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Contributed Paper

In vitro Anti-cancer Activity Comparison of the Freeze-dried and Spray-dried Bromelain from Pineapple Stems

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ABSTRACT

Bromelain is one of the proteolytic enzymes found in pineapple stems and fruits. It has been investigated for medical uses including anti-cancer activity. The objective of this study was to compare the *in vitro* anti-cancer activities of the freeze-dried and spray-dried bromelain from pineapple stems. Bromelain was precipitated from the pineapple stem juice by 95%v/v ethanol and then freeze-dried or spray-dried to get the dry powder. Both bromelain samples were investigated for anti-cancer activities including anti-proliferative by sulforhodamine B (SRB) assay and apoptotic induction by acridine orange (AO) and ethidium bromide (EB) double staining on seven human cancer cell lines. The highest anti-proliferation of the freeze-dried and the spray-dried bromelain was observed on A549 with the IC_{50} values of 18.31±5.11 and 26.36±9.76 μg/ml, respectively. For apoptotic induction, the highest activity of the freeze-dried and the spray-dried bromelain was observed on KB (7.53±3.51%) and A549 (6.29±0.28%) cell lines, respectively. The freeze-dried and the spray-dried stem bromelain exhibited higher immunostimulating activity of the bromelain samples performed in murine neutrophils using nitroblue tetrazolium (NBT) dye reduction test than the negative control of 4.37 and 4.40 times, respectively. This study has demonstrated not only the no significant different effect of the drying processes on the anti-cancer activities of the stem bromelain in seven cancer cell lines, but also the potential of the stem bromelain to be developed as an active compound for lung cancer because of its highest anti-proliferation and apoptosis induction in A549.

Keywords: bromelain, anti-proliferation, apoptosis, immunostimulation, drying process

1. INTRODUCTION

During processing, the crown and stem of pineapple (*Ananas comosus*) are cut off before peeling. The core is removed for further processing. The wastes including peel, core, stem, crown and leaves are generally account for 50% (w/w) of the total pineapple weight. Utilization of these wastes as sources of bioactive compounds, especially proteolytic enzymes, is an interesting value added alternative mean. Parts of pineapples such as peel, core, crown and leaves have been previously studied for bromelain extraction. Bromelain which is present in these parts of pineapple wastes is relatively smaller quantities than that in the stem [1, 2]. This study has extracted bromelain from the stem of pineapple waste because of its high quantity enzyme and it is a very cheap source as waste by-product. Bromelain from the stem of pineapples is a highly accepted phytotherapeutic compound which has anti-inflammatory, anti-tumor, anti-metastatic properties [3, 4], potent immunogenicity [5] and wound healing in burns [6]. In fact, the evidence of anti-cancer activity of bromelain was traditionally observed in Southeast Asia. Bromelain has been indicated the capacity to modify the key pathways that support malignancy. The *in vivo* evidence is consistent in demonstrating the tumor-inhibitory effects of bromelain. In chemically-induced mouse skin papillomas, topical application of bromelain reduced tumor formation, tumor volume and caused apoptotic cell death [7]. Báez et al. have demonstrated that bromelain exhibits antitumoral activity on various murine tumor models including P-388 leukemia, sarcoma (S-37), Ehrlich ascitic tumor (EAT), Lewis lung carcinoma (LLC) and ADC-755 mammary adenocarcinoma. Moreover, stem bromelain significantly reduced the number of lung metastasis induced by LLC transplantation [8]. Bromelain has also been reported to promote apoptosis, particularly in breast cancer cells, with the up-regulation of c-Jun N-terminal kinase and p38 kinase [9]. Bromelain is found to increase the expression of p53 and Bax in mouse skin, the well-known activators of apoptosis [8]. Anti-cancer activity of bromelain is due to its direct impact on cancer cells and their microenvironments, as well as on the modulation of immune, inflammatory, and haemostatic systems. Bromelain enhanced T cell receptor (TCR) and anti-CD28-mediated T cell proliferation in splenocyte cultures by increasing the costimulatory activity of accessory cell populations [10]. Bromelain can increase IFN-γ-dependent TNFα, IL-1β, and IL-6 production by human PBMC and enhance antigen-independent binding of T cells to monocytes *in vitro* [11]. Bromelain can enhance the acquired immune responses by activating T cells and induce the production of inflammatory cytokines. Bromelain also activates various innate immune cells including macrophages, dendritic cells and natural killer cells [12]. Eckert et al. have demonstrated that orally applied bromelain with a daily dose of 3000 F.I.P. units for 10 days stimulated the deficient monocytic cytotoxicity of mammary tumor patients [13]. However, only few anti-cancer studies of stem bromelain on human cancer cells have been reported.

