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In Vitro Antiproliferative Activity of Fresh Pineapple Juices on Ovarian and Colon Cancer Cell Lines

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Abstract The main component that contributes to the high value of pineapple is bromelain which is a proteolytic enzyme and has been scientifically identified as a therapeutic agent. This study was conducted to obtain high quantity of bromelain from pineapple and to investigate the anticarcinogenic activity of fresh pineapple juices against A2780 ovarian and HT29 colon cancer cell lines. It was found that homogenization, ultrafiltration, precipitation and dialysis contributed to heavy loss of bromelain. Therefore, fresh pineapple juices from the flesh (PJ-F), core (PJ-C) and stem (PJ-S) were selected as a source of crude bromelain. Various bromelain concentrations of PJ-F, PJ-C and PJ-S (1 μg/ml, 10 μg/ml, 100 μg/ml and 1000 μg/ml) were exposed to the cancer cells and the cell viability was determined using Methylthiazol Tetrazolium Assay (MTT assay) after 24, 48 and 72 h. Besides, IC_{50} values were measured. Using normal cell (HSF1184) comparison, it was found that a 100 μg/ml concentration of bromelain would efficiently inhibit the cancer cells without affecting the surrounding normal cells. Microscopic examinations were carried out to elucidate the modes of cell death on the basis of morphological alterations using florescent and inverted phase contrast microscopes. Furthermore, the colony forming abilities of fresh pineapple juices on A2780 and HT29 cells were examined. The results demonstrated that PJ-F, PJ-C and PJ-S effectively suppressed the colony formation in cancer cells. The findings suggest that PJ-F, PJ-C and PJ-S may have the potential to induce anticarcinogenic effects through an apoptosis to A2780 and HT29 cells in vitro.

Keywords Bromelain · Fresh pineapple juice · Anticarcinogenic · Colon cancer · Ovarian cancer

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Introduction

Pineapple has been used medicinally by natives of the tropics for centuries as a digestive aid and a wound healing agent. Some people nickname the pineapple as 'King of the Fruits' because of the potential and beneficial aspects of this plant for health purposes. A compound called bromelain, which was found to be highly concentrated in pineapple (Maurer 2001) has since been linked to the medicinal properties. Bromelain is the main component in pineapple fruit which is a 26 kDa proteolytic enzyme. It is

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typically produced via extraction process of pineapple juice that is a complex mixture of proteinase. Recent scientific research findings infer that bromelain may have multitudinous health benefits including digestive, anti-inflammatory, immunomodulatory and anticancer effects (Chobotova et al. 2010). Bromelain is highly favourable because of its inhibitory properties for platelet aggregation, anti-inflammatory action, fibrinolytic activity, modulation of cytokines and immunity, enhanced absorption of other drugs, skin debridement, digestive assistant, enhanced wound healing and anti-carcinogenic action (Kelly 1996; Bala et al. 2012; Pillai et al. 2013).

Clinical and preclinical researches, in vitro and in vivo studies as well as observations from conventional practices are considered as the evidence for the anticarcinogenic activity of bromelain (Chobotova et al. 2010). Bromelain is an orally prescribed drug for complementary tumor therapy based on the results recently obtained from pharmacological and preclinical studies. By raising the impaired immunocytotoxicity of monocytes against tumor cells, bromelain acts as an immunomodulator agent. It happens through induction of distinct cytokines production such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6, and IL-8 (Maurer 2001).

The major pathways and regulators implicated in cancer are affected by bromelain (Chobotova et al. 2010). Castell et al. (1997) reported that bromelain is absorbed into human intestine without degradation and without losing biological activity. Bromelain was reported to be able to inhibit platelet aggregation in vitro and in vivo as well as platelet-stimulated invasiveness of tumor cells (Tochi et al. 2008). Moreover, anti-tumor initiating effects of bromelain reported by Bhui et al. (2009) in 2-stage mouse skin tumorigenesis, but few experiments with human cancer cell lines carried out and reported (Bhui et al. 2009).

Two of high rated cancer types affecting women are colon cancer and ovarian cancer. Moreover, they are considered as the deadliest of all gynaecologic cancers among women. There has not been a specific study carried out to investigate the effects of bromelain on ovarian and colon cancer cell lines. Any parts of the pineapple can be the source of bromelain such as stem, crown, leave, stump and core. These parts usually represent the plant waste, contributing about 80 % of its industrial waste. Bromelain derived from the waste section is not suitable for direct consumption without an intensive purification process. Bromelain purification is time consuming as well as being expensive. Alternative to pure bromelain from the pineapple waste source, fresh pineapple juice from different parts of fruits which contain substantial concentration of bromelain could also be potentially beneficial for therapeutic purposes. Thus, the source of bromelain used in the study was obtained from different parts of the pineapple fruit namely, the flesh, stem and core. Although, many studies shed light on the pharmacological actions of this natural substance to treat cancer, there has not been specific study on ovarian and colon cancer cell lines. In this study, the crude bromelain in the pineapple juice was quantified and exposed to mainly colon and ovarian cancer cell lines. The aim of this study is to compare the ability of crude bromelain of juices pressed from different parts of pineapple to inhibit proliferation of human colon HT29 and ovarian A2780 cancer cell lines. "*Gandul*" type of pineapple was chosen in this study to extract bromelain due to its high bromelain content. We took advantage of this fact that "*Gandul*" is one of the five commercial pineapples in Malaysia which can be an abundant source of bromelain in juice form (Mashudin and Abdul Majid 2009).

Material

The pineapple source was obtained from Pineapple Cannery of Malaysia Sdn. Bhd. and Alor Bukit Malaysian Pineapple Industry Board plantation in Pekan Nanas, Johor, Malaysia. The bromelain standard was acquired from Sigma Aldrich (catalogue no. B-4882) and was exploited as the indicator to compare with the crude bromelain from fresh pineapple juice. Other chemicals used in this study were purchased from sigma in analytical purity and were used without further purification.

Methods

Screening Process for Highest Bromelain Yield

Bromelain extraction was conducted according to procedure described by Doko et al. (1991) with slight modifications according to Sankaran et al. (2011). The bromelain recovered at every processing step was evaluated. Briefly, 10 kg of pineapple cores were cut into small pieces and juiced to discard the fibre. Then, the juice was homogenised with triple loop homogenisation and single-loop homogenization at 100 bar (3 × 100 bar). The homogenisation process using homogeniser from APV Homogeniser GmbH was carried out to disrupt pineapple cells, to decrease juice viscosity and to release the intracellular enzyme. Then the homogenised juice was centrifuged at 4 °C for 5 min at full speed using refrigerated centrifuge (HETTICH 32R). Pellet was discarded and the supernatant was collected. After centrifugation, the juice was filtered using Millipore ultra-filtration unit (316 L) to obtain a clear juice.

Salt Precipitation Ammonium Sulphate of Bromelain

The precipitation of bromelain from clarified pineapple juice was carried out by addition of 55 % ammonium sulphate [(NH4)2SO4] at 4 °C under constant stirring overnight. Then, centrifugation process was carried out at 5200 rpm, 4 °C for 30 min and the supernatant was discarded (Sankaran et al. 2011). The processed juice from each step was collected and analysed for total protein (Bradford assay) and for bromelain activity and total recovery.

Purification of Bromelain by Dialysis

The precipitated protein from the salt precipitation contains our desired enzyme bromelain and other impurities along. The extracted bromelain were purified by dialysis (Sankaran et al. 2011).

