

# Soybean peptide fractions inhibit human blood, breast and prostate cancer cell proliferation

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**Abstract** In this study, we examined in vitro the bio-activity of peptide fractions obtained from soybeans against blood (CCRF-CEM and Kasumi-3), breast (MCF-7), and prostate (PC-3) cancer cell proliferation. Gastro-intestinal treated peptide fractions (<5, 5–10 and 10–50 kDa) prepared from seed proteins of two high oleic acid soybean lines—N98-4445A, S03-543CR and one high protein line—R95-1705, were tested for anticancer activity against human breast, blood and prostate cancer cell lines. Anti-proliferative cell titer assay was conducted to assess the inhibitory effects of the peptide fractions, while trypan blue dye exclusion assay was used to determine the dose response of most effective fractions. Results showed that the peptide fractions inhibited the cancer cell lines up to 68.0% and the minimum concentration to get 50% inhibitory activity ( $IC_{50}$ ) ranged between 608 and 678  $\mu\text{g}/\text{mL}$ . This multiple site in vitro cancer inhibition by GI friendly peptides could have the potential use as food ingredients or nutritional supplements in an alternative cancer therapy.

**Keywords** Bioactive peptides · Cytotoxicity · MTT assay · Trypan blue dye exclusion assay · Cancer cell lines

## Introduction

Cancer is now recognized as one of among the leading causes of death worldwide. The World Health Organization estimates that there were approximately 14 million new cases of cancer and 8.2 million cancer related deaths in 2012. According to the American Cancer Society cancer is the second most common cause of death in the United States with around 589,430 deaths in 2015 (ACS 2015). Conventional ways of cancer treatment includes surgery, chemotherapy and radiation therapy which are all expensive with various adverse side-effects and complications. The use of alternative methods in treating diseases like cancer has been on the rise in recent years. Advanced cancer treatment methods employ a holistic approach by incorporating dietary or nutritional, spiritual and physical therapies along with drugs and radiation. As part of nutritional supplementation, bioactive peptides have a prominent role in alternative medicine which involves controlling diseases using food derived macro molecules (Zhang et al. 1998; Korhonen and Pihlanto 2003; Kannan et al. 2008). Certain other bioactive molecules are from natural sources like plants (Ng et al. 2003; Ohba et al. 2004; Silva-Sánchez et al. 2008). One of the major approaches to produce bioactive peptides was enzymatic hydrolysis or fermentation, especially from sources like soybean, wheat, corn, rice, sunflower, milk, eggs, and meat (Gibbs et al. 2004; Wang and de Mejia 2005; de Mejia and de Lumen 2006; Kannan et al. 2008, 2009, 2010; Hettiarachchy 2013).

Although it is used as a low cost animal feed, the potential nutritive nature, especially of the protein obtained from soybean meal that is the by-product of soy oil industries is well documented (Gallagher et al. 2004). With studies conferring bioactivities to proteins, we found it

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necessary to test soybean protein hydrolysates for reducing the risk of cancer disease on cell culture models. Soybean meal has  $\geq 45\%$  protein with possible unique amino acid types and sequence that could render bioactive nature by mitigating apoptosis in cancer cells (Hernandez-Ledesma et al. 2009; Hsieh et al. 2010). It is also of significant importance for the bioactive peptides to possess resistance to gastrointestinal environment when ingested.

Soy has several value-added uses and has proven to be a source of peptides derived by enzymatic hydrolysis that have shown nutraceutical and functional properties (Rayaprolu et al. 2013, 2015). However, studies are lacking on the effect of peptides from high oleic acid lines on cancers like blood cancer (leukemia), breast cancer and prostate cancer, which are estimated to cause a total of more than 92,000 deaths in the United States in 2015 (ACS 2015). This is the first study that utilized high oleic acid soy meal as a starting raw material to produce peptides with anti-proliferation activity against breast, blood and prostate cancers that can have a significant impact in their treatment. Our previous studies have shown activities against colon, liver and lung cancer cells (Rayaprolu et al. 2013). The present study is in continuation to evaluate anti-proliferative activity against blood (CCRF-CEM, Kasumi-3), breast (MCF-7) and prostate (PC-3) cancer cell lines. Utilization of a low cost soybean meal to produce effective peptides against proliferation of cancer cells is the significant achievement of this research. The objectives of this research were to select two high oleic acid and one high protein soybean lines to prepare peptides, prepare protein hydrolysates with enzyme alcalase under optimum conditions, treat peptides with simulated gastro-intestinal juices, fractionate the peptides using ultrafiltration and to investigate the anti-proliferation activity of GI resistant fractions on the human blood, breast and prostate cancer cell lines.

