Discussion
5. DISCUSSION

The process of carcinogenesis in case of large number of chemical carcinogens involves pharmacokinetic as well as pharmacodynamic reactions. These carcinogens undergo metabolism under the influence of Phase I and Phase II enzyme systems and this results in activation and detoxification of the carcinogen. The activated metabolites, because of their high reactivity, tend to interact with macromolecules like DNA, RNA, and proteins and thereby producing a variety of adducts. Such adducts are important for several reasons. Firstly, they distort the shape of the DNA molecule, potentially causing mistranslations. Secondly, during the DNA replication, an adducted base can be misread causing a mutation in the new strand. Thirdly, repair of bulky adducts can result in breakages of the DNA strand which can, in turn, result in mutations or deletions of genetic material.

In the cell there is DNA-repair system. The replication of adducted DNA before its repair may fix the mutation in either one of the daughter cells or both. Mutations are likely to be more in the cells which are dividing rapidly or in the cells where DNA repair mechanisms are defective. Mutations and deletions in molecules such as oncogenes, tumour-suppressor genes and DNA-repair genes resulted from adduct formation and faulty repairs are known to be key steps in carcinogenesis. Therefore, the process of carcinogenesis involves an accumulation of genetic mutations. In case of cervical cancer, there are preceding abnormalities in the cervical cells and tissue known as dysplasia, which presupposes some changes in the DNA. The development of cancer from the stage of dysplasia is characterized by the events such as an increase in genetic abnormality, higher level of aneuploid cell population, higher chromasia and higher rate of mitosis.

Any factor which inhibits or induces these mutations/repairs in the DNA and their effects can modulate the process of carcinogenesis. This action can be exerted, among others, through modulating the activity of Phase I and II enzymes (detoxifying system in the cells), influencing hormones, inducing apoptosis, modifying the mitosis rate.

Diet is a factor which plays an important role in cancer of various origins as it can modify the process of carcinogenesis. Modulatory influence of diet on carcinogenesis could be examined by using single active ingredient or the whole plant. A whole or partially
purified extract of a plant is known to offer advantages over a single isolated ingredient. Synergistic interactions are of vital importance in phytomedicines. There are studies supporting the occurrence of synergy in phytomedicines (Williamson, 2001). In the present study we have examined chemopreventive potential of important six plants.

5.1 *Trigonella foenum-graecum* (fenugreek, methi)

The high steroid, phenolic and flavonoid content in fenugreek seeds make this plant very attractive to be studied and analyze its preventive potential. Fenugreek seeds exhibit hypoglycemic effect in experimental diabetes as well as in diabetic patients, it is shown to reverse the reduction in hepatic P450 and b5 level, as well as the activities of SOD-catalase, GST and DTD, after the induction of carcinogenesis by methylnitroso-uria (MNU) in rats. Lipid peroxidation, which is associated with various forms of carcinogens, was effectively attenuated in rats by fenugreek. It also reduced the number of tumors per tumor bearing rat (Mallikarjuna, 2002). Devaseva and Menon, (2003) asserted the colon carcinogenesis inhibitory effect of fenugreek seeds by modulating the activities of β-glucoronidase and mucinase, and postulated that the beneficial effect may be attributed to the presence of fibre, flavonoids and/or saponins in the seeds. Hibasami et al (2003) observed that protodioscin from fenugreek inhibited the growth of human leukemia cells by inducing apoptosis. However nothing has been reported so far on the preventive effect of fenugreek seeds or its constituents in cervical carcinogenesis.

In the present study, when female mice were given a 5% fenugreek seeds diet, the incidence of cervical cancer was significantly decreased (p<0.05) (Table 4.1). Joining this result with the fact of not finding any case of dysplasia, it seems fenugreek can act as preventive agent at early stage of the successive genetic changes as part of the chain of the events related to carcinogenesis and not allowing the cells to arrive even to the dysplasia stage. That is why it seems that the protective effect of fenugreek on cervical cancer is not only through an induction of apoptosis, since a higher incidence of dysplasia would occur; other early mechanisms for chemoprotection on cervical carcinogenesis must be exerted by fenugreek.
The protective action of fenugreek against cervical carcinogenesis could be due to its high phenolic and flavonoid content. The antioxidants present in fenugreek might also induce the Phase I and II enzyme systems and detoxify the carcinogens before these ones interact with the DNA. In addition, flavonoids and other phenolic components of fenugreek are estrogenic compounds. These natural phytoestrogens are antiviral, antiproliferative and growth inhibiting factors. These phytoestrogen compounds might bind to estrogens receptors reducing the estrogenic activity in the cells and in turn block the process of the development of cervical cancer (Nagata et al, 1997).

