ANALYSIS OF THE EFFECTS OF *CURCUMA LONGA* LINN. ON LYMPHOCYTES AND MALIGNANT CELLS IN MURINE MODEL FOR IMMUNOTHERAPY

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This is to certify that Smt. Hadida Yasmin, M.Sc. worked in my laboratory since June, 2001 under my supervision. She joined the ICMR fellowship as Senior Research Fellow which she is still continuing. She worked on the topic "Analysis of the effects of *Curcuma longa* Linn. on Lymphocytes and Malignant cells in murine model for immunotherapy" for fulfillment of the requirements of the Degree of Doctor of Philosophy (Science) of the University of North Bengal.

Smt. Yasmin performed all the experiments thoroughly and meticulously. She is conversant with the techniques and literature cited in the dissertation. It seems that the thesis is fit for submission for Ph.D. and she is worthy for award of the degree.

A Chakravarty

Prof. A. K. Chakravarty

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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.

LITERATURE REVIEW ON TURMERIC/CURCUMIN AND THEIR DIFFERENT BIOLOGICAL ACTIVITIES

Turmeric was described as *Curcuma longa* by Linnaeus and its taxonomic position as follows:

Class	Liliopsida
Subclass	Commenlinids
Order	Zingiberales
Family	Zingiberaceae
Genus	*Curcuma*
Species	*Curcuma longa*



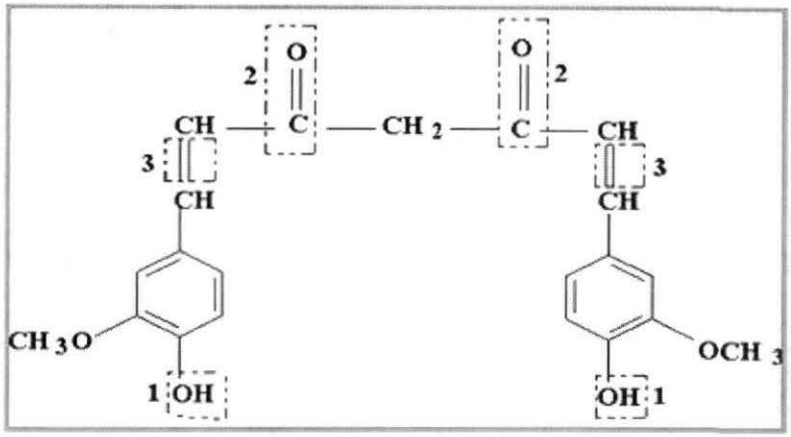
DISTRIBUTION: Cultivated throughout Asia including India, China and tropical countries.

DESCRIPTION: A rhizomatous perennial herb that measures up to 60-90 cm in height with short stem and tuft of erect leaves. Its rhizome is cylindrical, ovate, orange coloured often short branched. The flowers are pale yellow in spikes concealed by the sheathing petioles and with green flowering plates.

CHEMICAL COMPOSITION:

The important constituent of *Curcuma longa* L. is **curcumin** a diferuloylmethane (3-4%), responsible for the yellow colour. Curcumin was first isolated in 1815

and its chemical structure was determined by Roughley and Whiting in 1973. Curcumin has a melting point at 176- 177^{0}C and forms red brown salt with alkali. It is soluble in ethanol, alkali, ketone, acetic acid and chloroform. The main chain in the curcumin molecule is the aliphatic chain, unsaturated and aryl group can be substituted.



Chemical structure of curcumin

[1.Parahydroxyl groups, 2. Keto groups, 3.Double bonds]

Curcumin comprises of curcumin I (94%), curcumin II (6%) and curcumin III (0.3%). Demethoxy and bisdemethoxy derivatives of curcumin have also been isolated.

Turmeric also contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). The abundant starch is largely gelatinized. A complex acidic arabinogalactan, ukonan A, is also present. It also contains 2-7% essential oil, comprising mainly bisabolane, guaiane, and germacrane sesquiterpenes: turmerone, ar-turmerone, zingiberene, curlone, etc.; the high content of bisabolane derivatives distinguishes turmeric from other Curcuma species (Tomoda. et. al., 1990).

BIOLOGICAL PROPERTIES OF *CURCUMA LONGA* L.

ANTI-INFLAMMATORY

In traditional medicine, turmeric has been used as a potent anti-inflammatory agent and there are many papers relating the activity of compounds extracted from *C. longa* L. being potent inhibitors of inflammation. Curcumin was effective against carrageenin-induced oedema in rats (Ghatak & Basu, 1972; Srivastava & Srimal , 1985; Brouet & Ohshima, 1995) and mice (Srimal & Dhawan, 1973). Mukhophadhyay *et al.* (1982) demonstrated the anti-inflammatory activity of curcumin and other semi-synthetic analogues (sodium curcuminate, diacetyl curcumin, triethyl curcumin and tetrahydro curcumin) in carrageenin-induced rat paw edema and cotton pellet granuloma models of inflammation in rats. Arora *et. al* (1971) investigated the anti-inflammatory activity in different fractions of the petroleum ether extract of the rhizomes of turmeric (fractions A and B) in animal models and found effective in reducing granuloma growth and no toxic effects were observed. Anti-inflammatory and anti-arthritic actions of volatile oil of *C. longa* L. were also observed by Chandra and Gupta (1972).

Curcumin, in case of atopic asthmatics in response to house dust mites, inhibited Dermatophagoides farinea (D*f*)-induced lymphocyte proliferation and production of IL-2, IL-5, GM-CSF, and IL-4 in a concentration-dependent manner (Kobayashi, et.al., 1997) .Curcumin also inhibited experimental allergic encephalomyelitis (EAE) in association with a decrease in IL-12 production from macrophage/microglial cells and differentiation of neural Ag-specific Th1 cells (Natarajan & Bright, 2001). Patients with 'definite' rheumatoid arthritis were studied to compare the anti-rheumatic activity of curcumin and showed significant improvement

in morning stiffness, walking time and joint swelling, following two weeks of curcumin or phenylbutazone therapy (Deodhar 1980).

The anti-inflammatory role of curcumin was also mediated through downregulation of cyclooxygenase-2 and inducible nitric oxide synthetase through suppression of NF-κB activation (Surh. et.al., 2001). Curcumin also enhances wound-healing in diabetic rats and mice and in H_2O_2 - induced damage in human keratinocytes and fibroblasts (Phan *et. al,* 2001).

ANTI-TUMOR/ ANTI-CARCINOGENIC

The anti-tumor and antiproliferative effect of curcumin, against a variety of transformed and nontransformed cell types were investigated. 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; Soudamini & Kuttan 1989; Deshpande, Ingle and Maru. 1997, 1998).

The effect of turmeric and curcumin were also observed in different experimental tumorigenic models. The modulating effects of turmeric (T), ethanolic turmeric extract (ETE) and curcumin-free aqueous turmeric extract (CFATE) on the initiation or post-initiation phases of DMBA-induced mammary tumorigenesis were investigated in female Sprague-Dawley rats. Dietary administration of 1% T / 0.05% ETE resulted in significant suppression of DMBA-induced mammary tumorigenesis as seen by reduction in tumor multiplicity, tumor burden and tumor incidence (Deshpande.,Ingle and Maru. 1998). The effects of curcumin in oral cancers were assessed in experimental tumorigenesis using Syrian golden hamster cheek pouch model. Cheek pouches were painted with the carcinogen dimethyl benathracene (DMBA) and were fed with curcumin through diet. At the end of 14 weeks, animals given curcumin showed lower percentage of microscopic tumors as compared to controls (Krishnaswamy et al., 1998). Effects of curcumin and its derivative, tetrahydrocurcumin (THC), on development of putative, preneoplastic aberrant crypt foci (ACF) in colons of mice initiated with 1,2-dimethylhydrazine dihydrochloride (DMH) was also judged and were found to have potential chemopreventive activity against colon carcinogenesis (Kim et.al., 1998). The effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoyl-13-acetate was also studied by Huang et al. (1988), and observed that all these compounds inhibited the epidermal ornithine decarboxylase (ODC) and epidermal DNA synthesis, being curcumin the most efficient. The anticarcinogen potential of turmeric exract and curcumin was also substantiated by the reduction in tumour formation induced by subcutaneous injection of 20-methylcholanthrene (Soudamini & Kuttan.1989).

Oral treatment with T or ETE or CFATE did not show any toxicity as judged by body weights, liver weights or liver/body weight ratios (Deshpande.,Ingle and Maru. 1998). Chemopreventive

action of dietary curcumin on 7,12-dimethylbenz[a]anthracene (DMBA)-initiated and 12-O tetradecanoyl-phorbol-13 acetate (TPA) promoted skin tumor formation in male swiss ablino mice was investigated where the dietary administration of curcumin significantly inhibited the number of tumors per mouse and the tumor volume (Limtrakul, Lipigorngoson, Namwong, Apisariyakul and Dunn. 1997). Pretreatment of rats with 1% turmeric through the diet resulted in a significant decrease in induction of B(a)P-induced CYP 1A1 and 1A2 and phenobarbitone (PB)-induced CYP 2B1 in liver, lung and stomach, although the extent of the decrease was different. These results suggest that turmeric/curcumin(s) are likely to inhibit activation of carcinogens metabolized by CYP 450 isozymes, namely, CYP 1A1, 1A2 and 2B1. Topical application of low doses of curcumin inhibited 12-*O*-tetradecanoylphorbol-13-acetate -induced tumor promotion (Huang *et.al.*, 1988).

ANTI-MUATGENIC

The anti-mutagenic effects of turmeric on the levels of benzo [a] pyrene induced DNA adducts in the livers of rats were studied, where turmeric at levels of 0.1-0.5% in the diet decreased DNA adducts and mutagen load (Mukundan, Chacko, Annapurna and Krishnaswamy, 1993). Curcumin was also to inhibit the mutagenesis induced by aflatoxin B_1 (AFB_1). Dietary administration of curcumin to rats significantly reduced the number of gamma-glutamyl transpeptidase-positive foci induced by AFB_1 which is considered as the precursor of hepatocellular neoplasm (Kapoor & Priyadarsini. 2001).

Curcumin as well as its two natural analogues i.e demethoxy curcumin (dmC) and bisdemethoxycurcumin (bdmC) were found to be highly effective in suppressing genotoxicity of cooked food mutagens in a dose-dependent manner, in both the frame shift (TA98) as well as

base pair mutation sensitive (TA 100) strains of *S. typhimurium*. However, bdmC appeared to be a relatively less active antimutagen compared to C and dmC .These analogues were also found to be potent inhibitors of S9-mediated mutagenicity of hetorocyclic amines (Shishu, Singla and Kaur. 2002:).

ANTI-OXIDANT

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. Curcumin present in turmeric is an active phenolic compound and scavenge hydroxyl and superoxide anions (Ruby., Kuttan., Babu., Rajasekharan and. Kuttan. 1995). Its antioxidant property, has further been shown by its capacity to inhibit lipid peroxidation in mouse red blood cells (Toda et.al., 1998), in rat liver (Reddy and Lokesh, 1994). The effect of retinol deficiency and curcumin/turmeric on lipid peroxidation and fatty acid profile was studied in liver, kidney, spleen and brain microsomes of rats. Feeding 0.1% curcumin or turmeric for three weeks in diet to retinol deficient rats reduced the lipid peroxidation respectively to 12.5 or 22.6%, in liver, 23.7 or 24.1% in kidney, 14.4 or 18.0% in spleen and 16.0 or 31.4% in brain. 18. The inhibitory effects of curcumin and tetrahydrocurcumin (THC), one of the major metabolites of curcumin were also examined on the lipid peroxidation of erythrocyte membrane ghosts induced by tertbutyl-hydroperoxide. THC showed a greater inhibitory effect than curcumin (Song *et.al.*,2001). Studies showed that curcumin is a good scavenger of hydroxyl radical at high concentrations but at low concentrations activated the Fenton system to generate an increased amount of hydroxyl radical. Curcumin was also studied for superoxide scavenging activity and was found to be a potent scavenger. Curcumin was able to reduce ferric ions to promote the

Fenton type reaction to generate a hydroxyl radical in the presence of hydrogen peroxide ((Kunchandy & Rao, 1980). Curcumin inhibited induction of iNOS in macrophages activated with lipopolysacharides and IFN-γ (Brouet & Ohshima.1995) and was able to protect hemoglobin from nitrite-induced oxidation to methemoglobin (Umakrishnan & Rao, 1995). Curcumin was also able to reduce the amount of nitrite formed by the reaction between oxygen and nitric oxide generated from sodium nitroprusside (Sreejayan and Rao, 1997).

Curcumin (CUR) also prevented the glutathione loss occurring in dexamethasone-treated thymocytes by enhacing intracellular glutathione content in treated cells. Results showed that CUR treatment elevated the concentrations of glutathione and non-protein sulfhydryl groups, thus preventing their decrease in apoptotic thymocytes (Jaruga, *et.al.,* 1998) .Protective effects of curcumin (U1), tetrahydrocrcumin (THU1), against ferric nitrilotriacetate (Fe-NTA)-induced oxidative renal damage were also studied in male ddY mice, which showed suppression of oxidative stress-induced renal damage by dietary U1 and THU1. The *in vivo* antioxidant effects of THU1 were greater than those of U1 probably because THU1 may be more easily absorbed than U1 from the gastrointestinal tract and also had some advantages as a food additive because it is colorless. (Okada, *et. al,* 2001). Curcumin is able to repair most of the oxidized amino acid, with a great efficiency. In addition, cucumin also reacts with thiol radicals, indicating curcumin can be a powerful antioxidant to repair both oxidative and reductive damage caused to proteins by radiation (Kapoor & Priyadarshini.2001).

ANTI-BACTERIAL

Curcumin showed antibacterial property by inhibiting growth of *Streptococcus aureus* and *Staphylococcus albus* (Chopra et. al., 1941). Bhavani Shankar and Murthy (1979) investigated

the activity of turmeric fractions against intestinal bacteria such as *Lactobacillus.* Curcumin also inhibited the growth of H. pylori cagA+ strains *in vitro* (Mahady ,Pendland., Yun and Lu. 2002).

ANTI-VIRAL

The anti-viral effects of curcumin were determined on purified human immunodeficiency virus type 1 (HIV-1) integrase, where curcumin showed inhibition of an integrase deletion mutant containing only amino acids 50-21 probably by interacting with the integrase catalytic core (Mazumder, Raghaban, Weinstein, Kohn and Pommer. 1995). Curcumin also acted as an efficient inhibitor of Epstein-Barr virus (EBV) key activator Bam H fragment z left frame 1 (BZLF1) protein transcription in Raji DR-LUC cells. EBV inducers such as 12-0- tetradecanoylphorbol-13-acetate, sodium butyrate and transforming growth factor-beta increase the level of BZLF1 m-RNA at 12-48 hr treatment in these cells, which was effectively blocked by curcumin (Hergenhahn *et. al*, 2002). Curcumin was also capable of downregulating HPV18 transcription (Prustry and Das, 2005).

ANTI-PROTOZOAN

The ethanolic extract of the rhizomes was found to have anti- *Enatamoeba histolytica* activity. Curcumin was also found to have anti-*Leishmania* activity *in vitro* (Koide et. al., *2002).* Anti-*Plasmodium falciparum* and anti- *L. major* effects of curcumin have also been reported (Rasmussen et. al., 2000)

ANTI-VENOM

Ar-tumerone, isolated from *C. longa,* was found to neutralize both haemorrhagic activity of *Bothrops* venom and 70% lethal effect of *Crotalus* venom in mice (Araujo & Leon 2001

MATERIALS AND METHODS

ANIMALS

Inbred adult Swiss mice of both sexes, 8-14 weeks of age, were used for all experiments. Breeding nuclei were obtained from Indian Institute of Chemical Biology, Calcutta and are maintained with food and water *ad libitum* in our animal house by inbreeding for many generations.

TUMOR INDUCTION

Ehlrich ascitic carcinoma and Ascitic fibrosarcoma cell lines were obtained from Chittaranjan National Cancer Research Institute, Calcutta and maintained in our laboratory by serial passages. These two lines have been used for the initial experiments, the results were comparable, and we continued with Ehlrich ascitic carcinoma cells, for further experiments.

MAINTENANCE OF ASCITIC TUMOR CELL LINE

Tumor cells were collected from peritoneal exudates of the mice bearing ascitic tumor by aspiration with a syringe fitted with 27 gauge needle. After centrifugation tumor cells were washed twice with cold sterile PBS and resuspended in PBS at a concentration of 10^6 cells in 0.1ml PBS and injected intraperitoneally to the mouse for induction of ascitic tumor. Within 10 to 15 days full grown ascitic tumor develops. Serial passage was carried out after every 20 days. Average life spans of ascitic tumor bearing mice are 28 ± 4 days.

SOLID TUMOR INDUCTION

To induce a solid tumor, 10^6 tumor cells suspended in 0.1ml PBS were injected subcutaneously at the base of the left leg of a normal mouse. The mean time for appearance of palpable tumor was 14 days at the site.

ETHANOL TURMERIC EXTRACT (ETE) PREPARATION

Fresh rhizomes of turmeric *(Curcuma longa* L.*)* were obtained from local market. After cleaning properly with water; 10 gms of sample was crushed to a paste with mortar and pestle and 10 ml of absolute alcohol was added to this paste and kept in a refrigerator at 4^{o} C for overnight. The alcoholic turmeric extract was then filtered through Whatman filter paper 1; the filtrate was refiltered again through cellulose acetate Millipore filter paper (0.2 μm porosity, Sartorius) for sterilization, and the final solution obtained was aliquoted and stored at 4^{o} C in ml

The total amount of ETE prepared during each batch was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^{0} C to determine the percentage yields of the extract.

DOSES AND ROUTE OF ADMINISTRATION OF TURMERIC EXTRACT

Different doses of turmeric extract 10, 15, 20, 25, 50 and 100μl have been used for *in vitro* viability assay both for lymphocytes and tumor cells. The dose in which lymphocytes survive well but tumor cells become moribund was used for experiments. For *in vivo* experiments, turmeric extract was injected intravenously in lateral tail vein of mice.

As the turmeric extract was made in ethyl alcohol, the equivalent amount of ethanol for particular dose of turmeric was used for control, and this protocol was maintained for all the experiments.

SERUM COLLECTION

Goat (*Capra bengalensis*) serum was used for *in vitro* supplementation of culture medium. This serum was found to be effective as Fetal Calf Serum (FCS) for *in vitro* culture of lymphocytes (Chaudhuri & Chakravarty, 1983). Goat blood was collected aseptically from

jugular vein and was allowed to stand at room temperature for 45 mins for clotting and then at 4^0C for the next three hours. Serum was gently collected and then centrifuged at 1000 rpm for ten minutes. The clear serum was preserved in aliquots at -20^0C until use. When required, the serum was thawed, heat inactivated at 56^0C for 45 mins and sterilized by passing through Millipore filters (0.45 μm, Sartorius).

SEPARATION OF MACROPHAGES (MϕS)

Spleen and lymph node were collected aseptically from mice and the cells were dissociated in phosphate buffered saline (PBS, pH 7.2) with the help of stainless steel wire mesh; further dissociation of the cells was done by passing through a syringe fitted with 27-gauge needle. It was then transferred to sterile plastic petri plates and incubated at 37^0 C for 45 mins in humidified atmosphere of 5% CO_2 in air. After incubation freely suspended non-adherent cells (mostly lymphocytes) were collected in a centrifuge tube for further separation of B and T cell, while the adherent macrophages (Mϕs) remained attached to the petridish surface. The petri dish surface was given three mild flushes of chilled PBS with Pasteur pipette to obtain the Mϕs.

SEPARATION OF B AND T CELLS

The non-adherent cell types, depleted of Mϕs, were washed in PBS by centrifugation at 1500 rpm for 5 mins, and resuspended in RPMI 1640 with 10% goat serum.

In another approach Ficoll and Hypaque gradient was employed for the separation of lymphocytes from the total cell suspension. Spleen and lymph node cell suspensions in 3ml of PBS were layered on Ficoll and Hypaque solution (Type IV, Sigma Co., USA) and centrifuged at 3000 rpm for 10 mins. The band of lymphocytes at the junction of Ficoll Hypaque and PBS was taken out and washed twice with PBS. The ficoll-hypaque purified lymphocytes were finally resuspended in prewarmed RPMI 1640 with 10% goat serum. Then

the lymphocyte preparation was poured on a nylon wool fiber column for separation of B and T cells, as outlined by Julius and co-worker (1973).

For preparing the column 0.1gm teased and sterilized nylon wool (Robins' Scientific Corporation, USA), soaked in RPMI was gently packed in a 1ml syringe. The columns were loaded with cell suspensions (6 X 10^6 lymphocytes in 1 ml) and incubated at 37^0C for 1 hr. Non adherent T cells were eluted out with an excess amount of warm RPMI and re-suspended in fresh medium. The column was then filled up with chilled RPMI and further incubated in ice for 10 mins. Thus Nylon wool adherent B cells were eluted out with an excess amount of cold RPMI by agitation of the wool and then re-suspended in fresh medium. T and B cells were counted with the help of haemocytometer.

IN VITRO CELL CULTURE MEDIUM

The cells were suspended in Minimum Essential Medium (MEM, Hi-Media, Mumbai) or RPMI 1640 supplemented with glutamine, HEPES buffer, 200 mg $NaHCO_3$/100 ml, 100 U of penicillin/ml, 100 mg/ml streptomycin, 50 mg/ml nystatin and 10% heat inactivated sterile goat serum.

IMMUNIZATION

Sheep's erythrocytes were used as model antigen for immunization. Blood from jugular vein of a sheep was collected in Alsiever's solution and then two washes were made with PBS. The final pellet was diluted on volume to volume basis with PBS to make 25% of Sheep RBC (SRBC). For primary immunization 0.1ml and for secondary immunization 0.05 ml of the 25% SRBC were injected in mice intravenously in lateral tail vein.

IN VITRO VIABILITY ASSAY

Different doses of ethanolic turmeric extract were added to splenic lymphocytes, ascitic fibrosarcoma cells and Ehlrich ascitic carcinoma cells in glass culture tubes (Borosil) of

volume 5ml at a density of $1x10^6$ cells in 2 ml of culture medium. Cell survival at different hours (4, 6, 8, 12, 16, 24, 48 and 72 hr) of culture was judged by trypan blue dye exclusion test. Counting of the cells was made with haemocytometer.

LYMPHOCYTE PROLIFERATION ASSAY

Lymphocyte proliferation during primary and secondary immune responses to SRBC was judged after intravenous injection of turmeric, one day prior to primary immunization. Number of viable B and T lymphocytes and also Mϕs from the spleen of the immunized mice were counted by trypan blue dye exclusion test. Simultaneously the weight of spleen was also taken into account.

STUDY OF ANTIBODY MEDIATED PRIMARY AND SECONDARY IMMUNE RESPONSE

Antibody mediated immune response has been measured in different ways:

Plaque Formation Cell Assay (PFC)

Antibody response can be quantified in terms of the number of antibody secreting cells in the haemolytic plaque assay following Cunningham and Szenberg's (1968) PFC method with some minor modifications (Chakraborty and Chakravarty 1983). Suspension of spleen cell from a mouse, immunized with SRBC in 0.1 ml of PBS was mixed with 50µl of SRBC and 50µl of rabbit complement. After thorough mixing with the micropipette, the mixture was transferred into the micro-chambers made by two slides, fixed face to face with a bigummed tape (3M, Minnesota Mining and Mfg. Co., USA). Two slides of the chambers were sealed by dipping in a molten mixture of paraffin and petroleum jelly at 50^0 C. After 8 hrs of incubation of the slides at 37^0 C, typical plaques of lysed RBCs surrounding antibody secreting cells develop and they were counted under binocular microscope with transmitted light.

Haemagglutination Assay

The kinetics of primary and secondary humoral immune responses can easily be measured by the antibody titer technique. Agglutination reactions produce visible clumps of cells or particles bearing antigenic epitopes and antibody directed to them.

For haemagglutination assay, the first well contains 1:10 dilution of the immunized and heat inactivated serum and second well onward two fold serial dilution (1:20 to 1:20480) was made with saline in a 96 well microtitre plate. The first well contained 0.18 ml of PBS and 0.02 ml of serum. An amount of 0.02 ml of 1% SRBC as antigen was added to each well. The microtitre plate was kept at 37^{o} C overnight in humidified atmosphere. Different degrees of haemagglutination of SRBC occurred with antibody titers down the row of wells. Reciprocal value of the serum dilution in the last well giving positive haemagglutination reaction has been considered as agglutination titer value. On the right of this well the absence of haemagglutination revealed as precipitation of SRBCs at the bottom of the well as a red tiny button.

Haemagglutination reaction in presence of 2- merceptoethanol (ME)

2- mercaptoethanol (ME) reduces IgM molecules, but IgG molecules are resistant to this compound and can agglutinate erythrocytes even in the presence of this compound. Thus, whether Ab response was of primary(IgM) or secondary (IgG) nature was determined by adding 5µl of 0.1M 2-ME in a series of wells.

Immunoglobulin G (IgG) estimation by ELISA

Mouse IgG was assayed on microplates precoated with 100µl of serum samples diluted in coating buffer (8µl of serum sample and 1992µl of coating buffer) and incubated overnight at 4^{0}C. After incubation, wells were aspirated and washed 3 times with wash buffer (300µl/well). Then the plates were blocked with 200µl of 10% bovine serum albumin (BSA) and kept at room temperature for 1 hr. After incubation, plates were washed 4 times with wash buffer and then 100µl of standards (10pg/ml), controls and rabbit anti-mouse IgG primary antibody (GeneI, Bangalore) was pipetted into the wells and incubated at room temperature for 2hrs. Again the wells were washed 4 times with wash buffer and horseradish

peroxidase linked polyclonal goat anti-rabbit IgG (Genel, Bangalore) was added to the wells and incubated for 1 hr. Following a wash cycle to remove any unbound antibody-enzyme reagent, a substrate solution (hydrogen peroxide and tetramethyl- benzidine) was added to the wells and incubated for 30 mins. The enzyme reaction yielded a blue product that turned yellow on addition of stop solution (1 M H_3PO_4). The optical density of the plate was read at 450 nm in an ELISA reader (Biotech, MIOS). Readings of the different dilutions of standard mouse IgG were used for determining the concentration of IgG on the basis of Beer Lambarts' Law.

