSM was collected. Next, DKSM was refluxed in 50% of aqueous ethanol to obtain the crude ethanolic extract. For fractionation, crude ethanolic extract was dispersed in distilled water and partitioned with *n*-hexane to remove the residual lipids. Then, the *n*-hexane layer was removed and the aqueous layer was partitioned with *n*-butanol. *n*-Butanol fraction was then pooled and concentrated under the reduced pressure. Finally, aqueous fraction left from this partitioned layer was subjected to lyophilization [8].

2.2.2. Cell culture

MCF-7, MDA-MB-231, 3T3, HT29, HeLa and A549 cell lines were cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin). The cultured cells were maintained in an incubator at 37 °C, and in humidified atmosphere of 5% CO_2.

2.2.3. Determination of cytotoxicity

Cytotoxicity of the tested materials was determined using the MTT assay. The assay measures the metabolic activity of cells based on the reduction of mitochondrial succinate dehydrogenase from yellow (tetrazolium bromide) to dark purple (formazan) [9]. Briefly, the cells were seeded in 96-well plates at cell density of 1.0×10^5 cells/mL in 100 μL complete growth medium. After 24 h, the cells were treated with crude ethanolic extract, *n*-butanol fraction and aqueous fraction at various concentrations (15.625, 31.250, 62.500, 125.000, 250.000, 500.000 and 1 000.000 μg/mL) in triplicate. Untreated cells served as control. The cells were incubated at 37 °C in an incubator with humidified atmosphere of 5% CO_2 for 72 h. Subsequently, 20 μL of MTT solution (5 mg/mL in phosphate-buffered saline) was added. Three hours later, the medium was discarded and 100 μL of dimethylsulfoxide was added to dissolve the purple formazan product. The absorbance was measured at wavelength of 570 nm and 630 nm (as background) by using a microplate ELISA reader (BioTek EL 800, United States).

The percentage of cell viability was obtained by using the following formula:

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

The dose response curve of percentage of cell viability versus the extract concentration was plotted to determine the IC_{50}. The cytotoxic effects of the extracts were expressed as IC_{50} value (the extract concentration reducing the absorbance of treated cells by 50% with respect to untreated cells) [10].

2.2.4. Morphological analysis

Based on the IC_{50}, *n*-butanol fraction was found to be the most potent towards colon cancer cell line (HT29). Further experiment was then conducted by using *n*-butanol fraction only. HT29 cells were seeded at cell density of 0.5×10^5 cells/mL in 6-well plates. After 24 h incubation, the cells were treated with *n*-butanol fraction at concentration of 200, 300, 400, 500 and 600 μg/mL for 24, 48 and 72 h. Untreated cells were included as a control. The cell morphology was examined under magnification of 10× and 40× by using an inverted light microscope (Olympus, Tokyo, Japan). The same spot of the cell was captured at different time interval by drawing the cross under the plate as reference.

2.2.5. Statistical analyses

All data were expressed as mean ± SD. The data were analyzed with One-way ANOVA and Dunnett *post-hoc* test

using SPSS version 22.0. Probability of $P < 0.05$ was considered significant.

3. Results

3.1. Cytotoxicity of crude ethanolic extract, n-butanol fraction and aqueous fraction of DKSM

Crude ethanolic extract, *n*-butanol fraction and aqueous fraction suppressed the growth of all the cell lines in a dose-dependent manner. The IC_{50} values of the extract/fraction toward all the cancer cell lines are shown in Figure 1 and Table 1. The dose–response graph obtained shows a significant decrease in the percentage of cell viability ($P < 0.05$) of HT29 treated with *n*-butanol fraction. *n*-Butanol fraction was more cytotoxic towards all the cell lines compared to crude ethanolic extract and aqueous fraction. *n*-Butanol fraction was most cytotoxic towards HT29 with the IC_{50} value of (780.00 ± 28.28) µg/mL. Based on this, further analysis was carried out only on HT29 cell line.