Although freeze drying is less attractive than spray drying due to the high energy consumption and long processing time, it is recognized as the best method to produce high-quality dried food products because of the protection of various heat labile bioactive compounds including protease enzymes. As known, the limitations of spray drying are the efficient particle collection and the potential instability of materials sensitive to high temperatures. No work has been done on the comparison of anti-cancer activity of

bromelain prepared from the freeze drying and spray drying processes. This study has aimed to compare the anti-cancer activities including anti-proliferation and apoptotic induction activity in various human cancer cell lines as well as immunostimulating activity of the stem bromelain prepared by the freeze drying and spray drying processes.

2. MATERIALS AND METHODS

2.1 Materials

The stem pineapple was obtained from Welltech Biotechnology Co., Ltd., Bangkok in Thailand. The voucher specimens of the plant samples were authenticated by a botanist at Natural Products Research and Development Center (NPRDC), Science and Technology Research Institute (STRI), Chiang Mai University, Chiang Mai, Thailand. Acridine orange (AO), ethidium bromide (EB), sulforhodamine B (SRB), lipopolysaccharide (LPS) from *Escherichia coli* and EDTA were purchased from Sigma (St. Louis, MO, USA). Niroblue tetrazolium (NBT) dye and basic fuchsin were from Biobasic Inc., NY, USA. Polymorphprep™ was from Axis-shield, Oslo in Norway. Trypsin was prepared at 0.25% solution in phosphate buffered saline. Completed DMEM medium was prepared from Dulbecco's Modified Eagle Medium (GIBCO, Invitrogen 95 Corporation, NY, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). All other chemicals and reagents were of analytical grade.

2.2 Preparation of the Stem Bromelain

The stem pineapple samples were cut into small pieces and crushed with a blender. The crushed mass was pressed and which was filtered through a cloth to remove fibrous materials. The filtrate was centrifuged (Hettich: Universal 32 R, UK) at 10,000 × g for 10 min to remove insoluble materials. 1%w/v L-cysteine was added into the clear supernatant and the pH was adjusted to 4.3 [14]. The stem bromelain was seperated by adding 95%v/v ethanol with constant stirring [15]. The stirring was continued for 30 min to allow the equilibration between the dissolved and the aggregated protein. Then, the precipitated enzymes were collected by centrifugation at 10,000 ×g for 15 min and then divided into two portions to get the dry powder by freeze drying and spray drying. Maltodextrin at 5%w/w of the wet precipitate was used as a carrier for the spray drying process [16]. The aseptic condition during the drying process was performed by spraying 70% ethanol on all equipments, machines and operator's hands to reduce the risk of bacterial contamination. The protease activity of bromelain was determined according to the casein digestion unit (CDU) method [17].

2.3 Cell Cultures

The human colon adenocarcinoma (HT-29) cell lines were provided from Medicinal Microbiology Department, Faculty of Biology, University of Tuebingen, Tuebingen in Germany. Human lung adenocarcinoma epithelial (A549) and human duodenum adenocarcinoma (HuTu-80) cell lines were from College of Science and Technology, Nihon University, Tokyo in Japan. Hepatocellular carcinoma (HepG2) was obtained from Faculty of Tropical Medicine, Mahidol University in Thailand. Human prostate cancer (DU145), human mouth epidermal carcinoma (KB) and human cervical cancer (HeLa) cell lines were purchased from American Type Culture (ATCC). HT-29, HuTu-80, HepG2 and HeLa cell lines were cultured in DMEM medium, while A549 and DU145 were cultured in RPMI medium. DMEM and RPMI medium were supplemented with 10%(v/v) fetal bovine

serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). All cells were maintained in a humidified 5% CO_2 incubator (Contherm mitre 4000, Contherm Scientific, Hutt city, New Zealand) at 37°C. The cells were trypsinized and counted with a haemacytometer.