In the present study high dose of fenugreek surprisingly showed reduced chemopreventive effect as compared to lower dose and needs to be understood. It would be useful to know the biological significance of fenugreek.

5.2 *Brassica* sp. (mustard seeds)

The medicinal properties of *Brassica* sp. have been known for many centuries. These properties may be due to the beneficial contribution of several of its components like allyl isothiocyanate, sinigrin, thiamine and carotene. Some of them have been used to examine their chemopreventive activity against carcinogenesis in different sites, but no investigation has been done so far to study the chemopreventive activity of the whole mustard seed on cervical cancer.

In mustard seeds, allyl isothiocyanates are present accompanied by large quantities of their cognate glucosinolates. As it was previously said, isothiocyanates have been implicated in various pharmacological and toxic activities, chromosomal abnormalities and neoplasia as well as blocking of carcinogenesis (Zhang and Talalay, 1994; Hecht, 1995). Naturally occurring and synthetic isothiocyanates are among the most effective chemopreventive agents. A wide variety of isothiocyanates prevent cancer in the rat lung, mammary gland, esophagus, liver, small intestine, colon, and bladder. Mechanistic studies have shown that the chemopreventive activity is due to favorable modification of Phase I and phase II carcinogen metabolism, resulting in increased carcinogen excretion.
or detoxification and decreased carcinogen DNA interactions (Hecht, 1995; Munday and Munday, 2002). It is important that recently allyl isothiocyanate were shown have no genotoxic properties (Bechtel et al, 1998) even at high toxic doses. Sulforaphane, a natural occurring isothiocyanate from Brassica vegetables, has been reported to induce cell death by apoptosis, this induction correlated with the one of caspase-3 and -9 activities (Gingras et al, 2004). Another isothiocyanate, phenyl isothiocyanate, has anticarcinogenic effect against N-nitrosomethylbenzylamine induced cancer (Solt et al, 2003). High phenolic and flavonoid content of mustard seed are also good antioxidant components that contribute to the fast detoxification of carcinogenic compounds.

Mustard oil given to gestating females to which also DMBA was given, significantly reduced the incidence of tumors at multiple sites in the F1 progeny. The similar chemopreventive effect was observed when mustard oil was given to lactating mothers to which DMBA was given as well. Mustard oil suggested to exert its effect by inducing the enzymes of drug detoxification and also by changing the profile of the antioxidant defence system (Hashim et al, 1998). On the other hand the carotenes present in Brassica sp. contribute to its chemopreventive activity by regulating the cell growth and the cell differentiation (Tossetti et al, 2002)

Thiamines also contribute significantly to the medicinal properties of Brassica sp. A down-regulation of thiamine transporter THTR2 gene expression has been observed in breast cancer and it has been associated with resistance to apoptosis mediated at least in part by a caspase-3-dependent pathway (Liu et al, 2003). This result shows that an increase of thiamines could make up for at least part of the down regulation of thiamine transporter THTR2 gene expression and also re-establish normal rate of apoptosis.

What has been said so far supports the results of the present study. Brassica sp. in diet (5% and 7.5%) significantly reduces cervical carcinogenesis incidence (p<0.005) (Table 4.2) most probably through several pathways like inducing Phase I and II enzymes, increasing the antioxidant state of the cell, reinforcing the regulation of the cell growth and cell differentiation, and induction/maintenance of normal apoptosis rate.
In this study it was observed that 5% *Brassica* sp. in diet had a better chemoprotective effect than 7.5%. In support of our findings Musk *et al.* (1995), showed that sinigrin (an allylglucosinolate present in mustard seeds) in high concentrations had genotoxic activity. In our study, animals on higher dose of *Brassica* showed reduction in the body weight which could be due to reduction in intake of food fortified with higher content of *Brassica* (possibly due to distastefulness and impalatability at this dose level). Hence reduced intake of *Brassica* at this higher dose level would obviously fail to achieve higher degree of chemopreventive action.

It has been reported that ethanolic extract of mustard seed significantly reduced tumour incidence of DMBA-induced skin papillomagenesis in mice and significantly increased the latency period in the experimental group (Qiblawi and Kumar, 1999). These results support our findings regarding the high incidence of dysplasia found in both experimental groups (5% and 7.5% of *Brassica* sp. in diet). This increased latency period may correlate with a higher incidence of dysplasia. Therefore, *Brassica* sp. chemopreventive effect may act, at least partly, blocking the carcinogenesis process at dysplasia stage. Further studies are required to analyze the blocking effect of this agent.

