Chapter 5

DISCUSSION
Cancer is the leading cause of mortality worldwide and the new approaches for its treatment are critically needed. “Carcinogenesis is a multistep process regulated by multiple signaling pathways and is the target of numerous anticancer therapies. Many of the cellular pathways overlap, showing a high degree of redundancy within the system. Therefore, targeting a single molecule might ultimately have little or no effect, resulting in the need for either combination therapy or multitargeted therapy. Nutraceuticals are inexpensive, safe, and readily available and have multitargeted potential and have thus drawn considerable attention during the past” (Gupta et al., 2010). “Phytochemicals are able to contribute to cancer prevention by acting at different stages of the carcinogenic process, from tumor initiation through all the hallmarks of cancer i.e. cell proliferation, apoptosis, invasion and metastasis, angiogenesis, immortality, inflammation, immunity, genome instability, mutations, cell energetic and metabolism. Although, chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, but their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention.” Among the most extensively investigated phytochemicals, the hydrolytic products of glucosinolates, which occur naturally in a variety of cruciferous vegetables including cabbage, broccoli, and mustard needs to be explored for their potential as anticancer agents (Asakage et al., 2006). “The vegetables are good source of antioxidants that may be more effective and economical than the supplements available for protecting body against oxidative damage (Cohen, et al., 2000)”. Broccoli is rich source of glucosinolates that are known to be ineffective in their intact form, whereas their hydrolytic products reduce the risk of cancer progression.

“Many epidemiological studies demonstrated that increased consumption of cruciferous vegetables rich in isothiocyanates is associated with reduced incidence of human cardiovascular disease, atherosclerotic coronary heart disease and inflammatory and oxidative stress-related disorders (Nallasamy, et al., 2014)”. The health promoting effects of diet rich in Brassica vegetables have been known for several decades and seem to be a promising starting point for the development of chemopreventive and anti-inflammatory functional foods, dietary supplements, and drugs. “Sulforaphane is a key and active isothiocyanate that is present in large amounts in Brussels sprouts and has been suggested to exert cardiovascular protective effects (Nallasamy, et al., 2014)”.

Discussion
cancer cells contain multiple aberrant signaling pathways that lead to drug resistance and therapy failure in many patients. The combination therapy is known to kill cancer cells more effectively through diverse mechanisms simultaneously. Isothiocyanates (ITCs) exhibit a diverse range of cellular targets for anticancer effect. This property of ITCs makes them highly desirable for combinatorial therapeutic approaches. Several combination strategies have been tested in preclinical studies by combining ITCs among themselves or with conventional or new anticancer therapies (Gupta, et al., 2014). There is now a wealth of evidences regarding the protective effects of isothiocyanates via induction of cytoprotective enzymes, inhibition of inflammatory processes, and modulation of signaling pathways that are among the many diverse pharmacological outcomes of their exposure (Dinkova-Kostov and Kostov, 2012). “They have also been shown to exert their antitumor effects through multiple targets. Because cancer development is driven by several signaling pathways therefore multitargeted therapies are theoretically more efficient and could potentially evade the drug resistance that occurs when cancer cells develop new mutations” (Gonzalez -Vallinas et al., 2013). Chou et al., (2013) reported that “The use of multiple drugs may target multiple targets, multiple subpopulations, or multiple diseases simultaneously. The use of multiple drugs with different mechanisms or modes of action may also direct the effect against single target or a disease and treat it more effectively. The possible favorable outcomes for synergism include 1) increasing the efficacy of the therapeutic effect, 2) decreasing the dosage but increasing or maintaining the same efficacy to avoid toxicity, 3) minimizing or slowing down the development of drug resistance, and 4) providing selective synergism against target (or efficacy synergism)”.

Among the different mechanisms known for the plants extracts/products to exert their anticancer effects, one of the important mechanisms is by scavenging the free radicals or by enhancing the antioxidant defense system. Keeping in view the potential therapeutic importance of hydrolytic products of glucosinolates coupled with the fact that there is no report on the degree of variability in its bioactivities at different developmental stages of broccoli, the present study was planned to explore its different varieties for their anticancer potential along with antioxidant and anti-inflammatory activities.
“The extracts of Palam Samridhi (PS) and Palm Vichitra (PV) seeds, sprouts (3, 5 and 7 day), leaves and florets in addition to Punjab 1 seed and sprouts (3, 5 and 7 day) of *B. oleracea* italica varieties were obtained by method of Liang *et al.*, (2006). The different extracts were evaluated in different *in vitro* antioxidative assays before carrying out their anticancer and anti-inflammatory activities. The anti-radical effects was done using *i.e.* molybdate ion reduction assay for total antioxidant capacity, DPPH free radical scavenging assay and superoxide anion radical scavenging for hydrogen or electron donating potential and plasmid nicking assay for hydroxyl radical scavenging potential (Chaudhary *et al.*, 2014).”

