INTRODUCTION

Cancer cells bear an indefinite proliferative capacity, being able to elude the commitment to terminal differentiation and postmitotic quiescence that normally regulate tissue homeostasis in an organism. A cancer may require, in multiple steps, as many as ten or more mutations to develop its full devastating malignant character (Weber, 2002). During this progression of cancer, sequential mutations result in changes in growth structure, hormone dependence, enzyme and cytokine production and expression of surface antigens.

Several authors demonstrated the presence of antigenic determinants on the tumor cell surface or the tumor associated antigens (TAAs), which can induce immunological response involving several arms of host's immune system (Folly, 1953; Prehn and Main, 1957; Brawn, 1970; Coggin and Anderson, 1974; Alexander, 1975). Several other types of antigens have also been detected on tumors, including organ-specific, histocompatibility, oncofetal, and tumor specific antigens. Involvement of macrophages in tumor immunity has been observed by several authors (Hibbs *et. al,* 1972; Evans, 1976; Horwitz *et. al,* 1979; Talibaue *et. al,* 1979; Krishnan and Orwell, 1979; Evans and Lawler, 1980). Some of the cytolytic or cytostatic affects of macrophages on tumor cells involve cell contact or the secretion of various cytotoxic substances or both, and phagocytosis may also play an important role. Activated macrophages release a wide variety of cytokines such as IL-1 and TNF (Old, 1985; Matsushima *et. al.,* 1985), having pleiotropic effects (Mace *et. al.,* 1988) and also exhibit profound effects on tumor. TNF produced by activated macrophages can account for all the classical tumoricidal effects against some tumors *in vitro.* Activated macrophages synthesize nitric oxides from L- arginine, and these reactive nitrogen intermediates also appear to be important mediators of killing of tumor cells (Hibbs, *et. al,* 1987, Gladwin *et. al,* 2004). Macrophages from tumor bearing host also suppress autoreactive T cell proliferation by producing suppressor molecules prostaglandins (Alleva, *et.al.* 1994).

Hellstrom and co-workers have shown that tumor bearing host often possesses lymphocytes which are cytotoxic to its tumor cells *in vitro* and this number also declined in hosts having progressive tumors (Hellstrom and Hellstrom, 1969; Hellstrom *et. al,* 1971; Russell *et. al,* 1976;

Vose *et.al,* 1977; Vose, 1980). The cytotoxic cells generated *in vitro* or *in vivo* against experimental tumor cells were always found to be Thy-1^+ or T lymphocytes (Fredman 1972; Cerottini and Bruner, 1974; Wybran *et al,* 1974; Ting, 1976; McClusky and Bhar, 1977; Weistein and Okan, 1980; Vose, 1980; Green, 1981; Kedar and Weiss, 1981). At the same time NK cells do also have the ability to express cell mediated cytotoxic activity in a non-MHC restricted way, against a wide variety of infected cells and tumor cells (Lala *et. al,* 1985). Besides stimulating the cytolytic function, activation of NK cell induces production of interferon (IFN-γ), granulocyte macrophage colony stimulating factors (GM-CSFs), tumor necrosis factor (TNF-α), IL-8 and other cytokines.

Inspite of having a well organized immune system in higher vertebrates; malignant cells often evade immune surveillance as tumor associated antigens are found be antigenically very poor. Cancer cells are genetically and phenotypically less stable than normal cells and can rapidly change to escape immune destruction. Tumor cells may express mutant proteins that are tumor specific, but these mutant proteins may not serve as antigen. Antigenically poor tumor cells cause weak stimulation that produce incomplete or partial stimulation of TCR mediated T cell activation and thus these non-immunogenic type tumors grow progressively causing death to the host. Antibodies secreted against TAA often mask the antigens and deprive T cells from being activated against target cells or from recognizing target cells for destruction (Mansson, 1991). This antibody masking explains one of the mechanisms by which tumor cells escape immune surveillance mechanism despite the humoral immune response.