2.3.1 Anti-proliferative activity by the sulforhodamine B assay

Cells (10^4 cells/well) were plated in 96-well plates and incubated overnight in a humidified 5% CO_2 incubator at 37°C. An amount of 20 μl of the bromelain samples and the four standard anti-cancer drugs (cisplatin, doxorubicin, fluorouracil and vincristine) at 0.1-1000 μg/ml were added to the cells. After 24 h of incubation, the cells were fixed with 50% trichloroacetic acid solution, incubated at 4°C for 1 h and washed with distilled water. Excess water was drained off and the plates were air-dried for 24 h. The cells were stained with 50 μl of 0.4% SRB solution in 1% acetic acid for 30 min at room temperature (27±2 °C). After incubation, the SRB solution was poured off and the plates were washed with 1% acetic acid. The plates were air-dried and 100 μl of 10 mM Tris-base solution were added to each well to solubilize the dye and shaked for 30 min at room temperature (27±2 °C). The absorbance at 540 nm was determined by the microplate reader (Bio-Rad, model 680 microplate reader, USA). All experiments were performed in triplicate. The percentages of cell growth (%G) were determined using the following equation: % Cell growth (%G) = (A-C/B-C) × 100, where A was the optical density of the extracts, B was the optical density of the control and C was the optical density at time zero. The IC_{50} values were determined by plotting the percentages of cell growth (%G) versus the concentrations of the samples. Extracts which exhibited potential anti-proliferative activity were selected for the apoptotic test.

2.3.2 Apoptotic assay by acridine orange (AO) and ethidium bromide (EB) double staining

The three final concentrations (1000, 100 and 10 μg/ml) of the bromelain samples and the standard anti-cancer drugs (cisplatin, doxorubicin, fluorouracil and vincristine) at 100 μg/ml were used. Cells without the extract were served as the negative control. An amount of 10 μl of the extracts and the standard anti-cancer drugs at the above concentrations were added to the wells and incubated for 24 h. After that, 10 μl of the AO/EB dye mix (100 μg/ml of AO and 100 μg/ml of EB in PBS) were added to each well. The apoptotic, necrotic and live cells were observed and counted under the fluorescent microscope (Olympus CK40/U-RFLT 50, Olympus, Japan). All experiments were repeated for 3 times and at least 100 cells of each experiment were counted [18].

2.4 *In vitro* Immunostimulating Activity

2.4.1 Neutrophil separation

Murine neutrophils were separate from blood obtained from male Sprague Dawley (SD) rats, weighing between 250 and 300 g from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom in Thailand by a cardiac puncture under deep anesthesia condition. All collected blood samples were immediately transferred to the tubes containing EDTA (final concentration 1-2%) as an anticoagulant. Neutrophils were obtained from Polymorphprep™ centrifugation. Briefly, 5 ml of the blood samples with EDTA were carefully laid over 5 ml of Polymorphprep™ in a 15 ml centrifuge tube and centrifuged at 500×g for 30-35 min. Then, the band of the

polymorphonuclear (PMN) leukocytes was harvested and mixed with an equal volume of the half-strength saline solution to return to an iso-osmotic condition to the PMN suspension. The cell suspension was centrifuged at 400×g for 10 min and resuspended in the phosphate buffer saline (PBS).

2.4.2 Nitroblue tetrazolium (NBT) dye reduction test

The suspension of leukocytes (5×10^6/ml) was prepared in 0.2 ml PBS solution in 1.5 ml microcentrifuge tubes. An amount of the bromelain samples dissolved in PBS at 10 mg/ml at 0.1 ml was added to the leukocyte suspension. Serum containing LPS at the final concentration of 20 μg/ml and the PBS solution were used as the positive and negative control, respectively. An amount of 0.2 ml of the freshly prepared 0.15% NBT solution was added to each tube before incubation at 37°C for 20 min. After incubation, the leukocytes were harvested by gently centrifugation at 400 ×g for 3-4 min. An amount of 40-50 μl of PBS was added to resuspend the cells. One drop of the cell suspension was put on a microscope slide, dried, fixed with methanol and counter stained with the diluted carbol-fuschin (0.3%) for 15 sec. The slides were washed with tap water, dried and observed under the microscope (Olympus Optical Co. Ltd, Tokyo, Japan) using an oil immersion objective. Cells were scored as positive when the cells ingested the particles which were stained with blue-black by the precipitated formazan (the oxygen dependent reduction product of NBT). At least 300 cells were counted for each determination [19].

