# DISCUSSION

It is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, such as plants, marine organisms and micro-organisms (Newman et.al., 2003).

The rhizome of *Curcuma longa* has been widely used as a yellow color agent and spice in many foods, and it has been used in indigenous medicine for the treatment of inflammatory and other disorders. Curcumin, a diferuloyl methane, the major pigment in turmeric inhibits proliferation of a wide variety of transformed cells such as HeLa cells, (Huang et. al., 1997 ), Jurkat cells (Piwocka, Jaruga, Skierski, Gradzka and Sikora, 2001), prostrate cancer cells (Mukhopadhyay et. al., 2001) MCF-7 cells (Henry et. al., 1998), AK-5 tumor cells (Khar, Ali, Pardhasaradhi, Begum and Anjum. 1999) and many others. Several studies in recent years have also shown the inhibitory effect of turmeric and curcumin in different experimental tumorigenic models (Huang, M.T, 1994) and it has been found to be a potent inhibitor of the initiation and promotion of chemical carcinogen (12-O tetradecanoyl-phorbol-13 acetate (TPA), 1,2-dimethylhydrazine dihydrochloride (DMH), 20-methylcholanthrene, dimethyl benathracene (DMBA), benzo[a]pyrene, 7,12-dimethylbenz [a] anthracene etc.) induced tumor formation in animals (Huang, Smart, Wong and Conney,1988; Kim et. al., 1988; Soudaminin & Kuttan 1989; Deshpande, Ingle and Maru. 1997, 1998).

## ANTITUMOR ACTIVITY

In the present study we showed the antitumor activity of ethanolic turmeric extract (ETE), for ascitic fibrosarcoma cells as well for Ehlrich ascitic carcinoma cells. ETE showed propensity to induce cell death in both the cell type. The first evidence for it was the trypan blue exclusion test where within 16 hrs of ETE treatment the tumor cells exhibited 100% mortality at 25µl dose (Fig 2). ETE also showed its antiproliferative effect on tumor cells during cell cycle progression. In both the tumor cell lines it induces S phase arrest and accumulation of cells in the $G_2$-M phase and by doing so it inhibited the tumor cells for entering into the mitotic cycle ( Tab. 4; Fig. 13 & 14).

Several studies have shown that apoptosis leading to cell death could be induced by chemotherapeutic agents such as cisplatin, camptothecin, amasacrine, etoposide and teniposide. The present study also documents the induction of apoptosis towards tumor cells as revealed in FACS analysis. The percentage of tumor cells at sub $G_0$-$G_1$ phase, representing the apoptotic phase was higher than the controls (Table 3 & 4). Initiation of apoptosis was earlier within 16 hrs of ETE treatment in ascitic fibrosarcoma cells (Table 3).The process of apoptosis in tumor cells in presence of ETE has also been documented with scanning electron microscopy (Fig. 17 & 18). Initiation of apoptosis was within 10 mins of *in vitro* ETE treatment, tumor cells exhibited typical cytoplasmic blebs on the surface (Fig. 17B) which is indicative of apoptosis. With time, plasma membrane of tumor cells disintegrated and the cell volume shrank (Fig 17 D & E). Transmission electron microscopy also supported the incidence of apoptosis in tumor cells with ETE treatment, where tumor cells exhibited chromatin condensation, nuclear fragmentation, surface blebbings

and even extensive vacoulation through out the cytoplasm (Fig. 20). The direct cytotoxic effect of ETE against $^{51}Cr$ labeled tumor cells *in vitro* was also documented (Fig. 26).

Tumoricidal effect of ETE was judged *in vivo* situation after intravenous or oral application. By this procedure the tumor load could be curbed and the life span of the host could be increased. Suggested mechanisms for this inhibition of tumor growth are varied. Liu and coworkers (1993 ) showed that curcumin exerts inhibition through the serine/threonine protein kinase C pathway. Reddy & Aggarwal (1994) opined inhibition through protein tyrosine kinase transduction pathways. Others considered that the antiproliferative property of curcumin is partly mediated through inhibition of c-myc, c-jun, c-fos mRNA expression and even bcl-2 mRNA expression ( Kakar and Roy 1994; Lu, et. al., 1994; Chen and Huang. 1998).