MEASURE OF *IN VITRO* BLASTOGENESIS

The transformation of T and B cells into blasts after *in vitro* activation by ethanolic turmeric extract was studied. Blastogenic activity of turmeric was also compared with that of Concanavalin A (Con A), a polyclonal activator. Con A type IV (Sigma Chem. Co., USA) at a concentration of 5μg/ml was used for blastogenic transformation of murine T cells (Chakravarty and Chaudhury, 1981, 1983; Chakravarty and Maitra, 1990). The percentage of blast was enumerated with a haemocytometer in presence of trypan blue. Cells with diameter over 6 μm were considered as blast.

MEASURE OF DNA SYNTHESIS

Blast transformation is usually accompanied by DNA synthesis and cell proliferation. DNA synthesis at different hours of turmeric treatment was measured by incorporation of ^{3}H-thymidine (^{3}H-TdR) into DNA. Cells were obtained from mice injected (i.v.) earlier with turmeric extract and suspended at a concentration of 2 x 10^6 cells/ml in culture medium (RPMI-1640), of which 200μl of cell suspension was aliquot in each well of a 96-well micro-culture plate. The micro-culture plate was incubated for 8 h at 37° C in humidified atmosphere containing 5% CO_2 in air in the presence of 1 μCi of ^{3}H-thymidine (Sp. Act. 18.5 Ci/Mm, Bhaba Atomic Research Centre, Mumbai) per well. At the end of the culture

period, cells were harvested with a PHD Cell Harvester (Cambridge, MA) onto glass fiber filters, washed with methanol, dried and kept in Standard Scintillation vials (Beckman, USA). At the time of radioactivity count, 5 ml of scintillation fluid (6 gm PPO, 0.5 gm POPOP/lt of Toluene) was added into each vial. Radioactivity was counted in β scintillation counter (LS 1800 BECKMAN, USA). All assays were done in triplicate and the level of ^{3}H-TdR incorporation was expressed as counts per minute.

CELL CYCLE ANALYSIS BY FACS

For cell cycle analysis, mice were injected intravenously with turmeric extract and at different intervals (16 and 24h), spleen cell and ascitic tumor cell suspensions were prepared in PBS separately. To 1 ml of cell suspension, 1 ml of 80% ethanol was added and the cells were fixed overnight at 4^{o} C. Fixed cells were centrifuged, the supernatant was decanted off, and 0.5µl of 500 µg/ml (Standard 250 µg/ml) RNAse A was added, followed by incubation for 45 mins at 37^{0}C. 69 mM ethidium bromide was prepared in 38 mM sodium citrate. The cells were centrifuged and suspended in 0.5 ml of 69 mM ethidium bromide (a fluorochrome which stains nuclear DNA) at room temperature for 30 min. Finally the cell cycle analysis was done in fluorescence activated cell sorter (FACS, Caliber, Becton Dickinson), in which a laser beam and light detector were used to count the DNA content of single intact cells in suspension. The peak in the DNA histogram refers to percentage of cells at a particular stage of cell cycle.

SCANNING ELECTRON MICROSCOPY (SEM)

Cells treated with turmeric extract *in vitro* and *in vivo* were fixed in Karnovsky fixative for 3 to 4 hrs at 4^{o}C. A drop of cell suspension was taken on clean glass stubs (approximately 18x18 mm) and waited for 5-10 min to allow the cells to settle down and were air dried. The cells were washed in cocodylate buffer twice for 10 mins. The cells were then dehydrated with an ascending grade of acetone (30-50-70-80-90-95% twice for 10 min each) at room

temperature and finally kept in dry acetone. After dehydration the cells were dried by critical point drying method, substituting dry acetone from the cells by carbon dioxide. After drying, cells were coated with gold in a fine coat ion sputter (J.C.F. 1100) by mounting the glass stub using the cells on a brass stub with electro-conducting paints. Cells were then examined and photographed under Scanning electron microscope (Leo 435 VP) at AIIMS, New Delhi.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

For Transmission Electron Microscopy cells treated with turmeric extract *in vitro* and *in vivo* were fixed in karnovsky fixative for 2 hrs and then washed with 0.1 M PBS three times at 4^0C. Post fixation was done with 1% osmium tetraoxide for 2 hrs and the cells were again washed with 0.1 M PBS three times. The cells were then dehydrated with an ascending grade of acetone (30-50-70-80-90-95% twice for 10 mins at 4^0C and finally kept in dry acetone for 30 mins room temperature. After dehydration, cleaning was done with toluene twice for 30 mins, followed by infiltration with embedding medium and toluene. After this cells were embedded in pure embedding medium containing araldite (CY212), dodecenyl succinic anhydride (DDSA), 2,4,6 tridimethylamino methyl phenol (DMP-30) and dibutyl phthalate. After this, polymerization was done by keeping the blocks at 50^0C for 12 to 14 hrs and then for 24 to 48 hrs at 60^0C. Finally 0.5 to 2μm ultrathin sections were cut with ultramicrotome. These sections were then placed over copper grids. Cells were double stained firstly with uranyl acetate for 10mins at dark, then again with lead citrate for 10 mins. The prepared grids were then photographed under Transmission electron microscope (Philips CM10, Netherlands) at AIIMS, New Delhi.

ESTIMATION OF NUMBER OF CONJUGATES BETWEEN EFFECTOR LYMPHOCYTES AND TUMOR TARGET CELLS AND THE VIABILITY OF TUMOR CELLS.

Ehlrich ascitic carcinoma cells were collected from peritoneum of mice as mentioned earlier. Cell number was adjusted as 10^5 cells in 0.5ml culture medium. The effector T cells were

obtained from mice injected intravenously with 25µl of ethanolic turmeric extract, following the earlier mentioned protocol. Effector T lymphocytes in 0.5 ml were added in two different target : effector ratios (1:5, 1:10) to the fixed number of 10^5 tumor target cells in 0.5ml, and incubated in complete culture medium for 2hrs at 37° C in humidified atmosphere containing 5% CO_2 in air. After 2hrs of incubation, the number of conjugate formed by association of tumor target cell and one or more T effector cells were estimated microscopically under a phase contrast microscope. Sometimes tumor target cells were found with blebbings and also in moribund condition, which were judged by trypan blue dye exclusion test.

^{51}CR- RELEASE ASSAY (CYTOTOXICITY ASSAY)

Cytotoxic ability of T lymphocytes was determined by using ^{51}Cr release assay. This assay is based upon the finding that radioactive chromium ions ($^{51}Cr_3O_4^-$) diffusing into a cell are retained in the cytoplasm for a considerable period of time. This internal ^{51}Cr is released into supernatant fluid following cell membrane damage caused by cell mediated cytotoxic response of the effector lymphocytes.

In this study tumor target cells (Ehlrich ascitic carcinoma cells) were pre-labeled by sodium chromate ($Na_2\,^{51}CrO_4$, Sp. Act. 50 mCi/mg, BARC, Bombay). An amount of fluid containing 200 µCi $Na_2\,^{51}CrO_4$ was added in 1ml of tumor cell suspension containing 10^7 cells and were incubated for one and half hour at 37° C in humidified atmosphere containing 5% CO_2 in air. The tubes containing the cells were shaken thrice during incubation for proper labeling. After incubation the cells were washed three times with PBS and the number of cells was adjusted to $1X10^3$ cells in 0.25 ml. These radioactive chromium labeled cells were used as target cells for cytotoxic assay.

Effector T lymphocytes were collected from mice treated earlier with 25 µl of ETE for 48 hrs. To 1 ml of effector cells, 250 µl of ^{51}Cr labeled target cell suspension was added in 3 different target: effector ratios (1:100, 1:50 and 1:10) and the mixture was incubated for 6 hours.

The aliquots of 250 µl containing $1X10^3$ target cells only were taken separately for spontaneous and maximum release. In the tubes for spontaneous release of isotope from labeled target cells, no effector cells were added. In the set of tubes for maximum release of radioactivity from the target cells, 1 ml of distilled water (keeping the volume same with experimental tubes) was added instead of medium.

After 6 hrs incubation culture tubes were centrifuged and 1 ml of supernatant was collected from each tube and the amount of ^{51}Cr released into the supernatant was assessed by using gamma-ray spectrometer (Model No. GR532A, ECIL, India). The percentage of cytotoxicity by the effector cells was calculated using the following formula:

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

EFFECT OF ETE ON TUMOR GROWTH AND SURVIVALITY OF HOSTS

The efficacy of ETE in controlling the growth of tumors was also investigated. For this the lymphocytes in tumor bearing mice were stimulated by repeated intravenous injections as well as by oral administration with ETE (25µl) by following different schedules:

Schedule I: Two doses of ETE was administered intravenously with an interval of one week followed by subcutaneous injection of tumor cells (10^6cells in 0.1ml of PBS) on the 4^{th} day after 2^{nd} dose of ETE.

Schedule II: Oral administration of ETE was done thrice on 0 day, 3^{rd} day and 6^{th} day, and tumor cells were injected subcutaneous on 10^{th} day. In this experiment three different concentrations of tumor cells were used; 10^6 / 10^5/ 10^4 cells in 0.1ml of PBS.

Schedule III: ETE was administered intravenously twice with a gap of one week; after 7 days

10^6 tumor cells in 0.1ml of PBS were injected intraperitoneally.

Solid tumor growth of individual mice was measured every 7^{th} day using a slide caliper. Size of a tumor was determined as an average of two readings at right angles by the slide caliper and expressed in cm^2. For growth of ascitic tumor as per schedule III, weight (gm) and girth (cm) of the mice were taken into consideration. Each schedule consisted of 6 mice and the experiments were repeated thrice.

DELAYED TYPE HYPERSENSITIVITY ASSAY WITH 2, 4- DINITROFLUORO BENZENE (DNFB)

The delayed hypersensitivity type reaction depends on overall T- lymphocyte function. Primary sensitization of the mouse was carried out by applying 0.025 ml of 0.0001% DNFB made in acetone in the right foot pad subcutaneously, and then resensitization was made with 0.025 ml of 0.000001% DNFB in the left foot pad on 8^{th} day. Turmeric was administered intravenously 1hr prior to resensitization. The degree of erythema and induration was measured in terms of the diameter of the resensitized paw in cm from the ninth day onward. Size of the reaction spot was measured twice at right angles by a slide caliper and the average was taken as index for the reaction.

DIFFERENTIAL LEUCOCYTE COUNT FROM DTH MICE

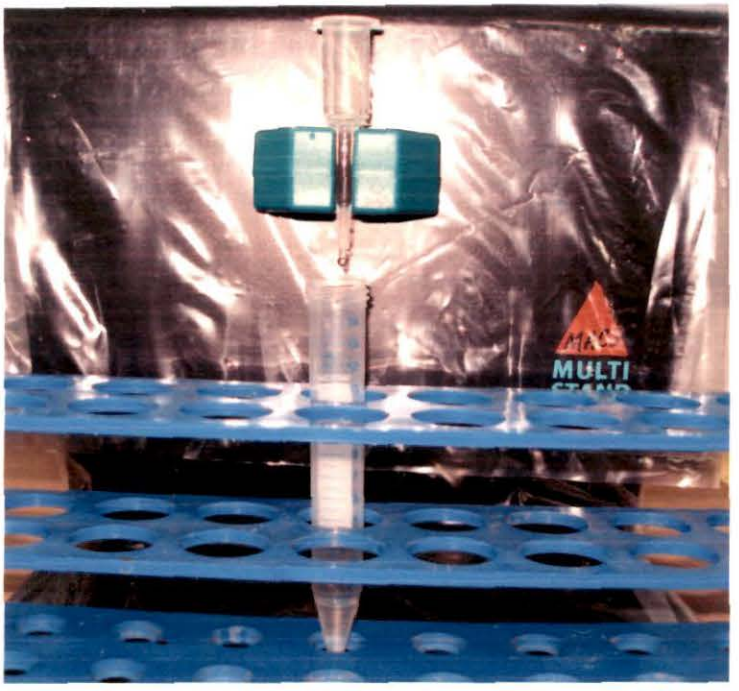
Differential count of the leucocytes at the inflammation site was made, to understand the ratio of WBC in the DTH mice treated with ETE after 24 and 48 hrs of resensitization. For this a thin and uniform blood film was prepared, stained with Leishman's stain and finally counting was done under microscope. The numbers of different types of white blood cells were expressed in percentage.

SEPARATION OF $CD4^+$ HELPER T CELLS FROM THE DTH MICE THROUGH MAGNETIC ASSORTED CELL SORTER (MACS) AND THEIR ENUMERATION

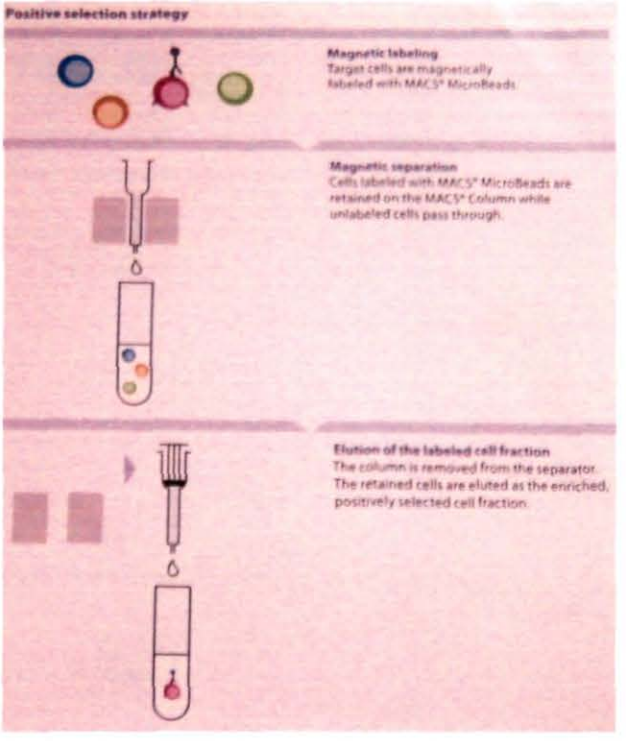
MACS (Mitenyi Biotech, Germany) technology is an extremely efficient magnetic separation method, for cells with specific antigenic markers. With MACS technology, cells of interest were specifically labeled with super paramagnetic MACS microbeads. Then the cells were passed through a MACS column, placed in a strong permanent magnet. The magnetically labeled cells are retained in the column and separated from the unlabeled cells, which passed through the column. After removing the column from the magnetic field, the retained cellular fraction was eluted.

Briefly, lymphocytes isolated from the spleen of DTH mice after 24 and 48 hrs of resentization were suspended in PBS for separating $CD4^+$ helper T cells. Cell suspension was then centrifuged at 1000 rpm for 10 mins and the cell pellet was resuspended at a concentration of 10^7 cells in 80µl of fresh PBS. To this 10^7 cells 20µl of $CD4^+$ (L3TH) microbeads (130-049-201) were added and refrigerated for 15 mins at 4 to 8°C for magnetic separation.

The magnetic separation (MS) column was prepared by rinsing with PBS. 500 µl of PBS was applied on the top of the column to make it wet and finally the effluent was discarded. As soon as the column was prepared the cell suspension containing the microbeads were poured into the column. The unlabeled cells that passed through the column were collected. The column was then washed with 3 times with 500µl of PBS. After collecting the total effluents of the unlabeled cell fractions the MS column was removed from the separator and placed in a suitable collection tube. 1ml of PBS was pipetted onto the MS column and the magnetic labeled cells were immediately flushed out from the column by firmly pushing a plunger into the column. And thus the magnetic labeled cells were collected which were then enumerated by counting them with the help of haemocytometer.



The MiniMACS Separation Unit attached to a MACS Multistand and shown with an MS Column



Digramatical representation of separation strategies with MACS

ESTIMATION OF TNF- α BY ELISA

Solid phase sandwich ELISA KIT (PharMingen, USA) was utilized for the evaluation of TNF-α. Mouse TNF-α was assayed on microplates precoated with affinity purified polyclonal antibody specific for mouse TNF-α. 100µl of standards (ranging from 500pg/ml- 15.6pg/ml), controls and experimental samples was pipetted into the wells and incubated at room temperature for 2hrs. The wells were washed 4 times and horseradish peroxidase linked polyclonal specific for mouse TNF-α was added to the wells. Following a wash cycle to remove any unbound antibody-enzyme reagent, a substrate solution (hydrogen peroxide and tetramethyl- benzidine) was added to the wells. The enzyme reaction yielded a blue product that turned yellow on addition of stop solution. The optical density of the plate was read at 450nm. Concentration of TNF-α was extrapolated from the standard curve of TNF-α (Paul *et al.*, 2001).

BIOCHEMICAL ESTIMATION OF FREE RADICAL

SUPEROXIDE SCAVENGING ASSAY

Superoxide radical (O_2^-) was generated from autoxidation of hematoxylin and was detected by an increasing absorbance at 560 nm wavelength in a UV-visible spectrophotometer (ELICO, S L164). The reaction mixture contained 0.1 M phosphate buffer (pH-7.4), 0.1mM EDTA, 50µM hematoxylin, 25µl of ETE. The final volume of the reaction mixture was adjusted to 2.5ml by adding double distilled water (Martin, Daiby and Sugrman, 1987). The inhibition of autoxidation of hematoxylin in presence of extract over the control was calculated.

HYDROXYL ION GENERATION

Hydroxyl radical was generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fentons' reaction) which attacks the deoxy D-ribose and a series of reaction that eventually resulted in the

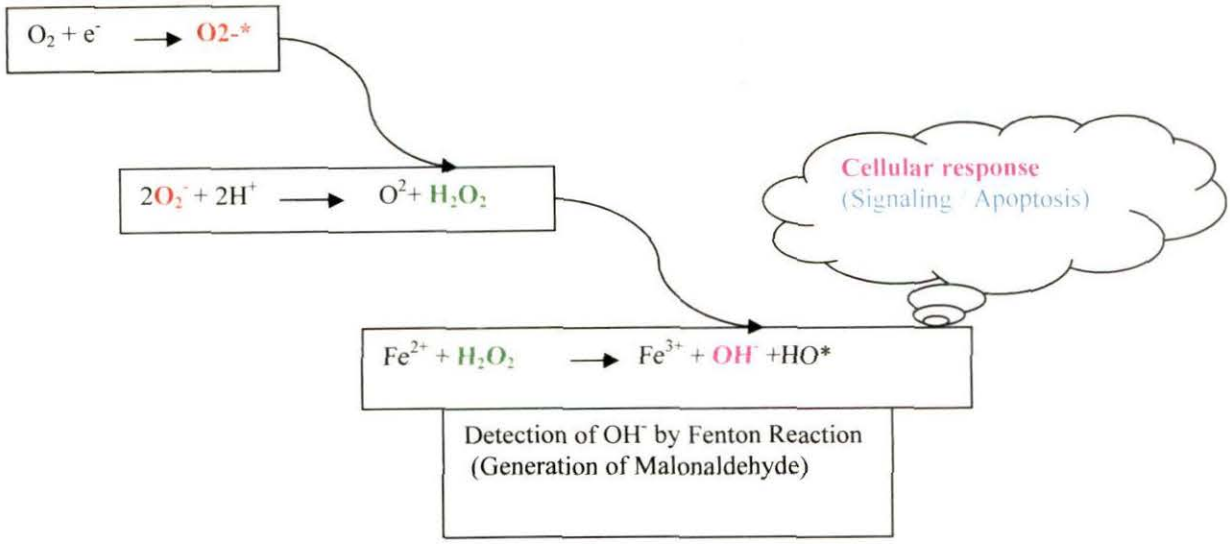
formation of malonedialdehyde (MDA). The reaction mixture contained 2.8 mM 2-deoxy D-ribose, 20mM of KH_2PO_4 - KOH (pH-7.4), 100 mM $FeCl_3$, 104 µM EDTA, 1mM H_2O_2, 1mM ascorbic acid and 25 µl of turmeric extract. The reaction mixture was incubated at 37^0C in humidified atmosphere containing 5% CO_2 in air for 1 hr. Then 2 ml of TBA-TCA reagent was added in each tube and boiled for 15 min. The color of the reaction mixture changes to a pink MDA-TBA chromogen which was finally measured at 532 nm in UV-spectrophotometer (ELICO, S L164). The level of hydroxyl radical generation was expressed as nM of MDA generated/hr (Halliwell, Gutteridge and Aruma, 1987).

LIPID PEROXIDATION

Lipid peroxidation of lymphocytes and tumor cells with the influence of turmeric extract was estimated separately according to Miller and Aust, 1989. Lipid peroxidation was induced by copper - ascorbate system and estimated as thioburbituric acid reacting substances (TBARS). The thiobarbituric acid assay is the most frequently used method for determining the extent of membrane lipid peroxidation *in vitro*. Malonedialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. MDA has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red species absorbing at 535 nm.

The reaction mixture contained $1X10^6$ packed cells in 0.2M phosphate buffer pH (7.4), with 20mMTris-HCl, 2mM $CuCl_2$, 10mM ascorbic acid and 25µl of ethanolic turmeric extract and were incubated for 1hour at 37^0 C in humidified atmosphere containing 5% CO_2 in air. Lipid peroxidation was measured as malonedialdehyde (MDA) equivalent using trichloroacetic acid (TCA) , thiobarbituric acid (TBA) and HCl (TBA-TCA reagent : 0.375% w/v TBA, 15% w/v TCA and 0.25 N HCl).

After incubation 2ml of TBA-TCA reagent was added and the mixture in each tube was shaken thoroughly. The tubes were then placed in a water bath for 15 mins and then centrifuged for 10 min at 1000 g. Finally the supernatant from each tube was taken turn wise in a cuvette and the OD value was determined spectrophotometrically at 535 nm. Results of lipid peroxidation have been expressed as nanomols of MDA produced/hr/10^6 cells.



NITRIC OXIDE SYNTHASE (NOS) ACTIVITY

NOS activity was determined by measuring the conversion of oxyhemoglobin to methemoglobin according to Jia *et al.*, 1996[35]. L- arginine was found to be the precursor for the synthesis of NO by vascular cells. Cytosolic NAPDH dependent monoxygenase is responsible for the conversion of L-arginine to NO. L–arginine first undergoes monohydroxylation to N^G- hydroxyl-L-arginine which is then oxidized to L-citrulline and produces NO. This NO then undergoes oxidation with oxyhemoglobin (HbO_2) and produces methemoglobin (met Hb). Thus the formation of metHb indicates the production of NO.

Briefly $1x10^6$ packed cells (lymphocytes or tumor cells) were incubated for 2 hr with 50 mM Tris-HCl buffer (pH 7.4), 10mM L-arginine, 64mM hemoglobin, with 25µl of turmeric extract at 37° C in humidified atmosphere containing 5% CO_2 in air. After incubation reaction mixture was centrifuged at 1000rpm for 5 min and the optical density of supernatant was measured in UV- spectrophotometer (ELICO, S L164) at 535nm. Results of NO production were expressed as pmol of NO produced/hr.

To confirm that the production of NO was actually due to the activation of NOS, a competitive inhibitor of nitric oxide synthase (NOS), 10µM N^G methyl- L-arginine acetate ester (NAME) was added in a separate set of experimental tubes.

ISOLATION AND CHARACTERIZATION OF THE DIFFERENT FRACTIONS OF ETE

Crude ethanolic turmeric extract (ETE) was first monitored on a fluorescent bound silica TLC plate (E Merck, Germany) with the solvent mixture containing 1:1 petroleum ether / ethyl acetate. The ethanolic turmeric extract was subjected to chromatographic separation over silica gel (60-120 mesh, SRL, India). Ethanolic turmeric extract was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^0C. The dried extract was then dissolved in a low polar solvent (Petroleum ether, BP-60 to 80^0C) and was added into the column with the help of a pipette. Finally column space above the adsorbent is filled with solvent with varying ratios of petroleum ether:ethyl acetate. The samples separated were finally collected in different small conical flasks. Fractions were dried under reduced pressure (Rotary Vaccum , EYELA, Japan) at 55^0 C to ascertain their respective dry weight in the ethanolic turmeric extract. The fractions were then dissolved in ethanol keeping the dry weight same as they were present in the ETE for further experimentations.

To characterize the crude extract as well as the fractions eluted, UV spectrophometric analysis was done by UV-Spectrophotometer (Shimadzu, UV-160, Japan).

IMMUNOLOGICAL ASSAYS TO JUDGE THE POTENT FRACTION

The potent fraction was judged through the biological assays mentioned earlier such as *Lymphocyte Proliferation Assay, Study of Antibody Mediated Primary and Secondary Immune Response in terms of antibody-secreting cells by Plaque Forming Cell (PFC) Assay, Hemagglutination assay, Heamagglutination reaction in presence of 2- mercaptoethanol (ME) and Immunoglobulin G (IgG) estimation by ELISA.*

To ascertain the fraction equivalency aforesaid assays were also carried out with different concentrations of commercially available curcumin (ACROS, Belgium) in ethanol (10, 25, 50 and 100μg/ml).

STATISTICAL ANALYSIS

Each experiment was done in triplicate and repeated more than thrice. This was applicable for all experiments. Results are expressed as Mean±SD of *n* observations Statistical significance was analyzed using students t test as well as using ANOVA software package. Rate of tumor growth was drawn according to the least-square fit method, and the slope for the line was calculated.

RESULTS

IN VITRO VIABILITY STUDIES WITH DIFFERENT DOSES OF ETE

MURINE LYMPHOCYTES

Survivality of lymphocytes were judged with different doses (10, 15, 20, 25, 50 and 100µl) of ethanolic turmeric extract (ETE). At different hours of assay, percentage of the viable lymphocytes cultured with ETE was found higher than the alcohol treated (control) lymphocytes. The percentage of lower doses of ETE up to 25µl showed better results (Fig 1). In overall 25µl seems to be optimal in view of significantly higher percentage of viable lymphocytes in comparison to other doses and control (Fig.1). At the end of 72 hr, 41.23 % of lymphocytes were alive with 25µl of turmeric treatment, whereas the viability was zero in alcohol treated control. The lymphocytes didn't survive beyond 48 hrs in the controls with 10 to 25µl of alcohol and the cells were alive up to 24hrs in 50 and 100µl alcohol controls.