3. Results and Discussion

3.1 Characteristics of the Stem Bromelain

Although the purification of the stem bromelain by the traditional precipitation [15] gave the crude enzyme with the contamination of other proteases, it was the easy and low cost method. The prepared freeze-dried and spray-dried stem bromelain from this study was in light yellow and pale brown dry powder, respectively. The brown pigments may be from the amino-carbonyl reactions of the enzyme [20]. The spray drying process has higher dynamics, in which the temperature, pressure and particle size may change the color of the final product. The percentages of the solid content of the freeze-dried and spray-dried stem bromelain were 44.2 and 47.7%w/w of the wet precipitate, respectively. No yield difference was detected between the bromelain samples from the two drying techniques. However, the stem bromelain from the freeze drying (2,980.00±508.39 CDU/mg of the extract) gave higher enzymatic activity than that from the spray drying process (2,262.69±887.78 CDU/mg of the extract) which were the same as the study of Devakate et al. They have showed that the enzymatic activities of the freeze dried and spray dried protein were observed to be 482.3 and 462.5 casein digesting units (CDU)/mg, respectively. These different enzyme activities may be from the different parts used of the pineapple as well as the different bromelain precipitation and drying methods [21]. The lower enzymatic activity stem bromelain from the spray drying than the freeze drying may be due to the denaturation with structural changes under thermal stress of the process. Cabral et al. investigated effects of the drying parameters on the retention of the enzymatic activity and the physical properties of the spray-dried pineapple stem extract. They concluded that high processing temperatures yielded the bromelain product with smaller moisture content, particle size and lower agglomerating

tendency. Moreover, the product with insignificant losses of the proteolytic activity (≈ 10%) and low moisture content (less than 6.5%) has been reported to be produced at the selected spray drying conditions [22].

3.2 Anti-proliferative Activity of the Stem Bromelain

The anti-proliferative activity (IC_{50} values) of the stem bromelain and the four standard anti-cancer drugs on seven human cancer cell lines were shown in Table 1. The stem bromelain exhibited anti-proliferative activities on HepG2, HT-29, A549, KB, HeLa and HuTu-80 cell lines, except DU145. In addition, the two drying methods gave different potentials on the cancer cell lines. The highest inhibition of the freeze-dried and the spray-dried stem bromelain was observed on A549 with the IC_{50} values of 18.31±5.11 and 26.36±9.76 μg/ml, respectively which were considered as moderately active according to the National Cancer Institute (NCI). However, both bromelain from the two drying processes showed lower activity than the four standard anti-cancer drugs on all human cancer cell lines. A previous study has reported that incubation of sarcoma L-1 cells with bromelain significantly reduced their tumorigenic/metastatic capacities. Intraperitoneal or subcutaneous administration of bromelain significantly ($p<0.05$) reduced the local tumor weight, however, lung colonization was non-significantly reduced [23]. The anti-proliferative activity of the freeze dried stem bromelain on A549, KB and HeLa cell lines was higher than that from the spray-dried stem bromelain of 1.44, 2.18 and 2.86 times, respectively. While, the anti-proliferative activity of the spray-dried stem bromelain on HT-29 and HuTu-80 was higher than that from the freeze dried stem bromelain of 1.13 and 1.68 times, respectively. Although the enzyme activity of the spray dried bromelain is lower than that of the freeze dried bromelain, the cell anti-proliferation of the spray dried bromelain showed better activity. This agreed with the study of Romano et al. They have found that the anti-proliferative activity of bromelain was not related to its proteolytic activity, since proteolytically inactive bromelain also exerted anti-proliferative activity [24]. Infact, the anti-cancer activities were not only from the enzymatic effect of bromelain, but also from other several mechanisms such as antioxidative effects from the phenolic compounds [25] which are the impurity of the crude bromelain extract. Although bromelain is primarily comprised of sulfhydryl-containing proteolytic enzymes, it also contains escharase, peroxidase, acid phosphatase, glucosidases, cellulases, protease inhibitors, glycoproteins, carbohydrates and the organically bound calcium [26]. Some of these constituents are heat-labile compounds which may be destroyed in the spray drying process. However, several compounds which can inhibit cancer cell proliferation have been reported to be heat stable such as flavonoids, phenolic compounds, namely quercetin, flavones-3-ol, flavones and ferulic acid [27]. In addition, the anti-cancer activities were not only from the enzymatic effect of bromelain, but also from several other mechanisms such as antioxidative effect. Hsu et al. have reported that the cell proliferative inhibition of the phenolic acids was well correlated to their antioxidant activity [28]. A previous study has reported that quercetin showed growth inhibition on A549 human lung cancer cells [29]. Amini et al. have demonstrated that bromelain inhibited the growth and proliferation of human gastrointestinal carcinoma cell lines including HT29-5F12, HT29-5M21, MKN45 and KATO-III at the IC_{50} values of 29, 34, 94, and 142 μg/ml, respectively [30]. This study showed the

