RESEARCH COMMUNICATION

Mechanisms of Anticancer Activity of Sulforaphane from *Brassica oleracea* in HEp-2 Human Epithelial Carcinoma Cell Line

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Abstract

Sulforaphane (SFN) an isothiocyanate formed by hydrolysis of glucosinolates found in *Brassica oleraceae* is reported to possess anticancer and antioxidant activities. In this study, we isolated SFN from red cabbage (*Brassica oleraceae var rubra*) and evaluated the comparative antiproliferative activity of various fractions (standard SFN, extract and purified SFN) by MTT assay in human epithelial carcinoma HEp -2 and and Vero cells. Probable apoptotic mechanisms mediated through p53, bax and bcl-2 were also examined. The SFN fraction was collected by HPLC, enriched for its SFN content and confirmed. Expression of apoptosis-related proteins was detected by western blotting and RT PCR. Results showed that Std SFN and purified SFN concentration found to have closer IC_{50} which is equal to 58.96 microgram/ml (HEp-2 cells), 61.2 microgram/ml (Vero cells) and less than the extract which is found to be 113 microgram/ml (HEp-2 cells) and 125 microgram/ml (Vero cells). Further studies on apoptotic mechanisms showed that purified SFN down-regulated the expression of bcl-2 (antiapoptotic), while up-regulating p53 and Bax (proapoptotic) proteins, as well as caspase-3. This study indicates that purified SFN possesses antiproliferative effects the same as Std SFN and its apoptotic mechanism in HEp-2 cells could be mediated through p53 induction, bax and bcl-2 signaling pathways.

Keywords: Sulforaphane (SFN) - *Brassica oleracea* - apoptosis - p53 - bax and bcl-2

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Introduction

Epidemiological studies states that certain cruciferous vegetables consumption reduces various types of cancer due to its enriched content of Glucosinolates (GLS) (Gasper et al., 2007; Meyer & Adam, 2008). Over 120 glucosinolates have been identified to this date. Glucosinolates present in red cabbage were reported to be Glucoraphanin, Glucobrassicin, Sinigrin, Progoitrin etc. (Renuka & Thangam, 2010). Glucosinolates may break down to from isothiocyanates and nitriles in plant material during processing by the action of the endogenous enzyme myrosinase of within the gastrointestinal tract by the action of commensal micro flora (Verkerk et al., 2001).

Glucosinolates and their breakdown products are of particular interest in food research because of the alleged anti-carcinogenic properties The hydrolysis products include Isothiocyanates (ITC), Nitriles, Indoles and Oxazolidinethiones, from which ITC and indoles reported to have anticancer properties (Steven et al., 2004). The isothiocyanate sulforaphane (SFN) has attracted much recent interest, since it was found be most potent naturally occurring inducer of phase II detoxification enzymes and potent scavenger of reactive oxygen Species (ROS) including superoxide anions and hydroxyl radicals. SFN has been shown to inhibit cell cycle progression. The mechanism by which these agents may act include dilution and binding of carcinogens in the digestive tract (fiber), antioxidant effects inhibition of nitrosamine formation, inhibition of activation of pro-mutagens/pro-carcinogens, induction of detoxification enzymes, alteration of hormone synthesis (Davis & Finley, 2002).

Varieties of genes are involved in cell metabolism, cell cycle regulation and apoptosis. Both pro apoptotic (Bax, Bak, Bid, Noxa, etc.) and anti-apoptotic (Bcl-XL, Mcl-1, Bcl-w, etc) proteins have been reported to be key regulators of apoptosis. Genes transcriptionally up regulated by p53 are implicated in promoting apoptosis, which includes the bcl-2 family members (e.g. Bax, Bak) and Noxa gene proteins. The p53-dependent apoptotic pathway can lead to the cellular protein cleavage (e.g. PARP), DNA damage and cell death (Gamet-Payrastre et al., 2000). Two major pathways leading to apoptosis exist in cells: the extrinsic pathway, which involves the activation of the TNF/Fas death receptor family and the intrinsic pathway, which involves the mitochondria. In both the pathways, an apoptotic death stimulus results in the activation of caspases, the major executioners of this process, either directly or via activation of the mitochondrial death program (lllic et al., 1996).