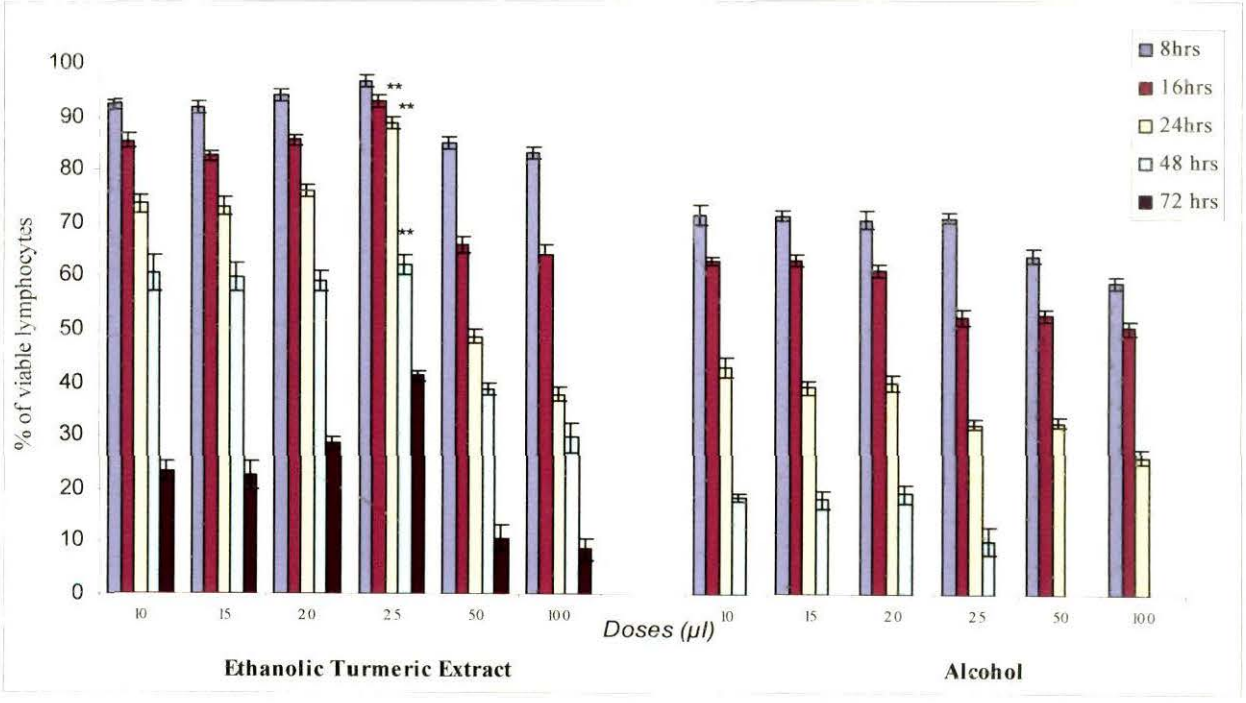


Fig. 1 Percentage of viable lymphocytes at different hours of treatment with ethanolic turmeric extract (ETE) and alcohol (control). Results are expressed as mean ± SD, **p< 0.01 compared to respective controls.

TUMOR CELLS

Ethanolic turmeric treatment causes significant level of death in Ehlrich ascitic carcinoma cells *in vitro* at 4 hrs (Fig. 2). Viability of tumor cells with turmeric fell dramatically after 8 hrs and continued to diminish with longer incubation period in comparison with the control (Fig. 2). With lower doses of ETE such as 10, 15, and 20µl all the tumor cells were dead after 16 hrs of incubation, whereas tumor cells were still alive in the control groups. The dose of 25µl ETE showed maximum killing of tumor cells, where viability fall to 0.0% after 12 hrs of incubation and were highly significant when compared to control. Higher doses were also inhibitory for tumor cells but the results were not very much significant when compared to control.

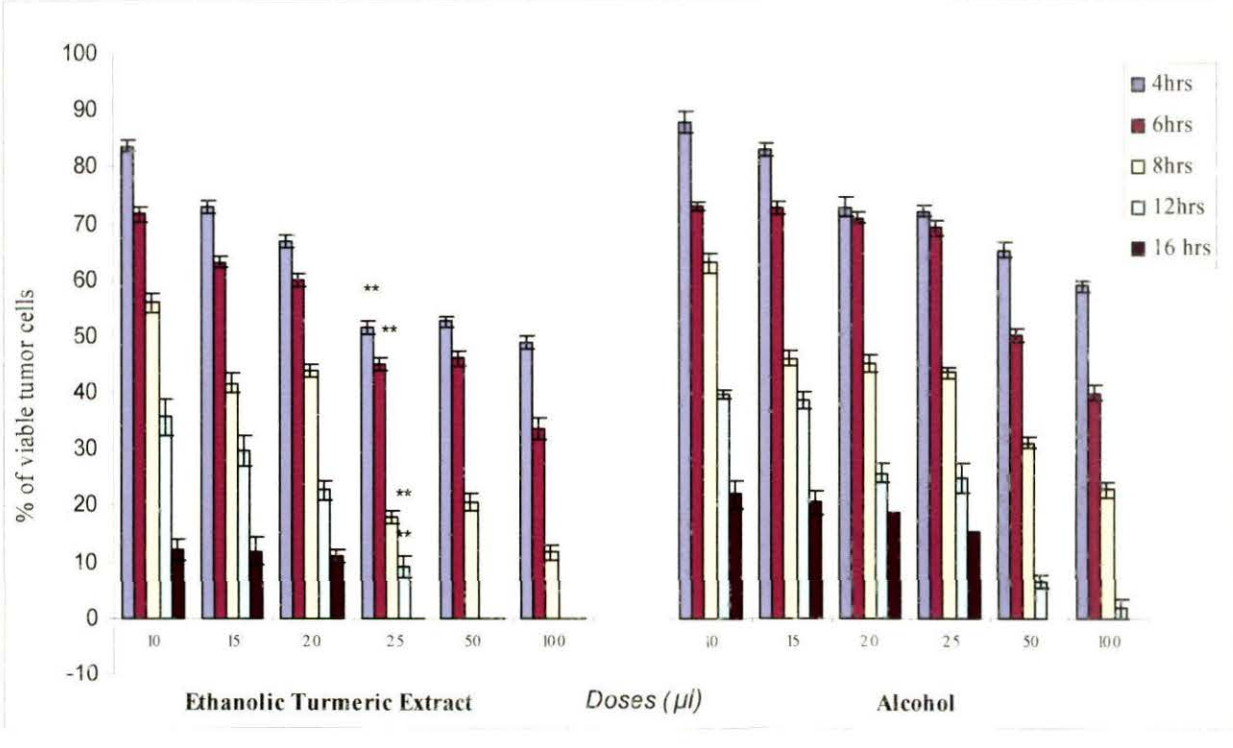


Fig. 2 Percentage of viable Ehlrich ascitic carcinoma cells at different hours of treatment with ethanolic turmeric extract (ETE) and alcohol (control). Results are expressed as mean ± SD, **p< 0.01 compared to respective controls.

EFFECT OF ETE ON PRIMARY AND SECONDARY IMMUNE RESPONSE

ESTIMATION OF SPLEEN WEIGHT AND TOTAL NUMBER OF VIABLE SPLENIC LYMPHOCYTES AND MACROPHAGES

No significant gain in the weight of spleen was observed in the ETE treated mouse over control in both primary and secondary immunization (Table 1). But ETE treatment caused higher total lymphocyte count than in the control groups both in primary and secondary immunization. T lymphocytes contributed much in the increment of the total count with ETE treatment, as revealed by 65.34%, and 61.96% T lymphocytes in primary and secondary immunization (Fig. 3 a & b).

The ETE treatment also increased macrophage count and this was much higher in secondary immune response (Table 1).

Table 1 Increment in the weight of spleen, total lymphocyte and macrophage count in SRBC immunized mice with ETE treatment in Primary and Secondary immune response. Results are expressed as mean ± SD, * $p< 0.05$ & ** $p< 0.01$ compared to respective controls.

	Primary Immune Response			**Secondary Immune Response**		
SRBC +	Weight of spleen (gm)	Total lymphocyte count x 10^7	Macrophage count x 10^7	Weight of spleen (gm)	Total lymphocyte count x 10^7	Macrophage count x 10^7
ETE	0.1673±0.032	5.210±0.093*	2.768±0.908*	0.116±0.017*	4.960±0.067*	6.406±0.078**
Alcohol	0.1405±0.0501	2.418±0.105	1.428±0.108	0.088±0.012	3.031±0.024	1.983±0.056
No Treatment	0.137±0.0463	3.476±0.113	1.983±0.370	0.090±0.009	3.06±0.032	2.24±0.037

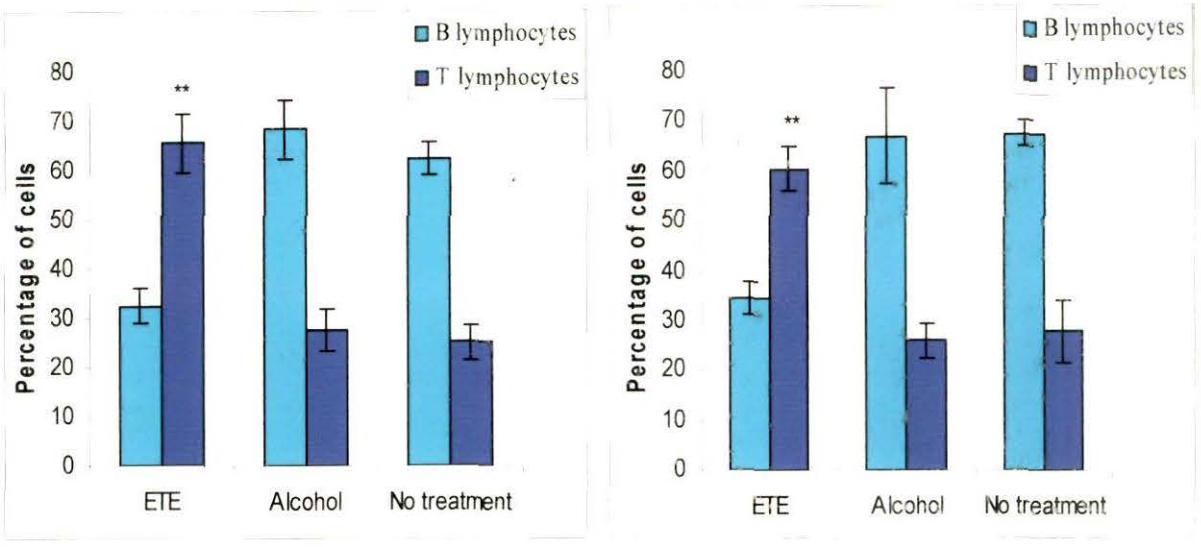


Fig. 3 Percentage of B and T lymphocytes from spleen of mice treated with ETE in ; a) Primary immune response, b) Secondary immune response. Results are expressed as mean ± SD, **p<0.01 compared to respective controls.

PFC RESPONSE DURING PRIMARY AND SECONDARY IMMUNE RESPONSE

ETE treatment stimulated T cell proliferation, but it could not boost the antibody response as measured by the number of antibody secreting cells. Significantly higher number of plaque forming cells was enumerated in control groups than in the ETE treated ones in primary immune response (Fig. 4). In secondary immune response the numbers of plaques in the control groups were also higher but statistically not significant (Fig. 4).

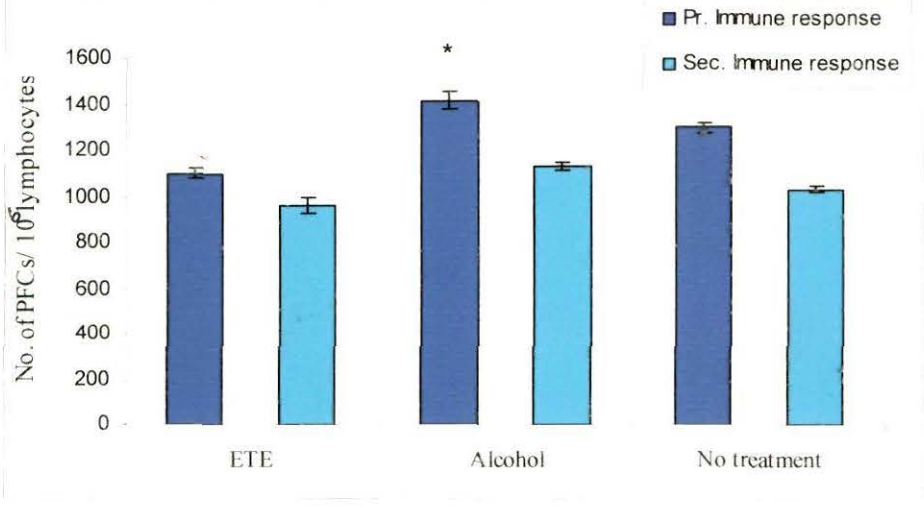


Fig. 4 No. of plaque forming cells (PFCs) in10^6 lymphocytes from spleen of mice treated with ETE in Primary and Secondary immune response. Results are expressed as mean ± SD, * $p< 0.05$ compared to ETE treatment.

ANTIBODY MEDIATED PRIMARY IMMUNE RESPONSE WITH ETE TREATMENT

The trend of PFC response was also reflected in the haemagglutination (HA) titre values, where titre with alcohol treatment was higher than with ETE (Table 2, Fig. 5), this was possibly due to the lower count of antibody secreting cells with ETE treatment as observed during PFC response (Fig. 4). The agglutination was not manifested in antibody excess zone or wells.

Table 2 Haemagglutination (Ab) titre value with ETE treatment in Primary response and Secondary immune response with and without ME.

SRBC +	Primary Immune Response	Secondary Immune Response	
	Ab. Titre value[a)]		
	Without ME	Without ME	With ME
ETE	5120	320	40
Alcohol	10240	> [b)]	40
No Treatment	2560	> [b)]	80

[a)]indicating last dilution of serum capable of agglutinating antigen. Higher value indicates better antibody response. [b)] sign to indicate titre value more than the dilution present in the 12th or last well of the row in the titre plate

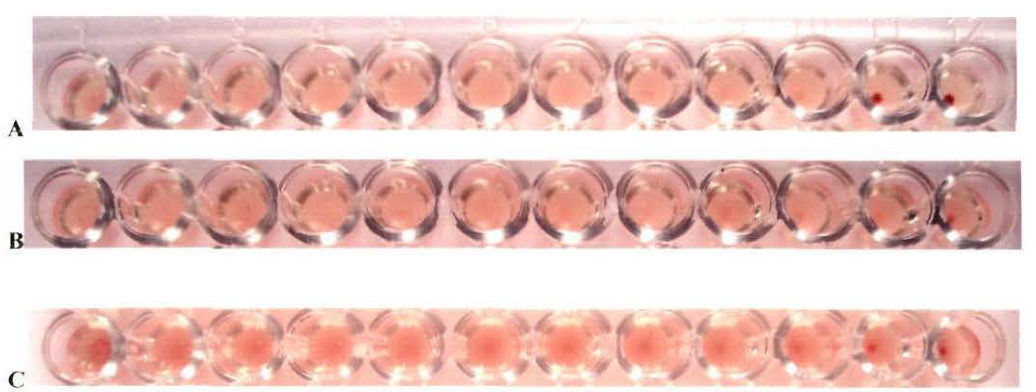


Fig. 5 Haemagglutination (Ab) titre with mouse antiserum towards SRBCs in Primary response: A) ETE treatment, B) Alcohol , C) No treatment (only SRBC). Wells with opaque look indicate agglutination of RBCs and well with distinct precipitation of RBCs at the bottom indicate no haemagglutination.

STUDY OF ANTIBODY MEDIATED SECONDARY IMMUNE RESPONSE WITH ETE TREATMENT

Circulatory antibody titre value in secondary immune response followed the same trend of primary immune response, higher response in control groups (Table 2, Fig. 6). No agglutination was in the control wells (both alcohol treatment and no treatment).

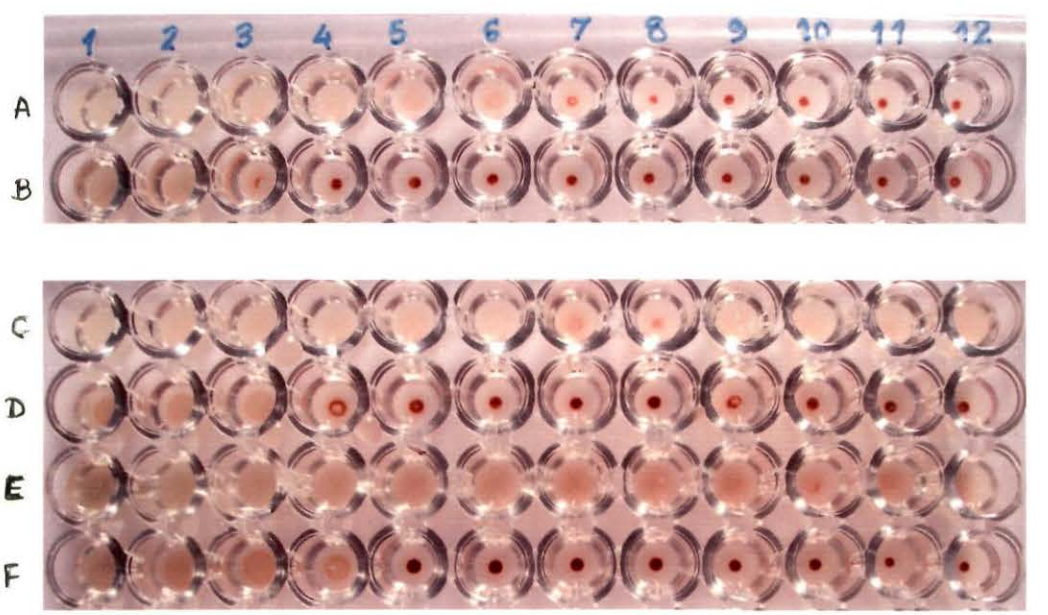


Fig. 6 Haemagglutination (Ab) titre with mouse antiserum towards SRBCs in secondary immune response: A) ETE, B) ETE + ME, C) Alcohol, D) Alcohol + ME, E) No treatment (only SRBC), F) No treatment + ME.

IgG mediated response as measured in presence of mercaptoethanol (ME) was also higher with control groups (Table 2, Fig. 6). This result has corroboration in marginal increase in the serum IgG level in ELISA test (Fig. 7).

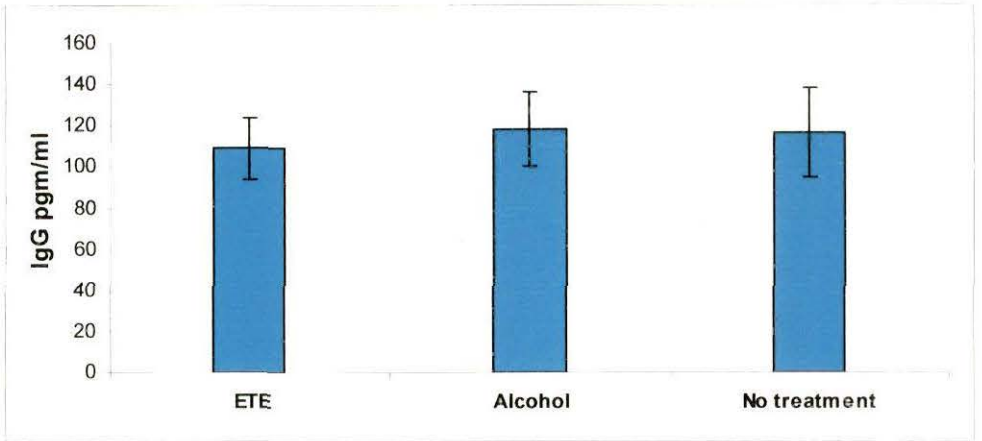
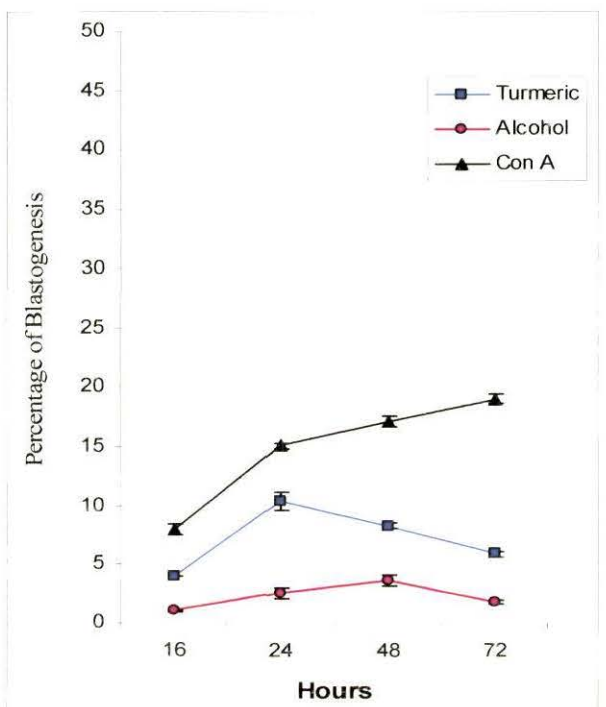


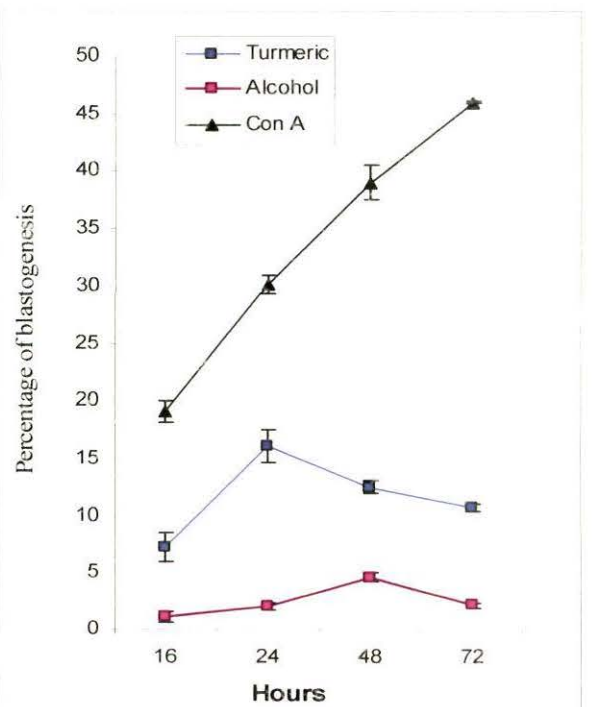
Fig. 7 Estimation of serum IgG level (pgm/ml) by ELISA in secondary immune response with ETE treatment, alcohol (control) and no treatment (only SRBC).

BLASTOGENIC RESPONSE OF LYMPHOCYTES WITH ETE TREATMENT

The treatment of ETE caused increase in number of blasts over the control (alcohol) with peak at 24 hrs. The blastogenic transformation of T cells (Fig. 8b & 9b) was higher than that with B cells (Fig. 8a & 9a).



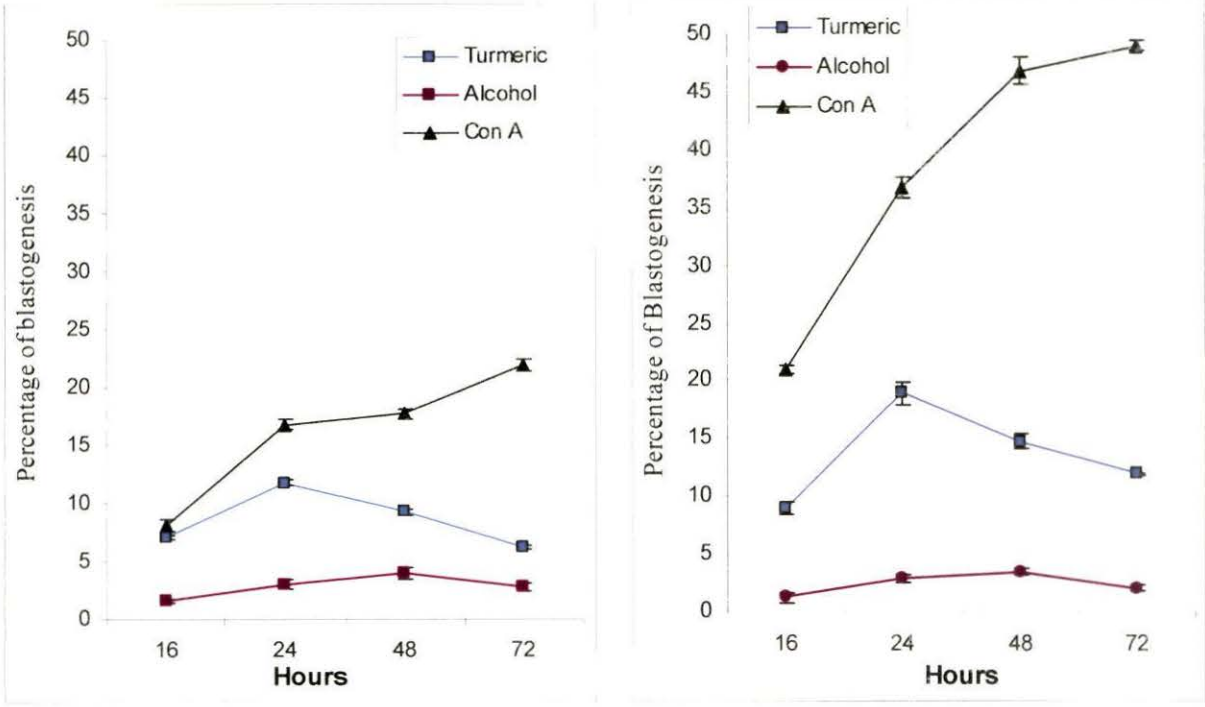
a)



b)

Fig. 8 Blastogenic responses of lymphocytes from spleen treated with ETE, alcohol and ConA: a) B lymphocytes, b) T lymphocytes. Results are expressed as mean ± SD, Two way ANOVA revealed all the treatment values are significant compared to control ($p < 0.01$).

Possibly that was reflected in the counts of the lymph node (Fig. 9a. &b) which was suppose to harbour more T cells in lymph node. The blastogenic response in presence of turmeric was reasonable but not as high as with Con A. Con A is a potent mitogen for murine T lymphocytes.