Curcumin affects the tumor cells not only being antiproliferative, simultaneously it can also induce apoptosis as presented in the present investigation. Curcumin induced apoptosis correlated with the activation of caspase-3 and caspase-8 and the downregulation of the expression of anti-apoptotic proteins, Bcl-2 (Mukhopadhyay et. al., 2001). Sikora et. al., (1993) found that curcumin activates the downstream caspase-9 and inhibited the matrix metalloproteinase-9 (MMP-9) enzymatic activity by inhibiting DNA binding activities of NF-κB and AP-1 transcription factors in course of induction of apoptosis. Curcumin downregulates Bcl-XL, release of cytochrome c, through blockade by N- acetylcysteine, indicating role of ROS in Caki cells' death (Woo et.al., 2003). Curcumin also downregulates $p^{53}$ gene expression, by modulating intracellular $Ca^{++}$(Chen et. al., 1996).

## IMMUNOSTIMULATORY PROPERTY

One immediate goal of research in cancer immunology is to come up with methodologies to enhance the body's natural defense against malignant tumors. This immunotherapeutic measure will be a powerful weapon in the arsenal of anticancer treatments. This was an objective of our laboratory for long. And thus, we got interested in turmeric.

Initially we investigated activation of immunocompetent cells in presence of turmeric which we thought would be the basis of boosting the immune system. The dose of 25µl of ETE, which was very effective in setting in apoptosis in tumor cells from 10 mins onwards, was tried for lymphocytes too. The survival indices of lymphocytes in this dose were better and found to be stimulatory in reference to the different criteria such as in *vitro* blastogenesis, DNA synthesis and cell cycle analysis by FACS in this study. This was necessary in an absence of detailed study in the area.

ETE treatment showed increment in the total lymphocyte count compared to control groups both in primary and secondary immunization (Table. 1). T lymphocyte seems to contribute the most in this increment and not the B cells (Fig. 3). Possibly that was the reason ETE cannot boost antibody production to the level of alcohol and no treatment controls (Table. 2; Fig.5 & 6). ETE was also able to activate macrophages as their count increased with the treatment and was much higher in secondary immune response, suggesting possible involvement of macrophage in cytokine production for this event (Table 1). Activated macrophages are also the effector cells for phagocytic elimination and presentation of antigens. Further study to measure the production

of cytokines in presence of ETE and their role in T cell proliferation and differentiation needs to be done in future.

Stimulation of blastogenesis by ETE showed better participation of T cells in comparison to B cells (Fig. 8 & 9). Possibly that is why the percentage of blasts was more with lymphocytes from lymph node which harbour more of T cells than they are in spleen. However the percentage of blast transformation was lower when compared with that induced by a polyclonal activator like ConA. Highest $^{3}$H-TdR incorporation with ETE treatment corroborates the blastogenesis data (Fig. 10 & 11).

Activation of lymphocytes towards blastogenesis can be much appreciated when one compares the results induced by ETE treatment in malignant cells. The distribution of lymphocytes at different cell cycle phases after ETE treatment indicates that the lymphocytes are driven towards mitotic cycle by activating $G_2$-M transition ( Fig. 12). In the same account the tumor cells remains arrested at S-phase causing accumulation in the $G_2$-M phase, inhibiting mitosis. This is a very significant point of this study to identify ETE in the same dose regime to be mitotic for lymphocytes and antimitotic and apoptotic for tumor cells. Thus, turmeric is a potential double edged sword to destroy the malignant cells directly and through the arm of cellular immunity.

Furthermore the condition of both the cells under ETE treatment was judged by electron photography. The blastoid transformation of lymphocytes and apoptotic condition of tumor cells were beautifully revealed under SEM. TEM images reveal large volume of chromatin material distributed throughout the nucleus of ETE treated lymphocytes suggesting the healthy nature of

the cell, whereas chromatin condensation, nuclear fragmentation and blebbings were prominent in the tumor cells.

To understand the way the turmeric is effective for lymphocytes and tumor cells, one may need to take into account the chemical nature of compounds present in it. Curcumin and its analogues are unique in having both phenolic and β- diketone functional groups on the same molecule (Ligeret, 2004; Tonnensen, Arrieta and Lerner, 1995). Like Vitamin E, curcumin is hydrophobic and a lipid soluble molecule and believed to be localized within the membranous sub cellular fraction of cells. This molecule easily passses the plasma membrane and spread throughout the lipid phase of membranous structures, such as endoplasmic reticulum (ER) and nuclear envelope (Ewa et.al, 1998). And so being in the cell membrane initiate the process of activation for lymphocytes and apoptosis in malignant cells. It might modulate various enzymatic activities including inhibition of protein Kinase C (PKC) in tumor cells. Signal transduction of T cell begins with the recruitment and activation of protein tyrosine kinases (PTKs) which remains associated at the cytoplasmic tail of polypeptide chains with TCR and activation of phosphotidylinositol phospholipase C (PLC). The present investigation provides impetus for further studies in this regard.