## Materials and methods

### Materials

Seeds from three soybean lines, S03-543CR and N98-4445A (high in oleic acid) and R95-1705 (high in protein, non-transgenic) were obtained from Dept of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR, USA. The gastro-intestinal enzymes pepsin and pancreatin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ultrafiltration system was purchased from Koch membrane systems (Wilmington, MA, USA). Cell lines and media were obtained from ATCC (Manassas, VA, USA) and Fetal Bovine Serum was purchased from Atlanta Biologicals (Atlanta, GA, USA). The reagents for cell proliferation inhibition were purchased from Sigma-

Aldrich (St. Louis, MO, USA) and Promega Inc. (Madison, WI, USA). The cells were observed for morphological changes using a Vistavision inverted phase contrast microscope (VWR, West Chester, PA, USA). All other chemicals were obtained from VWR (West Chester, PA, USA).

### Preparation of varying molecular size peptide fractions from soybean meal

Seeds from three soybean lines, S03-543CR and N98-4445A (high in oleic acid) and R95-1705 (high in protein, non-transgenic) were obtained from Dept of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR, USA. The soybean seeds were ground to flour and sieved (250  $\mu\text{m}$ ), lipid was removed with *N*-hexane to prepare the soybean meal. The meals were suspended in water (10% w/v) and protein isolates were prepared as described previously (Rayaprolu et al. 2013). The protein isolates were treated with the enzyme Alcalase (Novozyme Inc., Bagsvaerd, Denmark) at optimal conditions (pH 7.0, 1.675 AU of Alcalase, 55 °C for 45 min) to a 30% degree of hydrolysis using the central composite design of the response surface statistical method (Diniz and Martin 1996; Rayaprolu et al. 2013). The enzymatic protein hydrolysates were then treated with simulated gastric (pepsin at pH 2.0) and intestinal (pancreatin at pH 8.0) juices and fractionated using a membrane ultrafiltration system (Romicon, Koch membrane systems, Wilmington, MA, USA). Use of nominal molecular weight cut-offs (MWCO) of 5, 10, and 50 kDa sequential ultrafiltration columns yielded the peptide fractions <5, 5–10 and 10–50 kDa from the three soybean lines, which were tested for inhibitory activity against human blood (CCRF-CEM and Kasumi-3), breast (MCF-7) and prostate (PC-3) cancer cell lines.

### Propagation of human cancer cell lines

Human prostate cancer cell line PC-3 (ATCC# CRL-1435) was cultured in F-12K medium, the blood cancer cell lines Kasumi-3 (ATCC# CCL-2725) and CCRF-CEM (ATCC# CCL-119) were cultured in RPMI-1640 medium, and the breast (epithelial) cancer cell line MCF7 (ATCC# HTB-22) was cultured in Eagle's Minimum Essential Medium. The peripheral blood cells (ATCC# PCS-800-010) were cultured in the recommended Hank's Balanced Salt Solution and used for toxicity tests. All the media were devoid of antibiotics and supplemented with 10% fetal bovine serum. The cells were incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide. The growth phase of all cancer cells was monitored using an inverted phase contrast microscope. Upon achieving 80% cell growth,

they were treated with the peptide fractions, <5, 5–10 and 10–50 kDa, from the three soybean lines. The anti-proliferation assay was performed to determine the anticancer activity.

