RESEARCH ARTICLE

Cytokinetic Study of MCF-7 Cells Treated with Commercial and Recombinant Bromelain

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Abstract

Background: Breast cancer is a leading cause of death in women. The available chemotherapy drugs have been associated with many side effects. Bromelain has novel medicinal qualities including anti-inflammatory, anti-thrombotic, fibrinolytic and anti-cancer functions. Commercially available bromelain is obtained through tedious methods; therefore, recombinant bromelain may provide a cheaper and simpler choice with similar quality. Materials and Methods: This study aimed to assess the effects of commercial and recombinant bromelain on the cytokinetic behavior of MCF-7 breast cancer cells and their potential as therapeutic alternatives in cancer treatment. Cytotoxic activities of commercial and recombinant bromelain were determined using (sulforhodamine) SRB assay. Next, cell viability assays were conducted to determine effects of commercial and recombinant bromelain on MCF-7 cell cytokinetic behavior. Finally, the established growth kinetic data were used to modify a model that predicts the effects of commercial and recombinant bromelain on MCF-7 cells. Results: Commercial and recombinant bromelain exerted strong effects towards decreasing the cell viability of MCF-7 cells with IC_{50} values of 5.13 μg/mL and 6.25 μg/mL, respectively, compared to taxol with an IC_{50} value of 0.063 μg/mL. The present results indicate that commercial and recombinant bromelain both have anti-proliferative activity, reduced the number of cell generations from 3.92 to 2.81 for commercial bromelain and to 2.86 for recombinant bromelain, while with taxol reduction was to 3.12. Microscopic observation of bromelain-treated MCF-7 cells demonstrated detachment. Inhibition activity was verified with growth rates decreased dynamically from 0.009 h^{-1} to 0.0059 h^{-1} for commercial bromelain and to 0.0063 h^{-1} for recombinant bromelain. Conclusions: Commercial and recombinant bromelain both affect cytokinetics of MCF-7 cells by decreasing cell viability, demonstrating similar strength to taxol.

Keywords: Bromelain - cell viability - growth kinetics - MCF-7 cells - recombinant

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Introduction

Breast cancer is the second leading cause of cancer death among women. It is the most cancer frequently diagnosed in women worldwide with an estimated 1.4 million new cases and 458,400 breast cancer deaths occurred in women in 2008 (Desantis et al., 2009). In January of 2012, world cancer statistics diagnosed more than 2.9 million women living in the US with a history of invasive breast cancer and additional 226,870 women for new breast cancer cases were estimated (Society, 2013).

According to the World Health Organization, There are good precedents for looking to nature for drug discovery approximately 25 percent of modern medicines are made from plants that were previously used in traditional medicine. At the same time, many drugs used in chemotherapy were derived from bacteria and fungi. Taxol, one of the most powerful natural anti-tumor drugs originally comes from the bark of Pacific yew tree that grows in the Pacific Northwest, USA (Saville et al., 1995). However, the production of commercial taxol has always limited by its supply. Taxol also has been associated with various side effects including hypersensitivity in 41-44% of all treated patients (Mosley et al., 2007; Chik et al., 2010). Meanwhile, doxorubicin, a potent board spectrum inhibitor of human tumors, also exhibits severe adverse side effects. Therefore, the discovery of new compound from nature which is perceived to be safer and more effective is highly wanted.

Bromelain have been introduced as a medicinal compound due to its actions which includes: anti-tumor action, inhibition of platelet aggregation, fibrinolytic activity, anti-inflammatory action, modulation of cytokines and immunity, skin debridement properties, enhanced absorption of other drugs, mucolytic properties, digestive assistance, enhanced wound healing and cardiovascular

