Summary and conclusion

Colorectal cancer is the second most common cause of cancer related deaths around the world. Though the estimated incidence of colorectal cancer is low in India its prevalence is increasing at an alarming rate with changing life style. Colon cancer is frequently a pathological consequence of persistent oxidative stress, leading to DNA damage, mutations in cancer related genes and epigenetic silencing of tumour suppressor genes. Strategies for cancer prevention and therapy involving reduction or elimination of human exposure to environmental factors may not be always possible. However as an alternate approach, plant derived compounds that abolish the effect of carcinogen induced cancer development are currently evaluated to identify possible chemopreventive agents especially those that are naturally found in foods.

Radiation therapy (radiotherapy) is a form of cancer treatment which employs ionizing radiation to kill cancer cells and shrink tumour. Despite a good therapeutic index, radiotherapy can also cause normal tissue injury in long-term cancer survivors. Thus, a major challenge to radiotherapy is to increase normal cell tolerance by preventing their transformation to malignant cells, thus improving the quality of life of a patient. Over the years, a number of compounds have been tested for their radio modulating potentials, but with limited success due to their associated toxicity and lack of significant protective effects. Currently, however, only 2 modifiers are approved by the Food and Drug administration for clinical use. They are amifostine, a protector, and tirapazamine, a hypoxic cell cytotoxin. But both these drugs cause several side effects like nausea, fatigue, blood count depression, decreases parathyroid hormone level and systolic blood pressure. In view of these facts, the search for agents which increase the patient’s tolerance to radiotherapy still continues. Since free radicals play a major role in the initiation and progression of radiation induced toxicity, the use of antioxidants either in the diet or as a therapeutic agent might offer protection
against radiation induced damage. The use of naturally occurring compounds as radio-modifiers is currently becoming an important strategy in the field of radiotherapy.

**Carvacrol** (2-methyl-5-(1-methylethyl-phenol; CVC), is a prevalent herbal compound used in food products, besides a long history as a medicinal plant, since ancient times. CVC has been shown to possess very good pharmacological activities like antioxidant, anti-inflammatory and antiproliferative properties which are linked to anticancer activity. In this above context, we made an attempt to study the radiosensitizing potential of a natural compound CVC against DMH induced colon cancer in rats.

The present study was conducted in three phases to explore the radioprotective and radiosensitizing efficacy of CVC in normal lymphocytes and also by the use of *in vitro* and *in vivo* colon cancer models. To study the radioprotection, human blood lymphocytes were exposed to 4 Gy radiation and pretreatment with CVC. Next to, study the radiosensitizing efficacy in colon cancer cell lines we selected two cell lines HT-29 and SW480, which were treated with CVC and were exposed to 4 Gy radiation. Further, to study the radiosensitizing potential of CVC (*in vivo* long term study), rats were injected with DMH for 15 weeks and were supplemented with CVC (40 mg/kg b.w.). Two days after the last injection of the carcinogen the rats were supplemented with CVC throughout the study period (32 weeks) and/or irradiated with 6 Gy radiation at the 31st week.

**Phase-I: Radioprotective efficacy of CVC (*in vitro*)**

In this study we performed *in vitro* assays using lymphocytes (study design I: Human blood lymphocytes). Before conducting experiments to fix the effective dose of CVC and radiation, MTT assay was performed. Lymphocytes were pretreated with CVC 30 min before radiation exposure and its radioprotective effect was evaluated by adopting specific *in vitro* techniques. Based on the MTT assay, the optimum dose of CVC was fixed as 100 μg/mL and the radiation dose as 4 Gy. DNA damage was evaluated using dicentric
aberration assay, comet assay, DNA fragmentation assay and micronuclei (MN) assay. The results indicated the presence of increased number of dicentrics and acentric fragments, increased tail length and comet parameters, increased formation of DNA fragments and MN frequencies in the radiation treated groups. Pre-treatment with the effective dose of CVC reversed this effect due to its inherent antioxidant potential.