Quantification of Bromelain Concentration

All samples in each stage were analysed by high-performance liquid chromatography (HPLC) (Perkin Elmer Series 200) using a TSK gel Bio Assist S column (4.6 mm/D × 5 cm) consisting of a cation-exchange column loaded with polymer containing sulfopropyl groups as an ion-exchange structure. Linear gradient of NaCl from 1 to 0.5 M in 20 mM sodium phosphate buffer, pH 7 with a flow rate 0.8 ml/min was employed for elution. A UV 6000 wavelength detector at 260 nm was used to detect and quantify bromelain. The concentration of bromelain was determined from standard curve using bromelain standard. The standard curve of bromelain standard concentration was obtained by plotting bromelain concentrations against the area (uV) of four measurements for each concentration. Then, for quantification and detection of bromelain, the highest bromelain concentration was chosen to further the study after analysis.

Confirmation of Bromelain Purity and Molecular Weight

A 15 % SDS-PAGE was used for observation of bromelain purity and its molecular weight in PJ-F, PJ-C and PJ-S extracted from fresh pineapple juice. Bromelain standard (Sigma) was used as control. Briefly, after adding the solution into the gel sandwich until 1.5 cm from the top of the front plate, gel allowed for polymerization for 30–60 min. Stacking gel was prepared and pipetted onto separating gel until the solution reached the top of the front plate and then a comb was inserted. Later, the gel was placed in the polymerisation chamber. Electrophoresis buffer was added and the samples were loaded. The SDS-PAGE system was left running for 60 min after which the gel was transferred to a small container. The gel was gently agitated in Coomassie blue staining solution for 30 min using a rotary shaker. Then the solution was poured out and the gel was rinsed with water. Coomassie distaining solution was added and agitated for overnight.

In Vitro Study

Cell Culture Growth and Maintenance

The source to obtain human ovarian cancer cell line (A2780), human colon cancer cell line HT29 and human skin fibroblast cell line (HSF1184) was European Collection of Cell Cultures (ECACC), Salisbury, United Kingdom. HSF1184 cells were grown as monolayer in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 7 g/L sodium bicarbonate ($NaHCO_3$), 1 % penicillin–streptomycin, 10 % fetal bovine serum (FBS) at 37 °C with 5 % CO2 and 95 % humidity for 1 day while A2780 and HT29 cells were cultured as monolayer in 75cm2 flasks in Roswell Park Memorial Institute (RPMI) culture medium and McCoy's 5A culture medium respectively at 37 °C with 5 % CO_2 and 95 % humidity for 7 days whereas growth medium was renewed every 2 days.

Cells Proliferation Assay

The potential cytotoxicity of PJ-F, PJ-C and PJ-S on A2780 and HT29 cells was evaluated by Methylthiazol Tetrazolium Assay (MTT) assay using various concentrations of bromelain (1, 10, 100 and 1000 µg/ml). The cells were inoculated on 96- well plates with a cell density of 1×10^5 cells/well in 200 µl, and incubated in a humidified atmosphere containing 5 % CO_2 at 37 °C for 24 h to allow the cells to adhere to the bottom of the well. Medium, untreated cells and bromelain standard served as the respective controls in this study. After cell adherence, media was removed and 200 µL of treatment sample was added to each well to obtain the final concentrations of 1, 10, 100 and 1000 µg/ml of bromelain.

In the subsequent 24, 48 and 72 h of incubation, the cells viability were evaluated by the MTT assay (n = 6). 20 µl 3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide solution (5 mg/ml) in phosphate buffered saline (PBS) (pH 7.4) was added to each well. The incubation was continued for another 4 h, and then the solution was aspirated cautiously from each well. Thereafter, the MTT derivative was dissolved with 200 µl Dimethyl Sulfoxide (DMSO) completely, and the optical density (OD)

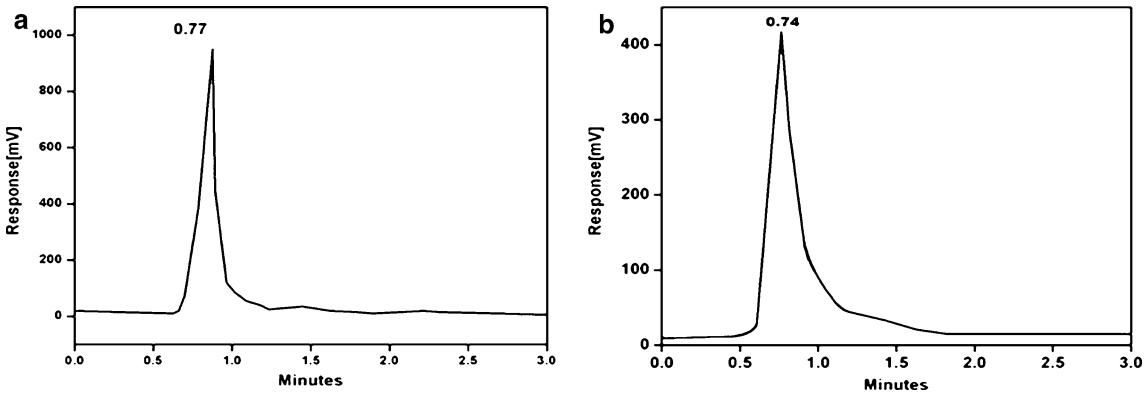

Fig. 1 Gradient HPLC chromatograms of the **a** bromelain standard and **b** bromelain extracted from ultrafiltration process

Table 1 Bromelain concentration in different processing steps

Process	Bromelain concentration (mg/ml)	Volume
Juicing	2.8345	5 L
Homogenization	2.5981	5 L
Centrifugation	2.5989	4.8 L
Ultrafiltration	2.8282	1.2 L
Precipitation	0.9549	20.54 g
Dialysis	0.0419	120 ml in acetate buffer

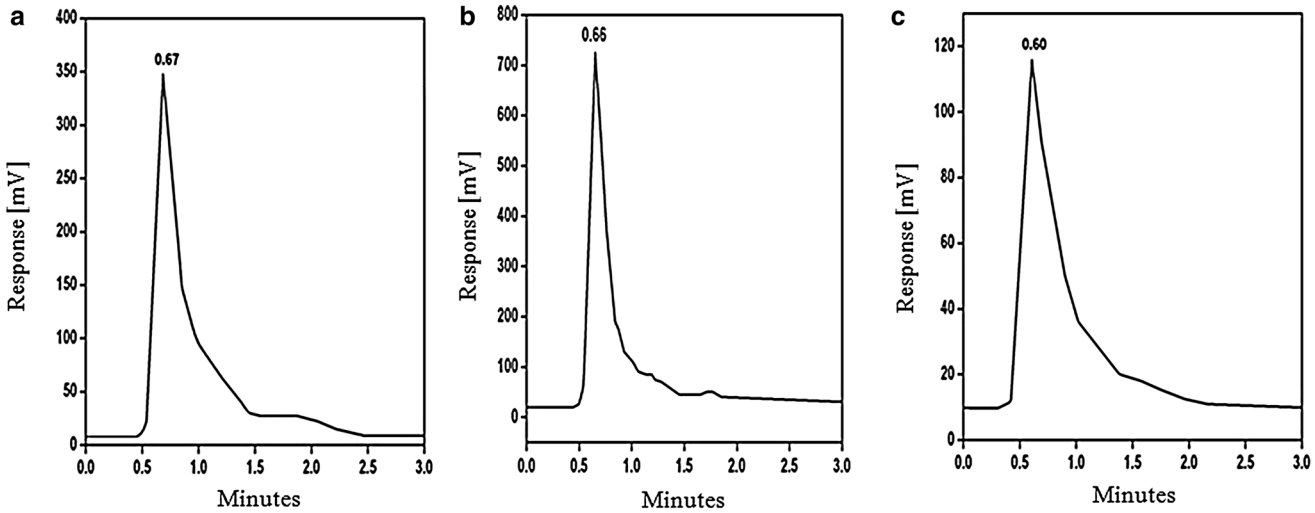

Fig. 2 HPLC chromatograms of bromelain **a** PJ-C **b** PJ-F **c** PJ-S

of the solution was measured using a microplate reader (Multiskan MK3, Thermo Electron Corporation, USA) at 570 nm. All the experiments were performed in triplicate (Doko et al. 1991).