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and circulatory improvement (Maurer, 2001). Bromelain is an aqueous extract of pineapple that contains a complex mixture of thiol proteases and non-protease components. Proteases constitute the major components of bromelain and its constitute of stem bromelain (80%), fruit bromelain (10%), and ananain (5%). Among the non-protease components are phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates (Chobotova et al., 2010). The evidences of the anti-cancer activity of bromelain come from traditional remarks (in Southeast Asia) and studies of animal-and cell-based models (Hale et al., 2005b). The anti-cancer activity of bromelain is qualified mainly to its protease components (Hale et al., 2005b; Chobotova et al., 2010). This support previous studies that showed anti invasive property of bromelain is dependent on its proteolytic activity (Tysnes et al., 2001). Bromelain can be absorbed in human intestines without degradation and without losing its biological activity (Castell, 1995). Anecdotal clinical studies of bromelain in the 1970s propose early evidence suggesting the efficiency of high dosages of bromelain (1-2.4 g/day) for treating some cancers, including breast and ovarian (Zavadova et al., 1995). However, bromelain has not been the subject of randomized controlled clinical studies as a cancer cure to this moment (Chobotova et al., 2010). While, the available commercial bromelain is obtained through tedious and costly purification method which yields bromelain at different degrees of purity, the use of recombinant bromelain is cheaper with similar degree purity of commercial bromelain. Moreover, recombinant bromelain provides substantial quantity which has resistant to natural inhibitors like pH and temperature (Amid et al., 2011).

In this cytokinetics experiment study, we demonstrated that bromelain and recombinant bromelain have good prospect as a strong anti-cancer agent and thus suitable to be used as alternative treatment for cancer. However, there are no commercial uses for recombinant bromelain and they may be readily available as natural sources for new discoveries in the treatment of cancer. In order to strongly understand the mechanism of bromelain and recombinant bromelain on cancer cells behavior, detailed understanding of the cell growth and especially its effects towards the treatment sensitivity become important tools for its pharmacological analysis. Most of the principal features of a cancer are a tendency to increase in size, to spread, and to destroy the function of normal organs. All these behaviors are reliant on the reproduction of its cells. Due to that, growth kinetic concepts pass through clinical thinking in both explicit and ambiguous ways (Dang et al., 2003). This experiment studied the cells growth patterns and presumed sites of action of anti-cancer drugs. It helps to respond the relationship between bromelain and recombinant bromelain treatments and the rate of tumor regression.

Materials and Methods

Production of recombinant bromelain

An *E. coli* strain BL21-AI harboring recombinant bromelain was grown in LB broth at 37°C, 225 rpm in the incubator shaker for 12 hours. The recombinant bromelain was over-expressed by L-arabinose induction when it reached mid-log phase (approximately 3-hour after incubation time). After centrifugation at 5000g, 4ºC/10 min, the pellet was collected by the fermentation product. The cell pellet was re-suspended in lysis buffer and sonicated about 20 seconds. The supernatant was then collected by centrifuging at 12000g for 30 minutes and purified by affinity chromatography using Nickel column. Finally, the purified supernatant was freeze dried to obtain the dry powder (Amid et al., 2011; Bala et al., 2011).

Preparation of bromelain, recombinant bromelain and taxol

About 0.1mg of bromelain and recombinant bromelain were dissolved in 1ml of PBS (phosphate buffered saline), respectively. Taxol was used as a positive control and 50μg of taxol was dissolved in PBS.

Cell line, culture condition

MCF-7 breast cancer cell line (ATCC No: HTB-22) was obtained from American type Collection Culture. Frozen MCF-7 breast cancer cells were thawed and inoculated into 5ml media (Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100μg/ml streptomycin and 100U/ml penicillin. The cells were cultured in T-25 flasks at 37ºC, 5%CO_2 and allowed to grow until 60-80% confluent.

