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Cytotoxic and Apoptotic Effects of Sprouted and Non-sprouted Lentil, Green and Yellow Split-peas

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ABSTRACT

Legumes such as lentils, green and yellow split-peas have been reported to provide health benefits against colon cancer due to nutrients and non-nutrient phytochemical compounds present in them. The aim of this study was to investigate the effect of selected legumes in inducing cytotoxicity, apoptosis and/or necrosis in human epithelial colorectal adenocarcinoma cells. Methanolic extracts of sprouted and non-sprouted legumes (Lentils, green peas, yellow peas) were prepared. Caco-2, human colonic carcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were incubated with legume extracts at selected concentrations (1.5, 3, 4.5, 6, 7.5 and 10 mg mL⁻¹) for determination of lactate dehydrogenase (LDH) release (cytotoxicity %). The ability of legume extracts in inducing cell apoptosis was analyzed at selected concentrations of 1.5, 3, 6 and 12 mg mL⁻¹ by quantifying DNA fragmentation. Morphological changes in cells were observed microscopically. The activity of caspase-3 enzyme was also determined. Results showed that legume extracts induced cytotoxicity in caco-2 cells in a dose-dependent manner. A 20-80% increase in release of LDH was observed in cells incubated with extracts compared to untreated cells. Total fragmented DNA (%) was higher in cells treated with sprouted legume extracts compared to their non-sprouted extracts. More than 40% of total DNA fragmentation was observed in cells treated with sprouted legumes. Caspase-3 activity was highest in cells incubated with non-sprouted yellow split peas at 6 mg mL⁻¹. Specific morphological changes related to apoptosis such as: surface blebbing, formation of apoptotic bodies, blisters and echinoid spikes and cell membrane destabilization were observed in cells incubated with extracts. These results suggested the cytotoxic and apoptosis inducing potential of legume extracts. Bioactive components in these selected legumes may be used as chemopreventive agents.

Key words: Clastogenic, legumes, apoptosis, necrosis, Caco-2

INTRODUCTION

Cancer is characterized by uncontrolled growth of cells resulted from damaged DNA and by the spread of abnormal cells into surrounding areas. Genetic changes may be caused by external factors such as tobacco, chemicals, diet, radiation and internal factors such as inherited mutations, hormones and immune conditions (ACS., 2010). Although, cancer death rates have been dropping since 1992, it remains a major public health problem affecting more than 1 million people every year in the US and still accounts for 1 in every 4 deaths (NCI., 2008). Colon cancer is the third most common form of cancer with occurrence of 103,170 new cases and 51,690 deaths during 2012 in the US (Siegel *et al.*, 2012).

Cells with damaged DNA which cannot be repaired normally undergo apoptosis, a process by which the cell kills itself. This process controls cell cycle and cell numbers at the early stage of the carcinogenesis. By apoptotic process, cells with damaged DNA are not allowed to participate in the normal process of cell division. Induction of apoptosis by chemopreventive agents is one of the mechanisms to reduce cell growth and proliferation. Apoptosis is mediated by a family of proteases called caspases made of initiators (Caspase 8, 9, 10, 12) of cell death process and effectors (Caspase 3, 6 and 7). Dietary compounds have been implicated in the protection against cancer by enhancing the elimination of initiated cells via the induction of apoptosis (Watson *et al.*, 2000). Food constituents such as short chain fatty acid (butyrate) and phytonutrients have the ability to regulate programmed cell death to provide protection against induction of neoplasia via the removal of initiated cells before they participate in cell division and give rise to precancerous lesions (Johnson, 2001).

