The Effects of the Herbal Enzyme Bromelain Against Breast Cancer Cell Line GI-101A

Honors Thesis

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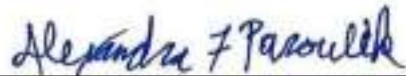

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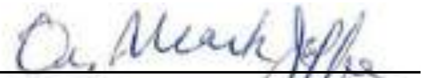

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Abstract

Bromelain is a proteinase derived from the stem of pineapple and has been studied for its anti-inflammatory, antithrombotic, and antimetastatic properties. Bromelain has also been known to significantly reduce local tumor growth and to raise the impaired cytotoxicity of monocytes in the immune system against tumor cells. GI-101A, the cell line used in this experiment, was derived from a xenograft of a 57-year-old female breast cancer patient who had a recurrent ductal adenocarcinoma. The goal of this project was to advance the mechanistic knowledge of herbal remedies and to confirm the already known antimetastatic properties of Bromelain. The MTS assay method was used 24 hours after Bromelain treatment to detect the cell death. The data show that after 1 μM of Bromelain treatment, the population of GI101A cells is significantly reduced. Using the M30-Apoptosense ELISA, levels of the neo-epitope CK18Asp396 from the protein cytokeratin 18 (CK18) were measured to detect any activity of apoptotic cell death. After 10 μg of Bromelain treatment, CK18Asp396 neo-epitope levels increased and a large number of apoptotic cell bodies were observed. The antitumor effects of Bromelain appear to be mainly involved in killing cancer cells by the induction of apoptosis.

Introduction

Breast cancer is the second leading cause of cancer death among women. It is mostly prevalent between the ages of 35-70, with the median age being 61 years old. The mortality rate in the U.S. from 2001-2005, all ethnicities considered, was 126 per 100,000

women per year diagnosed with breast cancer while 25 per 100,000 women per year died from breast cancer (Ries et al, 2008).

Cancer begins as abnormal cell growth in a particular area of the body, which if not caught in time, has the potential of producing tumors and spreading to other parts in the body. In the breasts, it is commonly found in either the ducts or the lobes. Ductal carcinoma begins in the lining of the milk ducts, and either staying in localized (in situ) or metastasizing and spreading to other breast tissue (invasive). Cancer cells that begin growing in the lobes, where milk is produced, may also remain in situ or become invasive (CDC, 2006). Breast cancer can also be influenced by women naturally producing estrogen. This type is known as estrogen receptor-positive breast cancer, and can easily be treated by stopping the production of estrogen in the body. Estrogen receptor-negative breast cancer is more difficult to treat because the cancer cells are not dependent on estrogen and may be fueled by angiogenesis, the formation of new blood vessels to supply growing tumors.

There are many risk factors associated with breast cancer, but the most common ones women are told to look out for include having a family history of breast cancer, a late first pregnancy, early menarche, late menopause, long term use of hormone replacement therapy, and use of oral contraceptives. Women who have had a family history of breast cancer can now be genetically screened for their BRCA1 and BRCA2 genes, which are involved in breast cancer development. Signs of cancer development are lumps in the breast or underarm, nipple discharge of blood, changes in shape or size of breasts, and dimpling of the breast skin (CDC, 2006).

As with most cancers, there is treatment available to help stop the cells from further metastasizing within the body. Current treatments include radiation therapy and chemotherapy. Radiation therapy uses high-energy X-rays that induce double stranded breaks in the DNA of cells, more so those cells undergoing mitosis. The DNA in cancer cells is broken up by the X-rays, making DNA repair impossible and, ultimately, destroying those cells. The skin on the chest and underarms are exposed to a carefully focused beam of radiation, leaving the treated area looking sun burnt. Women undergoing this therapy can be exposed to radiation every day for five days for up to six weeks. The side effects of radiation include fatigue, dry and itchy skin, and possible heart problems. (WebMD, 2007).

Chemotherapy, on the other hand, uses a combination of medicines that target rapidly dividing cells in the body. Combinations that are commonly used are Adriamycin and Cytoxan, or Cytoxan, Methotrexate, and 5-FU. For estrogen receptor-positive breast cancer, Tamoxifen is generally given to stop estrogen production. Side effects generally depend on the medicines taken and vary for each person. Cancer cells are not the only rapidly dividing cells in the body, however. Hair cells, red and white blood cells, and cells that line the digestive tract are also rapidly dividing cells. Losing these cells can cause loss of hair, being more prone to infections, bruising easily, loss of appetite, nausea, diarrhea, and mouth sores. In women, chemotherapy can cause damage to the ovaries, producing menopausal symptoms of hot flashes and vaginal dryness as well as infertility (Erstad, 2007).

Western medicine has vastly developed to treat terminal illnesses and add more years to patients' lives, but not without the risks from side effects that are caused by

strong medicine. From the early years of humans to the present day, herbs have played a major role in the existence of medicine. Though they are presently seen as alternative medicine and dietary supplements, herbs do have some medicinal effects on the body. Common herbs and their uses include: Echinacea for stimulating the immune system, Acacia for malaria and worms, and Licorice for upper respiratory infections (Buhner).