Determination of IC_{50} and cell viability using sulforhodamine B (SRB) assay

SRB Assay remains as one of the most used method for in vitro cytotoxicity screening. In this study it was employed to evaluate tumor cell viability in the presence or absence of treatment and then to determine the IC_{50} of commercial bromelain, recombinant bromelain and taxol. First, 1×10^6 cells/ml of MCF-7 cells was loaded into each well of 96-well plates. The plates were incubated for 24 hours to allow cells to stabilize prior to adding sample. Commercial and recombinant bromelain were prepared in six intervals range between 3.125 and 100μg/ml. Meanwhile, taxol concentration was between range 0.5 and 0.016μg/mL. The cells were then incubated for a further 48 hours. After incubation, trichloroacetic acid was used to fix the cells to the plate. After few numbers of washes, the cells were treated with the protein stain Sulforhodamine B. Then, the absorbance (OD) was measured using TECAN Infinite M200 multimode microplate reader at the optimal wavelength of 510nm (Lednicer and Narayanan, 1993). The formula used to calculate the number of viable and non-viable cells is:

%Cells growth=[Mean OD (sample)/Mean OD (sample)]×100 **(1)**

or,

%Cell killed=100-%Cells growth **(2)**

The half maximal inhibitory concentration (IC_{50}) of bromelain, recombinant bromelain and taxol were

determined based on the graph percentage inhibition against concentration of samples used. For cell viability assay, MCF-7 cells were harvested and seeded into T-25 flasks at 2×10^5 cells/mL. After 24 hours, media were removed and fresh media containing bromelain and recombinant bromelain were added into respective flasks. Cells were treated using bromelain at IC_{50} value of 5.125μg/mL and recombinant bromelain at IC_{50} value 6.25μg/mL. Cells were incubated for a further 24 hours before trypsinization. Cells were stained with trypan blue dye and counted using hemocytometer. Cell images were observed and documented using phase contrast microscope at 4× magnification (Freshney, 2010).

Growth kinetics study

The growth kinetic study was established to modify a model that predicts the effect of bromelain and recombinant bromelain on MCF-7 cells. Again, MCF-7 cells were harvested and seeded into T-25 flasks at 2×10^5 cells/ml. There were four batches of flasks in this study: *i*) Untreated MCF-7; *ii*) Taxol-treated cells at IC_{50} value of 0.063μg/mL; *iii*) Bromelain-treated cells at IC_{50} value of 5.125μg/mL; *iv*) Recombinant bromelain-treated cells at IC_{50} value of 6.25μg/mL. Each batch has 20 flasks to report sampling at 8-hourly intervals (between 0-152h). Each flask was performed in three independent experiments. After 24 hours, media were removed and fresh media containing samples as described above were added in respective flasks. Cells were incubated from 0-152 hours where at 8 hour intervals thereafter, one flask containing cells was harvested and quantified using trypan blue dye exclusion assay. The viable number was calculated as the equation below:

$$C=n\times2\times10^4 \quad (3)$$

Where ***n*** is the average cell count, **2** is the dilution factor and is the conversion of volume 0.1 mm^3 to mL.

The data were later used to plot growth curve and calculate the cell generation number using the following equations (Michaelis and Ratain, 2006; Gwyther and Schwartz, 2008):

$$X=(log_{10}N-log_{10}N_0)/log_{10}{}^2 \quad (4)$$

$$log_{10}N=log_{10}N_0+\mu t \quad (5)$$

$$t_d=log_{10}{}^2/\mu=0/301/\mu \quad (6)$$

Where, ***X***=the number of generations; ***N***=the final cells volume; N_0=the initial cells volume; μ=the specific growth rate (slope); ***t***=the duration of treatment; t_d=the doubling time.

Statistical analysis

All statistical analyses were performed using Design Expert software version 8, (Stat-Ease, Inc, USA). The data was signified the mean plus or minus the standard error of means of three samples and are representative of three independent experiments. Differences between two means were analyzed by Student's t-test. Values of p<0.05 were determined to be significant.

Results

IC_{50} of bromelain against MCF-7 cancer cells

The IC_{50} values of bromelain and recombinant bromeleain obtained from SRB assay were 5.125μg/mL and 6.25μg/mL respectively as seen in Figure 1A. While, Figure 1B shows IC_{50} value of taxol at 0.063μg/mL which is in agreement with other previous studies where its IC50 were 0.039 μg/mL and 0.043μg/mL (Mosley et al., 2007; Chik et al., 2010).