The similarity in several antigens between tumor cells and normal cells, (i.e. TAA might resemble an autologous major histocompatibilty complex (MHC) coded product) and the fact that tumor cells are normally masked by a sialomucinous glycocalyx, so that the immune system of the host is normally not sensitized against the antigens, suggest that most tumors are not strongly immunogenic and so remains the risks of malignancy. Thus, the possibility of activation of immunity to malignancies by immunization with tumor cells was not favored. The higher rate of mutation of tumor cells (Weber, 2002) which circumvent ongoing immune responses and the finding that many tumors down-regulate MHC antigens and make poor target for cytotoxic cells and also, are able to avoid the immune response by releasing certain suppressing factors which

can even suppress macrophage mediated cytotoxicity (Cameron, 1983), makes cancer altogether difficult, to treat.

Surgery is the most frequent primary treatment for cancer. Surgery and anesthesia are associated with transient suppression of the cellular immune system correlating with degree of tissue trauma (Cole & Humphrey, 1985). Radiation therapy is also suppressive for the cellular immune system and even induces losses of CD4 T cells which do not recover over months of observation (Ehrke *et.al,* 1996). Chemotherapy, particularly multi-drug chemotherapy, as employed in many cancers, is generally accepted to be not only myelosuppressive but immunosuppressive as well. Added support in this area has also come from organ transplant patients maintained in high dose of immunosuppressive drugs, who were found to be more prone to develop neoplasms than the normal population (Faanes *et.al,* 1980).

Advances in tumor immunology have directed emphasis toward T-cell mediated cytotoxicity and DTH type of reaction as critical mechanisms of host resistance to cancer (Oliver and Nouri, 1991; Roth *et. al,* 1994; Boon *et. al,* 1994; Toes *et. al,* 1994; Van *et al,* 1995; Wang, Rosenberg, 1999) Failure to effectively activate T-cells may result from defective antigen processing and presentation by antigen presenting cells (Wagner, 1973; Wagner and Rollinghoff, 1976, 1978; Waterfield, Waterfield and Moller, 1976) . Thus, the interaction of the host's immune system with the tumor provides a spectrum of factors potentially contributing to impairment of T lymphocyte and monocyte reactivity, and the abrogation, not only of specific responses to tumor antigens, but also cellular immunity in general. Thus, the interaction between the tumor and the immune response represents a severe local and mild to moderate generalized cellular immune deficiency which, if corrected, might favour tumor eradication or at least inhibition of tumor progression (Penn, 1994, 1995; Hadden, 2003).

One immediate goal of research in cancer immunology is the development of methods to harness and enhance the body's natural tendency to defend against malignant tumors. Immunotherapy represents a new and powerful weapon in the arsenal of anticancer treatment; it offers the tantalizing possibility of a fourth modality for the treatment of cancer, in addition to surgery, chemotherapy and radiotherapy. Immunotherapy implies immunologic manipulation of the host such that the host can mount both cellular and humoral attacks against the cancer

(Ray, 1982; Lotze and Finn, 1990; Old, 1996; Mitchell, 2003). It strengthens the overall activity of the immune system including those elements most able to combat cancers. T cells are considered to be more effective to combat tumor growth than other different cell types; therefore stimulation of T cells is likely to inhibit the tumor growth in a much effective way. At the same time in view of heterogeneous nature of tumor antigens, stimulation of several clones of T cells rather than a specific clone would be more fruitful. Polyclonal activating agents like Con A or PHA, which can generate large pool of activated T cells, both *in vitro* and *in vivo* were found to be effectively generating cytotoxic T cells against malignant cells with diverse array of TAAs (Heininger *et. al,* 1976; Waterfield & Waterfield, 1976; Waterfield, *et. al,* 1981; Chaudhury & Chakravarty 1983). Chakravarty and Maitra (1983, 1990), also demonstrated that the tumor induced angiogenesis in the anterior eye chamber of mice and growth of chemically induced fibrosarcoma can be inhibited in the presence of lymphocyte activated with Con A.