### 5.3 Cuminum cyminum (cumin seeds)

Cumin is one of the most extensively used condiments in the world. It is also widely used in ayurvedic medicine for the treatment of various diseases. Many studies have been carried out on its possible antitoxic or cancer chemopreventive effect. However, no information is available on the chemopreventive effect of cumin seeds on cervical carcinogenesis.

The results of the present investigation show that cumin significantly reduces cervical carcinogenesis in murine model system, when given in 7.5% diet (p<0.005) (Table 4.3). Cumin seeds were also shown to reduce significantly the incidence of Benzo [a]pyrene-
induced neoplasia in the stomach of Swiss albino mice and also the 3’-methyl-4-dimethylaminoazobenzene-induced hepatomas (Aruna and Sivaramakrishna, 1992).

The chemopreventive action of cumin may be due to the beneficial effect of several of its components. Among its constituents there are a large number of terpenoids (thyme, cuminol, corvone, cynol, terpene, etc.). The inhibitory action of some terpenoids on the effect of a potent chemical carcinogen was noted several decades ago. Terpenoids were shown to suppress the activation of nitrosamines (Wattenberg and Coccia, 1991) and azoxymethane (Kawamori et al, 1996), induce glutathione-S-transferase and inhibit oncogene activation by depressing the isoprenylation of oncogene products (Hudes et al, 2000).

D-carvone inhibit the occurrence of tumors in the forestomach, lung, skin, and mammary gland (Wattenberg et al, 1989)

Riboflavin is present in cumin seeds. When women with precancerous lesions were treated with riboflavin, the results indicated that local application of riboflavin could serve as chemopreventive agent for cervical cancer (Chen, 1993). Another component present in cumin seeds is thiamine which was found to have a clear anticarcinogenic activity (Liu et al, 2003). Cumin has a high phenolic content and moderate flavonoid content (Nair et al, 1998) as mentioned earlier, these components are good antioxidants and enhancers of Phase I and II enzymes and might contribute to chemoprevention of cancer.

It may be mentioned that cumin seeds have been tested in our laboratory for its modulatory effect on Benzo(a)pyrene-induced stomach carcinogenesis in Swiss albino mice. It was found to be effective in prevention of the stomach carcinogenesis. Further, it significantly enhanced the activity of GST, DTD, SOD and CAT in the liver. The increased antioxidant potential in the cells is likely to reduce the mutations and in turn carcinogenesis (Gagandeep et al, 2004).
5.4 *Piper longum* (pipli)

In spite of the well known medicinal properties of *Piper longum*, the chemopreventive effect of its fruits, used as a spice and also in pickles and preservative, has hardly been studied and analyzed. Other plants of the same family more ubiquitous than *pipli*, like *Piper methysticum* (Kava-kava) or *Piper nigrum* have been extensively studied (Steiner, 2000; Hashimoto *et al.*, 2003). There is no bibliography on research of the chemopreventive effect of *Piper longum* in cervical carcinogenesis.

Main constituents of *Piper longum* are alkaloids like piperine and piplartine. Some synthetic alkaloids like topotecan and irinotecan that have been tested for chemotherapy against cervical cancer showed inhibitory effects (Fiorika, 2003; Machida *et al.*, 2003).

Piperine effectively suppressed B(a)P-induced lung cancer. The chemopreventive effect of piperine has been ascribed to modulation of lipid peroxidation, augmenting antioxidant defense system (superoxid dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants levels (reduced glutathione, vitamin E and vitamin C) (Selvendiran *et al.*, 2003; Selvendiran *et al.*, 2004) and an antimetastatic activity. The inhibitory effect of piperine on lung cancer metastasis induced by B16F-10 melanoma cells was clearly seen in C57BL/6 mice. It also showed the antimetastatic activity. A significant reduction in tumor nodule formation, the lung collagen hydroxyproline in the metastasized lungs, the amount of uronic acid in the metastasized control animals when treated with piperine and significant reduction of the elevated levels of serum sialic acid and serum gamma glutamyl transpeptidase activity in the animals treated with piperine were noticed (Pradeep and Kuttan, 2002).

All these previous reports are in confirmation with the results of the present study where both 1% and 2% of *Piper longum* in diet significantly reduced tumor incidence (p<0.01 and p<0.05 respectively) in MCA-induced cervical carcinogenesis in Swiss albino mice. The high percentage of dysplasia shows a late chemoprotective effect of *Piper longum*.
letting mutations to take place and developing the preneoplastic lesions in the cervix. An early intake of *Piper longum* may yield better results (Table 4.4).