**Chemical Characterization and Isolation**

The different varieties of *Brassica oleracea* are known to be rich in glucosinolates as well as other orangosulphuric compounds. The seeds, sprouts (3, 5 and 7 day) of Palam Samridhi, Palam Vichitra and Punjab 1 varieties and Leaves and Floret extracts of Palam Samridhi and Vichitra varieties were explored for the presence of different phytochemical by Gas Chromatography Mass Spectroscopy. The analysis of different extracts showed a wide variation in the hydrolytic products of glucosinolates as well as other phytochemicals. Our results showed the presence of 3-butenyl isothiocyanate in all the extracts of different varieties except PS5, PS7, PVS, PV5, PVL and P17 of *Brassica oleracea* italica. The organo sulphuric compounds were also present in different extracts. “The individual component of different plant extracts was identified by comparison of their mass spectra (MS) with NIST database and Adams libraries (NIST/EPA/NIH, 1998 and Adams RP, 2004)”. Literature survey has revealed “that cruciferous sprout are rich in many sulfur-containing compounds, such as allyl methyl sulfide and allyl propyl sulfide which act as strong antiproliferative agents” (Brandt, *et al.*, 2004). “A similar study was conducted by Jirovetz *et al.*, (2002) for the aroma compound analysis of *Eruca sativa* (*Brassicaceae*) SPME Headspace of leaf samples using GC, GC–MS, and olfactometry and identified the compound *viz*., 4-methylthiobutyl isothiocyanate (14.2%), cis-3-hexen-1-ol (11.0%), cis-3-hexenyl butanoate (10.8%), 5-methylthiopentyl isothiocyanate (9.3%), cis-3-hexenyl 2-methylbutanoate (5.4%), and 5-methylthiopentanenitrile (5.0%) in higher concentrations”. Likewise, Chiang *et al.*, (1998) found that under split/splitless conditions using a gas chromatography/mass spectrometry (GC/MS) system operated
revealed that ≈80% of sulforaphane was degraded to 3-butenyl isothiocyanate. Among three varieties used in the present study, the extract of variety Palam Samridhi seeds (PSS) was subjected to column chromatography for further fractionation. After fractionation, the fractions with similar Rf values in TLC were pooled and identified by chromatographic and spectroscopic techniques such as FTIR (Fourier Transformation Infrared Spectroscopy), NMR (\textsuperscript{1}H, \textsuperscript{13}C & Dept-135), mass and three fractions/compounds (coded as ASM2, ASM3 and ASM5) were isolated. The preliminary identification led to the elucidation of three compounds viz. ASM2 (Methanesulfinic acid (6-hydroxyl-pentyl)-amide), ASM3 (N-(4-methanesulfinyl-2-methyl-butyl)-acetamide and ASM5 (1-Amino-4-metanesulfinyl-butan-1-ol).

**In vitro Antioxidatives Studies**

“A living system cannot escape from free radicals as they are byproducts of normal metabolic processes of body or can be produced through external factors such as pollution, food, stress and radiations. As free radicals are highly unstable due to the presence of unpaired electrons in their outer orbital, they can cause damage to different cellular components such as DNA, protein, amino acids and cell membranes by stealing their electron through a process called oxidation. Hence, these important cellular components lose their ability to function normally and thereby the risk of disease occurrence increases. Plants provide a rich source of compounds with promising antioxidant, cancer preventive and cancer therapeutic potential. Epidemiological studies have suggested that the content of phytochemicals such as polyphenols and glucosinolates derived products that are predominately present in broccoli help in reducing the risk of cancer through their role in the metabolism of carcinogens, in regulation of antioxidant enzymatic activities and in scavenging of free radicals (Fahey and Talay, 1999)”.

The molybdate ions reduction ability of extracts was measured by taking ascorbic acid as standard. The tendency of different extracts to reduce molybdate ions in phosphomolybdenum complex was expressed in terms of number of Ascorbic Acid Equivalents (AAE) in mg / 100mg weight of extract as calculated from the standard curve obtained for ascorbic acid. Thus, it was observed that the extracts of broccoli exhibited the good reduction ability in terms of AAE/ 100mg dry weight of extract. Our results
indicated the variations in the antioxidant capacity for different developmental stages of the plants. In molydate ion reduction assay (total antioxidant capacity) the activity was found to be highest in floret extract of Palam Samridhi (PSF) i.e. 30.05 mg in terms of AAE/ 100mg dry weight of extract (Chaudhary et al., 2014). In case of Palam Vichitra, the extract of leave (PVL), exhibited the highest reduction ability of 23.85 mg in terms of AAE/ 100mg dry weight at 125µg/ml concentration. In case of Punjab 1, the extract of seeds (P1S) exhibited the highest reduction ability of 17.94 mg in terms of AAE/ 100mg dry weight of extract at 125µg/ml concentration. Gülçin, et al. (2004) also studied the “water and ethanol extracts of broccoli florets for in vitro anti-oxidant properties: total antioxidant activity, reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activities and found that both extracts exhibited strong total antioxidant activity”.