Cytokines may prove more valuable in combination with one another or with other treatments in curbing malignancy. IL-2, found to be immunostimulant in cancer patients (Rosenberg, *et.al.*1993, Rosenberg, 1986) is a useful component of combination immunotherapy, such as with melanoma peptide vaccines, or with interferone-α-2b (rIFN-α), as a dual combination or part of biochemotherapy regimen (Mitchell, 2003). IL-2 therapy though promising (Lotze *et. al,* 1985) has its own limitation as high dose IL-2 therapy is seen to cause many systemic side effects in human (Margolin *et.al*, 1989; Kragel *et. al,* 1990). However IL-2 infusions *in vivo* leads to generation of lymphokine activated killer (LAK) cells, which can lyse a wide variety of fresh, NK resistant tumor cells (Rosenstein *et.al.* 1984) and freshly isolated autologous and allogenic tumor cells. With the expectations to get better results, the use of lymphokine activated killer (LAK) cells to combat malignancy has been extensively studied in both mouse and human models (Lotze, *et al.,* 1981; Grimm *et.al.,* 1982a, 1982b, 1982c; Rosenberg *et.al.,* 1993). LAK cell therapy also mediates the regression of established metastasis from a variety of immunogenic and non immunogenic tumors in tumor bearing animals (Mule *et.al.,* 1985, Salup and Wiltrout, 1986) and man (Rosenberg *et.al.*, 1986, 1993). Although LAK cell therapy seemed promising and effective in tumor regression, generation and isolation of LAK cells is cumbersome. Adoptive immunotherapy with tumor infiltrating lymphocytes (TILs) is gaining much attention as these are believed to be 50 to 100 times more potent than LAK cells to be used for immunotherapy (Rosenberg *et.al,* 1986). Das (1997) also showed that adoptive

transfer of activated TILs in mice bearing palpable tumors, causes 37.5% of the mice free of tumors, and in the remaining mice, rate of tumor development was slower and their life span increased.

Immunomodulatory agents including plant products can activate the immunological responsiveness of an organism directly at cell level or by inducing production of mediators (Upadhyay, 1997). Dahanukar and his co-workers have reported that the extracts from plants like *Tinospora cordifolia, Asparagus racemosus* and *Withania somnifera*, provide protection against bacterial infections in mice by specifically stimulating macrophages for enhanced phagoctic activity and intracellular killing ability. Methanol and chloroform extracts from the roots of *Ancistrocladus tectorius* were not only found to be antibacterial against eleven species but also anti-viral (HSV-type) and the extracts were able to enhance cytotoxicity (ADCC) of antibody opsonized sheep red blood cells. Alcoholic extract of the fruits of the plant *Piper longum* and its component piperine were found to stimulate the hemopoietic system of mice and also showed an increase in bone marrow cellularity and α-estarase positive cells indicating its effect on stem cell proliferation. This extract caused increase in antibody forming cells and circulating antibody titre (Kuttan, Sunila, 2004). *Azadirachta indica* (neem) activates the immune system non-specifically which responds more dynamically to subsequent mitogenic and antigenic challenge. It primarily activates macrophages and expression of MHC-II antigens on them indicating efficient antigen-presentation. Mice splenocytes treated *in vitro* with neem extract produce IL-2, IFN-γ and TNF-α indicating activation of T_{HI} type of cells (Upadhyay, *et. al,* 1999). Aqueous extract of *Albezzia lebbeck* stimulates IgG production in mice (Barua *et. al*, 2000).

Plants derived compounds have also been an important source of several clinically anti-cancer agents. Vegetables, fruits, whole grains, herbs, nuts and seeds contain an abundance of phenolic compounds, terpenoids, sulphur compounds, pigments, and other natural antioxidants and have been associated with protection from and treatment of cardiovascular diseases and cancer (Cragg & Newman, 2005). Watery extracts of *Phyllanthus embilica* has been found to enhance natural killer cell activity and antibody dependent cellular cytotoxicity (ADCC) in syngeneic BALB/c mice, bearing Dalton's lymphoma ascites (DLA) tumor. Green tea has also been found to enhance the humoral and cellular mediated immunity and decreasing the risk of certain

cancers (Dureja, Kaushik and Kumar, 2003). Ginseng (*Panax ginseng*) enhances production of macrophages, B and T cells, NK cells and colony-forming activity of bone marrow (Klein *et. al,* 2000). Alcoholic extract of *Piper longum* fruits was found to be toxic to Dalton's lymphoma ascites (DIA) cells and to Ehlrich ascites carcinoma (EAC) cells. Administration of this extract was also found to inhibit solid tumor development and increase the life span of tumor bearing mice (Sunila and Kuttan, 2004).