The viability was determined as the ratio between viable treated cells against untreated control cells. Moreover, IC50 values measured.

Morphological Observation of A2780 and HT29 Cells

The morphological apoptosis of cells after treatment was observed under inverted microscope. Besides, the qualitative study carried out by observation of morphological apoptosis using the acridine orange and ethidium bromide

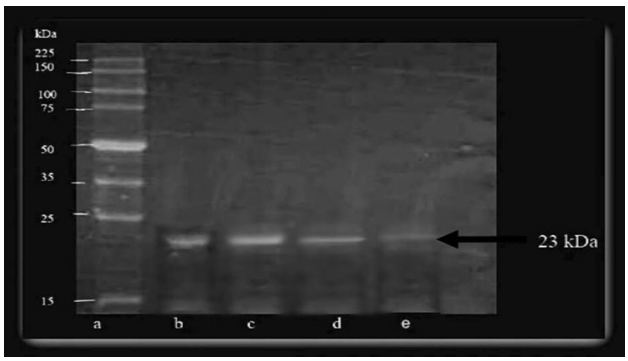

Fig. 3 Molecular weight by SDS-PAGE *a* marker, *b* PJ-C, *c* PJ-F, *d* standard bromelain (2 mg/ml), *e* PJ-S

Table 2 IC_{50} (μg/ml) values of bromelain against A2780 and HT29

Samples	A2780		HT29	
	24 h	48 h	24 h	48 h
Standard	549.53	76.91	88.71	–
PJ-F	280.54	274.15	–	104.95
PJ-S	280.54	195.88	267.30	887.16
PJ-C	231.74	195.99	324.34	146.89

(AO/EB) staining method under fluorescence microscope (Salti et al. 2000).

The 10^4 cells/ml were seeded in 24 well plates. At 70 % confluence of the cells, the cells were treated with