Clinical studies have linked diet with the incidence of colorectal cancer. Researches of Hu *et al.* (2001), Hu and Willett (2002) and Moore *et al.* (2001, 1999) have suggested that high intake of fruits, vegetables and dietary fiber may reduce the risk of chronic diseases such as cardiovascular diseases, stroke and type 2 diabetes. Legumes such as lentils, split peas (green and yellow) play a significant role in human nutrition as a source of minerals, vitamins (E, D and folate) and as a source of fiber. Antioxidant nutrients such as vitamin E and D and phytochemical compounds found in lentils, green split peas and yellow peas have been linked to the reduction in the risk of major chronic diseases because they can protect the body against harmful effects of free radicals (Adom and Liu, 2002; Steinmetz and Potter, 1996; Sun *et al.*, 2002). Sprouted foods are good source of antioxidants, vitamin A, E and C which play a crucial role in scavenging free radicals (Shipard, 2005). There is a lot of interest in plant foods to promote prevention of cancer. However, there was not much known about the mechanism involved in anticancer properties of legume extracts. The aim of this study was to investigate the cytotoxic and apoptotic activities of sprouted and non-sprouted legume extracts in Caco-2 cell line.

MATERIALS AND METHODS

Preparation of sprouted and non-sprouted legumes (lentil, green and yellow split-peas) extracts for tissue culture: Sprouted and non-sprouted lentils, green and yellow split-peas (5-10 g) extracts were prepared by shaking overnight with 100 mL of 80% methanol by following the method described by Nam *et al.* (2005). The concentrate was made to a final volume of 10 mL and stored at -20°C until further analysis.

Cell culture maintenance: Human colonic carcinoma cells (Caco-2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂ until development of monolayer. Cells (5×10⁴ cells/well) were seeded and grown to confluence in a 24 well plate and treated with sprouted and non-sprouted legume extracts. Selected legume extracts were prepared in media and used for experiments.

Cytotoxicity assay: The cytotoxic effects of selected legume extracts were measured by the release of lactate dehydrogenase (LDH) from the Caco-2 cells into the culture supernatant. Cells were incubated with the legume extracts (1.5, 3, 4.5, 6, 7.5 and 10 mg mL⁻¹) for 12 and 24 h. A 10% triton-X was used as a positive control, media was used as negative control. The LDH activity was

measured by a colorimetric cytotoxicity detection kit (LDH) (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. The absorbance was read at 490/600 nm wavelength in microplate reader

Determination of cell death by apoptosis

DNA fragmentation assay: DNA fragmentation assay was performed as described by Traore *et al.* (2001) and Wu *et al.* (2004) with some modifications. Caco-2 cells were treated with legume extracts at 1.5, 3, 6 and 12 mg mL⁻¹ for 24 h. After incubation, 500 µL of lysis buffer (10 mM Tris-HCl, pH 8/5 mM EDTA/0.6% Triton X-100) was added to cells to release nuclear contents. Lysed cells were centrifuged (12,000×g for 15 min), supernatant which represents the fragmented DNA was collected. Intact DNA from the pellet was further solubilized and collected by addition of 0.5 N per chloric acid followed by heat treatment. The amount of DNA content in both fractions was quantified by using a diphenylamine assay. Two volumes of DPA reagent (0.088 M DPA, 98% (v/v) glacial acetic acid, 1.5% (v/v) sulfuric acid and 0.5% (v/v) of 1.6% acetaldehyde solution) was added to the DNA fractions and stored at 4°C for 24 h. Optical density was measured at 560 nm in multiplate reader and results are expressed as the percentage of fragmentation by following this equation:

$$\text{DNA fragmentation (\%)} = \frac{\text{OD supernatant}}{\text{OD total (supernatant+pellet)}} \times 100$$

Morphological changes: Caco-2 cells were incubated with legume extracts (6 and 12 mg mL⁻¹) for 24 h and stained with Giemsa stain (0.1% in PBS) to observe morphological changes. Cells were washed with PBS and the plates were dried at 37°C. Microscopic changes in cells were observed under phase contrast microscope (Nikon CT 120, Nikon Instruments Inc., Melville, NY) and photographs were taken using a SPOT 2.0 Digital Camera at 40X magnification.