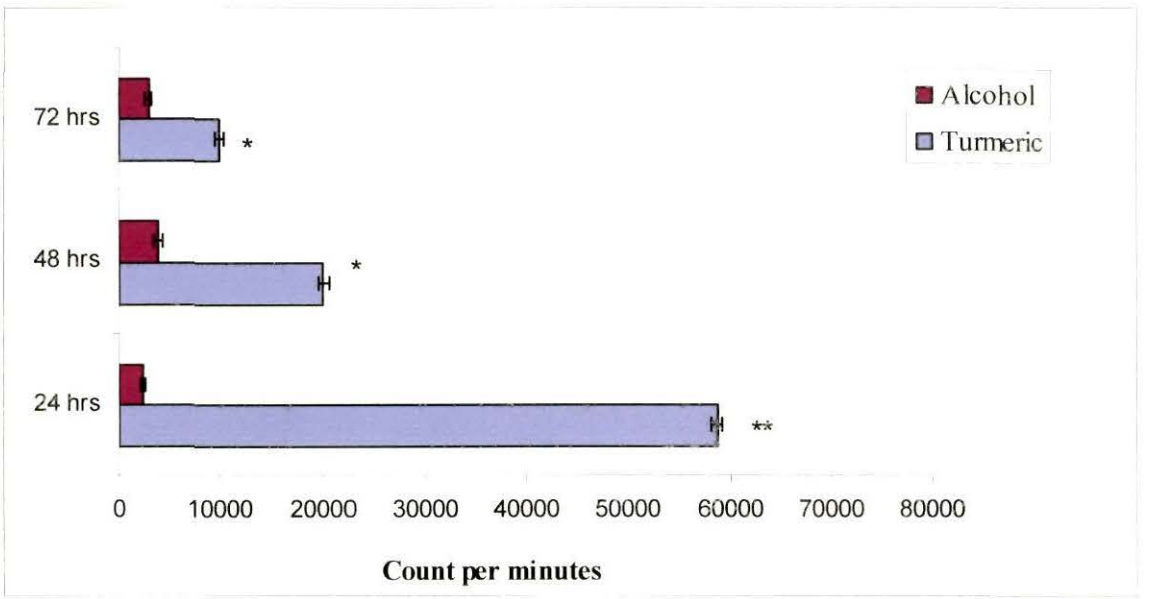
a) b)

Fig. 9 Blastogenic responses of lymphocytes from lymph node treated with ETE, alcohol and ConA: a) B lymphocytes, b) T lymphocytes. Results are expressed as mean ± SD, Two way ANOVA revealed all the treatment values are significant compared to control ($p < 0.01$).

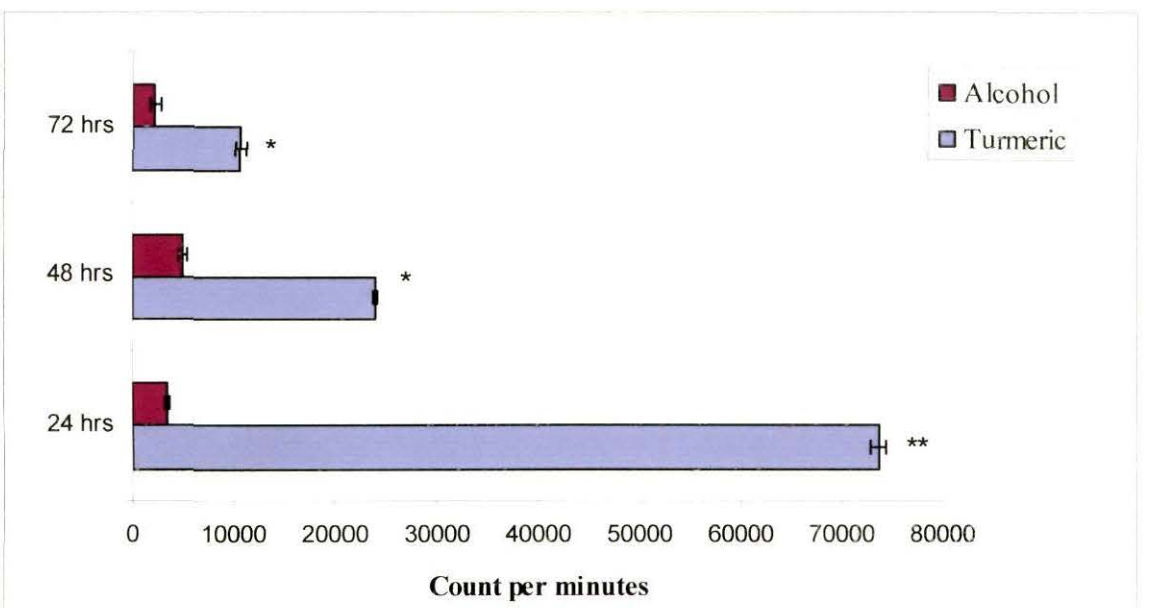
DNA SYNTHESIS BY ^{3}H-TDR INCORPORATION

The kinetics of DNA synthesis is in agreement with the blastogenesis. At 24 hrs, the ^{3}H-TdR incorporation was highest in lymphocytes of spleen and lymph nodes treated with 25 µl dose of turmeric. Again lymphocytes from lymph nodes showed better response (Fig. 10 A& B).

The result with 50µl turmeric was lower than that with 25 µl dose, and 100 µl dose could not elicit response higher than that of control (Fig. 11).



A)



B)

Fig. 10 Pattern of incorporation of ^{3}H-thymidine by lymphocytes treated for different hours with ETE : A) lymphocytes from spleen, B) lymphocytes from lymph node. Results are expressed as mean ± SD, * $p<0.05$ & ** $p<0.01$ compared to respective controls.

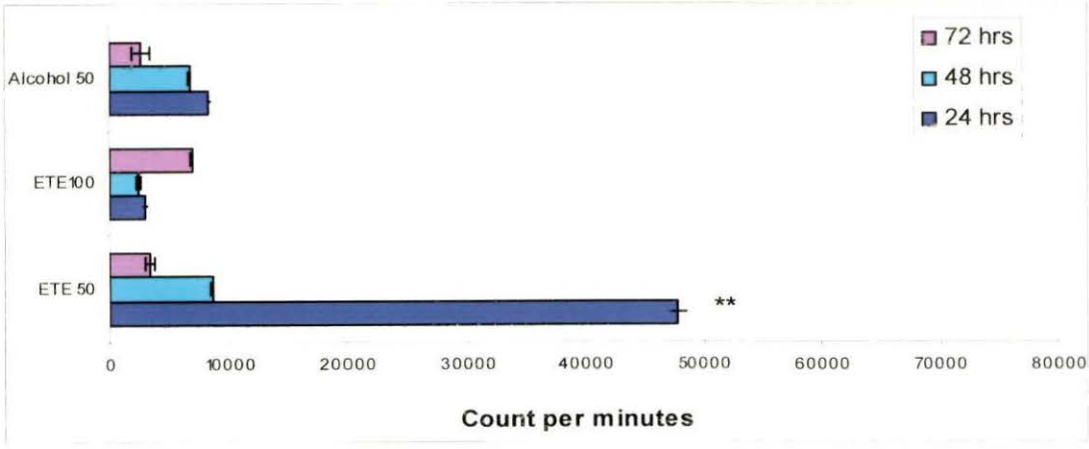


Fig. 11 The level of incorporation of ^{3}H-thymidine by lymphocytes treated for different hours with different doses (in µl) of ETE. The control values with ethanol are at the extreme. Results are expressed as mean ± SD, **$p<0.01$ compared to respective controls.

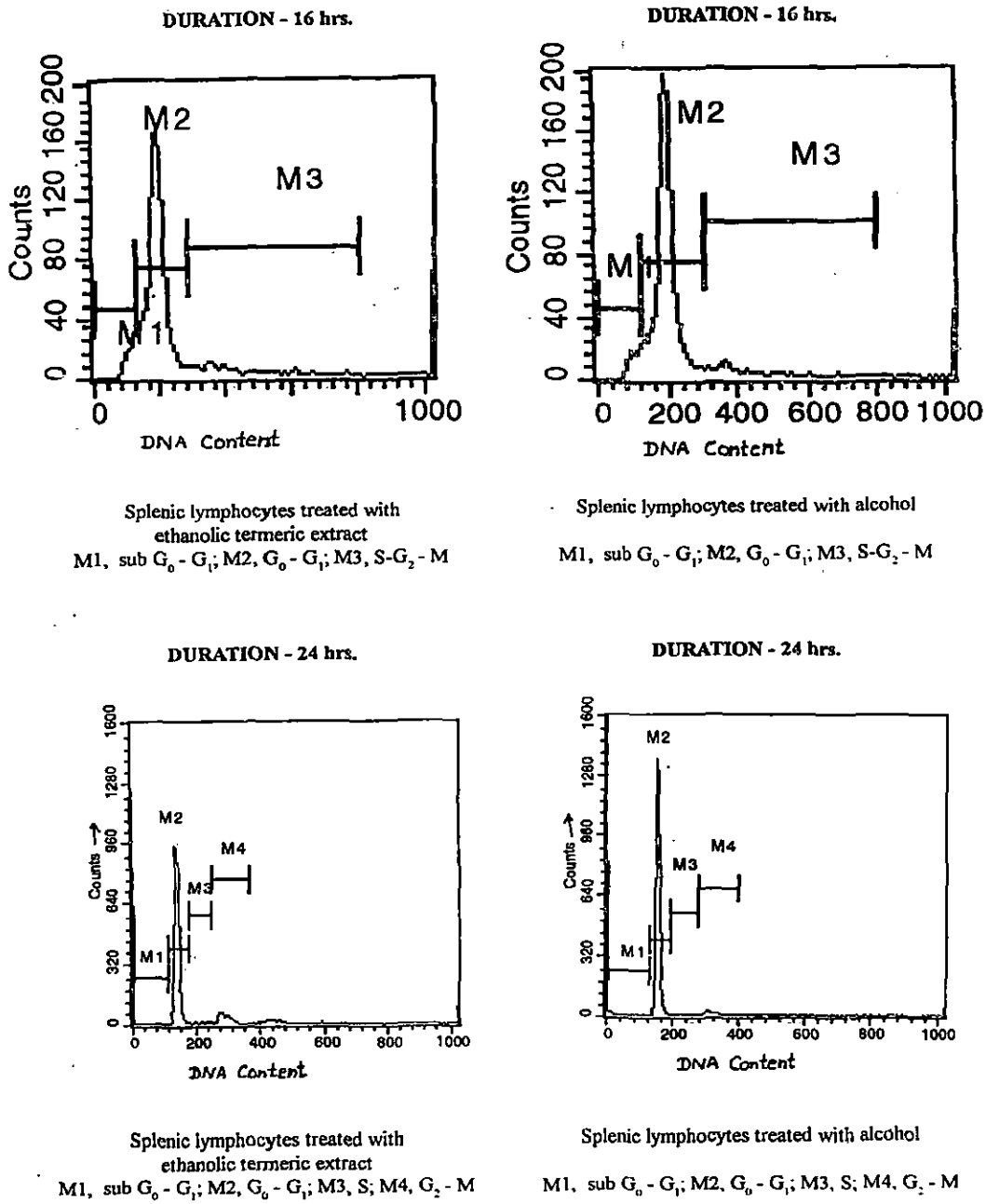
CELL CYCLE ANALYSIS WITH ETE TREATMENT

In cell cycle analysis by FACS, DNA replication was studied by tagging the fluorochrome ethidium bromide (EB). EB staining of permeabilized cells (DNA content) was plotted against cell numbers in the DNA histograms. In the text "index" refers to the percentage of cells at a particular cell cycle stage, represented by M1 to M2.

MURINE LYMPHOCYTES

Lymphocytes after 16 hrs of *in vivo* turmeric treatment did not show any significant differences in the percentage of cells at different stages of cell cycle (Fig.12, Table 3). The difference became apparent after 24 hrs of turmeric treatment, where the M1 peak (apoptotic phase) index was at 0.96 for lymphocytes against an index of 2.36 of the control (Fig.12, Table 4).This indicates higher percentage of apoptotic cells in alcohol control. The M2 peak showing the G_0-G_1 phase was also lower in the turmeric group in comparison to the control; suggesting turmeric possibly has driven the cells quickly into the next phase, i.e., S-phase (M3 peak). At G_2 -M phase, i.e., the M4 peak, the turmeric treated lymphocytes showed an index of 12.63 in

comparison with 3.34 in the control (Table 4). This indicated that turmeric has driven majority of the lymphocytes towards mitotic stage.



Splenic lymphocytes treated with ethanolic termeric extract
M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S-G_2 - M

Splenic lymphocytes treated with alcohol
M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S-G_2 - M

Splenic lymphocytes treated with ethanolic termeric extract
M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S; M4, G_2 - M

Splenic lymphocytes treated with alcohol
M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S; M4, G_2 - M

Fig. 12 DNA histograms by FACS for cell cycle analysis of splenic lymphocytes

ASCITIC FIBROSARCOMA CELLS

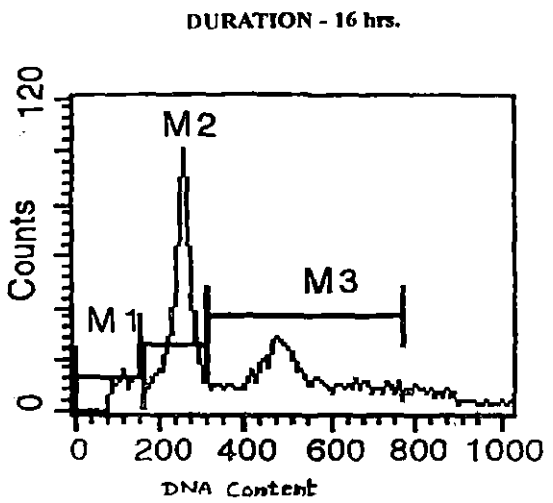
The turmeric treatment works in reverse mode for the ascitic fibrosarcoma cells. At 16 hrs, the indices for M-1 peak were 6.39 with turmeric and 0.00 in control (Fig.13, Table 3). This probably signifies that majority of the tumor cells had entered into the apoptotic state with turmeric treatment. The turmeric treatment for 24 hrs caused arrest of the cell cycle at S-phase as represented by the M3 peak (Fig. 13, Table 4). Thus, a fall in the M4 peak at G_2 -M phase have been observed, suggesting that the tumor cells have not entered into the mitotic stage.

Table. 3 Percentage of cells in different stages of cell cycle after 16 hrs treatment, shown by FACS.

Cell Cycle Stages (Peak)	**Lymphocytes (%)**		**Ascitic Fibrsarcoma cells (%)**	
	Turmeric	Control	Turmeric	Control
Sub G0-G1(M1)	1.89	2.72	6.39	0.00
G0-G1(M2)	84.10	88.92	46.41	34.27
S-G2-M (M3)	12.17	8.23	41.57	63.92

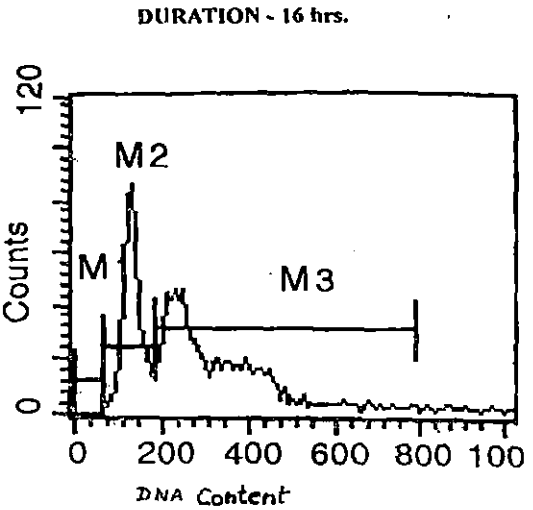
EHLRICH ASCITIC CARCINOMA CELLS

The results with turmeric treatment of Ehlrich ascitic carcinoma cells were comparable with that of ascitic fibrosarcoma cells. ETE treatment for 24 hrs significantly reduced the proliferation rate of tumor cells. That is why accumulation of 5.04 percentage of tumor cells were found at sub G_0-G_1 phase (M1 peak) in comparison to 0.30% of the control (Fig.14, Table. 4). The comparable trend remained for the next G_0-G_1 phase (M2 peak). Accumulation of arrested cells in this phase and decline in the percentage of cells at S-G_2-M phase (M3 peak) indicates that tumor cells are arrested from entering the mitotic cycle.



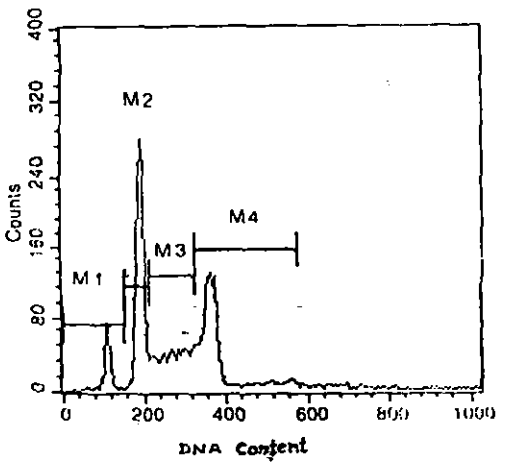
Ascitic Fibrosarcoma cells treated with ethanolic turmeric extract

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S-G_2 - M



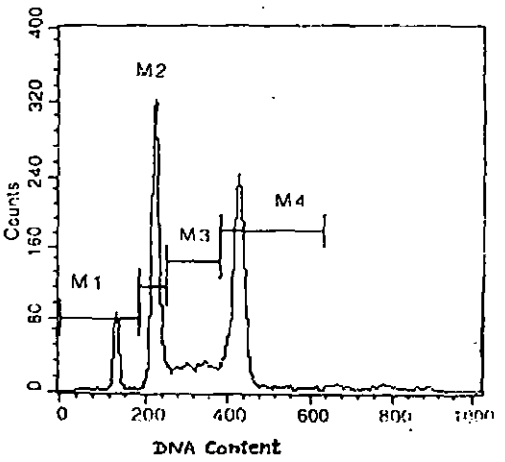
Ascitic Fibrosarcoma cells treated with alcohol

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S-G_2 - M



Ascitic Fibrosarcoma cells treated with ethanolic turmeric extract

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S; M4, G_2 - M



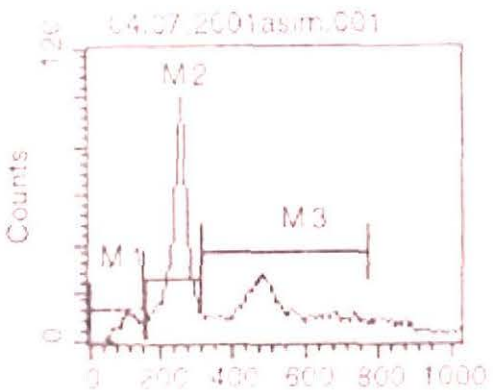
Ascitic Fibrosarcoma cells treated with alcohol

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S; M4, G_2 - M

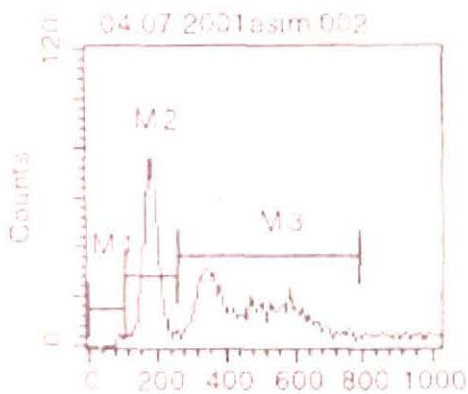
Fig: 13 DNA histograms by FACS for cell cycle analysis of ascitic fibrosarcoma cells

Table. 4 Percentage of cells in different stages of cell cycle after 24 hrs treatment, shown by FACS.

Cell Cycle Stages (Peak)	**Lymphocytes (%)**		**Ascitic Fibrosarcoma(%)**		**Ehlrich ascitic carcinoma (%)**	
	Turmeric	Control	Turmeric	Control	Turmeric	Control
Sub G0-G1(M1)	0.96	2.36	5.59	5.32	5.04	0.30
G0-G1(M2)	67.83	73.88	33.6	31.97	47.84	38.34
S (M3)	2.39	1.25	22.2	15.92	41.80 (S-G2-M under M3 peak)	57.80 (S-G2-M under M3 peak)
G2-M (M4)	12.63	3.34	33.21	41.60		



Tumor cells + ETE



Tumor cells + Alcohol

Fig: 14 DNA histograms by FACS for cell cycle analysis of Ehlrich ascitic carcinoma cells. [Sub G0-G1(M1); G0-G1(M2); S-G2-M (M3 peak)

In summary, FACS analysis reveals that turmeric is promotional for murine lymphocytes, by activating the cell cycle stages, and on the other hand it is inhibitory for cell division and induces programmed cell death in both Ascitic fibrosarcoma cells and Ehlrich ascitic carcinoma.

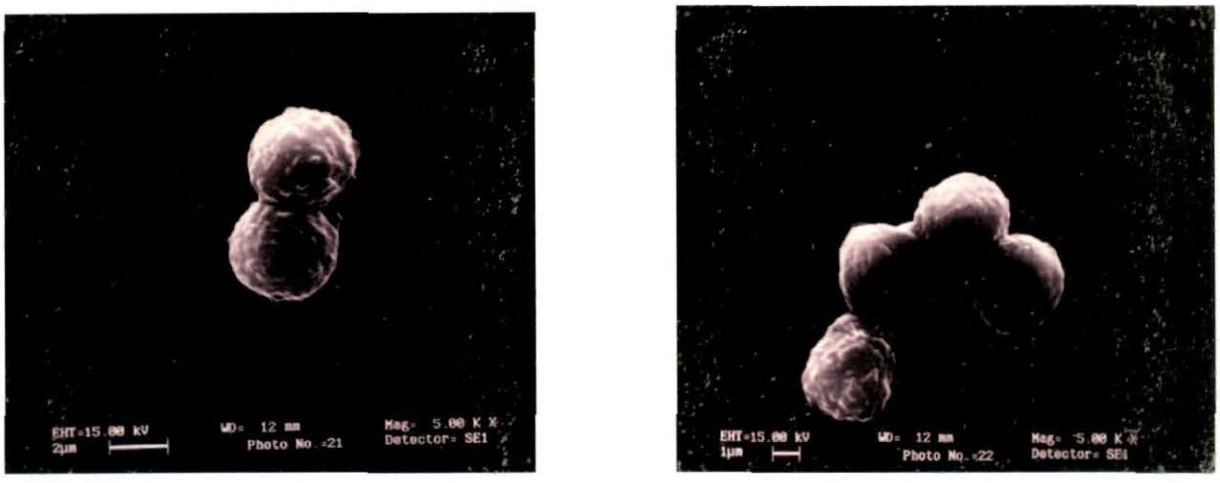
ELECTRON MICROSCOPIC STUDY

FACS results were very much intriguing as it showed ETE produced opposite effects on lymphocytes and tumor cells. Thus, it became imperative to study the actual state of the cells under electron microscope at different hours of ETE treatment. Scanning electron microscopy was done to study the cell surface and transmission electron microscopy for the internal milieu.

SCANNING ELECTRON MICROSCOPY (SEM)

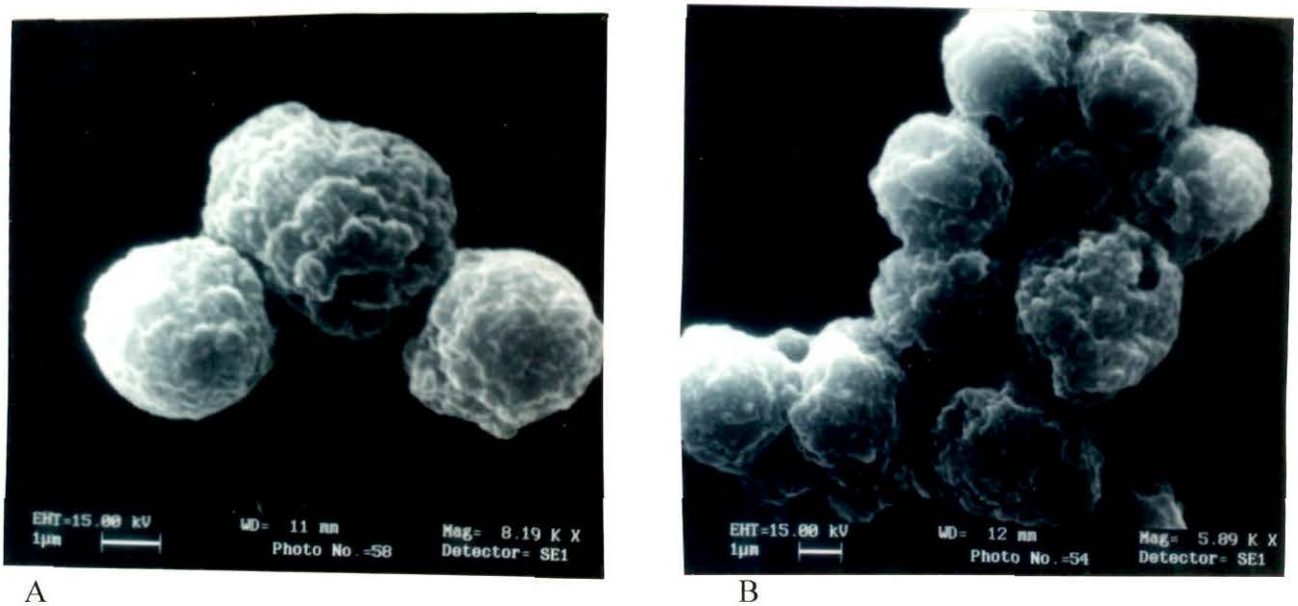
Murine lymphocytes

Scanning Electron micrograph of murine lymphocytes with *in vitro* turmeric treatment did not show notable changes on its surface topography, rather there is an increment in the diameter of the turmeric treated lymphocytes than the control after 24 hrs (Fig. 15 A & B). *In vivo* turmeric treatment also followed the same pattern by showing increment in the diameter of the ETE lymphocytes after 24 hrs (Fig.16 A& B), suggesting lymphocytes activation with turmeric treatment. This increment is possibly indicative for blastogenesis of treated cells, suggesting lymphocyte activation as observed earlier in case of blastogenesis (Fig. 8 & 9) and DNA synthesis (Fig. 10 & 11).



A B

Fig. 15 Scanning electron micrographs of murine lymphocytes from spleen after 16 hrs of *in vitro*, A) ETE treatment, lymphocytes showing no significant changes on its surface topography, B) Alcohol treated (control).