In this study we investigated whether sulforaphane isolated from red cabbage could contribute to the antiproliferative activity and apoptosis in human larynx cancer epithelial cell line. The main aim of the study was

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to determine possible mechanisms behind the initiation of apoptosis.

Materials and Methods

Extraction and separation of sulforaphane from Brassica

Red cabbage (*Brassica oleracea var rubra*) was obtained from local market. Sulforaphane standard was purchased from Sigma Chemical. Acetonitrile was HPLC grade, and methylene chloride and anhydrous sodium sulfate were reagent grade.

Extraction of sulforaphane from Brassica

Sulforaphane was isolated from red cabbage as described (Liang et al., 2006). Five grams of freshly harvested cabbage was homogenized for 5 min. The vegetable meal was left to autolyze at room temperature for 30 min. After autolyzing, the meal was extracted two times with 50 mL methylene chloride, which was combined and salted with 2.5 g anhydrous sodium sulfate. The methylene chloride fraction was dried at 30 °C under vacuum on a rotary evaporator. The residue was dissolved in acetonitrile and was then filtered through a 0.22 mm membrane filter prior to injection into HPLC. The samples were analyzed with a HPLC apparatus equipped with pumps and tunable absorbance detector, and reverse phase 18 columns. The solvent system consisted of 20% acetonitrile in water; this solution was then changed linearly over 10 min to 60% acetonitrile, and maintained at 100% acetonitrile for 2 min to purge the column. The column oven temperature was set at 30 °C. The flow rate was 1 mL/min. Sulforaphane was detected by absorbance at 254 nm. The representative chromatograms of the HPLC elution profile of standard, extract and purified sulforaphane from cabbage sample are shown in fig. The sulforaphane fraction was collected, enriched and confimed with the Std. graph.

Quantification of sulforaphane

Quantification was based on the external standard method. A stock solution was prepared with 5 mg of sulforaphane reference standard, which was dissolved and diluted to 10ml with acetonitrile. Aliquots of the standard stock solution of sulforaphane were pipetted into different 10mL flasks and diluted to mark with acetonitrile. The final concentrations of sulforaphane were in the range of 2.5-17.5 microgram/ml

Cell Culture

Human larynx epithelial carcinoma cell line (HEp-2) and Normal Vero cell line (African Green Monkey Kidney) were obtained from NCCS (National Centre for Cell Science, Pune). Cells were grown in DMEM medium supplemented with 100 microgram foetal bovine serum, with antibiotic at 37 °C in an incubator containing 5% CO_2.

MTT assay

MTT assay was performed as described (Edmondson et al., 1988; Pledgie-Tracy et al., 2007). Briefly cells (HEp-2 and Vero) were seeded at a concentration of $2x10^4$ cells/ml in a 96-well plate. After monolayer formation, 0, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 microgram/ml concentration of purified sulforaphane was added. The entire test was done in triplicates. These cells were incubated in a humidified atmosphere with 5% CO_2 for 2 days for HEp-2 and 3 days for Vero cell lines. Then, 20 microlitre MTT (3-4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) solution (4.14mg/ml) was added to each well and incubated at 37 °C for 3 hours. The medium was removed and formazan was dissolved in DMSO and the optical density was measured at 590 nm using Bio-assay reader (BioRad, USA)

Cell cycle analysis

Drug effect on cell proliferation was evaluated by measuring the distribution of the cells in the different phases of the cell cycle by flow cytometry. This determination was based on the measurement of DNA content of nuclei labeled with propidium iodide. Cells were seeded onto 75 cm^2 cell culture flasks and treated with 62.5 microgram/ml concentration of ensured sulforaphane fraction (Sreenivasa et al., 2001; Yina et al., 2004; Matsui et al., 2006). After 48 hr of the treatment, the cells were harvested and fixed in ethanol, washed with PBS and stained with 0.5% propidium iodide for 30 min at 4°C and analyzed using FAC scan flow cytometer (BD, Bioscience). The cell cycle distribution was evaluated on DNA plots.