### Determination of anti-proliferative activity of the peptide fractions

A [3-(4,5-dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTT)-based cell titer assay was used to determine the effect of soybean peptide fractions on the cytotoxicity against cancer cells. The blood cancer cell lines, CCRF-CEM and Kasumi-3, were allowed to grow in suspension (medium) since they do not attach to the well surface. Fresh growth media was added by separating the cells using centrifugation (1000g for 5 min). The pellet was rinsed and re-suspended in fresh media before transferring into the 96-well plate for 36 h incubation at 37 °C and 5% CO<sub>2</sub>. The prostate (PC-3) and breast (MCF-7) cancer cells were cultured in the 25 cm<sup>3</sup>-flask with the growth media to form a monolayer on the flask surface. The cells were checked for conformation (trypan blue dye exclusion test) for minimum counts of ≥3000. They were detached from the flask surface by adding trypsin–EDTA and re-suspended in respective fresh media. The four types of cancer cells were transferred to separate 96-well plates (200 µL/well) for a 36 h incubation at 37 °C and 5% CO<sub>2</sub>. The nine soybean peptide fractions dissolved in water (800 µg/mL) were added to the wells with the cancer cells. A column of wells were treated with saline (negative control) while genistein (200 µg/mL) was used as positive control for comparison of growth inhibition. The cancer cells were incubated for 18–36 h while being observed for cell death due to the test compounds. The MTT dye was added to determine cell survival and termination was observed by the formation of colored formazan product. The 96-well plate was read at 490 nm in a microplate reader (Bio-Rad Inc., Hercules, CA, USA) and the percent inhibition was determined based on the absorbance value (Rayaprolu et al. 2013).

### Dose response evaluation of the peptide fractions

The cancer cell growth inhibition based on dosage of GI resistant soy peptide fractions was established based on the trypan blue dye exclusion assay. This was conducted for the GI-treated peptide fractions that showed highest cytotoxicity against the four cancer cells tested. For trypan blue staining, 500 µL of cells from each cancer cell culture were aseptically transferred to a 24 well plate and incubated for 72 h. The peptide fractions with concentrations ranging from 200 to 1000 µg/mL were added to the wells along with media and incubated for 48–60 h at 37 °C and 5%

CO<sub>2</sub>. The wells were treated with 0.4% (w/v) trypan blue solution prepared in 0.81% NaCl and 0.06% (w/v) dibasic potassium phosphate. Cell counts were determined using a dual-chamber hemocytometer and a phase contrast microscope. Numbers of viable cells were recorded based on the stain retention.

### Statistical analysis

All tests were conducted in triplicate to minimize experimental error and were repeated for reproducibility. The JMP 9.0 statistical software (SAS institute, Cary, NC, USA) was used for all data analyses with analysis of variance (ANOVA) at a significant level of 5% (statistically different if  $P < 0.05$ ). The Tukey HSD was used to compare means for post hoc test.

## Results and discussion

### Protein hydrolysate preparation

Protein isolates prepared from the meal (defatted flour) of the R95-1705, S03-543CR and N98-4445A soybean lines had a purity of 90% or higher which reinforced the alkali extraction procedure for preparing isolates with high protein (Rayaprolu et al. 2013). Alcalase, a food grade enzyme, was used to produce protein hydrolysates from the isolates. The degree of hydrolysis (30%) was used as the response variable in the modelling of enzymatic hydrolysis and varying molecular sized protein fragments were collected (Nielsen et al. 2001). This was in congruence with the objective of the study which is to determine the bioactivities of peptides with various lengths and amino acid sequences. The simulated GI treatment would produce the peptides that are resistant to digestion in the GI tract and will remain bioactive without undergoing any degradation when consumed. The protein content of the GI-treated fractions from the three soybean lines ranged between 86 and 89% purity using a HPLC analysis (a linear gradient of 0.1% trifluoro acetic acid in 10 mM phosphate buffer at pH 7.3 and 0.1% trifluoro acetic acid in 50% acetonitrile in water for 90 min run through a C18 protein separation column).

### Anti-proliferative activity of GI-treated peptide fractions against cancer cell lines

Previous studies have shown that protein products are effective against various cancers (Bylund et al. 2000; Badger et al. 2005). All the four cancer cell types had counts ranging from 3000 to 5000 during the growth phase in the culture flasks. The morphological confirmation of the

blood, prostate and breast cancer cells during initial proliferation showed no shrinkage or nuclear blebbing, which was essential for the experiment.