**AUGUMENTATION OF CELL MEDIATED IMMUNE RESPONSE**

Our present study also showed that in addition to the ability to stimulate T lymphocytes to synthesise DNA and divide, ETE can drive at least some of the cells further down the pathway of cytotoxic differentiation. Conjugate formation is the prelude to target cell lysis, which was nicely demonstrated with ETE activated lymphocytes (Fig. 21A & 22) Induction of violent

blebbings (Fig. 21B & 23A) and fragmentation of nucleus and cytoplasm in the tumor target cells by the ETE activated lymphocytes leading to the formation of apoptotic bodies could be clearly observed under phase contrast light microscope (Fig. 24). The death of the tumor target cells was further revealed in $^{51}$Cr-release assay (Fig. 25).

The functional relationship between inflammation and cancer is often raised. It is now clear that proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA damage promoting agents, together certainly potentiates and further promotes neoplastic risk. So in a way it can be said that tumors acts as wound that fail to heal (Dvorak 1986). So any drug which can heal the inflammatory wound faster can act as a wonderful antidote to cure malignancy. And in this regard we found ETE to be an excellent inhibitor of delayed type hypersensitivity reaction induced in mouse paw by 2,4 DNFB and also healed the wound very faster compared to control ( Fig. 31 & 32) . *Oliver & Nouri (1991) proposed DTH type of reactions are critical for host* resistance to cancer. Inhibition of DTH response by ETE as shown by us might help it to get access in the list of anticancer agents.

Inflammatory cells such as neutrophils, monocytes, eosinophils contribute to malignancies by releasing extracellular proteases, pro-angiogenic factors and chemokines at the inflammatory sites (Coussens and Werb,2002; 1999, Kuper, Adami and Trichopoulous, 2000). The functional significance of macrophage recruitment at the site of neoplastic growth has been documented in various transgenic models (Lin et. al., 2001). Whereas ETE treatment efficiently reduced the degree of both neutrophil as well monocytes infiltration during the earlier stages of inflammation, ( Table 5 and Fig. 33), this may curb the promotion of malignancy.

The dominating role of $CD4^+$ T cells during DTH response demands the estimation of the cells in course of the reaction and was carried out with the help of Magnetic Assorted Cell Sorter (MACS). CD $4^+$ T cells in the DTH mice increased considerably over the control with ETE treatment and probably this increment contributes to the inhibitory role of ETE to DTH response.

The pleiotropic cytokine TNF-α (Mace *et al.,* 1988; Torisu *et. al,* 2000) play important roles in the immune regulation such as lymphoid cell development, activation, cell proliferation, cell death and in pro-angiogenic activities ( Rossi and Zlotnik, 2002; Balkwill and Mantovani, 2001). In the present investigation ETE could significantly inhibit TNF-α production in the DTH mice (Fig. 37), and thus by inhibiting a proinflammatory cytokine possibly contributes here towards wound healing. We observed that curcumin can do as well in inhibition of TNF-α and DTH reaction (Fig. 37, 31 and 32).

**SCAVENGING OF FREE RADICALS**

One of the recognized features of curcumin is its antioxidant property. Superoxide is the most abundantly produced free radical which dismutases into molecular oxygen and hydrogen peroxide in the presence of proton. This hydrogen peroxide then induces cellular damage in the presence of ferrous ions by a Fenton reaction, resulting in the formation of $OH^-$ free radicals and further aggravates the peroxidation of lipid membrane. Generation of superoxide and hydroxyl ion was inhibited by ethanolic turmeric extract (Fig. 38). Furthermore, ETE inhibits copper-ascorbate induced lipid peroxidation effectively in lymphocytes than in tumor cells (Fig. 39).