Herbs used in conjunction with chemotherapy can improve the management of stress from side effects and help fight off infections due to the decreased levels of immune efficiency. Curcumin, the active compound in the yellow spice turmeric, and Resveratrol from grapes can inhibit further tumor growth and angiogenesis by blocking the transcription of vascular endothelial growth factor (VEGF). Green tea also has anti-angiogenic effects by inhibiting VEGF and reducing the density of tumor vessels. Panax Ginseng also targets angiogenesis, but can induce tumor cell apoptosis (Sagar, Yance, & Wong, 2006).

Bromelain, a proteinase found in the stem and fruit of pineapple, is another preparation that has been shown to produce anti-tumor effects. Preliminary studies have given some insight into what kinds of properties Bromelain contains that may be able to cease tumor growth and cancer cell reproduction. Bromelain has mainly been studied for its anti-inflammatory, antithrombotic and its antimetastatic properties as well as its aid in digestion. A study conducted by Beuth and Braun found Bromelain to significantly reduce local tumor growth and experimental lung metastases in mice. In a separate study by

Figure 1: Bromelain can be found in both the stem and the fruit of pineapple.

Guimaraes-Ferreira et al., Bromelain inhibited metastasis-associated platelet aggregation and tumor cell invasiveness in the B16F10 murine (mouse) melanoma cell line. Bromelain is also one of a few herbs that can increase eicosanoid production. Eicosanoids are signaling molecules generally derived from omega-3 and omega-6 fats and control bodily systems, most importantly those of inflammation and immunity (Wallace, 2002). In a German study by Eckert et al. using oral Bromelain, the cytotoxicity of monocytes increased in breast cancer patients compared to healthy donors. Increased production of cytokines such as tumor necrosis factor-α (TNF-α) by Bromelain was found by Mynott et al. TNF-α is involved in the body's systematic inflammation response where it can induce inflammation and apoptosis, and inhibit tumor growth.

The breast cancer cell lines used for experimentation, which are few, do reflect the characteristics of cancer cells found in vivo (Lacroix & Leclercg, 2004). The cell lines commonly used are MCF-7, MDA-MB-231, SK-BR-3, and Hs578T. These cell lines originated from tumors taken from breast cancer patients. GI-101A, the cell line used in this study, is a mammary xenograft derived from a recurrent, Stage IIIa ductal carcinoma tumor isolated from a 57-year-old female breast cancer patient (Rathinavelu, 1999). GI-101A is an estrogen receptor-positive tumor cell line that is metastatic to both the lungs and lymph nodes. However, its expression of the pS2 protein makes GI-101A resistant to the anti-estrogen tamoxifen. High levels of pS2 protein correlate with ER+ status, but the estrogen receptor gene that is transcribed in GI-101A must be a variant to make the cell line resistant to anti-estrogen drugs (Morrissey & Raney, 1998). GI-101A cell line also expresses the oncogene MDM2, which promotes tumor growth by

inactivating p53, the tumor suppressor protein found in most cells that can lead to the induction of apoptosis (Zell et al., 2002).

In the present work, the anticancer effects of Bromelain were tested against GI-101A to determine whether Bromelain has the potential of being used as a therapeutic agent against cancer. The specific aims of this study were to observe Bromelain's anticancer effects, thereby, confirming data from earlier studies, and secondly, by completing this project it was expected to advance my knowledge of research methodology.

Experimental Section

Proteinase

Pure, freeze-dried Bromelain powder was purchased from Sigma Chemical Co. (St. Louis, MO) and was freshly prepared as a 10 μg/ml solution in PBS in each experimental trial. Bromelain is best stored at -20°C.

Figure 2: Preparation of Bromelain from powdered ingredient to liquid solution.

Cells and Cell Culture

GI-101A cell line was provided by the Rumbaugh-Goodwin Institute for Cancer Research (Plantation, FL). Cells grew to 60-80% confluency as a monolayer in 25 cm^2 flasks in RPMI growth medium containing 10% fetal bovine serum, 1% anti-bacterial/anti-microbial (Penicillin and Streptomycin) and 0.05% Progesterone/Estrogen.

The cells were incubated in 5% CO atmosphere at 37°C, and weekly sub-cultured using 0.05% trypsin-EDTA.

Trypan Blue Exclusion Test

This test uses trypan blue dye to determine the number of viable cells in a cell suspension. The cell membranes of living cells are selective in what they allow entering the cell and so can block out certain dyes, while dead cells have degraded cell membranes which will take up the dye. The cell suspension mixed with the trypan blue dye is loaded onto a hemocytometer, a thick glass microscope slide that contains an indented chamber with a grid of perpendicular lines. Live cells will be seen as having a clear, white cytoplasm while dead cells will appear to have a blue cytoplasm. The total number of cells per milliliter in the suspension is calculated with the formula

$$\text{viable cells (\%)} = \frac{\text{total number of viable cells per ml of aliquot}}{\text{total number of cells per ml of aliquot}} \times 100$$

where the total number of viable cells is multiplied by two to account for the dilution factor of trypan blue.