“The 2, 2’-diphenyl- 1-picryl hydrazyl (DPPH) assay is also the most commonly used method for evaluation of hydrogen donating potential of extracts due to its simplicity, less duration and accuracy. DPPH assay involves the use of commercially available radical i.e. DPPH. This is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen to become a stable diamagnetic molecule (Jamuna et al., 2010)”. “The methanolic solution of DPPH is violet in color due to delocalization of the spare electron over the molecule as a whole. The solution of DPPH is mixed with a substance that can donate a hydrogen atom or electron, the color of the DPPH solution changes to pale yellow due to formation of corresponding hydrazine. The degree of decolorization indicates the radical scavenging potential of the extract in terms of hydrogen donating activity (Rajan et al., 2011)”. All the extracts showed more or less equal activity in DPPH assay, though the level of activity was not very pronounced at low concentrations. It was observed that the floret extract of Palam Samridhi (PSF) at high concentration of 2 mg/ml exhibited only 29.07%, floret extract of Palam Vichitra (PVF) showed 30.76% and P17 showed 16.37 activities. Our finding is in concordance with work done by Guo et al., (2001) who also “reported that methanolic extract of broccolis showed antioxidant activities only at higher concentrations (0-20 mg/ml)”. Furthermore, Guo et al., (2001) showed that methanol and water extracts of flower, stem and leaves of broccoli exhibited a higher reducing power while acetone extract was least effective.
“Buckwheat was extracted using methanol, acetone, butanol, ethanol, and ethyl acetate (Sun & Ho, 2005). The methanol extract showed the highest antioxidant activity tested by the β-carotene bleaching method and the acetone extract showed the highest antioxidant activity determined by the DPPH method”. Thus, the properties of the extracting solvents significantly affected the antioxidant activity of extracts. Jahan et al., (2010) “has also reported that methanol extract of broccoli (flower and stem) exhibited high DPPH radical scavenging activity as well as ion chelating activity”. “The ethanolic extract was more active than the aqueous extract with IC$_{50}$ values of 0.44 and 2.13 mg/ml, respectively (Bidchol et al., 2011)” Kaur and Kapoor (2002) analyzed “the antioxidant activity of extracts from 34 vegetables and also concluded that broccoli belongs to the group of vegetables with a high antioxidant activity (>70%)”. Sun et al. (2007) reported that, “methanol and acetone extracts had higher antioxidant activity than water extracts of asparagus, broccoli and their juices, because flavonoids are major antioxidants in both vegetables and are more soluble in methanol and acetone than water. Thus water alone is not a good solvent to extract the antioxidants of asparagus or broccoli”. Moure et al. (2000) used “five solvents viz. water, methanol, ethanol, diethyl ether and acetone for extraction of Gevuina avellana hulls and found that ethanol and methanol extracts showed the highest antioxidant activity while the water extract had the lowest antioxidant activity determined by DPPH method”. Vinson et al., (1998) concluded that “antioxidant activity depends more on which vegetable the polyphenols were extracted from rather than on which solvent system was used for extraction”. Therefore, solvent extraction as well as concentration has impact on scavenging activity of the extract. According to Fahey and Talalay (1999), “ITCs are unlikely to have direct antioxidant activity because of the central carbon atom of the N=C=S group which reacts readily with sulfur-, nitrogen- and oxygen-based nucleophiles. Their overall protective effect has been generally attributed to the modulation of xenobiotic metabolizing enzymes and enzymatic antioxidative defense system. However, whether these effects can mainly be ascribed to single class of molecules, e.g., GLs or ITCs, or to their complex interactions with other phytochemicals present in the whole vegetable still remains unknown”. “Erucin does not possess any chain breaking antioxidant activity” (Barilleri et al., 2005). Beevi et al., (2010) showed that “chloroform and hexane extracts of Raphanus sativus
displayed the weakest activity with IC$_{50}$ of over 1.0 mg/ml”. Farag and Motaal, (2010) reported that chloroform extracts of red cabbage and chinese kale exhibited good DPPH radical scavenging activity with percentage inhibition values of 73 and 54 respectively at highest dose i.e. 10mg/ml. But other extracts like Turnip, Brussels sprouts and broccoli have weak to moderate antioxidative effect. They ascribed this activity to the presence of anthocyanins, tocopherol and flavonoid. However, no radical scavenging activity was seen even in case of sulforaphane at the dose of 10 mg/ml. Papi et al., (2008) observed the “raphastin (4-methylthio-3-butenyl isothiocyanate) to be good in quenching DPPH radicals followed by 2,6-di-tert-butyl-4-methoxyphenol (BHA) or 2,6-di-tert-butyl-4-methylphenol (BHT) (common synthetic phenolic antioxidants)”. Similarly, Hamed et al., (2007) reported that “Capparis deserti did not show antioxidant activity at low concentrations in DPPH radical scavenging assay”. On the contrary, it has been reported that methylthioalkyl ITCs exhibit good antioxidant activity which has been explained by the fact that direct radical scavenging ability can be attributed only to and cannot be considered a universal property for all ITCs. Morimitsu et al., (2002) purposed the hypothesis that the oxidation state of sulphur and the number of methylene groups is the link between the thiomethyl and ITC moieties for observed activity of methylthio ITCs. Valgimigli and Iori (2009) suggested that radical scavenging ability of ITCs is due to side chain rather than N=C=S group.

“Superoxide anion plays an important role in the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induces oxidative damage in lipids, proteins and DNA (Halliwell and Gutteridge 1989; Pietta 2000)”.”The superoxides are also known to initiate lipid peroxidation indirectly as a result of H$_2$O$_2$ formation, creating precursors of hydroxyl radicals (Meyer and Isaksen 1995)”. The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce nitroblue tetrazolium (NBT). It was noticed that all the extracts exhibited pronounced scavenging activity of hydrogen peroxide in an amount-dependent manner. In the present study, Palam Samridhi sprouts extract (PS3) showed superoxide anion scavenging ability of 94.25% at 2mg/ml concentration (Chaudhary, et al., 2014). In case of Palam Vichitra superoxide anion scavenging ability of 5 day sprouts (PV5) extract was 81.38% at 2mg/ml concentration.
In Punjab 1 variety, it was noticed that at 2mg/ml concentration, P17 showed inhibition of 87.11%. Similarly, Harish and Chauhan, (2014) showed “the inhibition of superoxide anion and metal chelation of aqueous and ethanolic extracts of brussel sprouts with IC$_{50}$ concentration of 0.5 mg/ml, 0.6 mg/ml and 0.2 mg/ml, 0.3 mg/ml respectively”. Beevi et al., (2010) showed that the superoxide radical (O$_2^•$) scavenging ability of *Raphanus sativus* chloroform extract (leaves and stem) showed (0.80 and 0.96 mg/ml) IC$_{50}$ and hexane extracts (leaves and stem) showed (1.85 and >2 mg/ml) IC$_{50}$ respectively. Bidchol, et al., (2011) reported that “*Brassica oleracea* L. var. *italica* effectively scavenged superoxide in a concentration-dependent manner with IC$_{50}$ values for the aqueous and ethanolic extracts was 0.93 and 0.25 mg/ml, respectively. They also concluded that ethanolic extract of *Brassica oleracea* L. var. *italica* exhibited higher antioxidant activity in DPPH radical and superoxide anion scavenging assays than that of aqueous extract”. Zhou and Yu (2006) showed that “tomato samples quenched about 25–73% of radical O$_2^•$ in the reaction mixture, while the potato, carrot, green bean, broccoli, spinach, and kale extracts quenched 15–36%, 24–29%, 38–39%, 53–55%, 49–61%, and 72–76% O$_2^•$ in the system under the same testing conditions”.