anti-proliferative activity of bromelain prepared by the freeze drying and spray drying on HT-29 with the IC_{50} values of 91.76±70.1 and 103.45±109.05 μg/ml, respectively. This difference may be from the different strain of the cancer cell lines. The mechanism of bromelain against these cancers is not fully known. Nevertheless, it has been suggested that the proteolytic enzyme of bromelain may kill the cancer cells by breaking down the fibrin coat of the cancer cell wall and the protein into amino acids [31]. Bromelain exhibited the reduction in proliferation of human epidermoid carcinoma-A431 and melanoma-A375 cells and suppressed their potential for the anchorage-independent growth. Bromelain caused depletion of the intracellular glutathione and generation of reactive oxygen-species followed by mitochondrial membrane depolarization. This led to bromelain-induced cell-cycle arrest at G(2)/M phase which was mediated by modulation of cyclin B1, phospho-cdc25C, Plk1, phospho-cdc2, and myt1. This was subsequently followed by the induction of apoptosis [32].

Table 1. Comparison of anti-proliferative activity of the stem bromelain prepared by the two different drying processes on seven human cancer cell lines.

Samples / Cancer cell line	IC_{50} (μg/ml)						
	DU145	HepG2	HT-29	A549	KB	HeLa	HuTu-80
Freeze dried stem bromelain	ND	111.16± 55.65	103.45± 109.05	18.31± 5.11	126.78± 48.57	56.44± 22.16	147.27± 62.78
Spray dried stem bromelain	ND	112.45± 36.22	91.76± 70.11	26.36± 9.76	276.81± 83.17	161.51± 37.24	87.67± 57.33
Standard anti-cancer drugs							
Cisplatin	ND	1.73± 0.32	1.94± 0.29	8.61± 0.50	2.16± 0.31	ND 7.59±	62.51± 0.49
Doxorubicin	1.37± 0.58	0.39± 1.23	8.21± 0.72	0.83± 0.019	0.21± 0.73	0.39	2.22± 0.37
Vincristine	ND	0.22± 0.97	2.11± 2.88	0.36± 0.10	0.28± 0.99	ND	0.37± 0.11
Fluorouracil	5.00± 0.77	3.31± 2.27	0.12± 0.05	13.12± 3.00	5.38± 2.34	178.12± 0.34	106.58± 0.03

Note : ND = not dectactable, DU145 = human prostate cancer cell lines, HepG2 = hepatocellular carcinoma cell lines, HT-29 = human colon adenocarcinoma cell lines, A549 = human lung adenocarcinoma epithelial cell lines, KB = human mouth epidermal carcinoma cell lines, HeLa = human cervical cancer cell lines, HuTu-80 = human duodenum adenocarcinoma cell lines.

3.3 Apoptotic Induction Activity of the Stem Bromelain

In fact, defects in apoptosis are common phenomena in many types of cancer and are also the critical step in tumorigenesis and resistance to therapy. Thus, the assay of the apoptosis is an important target to evaluate the cancer treatment. The percentages of apoptotic cells induced by the stem bromelain and the standard anti-cancer drugs on seven human cancer cell lines were shown in Table 2. The stem bromelain exhibited apoptotic induction activity on DU145, HT-29, A549, KB, HeLa and HuTu-80 cell lines, except HepG2. Both bromelain from the two different drying processes indicated a concentration-dependent manner of apoptotic induction activity in all cancer cell