Western blot analysis

HEp-2 cells were plated and cultured in complete medium and allowed to grow for 24hr followed by the addition of 62.5 microgram/ml ensured concentration of sulforaphane and incubated for 12, 24, 48 and 72 hr. Control cells were incubated in the medium with 0.1% DMSO for the same period. After the indicated incubation period, the cells were harvested and collected by centrifugation. Cells were re-suspended in Tris-HCL and lysed using 4% SDS. Cell lysate were subjected to 100 microgram SDS and eletrophoretically transferred to nitrocellulose membrane. The membrane was blocked with 5% non fat dry milk and incubated with primary antibodies for 1 hr at room temperature. The primary antibodies used were anti-p53, bax and casp-3 (Myzak et al., 2006). After treatment with secondary antibodies and bands were visualized using chemiluminescence methods.

Real time PCR

Total RNA was extracted from HEp-2 cells previously treated with 62.5 microgram/ml concentration of sulforaphane.Treated and untreated cells were harvested by trypsination and washed with PBS. To this 1ml of trizol reagent was added and RNA was precipitated using isopropanol. Level of mRNA was quantified using reverse transcriptase kit (Invitrogen, India). Levels of Bcl- 2 mRNA were quantified by real time PCR and normalized to glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Dashwood, 1988; Pappa et al., 2006). The sequence of the bel-2 forward primer 5' AGGAAGTGAACATTTCGGTGAC3' and reverse primer5' GCTCAGTTCCAGGACCAGGC3'. PCR was conducted over 40 cycles (95 °C for 10 s 56 °C for 20 s

72 for 20 s) using SYBR Green I dye.

Statistical analysis

T-test (Unequal variance) was used to compare the cytotoxic activity of Std, extract and purified sulforaphane in Vero and HEp-2 cell line. All the samples were analyzed by T-test (Equal variance) to check the significance. All analysis was performed with a = 0.05 i.e. Confidence interval of 95%.

Results

Extraction and separation of sulforaphane from Brassica.

In our study HPLC was used to separate sulforaphane from Red cabbage and quantify using standard graph method. The chromatograms of Extract and standard were shown in Figure 1A, 1B. The sulforaphane (SF) fraction was collected and enriched for its content. The enriched fraction later injected to HPLC and confirmed using Std graph as shown in Figure 1C. The quantification of sulforaphane in the enriched fraction was done using external Std method (Figure 1D).The amount was found to be 75 microgram/ml .

Cell Proliferation Assay.

The effect of various concentration of sulforaphane on cell growth was examined using HEp-2 (human lung cancer) cell line and Vero (Normal African Green Monkey Kidney) cell line as shown in Table 1, 2 and Figure 2 under the experimental conditions used in 48 hr treatment for HEp-2 cells and 72 hrs for Vero cells. Purified SFN Sulforaphane exerts a significant growth inhibitory effect on HEp-2 cells than compared to Vero cell line as shown in the Figure 2 C, D. The IC_{50} values of Std, Extract and Purified fraction were shown in Table 1 and 2.

IC_{50} for Std and purified SFN was found to be close (60microgram/ml) in Vero cells compared to (58 microgram /ml in HEp-2 cells) IC_{50} for extract was found to be higher than the std which may be due to the presence of other constituents like Indoles, anthocyanidins etc. Results revealed that apart from its cytotoxic effect SFN could induce apoptosis which has been depicted in the Figure 3 A, B. Previous studies in broccoli and white cabbage suggested the induction of apoptosis was mediated through p53 pathway by SFN.