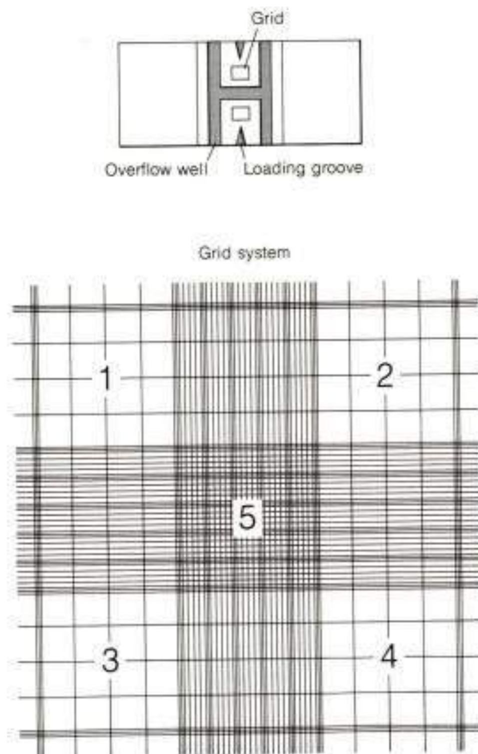


Figure 3: The hemocytometer and its grid lines used to determine the total number of cells in a cell suspension.

MTS Assay for Live Cells in Cytotoxicity Experiments

GI-101A cells were harvested from the tissue culture flasks by using 0.05% trypsin-EDTA, washed and resuspended in growth medium, counted using the trypan blue exclusion test, and diluted to 100,000 cells/mL. From that dilution, 100 µl containing 10,000 cells were plated into the wells of a 96-well microplate, with the first column containing media alone and incubated for 24 hours. The next day, 100 µl of Bromelain dilution was added in concentrations of 0.1 µg, 1 µg, 10 µg, and 100 µg, so that the plate setup was of wells with media alone, control with cells alone, and the five Bromelain concentrations. After the second incubation period of 24 hours, 20 µl of MTS assay reagent was added to all wells with a 4 hour incubation period. The absorbance in each well was read at 490nm with a microplate reader.

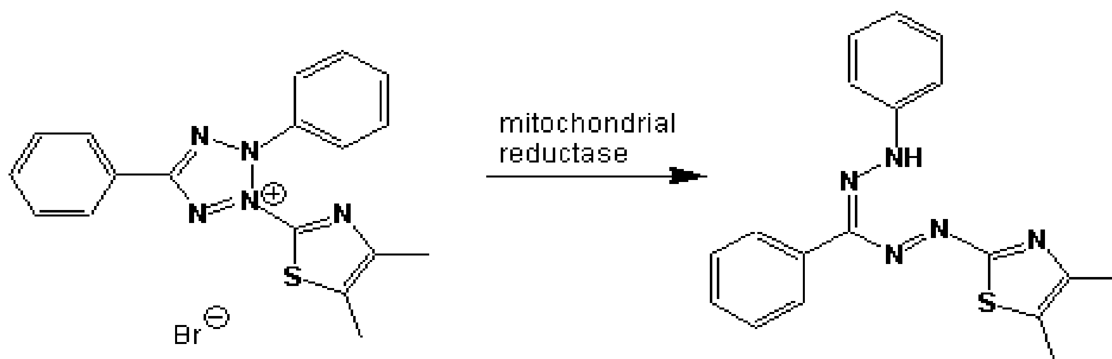
M30-Apoptosense ELISA

GI-101A cells were seeded into 6-well plates with the plate setup of two controls, two with 10 μg Bromelain concentrations, and two with 20 μg Bromelain concentrations. After 48 hours of incubation, cells were placed into the microplate with wells coated with mouse monoclonal CK18 antibody M5. Diluted M30 HRP Conjugate solution (M30 antibody and horseradish peroxidase in phosphate buffer with protein stabilizers) was added to each well. The wells were covered with sealing tape in incubated on a shaker for four hours. They were then washed and added with TMB Substrate (3,3',5,5'-Tetramethylbenzidine) and incubated in darkness for 20 minutes. Stop solution (1.0 M sulfuric acid) was added and left for five minutes. Absorbance was read at 450nm in a microplate reader.

Results and Discussion

MTS Assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl) -2-(4-sulfophenyl)-2H-tetrazolium) assay is a colorimetric method to determine the viability of cells in the presence of a cytotoxic test substance. MTS reagent contains this tetrazolium compound and an electron coupling reagent phenazine methosulfate (PMS), which combines with MTS to produce a stable solution. In living cells, MTS is reduced to a water-soluble formazan product that has an absorbance maximum at 490-500 nm in phosphate-buffered saline. These reductions take place only when reductase enzymes are active, since cells rapidly lose their ability to reduce tetrazolium products at death.



Therefore, the production of formazan is directly proportional to the number of live cells in culture. The maximum absorbance of formazan product is easily measured using a micro-well plate reader or a colorimeter. This assay is one of the most reliable assays for measuring cell viability and cell proliferation.