This signifies better protection of lymphocytes from lipid peroxidation. Probably that is why survivality of lymphocytes was always better with turmeric treatment *in vitro* (Fig. 1)

Lipid peroxidation is actually the oxidative deterioration of polyunsaturated lipids. This leads to decrease in membrane fluidity, increase the leakiness of the membrane to substances like inactive membrane- bound enzymes, $Ca^{2+}$ etc. Turmeric, we found inhibits lipid peroxidation by scavenging and neutralizing free radicals, such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^-$), contributing to cell membrane integrity. The phenolic and β-diketone (Tonnensen, Arrieta and Lerner, 1995) groups in curcumin play the role of the antioxidant.

**NITRIC OXIDE SYNTHASE ACTIVATION**

NO is pleiotropic molecule and mediates diverse functions by acting in most of the body cells through interaction with different molecular targets from superoxide anion to protein macromolecules, which can either be activated or inhibited through oxidation of thiols, hemes, Fe-S clusters, and other nonheme iron prosthetic groups of macromolecules (Fleming,1999; Ignarro, *et. al.*,1996; Moncado and Higgs, 1995; Furchgott and Jothianandan 1991; Nathan, 1992; Moncada, 1999). This NO being designated as a messenger molecule of different biological functions ( Bredt and. Snyder 1994; Gladwin, Crawford and Patel, 2004) can also acts as protector from cytotoxicity associated with oxyradical (Ignarro, 1989). We observed L-arginine derived NO production in both lymphocytes as well as tumor cells with turmeric treatment. Lymphocytes collected from tumor bearing mice produced more NO then the normal lymphocytes (Fig. 40). Increase in the NO production in lymphocytes possibly leads the cells

towards cytotoxic differentiation. The higher NO level in lymphocytes from tumor bearing mice supports the contention to an extent. In all likelihood the lymphocytes in tumor bearing host were to mount response towards tumor cell and ETE hightened the response.

On the other hand, L-arginine derived NO production is also critical for tumor cells (Hibbs, Taintor and Vavrin, 1987; Xie and Fidler 1998) which was more with ETE treatment (Fig. 40). Excessive NO results in limitation of angiogenesis and in some tumor cells increases apoptosis (Hung and Xie, 2003; Hofseth *et. al.*, 2003). So, it can be suggested that in case of tumor cells, increase in NO with ETE is for inducing apoptosis. The phenomenon of apoptosis were documented in figures 13, 14, 17, 20 and 24 and $^{51}Cr$ release from ETE treated labeled tumor cells *in vitro* (Fig. 26).

**INHIBITION OF TUMOR *IN SITU***

These observations suggested to stimulate lymphocytes of the host to combat the malignant growth. ETE injected intravenously could efficiently reduce the tumor growth and thereby increased the longevity of the tumor bearing hosts (Fig. 27). ETE when given orally found to be less effective in restricting solid tumor growth (Fig. 28) probably due to poor absorbtion of curcumin by the gastrointestinal tract (Ammon and Wahl, 1991). Evidences suggest that curcumin is biotransformed in the intestinal tract of humans and rodents and its systemic availability is also poor in them.

## TURMERIC AND CURCUMIN

All the findings so far suggested the potential role of ETE on lymphocytes and tumor cells which might be very important from immunotherapeutic point of view. So we carried out fractionation of ETE by thin layer and column chromatography and compared the efficacy of ETE and fractions with commercially available curcumin. Two major bands at 421 nm and 234 nm were separated by column chromatography and named F1 and F2 respectively and their percentages being 37.61% and 3.62% of ETE. The commercially available curcumin (Acros, Germany) showed a wavelength at 210nm and as per the available literature curcumin constitutes about 3 to 4 % in the turmeric (Teyyam, Health, Al-Delaimy and Rock, 2006). Hence our study suggests F2 is more to correspond with curcumin present in ETE.

When increase in number of lymphocytes in primary and secondary immune responses was studied with two fractions of ETE and curcumin, the fractions were found to do the job better (Table 7). T lymphocytes contributed more in the increment (Fig. 43). B cell count was boosted better by the curcumin than ETE and its fractions. Notable increase in macrophage count with ETE treatment had already been observed (Table.1). The fractions separately were not effective as that of ETE in stimulating T lymphocytes. Overall performance of ETE seems to be better than two fractions and curcumin. Mass spectroscopy and NMR will help us to identify curcumin, carbohydrate, proteins, fats, mineral and moisture contents in ETE and its fractions. That will allow better comparison between these and curcumin and might strengthen the use of ETE or a particular fraction for immunotherapeutic purpose.