The results showed that 68% of CCRF-CEM blood cancer cells were inhibited by the 10-50 kDa fraction from the N98-4445A soybean line (Fig. 1) which was the highest among all fractions tested. The 5–10 kDa fraction from the same soybean line showed 52% cytotoxicity which was significantly different from the 5–10 kDa fraction of S03-543CR (41% inhibition). On the Kasumi-3 blood cancer cell line, <5 kDa (52%) and 10–50 kDa (49%) fractions from the N98-4445A soybean line and 5–10 kDa (55%) fraction from S03-543CR, inhibited the cell growth with no statistically significant difference ( $P > 0.05$ ) amongst them (Fig. 2). However, the 5–10 kDa of S03-543CR had the highest inhibition among all the fractions. The 5–10 kDa fractions from S03-543CR and R95-1705 soybean lines showed 41% (CCRF-CEM) and 37% (Kasumi-3) inhibition, respectively, which was the only other significant reduction observed during the MTT assay on the two blood cancer cell lines (Figs. 1, 2).

The 5–10 kDa fraction from S03-543CR soybean line showed 63% inhibition against the PC-3 prostate cancer cells, while the 10–50 kDa fraction from N98-4445A soybean line inhibited 58% of the cells (Fig. 3). Similar sized fraction (5–10 kDa) obtained from R95-1705 soybean line also showed 38% inhibition of the PC-3 cells but this reduction was found to be significantly lower. The other fractions that were tested demonstrated less than 22% or no activity against the prostate cancer cells. This is the first study that revealed the efficiency of GI resistant fractions from a seed protein in inhibiting in vitro prostate cancer cell proliferation.

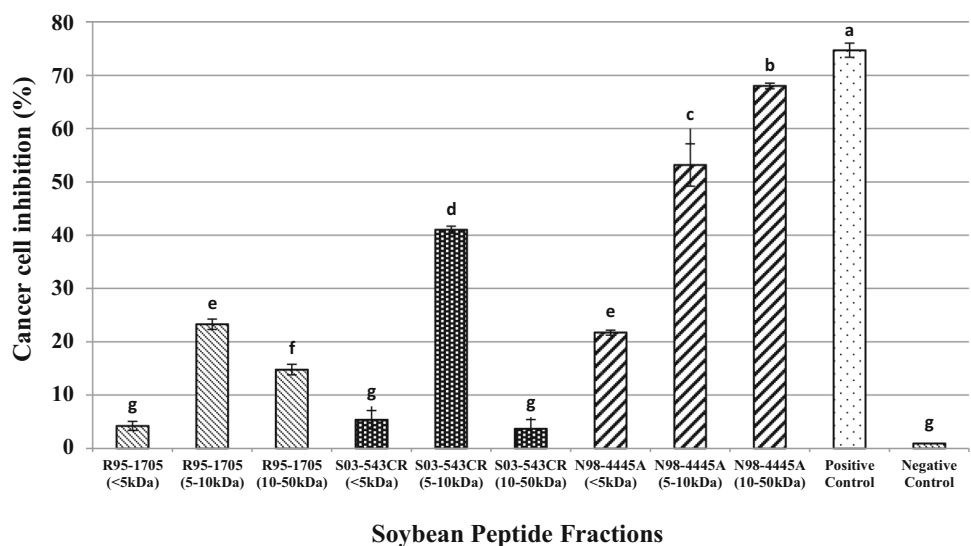
The MCF-7 breast cancer cells were used as a model system for testing anti proliferative activity and

biochemical pathways by various researchers (Doyle et al. 1998; Kannan et al. 2009; Chen et al. 2012; Li et al. 2014a). The 5–10 kDa fraction from the R95-1705 soybean line showed a significantly high activity among all the fractions with 63% inhibition of MCF-7 cells (Fig. 4). This was not statistically different ( $P = 0.8941$ ) from the positive control. This result has a major impact on future studies involving agents that inhibit proliferation of cancers from plant sources since R95-1705 is a non-transgenic (non-GMO) soybean line. The <5 and 10–50 kDa from the R95-1705 soy line also inhibited the MCF-7 cells and the activity was significant higher in comparison to the other fractions. The three fractions from the S03-543CR high oleic acid line showed extremely poor activity against the breast cancer cells while the fractions from N98-4445A soybean line showed 18–30% inhibition of the MCF-7 cells.