DNA fragmentation by agarose gel electrophoresis: Caco-2 cells (1.8-2 million cells) were exposed to legume extracts at 1.5, 3, 6 and 12 mg mL⁻¹ for 24 h. The assay was performed using the dimethyl sulphoxide (DMSO Sodium Dodecyl Sulphate (SDS) Tris-EDTA (TE) method as described by Suman *et al.* (2012). After incubation with extracts, cells were dislodged and washed with cold PBS. The DMSO (100 µL) was added to cell pellet and mixed well. Equal volume (100 µL) of TE buffer (pH 7.4) with 2% SDS was added, centrifuged at 12000×g at 4°C and finally 40 µL of the supernatant was loaded on 1.8% agarose gel containing 1 µg mL⁻¹ of ethidium bromide.

Caspase-3 activity: Caco-2 cells were treated with legume extracts (1.5, 3, 6, 12 mg mL⁻¹) for 24 h. Cells were extracted with PBS (pH 7) and centrifuged at 10,000×g for 15 min. Cell lysis buffer was added, incubated for 10 min and centrifuged at 15,000×g for 15 min. The supernatant was collected and caspase-3 activity was determined using a caspase kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions.

RESULTS

Cytotoxicity of legume extracts: The cytotoxicity of LS, LNS, GS, GNS, YS and YNS extracts at 1.5, 3, 4.5, 6, 7.5 and 10 mg mL⁻¹ on Caco-2 cells was determined after an incubation period of 12 and 24 h by measuring lactate dehydrogenase (LDH) release. Low LDH release was observed after 12 h of incubation with low (3 and 6 mg mL⁻¹) and high concentrations (7.5 and 10 mg mL⁻¹) of extracts (Fig. 1). There was no difference in LDH release between sprouted and non-sprouted

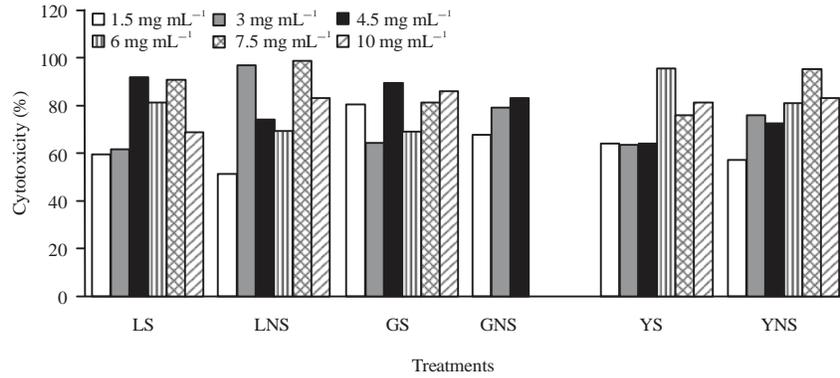


Fig. 1: Effect of sprouted and non-sprouted legumes on LDH release (12 h), LS: Lentil sprouted, LNS: Lentil non-sprouted, GS: Green split-peas sprouted, GNS: Green split-peas non sprouted, YS, Yellow split-peas sprouted and YNS: Yellow split-peas non sprouted

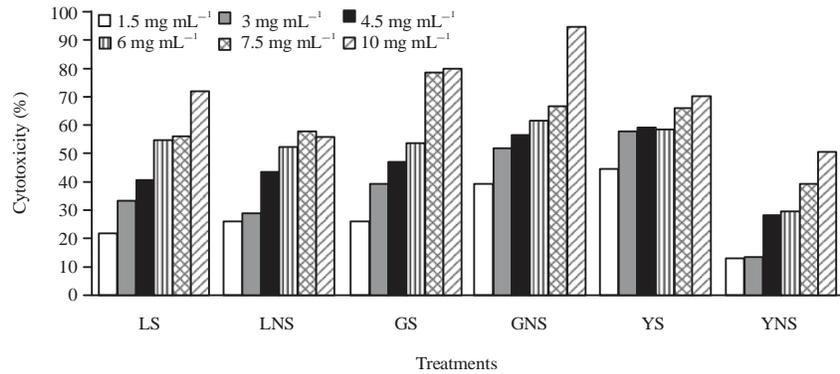


Fig. 2: Effect of sprouted and non-sprouted legumes on LDH release (24 h), LS: Lentil sprouted, LNS: Lentil non-sprouted, GS: Green split-peas sprouted, GNS: Green split-peas non sprouted, YS: Yellow split-peas sprouted and YNS: Yellow split-peas non sprouted

legume extracts with incubation of 12 h. A dose dependent release of LDH was observed with increase of concentrations with incubation of extracts for 24 h with extracts (Fig. 2). Low LDH release was observed in cells treated with non-sprouted legumes except green split-peas compared to their sprouted counterparts. The LDH release was decreased with increase in incubation time of extracts. Percentage of viability was reduced in legume treated cells compared to normal cells.