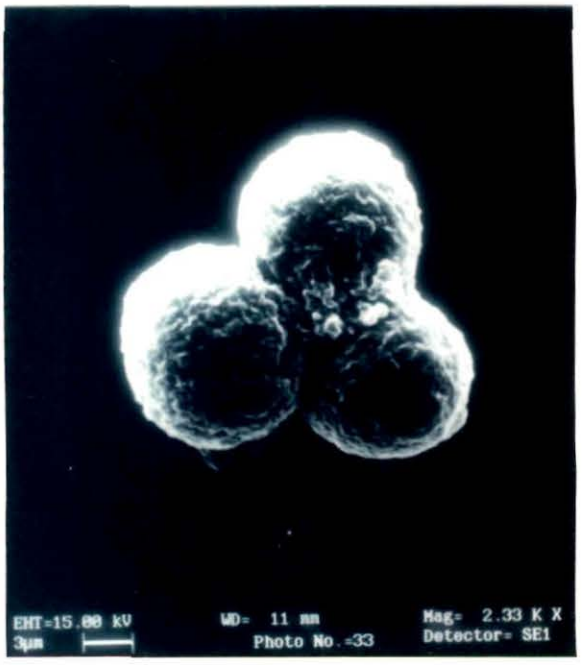


A B

Fig. 16. SEM images of murine lymphocytes from spleen after 24 hrs of **in vivo** treatment with A) ETE, B) Alcohol (control)

Ehlrich ascitic carcinoma cells

On the other hand, *in vitro* turmeric treatment caused progressive changes in cell surface of Ehlrich ascitic carcinoma cells (Fig. 17 B-E). Ruffles on cell surface were distributed evenly prior to treatment (Fig. 17A). Within 10 min of ETE treatment cytoplasmic blebs formed on tumor cell surface (Fig. 17B). These changes accentuated within 4 h of treatment; particularly the cytoplasm blebs were broader and conspicuous (Fig. 17C). After 8 h of turmeric treatment, the blebs became numerous and the cell volume tends to decrease, this was indicative of initiation of disintegration of cell structure (Fig. 17D). Loss of cellular organization and disintegration of plasma membrane was more pronounced at 16 h of treatment (Fig: 17E). These changes clearly showed the onset of apoptosis in ETE treated tumor cells; when tumor cells treated with alcohol (control) remain unchanged (Fig.17F). The tumor cells showed similar trend of changes with *in vivo* treatment of ETE, showing shrinkage in the cell volume and numerous blebs on the cell surface occurred. These changes are very much indicative of apoptotic process in tumor cells (Fig. 18A). Whereas tumor cells treated with alcohol (control) as usual remain almost unchanged, maintaining well its round shape and its volume. (Fig. 18 B).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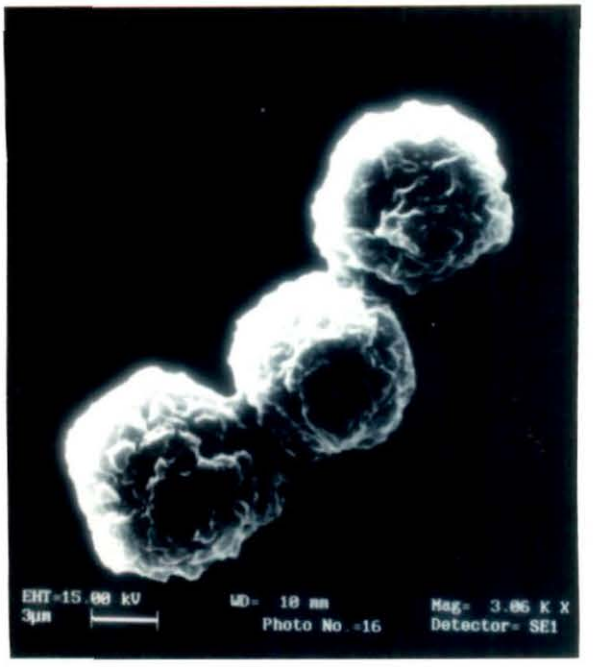
WD= 11 mm
Photo No.=33
Mag= 2.33 K X
Detector= SE1

A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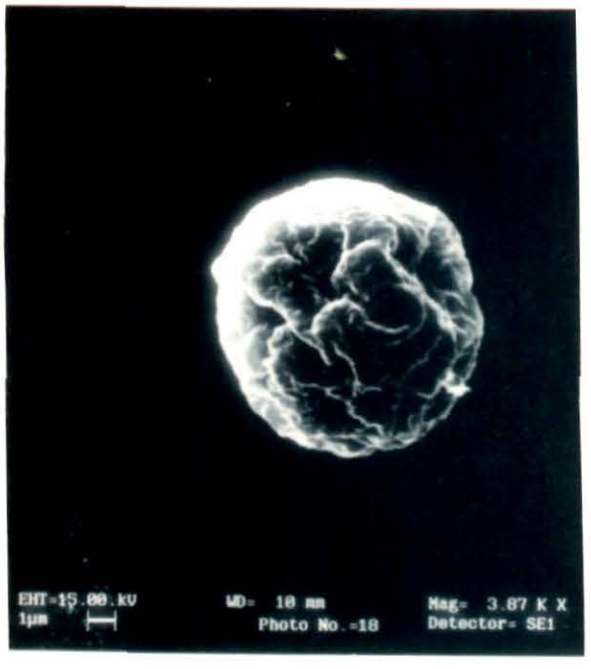
B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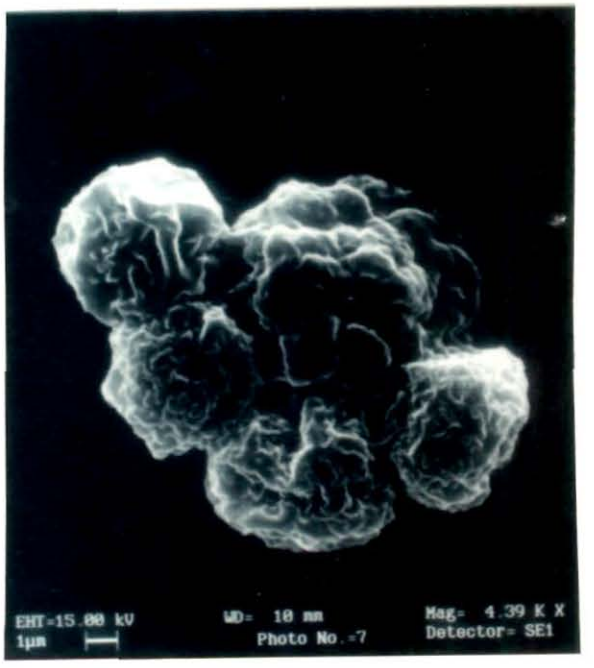
C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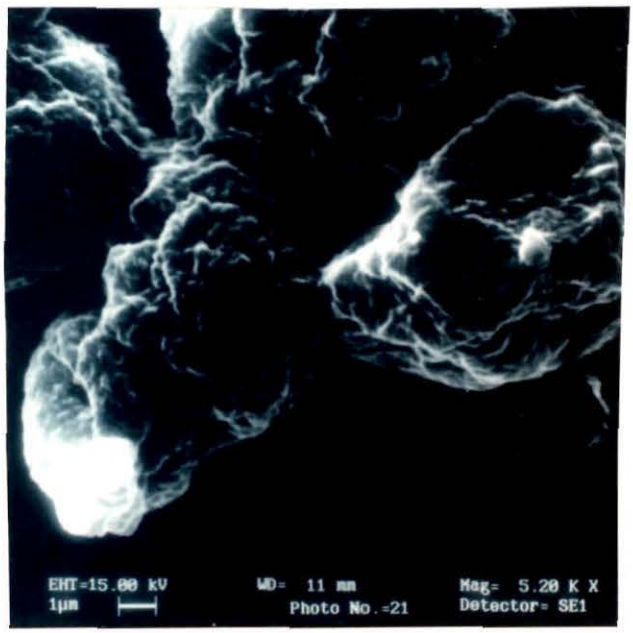
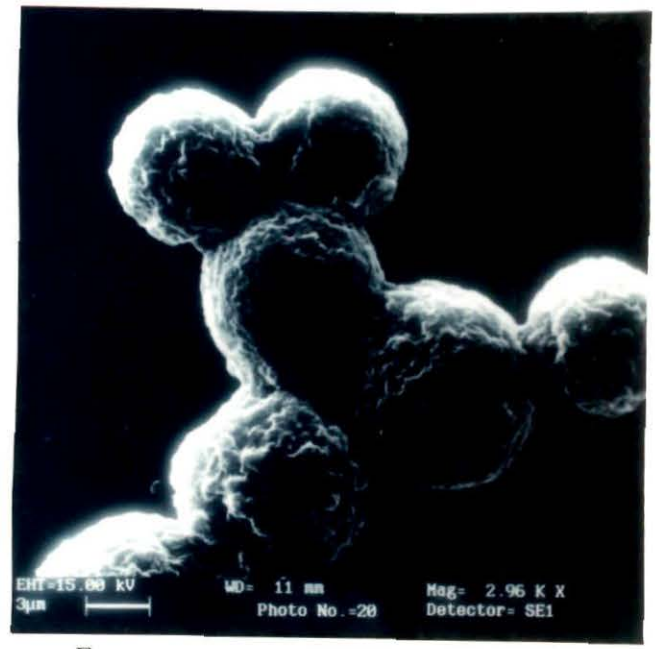
D

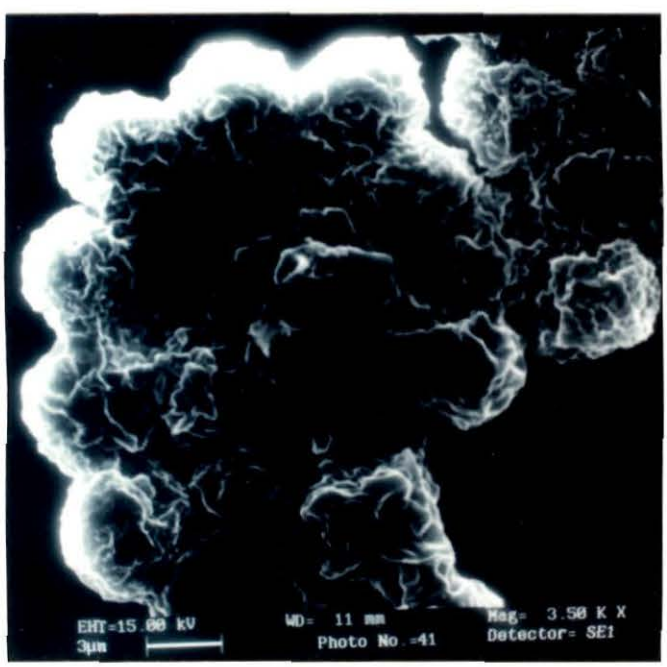


E

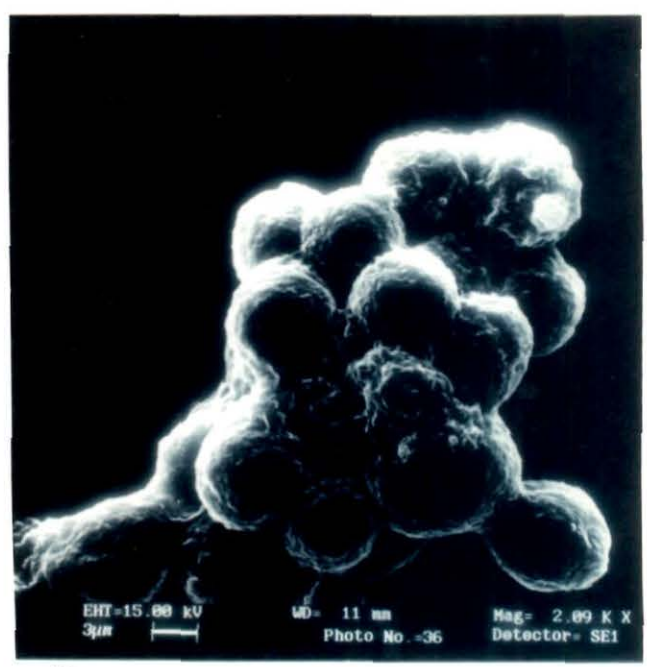


F

Fig. 17 Scanning electron micrographs of Ehlrich ascitic carcinoma cells. A) Tumor cells prior to turmeric treatment, showing ruffles distributed all over the surface; B) after 10 min of turmeric treatment, cells showing formation of cytoplasmic blebs; C) after 4 h of turmeric treatment, cytoplasmic blebs were broader and conspicuous; D) After 8 h of treatment, cytoplasmic blebs, became numerous, cell volume tends to decrease; E) after 16 h of turmeric treatment, loss of cellular organization with disintegration of plasma membrane; F) control, after 16 h of culture tumor cells remains unchanged.



A



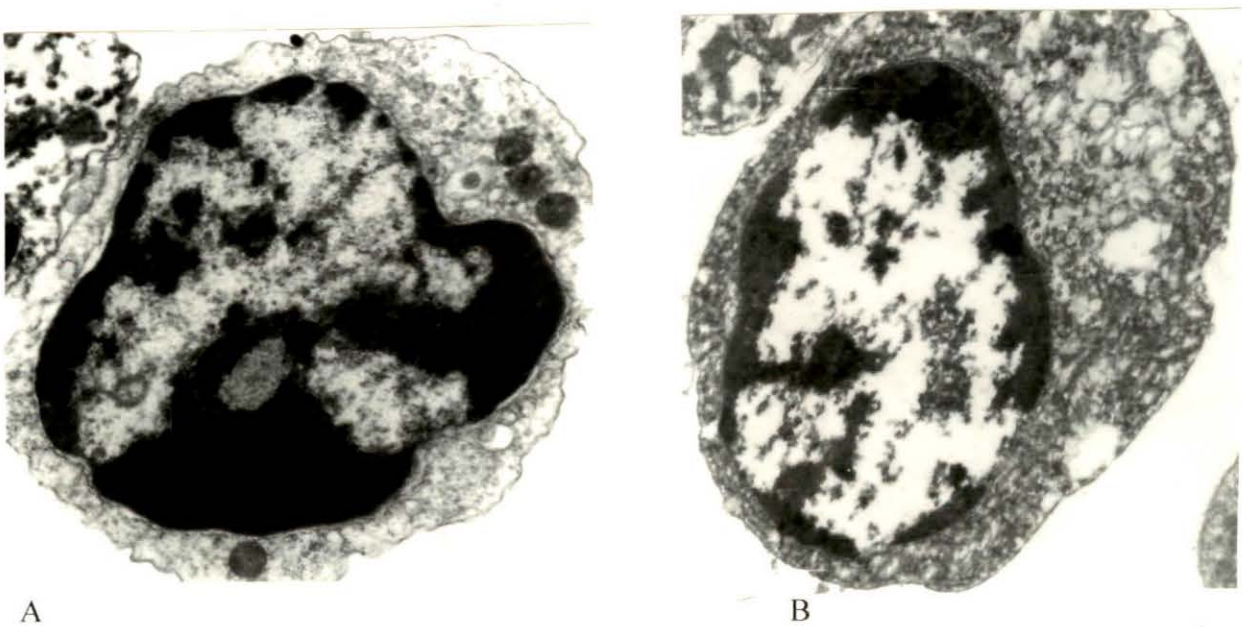
B

Fig. 18 SEM images of Ehlrich ascitic carcinoma cells after 24 hrs of *in vivo* treatment with; A) ETE, B) Alcohol

TRANSMISSION ELECTRON MICROSCOPY

Murine lymphocytes

Lymphocytes treated with ETE *in vivo* maintained their normal features with large volume of compact chromatin material distributed throughout the nucleus (Fig.19A). Whereas the alcohol treated ones showed much vacuolation in the cytoplasm (Fig. 19B).



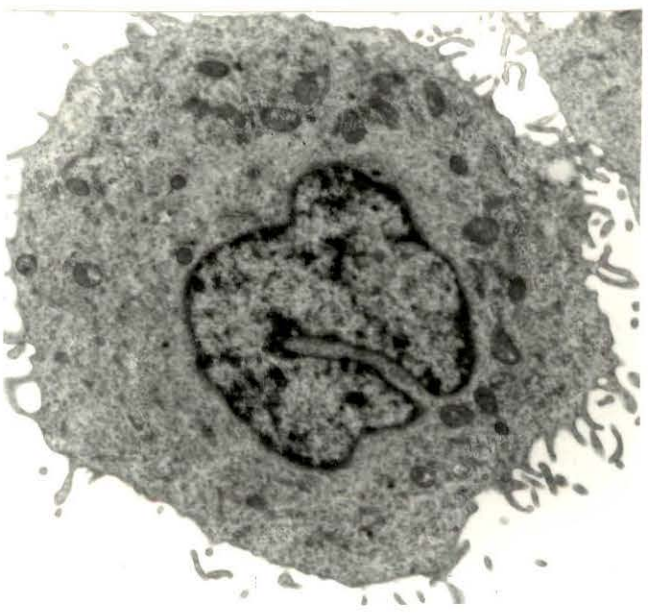
A B

Fig. 19 Transmission electron microscopic images of splenic lymphocytes after 24 hrs of in vivo treatment with, A) ETE (Mag. X 5400), B) Alcohol (Mag. X 5200)

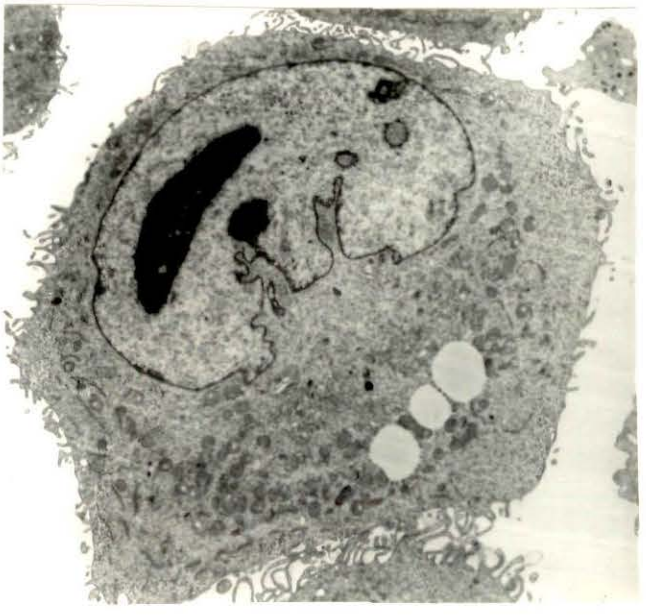
Ehlrich ascitic carcinoma cells

Ehlrich ascitic carcinoma cells show characteristics projections from plasma membrane under transmission electron microscope (Fig. 20A). Tumor cell from mice injected with ETE for 16 hrs, showed chromatin condensation as dark patches in the nucleus and indentation of the nuclear membrane (Fig. 20B). These characteristics suggests onset of apoptosis. Tumor cells after 24 hrs of ETE treatment showed fragmentation of nucleus and extensive vaculation in cytoplasm. The plasma membrane lost its characteristic outward projections, rather showed signs of blebbing (Fig. 20C). Further increase in the size of the vacuole in the cytoplasm was observed after 48 hrs

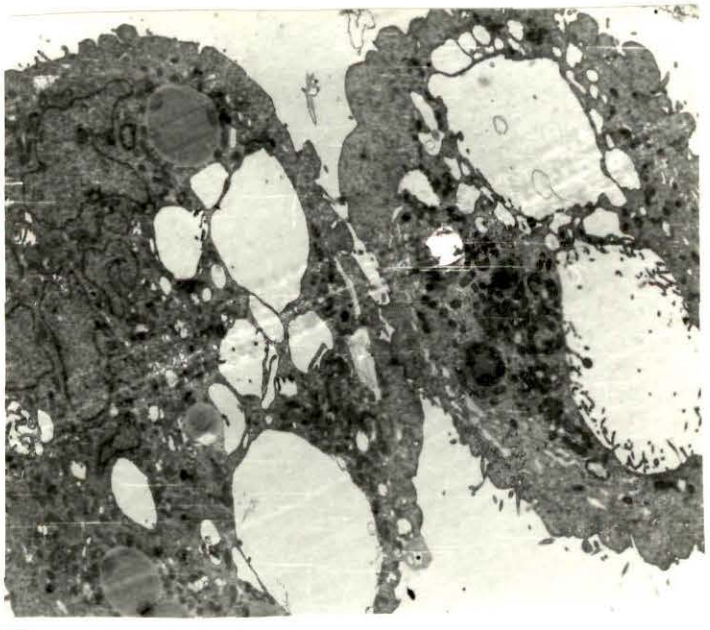
of ETE treatment (Fig. 20D). The Ehlrich ascitic carcinoma cells from mouse treated only with alcohol (control) showed the regular features of the tumor cells without any sign of fragmentation of nucleus, vacuolation and apoptosis (Fig. 20E).



A



B



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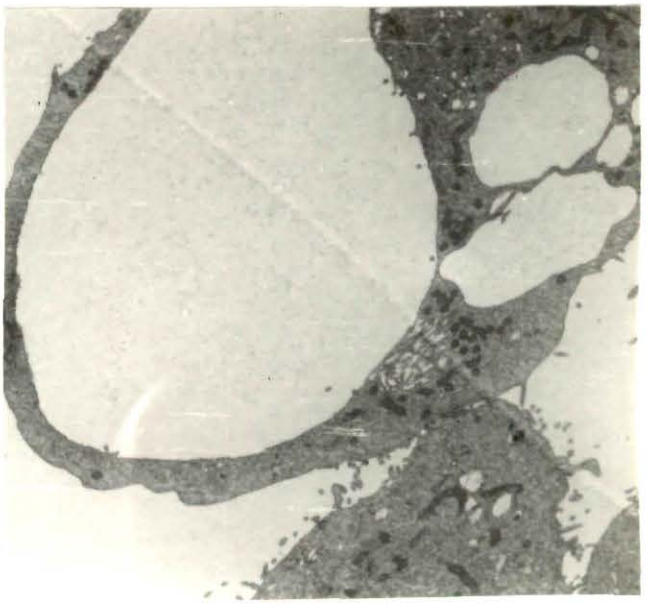

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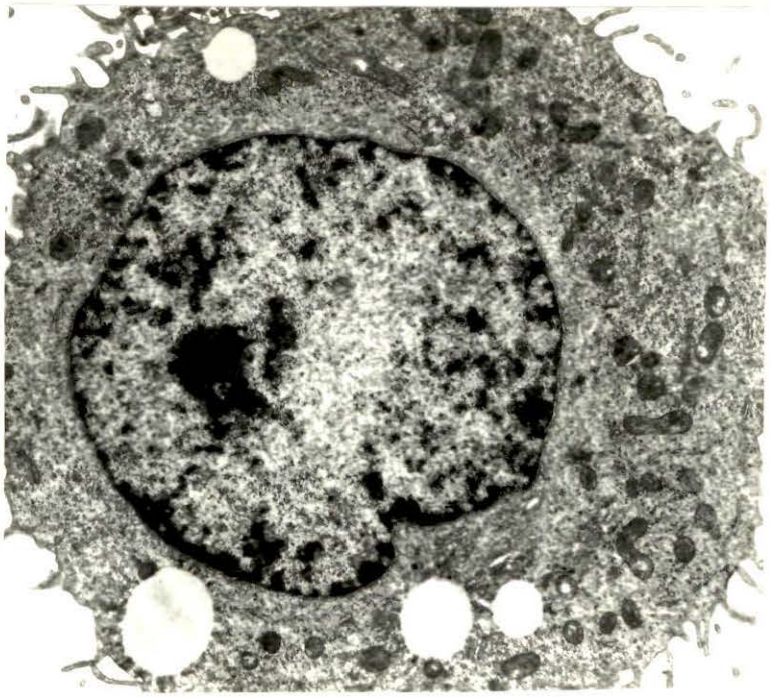


E

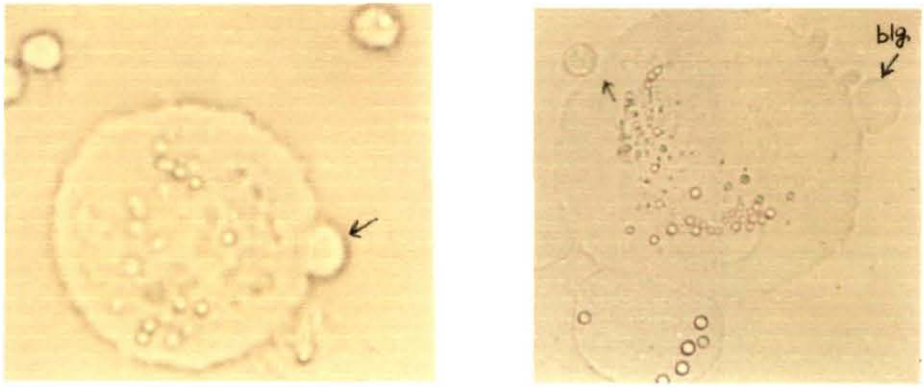
Fig. 20 TEM of Ehlrich ascitic carcinoma cells; A) Tumor cell prior to treatment, showing characteristic projections from plasma membrane (Mag. X1450), B) after 16 hrs of ETE treatment, showing chromatin condensation and nuclear membrane indentations (Mag. X1550), C) after 24 hrs of ETE treatment, plasma membrane showing blebbings and large number of vacuoles in the cytoplasm and fragmentation of nucleus observed (Mag. X 2100), D) after 48 hrs of ETE treatment, the size of the vacuoles increased (Mag. X 1550), E) after 48 hrs of alcohol treatment (control), tumor cell remain unchanged.

CELL MEDIATED IMMUNE RESPONSE

Cell mediated immunity is an important mechanism for surveillance against cancer, primarily mediated by T lymphocytes. So far it seems that ETE can activate the murine lymphocytes especially the T cells to a reasonable degree in terms of blastogenesis and DNA synthesis. The FACS and Electron microscopic images support the contention of activation of lymphocytes by ETE. So, it became quite necessary to study whether the ETE mediated activation can drive the T cells all the way to cytotoxic state against tumor cells. The cytotoxic state of T cells were determined by estimating number of conjugates formed between tumor target cells and effector cells and by ^{51}Cr- release from labeled tumor cells.