Hydroxyl radicals are considered to be one of the most detrimental free radicals owing to its high reactivity. It can react with almost all the biomolecules (Agnihotri and Mishra, 2009) and cause DNA damage (Balasubramanian et al., 1998), protein oxidation (Leeuwenburgh, et al., 1989) and DNA-protein crosslink (Dizdaroglu et al., 1992). In biological systems, hydroxyl radicals are produced by phagocytosis and prostaglandin biosynthesis. However, Fenton’s reaction has been used most widely and successfully for more than a century for *in vitro* generation of hydroxyl radicals. In a Fenton’s reaction, FeCl$_3$ react with H$_2$O$_2$ and forms `OH radicals. “It has been found that in this assay, when the plasmid DNA (pBR322 DNA) was dissolved in the fenton’s reaction mixture, there was a time dependent increase of single stranded and double stranded nicked and linear forms of DNA (Form II and Form III respectively) due to hydroxyl radicals generated in reaction mixture as reported in our own study (Chaudhary et al., 2014)”. The hydroxyl radicals generated in above reaction act as potent oxidant and react with the various hydrogen atoms of the deoxyribose of the DNA duplex resulting in oxidative damage of DNA molecules (Balasubramanian et al., 1998). Plasmid nicking assay is a sensitive
method for detection of antioxidant activity of extracts which can act against hydroxyl radicals generated via fenton reaction and thus protect the supercoiled DNA from the damaging effects of OH radicals. The three topologically different forms of supercoiled DNA (Form I), open circular DNA (Form II) and linear DNA (Form III) can be separated and analyzed by agarose gel electrophoresis on the basis of their differential mobilities. In the our study, it was found that extract Palam Samridhi, Palam Vichitra, Punjab 1 were more or less capable to maintain the integrity of supercoiled DNA indicated their protective effects at concentrations of 200 µg/ml against the DNA damage caused by hydroxyl radicals. It was seen that the amount of percent supercoiled DNA, in the presence of Palam Samridhi extracts was 82.1%, 51%, 62.2%, 48.7%, 46.3%, 48.5%, 49% and 82.1% in case of PSS, PS3, PS5, PS7, PSF, PSL and rutin (positive control) respectively (Chaudhary et al., 2014). Plasmid nicking assay showed that extract of seeds was least effective and three days sprouts (PS3) observed to have more pronounced activity as compared to other extracts at same concentration. The amount of supercoiled DNA, in the presence of Palam Vichitra extracts was 34.9%, 50.9%, 64.4%, 61.9%, 47.1%, 49.1% and 69.7% in PVS, PV3, PV5, PV7, PVF, PVL and rutin (positive control) respectively. In case of Punjab 1 the extracts recovered the supercoiled DNA by 48.1%, 39.9%, 47.2%, 49.7% and 50.4% in P1S, P13, P15, P17 and rutin (positive control) respectively. Manesh and Kuttan (2003) studied the hydroxyl radical scavenging effect of allyl and phenyl ITCs. Both of these ITCs were found to scavenge the hydroxyl radicals effectively in deoxyribose degradation assay. Allyl ITC was found to induce 61.9% inhibition of hydroxyl radical production and phenyl ITC by 69.7% at the concentration of 25µg/ml. Kwon et al., (2006) reported that “the closed circular form of plasmid pUC19 was converted efficiently to the nicked linear form after treatment with Fe^{2+}/H_2O_2, and in same experiments the relaxed form also was detected as a minor band. Each of the plant extracts attenuated the formation of nicked DNA in in vitro system. For example, when the linear form was normalized to the corresponding control treated with Fe^{2+}/H_2O_2 but no plant extract, Angelica keiskei, inhibited DNA nicking by ~50% at 20 µg/mL, Oenanthe javanica inhibited by >70% and Brassica oleracea inhibited by 40%”. Similarly, Kaur et al., (2013) reported that Lepidium latifolium “extracts showed ability to quench free radicals which is rich in glucosinolates in three in vitro assays namely

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DPPH, $O_2^-$ scavenging activity and $OH^-$ scavenging activity. All the extracts showed a remarkable radical scavenging with their highest activity observed in superoxide radicals. Results on plasmid DNA showed significant amount of DNA protective activity among the top leaf extracts of *Lepidium latifolium*. They observed nicked and linear forms are clearly visible where extracts have been added, in comparison to the control samples where no extracts have been added (Kaur et al., 2013).

A critical analysis of results of antioxidant assays showed that the antioxidant capacity of extracts of different varieties (Palam Samridhi, Palm Vichitra and Punjab 1) at different developmental stages was weak in DPPH assay. However, some extracts showed good hydroxyl radical scavenging activity in plasmid nicking assay. This activity was due to the presence of large number of phytochemicals in addition to ITCs. There is no universal criteria for presence or absence of antioxidant activity of different ITCs. Therefore, it is imperative that the antioxidative potential of extracts should be evaluated by different antioxidative methods because of diverse mechanism of action. “The results obtained in the *in vitro* models clearly suggest that, *Brassica oleracea* L. var. *italica* is a natural source for antioxidants, which could serve as a nutraceutical with potential applications in reducing the level of oxidative stress and related health benefits”. However, till date, no study except ours had considered the degree of variability in antioxidant and anticancer potential with respect to different developmental stages.

*In vitro anticancer Studies*

“The cytostatic effect of extracts on cancer cells was often much better than the effect of their particular biologically active compound individually (Vickers, 2002; Yano et al., 1994)”. “Chemotherapeutic drugs are known to induce cytotoxicity in tumor cells through diverse mechanisms, in which signaling events play an important role depending upon the cell type and stimulus. There is an utter need to find new anti-cancer drugs that can kill cancerous cells with minimal toxicity. The major approach in searching the potential anticancer agents over the last 50 years has been based on selective cytotoxic effects on mammalian cancer cell lines (Hoshino et al., 1991)”.