Cell cycle analysis

To understand the underlying mechanism of cell death SFN treated cells (62.5 microgram/ml) are stained with propidium iodide. Cell plots revealed that untreated cells have 15.1 apoptotic cells lower than that of 65.34 for treated cells (Figure 3C, D, Figure 4). To further study

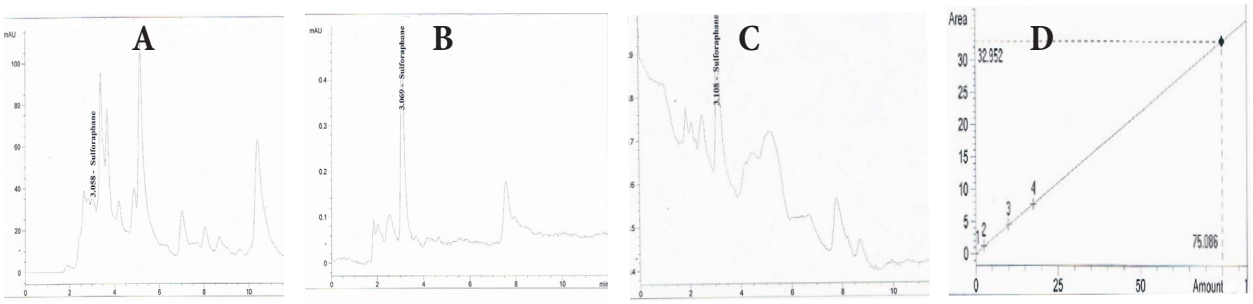

Figure 1. A) HPLC Chromatogram of Red Cabbage Extract, B) HPLC Chromatogram of Std Sulforaphane (SFN), C) HPLC Chromatogram of Purified Sulforaphane, D) Calibration Curve using Different Concentration of standard SFN with Concentration of SFN in the Extract Sample by Extrapolation

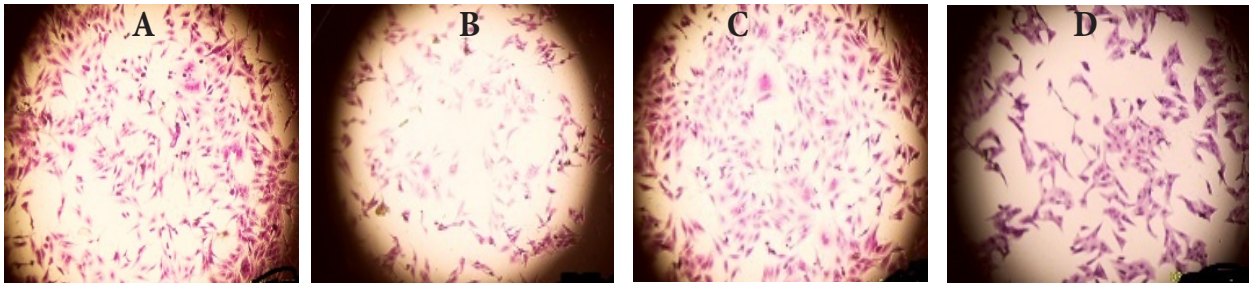

Figure 2. A) Normal Vero Cell Line, B) Vero cells with 50% Cytotoxicity, C) Normal HEp-2 Cell Line, D) HEp-2 cells with 50% Cytotoxicity.

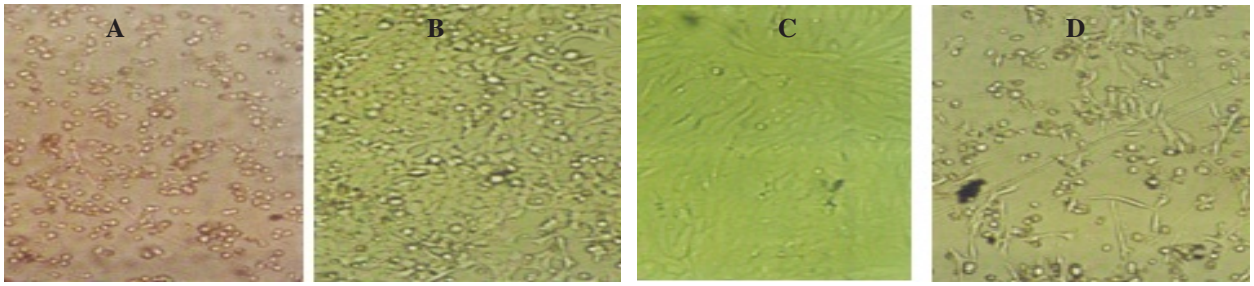

Figure 3. A) Normal HEp-2 cells. B) HEp-2 Cells Treated with Purified SFN Showing Massive Cell Apoptosis, C) Normal Vero cells, D) Vero Cells Treated with Purified SFN Showing Cell Apoptosis