CONJUGATE FORMED BETWEEN EFFECTOR AND TARGET CELLS AT DIFFERENT RATIOS WITH ETE TREATMENT

The conjugate formation is often considered a prerequisite for mounting cell mediated response. A typical conjugate formed between effector lymphocyte and tumor cell is presented in figure 21A. ETE stimulated lymphocytes participated well in conjugate formation with tumor cells as compared to control groups of alcohol and without treatment (Fig. 22). Consequent to conjugate formation, the tumor cells showed blebbings on their surface (Fig. 21B); the frequency of tumor cells with blebbings was higher with ETE (Fig. 23A), and so was the death (Fig. 23B). The blebbings and apoptotic bodies with ETE were also recorded in Giemsa stained slides (Fig. 24).



A B

Fig. 21 Phase contrast micrograph of typical conjugate between Ehlrich ascitic carcinoma cells and ETE activated lymphocytes *in vitro*; A) One lymphocyte (arrow) attached to the tumor cell surface (larger one), B) Lymphocyte probably getting detached from the tumor cell surface (arrow) after initiating apoptosis as indicated by blebbings (blg) on the tumor cell surface. Mag. X3200.

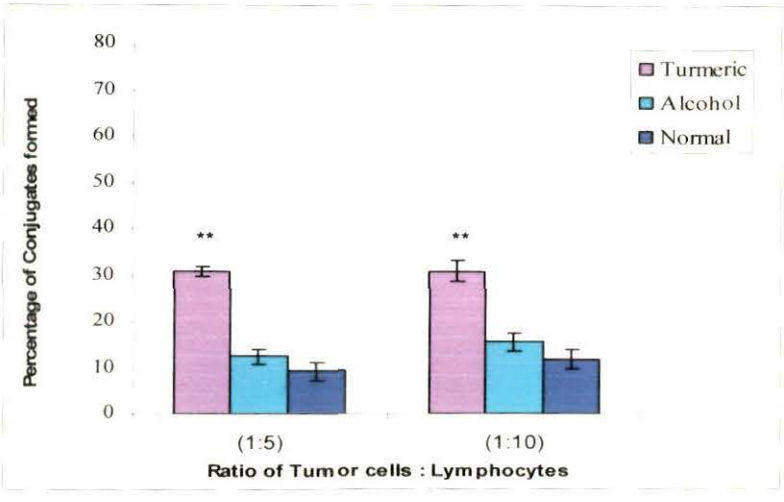
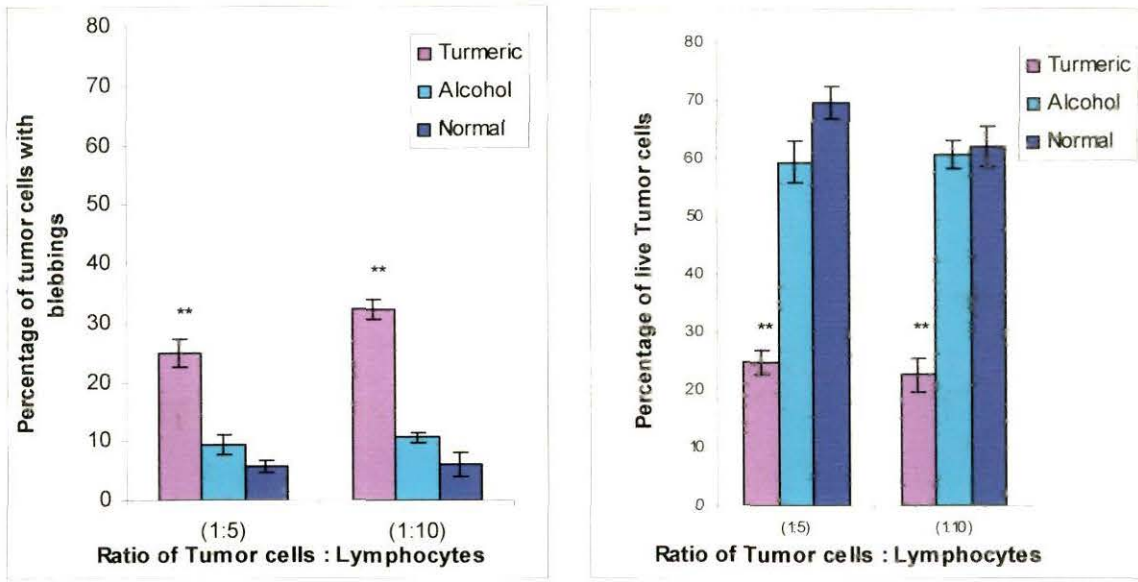
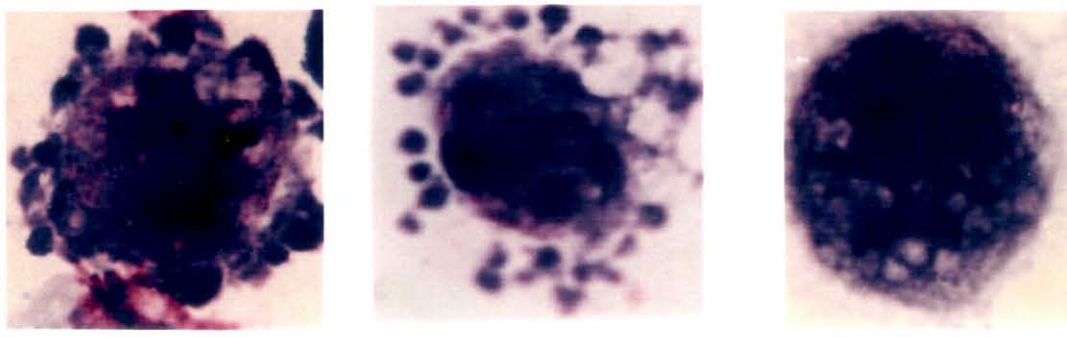


Fig. 22 Percentage of conjugate formed between tumor target cells and lymphocytes, at different ratios after 2 hrs of *in vitro* ETE treatment. Results are expressed as mean ± SD, Two way ANOVA revealed all the treatment values are significant compared to controls (**p< 0.01).



A B

Fig. 23 A) Percentage of tumor cells with blebbings after conjugate formation in presence of ETE for 2 hrs *in vitro* and B) subsequent drastic reduction in viable tumor cells. Results are expressed as mean ± SD, Two way ANOVA revealed all the treatment values are significant compared to controls (**p< 0.01).



A B C

Fig. 24 Photomicrographs of Ehlrich ascitic carcinoma cells stained with Giemsa with ETE treatment;
A) Transformation of cell membrane into deeply stained numerous cytoplasmic blebs, B) Formation of multiple apoptotic bodies, C) No change in the morphology of tumor cells in presence of lymphocytes treated with alcohol (control). Mag. X 2400.

^{51}Cr- RELEASE WITH ETE TREATMENT

Conjugate formation and subsequent apoptosis in presence of ETE treated lymphocytes instigated us to measure the cytotoxic ability of the effector lymphocytes against ^{51}Cr labeled tumor target cells. ETE treated lymphocytes showed significantly higher percentage of cytotoxicity towards the tumor target cells than the alcohol treated and the normal lymphocytes with all the three different target: effector cell ratios such as 1:100, 1:50 and 1:10. The cytotoxicity indices with different ratios (Fig. 25) produced a graded response, indicating ETE generated effector T cells perform just like the effector cells activated by other means (Heininger *et. al,* 1976; Waterfield et. al., 1975, 1976; Chaudhury & Chakravarty 1983, Chakravarty and Maitra 1983 & 1990).

In course of studying the effect of ETE on survivality of tumor cells and lymphocytes, we observed higher rate of mortality in tumor cells. So, an experiment was designed to measure the release of ^{51}Cr labeled tumor cells in presence of ETE only. Direct application of ETE caused reasonable amount of Cr-release from the tumor cells than the control (Fig. 26). This confirms our earlier observation referred in figure 2.

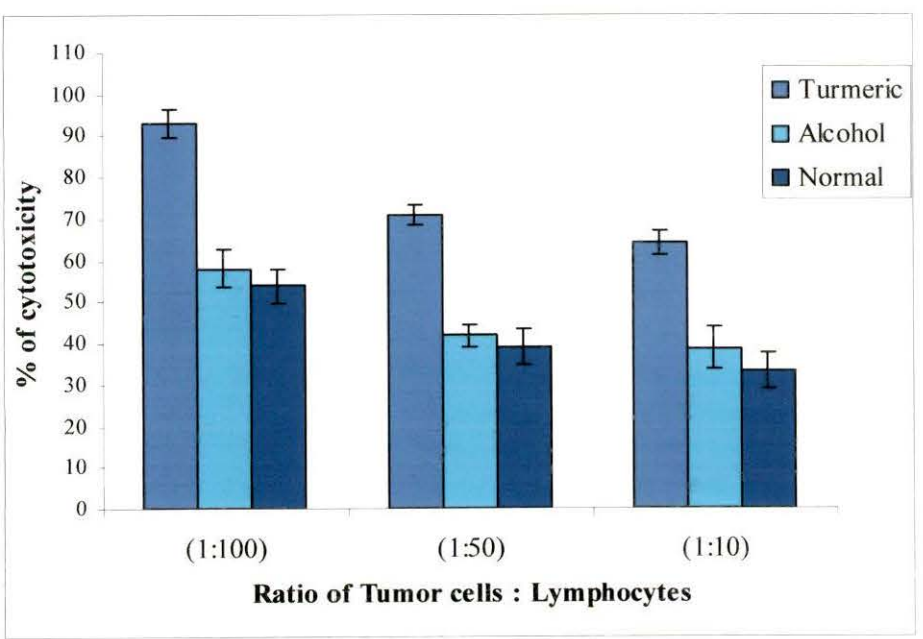


Fig. 25 Percentage of cytotoxic response mounted by ETE treated lymphocytes against the tumor target cells at three different ratios of target : effector cells. Results are expressed as mean ± SD, Two way ANOVA revealed all the treatment values are significant compared to control ($p < 0.01$).

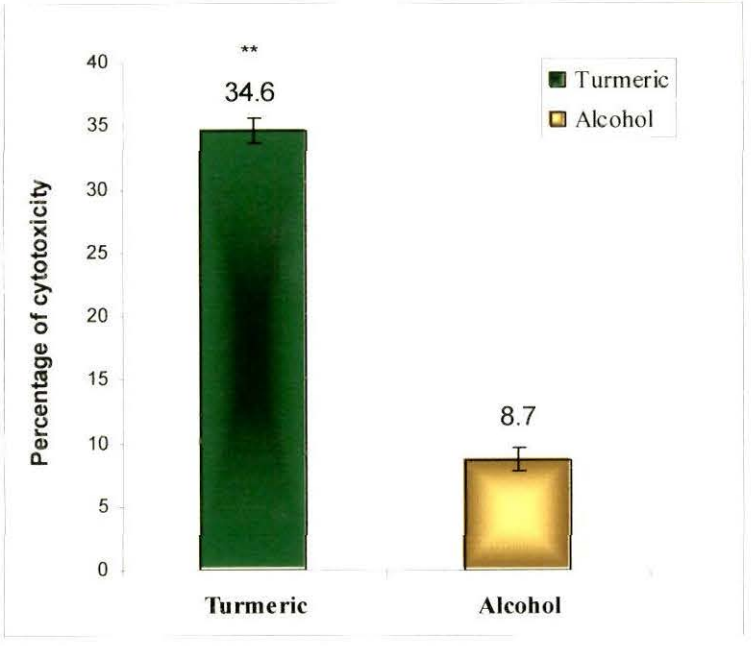


Fig. 26 Percentage of cytotoxicity towards tumor cells after 6 hrs of *in vitro* ETE treatment. Results are expressed as mean ± SD, **$p < 0.01$ compared to respective controls.

INHIBITION OF TUMOR GROWTH AND INCREASED SURVIVALITY OF HOSTS WITH ETE TREATMENT

Since cytotoxic activation of lymphocytes occurred by ETE treatment, it was necessary to study whether the treatment *in vivo* can restrict the tumor growth.

The rate of tumor development was relatively slower in all the three schedules of administration of ETE (see p.31) than the control mice, as evident from figure 27, 28 and 29. The figures also indicate the increase in survivality of the tumor bearing hosts with ETE treatment; this was possibly due to slower rate of tumor growth in all the cases.

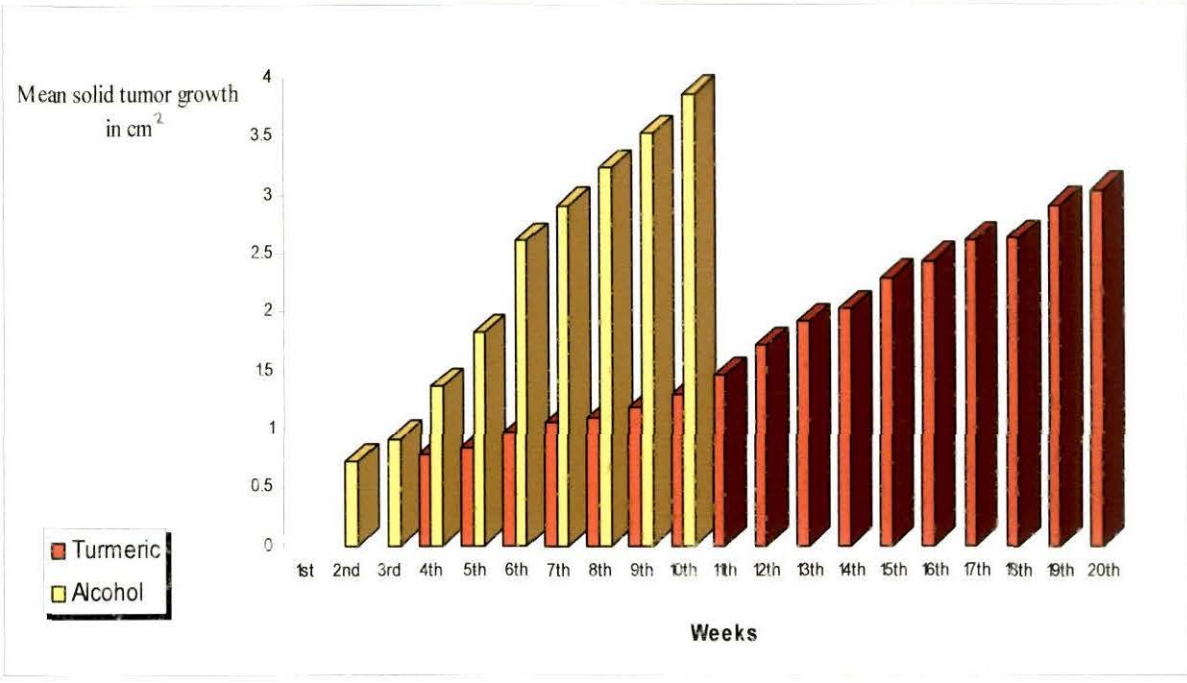
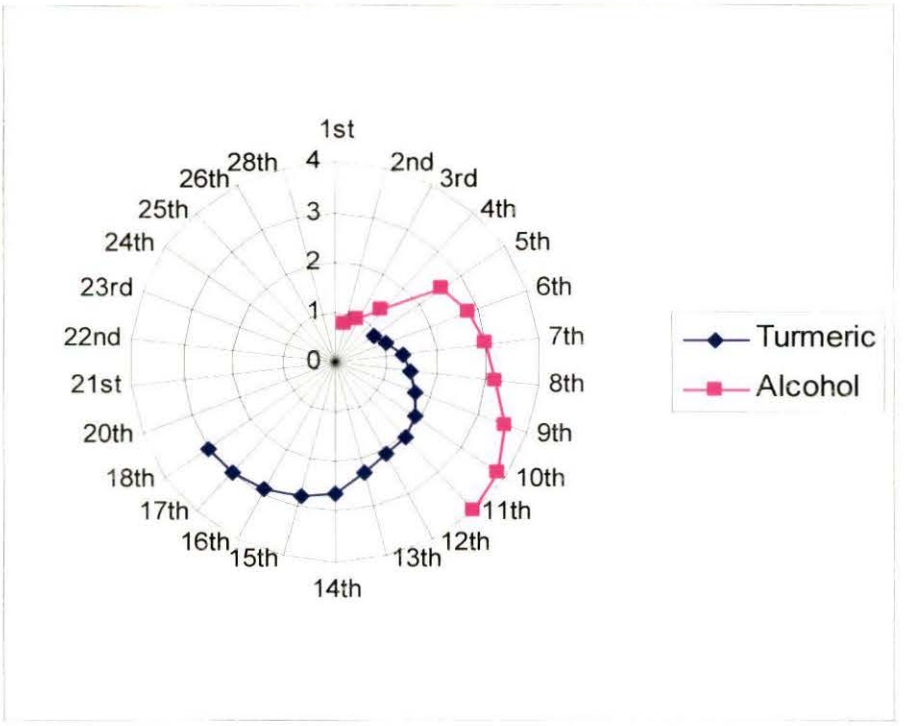


Fig. 27 Rate of solid tumor growth (in cm^2) with two intravenous injections of ETE twice before tumor induction (schedule I). Tumor growth was measured every week after induction.

The tumors were palpable but not measurable upto 4^{th} week in mice receiving ETE intravenously; the survivality of these mice were upto 20^{th} week (Fig. 27). Whereas tumors became palpable on 3^{rd} week and measurable from 5^{th} week when ETE was administered orally; the tumor bearing mice survived up to 18^{th} week (Fig. 28A). In both the cases 10^6 tumor cells were used for induction of tumor.

In two sets of experiment for oral administration, lower concentrations (10^5 and 10^4) of tumor cells were used for induction of solid tumor (Fig. 28 B & C). The lower concentrations could induce tumor with some delay. The tumor size did not reach to maximum of 3 cm^2 and survivality of mice were more with the lowest concentration of inducing dose of tumor cells.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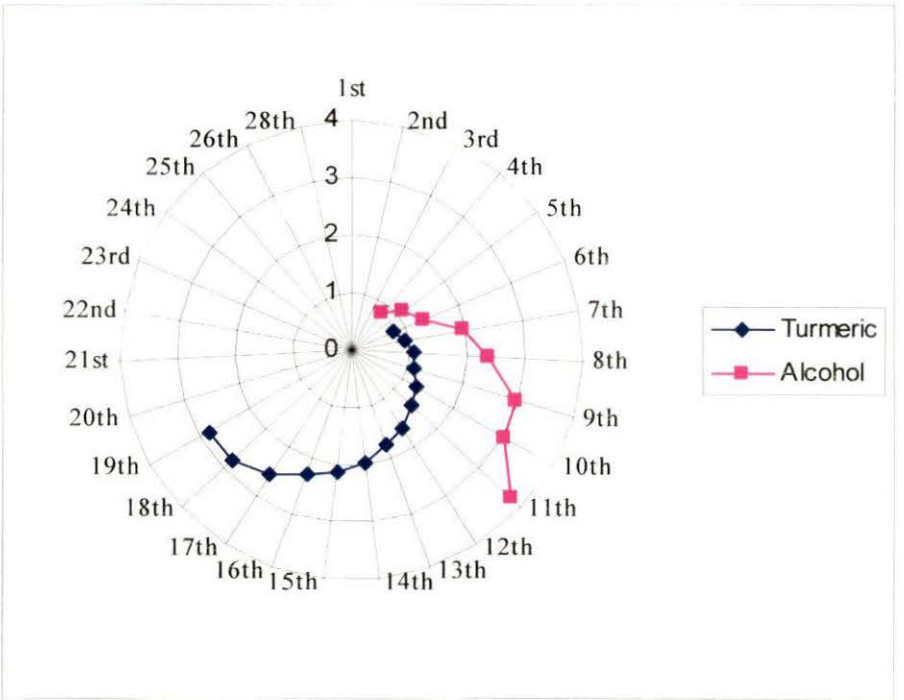
Turmeric
Alcohol

A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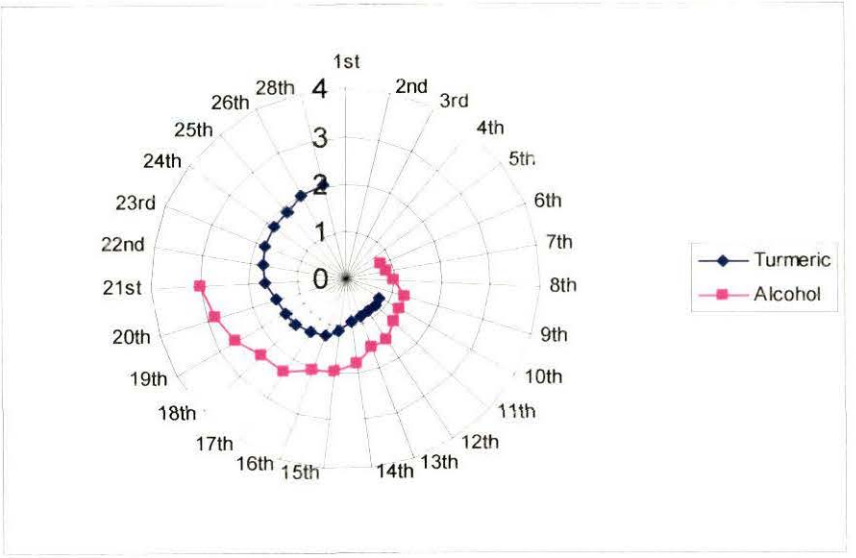
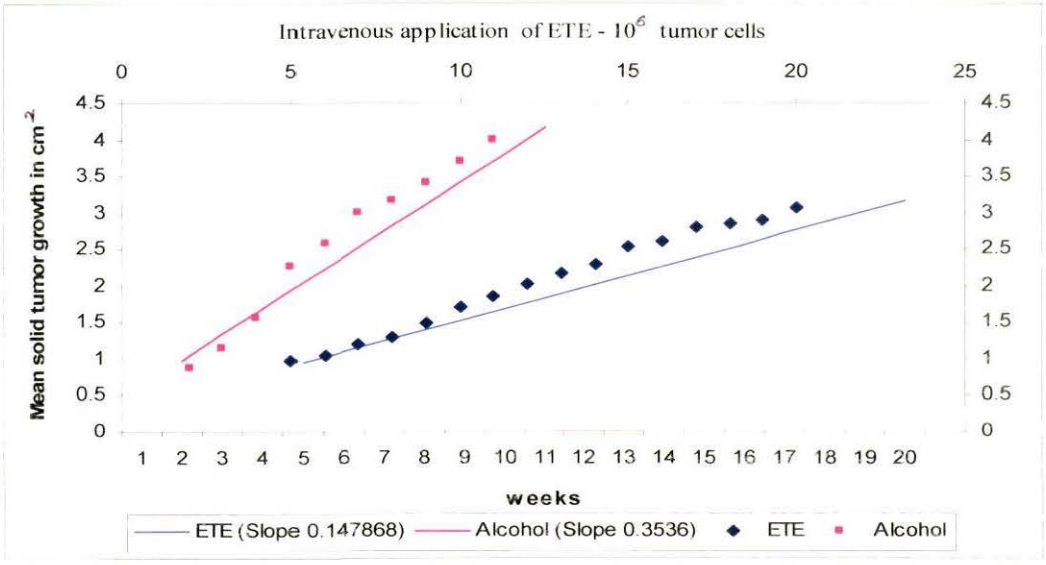


C

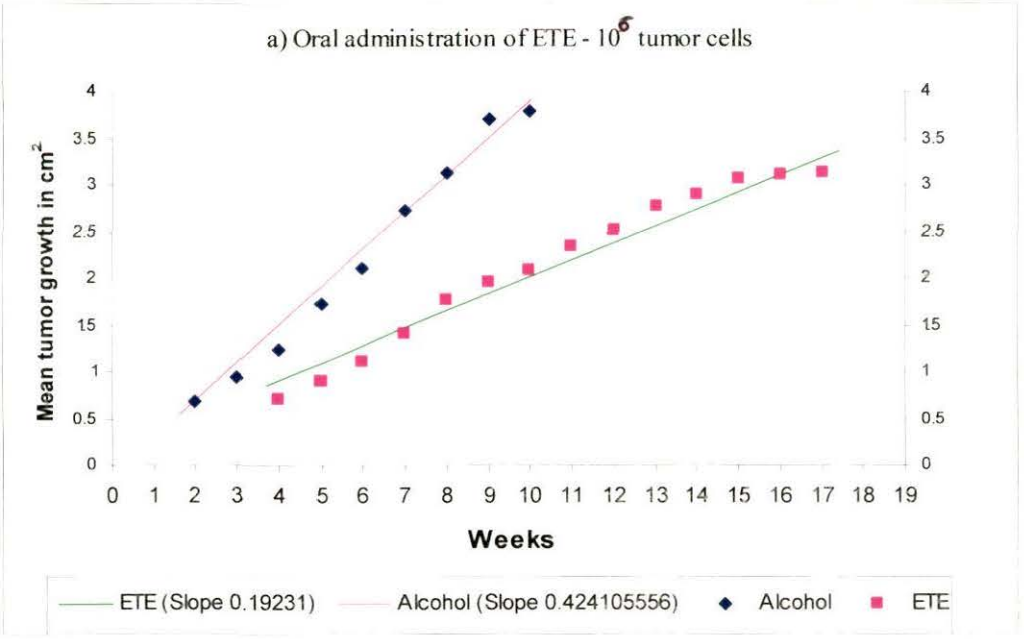
Fig: 28 Rate of solid tumor growth in mice administered ETE orally thrice (on 0, 3rd and 6th day) before tumor induction. Growth rate was measured every week marked clockwise on the circumference. Each concentric circle denotes 1cm^2 growth as indicated by 1-4. Three concentrations of tumor cells were used for induction of tumors (see schedule II, p. 31): A) 10^6, B) 10^5 and C) 10^4 tumor cells/0.1ml PBS.