Among the various plant derived compounds rhizome of turmeric is most extensively used as a spice for its color, taste and flavor and also for its medicinal properties. The colouring principle of turmeric was isolated in the 19^{th} century and was named **curcumin** after the plant, *Curcuma longa* Linn. from which it was extracted. The traditional uses of turmeric in folk medicines are multiple and many of these therapeutic effects have been confirmed by contemporary scientific research. Reasonably good number of papers suggest its broad spectrum of effects, including anti-tumor, anti-mutagenic, anti-carcinogenic, anti-inflammatory, antioxidant anti-bacterial, anti-viral and many other properties.[Literature review on different biological activities of curcumin have been cited, p. 12-20]

Research works that have been carried out with turmeric and curcumin till date are mostly to show its inhibitory property towards tumor growth, carcinogens, mutagens, viruses etc. Its effect on immunocompetent cells has not been looked into detail. The present investigation elucidates the effect of ethanolic turmeric extract (ETE) both on lymphocytes vis-à-vis tumor cells *in vivo* and *in vitro* to assess the effective dose of ETE, antibody mediated response during primary and secondary immune response, activation of lymphocytes in terms of blastogenesis and DNA synthesis, cell cycle study by Flourescence Activated Cell Sorter (FACS) to assess the proliferation of cells in activation and apoptosis, morphological changes through electron microscopy, cytotoxic functions of lymphocytes towards tumor cells and the growth of tumor. Changes in leukocyte and $CD4^+$ helper T cell counts and TNF-α regulation during delayed type hypersensitivity and also the biochemical estimation of free radical generation (superoxide ion, hydroxyl radical, hydrogen peroxide and nitric oxide), were taken into consideration. Efforts have also been made to elute out the active fractions present in the ethanolic turmeric extract.

Firstly, *in vitro* survivality assay of both lymphocytes and tumor cells have been carried to assess the effective dose of ETE.

Secondly the kinetics of the primary and secondary response of mice injected with ethanolic turmeric extract (ETE) were investigated in terms of antibody-secreting cells by plaque forming cell (PFC) assay, Haemagglutination assay, Haemagglutination reaction in presence of 2-merceptoethanol (ME) and Immunoglobulin G (IgG) estimation by ELISA. Sheep red blood cells (SRBCs) was used as antigen. Simultaneously the count for B and T lymphocytes and macrophages from the spleen of turmeric treated and control animals were taken into consideration, which will help to judge whether the treatment leads to increment in the cell number *in vivo* in course of heightened immune response with the ETE treatment during primary and secondary response.

Degree of activation and proliferation of lymphocytes treated with ETE was analyzed in terms of blastogenesis (blastoid transformation) of B and T lymphocytes and DNA synthesis through ^{3}H-thymidine incorporation.

Upon activation lymphocytes are programmed into cell cycle progression which can easily be judged through FACS analyses. FACS in addition to the measurement of DNA content per cell can also analyze the percentage of cells in a given population at different stages of mitotic cycle. Cells arrested in mitotic pathway may proceed towards apoptosis; this can also be analyzed by FACS (Hartwell *et. al*, 1994; Qin *et. al,* 2004). That is why effect of ETE on lymphocytes and tumor cells during cell cycle progression has been studied with FACS.

If ETE treatment leads to activation or apoptosis of a cell type, then that will definitely be reflected on their cellular morphology. The visual image of the cell surface and internal milieu of the ETE treated lymphocytes as well as tumor cells, were obtained through scanning electron microscopy and transmission electron microscopy.