Oxidative stress is a term denoting an imbalance between the production of oxidants and the counter effects by an effective defence system of an organism. In our study, we observed a proportional decrease in the activities of antioxidant enzymes (SOD, CAT, GPx & GSH) and increase in the levels of lipid peroxidation markers (TBARS, LOOH & CD) in the irradiated groups indicative of the damage caused by ionizing radiation. Pre-treatment with the effective dose of CVC (100 µg/mL) increased the activities of antioxidant enzymes and decreased the levels of lipid peroxidation markers significantly when compared to radiation control group.

Certain stages in the life cycle of a replicating cell are more prone to the vulnerable effects of ionizing radiation and in our study, we observed an increase in the apoptotic cells in the radiation control group. But pre-treatment with the effective dose of CVC significantly decreased the number of apoptotic cells. Thus CVC by means of its effective antioxidant potential and ROS scavenging properties protected the lymphocytes from free radical mediated damage and oxidative stress.

**Phase-II: Radiosensitizing potential of CVC (in vitro)**

We have evaluated the radiosensitizing effect of CVC on HT-29 and SW480 cell lines. The cytotoxic effect of CVC on HT-29 and SW480 cells was analyzed using MTT and the effective dose i.e., IC₅₀ concentration of CVC was fixed at 39.54 µg/mL (HT-29) and 34.77 µg/mL (SW480) at 24h, 48h and 72h in a time and dose dependent manner. The combined effect of CVC and radiation treatment was more significant as compared to the treatment with CVC alone or radiation alone to HT-29 and SW 480 cell lines.
which was confirmed by a) decreased cell viability, cell survival, b) marked induction of apoptosis [EtBr staining], c) alterations in nuclear morphology [Hoechst 33258 staining], d) collapse in mitochondrial membrane potential ($\Delta \Psi_{m}$) [JC-1 staining], e) extent of DNA damage [comet assay], f) increased ROS & TBARS and reduced the activities levels of SOD, CAT, GPx and GSH, g) increased proapoptotic protein Bax together with decreased antiapoptotic protein Bcl-2 and decreased cell proliferation marker cyclin D1. Thus, our in vitro study results showed that CVC sensitizes the cancer cells to radiation.

**Phase –III: Long term studies- Combined therapeutic efficacy of CVC and radiation (In vivo)**

Next, we proposed to study the effect of CVC especially its radiosensitizing potential in a long term in vivo study, which might help in augmenting the anticancer effects of radiation.

During the experimental period of 32 week, no adverse effects were observed in the CVC alone supplemented rats suggesting that CVC is well tolerated. Decreased body weight gain, growth rate, increased polyps/preneoplastic incidence and altered activities of oxidative stress markers in DMH alone exposed rats observed in the present study reflected the initiation and progression stages of colon carcinogenesis in rats. Exposure to 6 Gy radiation and supplementation with CVC to DMH exposed rats reversed the body weight changes and also blunted the ability of DMH to stimulate preneoplasia in the colonic epithelium. This inhibitory effect was apparent by the reduction in the number of polyps/preneoplastic lesions, reversion in the activities of biochemical markers (oxidative stress markers) and the reduction in the incidence of carcinoma. The protective effect was more pronounced in the rats supplemented with CVC (40 mg/kg b.w.) along with radiation (6 Gy) exposure. Thus under the present experimental conditions CVC (40 mg/kg b.w.) and 6 Gy radiation was shown to efficiently potentiate the antioxidant levels to counteract the oxidative stress and genotoxic insult elicited by DMH.
Xenobiotic metabolism is involved in the elimination of carcinogen and its metabolites. Exposure to radiation alone and supplementation with CVC alone to DMH injected rats during the promotional stages of carcinogenesis reduced the activities of phase I enzymes (cytochrome P450, cytochrome P4502E1, NADH-cytochrome P450 reductase, cytochrome b5, cytochrome P450 reductase) as compared to the DMH alone treated rats. The decline in the activities of phase I enzymes were even more pronounced in DMH + CVC + radiation treated rats. On the other hand, exposure to radiation alone and supplementation with CVC alone to DMH exposed rats increased the activities of phase II enzymes (UDPGT, GST and DTD) as compared to the DMH alone treated rats. The increased activities of phase II enzymes were even more significant in DMH + CVC + radiation treated rats.