This method was meant to determine the half maximal inhibitory concentration (IC_{50}) of commercial bromelain and recombinant bromelain towards the cancer cells.

Effect of bromelain on MCF-7 cell viability

As seen in Figure 2, the MCF-7 cell viability was significantly decreased by more than 50% as compared to control for bromelain (p=0.00008) and recombinant bromelain (p=0.0002), treatment respectively.

Effect of bromelain on MCF-7 cell growth kinetics

The growth kinetics of MCF-7cells untreated and treated with bromelain (5.125μg/mL) and recombinant bromelain (6.25μg/mL) was studied and compared. The growth behavior of MCF-7 cells and its changes after treatment can be seen in Figure 3. The cells were initially inoculated at 2×10^5 cells/mL. The lag phase of the

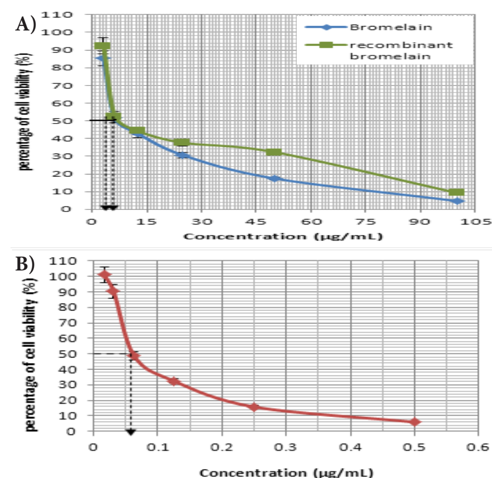

Figure 1. IC_{50} Values of A) Bromelain and Recombinant Bromeleain; B) Taxol against MCF-7 Breast Cancer Cells

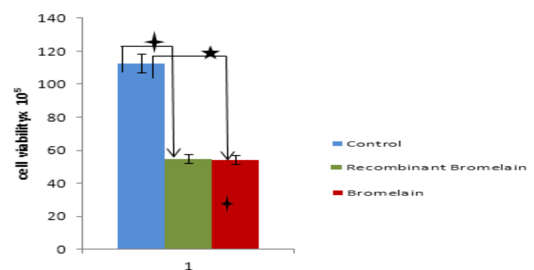

Figure 2. Effect of 24 hr Treatment of Bromelain and Recombinant Bromelain on MCF-7 Breast Cancer Cells. The results are 0.00008 for bromelain and 0.0002 for recombinant bromelain. ✦1.09×10^6 cells/mL, p values is 0.0002 for recombinant bromelain; ★1.08×10^6 cells/mL, p values is 0.00008 for bromelain

untreated (control) and treated MCF-7 cells were similar at first 8 hours. However, the cells started to proliferate after 16 hours and followed through exponential or log phase for 56 hours before the cells reached their stationary phase at 72 hours in untreated cells. However, in treated cells there was no stationary phase observed but the cells reduced height saturation density at 88 hours. Finally, the cell's growth started to decline (death phase) after 88 hours. Although the growth profile trend to all treatment and control was similar, number of viable cells in treated batches clearly showed some reduction as compared to the control. The highest cells number achieved by taxol, bromelain and recombinant bromelain treated cells were only 1.75×10^6, 1.45×10^6 and 1.4×10^6 cells/mL, respectively as compared to control (3.05×10^6 cell/mL) when reached at the stationary phase.

The exemplary photos of cells at 24, 48, 72, 96, 120 and 144 hrs for bromelain, recombinant bromelain and control were presented in Figure 4. The longer the incubation time, the more reduction in cell density of MCF-7 observed. This may be due to the anti proliferative activity of bromelain and recombinant bromelain towards MCF-7 cells growth.