The present study was aimed to investigate the effect of broccoli extracts on PC-3 cells. The viability of cells was assessed by MTT assay and it was seen that the extract exerted dose-dependent cytotoxic action on malignant cell line. “The plant constituents
exhibited antiproliferative effect by the stimulation of carcinogen detoxifying enzymes, scavenging of free radicals, anti-inflammatory activity, cell cycle arrest and triggering of apoptosis. The anti-proliferative activity was detected by MTT assay, a colorimetric method for determining the number of viable cells (Matic et al., 2013b). In the range of the doses used (10-100μg/ml), the crude sprouts, seed, leaves and floret extracts of broccoli showed anti-proliferative activity on the human prostate cancer (PC-3) cells. Our results demonstrated a significant dose-dependent and very good anti-proliferative effect on the human prostate cancer cells (PC-3) in the presence of different extracts. It was noticed in the present study that although, all the extracts showed significant anti-proliferative effect on PC-3 cells but the floret extract of Palam Samridhi (PSF) exhibited comparatively more effect with low IC$_{50}$ concentration. Likewise, Charoensin, (2014) showed “the antiproliferative activity of Moringa oleifera leaves (methanol and dichloromethane extracts) on HepG2, Caco-2, MCF-7 and human fibroblasts cell. Dichloromethane extract was found to be more cytotoxic than methanol extract. It showed IC$_{50}$ value of 120.37±2.55, 112.46±3.74 and 133.58±2.47 μg/ml in HepG2, Caco-2 and MCF-7 cell lines, respectively, while methanol extract exhibited less cytotoxicity to all cancer cell lines (IC$_{50}$ > 250 μg/ml)”. Tang et al., (2006) also showed that isothiocyanate-rich broccoli sprout extracts inhibited cancer cell growth very effectively. Yeh et al., (2006) studied “the influence of various Se compounds and Se-enriched broccoli extract on C6 cell proliferation by MTT assay and found significant growth inhibition of C6 cells at the concentration as low as 62.5 nM for 48 h and the increase in Se concentration did not further inhibit C6 cell proliferation”. Glucosinolates and their hydrolytic products are known to protect against cancer and upregulating detoxification enzymes and is reported to act against various types of cancers (Fahey et al., 1997; Vig et al., 2009). The results of the studies in literature revealed that broccoli have some potent antigenotoxic compounds (Anupama et al., 2008 and Rampal et al., 2010). The results of previous study (Jakubikova et al., 2005), “according to which the purified iberin was found to inhibit the growth of human colon carcinoma Caco-2 cells by inducing apoptosis”. “The literature studies revealed the lowest effective dose for HT-29 colon carcinoma cells was 0.25g eq ww/ml for broccoli and 0.50g eq ww/ml for cauliflower and brussels sprouts” (Ferrarini et al., 2012). “It is also reported to upregulate the
thioredoxin reductase1 expression in human MCF cells suggesting a role in maintenance of redox in cell homeostasis” (Wang, et al., 2005). “The treatment of neuroblastoma cells with iberin resulted in a dose- and time- dependent inhibition of growth, increased cytotoxicity, and G₁ or G₂ cell cycle arrest depending upon dose and cell type” (Jadhav et al., 2007). It was observed in our previous study that IC₅₀ values increased with increase in extract concentration both in seed extracts of Palam Samridhi (PS) and Palam Vichitra (PV). The minimum IC₅₀ value was found in PV at 87.25 µg/ml in cell line Colo-205. The PV extract also showed IC₅₀ of 131.4 µg/ml, 2090 µg/ml and 253 µg/ml in OVCAR-5, MCF-7 and PC-3 cell lines respectively. In case of PS extract the minimum IC₅₀ value was to be 221.5 µg/ml in cell line Colo-205. The PS extract also showed IC₅₀ value of 331.9 µg/ml, 1657.2µg/ml and 563.5 µg/ml in OVCAR-5, MCF-7 and PC-3 cell lines respectively. The positive control adriamycin (1 µM) showed % growth inhibition i.e. OVCAR-5 (60%), Colo-205 (59%), MCF-7 (72%) and PC-3 (66%) (Chaudhary et al., 2012). Jaing et al., 2003) studied “the effect of three detoxifying enzyme inducers, tert-butylhydroquinone (tBHQ), β-naphthoflavone (β-NF), and sulforaphane (SUL), which are potential chemopreventive compounds on seven well-established mammalian cell lines, which have different origins. Among tested mammalian cell lines, the mouse hepatoma Hepa1c1c7 cells were the most robust and sensitive cells, which had higher basal as well as upregulated enzymatic activities. In human cell lines, the prostate LNCaP and hepatic HepG2 cells were also very responsive to the inducers”.

The different extracts and isolated compounds were also evaluated for their morphological and nuclear changes at their respective IC₅₀ values in PC-3 cell line stained with 4’,6-diamidino-2-phenylindole (DAPI) using confocal microscope. DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA. The presence of apoptotic bodies in the cells treated with positive control and extracts/compounds further points out towards the fact that these extracts/compounds triggered cell death via apoptosis. The confocal microscopy revealed that among the different extracts of Palam Samridhi (PS), PSS and PSF showed comparatively more changes in morphology typical of apoptosis as compared to other extracts. The confocal microscopy revealed that among the different extracts of Palam Vichitra i.e. PVS and PV3 showed more visible features of apoptosis as compared to other extracts. In case of Punjab1 (P1), the extracts P1S and
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P13 were found to be effective in inducing the death via apoptosis as compared to other extracts. Among the compounds isolated from PSS, the compound ASM3 caused more cell shrinkage and cell rounding, followed by ASM5 and ASM2 as observed under phase and confocal microscope. Furthermore, the characteristic differences between control and treated PC-3 cells were also observed with scanning electron microscope (SEM). The treated cells as compared to control were observed to be less adherent, lost polygonal shape, significant shrinkages and a few prominent surface blebs, the characteristics of apoptosis. Similar studies in literature (Ramasamy et al., 2013; Kiechle & Zhang, 2002; Elango, et al., 2011) described cell rounding, cell shrinkage, chromosomes condensation and cell morphological changes after staining with different dyes PI, DAPI, Rhodamine 123 and DCFDA. Tang, et al., (2006) described that “broccoli sprout extract rich in isothiocyanates disrupt mitotic spindles and cause M-phase arrest in mammary carcinoma cells, presumably by inhibiting tubulin polymerization”. Thus, mitotic spindles were visualized under fluorescence microscopy after immunostaining of α-tubulin with an FITC-tagged antibody and nuclear DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI). Abdulah et al., (2009) “observed clear morphological changes on LNCaP prostate cancer cells when treated with selenium-enriched broccoli sprout extract and induced cell death more effectively than control”.