Gut microbial enzymes help to eliminate the toxic metabolites which are harmful to the colon. The activities of bacterial enzymes (β-glucuronidase, β-glucosidase, β-galactosidase, mucinase, nitroreductase and sulphate) and presence of colonic mucin in DMH treated rats exposed to radiation alone or supplemented with CVC alone were reduced as compared to the DMH alone treated rats. Supplementation with CVC to DMH treated rats and subsequent exposure to radiation decreased the activities of mucosal bacterial enzymes even more significantly as compared to the DMH alone treated rats.

Cell proliferation is a carefully orchestrated process and disruption of the same plays an important role in multistage carcinogenesis. PCNA can be used to mark cell proliferation activity and is helpful to study the dynamic changes taking place during morphogenesis. Cyclin D1 regulates the progression of G1 to S phase of the cell cycle; it is also involved in apoptosis, in addition to its role in cell proliferation and oncogenesis. In this study our results clearly indicate that DMH alone exposed rats showed increased number of AgNOR dots and enhanced expression of PCNA and cyclin D1. DMH treated rats on exposure to radiation alone or on supplementation with CVC
alone showed a decrease in the expression of cell proliferation markers viz., AgNORs PCNA and cyclin D1. A more pronounced reduction in cell proliferation markers was observed on combined treatment with CVC and radiation, reflecting the radiosensitizing effect of CVC.

Apoptosis, or programmed cell death, not only plays an important role in the development and maintenance of tissue homeostasis but also represents an effective mechanism by which abnormal cells, such as tumour cells, can be eliminated. Tumour cells often evade apoptosis by expressing anti-apoptotic proteins such as Bcl-2, while down-regulating and mutating proapoptotic genes such as Bax, caspase-3, caspase-9, cytochrome C and alternating p53 and CDX-2 pathways that give them survival advantage, thereby preventing and conferring resistance against therapy induced apoptosis.

Activities of Bax, p53, CDX-2, caspase-3, caspase-9 and cytochrome C were significantly reduced and Bcl-2 was significantly elevated in the DMH alone exposed rats in our study, making the colonic mucosa of these rats favourable for proliferation and resistant to apoptosis. In addition decreased expression of Bax and cytochrome C in DMH alone treated rats as compared to the control rats suggest impaired induction of apoptosis in the colon of these rats. CVC, at the dose of 40 mg/kg b.w. and 6 Gy radiation, effectively suppressed the initial phase of colon carcinogenesis probably by inhibiting the DMH induced carcinogenicity. The decreased expression of Bcl-2 and increased expression of Bax, caspases (3 and 9), cytochrome C, p53 and CDX-2 proteins in the colonic mucosa of rats supplemented with CVC and radiation following DMH treatment correlates with the proapoptotic effects of both CVC and radiation.

Chronic inflammation, a process associated with elevated levels of various cytokines and ROS, has also been regarded as a leading contributor to carcinogenesis. Investigations on the expression profile of IL-6 and COX-2 in the present study confirmed that DMH exerts its inflammation associated
tumourigenic effects in the colon of rats, by up-regulating their expressions along with mast cell recruitment, which in turn potentiates the toxicity of DMH and increases the onset of inflammation. Combined therapy with CVC supplementation and radiation exposure during the promotional stage of carcinogenesis counteracted these effects, by decreasing the number of mast cells and inflammatory response in the colonic tissue of DMH exposed rats, validating the antiinflammatory potential of CVC.

HIF-1α is the transcriptional regulator of cellular and developmental response to hypoxia. Overexpression of HIF-1α facilitates the angiogenesis and vascularization, cell survival, energy metabolism and tumour invasion. VEGF is a critical growth factor for tumour angiogenesis and is an important determinant to evaluate the radiosensitizing potential of a compound. Supplementation with CVC and subsequent exposure to radiation during the promotional stage of carcinogenesis showed significant reduction in the expression of the angiogenesis markers, HIF-1α and VEGF as compared to the DMH alone treated animals.