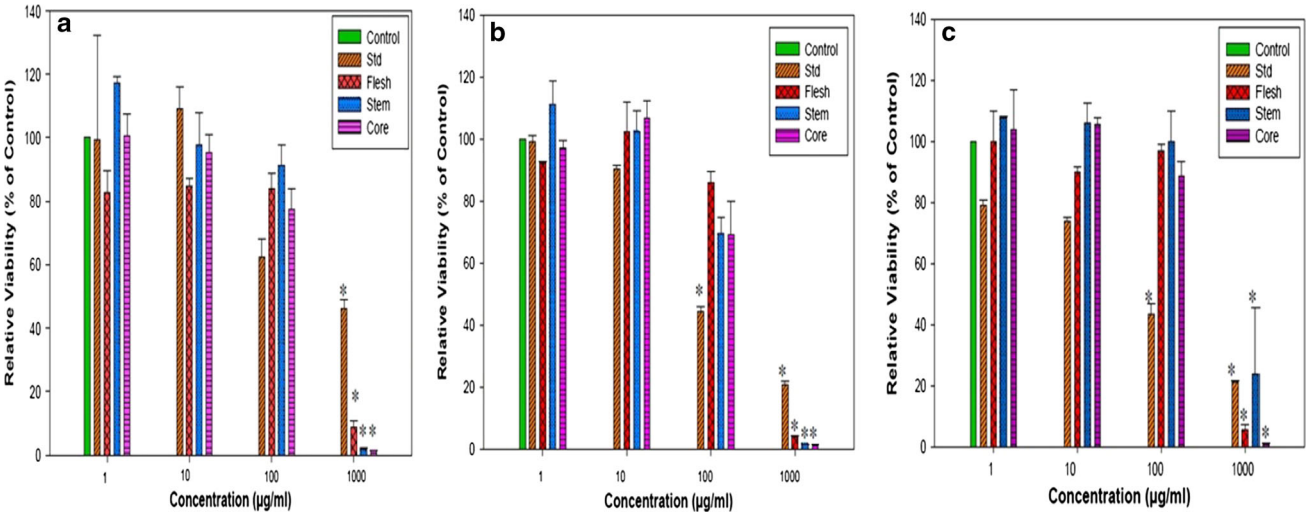

Fig. 4 A2780 cells MTT assay results after **a** 24 h, **b** 48 h and **c** 72 h

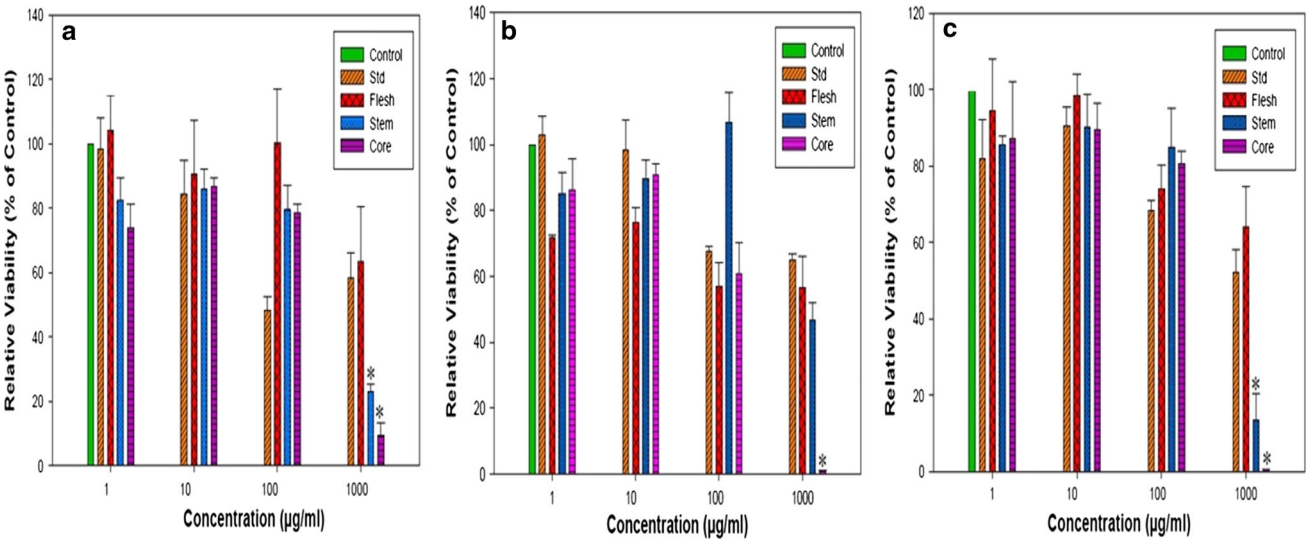

Fig. 5 HT29 MTT assay results after **a** 24 h, **b** 48 h, **c** 72 h

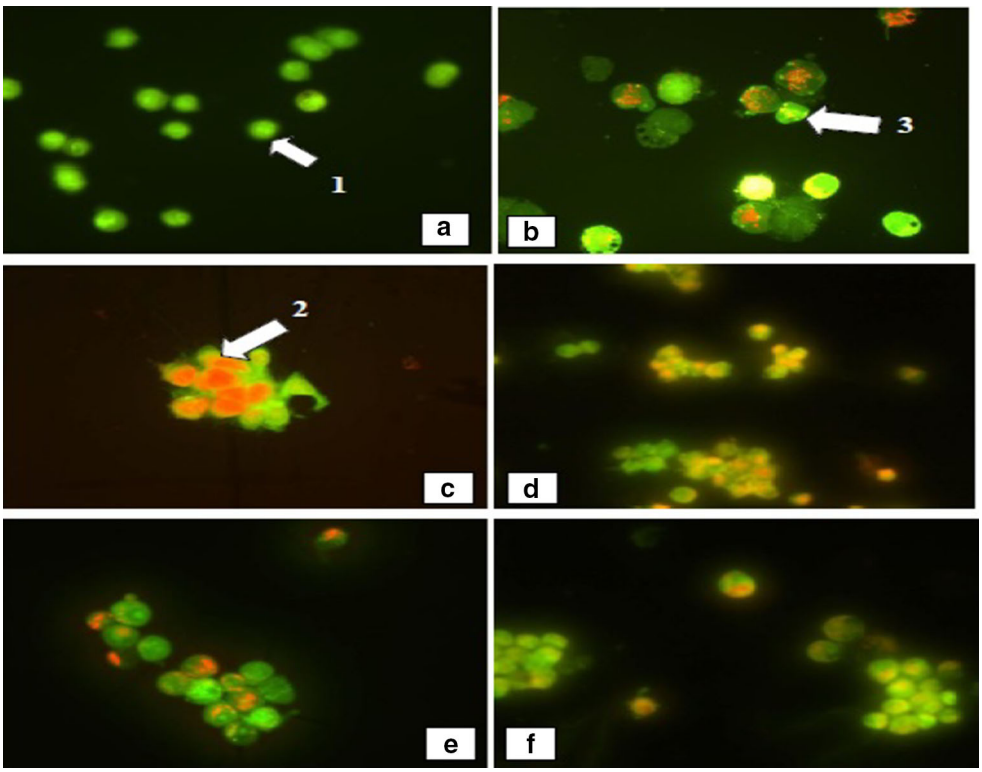

Fig. 6 Morphological observations of apoptosis in A2780 by fluorescence microscope at actual magnification ×400. Figure **a** negative control (untreated cells), **b** bromelain standard **c** cisplatin **d** PJ-F **e** PJ-S and **f** PJ-C. Cells labelled *1*, *2* and *3* are living, apoptotic and necrotic cells respectively

bromelain standard and cisplatin as positive apoptosis controls and PJ-F, PJ-C and PJ-S in different concentrations. The untreated A2780, HT29 and HSF 1184 cells were used as negative control. 48 h after treatment, the supernatant was transferred into 2 ml centrifuge tube and the cells were rinsed by PBS at pH 7.4. Then, Trypsin-Ethylenediaminetetraacetic acid (EDTA) solution was added to detach the cells. Trypsinised cells then transferred to centrifuge tubes and centrifuged at 3300 rpm for 10 min. The supernatant was discarded and 50 µl cold PBS at pH 7.4 was added to the pellets. The cells were then stained with 2 µl of 1 × working solution of nuclear stains consisting of ethidium bromide (500 µg) and acridine orange (100 µg) in 1 ml of PBS. After 10 min of incubation, the cells were observed under fluorescent microscope (Olympus) fixed with spectral imaging and photo activation. Dead cells stained bright orange while the viable cells stained bright green.

Cytotoxicity Assay

To determine the toxicity level of bromelain on the normal cells, HSF1184 cells were inoculated to each well of 96-well plates with a cell density of 1×10^5 cells/well in 200 µl DMEM medium, and incubated in a humidified atmosphere containing 5 % CO_2 at 37 °C for 24 h to allow the cells to adhere to the bottom of the well. After 24 h, cells were treated with various concentrations (1, 10, 100 and 1000 µg/ml) of PJ-F, PJ-C and PJ-S. After 24 and 72 h of incubation, the cells viability were evaluated by the MTT assay (n = 6). Bromelain standard, untreated HSF 1184 cultured cells and medium were served as the respective controls.

Clonogenic Assay

The clonogenic inhibition assay was performed as explained previously by (Franken et al. 2006). Briefly, log growth phase of A2780 and HT29 cells were trypsinized and initial cell concentrations of 1×10^3 cells/mL were seeded into 6-well plates in triplicate and allowed to adhere overnight. After incubation for 24 h, the medium was removed and fresh medium was added with different concentrations (1, 10, 100 and 1000 µg/mL) of the PJ-F, PJ-C and PJ-S. The cells were allowed to incubate at 37° C for 12 days with growth media replaced after every 2 days to examine clonogenic capacity. On day 13, the resulting colonies were washed with cold phosphate buffer saline before fixation with −20 °C cold methanol. Finally, the colonies were stained with 0.5 % trypan blue solution. The number of colonies with >50 cells were counted and colony formation expressed as a percentage of untreated control cultures.