lines, except the HeLa cell line. The highest activity of the freeze-dried and the spray-dried stem bromelain was observed on KB (7.53±3.51%) and A549 (6.29±0.28%) cell lines, respectively. This different response may be due to the different responses of different cell lines by the bromelain extracts even at the same conditions. In fact, it has been reported that the same agent may induce apoptosis in one cell line, but not in the other [33]. The spray-dried stem bromelain can inhibit most of the cancer cell lines higher than the freeze dried stem bromelain. As known, the crude stem bromelain contains a mixture of different thiol endopeptidases and other components such as phosphatases, glucosidase, peroxidases, cellulases, glycoproteins, carbohydrates, and several protease inhibitors [34]. Thus, the heat-stable compounds in the bromelain from the spray drying process may be responsible for the apoptotic induction in these cancer cells. However, both stem bromelain from the two drying processes gave lower apoptotic induction activity than all standard anti-cancer drug (100 µg/ml) on all cancer cell lines. The anti-cancer mechanisms of the stem bromelain may be different from those of the standard anti-cancer drugs. Several studies have reported the mechanism of bromelain against apoptosis cancer cell by the sub-G1 cell fraction with the apoptotic features like chromatin condensation and nuclear cleavage [35]. Bromelain has also been reported to promote cancer cell apoptosis via caspase system as well as the extranuclear p53 and decrease the cancer cell survival by inhibiting the Akt pathway and modulating various oncoproteins [30]. In addition, bromelain has been indicated to induce up-regulation of p53 and Bax and subsequent activation of caspase-3 and caspase-9 with a decrease in Bcl-2 expression [4]. Furthermore, bromelain may induce the apoptosis-related proteins along with the inhibition of -driven COX-2 expression by blocking the MAPK and Akt/protein kinase B signaling in DMBA-TPA-induced mouse skin tumors [7]. However, in this study, the freeze-dried and the spray-dried stem bromelain showed necrotic cells on KB, but not in other cancer cell lines of 6.73±2.07 and 7.31±2.26%, respectively which was an undesirable effect. This present work has demonstrated that the stem bromelain especially by the spray drying process seemed to be potential for lung cancer treatment since it gave high apoptotic induction with no necrosis.

Table 2. Comparison of the apoptotic cell percentages induced by the stem bromelain prepared by the two different drying processes and the standard anti-cancer drugs on seven human cancer cell lines.

Cancer cell lines	Samples	Concentrations (µg/ml)		
		1,000	100	10
DU145	Bro-FD	2.87±0.29	3.14±0.77	3.20±2.71
	Bro-SD	4.01±2.40	4.11±2.41	3.38±1.00
	doxorubicin	-	25.60±5.29	-
HepG2	Bro-FD	0	0	0
	Bro-SD	0	0	0
	doxorubicin	-	13.73±1.59	-

Table 2. Continued.

Cancer cell lines	Samples	Concentrations (µg/ml)		
		1,000	100	10
HT-29	Bro-FD	4.36±1.22	0	0
	Bro-SD	4.01±1.78	0	0
	doxorubicin	-	21.84±4.71	-
A549	Bro-FD	4.88±1.59	4.10±2.83	3.06±1.01
	Bro-SD	6.29±0.28	5.37±2.46	3.04±2.70
	cisplatin	-	34.92±2.81	-
KB	Bro-FD	7.53±3.51	5.28±1.72	0
	Bro-SD	5.39±2.18	5.07±1.00	0
	cisplatin	-	88.65±4.91	-
HeLa	Bro-FD	0.82±0.87	0.59±1.02	1.19±1.05
	Bro-SD	1.83±0.82	1.09±1.06	1.45±0.74
	doxorubicin	-	95.91±1.53	-
HuTu-80	Bro-FD	2.61±1.02	1.33±0.68	1.22±0.45
	Bro-SD	3.31±1.26	1.76±1.46	0.52±0.91
	cisplatin	-	53.83±3.95	-

Note : Bro-FD = freeze dried stem bromelain, Bro-SD = spray dried stem bromelain

As known, anti-cancer drugs have a narrow therapeutic index and the dosage needed to achieve a therapeutic response is usually toxic to the body's rapidly proliferating cells. Normal tissues adversely affected by these drugs including the bone marrow, gastrointestinal tract and hair follicles. Some anti-cancer drugs such as methotrexate, cisplatin, vincristine and 5-fluorouracil have organ specific toxicities. But, bromelain has very low toxicity and is safe even in high doses. The oral toxicity (LD_{50}) of bromelain has been reported to be more than 10 g/kg in rat and more than 12 g/gay in human [36]. Thus, although the activity of bromelain seemed to be lower than the anti-cancer drugs, bromelain has low toxicity and is safe even in high doses in comparing to the anti-cancer drugs.