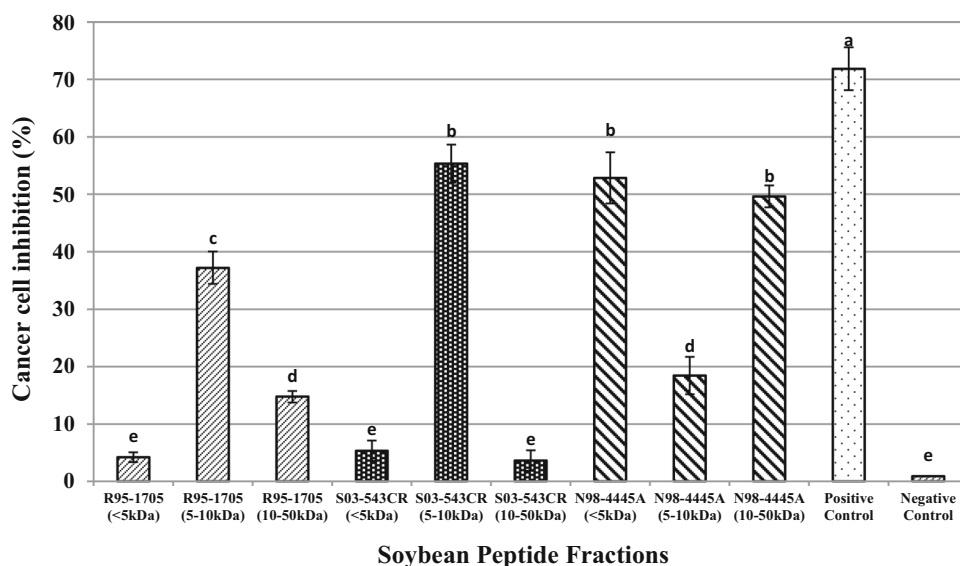
**Dose response study**

The trypan blue dye exclusion test has been used for numerically quantifying the cell counts. This test provided the cell counts against the dose response of the specific fractions that showed highest inhibitory activity based on MTT colorimetric method. The established cell counts based on trypan blue assay provided a quantitative scrutiny of the effectiveness of peptide fractions against the cancer cells. The results from the assay correspond to the cell survival/viability percentage calculated based on the counts in comparison to the known positive control, genistein (Fig. 5). Among the fractions, the 5–10 kDa fraction from S03-543CR soybean line showed the best growth inhibition of both prostate and Kasumi-3 blood cancer cell lines. The dose response showed significant increase in activity at 600 µg/mL or higher which were observed on PC-3,

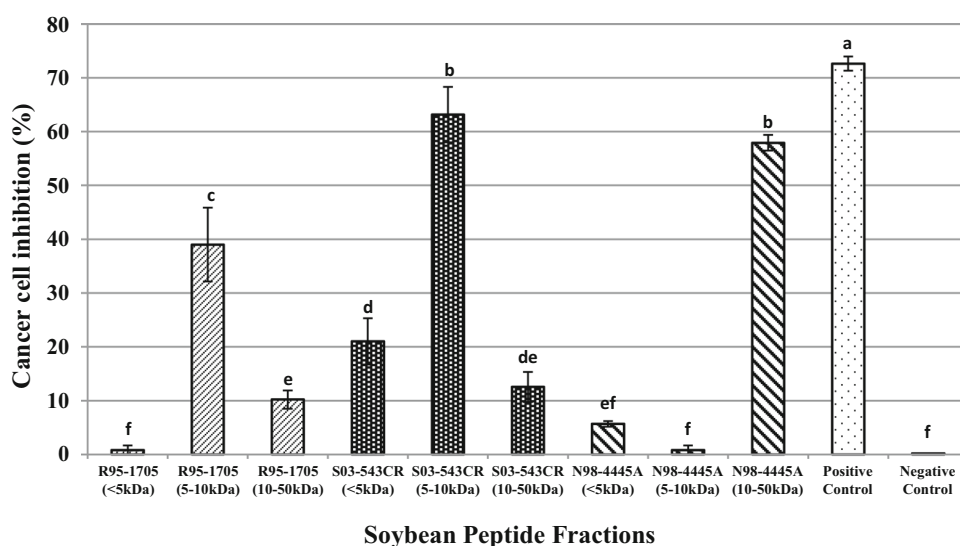
**Fig. 1** Cancer cell inhibitory activity of peptide fractions prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against CCRF-CEM human blood cancer cells. Positive control: genistein 200 µg/mL; Negative control: Saline solution. The bars represent % inhibition values expressed as mean ± standard deviation (of triplicates) and those connected by different letters are significantly different ( $P < 0.05$ )