“The apoptosis is a molecular linkage between cell death and cell cycle which has become an object of great concern research in recent years. Cancer cells undergo apoptosis either by generation of free radicals or by depletion of endogenous antioxidants. The regulation of the cell cycle involves processes crucial to the survival of cell as well as the prevention of uncontrolled cell division. The unregulated cell proliferation leading to cancer and differential sensitivity to apoptosis is linked to the distinct phases of the cell cycle (Pal, et al., 2010 and Buttke and Sandstrom 1994)”. It was observed that the PC-3 cells treated with plant extracts/compounds at their corresponding IC\textsubscript{50} values for 12 h restricted the cells in hypo-diploid (sub G\textsubscript{0}) phase of the cell cycle as compared to the control. The specific characteristics of cell apoptosis like DNA fragmentation, induced by extracts lead to the appearance of hypo diploid cells (sub G\textsubscript{0} population). The phase of the cells with DNA content less than in G\textsubscript{1} phase of the cell cycle are counted as the hypo diploid cells. The sub G\textsubscript{0} population of cells was 25.7%
in control cells, which increased to 75.0% for standard camptothecin at the IC\textsubscript{50} value. Among all the Palam Samridhi, extracts PS5 showed highest percentage of sub G\textsubscript{0} population \textit{i.e.} 64.4%. The compounds isolated from PSS extract showed a dose dependent increase in sub G\textsubscript{0} population. It was seen that ASM3 compound had 32.1\% (IC\textsubscript{30}), 40.9\% (IC\textsubscript{50}) and 42.4\% (IC\textsubscript{70}) DNA in sub G\textsubscript{0} phase. The ASM5 compound had 28.1\% (IC\textsubscript{30}), 34.9\% (IC\textsubscript{50}) and 40.1\% (IC\textsubscript{70}) DNA content in sub G\textsubscript{0} phase and ASM2 compound showed 23.1\% (IC\textsubscript{30}) 31.7\% (IC\textsubscript{50}) and 36.4\% (IC\textsubscript{70}). It was observed that the cells treated with PVS extracts have highest percentage of DNA content in sub G\textsubscript{0} population \textit{i.e.} 77\%. In case of Punjab 1, the cells treated with P17 have 69.4\% of DNA content in sub G\textsubscript{0} population. All the extracts elevated the DNA content in sub G\textsubscript{0} population as compared to control. It has also been observed that all compounds arrested the cells in G2/M phase of cell cycle in dose dependent manner. “Literature survey has revealed that cell growth and proliferation of mammalian cells are dependent on cell cycle progression” (Schwartz & Shah, 2005). Hu \textit{et al.,} (2010) has also reported that anti-cancer “agents arrest the cell cycle at the G\textsubscript{0}/G\textsubscript{1}, S, or G\textsubscript{2}/M phase and then induce apoptotic cell death”. “The cell cycle arrest has become an appreciated target for management and treatment of tumor cells with cytotoxic agents (Schwartz & Shah, 2005)”. “The fluorescence intensity of sub G\textsubscript{0} cell fraction represented the apoptotic cell population (Krishan, 1975, Pal \textit{et al.,} 2010 and Jain \textit{et al.,} 2013)”. The current study showed that apoptotic cell death increased significantly after treatment with extracts for 12 h and all extracts showed increased sub G\textsubscript{0} DNA fraction in PC-3 cells. All the extracts have distinct G\textsubscript{2}/M phase, which might arrest the cells in this phase as compared to camptothecin and untreated cells. Pervious findings revealed that “benzyl isothiocyanate (BITC) induces G\textsubscript{2}/M phase arrest and apoptosis in human melanoma A375.S2 cells through reactive oxygen species (Huang, \textit{et al.,} 2012)”. Likewise, Stan \textit{et al.,} (2013) showed that “PEITC treatment (10 \textmu mol/L) led to a statistically significant increase in the fraction of MIAPaca2 cells in G\textsubscript{2}/M phase as compared with DMSO-treated (control) cells. The G\textsubscript{2}/M arrest was associated with decrease in the percentage of cells in G\textsubscript{0}/G\textsubscript{1} phase and an increase in the percentage of cells in SubG\textsubscript{0}/G\textsubscript{1} and S phases. They further confirmed the mitotic arrest induced by phenethyl isothiocyanate (PEITC) by determining the levels of (Ser 10) phospho-histone H3, a marker for mitotic cells via
Western blotting (Hendzel et al., 1997). PEITC treatment caused a dose-dependent increase in the level of phospho-histone H3 (Ser10) in PEITC-treated MIAPaca2 cells. Moreover, flow cytometric analysis showed a significant increase in the fraction of subdiploid (apoptotic) cells after exposure to 10 μmol/L PEITC for 24 h”. de-Oliveira et al., (2014) also reported that “sulforaphane (SFN) increased the percentage of cells in the G2/M phase and decreased the percentage of cells in G1/G0 in a dose dependent manner. Under the conditions tested, SFN treatment significantly downregulated the expression of the Chk1 kinase-encoding gene and also decreased the expression of CDC25C, encoding the M-phase inducer phosphatase. Moreover, a non significant decrease in the expression of the cyclin B1-encoding gene CCNB1 was observed for the SFN-treated cells. SFN was also found to upregulate the expression of CDK1. Among all the markers tested, the most pronounced effect observed in MG-63 cells upon SFN treatment was downregulation of CDC25C”.