3.2. Morphological changes of HT29 cells following treatment with n-butanol fraction of DKSM

As shown in Figure 2, treatment with the *n*-butanol fraction caused changes in HT29 cells. Reduction in cell number was noted at the highest concentration (600 µg/mL), with majority of the cells detached from the substratum as early as at 24 h. The cell debris was abundant. Figure 3 shows the apoptotic characteristics of HT29 cells after treatment with *n*-butanol fraction after 24, 48 and 72 h that include membrane blebbing, detachment of the cells from substratum, cell shrinkage as well as formation of apoptotic bodies.

Table 1

Cytotoxicity of extract/fractions of kenaf towards selected cell lines after 72 h as reflected by the IC_{50} value determined by MTT assay.

Cell line	IC_{50} (µg/mL)		
	Crude ethanolic extract	*n*-Butanol fraction	Aqueous fraction
HT29	> 1 000	780.00 ± 28.28	> 1 000
MCF-7	> 1 000	895.00 ± 7.07	> 1 000
MDA-MB-231	> 1 000	> 1 000	> 1 000
A549	> 1 000	> 1 000	> 1 000
HeLa	> 1 000	> 1 000	> 1 000
3T3	> 1 000	> 1 000	> 1 000

Values are the means of duplicate samples (n = 3), and are presented as mean ± SD.