Based on equation 4 that used on the growth curve obtained in Figure 3, the cell generation number (Table 1), cell growth at exponential phase and cell growth at death phase (Figure 5) were obtained for all treatment. The cell generation number was reduced from 3.91 generations in control to 2.81 generations in bromelain treated cells and 2.86 generations in recombinant bromelain treated cells. Meanwhile, taxol reduced the number of cells generations from 3.91 generations to 3.12. This result is significant when the cell generations number were compared between the three different treatments (taxol, bromelain, and recombinant bromelain ($p<0.05$).

The relationship between growth rate and the volume of cancer cells has been shown to give good approximation of cancer growth model for some types of cancers (Mehrara et al., 2007). Thus, to evaluate the bromelain effect on the specific growth graph, the log of viable cells number vs. time were consulted and the regression slope was taken as μ. The growth rate was observed to decrease significantly from $0.009h^{-1}$ in control to $0.0059h^{-1}$ in bromelain and $0.0063h^{-1}$in recombinant bromelain.

Discussion

This research was undertaken to investigate the prospect of commercial and recombinant bromelain which is more effective, non-toxic and safe as alternative therapeutic to available breast cancer treatment. The IC_{50} value of bromelain and recombinant bromelain were higher than taxol, supportive a milder effect of native-based product compared to highly toxic taxol. Taxol, a common anti-cancer drug has been known to give long term side-effects to the cancer patients due to its ability to kill normal/non-cancerous cells. Besides, there are many

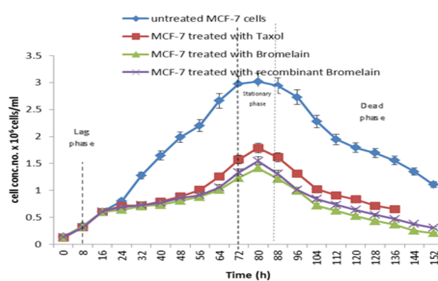

Figure 3. MCF-7 Growth Profiles of Untreated (control) and Treated with Bromelain (IC_{50}=5.125 μg/mL), Recombinant Bromelain (IC_{50}=6. 25 μg/mL) and Taxol (IC_{50}=0.063 μg/mL).

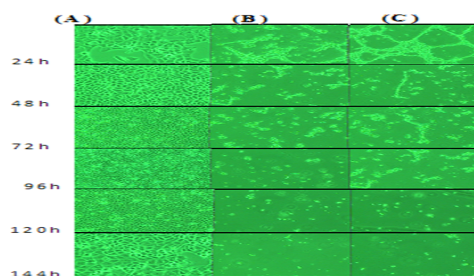

Figure 4. Representative Photographs of: A) Untreated MCF-7 Cells, MCF-7 Cells Treated with B) Bromelain and C) Recombinant Bromelain at Designated Time Point

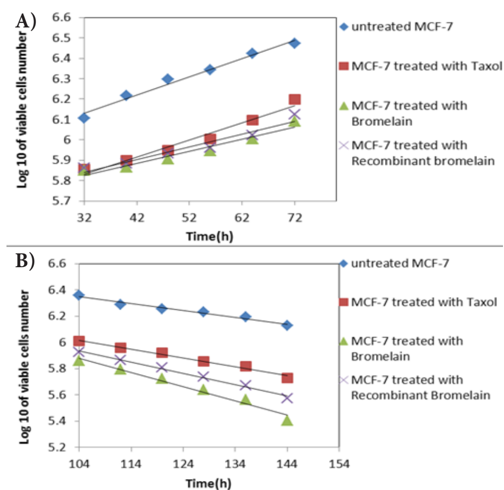

Figure 5. MCF-7 Cells Growth at A) Exponential Phase; B) Death Phase, before and after Treated with Bromelain, Recombinant Bromelain and Taxol

Table 1. Number of Generations (X) for Untreated MCF-7 Cells (control) and MCF-7 Cells Treated with Bromelain, Recombinant Bromelain and Taxol*