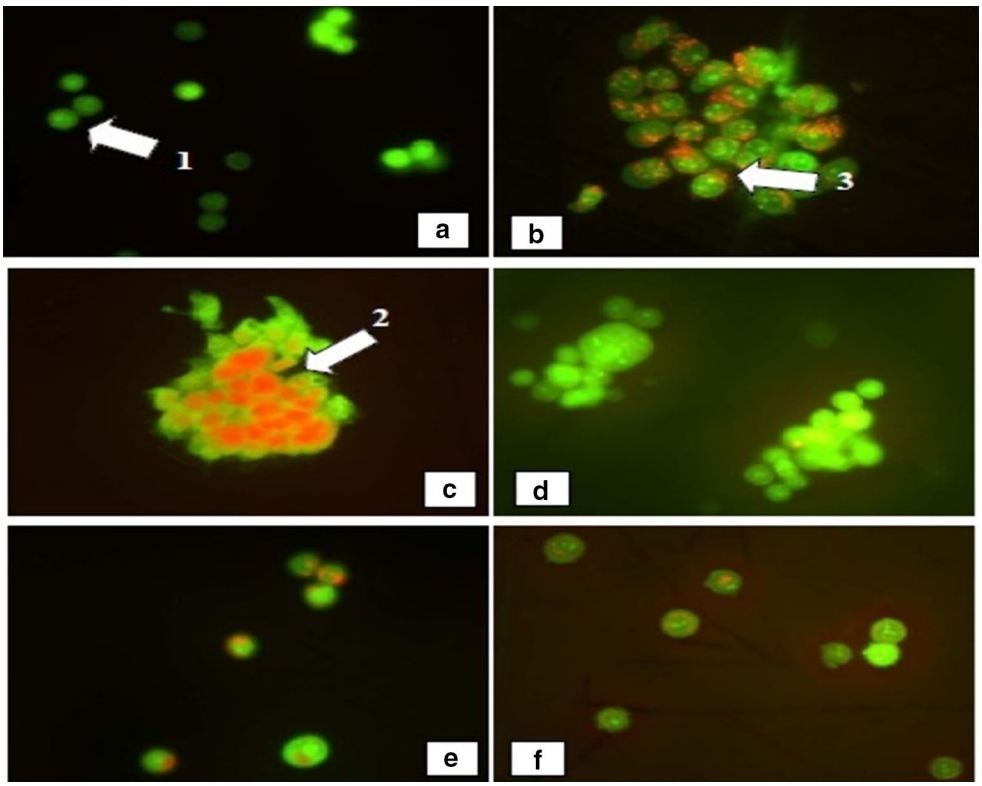

Fig. 7 Morphological observations of apoptosis in HT29 by fluorescence microscope at actual magnification ×400. Figure **a** negative control (untreated cells), **b** bromelain standard **c** cisplatin **d** PJ-F **e** PJ-S and **f** PJ-C. Cells labelled *1*, *2* and *3* are living, apoptotic and necrotic cells respectively

Statistical Analysis

Data collected were compared by one-way analysis of variance and Student's *t* test to determine statistical significance. The analysis was carried out using Sigma Plot 10.0. The values were expressed as mean ± SEM. Differences with *P* values of <0.05 were considered significant.

Results and Discussion

Highest Bromelain Yield

Bromelain was extracted from pineapple core using a series of processing steps; juicing, homogenisation, centrifugation, ultrafiltration and dialysis (Doko et al. 1991, Sankaran et al. 2011). The aim of this process was to extract the concentrated bromelain. Therefore, the steps of the process especially the purification were to remove excess pulp including fibres and the overall non-protein substances.

Five litres of juice was obtained from 10 kg of pineapple core. The yield of the juice was about 50 % of solid pineapple core weight. The final yield of wet solid bromelain was about 20 g out of 10 kg of pineapple core. The bromelain concentration and purity in different processing steps was determined by HPLC at 280 nm. The bromelain profile using HPLC is presented in Fig. 1.

Table 1 shows that bromelain concentration was further reduced at every processing step. The juice contained 2.83 mg/ml of bromelain while the crude bromelain (after dialysis) was around 1.0 mg/ml only. The losses are due to increasing absorption and the decay is due to the physical destruction of bromelain. These are the factors that should be taken into account during the process. The selection of 55 % ammonium sulphate precipitation mode could also contribute to heavy loss of bromelain during processing. Other factors could also contribute such as pH, temperature and pressure (Doko et al. 1991).

From the study, it was indicated that fresh juice which was from juicing process showed higher bromelain concentration compared to the other processed juices during the extraction process. Bromelain concentration in PJ-F, PJ-C and PJ-S were 8.65, 1.78 and 3.23 mg/ml respectively. The purity of bromelain in different samples was determined using SDS-PAGE and chromatography. The HPLC profile for each part is shown in Fig. 2. The Bradford assay results confirmed the results obtained in HPLC. The highest concentration of bromelain was in PJ-F whereas the lowest concentration was in PJ-S of the same *"Gandul"* type. This result is supported by Hale et al. (2005) that reported the highest major

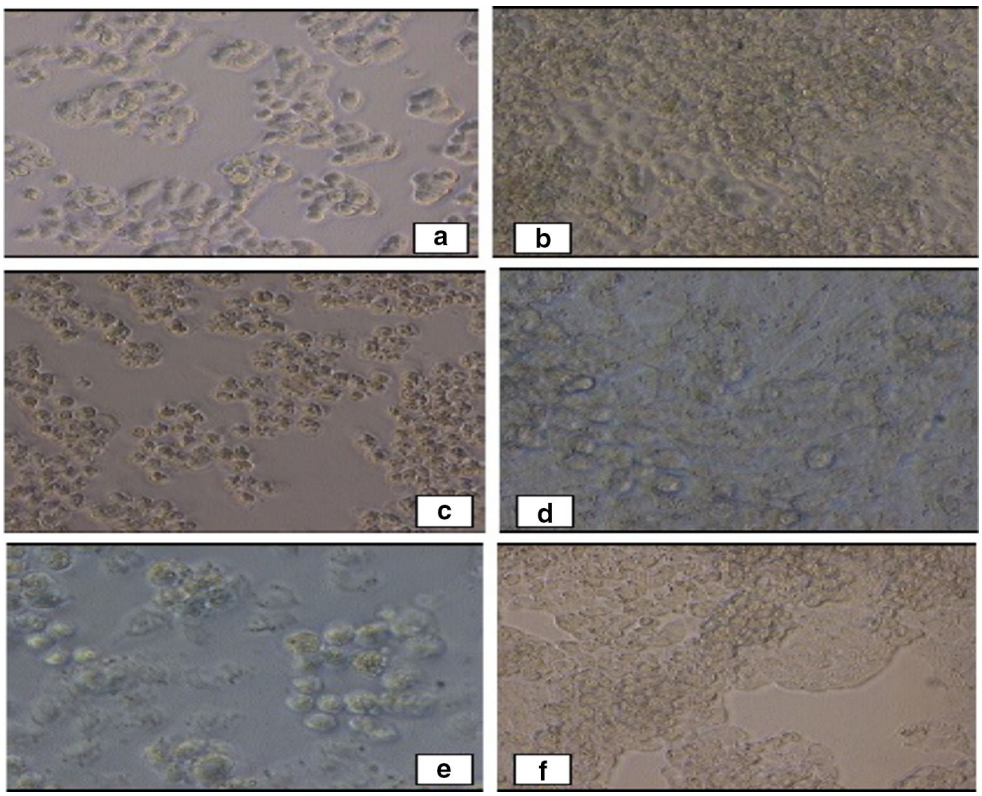

Fig. 8 Morphological study of apoptosis in A2780 by inverted microscope (×100). Figure **a** control (untreated cells), **b** bromelain standard **c** cisplatin **d** PJ-F **e** PJ-S **f** PJ-C at concentration of 100 μg/ml for 48 h