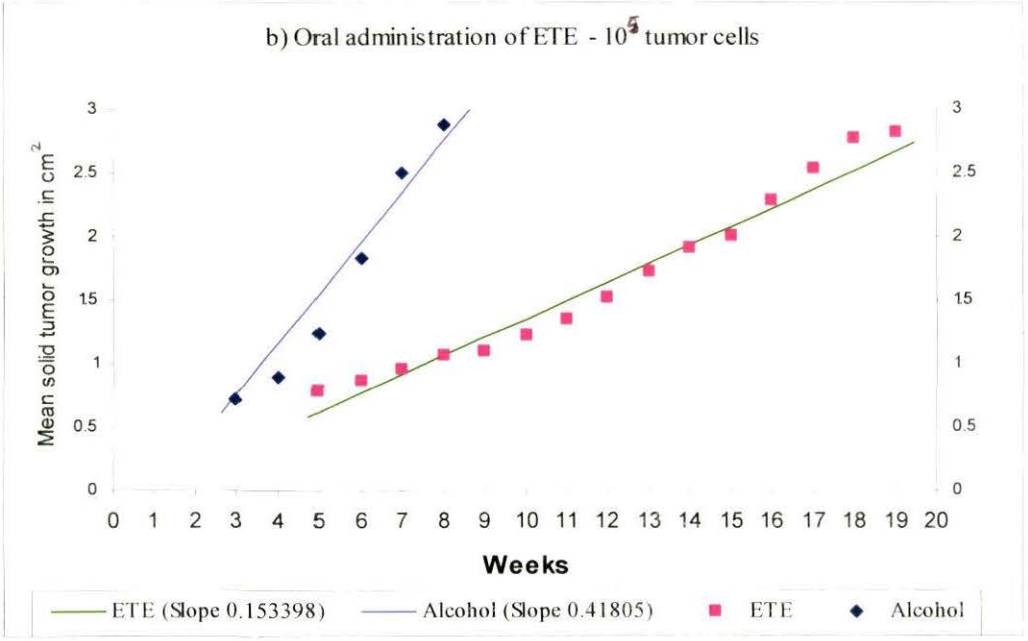
The results of the inhibition of the tumor growth with ETE treatment following three schedules, were also plotted with least square fit analysis (Fig. 29); to find out the regression slope for tumor growth.

A Schedule I



B Schedule II





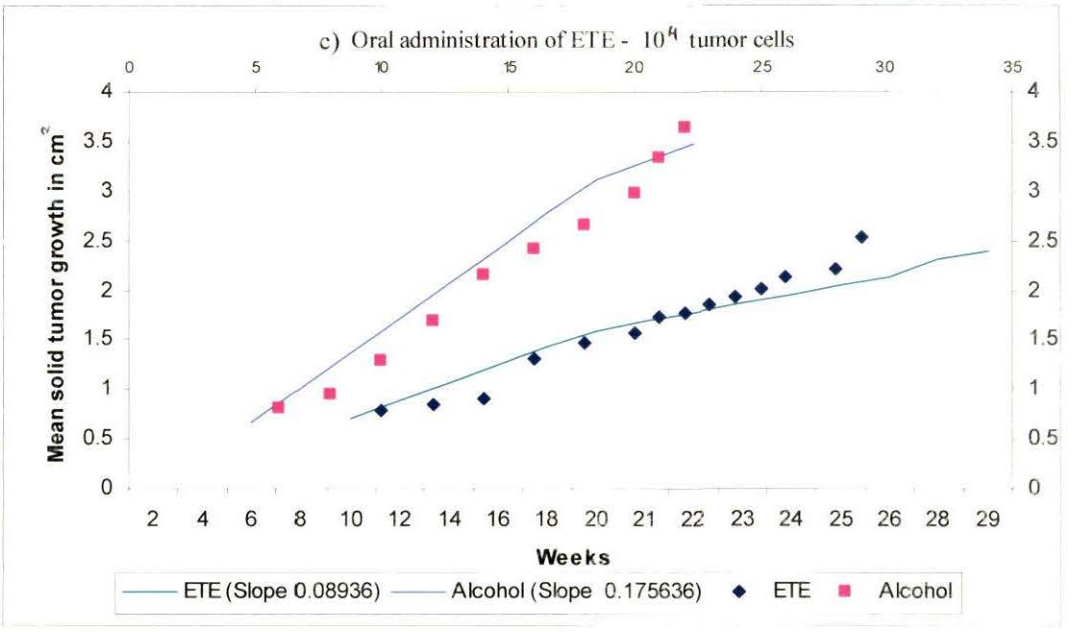
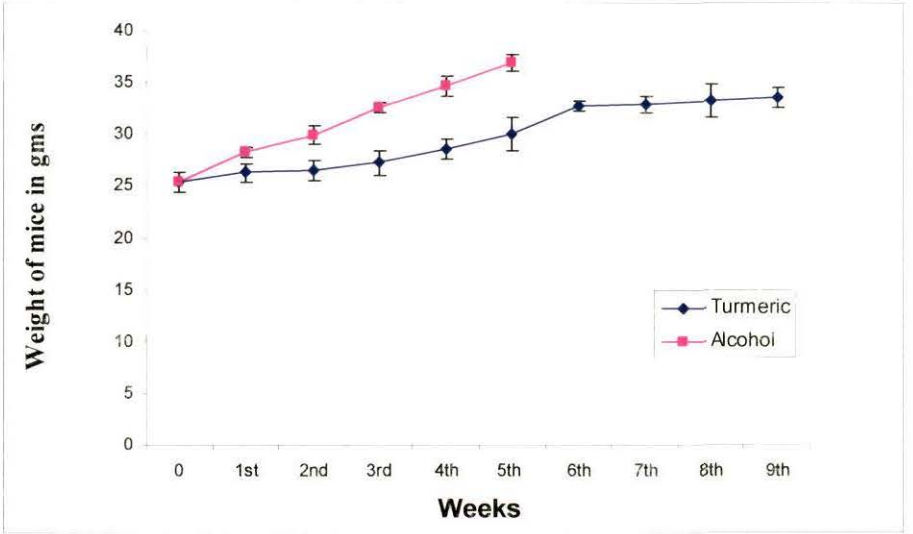
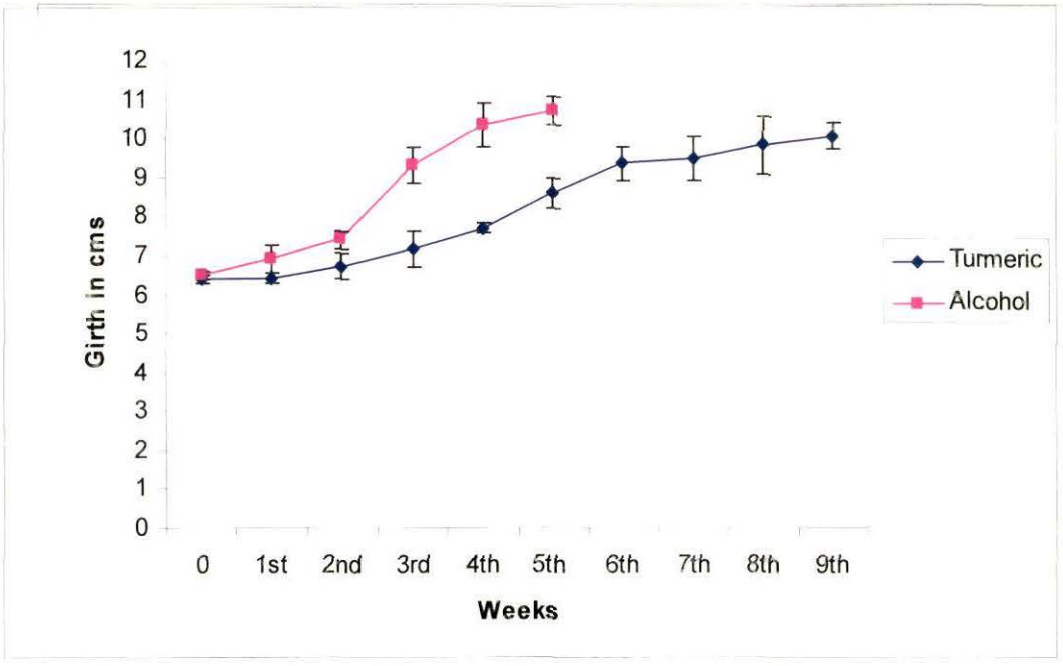


Fig. 29 Least square fit analysis of solid tumor growth following ; A) Schedule I, B) Schedule II with different number of cells/0.1ml PBS a)10^6, b) 10^5 and) 10^4 tumor cells/0.1ml PBS] The straight line for rate of tumor growth was drawn according to the least-square fit method, and the slope for the line was calculated.

ETE could also restrict growth of ascitic tumors, induced as per schedule III. In consequence the longevity of mice increased (Fig. 30).



A



B

Fig. **30** Rate of ascitic tumor growth with ETE, administered intravenously twice prior to the induction of tumor (see schedule III, p. 33); A) weight of mice in gm, B) Girth of mice in cm.

EFFECT OF ETE ON DTH RESPONSE INDUCED BY DNFB

ETE was found to activate cell mediated immunity so its effect on delayed type hypersensitivity (DTH) response was also studied. DTH reaction is supposed to be function of T lymphocytes and 2,4-DNFB was used here for initiating DTH.

The untreated mice had the swelling all through the study period of 9 days (Fig. 31). ETE and curcumin (10μM) treatment reduced the swelling noticeably at all the points compared to controls (Fig. 31).

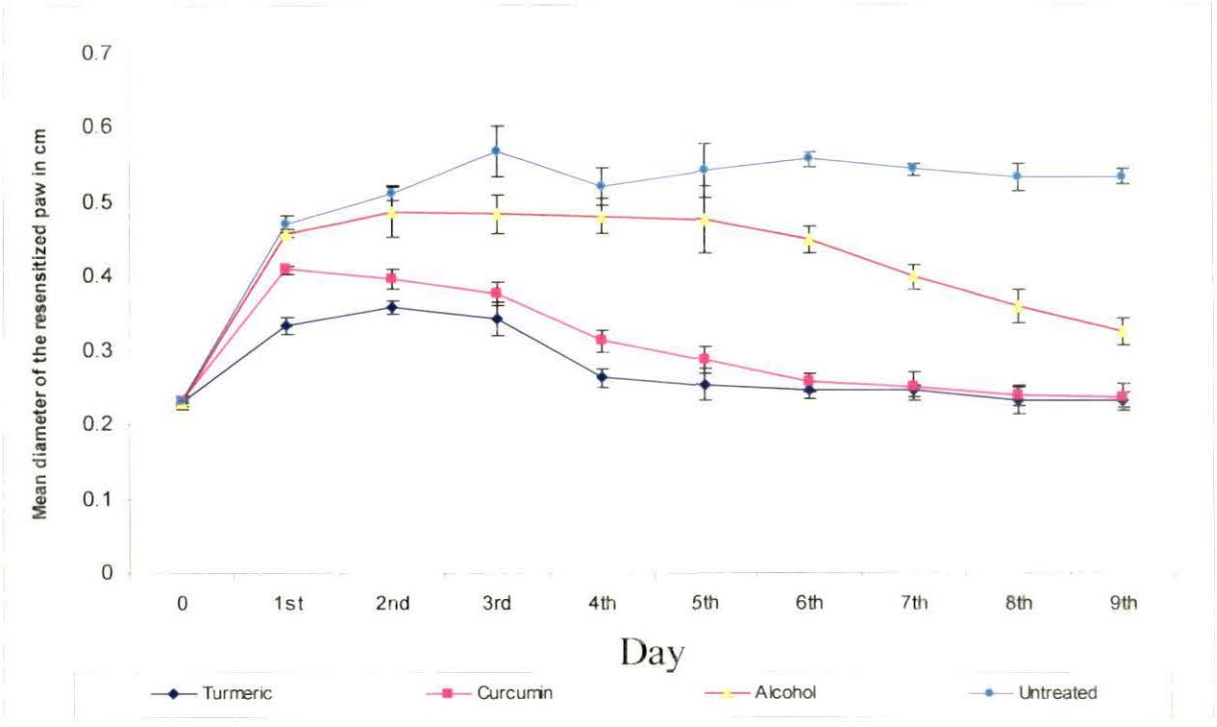


Fig. 31 Change in paw size of DTH mice with ETE and curcumin treatment given intravenously, 1 hr prior to resensitization (Day of resensitization indicated by 0) alcohol treated and untreated controls were maintained. Results are expressed as mean ± SD. Two way ANOVA revealed all the treatment values (ETE and curcumin were significant compared to controls ($p< 0.01$).

The inhibitory effect of ETE for DTH reaction was appreciable and remarkable from the photographs (Fig. 32). The untreated mice maintained severe inflammation, leading to loss of digits and foot pad (Fig. 32 A, 1st column). Alcohol control showed loss of foot pad. While with ETE and curcumin treatment the edema subsides within the 9th day of resensitization and the paw retains it normal size and digits(Fig. 32 C &D). In comparison to curcumin, ETE showed even better inhibition of DTH response (Fig. 31 & 32).

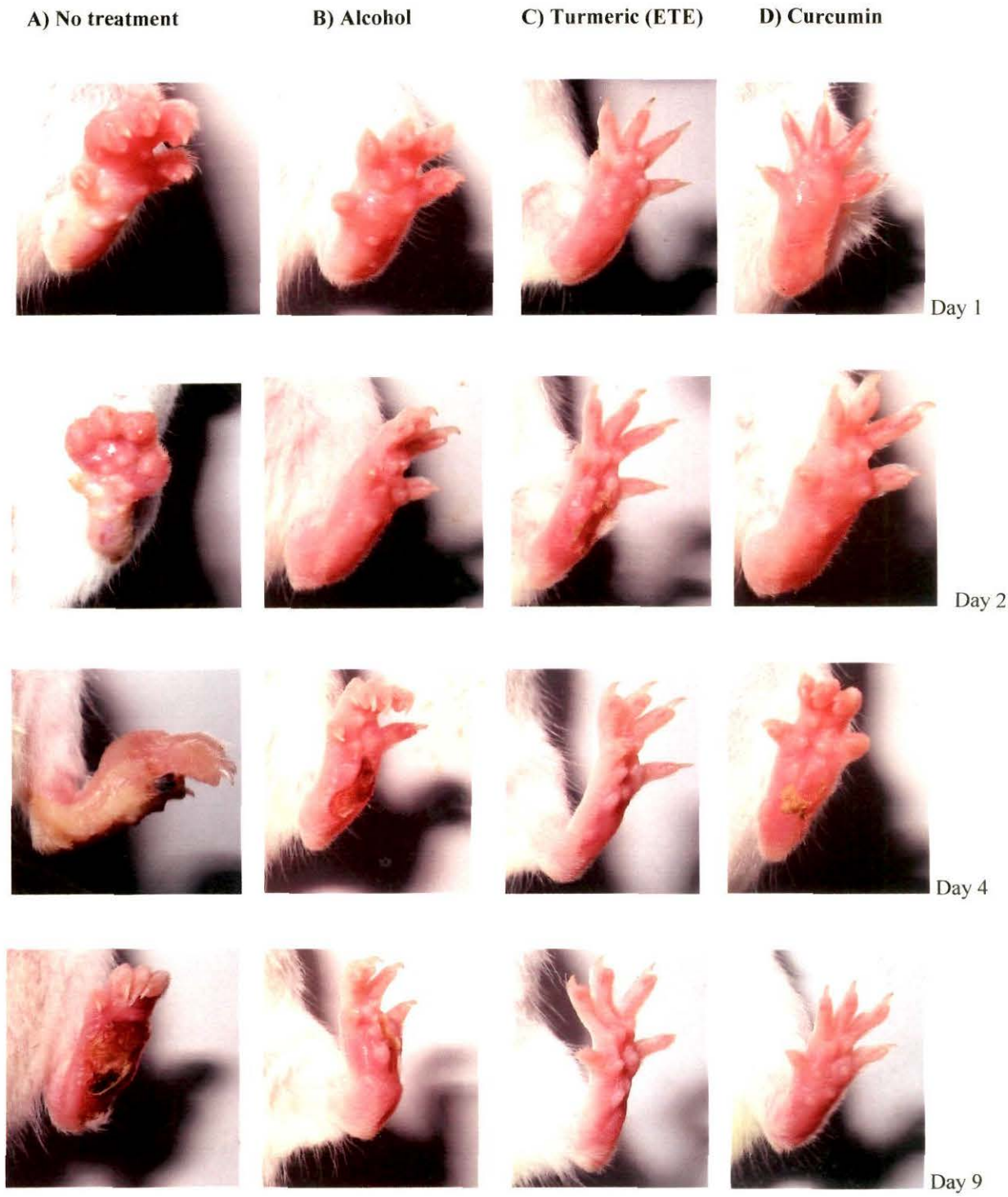
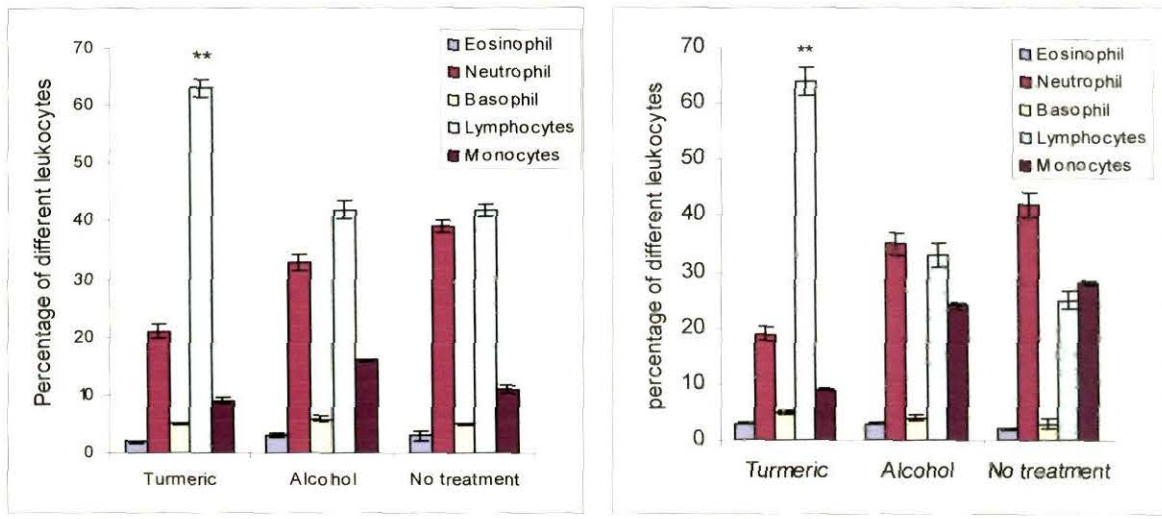


Fig. 32 Photograph showing the DTH reaction of paw in A) untreated, B) alcohol, C) ETE and D) curcumin treated mice in course of 9 day period of study.

DIFFERENTIAL LEUCOCYTE COUNT AT THE INFLAMMATION SITE OF DTH MICE WITH ETE TREATMENT

Usually a DTH response is accompanied with infiltration of leukocytes (neutrophils, basophil, lymphocytes and monocytes) from the venous and lymphatic system at the site of inflammation. And thus to know what are the cell types that infiltrated at the site of inflammation and effect ETE treatment, differential count of leucocytes was taken into account.

Increase in the percentage of lymphocytes and visa-a-vis decrease in neutrophils were noted in case of ETE treatment. Increase in monocyte population was found after 48 hrs of resensitization in controls (Fig. 33, Table 5). No appreciable change in the number of eosinophils and basophils after ETE treatment was observed when compared to controls.



A B

Fig. 33 Percentage of different leukocytes at the inflammation site (resensitized paw) with ETE treatment (1 hr prior to resensitization) ; A) 24 hrs and B) 48 hrs after resensitization. Results are expressed as mean ± SD, **p< 0.01 compared to respective controls.

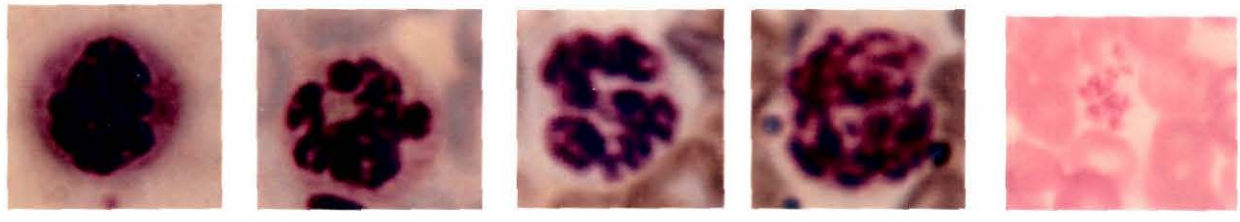
On further microscopic study, neutrophils were found in apoptotic conditions with disintegrating chromatin material. The numbers of apoptotic neutrophils were more in both the

control groups (Fig. 34a, Table 5). In these two groups, lymphocytes in apoptotic condition, with numerous projections on cell surface, were also observed (Fig. 34b, Table 5).

Table 5 Mean percentage of different leukocytes from the inflammation site of paw, after 24 and 48 hrs of resensitization for DTH. ETE and alcohol were injected i.v.1 hr prior to resensitization. Results are expressed as mean ± SD, * $p<0.05$ & ** $p<0.01$ compared to respective controls.

	Eosin	Neutro			Baso	Lymphocytes				Monocytes		
		N[a)]	A	Total		S	L	A	Total	S	L	Total
24 hrs of resensitization												
Turmeric	2±0.16	14±11.4	7±1.54	21±1.35	5±1.11	43±0.84*	20±1.23*	0	63±1.67 **	6±0.12	3±0.14	9±0.56
Alcohol	3±0.98	18±1.34	15±1.47	33±1.05	6±1.56	32±0.91	6±0.06	4±1.45	42±1.03	7±0.45	9±0.19	16±0.14
No treatment	3±0.54	17±1.06	22±2.21	39±0.97	5±1.89	28±0.46	4±0.75	10±0.7	42±1.65	7±0.14	4±0.10	11±0.23
48 hrs of resensitization												
Turmeric	3±0.65	16±0.91	3±0.19	19±1.06 *	5±0.12	38±0.56	26±1.76	0	64±2.45 **	7±0.67	2±0.56	9±0.13*
Alcohol	3±0.12	27±0.87	9±0.12	36±2.13	4±0.52	24±0.79	9±0.06	2±0.03	33±2.12	15±0.3	9±0.93	24±0.45
No treatment	2±0.43	29±1.03	13±0.56	42±2.24	3±0.10	10±1.23	6±1.12	9±0.56	25±1.98	18±0.4	10±0.4	28±0.42

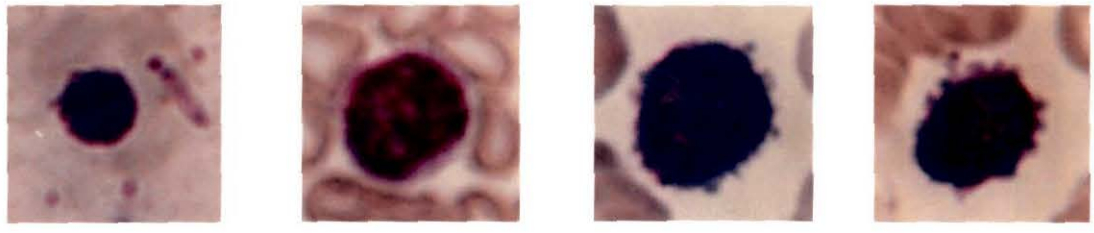
[a)] Abbreviations: N- Normal, A- Apoptotic, S- Small, L-Large



Normal

Apoptotic

a)

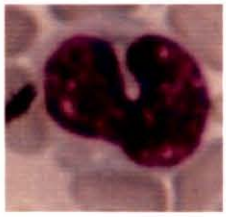


Small

Large

Apoptotic

b)



Small Large

c)

Fig. 34 Photomicrograph of different leukocytes at the inflammation site of DTH mice, stained with Leishmen's stain; a) neutrophils, b) lymphocytes, c) monocytes. (Mag×2400)

ETE treatment showed increase in lymphocyte count as in figure 33. In this increment, contribution of large lymphocytes was more (Table 5).

$CD4^+$ T CELL COUNT IN DTH MICE WITH ETE TREATMENT

As the number of lymphocytes in the DTH site increased considerably with ETE treatment compared to control groups, the contribution of $CD4^+$ T cells in this hike was studied from lymphocyte population of spleen. Incidentally $CD4^+$ T cells are important participant of DTH response. The living $CD4^+$ T cells from splenic lymphocyte population were isolated by MACS and photographed (Fig. 35).

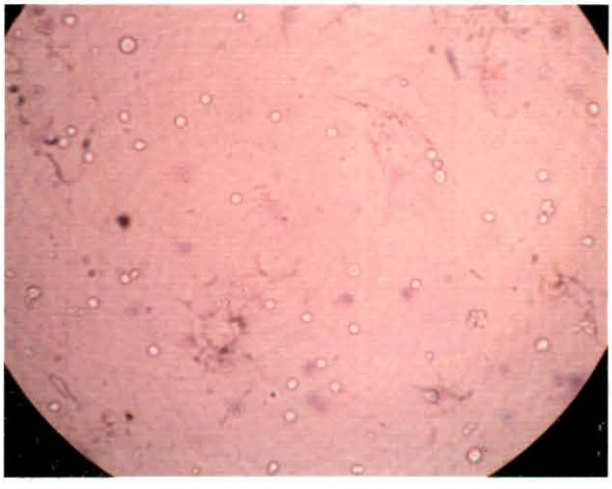
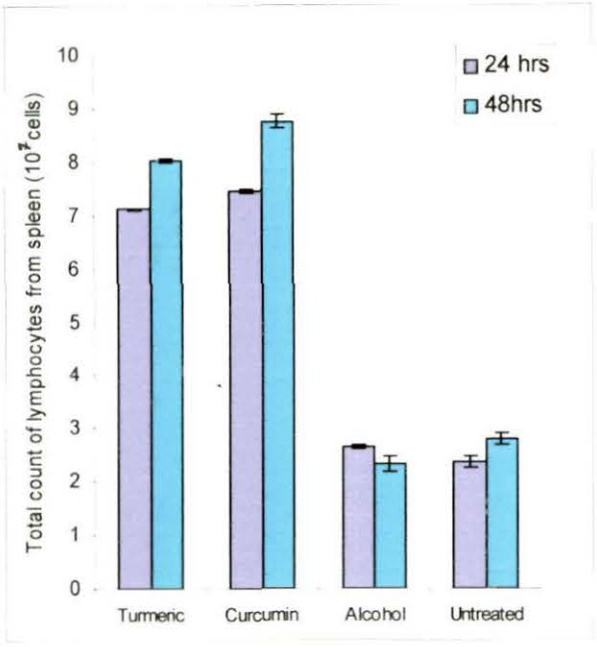
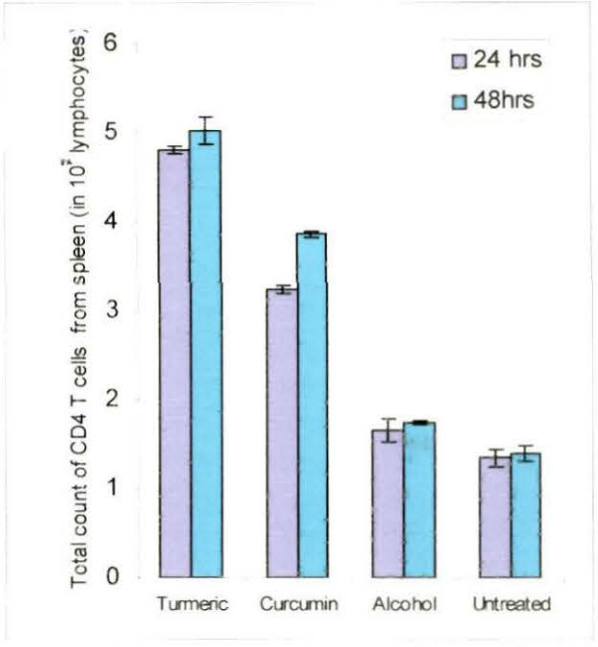


Fig. 35 Phase contrast image of $CD4^+$ T cells isolated from the spleen of DTH mice with the help of MACS(Mag×40)



A



B

Fig. 36 Total count of A) lymphocytes ($x10^7$), B) $CD4^+$ T cells (in 10^7 lymphocytes) isolated from spleen of DTH mice after 24 and 48 hrs of resensitization with ETE and curcumin treatment (10 μM). Results are expressed as mean ± SD. Two way ANOVA revealed all the treatment values (ETE and curcumin were significant compared to controls ($p< 0.01$).