“In addition to above mechanisms, apoptosis induction is also caused by reactive oxygen species (ROS) which play an important role in depolarizing mitochondria. The 2,7 dichlorofluorescein diacetate (DCFH-DA) a non-fluorescent cell-membrane permeable probe, reacts with cellular esterase/ROS and then metabolized into fluorescent DCF (Royall and Ischiropoulos 1993)”. It was observed that the PS7 extracts showed 31.0% DCF fluorescence respectively at IC70 value. The results revealed the concentration dependent increase in DCF positive cell population, thus indicating the generation of reactive oxygen species. Treatment with camptothecin at different concentrations was found to enhance ROS generation by 20.1%, 27.9% and 36.1% at their respective IC30, IC50 and IC70 values respectively as compared to low DCF fluorescence (7.1%) observed in untreated PC-3 cells as analyzed by flow cytometer. The isolated compounds from Palam Samridhi were also tested at different concentrations for ROS generation by spectrofluorometry at 485/20 nm excitation and 528/20 nm emission wavelengths. The DCF formation was expressed as fluorescent intensity units (FIU). In case of ASM3, a dose dependent increase of DCF formation was observed with maximum value of 457.3 FIU at IC70 concentration as compared to 134 FIU in untreated cells. Treatment with camptothecin (positive control) at different concentrations was found to enhance ROS generation by 381, 459 and 539.3 FIU at their respective IC30, IC50 and IC70 concentration
as compared to low fluorescence intensity units (134) in untreated PC-3 cells. It was observed that all the extracts of Palam Vichitra (PV) showed dose dependent increase in DCF fluorescence as analyzed by flow cytometer. In case of PVF extracts, the maximum DCF fluorescence of 39.30% and in case of P17 extracts maximum DCF fluorescence was 32.1% was observed at IC$_{70}$ value compared to low DCF fluorescence of 7.1% in untreated cells. The oxidative DNA damage involves a complex succession of cellular and molecular changes mediated by array of endogenous and exogenous stimuli (Loeb et al., 2003; Powell et al., 2005; Kaur et al., 2013). “The exact mechanism of apoptosis in PC-3 cells is still not known and the same might be mediated through enhanced production of ROS as reported by Sandhya and Mishra (2006) which is evident from the marked increase in ROS generation in treated cells”. “Literature studies support that the mechanism of cytotoxicity of tumor cells induced by gallic acid and certain polyphenols are mediated by intracellular production of ROS (Yano et al., 1994 and Elango et al., 2011)”. “These intracellular ROS are responsible for apoptosis through an oxidative stress-induced cell death. Mitochondria are an important source of ROS (Kowaltowski et al., 2009)”. Plant extracts may exert their cytotoxic effect on tumor cell lines through organo sulphuric component that have been demonstrated to generate large amounts of intracellular peroxides in tumor cells. “There are reports which show that sulforaphane exerts its anti-proliferative effect by arresting the cell cycle which has been documented in the colon, prostate, breast, bladder and T cells (Juge, et al., 2007)”. Cancer cells undergo apoptosis either by generation of free radicals or by depletion of endogenous antioxidants (Buttke and Sandstrom 1994). In the present study, elevated level of intracellular ROS was observed in the PC-3 cells with increase in the concentration of all the extracts. This clearly indicates that sprouts extracts induced cytotoxicity by generation of ROS. Likewise, the studies in the literature also revealed ROS mediated apoptosis by pomegranate peel extract and in sprouts of crucifers (Elango et al., 2011; Arimura et al., 2003). The comparative analysis of the crude extract on toxicological data (anti-proliferative and antioxidant activities) seems to reveals that high anti-proliferative activity could be correlated with high antioxidant activity, suggesting that the mechanistic action could be related to the redox cellular status. The results of MTT assay as well as
morphological changes of cancer cell support the results of cell cycle and intracellular measurement of ROS.

“Mitochondria are reported to be involved in active control of cell death processes (Borutaite, 2010)”. “Intracellular production of ROS active oxygen species such as \( \cdot \text{OH} \), \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) is associated with the arrest of cell proliferation. Generation of oxidative stress in response to various external stimuli has been implicated in the activation of transcription factors and to the triggering of apoptosis”. ROS functions as redox messengers in intracellular signaling and regulation at physiological low levels, whereas excess ROS can promote cell apoptosis. In present study, our finding showed that extract resulted in an increase of ROS at IC\(_{50}\) in PC-3 cells. The excessive ROS induced by extract lead to cell apoptosis of PC-3 cells. The rhodamine 123 fluorescence was also used to determine shift in mitochondria transmembrane potential in PC-3 cells following treatment with different concentrations of extracts and isolated compounds, at their respective IC\(_{30}\), IC\(_{50}\) and IC\(_{70}\) concentrations. The shift in mitochondria transmembrane potential was measured at 485/20 nm excitation and 528/20 nm emission wavelengths. The spectrofluorometric analysis revealed that the different extracts of Palam Samridhi (PS) exhibited a remarkable attenuation of mitochondrial membrane potential after 12 h exposure of PC-3 cells. The shift of membrane potential indicated the loss of mitochondrial integrity. The highest shift of mitochondrial membrane potential in extract of Palam Samridhi PS7 extract was 18.78% at IC\(_{70}\) concentration. The different extracts of Palam Vichitra (PV) exhibited highest attenuation of mitochondrial membrane potential of 58.21% in extract PV7 and in case of Punjab 1 (P1), extracts P17 exhibited 49.05% mitochondrial membrane potential at IC\(_{70}\) concentration. It was observed that all the untreated PC-3 cells were functionally active (100%) with high Rh-123 signals while camptothecin induced shift in mitochondria membrane potential in a concentration dependent manner.

Roy et al., (2008) showed “DIM-induced inhibition of F\(_0\)F\(_1\)-ATP synthase activity which causes depletion of mitochondrial ATP levels and stimulation of mitochondrial reactive oxygen species (ROS) production, followed by depolarization of mitochondrial membrane potential (\( \Delta \Psi \text{m} \)). Because \( \Delta \Psi \text{m} \) is the driving force for mitochondrial ATP synthesis, loss of \( \Delta \Psi \text{m} \) results in depletion
Discussion