### 5.5 *Camellia sinensis* (green tea)

Green tea is, after water, the most popular beverage in the world. Green tea contains polyphenols, which include flavonols, flavandiols, flavonoids, and phenolic acids accounting for up to 30% of the dry weight. Most of the green tea polyphenols are flavanols, known as catechins. These include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epicatechin (EC), (+)-gallocatechin, and (+)-catechin (Graham, 1992). The phenolic content of *Camellia sinensis* is 12460mg/100gm, and its flavonoid content is 1255mg/100gm (Nair *et al*, 1998).

Along with epidemiological studies which have shown decreased cancer occurrence in those individuals who drink tea regularly (Kazi *et al*, 2002). There are abundant literature supporting the chemopreventive effect of green tea against carcinogenesis in different sites. Green tea reduces the occurrence of skin cancer and act as a photochemoprotector (Ahmad and Mukhtar, 2000; Katiyar *et al*, 2000; Lu *et al*, 2001). The oral administration of green tea enhanced 2.5 fold the inhibitory effects of doxorubicin (a drug used in chemotherapy) on Ehrlich ascites carcinoma tumor growth (Sadzuka *et al*, 1998). Green tea consumption also affords protection against cancers induced by chemical carcinogens that involve the lung, forestomach, esophagus, duodenum, pancreas, liver, breast, prostate, colon and skin in mice, rats and hamsters (Mukhtar and Ahmad, 2000; Adhami *et al*, 2003). Green tea polyphenols have antiinflammatory and anticarcinogenic properties. These effects appear to correlate with antioxidant properties of green tea polyphenols. The major and most preventive constituent in green tea responsible for these biological effects is (-) epigallocatechin-3-gallate (EGCG) (Katiyar *et al*, 2000).

In a recent study it has been shown that EGCG induces apoptosis, cell-growth inhibition and cyclin kinase inhibitor WAF-1/p21-mediated cell-cycle dysregulation. EGCG
treatment of androgen sensitive cells resulted in induction of genes that functionally exhibit growth-inhibitory effects, and also repression of genes that belong to the G-protein signaling network (Adhami et al, 2003). EGCG suppresses extracellular signals and cell proliferation through epidermal growth factor receptor binding (Mukhtar and Ahmad, 2000). EGCG has also antimetastatic and antiangiogenic properties inhibiting matrix metalloproteinases (MMP-2 and MMP-9) and the expression of the vascular endothelial growth factor genes (VEGF), which are angiogenic growth factors and antimetastasis genes respectively. This could account, at least in part, for the tumor prevention effects observed in green tea (Kazi et al, 2002; Tosetti et al, 2002; Adhami et al, 2003; Pfeffer et al, 2003). Green tea polyphenols stimulate the transcription of phase II detoxifying enzymes (Mukhtar and Ahmad, 2000), and act as a chemopreventive agent (Wattenberg, 1985). Camellia sinensis has also tumor promotion inhibition activity. EGCG was found to inhibit mouse skin tumor promotion (Okuda et al, 1985).

In the present work, in both 1.25% w/v and 2.5% w/v doses of Camellia sinensis a significant reduction in cervical carcinogenesis incidence was found (p<0.001 and p<0.05 respectively) (Table 4.5). Such good chemopreventive effect could be explained on the basis of different pathways mentioned earlier through which green tea acts.

In the present study a low percentage of dysplasia incidence as compared to the other modulators was found. The reason of this may be that this modulator was given to the mice one week before the cancer induction, giving green tea more time to prepare the appropriate conditions in the cells for a better cancer chemoprevention. On the other hand, the low dysplasia incidence can be also due to the fact that Camellia sinensis acts at the same time as an inhibitor preventing formation/activation of carcinogens, as a blocking agents and as a suppressing agents.

5.6 Glycine max (soybean)

Epidemiological studies have revealed that Asians, who consume a traditional diet high in soy products, have relatively low incidences of breast and prostate cancers, while the
incidences are much higher in the Western world. Soybean seeds contain storage proteins; bioactive proteins including cytochrome c, lectin, lipoxygenase, urease, the Kunitz inhibitor of trypsin (KTI), and the Bowman-Birk inhibitor of chymotrypsin and trypsin (BBI); and secondary metabolites including isoflavones, saponins, phytic acid, flatus-producing oligosacarides, and goitrogens (Friedman and Brandon, 2001).