Total DNA fragmentation: The ability of sample extracts to induce breakage of chromosomes was studied in treated and untreated cells. The effect of legume (LS, LNS, GS, GNS, YS and YNS) extracts at selected concentrations was determined by calculating the percentage of total DNA fragmentation (Fig. 3). Higher DNA fragmentation was observed in cells treated with LS, LNS and GS extracts. Concentration of LS, LNS and GS extracts did not have any effect in inducing DNA fragmentation. On contrary, treating cells with YS and YNS extracts decreased DNA fragmentation with increase of extract concentration. The GNS treatment exhibited an increase in DNA fragmentation in a concentration dependent manner.

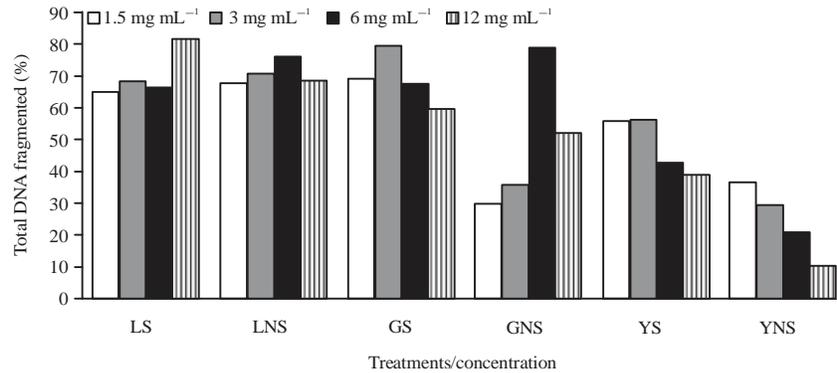


Fig. 3: Effect of sprouted and non-sprouted legumes on total DNA fragmentation (24 h), LS: Lentil sprouted, LNS: Lentil non-sprouted, GS: Green split-peas sprouted, GNS: Green split-peas non sprouted, YS: Yellow split-peas sprouted and YNS: Yellow split-peas non sprouted

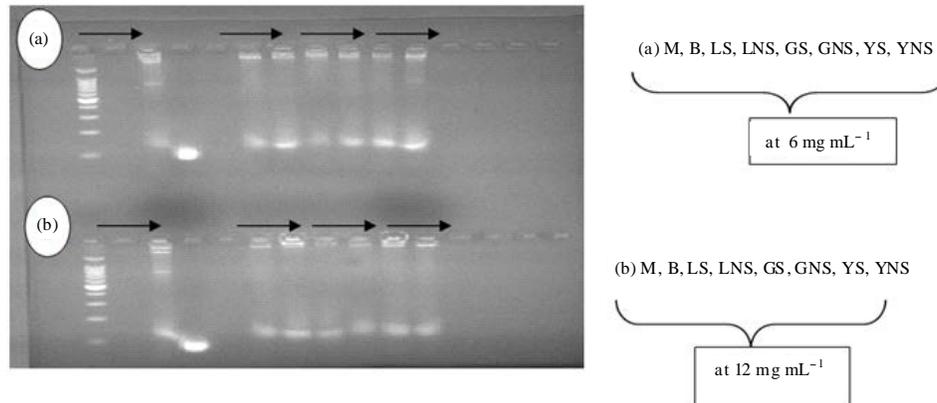


Fig. 4: Internucleosomal DNA degradation in cells treated with legume extracts at 6 and 12 mg mL⁻¹ 24 h, LS: Lentil sprouted, LNS: Lentil non-sprouted, GS: Green split-peas sprouted, GNS: Green split-peas non sprouted, YS: Yellow split-peas sprouted and YNS: Yellow split-peas non sprouted

DNA fragmentation by agarose gel electrophoresis: Cells treated with legume extracts showed DNA fragmentation and cells treated with YNS at 6 mg mL⁻¹ showed low degree of DNA degradation. Untreated cell's DNA was intact and lack degradation (Fig. 4).