The control wells had the darkest color out of all the other wells, since the cells were still viable and able to reduce the reagent into formazan due to the presence of high levels of active reductase enzymes. When Bromelain was added to the cells and incubated overnight, dead cells could be observed aggregated together and floating in the medium. Figure 6 shows that with the increases in Bromelain concentration, the absorbance readings, due to the color in the wells, decreased with less viable cells present. The wells with lighter colors showed that Bromelain was reducing formazan production by killing a majority portion of the cells. Counting the cells using the trypan blue dye exclusion test gave a more accurate number to the findings, as shown in Figure 5. At 0.1 µg of Bromelain concentration, a large decrease of living cells was found and more so with higher Bromelain concentrations. It was apparent that Bromelain had strong and direct effects on the GI-101A cells in order to prevent further cell growth and induce death and

Figure 4: Introduction of the MTS tetrazole reagent into the cell samples will initiate the reaction of enzymes in live cells to reduce the reagent into formazan. From the amount of formazan produced, absorbance readings can suggest the number of live cells still present in a sample.

aggregation of the existing cells even in small concentrations. However, the pathway in which Bromelain was able to destroy these cells was still not determined.

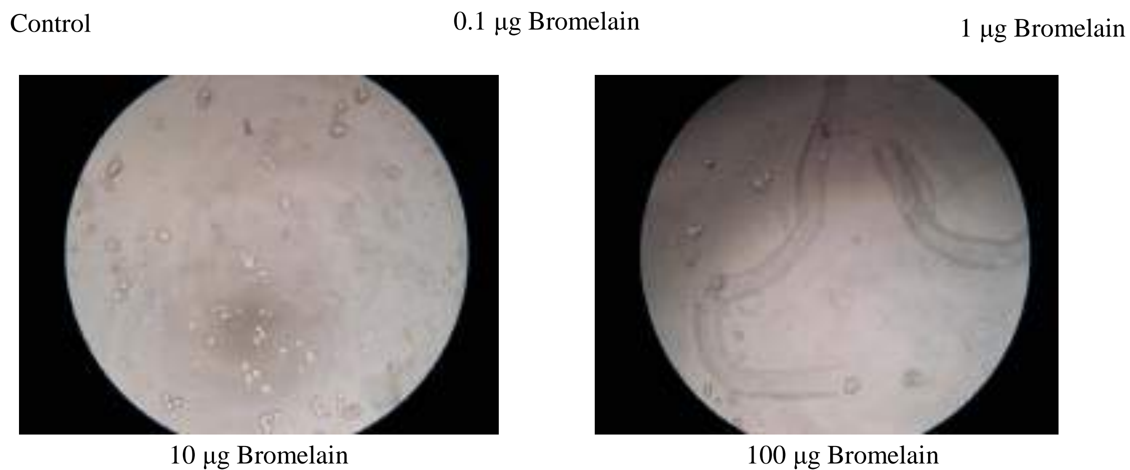


Figure 5
Microscopic view of GI-101A cells in the microplate wells after treatment.

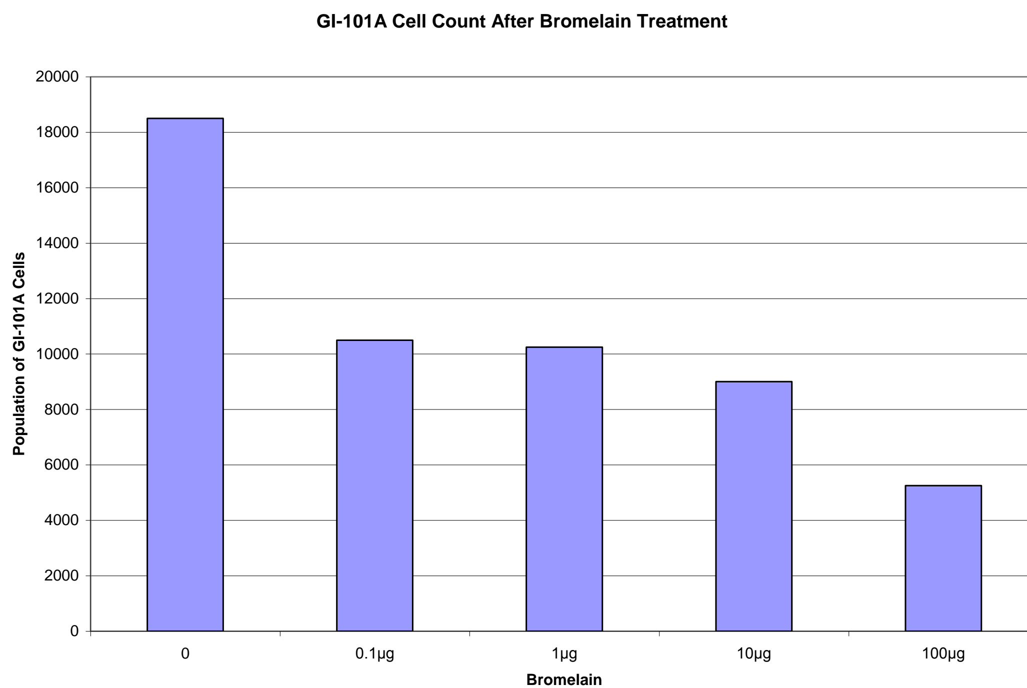


Figure 6

Cell count using the trypan blue exclusion test. Those cells that are alive will have their membrane in tact and will appear as white dots, while the membranes of dead cells will allow the blue dye to penetrate and make the cells appear as blue dots.