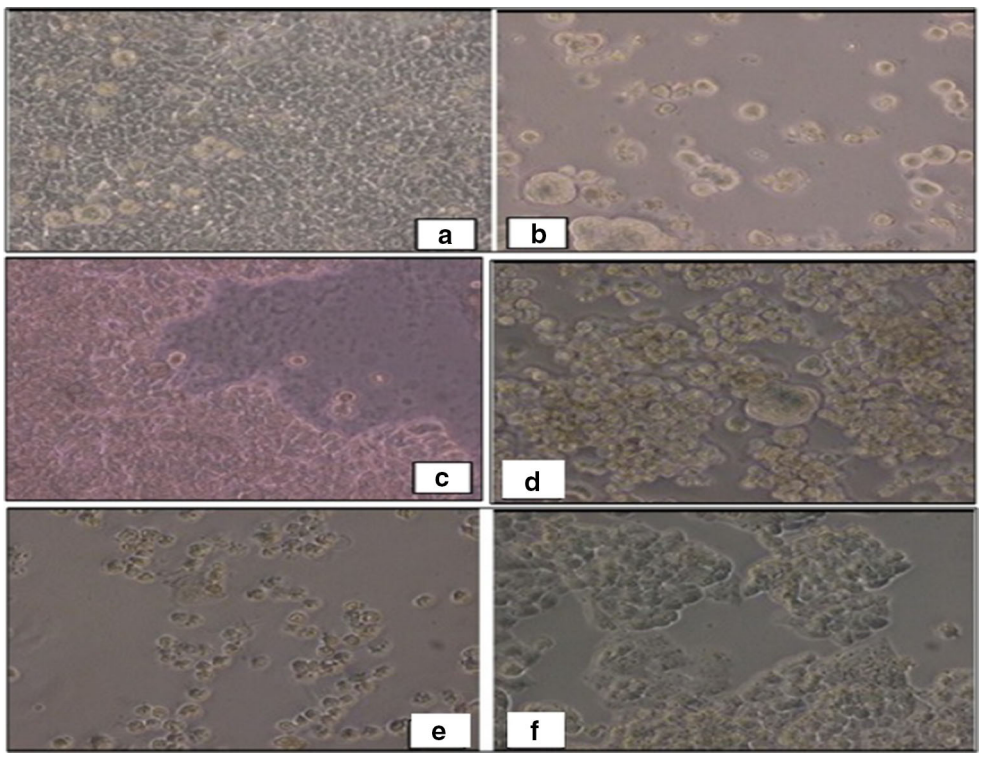

Fig. 9 Morphological study of apoptosis in HT29 by inverted microscope (×100). Figure **a** control (untreated cells), **b** bromelain standard **c** cisplatin **d** PJ-S **e** PJ-F **f** PJ-C at concentration of 100 μg/ml for 48 h

proteinase present in flesh and minor component in stem. In contrast, Umesh Hebbar et al. (2008) found the highest bromelain concentration present in the stem, followed by the flesh, core and crown. Using different types of pineapple could have been the reason for variation observed (Mashudin and Abdul Majid 2009).

According to the literature, bromelain extracted using a series of processing steps was not stable below 50 mg/ml during storage resulting potential difficulty for further experiment, but bromelain extracted from juicing process was stable at its natural condition (Umesh Hebbar et al. 2008; Hale et al. 2005).

The type of bromelain varies in each part of the pineapple that leads to different efficacy and activity. So, all three sources of bromelain from juicing process in flesh, stem and core were selected to further investigate the potential of bromelain to inhibit the growth of A2780 and HT29 cancer cell lines. Based on the above discussion, PJ-F, PJ-C and PJ-S were used to proceed in this study.

Bromelain Molecular Weight

The results demonstrated that PJ-F, PJ-C and PJ-S had similar molecular weight as bromelain standard. The band obtained found to be about 23 kDa (Fig. 3) lying close to the previously reported range of bromelain molecular weight (26–28 kDa) which is similar to the findings by Umesh Hebbar et al. (2008) and Sankaran et al. (2011). PJ-F presents the highest bromelain concentration with 8.66 mg/ml and showed the clearest band while PJ-S with the lowest bromelain concentration produced not a very clear band.

MTT Assay

The viability of A2780 and HT29 cells treated with bromelain standard, PJ-F, PJ-C and PJ-S were measured by MTT assay for 24, 48 and 72 h (Fig. 4). Bromelain standard was used as positive control, while untreated A2780, HT29 cultured cell were used as negative control.

The results indicated a linear decrease of A2780 cell exposed to bromelain standard over the period of 72 h. On the other hand, the reduction of cell viability was clearly observed after PJ-F, PJ-C and PJ-S treatment within 24 and 48 h but cells seemed to regrow after 72 h. However, it is not known whether accumulated cells after treatment are cancerous or otherwise. Molecular studies should be carried out to assess any possibility. There was noticeable reduction of cell number at concentration of 1000 µg/ml within 24, 48 and 72 h. The results indicated a considerable potential of PJ-F, PJ-C and PJ-S to reduce A2780 cell viability at 100 and 1000 µg/ml concentrations within less than 48 h that point out all three PJ-F, PJ-C and PJ-S may have anti-carcinogenic activity against A2780 cell.

On the other side, bromelain standard at 100 and 1000 µg/ml concentration significantly inhibited HT29 cell growth. As shown in Fig. 5, 100 and 1000 µg/ml of bromelain standard indicated more than 50 % reduction of cell viability and PJ-F, PJ-C and PJ-S showed noticeable reduction at the concentration of 1000 µg/ml within 24, 48 and 72 h. The results showed a remarkable potential of PJ-F, PJ-C and PJ-S to decrease the HT29 cells viability at concentration of 100 and 1000 µg/ml so that it can be concluded, all three PJ-F, PJ-C and PJ-S may have anti-carcinogenic activity against HT29 cell.

The IC_{50} (µg/ml) values of A2780 and HT29 cells treated with bromelain standard (positive control) and crude bromelain in juices summarised in Table 2.

Apoptosis Morphological Observation

Induction of apoptosis is one of the considerations in drug development. Most of the cytotoxic anti-cancer drugs in