Morphological changes: Morphology of Caco-2 cells after incubation with sample extracts at 6 and 12 mg mL⁻¹ was observed microscopically and results are in Fig. 5. Untreated cells were intact, confluent and did not show any morphological changes related to apoptotic activity (Fig. 5a). Cells treated with GS (6 mg mL⁻¹), YS (6 mg mL⁻¹) and YNS (6 and 12 mg mL⁻¹) exhibited morphological changes that are characteristic of apoptosis, such as chromosomal and cytoplasmic condensation, blebbing, echinoid spikes and blisters at membrane (apoptotic bodies) (Fig. 5b, c, d). Cells treated with GS and YS (12 mg mL⁻¹) showed morphological changes characteristic of necrosis including membrane destabilization, stretching and melting of cell membrane (Fig. 5e). Cells incubated with legume extracts were round, shrunk and lost surface contact.

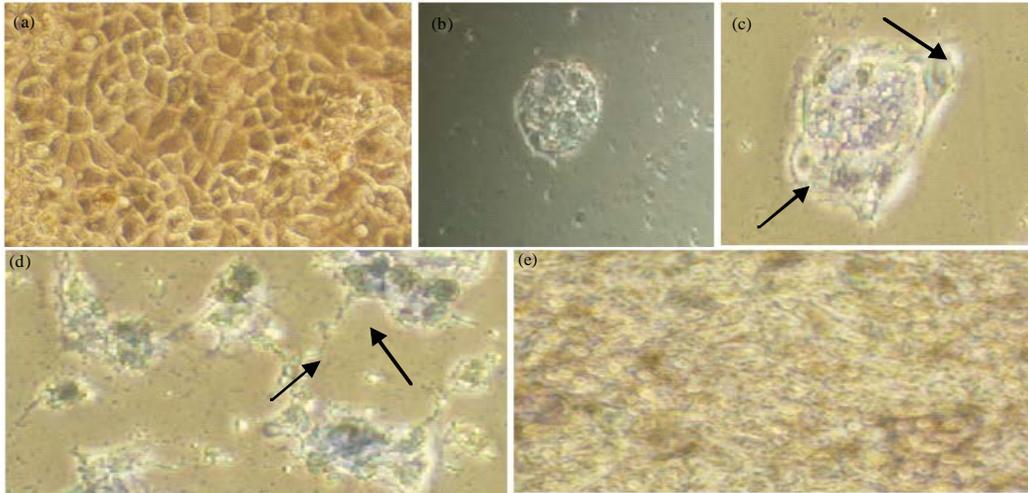


Fig. 5(a-e): Morphological changes of Caco-2 cells incubated with legume extracts (a) Morphology of untreated cells (intact cellular structure), (b, c) Surface blebbing and formation of apoptotic bodies, (d) Blisters and (e) Echinoid spikes