Cells growth	Maximum cells number (N)	Cell generations number (X)	p value
Untreated MCF-7 cells	3.05×10^6 cells/mL	3.91	-
MCF-7 cells+Bromelain (IC_{50}=5.125 μg/mL)	1.40×10^6 cells/mL	2.81	0.004
MCF-7 cells+Recombinant bromelain (IC_{50}=6.25 μg/mL)	1.45×10^6 cells/mL	2.86	0.004
MCF-7 cells+Taxol (IC_{50}=0.063 μg/mL)	1.75×10^6 cells/mL	3.12	0.004

*Cells intitial volume (No) was constant at 2×10^5 cells/mL for all cases

100.0
6.3
10.1
20.3
12.8

side effects such as neutropenia, leucopenia, anemia, hair loss, muscle pain or joint pain, nausea and vomiting as well as diarrhea has been documented in many clinical trials (Chik et al., 2010).

In this study, the anti-cancer effects of commercial and recombinant bromelain were investigated on adherent MCF-7 cancer cells. Result suggests that in the adhering cells, the anti-invasive effect of commercial and recombinant bromelain are cumulative of its effects at proteolytic and antimigration levels. Moreover, the representative photographs also showed the reduction of density of cells on the surface of 25 T-flasks at different time after treatment by either commercial or recombinant bromelain. From the previous studies, in the case of the proteolytic activity, protease may chelate the metal ions that required by matrix metalloproteinases (MMPs) to exert their proteolytic activity, thus inhibition the overall invasive (Koblinski et al., 2000). It could be due to inhibition of extracellular matrix proteolysis directly or due to inhibition of activation of a proteolytic cascade indirectly. The main mechanism of bromelain's action appears to be proteolytic in nature, while verification evidence suggests an immunomodulatory and hormone-like activity performing via intracellular signaling pathways (Hale et al., 2005a). Bromelain reduced cell surface receptors, such as hyaluronan receptor CD44 that associated with leukocyte migration and induction of pro-inflammatory mediators (Koblinski et al., 2000; Manhart et al., 2002). In order to understand the cytotoxic and anti-proliferative effects seen, the next phase of study focused on a general kinetic model that would predict the inhibitory effects of commercial and recombinant bromelain on cancer cell growth. From the growth curve, the numbers of generations for treated and untreated MCF-7 cells growth were found to be significantly reduced from control 3.92 generations to 2.81 and 2.86 for bromelain and recombinant bromelain treatment (Table 1). General models for MCF-7 breast cancer cells in response to commercial and recombinant bromelain were developed, expecting that an effective treatment may reduce the cell proliferation rate (cytostatic effect) and boost the cell death rate (cytotoxic effect). Most chemotherapeutic drugs interfere with cell division processes and are thus most successful on growing tumors. They act either by damaging DNA or by interfering with DNA synthesis (O'Reilly et al., 1997). This can be proved when, indeed, the doubling time was found to be expanded from 33.44 hours in control to 51 hours and 47.77 hours for commercial and recombinant bromelain, respectively, indicating cytostatic effect. Meanwhile, taxol had inhibited the MCF-7 cells growth by expanding the doubling time to 35.83. Additionally, other studies have shown that cancer growth rate may decrease with time which grades in non-exponential growth model of cancers (Afenya and Calderon, 2000). While the cytostatic effects were inferred to cells at exponential phase (Figure 5A) while the cytotoxic effects were inferred at death phase (Figure 5B).

According to American Cancer Society (ACS) (2011), a biopsy sample is normally used to achieve certain tests that can help the determination of how speedily a cancer is likely to grow and what treatments are likely to be successful. Thus, this is where the kinetics model with simple formulations and logical precision may help explain the size of a tumor as a function of time. For untreated MCF-7 breast cancer cells growth, the first order linear equation gives:

$$\log_{10}N=\log_{10}N_0+0.009t \qquad (6)$$

By treating the cells with bromelain, the first order linear equation can be changed to:

$$\log_{10}N=\log_{10}N_0+0.005t \qquad (7)$$

Moreover, by treating the cells with recombinant bromelain, the first -order linear equation can be changed to:

$$\log_{10}N=\log_{10}N_0+0.006t \qquad (8)$$

and the first-order linear equation of the MCF-7 cells treated with Taxol can be expressed as:

$$\log_{10}N=\log_{10}N_0+0.008t \qquad (9)$$

However, neglecting the natural growth characteristics of tumors tends to underestimate a treatment's effectiveness (Mehrara et al., 2007). Thus, a kinetics final growth model response to bromelain treatment can be expressed as:

$$\log_{10}N_f=(\log_{10}N_0+0.009t)-(\log_{10}N_0+0.005t) \qquad (10)$$

And the kinetics' final growth model response to recombinant bromelain treatment can be expressed as:

$$\log_{10}N_f=(\log_{10}N_0+0.009t)-(\log_{10}N_0+0.006t) \qquad (11)$$

And the kinetics' final growth model response to taxol treatment can be expressed as:

$$\log_{10}N_f=(\log_{10}N_0+0.009t)-(\log_{10}N_0+0.008t) \qquad (12)$$

Where, $\log_{10}N_f$=the final volume or size of the tumor after treatment, $\log_{10}N_0$=the initial volume or size of a tumor and t=the duration of a treatment (t_f-t_o)

The growth kinetics term (specific growth rate, μ) was then used to achieve growth kinetics model based on equation 4. Such model allows identification of tumor size in early stage (below than 1cc) and in prediction interpolation and extrapolation of sizes at other time. It appears that from the kinetic model constants, bromelain and recombinant bromelain treatment inhibited MCF-7 cancer cell growth.

A biopsy was taken from a breast cancer patient. The tumor size is 2×10^4 cells per centimeter cubic, which is less than 1 cc. Based on equation 6, our data shows the rate for a MCF-7 breast cancer cells to grow is 0.009 h^{-1} (0.201 d^{-1}). If left untreated at least for a week, a primary breast cancer initial 2×10^4 cells per cubic centimeter, could be increase to approximately 6.48×10^5 cells per cubic centimeter. It is typically dangerous if the size continues to arise more than 1 liter (1000cc=1×10^{12} cells per cubic centimeter). However, using kinetics model as described in Equation (10) and (11) for bromelain and recombinant bromelain, respectively, if the tumor being treated by

bromelain at early stage, possibly the size could be reduced to approximately 5.1×10^5 cells per cubic centimeter in a week and 4.45×10^5 cells per cubic centimeter in a week.

Finally, the death rate during the declining phase can be calculated from the same equation 4 and 5. Cell viability number was lowest during the decline or death phase of culture. The measured viable cell concentration decreases as the cells lysed and their intracellular metabolites were released into the growth medium (Gwyther and Schwartz, 2008). If $\mu<0$, then the cell number said to be having exponential reaction (Iwata et al., 2000). The death rate was increased to $0.0108h^{-1}$ for commercial bromelain and $0.0086h^{-1}$ for recombinant bromelain as compared to untreated MCF-7 cells ($0.005h^{-1}$). Meanwhile, taxol gave death rate up to $0.0068h^{-1}$ (Mehrara et al., 2007). Furthermore, Assessment of cancer response to chemotherapeutic drugs is necessary for evaluation of the efficacy of novel anti-cancer drugs in clinical trials (Deasy et al., 2003). Traditional anti-cancer agents exhibit cytocidal effect by actively destroying cancer cells and, therefore, cancer shrinkage has been used as measure of treatment efficacy (Bajzer, 1999).

In conclusion, this research upholds the anti-cancer activity of commercial and recombinant bromelain towards MCF-7 breast cancer cells. It also demonstrated their importance as anti-cancer therapeutic when compared to taxol. As well, it contributes to indicate the effectiveness and the similarity of recombinant bromelain as compared to commercial bromelain among all the results. Future approach will required to validate our hypothesis on bromelain proteolytic effect on extracellular matric and on anti-proliferation.

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