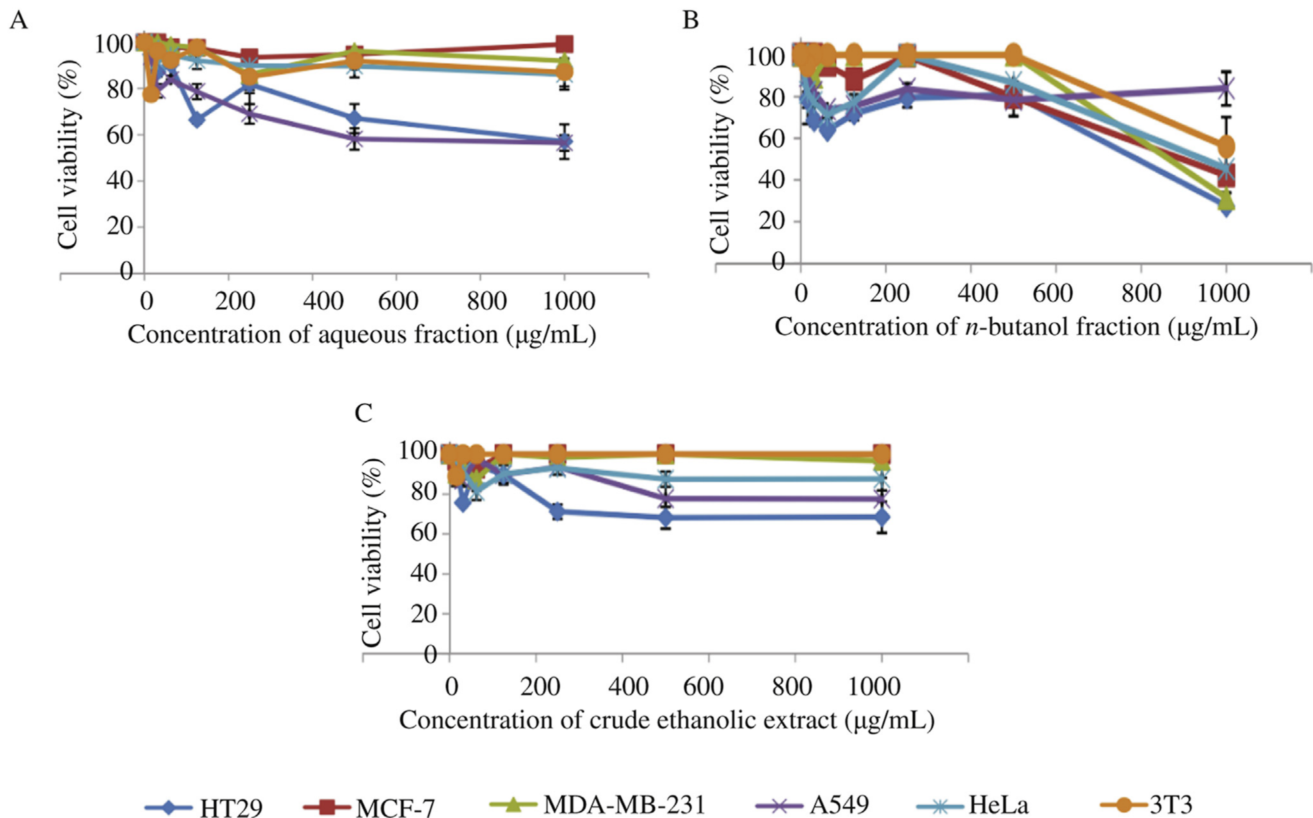

Figure 1. Dose-response curve of crude ethanolic extract (A), *n*-butanol fraction (B) and aqueous fraction (C) for HT29, MCF-7, MDA-MB-231, A549, HeLa and 3T3 cell lines after 72 h as determined by MTT assay. Values are the means of duplicate samples (n = 3) and are presented as mean ± SD.