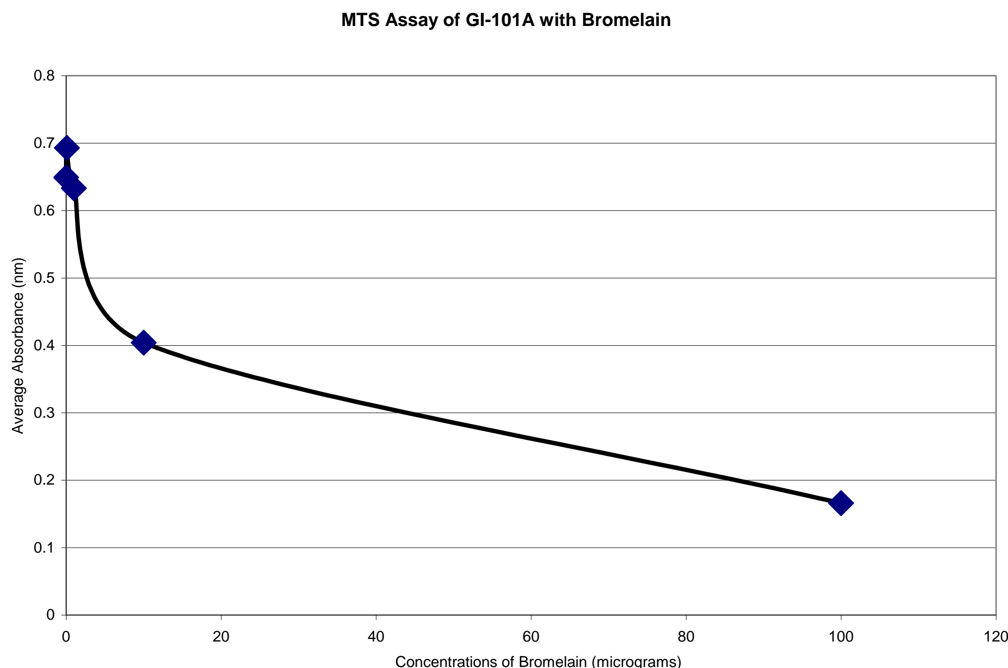


Figure 7
The recorded average absorbance from MTS assay. MTS assay is colorimetric, where the darker the color of the wells means high amounts of live cells and the lighter the color means little to almost no live cells.

M30-Apoptosense ELISA

The M30-Apoptosense ELISA helped determine in what ways Bromelain was affecting the cells. Apoptosense ELISA measures the levels of fragments of the intermediate filament protein cytokeratin 18 (CK 18) containing the CK18Asp396 neo-epitope. The neo-epitope levels increase when apoptosis is largely induced among the cells due to the activation of caspases. Expression of caspases 3 and 9 is important in executing apoptosis, which Bromelain was found to increase in the study by Kalra et al. and in the Apoptosense ELISA. In this immunoassay, the higher absorbance readings indicate high neo-epitope levels, which suggest that the majority of the cells have undergone apoptosis. For cells to become apoptotic, their p53 gene must be active for the

cell to initiate their own death. Because this gene is shut off in cancer cells, the cells continue to grow and metastasize with no direction of when to stop. GI-101A posses mutant p53 protein, and therefore has high VEGF levels and tumor angiogenesis. Bromelain must have direct contact with the cell's apoptotic pathway in order to induce cancer cells to turn on that defected pathway. From Figure 8, the 10 μg and 20 μg concentrations of Bromelain have high absorbance readings signifying high levels of CK 18 neo-epitope present, meaning more apoptotic bodies than live cells. Even though this first experiment using the Apoptosense ELISA was successful, repeated trials were not and the same results could not be obtained. A reason for this could have been that the assay reagents were exposed to light when taken out for use and deactivated. TMB substrate and M30 HRP Conjugate solution are especially sensitive to light. TMB substrate cannot be used after exposure to light, and the M30 HRP Conjugate is stable for only two weeks refrigerated at 2-8°C in the dark. Apoptosense experiments were conducted once a week using the same kit, which may have resulted in deactivating the reagents.