3.4 Immunostimulating Activity of the Stem Bromelain

The anti-cancer activity of bromelain consists in the direct impact on cancer cells and their microenvironments, as well as in the modulation of immune, inflammatory and haemostatic systems. Immunostimulating activity of the stem bromelain by the NBT test was shown in Table 3. The freeze-dried and the spray-dried stem bromelain gave the percentage of the NBT positive cells at 16.2±1.3 and 16.3±2.1% which were higher than the negative control of 4.37 and 4.40 times, respectively. Hence, the stem bromelain from different drying processes appeared not give any significant immunostimulatory activity difference. The intracellular reduction of nitroblue tetrazolium dye to formazan by the neutrophils has confirmed the intracellular killing and the preservation of the integrity of the neutrophils. The reduction of NBT to the insoluble blue formazan was used as a probe for superoxide generation, although it is not entirely specific for the O_2^- radical [37]. The NBT reduction assay estimated the ability of the neutrophils and macrophages to produce oxygen radicals (O_2^-, OH^-, O_3, H_2O_2).

The ability of macrophages to kill the pathogenic microbes is probably one of the most important mechanisms for the protection against diseases [38]. Bromelain has been shown to simultaneously enhance and inhibit the immune cell responses *in vitro* and *in vivo*. It has various immunomodulatory effects on leukocytes and can enhance IFN-γ-derived signals in both primary macrophages and macrophage cell lines. Bromelain also increased IL-2- and IL-12-mediated IFN-γ production by NK cells [12]. In human macrophages/monocytes and mixed lymphocyte culture, bromelain induced a significant increase in interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFNγ). Bromelain also enhanced IFNγ mediated TNF-α and NO production by murine macrophages [11]. Brakebusch et al. have analysed the bromelain effect on granulocyte and macrophage function in patients with a disorder of the humoral immunosystem X-linked agammaglobulinemia. They found an acceleration of phagocytosis, respiratory burst and killing in blood sample using *Candida albicans* as a target organism [39]. Zavadova et al. suggested that bromelain (as part of the multi-enzyme preparation Wobenzym) increases neutrophil activity, based on a study using healthy volunteers taking bromelain orally [40].

Table 3. Immunostimulating activity comparison of the stem bromelain prepared by the two different drying processes by the NBT test.

Samples	% NBT positive cells	Ratio to the negative control
Freeze dried stem bromelain	16.2±1.3	4.37
Spray dried stem bromelain	16.3±2.1	4.40
Positive control 20 μg/ml LPS in serum	9.0±1.0	2.43
Negative control (PBS)	3.7±0.3	-

4. Conclusion

This study has compared the *in vitro* anti-cancer activities of the freeze-dried and the spray-dried stem bromelain on seven human cancer cell lines. The percentage of the solid content of the freeze-dried and the spray-dried stem bromelain were 44.2 and 47.7%w/w of the wet precipitate, respectively. The two different drying methods gave different anti-cancer potential of bromelain on seven cancer cell lines. The highest anti-proliferation of the freeze-dried and the spray-dried stem bromelain was observed on A549 with the IC_{50} values of 18.31±5.11 and 26.36±9.76 μg/ml, respectively. For apoptotic induction, the highest activity of the freeze-dried and the spray-dried stem bromelain was observed on KB (7.53±3.51%) and A549 (6.29±0.28%) cell lines, respectively. However, it was unexpectedly found that the freeze-dried and the spray-dried stem bromelain exhibited necrotic cells on KB of 6.73±2.07 and 7.31±2.26%, respectively. There was no significant different immunostimulating activity of the stem bromelain prepared by the two different drying processes. This study has suggested that the stem bromelain from the spray drying process seems to be potential for lung cancer treatment because of its high anti-proliferative and apoptosis induction with no necrosis.

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