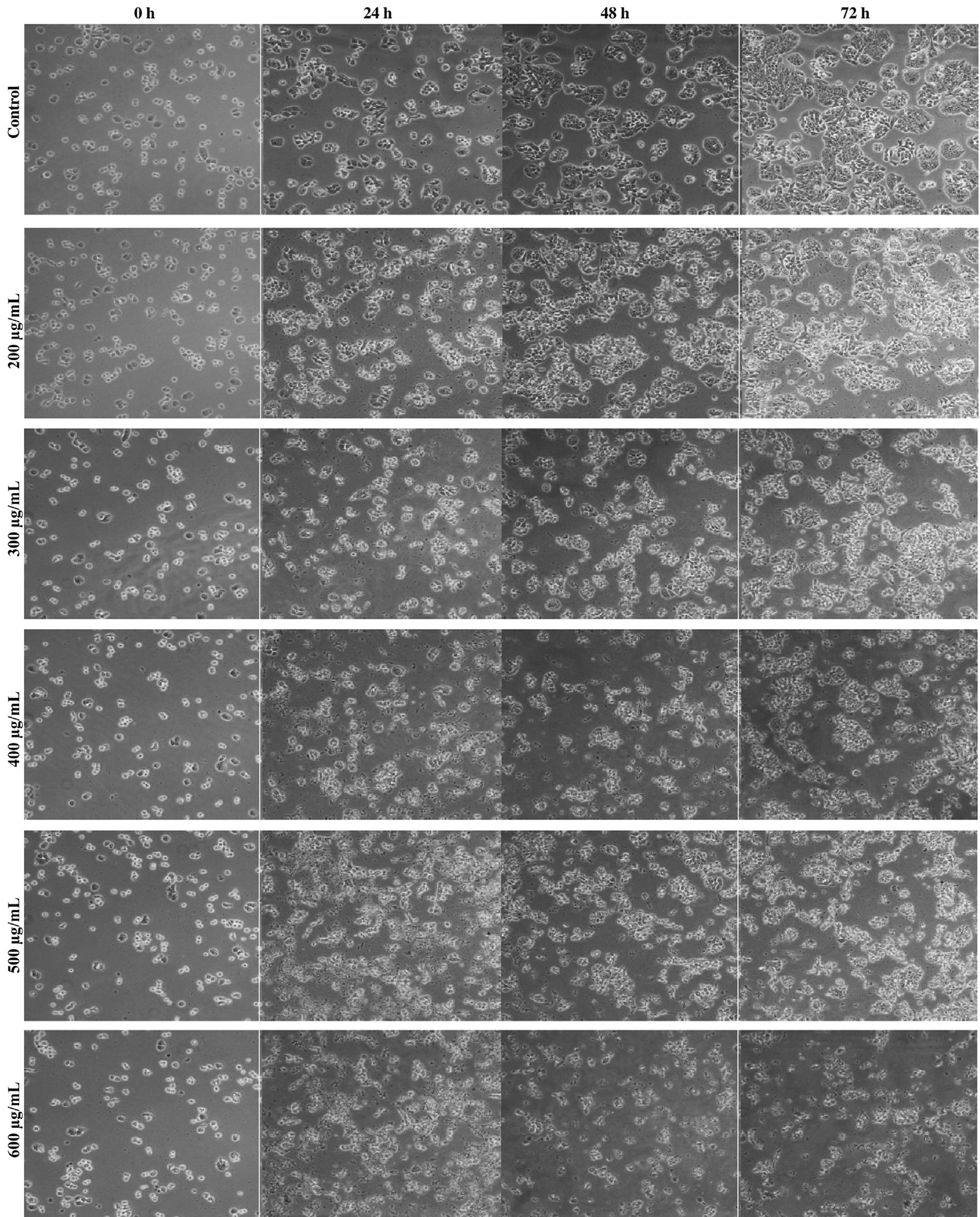

Figure 2. Morphological changes of HT29 cells treated with different concentration of *n*-butanol fraction observed under an inverted light microscope. Control was included (100× magnification).

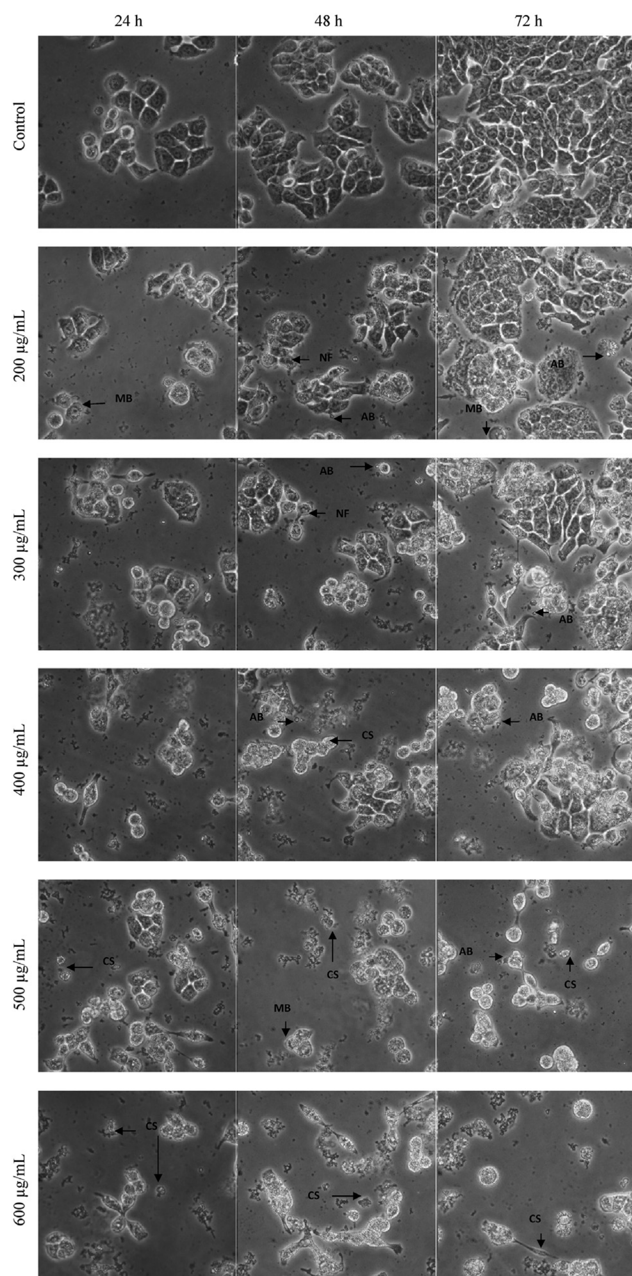

Figure 3. Close-up views of HT29 cells treated with *n*-butanol fraction at a range of concentrations.
CS: Cellular shrinkage; MB: Membrane blebbing; AB: Apoptotic bodies; NF: Nuclear fragmentation. 400× magnification.