The cellular arms of immune system play a key role in antitumor immunity. Cytotoxic T lymphocytes (CTLs) are leading candidate in most cases of tumor rejection, their generation is augmented during the rejection and cytotoxic activity against tumor target cells *in vitro*. We have

followed the cell mediated immune response in the first step by estimating the number of conjugates formed between effector lymphocytes and tumor target cells and subsequent viability of tumor cells. Then the cytotoxic activity of CTL was judged by ^{51}Cr- release assay after ETE treatment.

The enhancement or potentiation of host defense mechanism has been recognized as a possible means of inhibiting tumor growth without harming the host. Several laboratories have shown that stimulation of the reticuloendothelial system of host with certain non-specific agents like BCG (Mathe *et. al*, 1972) and other killed microorganisms (Milas, Gutterman & Basic, 1974; Purnell, Kreider & Bartlett, 1975; Ray, Cooper & Mark 1979) restrict the growth of malignant tumor. T cell-mediated immune response is considered the effector mechanism in rejection of grafts including neo-antigen-bearing malignant cells (Cerottini & Bruner, 1978; Green 1981). Concanavalin A, a polyclonal T cell stimulator, was found to activate the murine T cells, both *in vitro* and *in vivo* driving the cells all the way to cytotoxic killer cells against targets of H-2 nonidentity and tumor cells (Waterfield & Waterfield 1976; Chakravarty & Clark, 1977; Chakravarty & Maitra, 1983, 1990). Thus, with an hope whether turmeric can activate the immune system and restrict solid as well as ascitic tumor growth, it was administered intravenously as well as orally to the tumor bearing mice.

Inflammation considered as a critical component of tumor progression and the tumor environment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering tumor growth, stimulates angiogenesis, induces fibroblast migration and maturation, and enables metastatic spread via engagement with either the venous or the lymphatic networks. (Coussens & Werb, 2002) And thus anti-inflammatory therapy is suggested to be efficacious towards early neoplastic progression and malignant conversion. Several workers investigated the anti-inflammatory property of turmeric through various inflammatory models such as cotton pellet test, granuloma pouch test, or by looking into various cyclo-oxygenase and lipo-oxygenase pathways (Srimal & Dhawan, 1973; Srivastava & Srimal, 1985;Huang *et. al*, 1991; Goel , Boland, Chauhan, 2001). Here to judge the anti-inflammatory property of ETE, the rate of reduction in DTH induration from the first day of resensitization till

it subsides was taken into consideration. Delayed type hypersensitivity (DTH) reaction was induced in mice with 2,4 dinitro flurobenzene.

In response to tissue injury during inflammation, a multifactorial network of chemical signals initiates and maintains a host response designed to heal the afflicted tissue (Marx, 2004). This involves activation and directed migration of leukocytes (neutrophils, monocytes and eosinophils, as well as lymphocytes) from the venous system to the sites of damage. Differential counts of leukocytes from the inflammation site of the DTH mice have also been taken into account to understand the effect of turmeric on the cells responsible for DTH reaction.

The analysis of cell surface markers eventually indicated that the cells responsible for DTH are indeed $CD4^+$ T cells and of although sometimes T_C cells are also involved in DTH response (Black 2000). Activation of pre-sensitized T_{DTH} cells by antigens on appropriate antigen presenting cell (APC) results in secretion of various cytokines, including IL-2, TNF-α, MIF and also TNF-β. The overall effects of these cytokines were to draw the macrophages into the area and activate them to promote increased phagocytic activity and increased concentration of lytic enzymes for more effective killing, and secretion of cytokines. $CD4^+$ helper T cells produce many of these cytokines and stimulate the differentiation of $CD8^+$ T lymphocytes. The dominating role of $CD4^+$ cells during DTH response demands the estimation of the cells in course of the reaction. The separation of the $CD4^+$ T cells from mice treated with ETE was carried out with the help of Magnetic Assorted Cell Sorter (MACS) for their estimation during the DTH reaction.