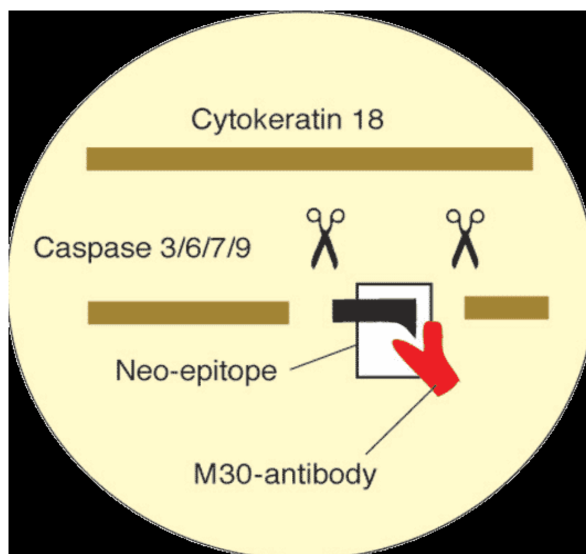


Figure 8: Caspases cleave CK18 into fragments, containing the neo-epitope that is recognized by the M30 antibody.

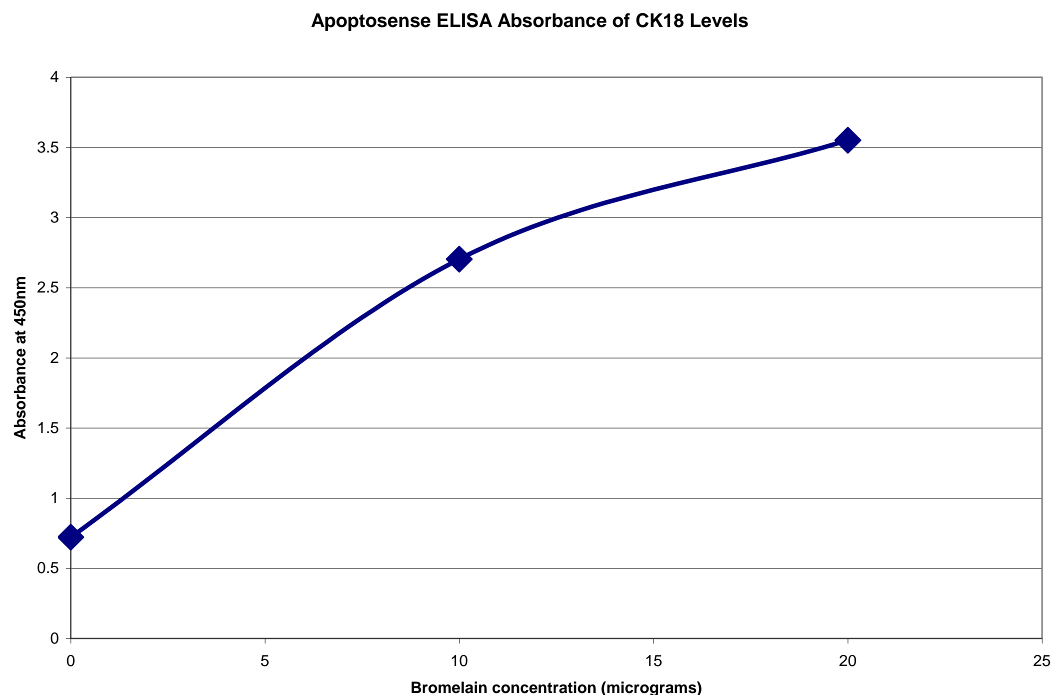


Figure 9
Absorbance of CK18 levels in the control and Bromelain samples. High absorbance indicates high CK18 in the sample which are released from apoptotic cells.

Statistical Analysis

Statistical analyses of the cell count after treatment and the absorbance readings from both MTS and Apoptosense assays were performed to compare the control with the increasing concentrations of Bromelain using one-way analysis of variance (ANOVA). For the cell count, the statistical significance for the decreasing amount of cells from the Bromelain treatment was $p < 0.05$. Increasing the Bromelain concentration, even though the only units used were micrograms (µg), had a considerable effect of reducing the GI-101A population compared to its growth in the control. The MTS absorbance readings also had a statistical significance for the decreasing absorbance readings due to higher

concentrations of Bromelain, along with the Apoptosense ELISA with a statistical significance for the increasing absorbance readings in direct proportion to the increasing Bromelain concentrations. The significance values for both assays were $p < 0.05$. The significance of Bromelain was on destroying most of the cells to not allow them to reduce MTS and for inducing apoptosis in existing cells, respectively.

Conclusion

Results have confirmed that Bromelain is very effective phytochemically towards combating breast cancer cells. In its role as a proteinase it can directly target the rapidly dividing cancer cell and interfere with cell division by turning on the apoptotic pathway.

Since our experiments have shown that Bromelain has apparent anticancer effects in the *in vitro* systems, *in vivo* studies should be conducted in order to test Bromelain's effects in the body. It appears that small concentrations in the range of 01. µg to 100 µg of Bromelain were enough to kill cancer cells in RPMI 1640 medium, but the abundance of enzymes, defense mechanisms, and the digestive tract within the body may pose a challenging environment for medicines like Bromelain to reach its target at low concentrations. Because pineapple does not contain high enough doses of Bromelain to have a medicinal effect, Bromelain must be extracted and given in high amounts in order to be effective in the body. The recommended dosage by the German Commission E is 80-320 mg two to three times per day, or higher for other specific conditions (Ehrlich, 2007). In the clinical study by Eckert et al., oral Bromelain was administered to patients for 10 days with a daily dose of 3000 F.I.P. units, a measurement of enzyme activity determined by the International Pharmaceutical Federation. Bromelain did increase the cytotoxicity of monocytes in breast cancer patients, but what route it takes in the body

and how it directly affects cancer cells amidst the body's enzyme rich environment is not well known. Therefore, further i*n vivo* experiments for Bromelain must take into account the dosages in proportion to body size, the pathway that might be taken if Bromelain is given orally or intravenously, and any adverse effects it may create aside from allergies to pineapple.