Table 1. Percentage Viability of Std SFN Extract Extract Purified SFn on HEP-2 Cell Line

	Concentration microgram/ml	Percentage viability with SD	IC_{50} microgram/ml
Std sulforaphane	500.00	21.33±0.577	61.2
	250.00	31.66±0.577	
	125.00	43.00±1	
	62.50	51.00±1	
	31.25	60.66±0.577	
	15.62	66.33±0.577	
	7.81	79.67±1.15	
Extract	500.00	24.66±1.52	125.0
	250.00	40.66±1.527	
	125.00	50.33±0.577	
	62.50	60.00±1	
	31.25	67.33±1.527	
	15.62	80.66±1.527	
	7.81	88.66±2.081	
Purified sulforaphane	500.00	17.66±0.577	60.0
	250.00	30.33±0.577	
	125.00	40.66±0.577	
	62.50	52.33±1.527	
	31.25	66.00±1	
	15.62	76.66±1.527	
	7.81	87.00±1	

*The data shown are means, means ± SD of three independent expriments

Table 2. HEP-2 Cell Viability of SFN Extract and Purified Std

	Concentration microgram/ml	Percentage viability with SD	IC_{50} microgram/ml
Std sulforaphane	500.00	20.33±1.527	58.96
	250.00	32.33±1.527	
	125.00	46.33±1.527	
	62.50	53.00±1	
	31.25	62.00±1	
	15.62	68.00±1	
	7.81	74.00±1	
Extract	500.00	27.00±1	113
	250.00	40.00±1	
	125.00	55.33±0.577	
	62.50	63.00±1	
	31.25	72.00±1	
	15.62	82.33±1.527	
	7.81	90.00±1	
Purified sulforaphane	500.00	20.50±0.577	58.9
	250.00	34.50±1.527	
	125.00	44.50±1	
	62.50	55.00±1.154	
	31.25	68.00±2	
	15.62	83.50±1	
	7.81	91.00±0.577	

the probable mechanism of apoptosis and its pathway the key regulators of apoptosis such as p53, bax, bcl-2 and caspase expression were studied.

Western blot and RT PCR analysis

Monolayer cells were treated with SFN Fraction to study the expression of p53, bax and Casp-3 by western blot. Tubulin protein level, which was used as the loading

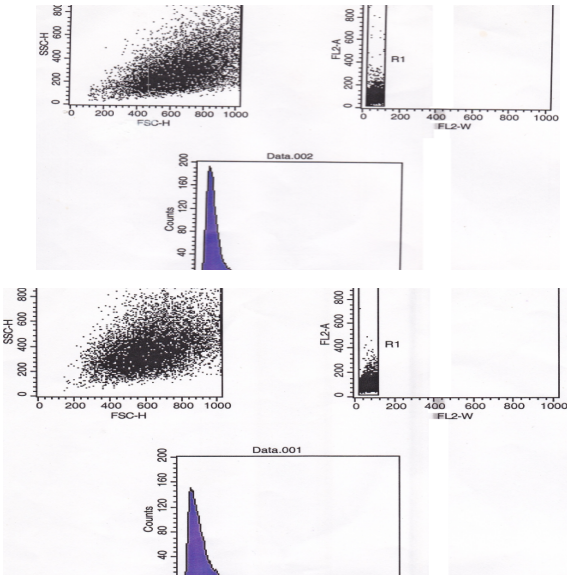

Figure 4. Cell Cycle analysis by Flow cytometry. A) Untreated HEp-2 cells, B) SFN Treated (62.5microgram/ml)