4. Discussion

A cytotoxic extract or fraction induces loss of cell viability either by decreasing cell survival or triggering cell death [11]. In this study, cytotoxicity of crude ethanolic extract, *n*-butanol fraction and aqueous fraction was measured by using the MTT assay. In MTT assay, the formazan accumulation directly reflects the mitochondrial activity in live cell (presence of mitochondrial dehydrogenase), which is an indirect measurement for the cell viability [9]. *n*-Butanol fraction was the most cytotoxic towards all the selected cancerous cell lines compared to crude ethanolic extract and aqueous fraction, probably due to its elevated level of saponin and phenolic compounds. Interestingly, *n*-butanol fraction was less cytotoxic towards 3T3 normal cell line.

n-Butanol fraction induced apoptosis in HT29 cells, which was characterized by membrane blebbing, nuclear fragmentation and cellular shrinkage. Apoptosis is a type of programmed cell death. Apoptosis is more favorable than necrosis because it is a well-planned, cell determined form of cell death and does not trigger inflammatory responses [12]. Agents with the ability to induce apoptosis in cancer cells have the potential to be used for anti-cancer therapy [13]. In addition, apoptosis induction is one of the potent defensive strategies against cancer progression [14].

Chan *et al.* reported that total phenolic content in *n*-butanol fraction was 6 times higher than that in aqueous fraction and 3 times higher than that in crude ethanolic extract that probably contributes to its better antioxidant activity [8]. In general, there are four major phenolic compounds including (+)-catechin, *p*-coumaric acid, gallic acid and caffeic acid, which increased in *n*-butanol fraction after fractionation of crude ethanolic extract. The elevated level of phenolics might contribute to the higher cytotoxicity of *n*-butanol fraction towards the cancerous cell lines. Previously, catechins from green tea inhibited vascular endothelial growth factor induction in human colon carcinoma cells (HT29) in athymic BALB/c nude mice [15], inhibited the growth of human cancer cell lines such as breast adenocarcinoma [16], lung, prostate and colon cancer [17], and exhibited anti-tumorigenic properties in rats with hepatoma [18]. In the study done by Chang and Shen [19], *p*-coumaric (trans-4-hydroxycinnamic) acid inhibited the growth of colorectal, breast and liver cancer cells, and inhibited angiogenesis process *in vivo* [20]. Moreover, gallic acid was reported to induce apoptosis in esophageal cancer cells (TE-2) [21], exhibit anti-tumor effect on two human osteosarcoma cell lines (U-2OS and MNNG/HOS) and directly decrease the xenograft tumor growth in BALB/c mice [22]. In addition, caffeic acid suppressed the growth of colon cancer cells via PI3-K/Akt and AMPK signaling pathways [23].

Through fractionation of crude ethanolic extract, saponins content of *n*-butanol fraction also increased in concomitant with the phenolics [8]. Saponins have been reported to inhibit cancer progression [24,25], to induce apoptosis [26], to exhibit anti-mutagenic action [27], and anti-tumor activities [24]. Saponins have also been successfully used in combination with dianthin-epidermal growth factor as an effective tumor-targeted toxin in a xenograft model for colon cancer [28], and as an anti-ulcer agent for ethanol-induced ulcer in rats [29]. Tin *et al.* reported that saponins were pro-apoptotic and showed anti-proliferative effects on HT-29 xenograft in nude mice [30]. Saponins inhibited development of colon cancer by decreasing β-glucuronidase in colonic mucosa of rats [31].

The *n*-butanol fraction/phenolics-saponins rich fraction was more cytotoxic than the crude ethanolic extract and aqueous fraction of DKSM toward all the selected cell lines in a dose-dependent manner. The stronger cytotoxic effect might largely be attributed to the elevated level of phenolics and saponins following the fractionation of the DKSM ethanolic extract. HT29 was the most sensitive towards *n*-butanol fraction compared to MCF-7, MDA-MB-231, A549, HeLa and 3T3 cell lines. *n*-Butanol fraction induced apoptosis in HT29 cells. Cytotoxic effects of *n*-butanol fraction towards HT29 cells indicate that it has the potential to be developed into a valuable nutraceutical product for management (prevention or curing) of colon cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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