Soybeans proteins are not of a very good quality due to its deficiency in the essential amino acid L-methionine and to its content on inhibitors of digestive enzymes which adversely affect the nutritional quality of soy proteins. Efforts are being made to develop soybean lines that overexpress methionine-rich proteins (Kho and Lumen, 1988; Sleister and Rao, 1988) and to inactivate the inhibitors by heat treatment during food processing or by partially remove them by fractionation. These two factors (deficiency in L-methionine and presence of inhibitors) may inhibit tumors in rats; another possible factor present in soybean seeds that may reduce tumor incidence is phytoestrogens (mainly genistein) (Dixon and Ferreira, 2002; Sarkar and Li, 2002).

Dietary protease inhibitors might contribute to the prevention of human cancer by blocking the formation of active oxygen species by stimulated neutrophils, inhibit tumor promotion, and prevent the digestion of proteins to aminoacids, thus depriving rapidly growing cancer cells of essentials aminoacids. There are many reports on possible beneficial anticarcinogenic effects of Bowman-Birk inhibitor (BBI) in animals and humans (Meyskens, 2001; Armstrong et al, 2003).

In vitro studies show that the BBI suppresses the production of superoxide anion free radicals in HL-60 differentiated cells (Ware et al, 1999); potentiates radiation- and cisplatin-induced killing of human (breast, cervical, head and neck, lung, and ovarian) cancer cells (Zhang et al, 1999); and also suppresses the growth of human prostate cancer xenografts in nude mice (Wan et al, 1999 b). The suppression of colon carcinogenesis in mice by BBI may be due to the observed uptake of the inhibitor by intestinal epithelial cells (Billings et al, 1991). The protective effect of BBI in human oral cancer (leukoplakia) may be due to the inhibition of serine proteases, which cleave the neu
oncogen protein, a cell surface biomarker for human cancer (Wan et al., 1999a). A possible consequence of protease inhibition is the accumulation of the neu protein on the cell surface, leading to enhanced immune recognition of the cancer cells. This in turn permits more efficient destruction of tumors by cytotoxic lymphocytes and natural killer cells. The selective inhibition of serine proteases important for the growth of tumor cells may be a key step in cancer prevention by BBI. The effect of BBI may also be related to its ability to reach intracellular target molecules (Friedman and Brandon, 2001). Lunasin, a bioactive polypeptid present in soybean seeds, arrests cell division and induces apoptosis in malignant cells (Mejia et al., 2003). Isoflavones are the biologically active compounds of soybeans which belong to the group of flavonoids. Having estrogen and antioxidant activities isoflavones are usable in prevention and treatment of cancer and other diseases as atherosclerosis, osteoporosis, etc. (Kaprel’iants et al., 2003).

Soy isoflavones have both hormonal and non-hormonal effects relevant to prostate cancer prevention. In vitro, the main soybean isoflavone, genistein, inhibits prostate cancer cell growth. In animals, most but not all studies show isoflavone-rich soy protein and isolated isoflavones inhibit prostate tumor development (Messina, 2003).

Genistein (4′,5,7-trihydroxyisoflavone) has been shown to inhibit the growth of cancer cells through the modulation of genes that are related to the homeostatic control of cell cycle and apoptosis. It has been found that genistein inhibits the activation of the nuclear transcription factor, NF-kappaB and Akt signaling pathway, both of which are known to maintain a balance between cell survival and programmed cell death (apoptosis). Genistein is known to have antioxidant property, and commonly known as phytoestrogen, which targets estrogen and androgen-mediated signaling pathway in the processes of carcinogenesis. Moreover, genistein is also found to be a potent inhibitor of angiogenesis and metastasis. For all this, genistein is a promising agent for cancer chemoprevention and/or treatment (Sarkar and Li, 2002).

Soybean constituents have shown many other beneficial effects besides the anticarcinogenic affect as well, some of them may be related to the chemoprevention
effect on cancer in an indirect way. Some of these beneficial effects are: induction of lowering of cholesterol; protective effect against obesity, diabetes, irritants of the digestive tract, bone and kidney diseases, heart diseases, etc. (Friedman and Brandon, 2001; Krauze-Brzosko et al, 2002).

In the present study the chemopreventive effect of soy bean seeds on cervical carcinogenesis is clearly shown. The reduction of tumor incidence goes together with the increase of the concentration of soybean in the food, being significant different with 7.5% dose (p<0.05) (Table 4.6).

The chemopreventive effect of soybean on cervical carcinogenesis may be due to its antioxidant and estrogenic properties. Apoptosis is another possibility, and this would explain the light increase in dysplasia incidence that we have observed in the present study.