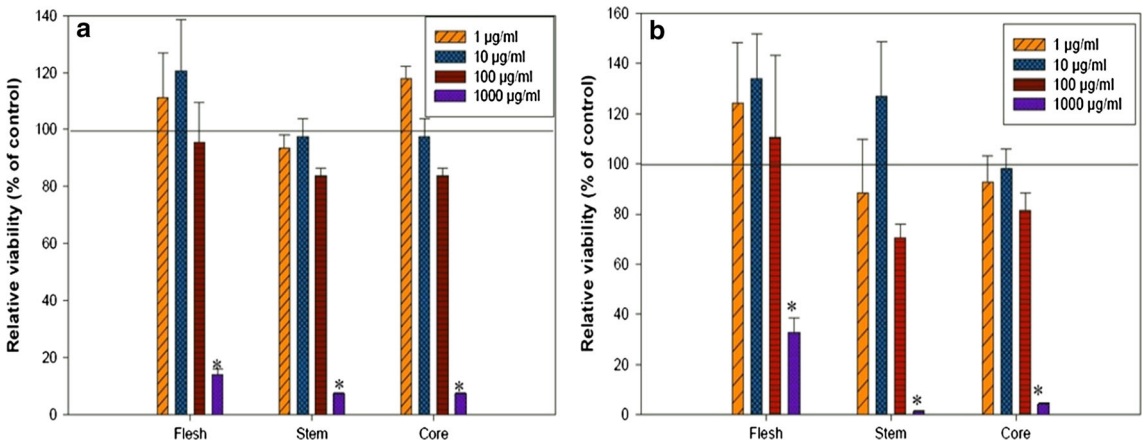

Fig. 10 HSF1184 cells MTT assay results after **a** 24 h and **b** 72 h

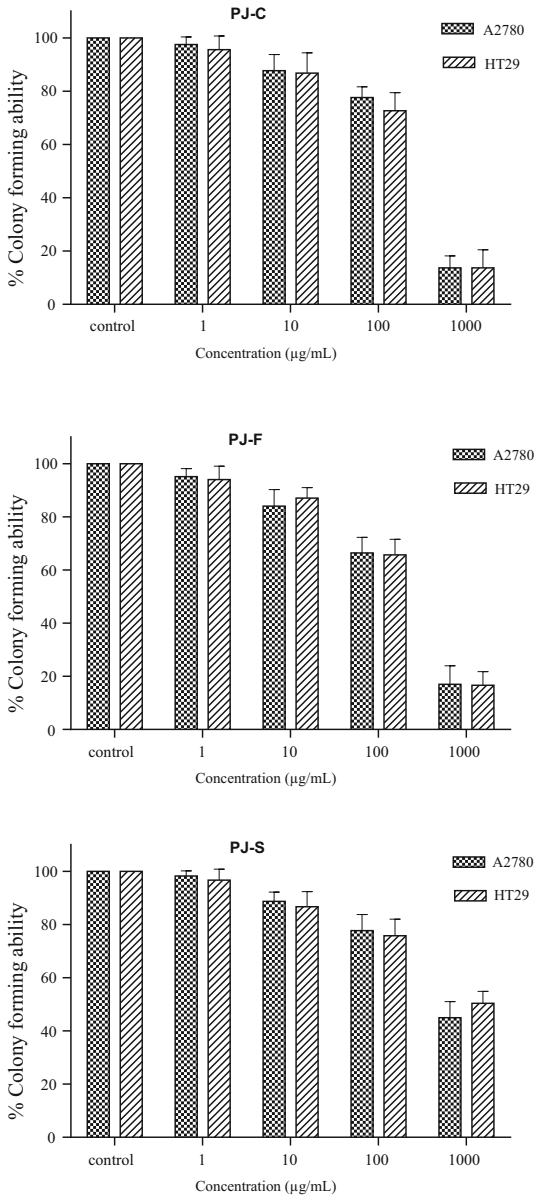

Fig. 11 Quantitative measurement of colony formation of fresh pineapple juices on A2780 and HT29 cells at different concentration (1–1000 µg/mL). Bromelain inhibited colony formation in a dose dependent manner. The colony forming potential of the cells at each concentration of bromelain in fresh pineapple juice is expressed in terms of percentage of control and reported as mean ± SEM. The experiment was repeated three times

current use have shown capability to induce apoptosis in susceptible cells (Hickman 1992; Kaufmann and Earnshaw 2000). Apoptosis is an active process of cell destruction and is an important model of cell death that occurs in response to a variety of agents including ionizing radiation or anti-cancer chemotherapeutic drugs (Fisher 1994).

Pervious researches indicated that bromelain increases expression of p53 as well as other apoptosis activators such as Bax in mouse skin papilloma (Kalra et al. 2008). Chobotova et al. (2010) reviewed that bromelain also decreases the activity of cell survival regulators such as protein kinase B known as Akt and extracellular signal-regulated kinases known as Erk thus promoting apoptotic cell death in tumor.

Since our preliminary results indicated that PJ-F, PJ-C and PJ-S at concentration of 1000 µg/ml significantly reduces the viability of both A2780 and HT29 cancer cells, those cancer cells were exposed to bromelain standard, cisplatin, PJ-F, PJ-C and PJ-S at 1000 µg/ml for 48 h in order to observe the morphological changes.

Figures 6 and 7 show that there was no distinct morphological change in the control group, but A2780 and HT29 cells exhibited condensed chromatin, fragmented nucleic and appearance of apoptotic bodies after treatment. By examining cell morphology, it was observed that bromelain caused cell detachment and formation of clusters and aggregates that still contained live cells.

Cell shape and its changes also can be observed clearly under inverted microscope. Figures 8 and 9 show the morphology of A2780 and HT29 cells after treatment with bromelain standard, PJ-F, PJ-C and PJ-S at concentration of 100 µg/ml. Treated cells showed obvious morphological changes. The cells have rounded up and detached from the culture plate, with many showing signs of necrosis.

As a result, PJ-F, PJ-C and PJ-S at concentration of 100 and 1000 µg/ml inhibited the proliferation of A2780 and HT29 cancer cell lines.

Cytotoxicity Test

The toxicity level of standard bromelain, PJ-F, PJ-C and PJ-S on the normal cells were investigated by MTT Assay. Figure 8 shows all samples at concentration of 1 and 10 µg/ml did not give reduction within 72 h. A 24-h observation determined that all samples at concentration of 1000 µg/ml showed low cell viability. The cells showed less affected and a slight reduction at 100 µg/ml after 24 and 72 h. Based on the findings, a concentration of 1000 µg/ml inhibited the normal cell, but a concentration of 100 µg/ml less affected with minor reduction of 20 % cell viability. In cancer treatment, chemotherapeutic agents also kill the normal cell. Ideally, anticancer drugs should specifically target only neoplastic cells to decrease cytotoxic effects on normal cell (Johnstone et al. 2002).

Effect of Fresh Pineapple Juices on Colony Formation in A2780 and HT29 Cells

Having established the anti-proliferative and cytotoxic activities of PJ-F, PJ-C and PJ-S in the A2780 and HT29 cells, next we further ascertained their effects on cell