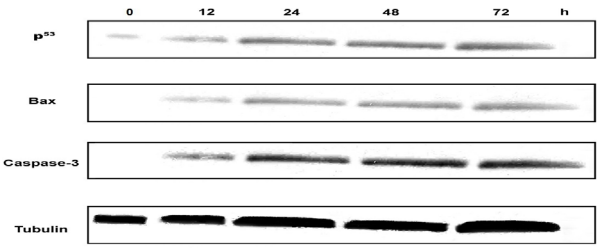

Figure 5. Effects of p53, Bax and Casp-3 in Sulforaphane Induced Cell Death. Cells were treated with 62.5microgram /ml purified sulforaphane for 12, 24, 48 and 72 h and then harvested for Western blotting analysis. Tubulin was used as a positive control

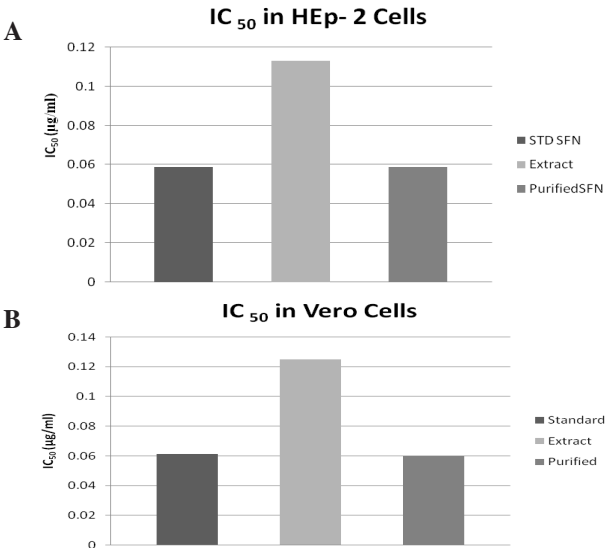

Figure 6. IC_{50} values of Percentage Viability Values of Std SFN, Extract, Purified SFN in HEp-2 Cells and Vero Cells. A) HEp-2, B) Vero Cells

control, remained unaltered under each tested condition. Western blot analysis revealed that SFN treatment increased the p53 protein concentration in HEp-2 cells. (Bonnesen et al., 2001; Talalay and Fahey, 2001; Riby et al., 2006; Jin et al., 2007). A marginal increase in pro-apoptotic signal such as bax concentration was also

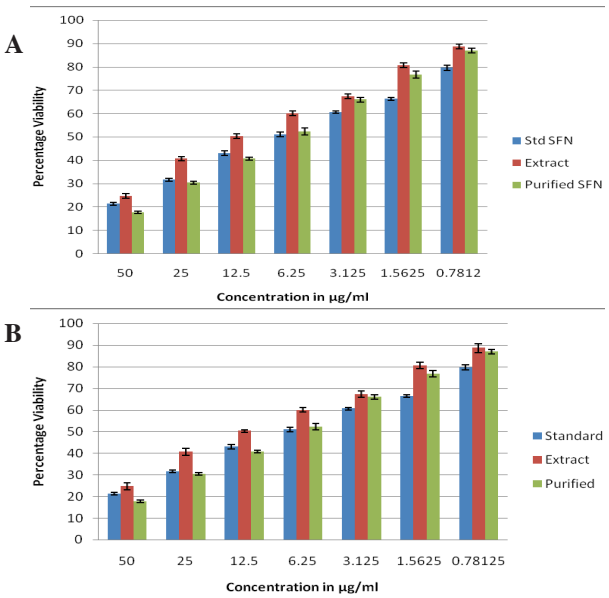

Figure 7. Percentage Viability for HEp2 and Vero Cells. A) Percentage Viability for HEp2 Cells. Statistical analysis by T-tests (a=0.05) showed the extract, pure fraction and standard has similar means in activity on HEp-2 Cells. The Significance of pure fraction to standard was more compared to extract to standard. B) Percentage Viability of Vero Cells. Statistical analysis by T-tests (a=0.05) showed the extract, pure fraction and standard has similar means in activity on Vero Cells. The significance of pure fraction to standard was more compared to extract to standard

observed which is coupled with down regulation of bcl-2 gene. Parallel amplification of GAPDH transcripts was used as a loading control for this experiment. Changes induced by SFN in bcl-2 and bax expression were associated with an increase in caspase-3 protein levels as shown in the blot study.