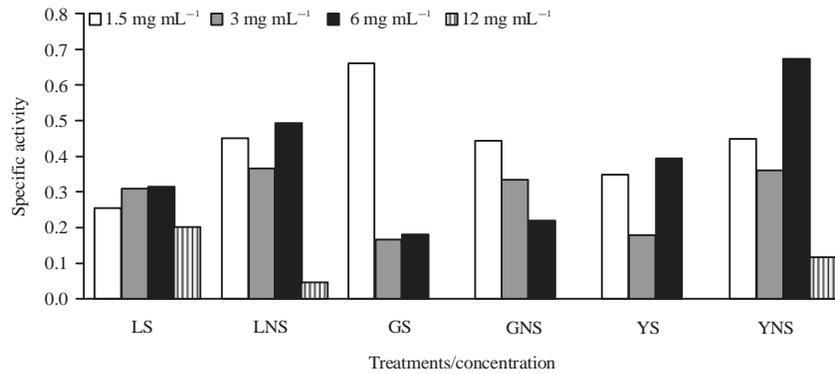


Fig. 6: Effect of sprouted and non-sprouted legumes on caspase-3 activity (24 h), LS: Lentil sprouted, LNS: Lentil non-sprouted, GS: Green split-peas sprouted, GNS: Green split-peas non sprouted, YS, Yellow split-peas sprouted and YNS, Yellow split-peas non sprouted

Caspase-3 activity: Treating cells with extracts exhibited high caspase-3 activity compared to untreated cells. Caspase-3 activity was lowest in cells incubated with LS among the cells treated with extracts. The highest caspase-3 activity was observed in cells treated with GS at 1.5 mg mL⁻¹ and YNS at 6 mg mL⁻¹. Among the tested concentrations of the extracts, lowest caspase-3 activity was observed in Caco-2 cells incubated with legume extracts at 12 mg mL⁻¹ (Fig. 6).

DISCUSSION

Non-nutritive compounds, flavonoids and polyphenols, provide much of the flavor and color to fruits, vegetables, grains and legumes (Hollman and Katan, 1999; Ross and Kasum, 2002). The antioxidative and cytotoxic activities of dietary compounds have generated interest in plant foods as potential sources of chemopreventive agents (Ampasavate *et al.*, 2010; Gerhauser, 2008;

McCann *et al.*, 2007). These chemopreventive agents may target cellular alterations associated with tumorigenesis by anti-oxidant and anti-inflammatory activities and also modulating cell signaling pathways which may lead to inhibition of cell proliferation, cell growth and induction of apoptosis (Gerhauser, 2008). Feeding non-nutritive compounds reduced certain type of cancers in animals via induction of Phase II enzymes, scavenging ROS, suppression of cell proliferation, inhibiting cell invasion (Tanaka *et al.*, 2001; Tanaka and Sugie, 2007) and induction of cytotoxicity (Fresco *et al.*, 2010). *In vitro* cell culture using the Caco-2 cell line is a well-developed model for studying the effect of phytochemicals on growth inhibition in human colonic adenocarcinoma as part of chemopreventive strategies. The present study was conducted to investigate the effect of sprouted and non-sprouted legume extracts on inducing cytotoxicity and apoptosis in Caco-2 cells.

Dietary chemopreventive agents induce cell specific cytotoxicity leading to inhibition of cell growth and proliferation. In this study, extracts of sprouted and non-sprouted legume extracts showed cytotoxic effects in Caco-2 cells in a dose and time dependent manner. The results exhibited the effect of legume extracts in inhibiting cell proliferation. Incubation period of 12 h with extracts showed a high induction of cytotoxicity compared to the 24 h incubation period. This may due to the high percentage of cell death in cells incubated for 24 h with extracts. The enzyme, LDH is released by live cells. Wolter *et al.* (2002) reported an inhibition in growth and proliferation of Caco-2 cells after treating with a polyphenol (piceatannol) extracted from an asian legume in a dose and time dependent manner. Their results showed reduction in cell count and LDH release after 24 h incubation period with legume extracts. In contrary, a dose dependent increase in LDH release of Caco-2 cells was reported when cells were incubated with dry beans ($0.5-25 \text{ mg mL}^{-1}$) for 24 and 48 h (Boateng *et al.*, 2008). Sochocka *et al.* (1994) also suggested a correlation between LDH release and cell death.