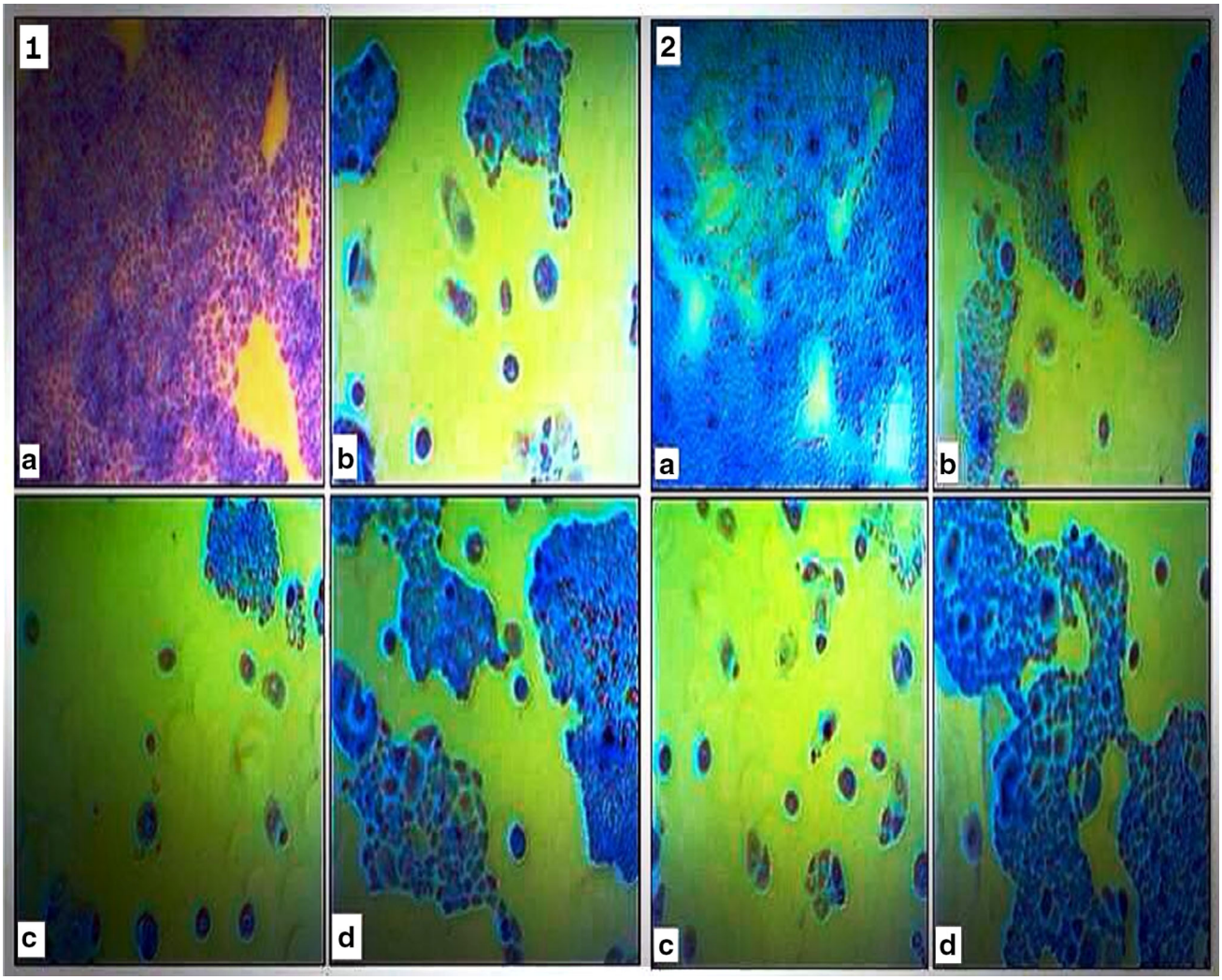

Fig. 12 Effect of bromelain fresh pineapple juices on colony-forming abilities of **1** A2780 and **2** HT29 cells. Figure *a* control (untreated cells), *b* PJ-C *c* PJ-F *d* PJ-S at concentration of 1000 µg/ml

viability by clonogenic inhibition assay against the same cell lines using various concentrations of bromelain in fresh pineapple juices. The clonogenic survival of treated cancer cells was proportional to the bromelain concentrations. As depicted in Figs. 10, 11 and 12, PJ-F, PJ-C and PJ-S causing a concentration dependent to decrease in number and size of growing colonies when compared to the negative control (untreated cells). Bromelain at concentration of 1000 µg/mL visibly modulated the size of growing colonies and significantly inhibited colony-forming abilities of cells. PJ-F inhibited the colony-forming abilities of cells better than the other samples. Lowest concentrations of extracts (1 µg/mL) did not have any significant effect on clonogenicity of any of the cell lines. These results demonstrated that PJ-F, PJ-C and PJ-S exhibited a cytostatic effect on long-term colony formation by A2780 and HT29 cells.

Conclusion

This study has come up with several findings. In summary, it was found that fresh pineapple juice has the highest bromelain concentration in comparison with processed juices. Although, there may be some other impurities in fresh juices from various parts of pineapple along with bromelain, particularly the flesh, stem and core of *Gandul* type contained substantial amounts of bromelain showing the potential to inhibit the growth of ovarian (A2780) and colon (HT29) cancer cells in vitro. The concentration of 100 and 1000 µg/ml crude bromelain in juices of flesh, stem and core of pineapple are recommended as suitable dosages to inhibit the growth of ovarian (A2780) and colon (HT29) cancer cells. The concentration of 1000 µg/ml of bromelain has toxic effects on HSF1184 normal cells so that further studies could be carried out to target bromelain

using as drug delivery vehicle providing less cytotoxic effects on normal cells and maximum toxic effect on cancer cells. To identify whether the fresh pineapple juices induced inhibition of cancer cells growth through apoptosis, acridine orange and ethidium bromide were used to observe the apoptotic features. Apoptotic induction was shown in treated cells, which revealed hallmark properties of apoptosis like nuclear fragmentation and cellular shrinkage. Moreover, it was found that PJ-F, PJ-C and PJ-S effectively inhibited the colony formation of A2780 and HT29 cells at bromelain concentrations of 100–1000 μg/ml. To conclude, all three PJ-F, PJ-C and PJ-S may act as an efficient inhibitor against ovarian (A2780) and colon (HT29) cancer cell growth. More researches are needed to investigate an accurate mechanism of cytotoxic activity of bromelain in fresh pineapple juices.

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Conflict of interest All the authors declare that there is no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Bala M, Ismail NA, Mel M, Jami MS, Salleh HM, Amid A (2012) Bromelain production: current trends and perspective. Arch Des Sci 65(11):369–399

Bhui K, Prasad S, George J, Shukla Y (2009) Bromelain inhibits COX-2 expression by blocking the activation of MAPK regulated NF-kappa B against skin tumor-initiation triggering mitochondrial death pathway. Cancer Lett 282(2):167–176

Castell JV, Friedrich G, Kuhn CS, Poppe GE (1997) Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. Am J Physiol 273:139–146

Chobotova K, Vernallis AB, Abdul Majid FA (2010) Bromelain's activity and potential as an anti-cancer agent: current evidence and perspectives. Cancer Lett 290(2):148–156

Doko MB, Bassani V, Casadebaig J, Cavailles L, Jacob M (1991) Preparation of proteolytic enzyme extracts from Ananas comosus L Merryl fruit juice using semipermeable membrane, ammonium sulfate extraction, centrifugation and freeze-drying processes. Int J Pharm 76(3):199–206

Fisher DE (1994) Apoptosis in cancer therapy: crossing the threshold. Cell 78(4):539–542

Franken NA, Rodermond HM, Stap J, Haveman J, Van Bree C (2006) Clonogenic assay of cells in vitro. Nat Protoc 1(5):2315–2319

Hale LP, Greer PK, Trinh CT, James CL (2005) Proteinase activity and stability of natural bromelain preparations. Int Immunopharmacol 5(4):783–793

Hickman JA (1992) Apoptosis induced by anticancer drugs. Cancer Metastasis Rev 11(2):121–139

Johnstone RW, Ruefli AA, Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell 108(2):153–164

Kalra N, Bhui K, Roy P, Srivastava S, George J, Prasad S, Shukla Y (2008) Regulation of p53, nuclear factor κB and cyclooxygenase-2 expression by bromelain through targeting mitogen-activated protein kinase pathway in mouse skin. Toxicol Appl Pharmacol 226(1):30–37

Kaufmann SH, Earnshaw WC (2000) Induction of apoptosis by cancer chemotherapy. Exp Cell Res 256(1):42–49

Kelly GS (1996) Bromelain: a literature review and discussion of its therapeutic uses. Altern Med Rev 1(4):120–123

Mashudin M, Abdul Majid FA (2009) Report on Nutritional Value of Commercial Pineapple Varieties in Malaysia. Project collaboration between Malaysia Pineapple Industry Board (MPIB) with Universiti Teknologi Malaysia

Maurer HR (2001) Bromelain: biochemistry, pharmacology and medicinal use. Cell Mol Life Sci 58:1234–1245

Pillai K, Akhter J, Chua TC, Morris DL (2013) Anticancer property of bromelain with therapeutic potential in malignant peritoneal mesothelioma. Cancer Invest 31(4):241–250

Salti GI, Grewal S, Mehta RR, Das Gupta TK, Boddie AW Jr, Constantinou AI (2000) Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. Eur J Cancer 36(6):796–802

Sankaran K, Vadanasundari V, Hemavathy RV, Thirunavukkarasu A (2011) A comparative study on determining of efficacy of salt precipitation and biphasic system in the extraction of bromelain from Ananas comosus. Asian J Sci Technol 2(1):016–022

Tochi BN, Wang Z, Xu SY, Zhang W (2008) Therapeutic application of pineapple protease (bromelain): a review. Pak J Nutr 7(4):513–520

Umesh Hebbar H, Sumana B, Raghavarao K (2008) Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes. Bioresour Technol 99(11):4896–4902