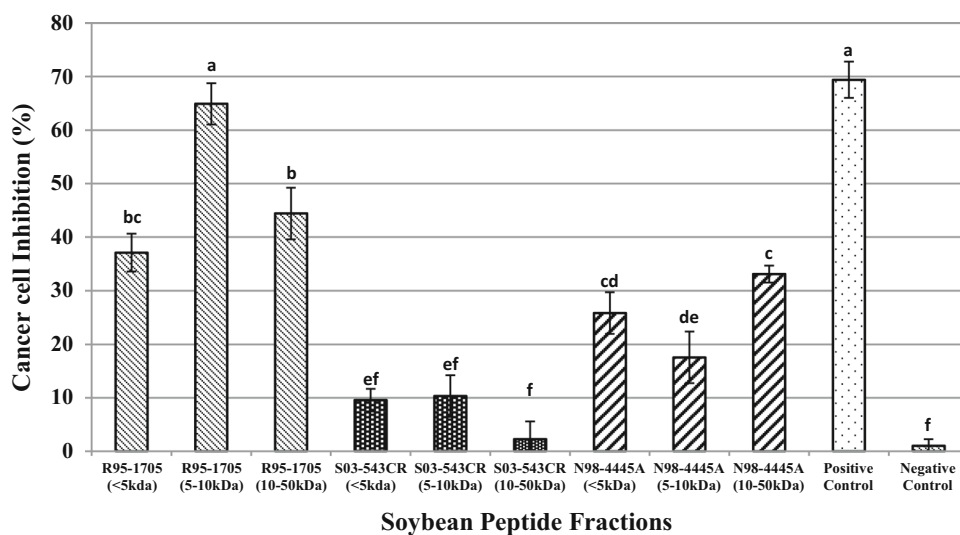
**Fig. 2** Cancer cell inhibitory activity of peptide fractions prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against Kasumi-3 human blood cancer cells. Positive control: genistein 200  $\mu\text{g}/\text{mL}$ ; Negative control: Saline solution. The bars represent % inhibition values expressed as mean  $\pm$  standard deviation (of triplicates) and those connected by different letters are significantly different ( $P < 0.05$ )

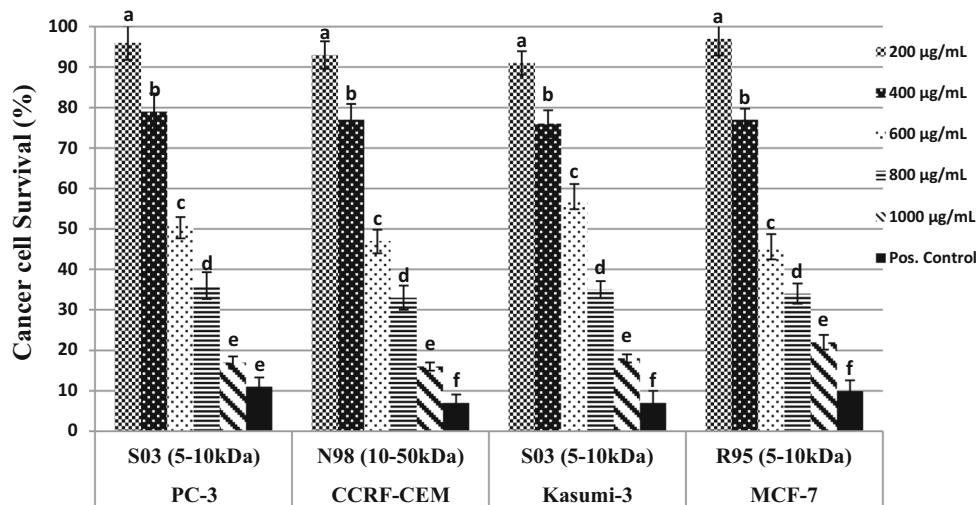


**Fig. 3** Cancer cell inhibitory activity of peptide fractions prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against PC-3 human prostate cancer cells. Positive control: genistein 200  $\mu\text{g}/\text{mL}$ ; Negative control: Saline solution. The bars represent % inhibition values expressed as mean  $\pm$  standard deviation (of triplicates) and those connected by different letters are significantly different ( $P < 0.05$ )