Acknowledgements

I would like to thank Dr. Appu Rathinavelu and Dr. Mark Jaffe for their guidance and support in this study, the Royal Dames of Fort Lauderdale and the staff at Rumbaugh-Goodwin Institute for Cancer Research for allowing me to use the labs to conduct my experiments, to Dr. Matthew He, Dean Don Rosenblum, the Honors program and Farquhar College for granting me the opportunity to conduct this research during my undergraduate career at NSU.

Appendix

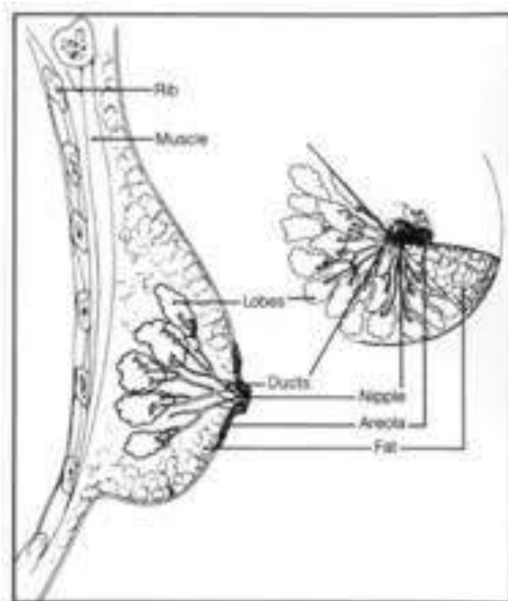


Figure 10
Source: Centers for Disease Control and Prevention. Diagram showing where cancer can develop in the lobes and ducts of the breast.

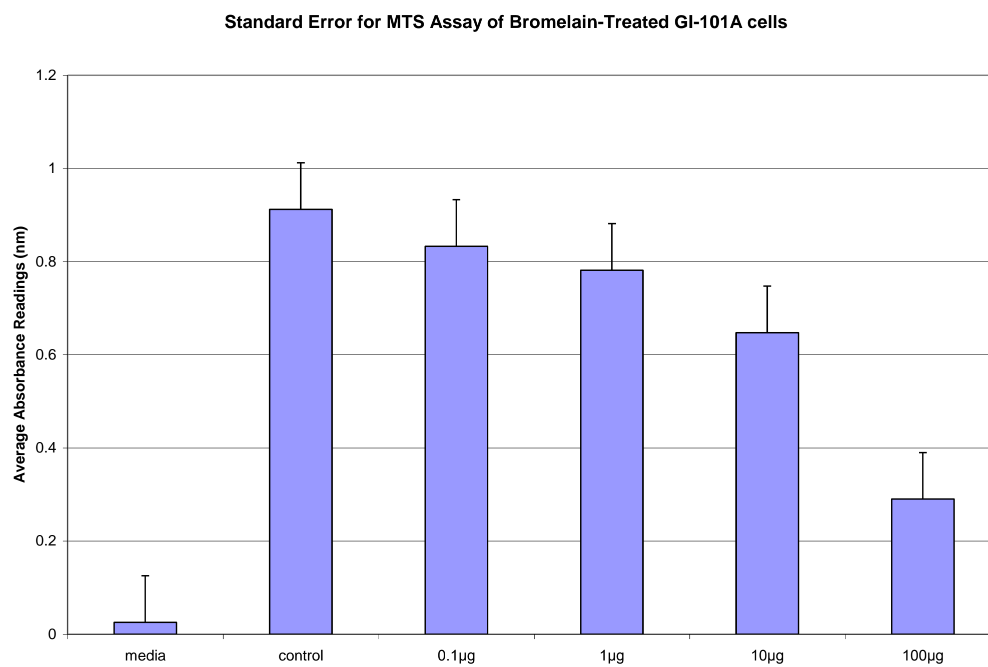


Figure 11
Standard error for average absorbance for MTS assay.