Discussion

Among the plant groups Brassica research continues to attain greater pace for the past 15 years (McNaughton et al., 2003; Han et al., 2007). Vegetables of the Brassicaceae family, in particular those of the Brassica genus (broccoli, cabbage, cauliflower, Red cabbage, mustard, etc.) received much attention, because of their anticancer activity in vitro and in vivo (Fimognari et al., 2002). Glucosinolates found in Brassica vegetables and their hydrolysis products like Indole and Isothiocyanates derivatives are considered to be cancer chemo-preventive agents, which act at several stages of carcinogenesis (Talalay & Fahey, 2001; Cawthon, 2002; Riby et al., 2006). They are known to inhibit the growth of cancer cells and to induce apoptosis, but the mechanisms are still only partially understood (Denoyelle et al., 2003). Previous studies have demonstrated that Indoles and Isothiocyanates from Brassica reported to have anticancer activity through p21 and down regulating the bcl-2 apoptotic pathways (Dashwood, 1988; Cawthon, 2002; Denoyelle et al., 2003; McNaughton & Marks, 2003; Yina et al., 2004; Pappa et al., 2006). Several investigations have attempted to characterize the pathways involved in the apoptotic responses in cancer cells exposed to indole derivatives, which are found in cruciferous vegetables. These studies found that such compounds have anti- carcinogenic activities and may affect many biochemical pathways (Brandi et al., 2005; Aamer et al., 2010). In this study, we investigated the anticancer activity of SFN isolated from Red cabbage in comparison with the standard by studying the antiproliferative activity of all the fractions in Vero and HEp-2 cell lines. SFN purified fraction found to have significant effect on inhibiting tumor cell growth. In addition to its tumor cell inhibition effect, SFN fraction showed a good ability in inducing cell apoptosis when compared to untreated cells. A reduction in cell growth and an induction in cell death are two major means to inhibit tumor growth (Firestone et al., 2003). Apoptosis is one of the important pathways through which chemo preventive and chemotherapeutic agents inhibit the growth of cancer cells (Ming et al., 2012). Over expression of bcl-2 aborts the apoptotic response while bax activity promotes cell death (Savli et al., 2004). Many reports suggest SFN could induce apoptosis mediated through p53, p21 pathways. Several studies have shown that bcl-2 family proteins is the central to apoptosis regulation. Over expression of bcl-2 aborts apoptosis response where bax, Bid, Bak activity promotes cell death (Steven et al., 2004; Myzak et al., 2006). The result revealed the enhanced expression of pro-apoptotic protein (bax) and down-regulation of anti-apoptotic protein (bcl-2) from RT PCR graph. Recent research also supported that Broccoli has exerted apoptosis through activated (PI3K/AKT) signaling pathway (Sreenivasa et al., 2001; Aamir Qazi et al., 2010). Our results displayed SFN from Red Cabbage fraction caused an enhanced expression of bax as well as triggering the down regulation of bcl-2 with a subsequent promotion of the apoptotic activity in HEp-2 cells. The present investigation has established an association between elevated expression of p53, bax, casp-3 and decreased expression of bcl-2 suggest their involvement in cell apoptosis cascade.

In conclusion, here in our study we demonstrated that the effects of the SFN fraction from Red cabbage were found to have significant anticancer activity. Standard and Purified SFN found to have closer IC_{50}, were found to have significant cytotoxic activity HEp-2 cells. Cell cycle analysis revealed the higher percentage of apoptotic cells when compared to control. Further insight to the mechanism of signaling molecules involved in apoptosis revealed that activated p53 caused up-regulation of bax, Casp-3 and downregulation of bcl-2 proteins modulated signal transduction. This may be a therapeutical insight in the treatment of cancers using Brassicaceae family.

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