To elucidate the transcriptional mechanisms underlying the antiinflammatory effects, we further examined the effect of CVC on the activities of NF-κB (p65) in DMH exposed rat colon. NF-κB is one of the prime components of the intracellular signalling pathways responsible for the up-regulation of proinflammatory proteins. There is accumulating evidence to show that Nrf2 is a key transcriptional factor that activates the antioxidant response element (ARE) and in turn regulates the expression of antioxidant and phase II detoxifying enzymes. Consistent with the these reports, our results also showed that Nrf2 expression in the DMH exposed rat colon was lower than those of the control, whereas CVC supplementation and radiation exposure induced Nrf2 activation and enhanced the expression of phase II detoxification enzymes. Moreover, CVC and radiation inhibited the expression of NF-κB (p65) in DMH exposed rats confirming its antiinflammatory effect.
Further, the results of the present study showed decreased immunoreactivity to TGF-β and Smad-4 in the colon of DMH alone treated rats as compared to the control rats reflecting the down-regulation of TGF-β pathway in tumour bearing rats. Treatment with both CVC and radiation resulted in the significant upregulation of TGF-β and Smad-4 in the colonic epithelial cells thereby inhibiting the transformation of cancer cells.

Stress induced by carcinogen and ionizing radiation can initiate the central MAPK pathway, thereby deregulating the various cellular events in the tumourigenesis. We observed increased expression of p38 and JNK in DMH alone injected rats. The expression patterns of p38 and JNK was strikingly reduced in the DMH treated rats supplemented with 40 mg/kg b.w. of CVC and subsequent exposure to 6 Gy radiation.

Efficient repair of DNA is an important mechanism by which therapeutic resistance is exerted by cancer cells. Thus altering the activity of DNA repairing genes using phytochemicals paves a way for radiosensitization. We observed upregulation of DNA repairing genes hOGG1 and XRCC1 in DMH alone injected rats. Pronounced downregulation of the DNA repairing genes was observed in the combined treatment with both CVC and radiation to DMH exposed rats.

Histology of the liver and colon displayed the various lesions induced by carcinogen and radiation. These tissue alterations and lesions were reduced in the combined therapy with CVC and radiation to DMH administered rats.
CONCLUSION

Based on the above facts, our findings demonstrate that the dietary compound, CVC, acts as an efficient radioprotector (in vitro) by exerting its protective effects against X-radiation induced alterations such as the formation of genetic damage and oxidative stress. CVC acts as an efficient radiosensitizer (in vitro), and the cancer cells which on exposure to radiation revealed increased DNA damage, apoptosis and alterations in cell cycle progression. In addition, CVC combined with radiation acts as an efficient radiomodulator (in vivo), in DMH induced rat colon cancer model by exerting its protective effects against DMH induced alterations such as the formation of preneoplastic lesions, cell cycle progression, oxidative stress, bacterial enzymes, inflammatory proteins, angiogenic markers, stress activated proteins, DNA repairing genes and histopathological changes. Further long term molecular studies are warranted to understand its mechanism of action before entering clinical trials, which would potentiate the development of CVC combined with radiation as a new therapeutic agent for colon cancer therapy.

Future directions

Our future directions will be concentrating on studying the effects of CVC and radiation on

1. DNA damage signalling pathways.
2. The expression patterns of other genes related to hypoxia.
3. Expression patterns of oncogenes which are responsible for colon cancer initiation.
Figure 47a: Summary of the mechanisms for the observed radioprotective effects of CVC (in vitro study)

Indicates inhibitory effect of CVC
Figure 47b: Summary of the mechanisms for the observed radiosensitizing effects of CVC (*in vitro* study). Radiation and CVC act synergistically on cancer cells

*In vitro*  
Cancer cell  

Cell viability  

ROS  

DNA damage  

Apoptosis  

Cyclin D1  

Cell cycle arrest  

Cancer cell proliferation  

Indicates inhibitory effect of CVC and radiation
Figure 47c: Summary of the mechanisms for the observed combined effects of CVC and radiation against DMH induced colon carcinogenesis (in vivo-long term study)

\[\text{Indicates inhibitory effect of CVC and radiation}\]