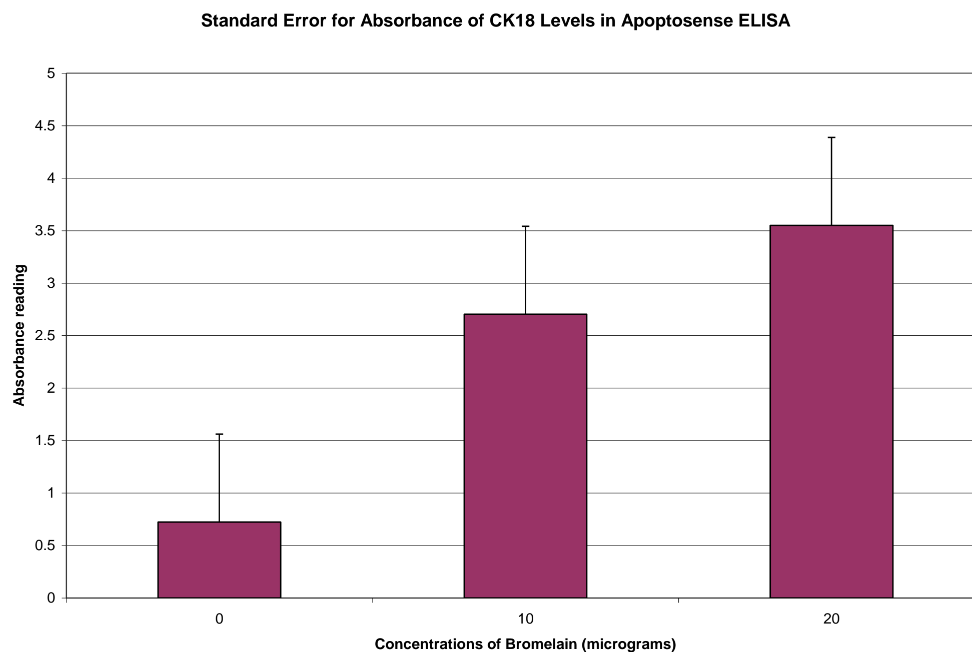


Figure 12
Standard error for absorbance of CK18 levels.

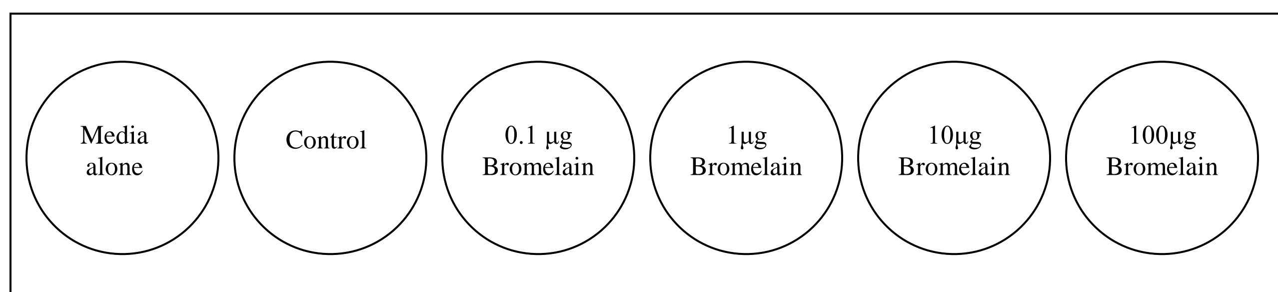


Figure 13
Microplate setup for MTS assay procedure.

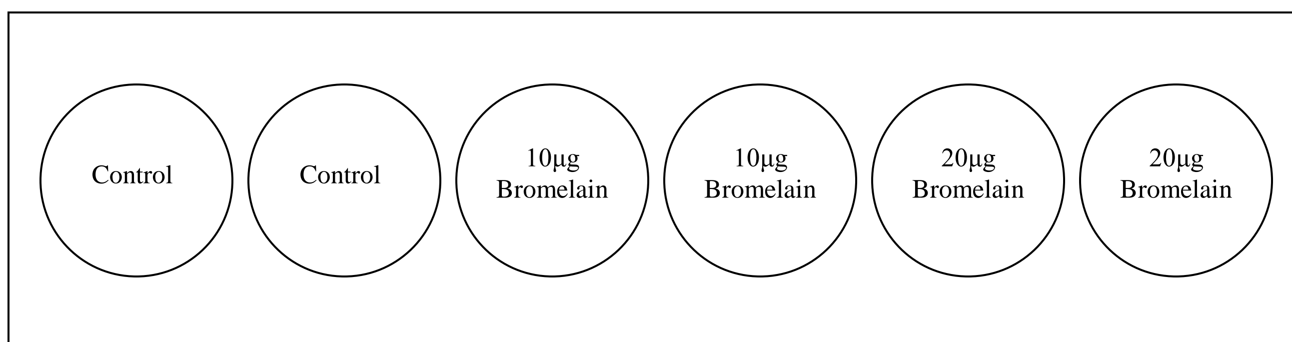


Figure 14
Microplate setup for M30-Apoptosense ELISA procedure.

Table 1: ANOVA: GI-101A Cell Count

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.85E+08	1	2.85E+08	24.34069	0.001144	5.317655
Within Groups	93682632	8	11710329			
Total	3.79E+08	9				

Table 2: ANOVA: MTS Absorbance Readings

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.556405	4	1.139101	7.858876	0.000018	2.472927
Within Groups	13.04501	90	0.144945			
Total	17.60142	94				

Table 3: ANOVA: M30-Apoptosense ELISA Absorbance Readings

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.203728	2	4.101864	69.72896	0.003056	9.552094
Within Groups	0.176478	3	0.058826			
Total	8.380206	5				

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