**Fig. 4** Cancer cell inhibitory activity of peptide fractions prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against MCF-7 human breast cancer cells. Positive control: genistein 200  $\mu\text{g}/\text{mL}$ ; Negative control: Saline solution. The bars represent % inhibition values expressed as mean  $\pm$  standard deviation (of triplicates) and those connected by different letters are significantly different ( $P < 0.05$ )





**Fig. 5** Dose response study of soybean peptide fractions with highest anticancer activity based on trypan blue dye exclusion assay. Positive control: genistein 200 µg/ml. PC-3: Prostate cancer; CCRF-CEM and Kasumi-3: Blood cancers; and MCF-7: Breast cancer. Soybean lines

are designated as: S03 = S03-543CR, N98 = N98-4445A, and R95 = R95-1705. Values expressed as mean  $\pm$  standard deviation (of triplicates) and those connected by *different letters* for each cancer type of each peptide fraction are significantly different ( $P < 0.05$ )

CCRF-CEM and MCF-7 cell lines with approximately 50% reduction in cell survival. The 5–10 kDa fraction from the S03-543CR soy line showed the highest reduction at 1000 µg/mL on PC-3 cell counts which was not significantly different in comparison to the reduction by genistein ( $P$  value = 0.5293). Although the cell survival percentage reduced to less than 20% in the blood cancer cell lines (CCRF-CEM and Kasumi-3), the positive control had the significantly higher inhibition ( $P$  values are 0.0121 and 0.0008, respectively). On the MCF-7 breast cancer cell line, the 5–10 kDa fraction showed a significant reduction in cell survival at the  $\geq 800$  µg/mL dose ( $P < 0.0001$ ). The minimum concentration to cause 50% inhibitory activity ( $IC_{50}$ ) for the 5–10 kDa (S03-543CR) fraction was found to be 608 µg/mL. The 10–50 kDa fraction from N98-4445A against CCRF-CEM blood cancer cells was calculated to be 678 µg/mL while the 5–10 kDa fraction of S03-543CR had an  $IC_{50}$  value of 611 µg/mL on the Kaumi-3 cells. On the MCF-7 breast cancer cell line, the 5–10 kDa fraction from R95-1705 showed a reduction in cell survival (21%) at a dosage of 1000 µg/mL dose with an  $IC_{50}$  of 654 µg/mL.

Separation of pure peptides from the fractions and testing them for anti-proliferative activity against cancer cells may provide enhanced results. Other researchers have demonstrated an effective anticancer activity of single peptides against breast cancer (Kannan et al. 2009; Li et al. 2014a, b). These results have demonstrated the immense potential of peptide fractions and peptides in reducing in vitro breast cancer cell proliferation. Continuation of this research with clinical trials may provide potential drug-free or supplemental therapeutic solutions in treating cancers.

## Conclusion

Enzymatic hydrolysis has been proven to be a promising approach to obtain peptides from protein sources that are considered a bio-waste. This study reports that utilization of soybean meal from two high oleic acid soybean lines and one high protein line to obtain bioactive peptides against multiple-site cancer cells. Soy peptide fractions obtained after proteolytic digestion using Alcalase and GI enzymes for GI resistance showed potent anti-proliferation activity against human blood, breast and prostate cells. The results showed that different peptide fractions from different soybean lines have varying inhibitory activities against each cancer cell line. The maximum inhibitory activity (68.0%) was found from 10 to 50 kDa fraction of soybean line N98-4445A against CCRF-CEM blood cancer cell. The minimum concentration to result 50% inhibitory activities by all the observed fractions ranged between 608 and 678 µg/mL. This multiple site in vitro cancer inhibition by GI friendly peptides could have potential use in food ingredients or nutritional supplements in an alternative cancer therapy. These GI resistant peptides could be suitable for use in food product applications due to their potential stable bio-availability.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

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