Tumor necrosis factor (TNF) play important roles in the immune system and are involved in the immune regulation such as lymphoid cell development, activation, cell proliferation, and even death. The pleiotropic cytokine TNF-α (Mace *et al.*, 1988; Torisu *et. al,* 2000) produced by macrophages, neutrophils, activated T cells, and smooth muscles cells, induces the production of IL-1 β, and, together, they play significant roles in many acute and chronic inflammatory diseases (Wahl & Kleinman, 1998; Kuper, Adami, Trichopoulos, 2000). TNF-α has been implicated in the pathogenesis of intracellular parasitic infections, artherosclerosis and autoimmune disorders and also an important regulator of T_{H1} immune response. The proinflammatory cytokine TNF-α is important in early events in tumors, regulating a cascade of

cytokines, chemokines, adhesions, and pro-angiogenic activities (Rossi & Zlotnik, 2002; Balkwill & Mantovani, 2001; Dvorak, 2002).Thus, TNF-α level in serum of the DTH mice with ETE treatment was estimated through ELISA.

Generation of free radicals by univalent reduction of O_2 is fundamental to any biochemical process and represents an essential part of the cellular metabolism (Bandyopadhyay, Das, Banerjee, 1999). There is a dynamic balance between the amount of free radicals generated in the body and cellular antioxidants to quench them and protect the body against their deleterious effects (Tiwari, 2001). So, any additional burden of free radicals can tip the pro-oxidant and anti-oxidant balance leading to oxidative stress which certainly has negative cytopathologic consequences (MacNee, Rahman, 1999; Rahaman, et. al, 2000). The unregulated and prolonged production of reactive oxygen species (ROS) in the form of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) during the metabolism of certain chemical carcinogens has been linked to mutation (oxidant-induced DNA damage), as well as modification of gene expression such as c-jun, c-fos etc. (Simonian & Coyle, 1996; Chandel, et.al, 2000; Vafa, et. al., , 2001; Klaunig & Kamendulis, 2004). ROS induce cell proliferation during the tumor promotion stage of carcinogenesis. Cellular receptors for growth modulator molecules are also affected by reactive oxygen species. The oxidizing molecule binds and activates epidermal and platelate derived growth factors and can activate downstream signaling cascade, which may contribute to carcinogenesis (Haddad, 2004). In the signaling pathways, oxidants mostly affect mitogen-activated protein (MAP) kinases/AP-1 and NF-κB (Haddad, 2002). The major pathways for cell signaling, which involve protein phosphorylation and redox dynamic fluctuations, may have a colossal impact on cellular functions ranging from proliferation and differentiation to regulation of cell cycle events, apoptosis and under extreme conditions, necrosis (Haddad, Olver and Land, 2000; Thannekal and Fanburg, 2002). Oxygen species therefore are important determinants of redox state and can interfere with the cells homeostasis and may lead to various pathophysiologic conditions.

Natural phenolic antioxidants from medicinal or edible plants have recently received much attention as promising agents for reducing the deleterious effects of oxidative stress-induced diseases (Tiwari, 2001; Naik, 2003). Curcumin present in turmeric is an active phenolic

compound and scavenge superoxide anions (Kunchandy and Rao, 1989; Kunchandy & Rao, 1990). Its antioxidant property has further been shown by its capacity to inhibit lipid peroxidation in rat brain homogenate (Rajakumar & Rao, 1994), in mouse red blood cells (Toda, Ohnishi, Kimura and Nakashima, 1998), in rat liver (Reddy and Lokesh, 1994) and also in renal epithelial cell (Cohly, Taylor and Angel, 1998). Curcumin also inhibits induction of iNOS in macrophages activated with lipopolysacharides and IFN-γ (Brouet & Ohshima, 1995). In the present investigation, the status of generation of free radicals, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) and nitric oxide (NO), by lymphocytes and tumor cells in presence of ETE has been studied which was not been so far looked into.

Initially we found that the ethanolic extract of turmeric is more effective than extract in water and the ETE was mostly used in this investigation. In course of the study we found that majority of researchers in recent time were working with the commercially available curcumin, the active component present in turmeric. So, few of our experiments were also carried out with curcumin. At the same time different fractions from ethanolic turmeric extract were isolated by Thin Layer Chromatography (TLC), followed by column chromatography (adsorption) and efficacies of the fractions eluted out were also judged through different immunological assays as mentioned earlier and the results were also compared with different concentrations of commercially available curcumin dissolved in alcohol.