of cellular ATP level. The loss of ΔΨm causes the cellular ROS generation and in turn leads to the oxidative DNA lesions followed by DNA fragmentation. In contrast, loss of ΔΨm leads to release of cytochrome c into the cytosol and subsequently activates the caspase-like proteases, which lead to oligonucleosomal DNA cleavage”. The cytochrome c release from altered mitochondrial structure is known as an early event of apoptosis (Xue et al., 2014). The level of cytochrome c in mitochondria of LNCaP cells was determined after exposure for 4 or 18 h to 8 μM PEITC compared to control. Sulfuraphane induces apoptosis through ROS-dependent disruption of mitochondrial membrane integrity in 5637 cells stained with DCFDA and JC-1 dyes, for ROS and mitochondria respectively (Park et al., 2014). “The ROS triggers the intrinsic apoptotic cascade via interactions with proteins of the mitochondrial permeability transition complex (Simon et al., 2000)”. “The loss in mitochondrial membrane potential, an early event in apoptosis, represents mitochondrial dysfunction which is an irreversible checkpoint in apoptosis. So, determination of attenuated mitochondrial membrane potential (ΔΨm) is often used to detect viability and apoptosis as well as to characterize various other processes inside the cells and draw proper conclusions from changes in ΔΨm that correspond to different stages of apoptosis (Kalbacova et al., 2003)” It has been reported that for the initiation of apoptosis, mitochondria plays an important role by releasing cytochrome c (Penninger and Kroemer, 2003; Ravagnan et al., 2002). Due to apoptosis, loss of intracellular water occurs which result in condensation of the cytoplasm and leads to the change of cell shape, size, and loss of adhesion. There are abundant evidences which suggest that mitochondria plays a key role in the initiation of apoptosis or involved in active control of cell death processes by releasing cytochrome c (Christodoulou et al., 1998; Ravagnan et al., 2002; Borutaite, 2010). In addition to cytochrome c, other factors such as apoptosis signaling molecules and apoptosis inducing factor (AIF) can be important that triggers apoptosis (O’Connor et al., 2006 ) It has been confirmed from cell cycle analysis that cell death occurred through apoptosis in G0 phase which may be due to release of cytochrome c from mitochondria. The result of current study support the hypothesis proposed by Circu and Aw, (2010)
“which verified that intrinsic mitochondrial apoptotic pathway triggers caspase-dependent or caspase-independent cytosolic signaling events”. “Loss of transmembrane potential is measured as a major determinant in the cellular commitment to death. “Mitochondria pathway of apoptosis is initiated by loss of mitochondria membrane potential (MMP), uncoupling of oxidative phosphorylation, the increased production of ROS, the depletion of ATP and distribution of the contents of the mitochondrial intermembrane space like cytochrome c into the cytosol (Bras et al., 2005)”. McStay et al. (2007) has well described “the mitochondria mediated apoptosis by cytochrome c binding with Apaf-1 and procaspase-9 to form the apoptosome”.

**Anti-inflammatory Activities**

*In vitro* COX-2 inhibitory activities of the extracts was evaluated using ‘COX (ovine) inhibitor screening assay’ kit (Cayman Chemicals Company, USA) with 96-well plates. The COX-1 is housekeeping enzyme which is constitutively expressed in a wide variety of cells all over the body and COX-2 inducible enzyme dramatically get up-regulated during inflammation. *In vitro* anti-inflammatory studies showed that different varieties of *Brassica oleracea* L. var. *italica* Plenck inhibited COX-2 selectively than COX-1 enzymes. In Palam Samridhi, PS5 extract selectively inhibited the COX-2 by 86.2% at 50µg/ml concentration as compared to inhibition of COX-1 by 0% and at 100 µg/ml it selectively inhibited the COX-2 by 88.33% compared to inhibition of COX-1 by 23.23%. In Palam Vichitra, PV3 extract also selectively inhibited the COX-2 by 95.51% at concentration of 50 µg/ml compared to inhibition of COX-1 by 15.56%. The PVF extracts selectively inhibited the COX-2 by 91.99% at concentration of 50µg/ml compared to inhibition of COX-1 by 7.56% and at 100µg/ml selectively inhibited the COX-2 by 99.85% compared to inhibition of COX-1 by 73.16%. In case of Punjab 1, P1S extract selectively inhibited the COX-2 by 93.58% at concentration of 50µg/ml compared to inhibition of COX-1 by 35.66% and at 100µg/ml selectively inhibited the COX-2 by 96.65% compared to inhibition of COX-1 by 40.15%. Our results showed that 50 µg/ml concentrations were more effective in inhibiting COX-2 selectively. *In vitro*
anti-inflammatory studies showed that different varieties of *Brassica oleracea* L. var. *italica* Plenck inhibited COX-2 enzyme selectively.

Among the different ways by which phytochemicals can protect the cells from over proliferation, one of the mechanisms is by scavenging the reactive oxidative species (ROS) that initiate carcinogenesis through oxidative damage of DNA. “Oxidative stress can damage DNA, lipids, proteins, and carbohydrates leading to impaired cellular function and enhanced inflammatory reactions. Several studies have revealed link between cancer and cyclooxygenase-2 because prostaglandins (PGs) are mediators of inflammation” (Kaur, *et al.*, 2010; Lobo, *et al.*, 2010; Sobolewski, *et al.*, 2010 and Ricciotti and FitzGerald, 2011). Similarly, Cheung, *et al.*, (2009) reported that “the combination of curcumin (anti-inflammatory effect) and sulforaphane (Phase II enzyme inducer) could be more effective than either used alone in preventing inflammation. Likewise, scopoletin could inhibit iNOS expression through NF-κB transcription factor but quercetin also suppresses iNOS expression through NF-κB and STAT-1 transcription factors. The association of chronic inflammation with development of human cancer is well recognized. There are number of reports of involvement of inflammatory process in the initiation and progress of cancer. COX-1 and COX-2 are involved in the control of inflammatory reactions and catalyze the conversion of arachidonic acid to PGH2, the precursor of PGs. Inhibition of COX-1, the constitutive form may result in gastric ulceration whereas COX-2 is inducible and is thought to be causative factor of cellular injury and may ultimately lead to carcinogenesis. There is great demand for natural COX-2 inhibitors with fewer side effects (Hamalainen *et al.*, 2007)”.

It has been found that cancer cells exposed to extracts/compounds undergoes apoptosis by different mechanisms *viz.* cell cycle arrest, fragmentation of nuclear DNA, reactive oxygen species and change in mitochondrial membrane potential (shown in figure 5.1).
Figure 5.1: A Generalized proposed mechanism for the induction of cell apoptosis by the extracts of different varieties (Palam Samridhi, Palam Vichitra and Punjab 1) of *B. oleracea*