Activation of apoptosis pathways is a key mechanism by which dietary phytochemicals inhibit tumor cell growth. Apoptosis is characterized by morphological and biochemical changes in cells such as cell shrinkage, DNA fragmentation mediated by caspases and caspase independent DNA cleavage (Debatin, 2004; Johnstone *et al.*, 2002). The inter-nucleosomal degradation of DNA (Caspase dependent fragmentation) is quantitatively measured via detection of histone-associated mono and oligonucleosomes, which are indicators of apoptosis (Seeram *et al.*, 2005). An increase in DNA fragmentation (histone related and caspase independent DNA degradation) was observed in cells treated legume extracts compared to untreated cells. The polyphenols in legume extracts might be responsible for induction of DNA fragmentation. An increase in DNA fragmentation was observed in HT-29 human colon adenocarcinoma cells incubated with flavanol, epigallocatechin gallate (EGCG), at doses $20-500 \text{ } \mu\text{mol L}^{-1}$ (Chen *et al.*, 2003). High DNA fragmentation in cells treated with low concentration of legume extracts compared to cells with high concentrations of legume extracts could be due to less viability of cells at higher concentrations. However, Hosokawa *et al.* (2004) reported a dose dependent increase in cellular DNA fragmentation in Caco-2 cells incubated with $22.6 \text{ } \mu\text{M}$ of dietary carotenoids from Fucoxanthin, found in brown algae, for 24 h, a 10-fold increase over that of untreated cells. At the molecular level, flavonoids modulate key elements in cellular signal transduction pathways associated with the apoptotic process including activation of caspases and up-regulation of Bcl-2 genes (Ramos, 2007). Formation of DNA ladder is one of the early signs of apoptosis and used as marker for apoptotic analysis. The ladder like DNA appearance in cells treated with legume extracts also confirmed the ability of legume extracts in inducing apoptosis.

Morphological changes in cells incubated with legume extracts at specific concentrations (Fig. 5) for 24 h showed surface blebbing, blisters and apoptotic bodies indicating the apoptotic

inducing activity of legume extracts. Similar morphological changes were observed in Caco-2 cells with incubation of various plant extracts (Verghese *et al.*, 2006; Gourineni *et al.*, 2010). Loss of cell shape is due to proteolysis of cytoskeletal protein (Debatin, 2004) while nuclear shrinking and budding occur after degradation of lamin (Lazebnik *et al.*, 1993; Martelli *et al.*, 2001). Necrotic cells, however, swell and lose the protective function of the plasma membrane (Martelli *et al.*, 2001). Characteristics of necrotic activity (stretching or loss of cell membrane) were also observed in Caco-2 cells incubated with legume extracts at specific concentrations (Fig. 5). These results indicated that legume extracts caused apoptosis and necrosis at the same time in Caco-2 cells. Apoptosis and necrosis can occur at the same time in cells but, intensity of the initial insult decides the prevalence of either apoptosis or necrosis (Formigli *et al.*, 2000; Nicotera *et al.*, 1999).

Intrinsic and extrinsic pathways of apoptosis trigger proteolytic enzymes, caspases, for induction of apoptosis. An increase in caspase-3 activity was observed in cells incubated with all tested legume extracts indicating the apoptotic inducing property of legume extracts. However, the activation of caspase-3 activity by legume extracts in Caco-2 cells varied among the treatments. Activity of caspase-3 increased in an inverse dose dependent manner, except for GS where the activity increased in a dose dependent manner. Lack of caspase-3 activity observed at the highest concentration (for GS, GNS and YS) of extracts could be due to coexistence of necrotic process. At the highest concentrations, the intensity of phytochemical compounds in extracts might have caused initial injury to cells. A study conducted by Gourineni *et al.* (2010) using soybean meal (25, 50, 100 $\mu\text{g mL}^{-1}$) reported high caspase-3 activity at all doses in Caco-2 cells after 24 h. Chen *et al.* (2003) reported an enhancement of caspase-3 activity initiated after 12 h and completed after 24 h in HT-29 cancer cells treated with dietary flavonoids (EGCG). McCann *et al.* (2007) reported a decrease in proliferation and induction of apoptosis in Caco-2 and HT29 cells treated with flavonoids from apples.

CONCLUSION

Results of this study suggested that the anti-cancer properties of bioactive compounds in sprouted and non-sprouted legumes by inducing LDH release, DNA fragmentation, morphological changes and activation of caspase-3 activity in Caco-2 cells.

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