The $CD4^+$ T cells count indeed significantly increased in spleen with ETE as well as with curcumin treatment (Fig. 36). For the purpose ETE treatment seems better than that with curcumin.

SERUM TNF- α LEVEL IN DTH MOUSE TREATED WITH ETE

DTH response not only influences the recruitment of various leukocytes at the inflammatory site but also modulates secretion of various cytokines from these inflammatory cells. One such proinflammatory cytokine TNF-α which plays significant role in many acute and chronic inflammatory diseases became our interest of investigation and was estimated by ELISA.

Intravenous administration of ETE 1 hr prior to resensitization inhibited TNF- α production in DTH mice (Fig. 37). The serum TNF- α level was much higher in the control groups and was maxima with alcohol treatment. Curcumin (10μM) also inhibited serum TNF-α. (Fig. 37)

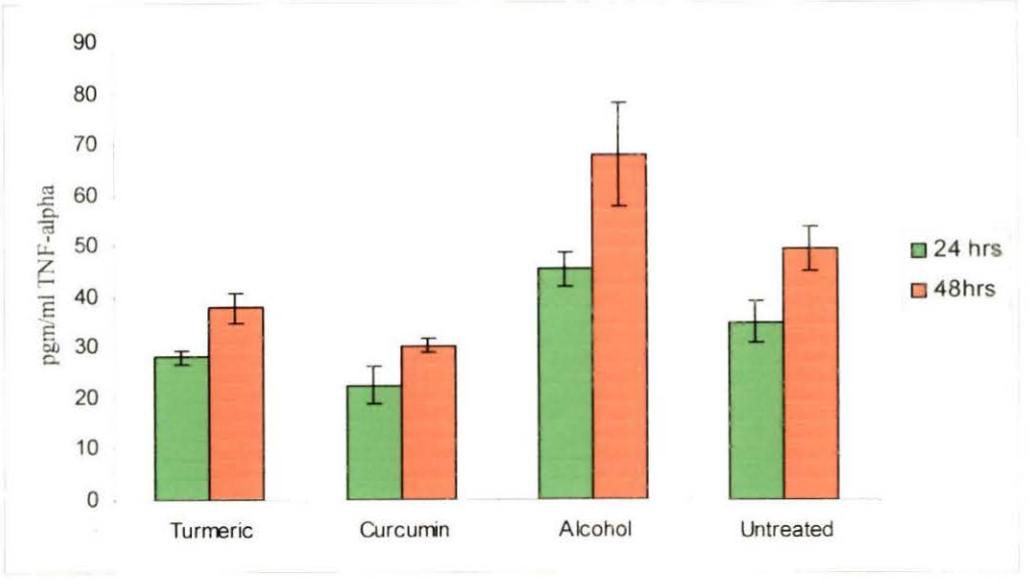


Fig. 37 Inhibition of serum TNF- α level in pgm/ml quantity with ETE and curcumin treatment after 24 and 48 hrs of resentization as judged by ELISA. Two way ANOVA revealed all the treatment values (ETE and curcumin) were significant compared to controls ($p < 0.01$).

EFFECT OF ETE TREATMENT ON THE GENERATION OF FREE RADICALS (Superoxide, Hydroxyl ion and Hydrogen peroxide) *IN VITRO*

The generation of free radicals affects the cellular metabolism in an extensive way. Thus, ETE's role in quenching the generation of free radicals was studied here. ETE could inhibit generation of superoxide by autoxidation of hematoxylin. The degree of inhibition was measured over the control which was considered 100%. Thus, ETE was effective in 45 % inhibition for superoxide generation (Fig. 38). Similarly ETE inhibited hydroxyl ion generation by 89% over the control, as measured in Fenton's reaction (Fig. 39). Thus, ETE was found to be potent as antioxidant.

Whether ETE can perform as inhibitor for generation of free radical within the cell, copper-ascorbate induced lipid peroxidation and malonaldehyde (MDA) production as index was carried

out for lymphocytes and tumor cells. H_2O_2 generation induces lipid peroxidation in the cell membrane which in turn produces MDA. ETE inhibits generation of H_2O_2 in lymphocytes (Fig. 39). Percentage of inhibition was at 43.33 % in reference to control. ETE can inhibit lipid peroxidation in tumor cells at best to 11.64 %. When inhibition of peroxidation is more in lymphocytes , is likely to buy more protection for the cells.

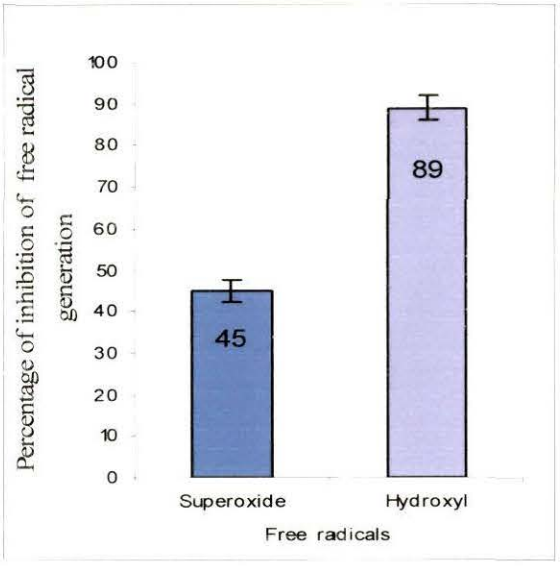


Fig. 38

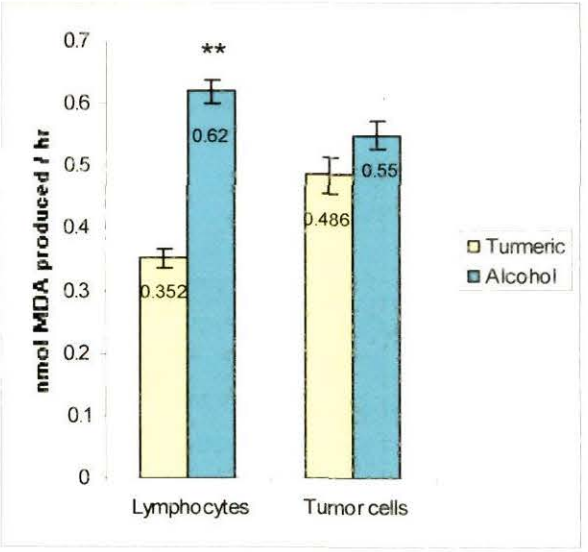


Fig. 39

Fig. 38 Inhibition of superoxide and hydroxyl ion generation by ETE treatment over the control (alcohol treated).

Fig. 39 Copper ascorbate induced H_2O_2 generation in lymphocytes and tumor cells in presence and absence of ETE. ETE treatment caused lower production of MDA than the control. Results are expressed as mean ± SD, **$p<0.01$ compared to respective controls.

NITRIC OXIDE SYNTHASE ACTIVATION WITH ETE TREATMENT

NO is one of the smallest molecular products of mammalian cells and a free radical, it functions in the regulation of many cellular events and in defending against various pathogens and tumorigenic transformation. The level of activity of cellular enzyme nitric oxide synthase (NOS) in generation of NO from L-arginine was measured in both

lymphocytes and tumor cells. In this experiment beside normal lymphocytes and tumor cells, lymphocytes from tumor bearing mice was also taken into consideration. ETE treatment could increase the NOS activity in all three types of cell, but the level of activity was least in the normal lymphocytes as revealed in Fig. 40. Addition of 10μM N^G methyl- L-arginine acetate ester (NAME) to the reaction mixture in all the cases completely inhibited NO production, thus suggesting NO production was due to the NOS activity.

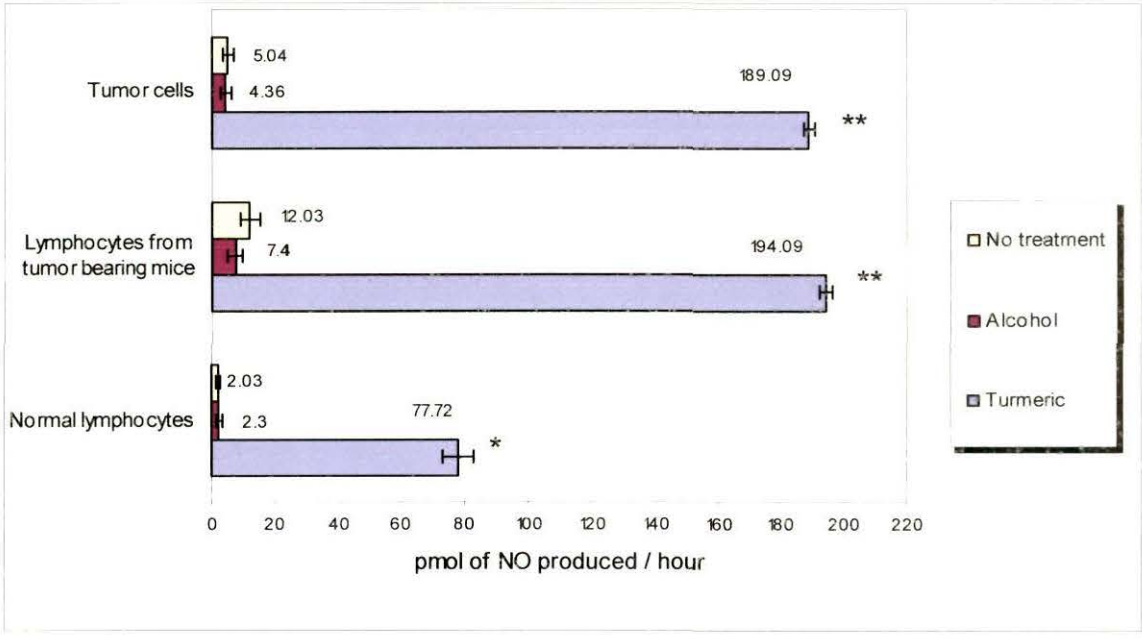


Fig. 40 L- arginine derived NO production in both lymphocytes as well as tumor cells. Activation of NOS by ETE treatment, as suggested by higher production of NO than the control. Results are expressed as mean ± SD, * $p<0.05$ & ** $p<0.01$ compared to respective controls.

SEPARATION AND CHARACTERIZATION OF THE DIFFERENT FRACTIONS OF ETE

All the findings so far suggested the potential role of ETE on lymphocytes and tumor cells which might be very important from immunological point of view. In this section ETE was fractionated through chromatographic techniques to obtain different components and to investigate their effect in certain immunological parameters such as lymphocyte and

macrophage counts&antibody responses. The results were compared with that of ETE and commercially available curcumin.

FRACTIONATION OF ETE

The yield of turmeric in ethanolic extract (ETE) of rhizome was 0.435 ± 0.032 mg/ml and showed two prominent bands on TLC with 50% ethyl acetate / pet. ether (Fig. 41A)

ETE was subjected to chromatographic separation over silica gel. The compound was adsorbed in silica gel column using small amount of petroleum ether. Elution with 40% ethyl acetate/ petroleum ether afforded the 1st fraction with a prominent yellow band on TLC (Fig. 41 B). The TLC was run on fluorescent bound silica. Further elution with 50% ethyl acetate/ petroleum ether furnished the 2nd fraction as brownish gummy material (Fig. 41 C). Both the fractions were UV active and apparently single spot on TLC. The fractions were dried and dissolved further in ethanol for UV spectra and experimentation.

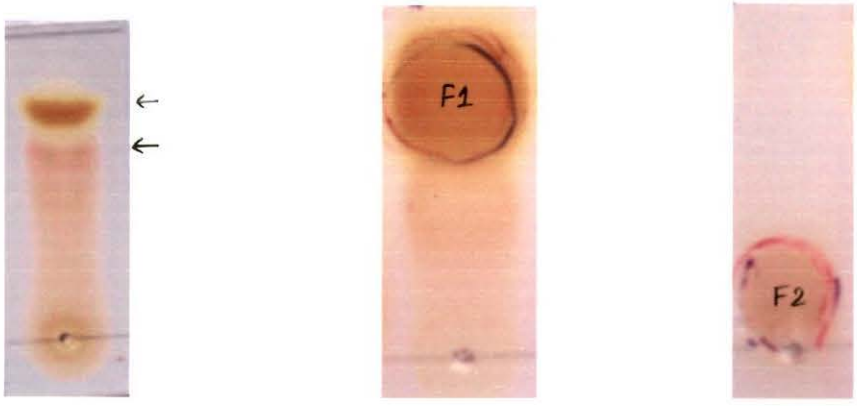


Fig. 41 TLC strip showing bands of A) ETE (shown byarrow), with 50% ethyl acetate / pet. ether, B) Fraction 1 (F1) with 40% ethyl acetate/ petroleum ether and C) Fraction 2 (F2) with 50% ethyl acetate/ petroleum ether

Then UV spectra were run for the ethanolic turmeric extract as well as for Fraction 1 (F1) and Fraction 2 (F2). While ETE aggravated two λ max values at 424nmand 234nm, F1 displayed the major band at 417 nm and the F2 gave the λ max value clearly at 229 nm (Tab.6).

Table. 6 Characteristics of ETE, its two fractions and commercial curcumin.

	Dry weight in mg in 1ml	**No. of bands in TLC** (**Solvent**- ethyl acetate/ Pet ether)	**UV spectra**
Ethanolic turmeric extract (ETE)	0.435 ± 0.032	**2** (yellow orange prominent band and faint brownish band)	424nm 234nm
Fraction 1(F1) of ETE	0.164 ± 0.018 (37.61% of ETE)	**1** (yellow orange prominent band)	417nm
Fraction 2 (F2) of ETE	0.0157± 0.0019 (3.62% of ETE)	**1** (faint brownish band)	229nm
Curcumin 10µM (Acros)	0.0036838	-	210nm

Fig. 42 Variations in the color of A) ETE, B) F1, C) F2, D) Curcumin (100µM), E) Curcumin (50µM), F) Curcumin (25µM), G) Curcumin (10µM) in the ethanol solutions.

IMMUNOLOGICAL ASSAYS WITH FRACTIONS AND CURCUMIN

The contributing role of two fractions of ETE and curcumin in primary and secondary immune response were studied in terms of cell count and antibody level after immunization with SRBC.

WEIGHT OF SPLEEN AND THE NUMBER OF LYMPHOCYTES AND MACROPHAGES

For increase in lymphocyte count with treatment of ETE and fractions, T lymphocyte seems to contribute the most (Table.7, Fig. 43). In primary immune response, the numbers of B lymphocytes with all different treatment were lower than the control of alcohol and no treatment. However B cell count was boosted better by the curcumin than ETE and its fractions. In secondary immune response count of B lymphocytes with curcumin, dose of 10μM and 25μM were almost similar to that of control.

Notable increase in macrophage count with ETE treatment had already been observed (Table.1). The fractions separately were not effective as that of ETE, even then they stimulated better index for macrophage count than other treatment except curcumin at the dose of 10μM (Table.7).

Table. 7 Weight of spleen, total lymphocyte and macrophage count in SRBC immunized mice treated with ETE, F1, F2, Curcumin (10 to 100 μM), alcohol (control) and no treatment (only SRBC) in A) Primary and, B) Secondary immune response. Results are expressed as mean ± SD, * $p<0.05$ & ** $p< 0.01$ compared to respective controls.

A. Primary

	Weight of spleen (gm)	Total lymphocyte count x 10^7	Macrophage count x 10^7
ETE	0.1673 ± 0.032	5.210 ± 0.093*	2.768 ± 0.908
F1	0.226 ± 0.0421	9.161 ± 0.165**a)	2.611 ± 0.132
F2	0.2734 ± 0.0548	9.338 ± 0.134**a)	2.505 ± 0.175
Cur 10μM	0.2148 ± 0.0312	3.359 ± 0.129	2.436 ± 0.094

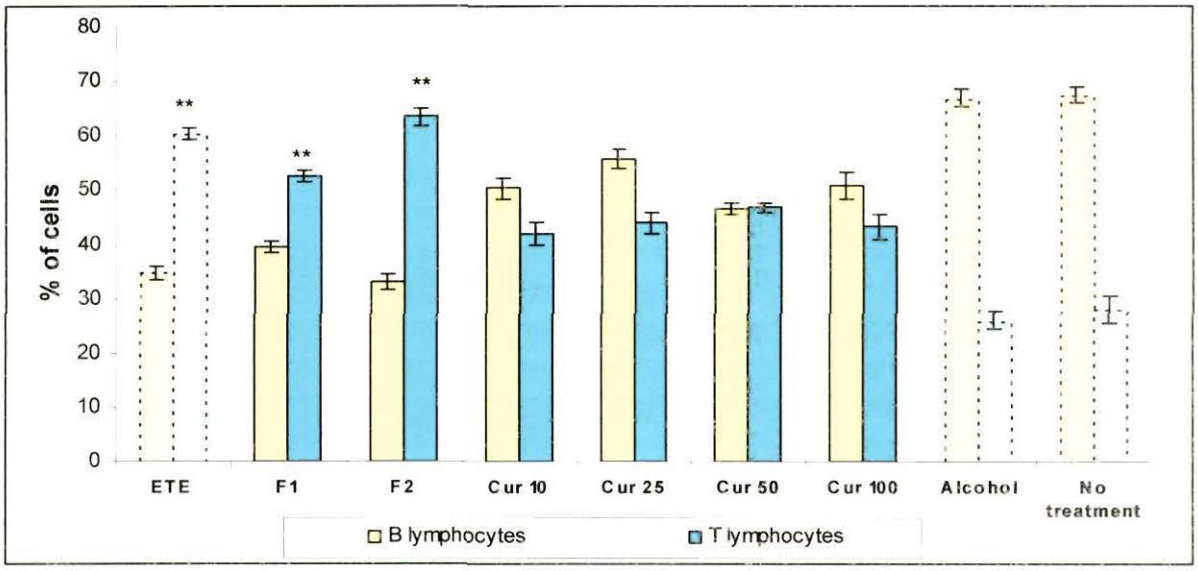
Cur 25 µM	0.206 ± 0.0421	3.240 ± 0.089	1.823 ± 0.083
Cur 50 µM	0.120 ± 0.063	2.412 ± 0.096	1.756 ± 0.082
Cur 100 µM	0.104 ± 0.054	2.168 ± 1.034	1.146 ± 0.142
Alcohol	0.1405 ± 0.0501	2.418 ± 0.105	1.428 ± 0.108
No treatment	0.137 ± 0.0463	3.476 ± 0.113	1.983 ± 0.370

[a] Note maximum increase with F1 and F2 fractions of ETE. Same is true in table 7B.

B. Secondary

	Weight of spleen (gm)	Total lymphocyte count x 10^7	Macrophage count x 10^7
ETE	0.116 ± 0.017	4.960 ± 0.067*	6.406 ± 0.078**
F1	0.145 ± 0.027	5.496 ± 0.089* [a]	3.91 ± 0.067*
F2	0.132 ± 0.043	6.262 ± 0.056** [a]	3.176 ± 0.065*
Cur 10µM	0.139 ± 0.019	2.246 ± 0.089	3.897 ± 0.086*
Cur 25 µM	0.118 ± 0.032	1.642 ± 0.098	1.876 ± 0.054
Cur 50 µM	0.100 ± 0.056	1.643 ± 0.232	1.803 ± 0.098
Cur 100 µM	0.090 ± 0.017	1.289 ± 0.143	1.753 ± 0.079
Alcohol	0.088 ± 0.012	3.031 ± 0.024	1.983 ± 0.056
Only SRBC	0.090 ± 0.009	3.06 ± 0.032	2.24 ± 0.037

A. Primary



B. Secondary

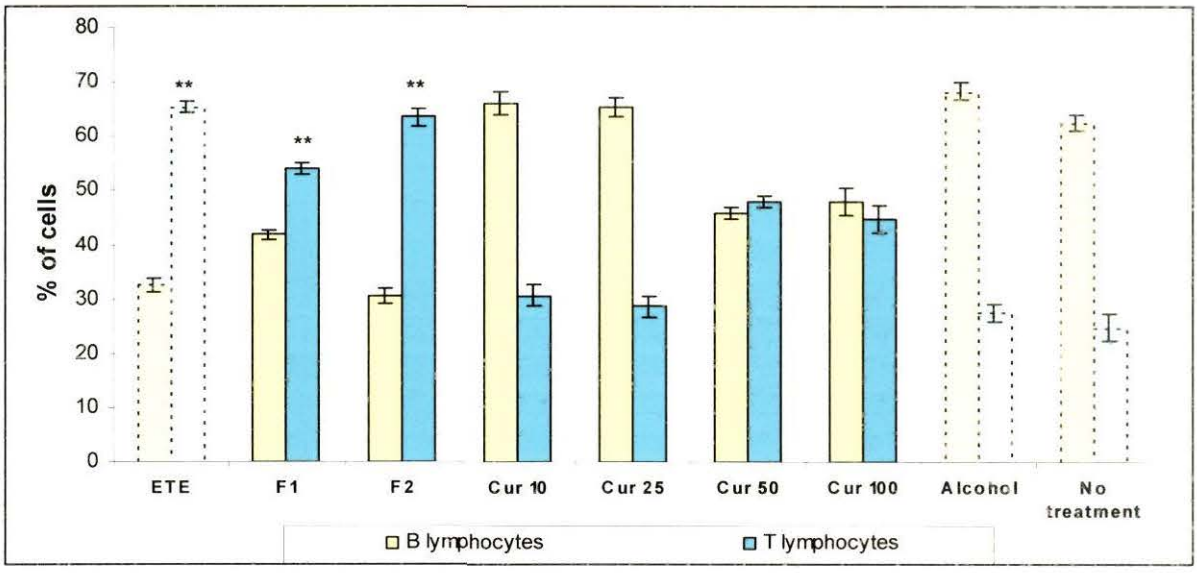
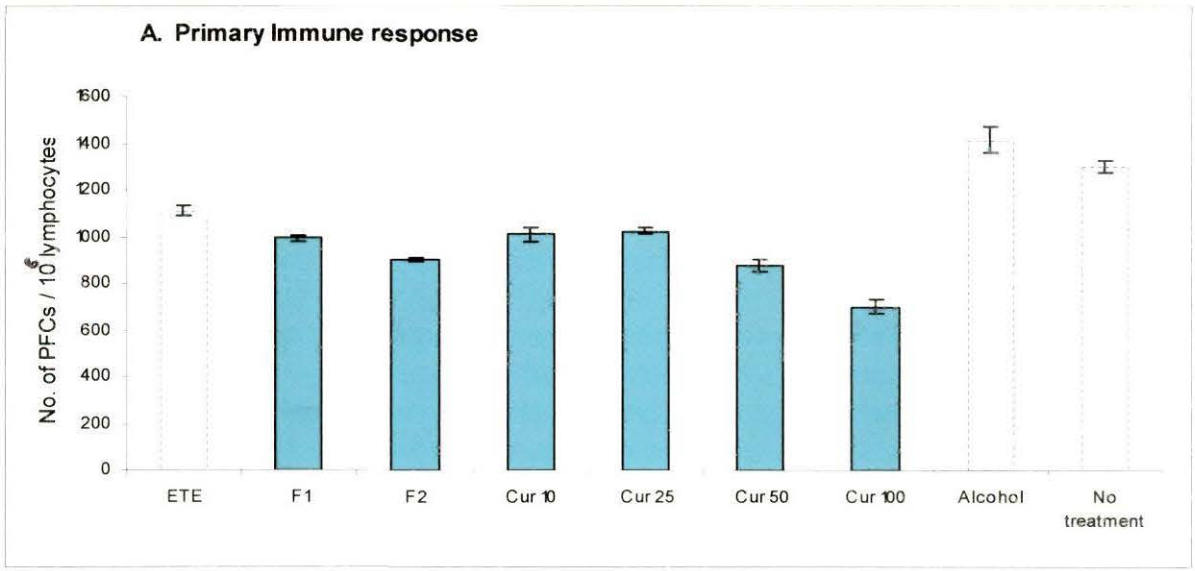


Fig: 42 Percentage of B and T lymphocytes from spleen of mice immunized with SRBC and treated with ETE, F1, F2, Curcumin (10 to 100 μM), alcohol (control) and no treatment in : A) Primary and, B) Secondary immune response. [Values of ETE and control groups are quoted here from earlier data (Fig.3) and presented in lighter shade and stippled boundary for better comparison of the results]. Results are expressed as mean ± SD, **p< 0.01 compared to respective controls.

It was observed that ETE as such could not boost antibody production to the level of alcohol and without any treatment (refer to Fig. 4, 5 &6; Table 2). The fractions F1 and F2 neither could do the job better as shown in figure 44 A & B. Low response with ETE and fractions were evident both from the number of the plaque forming cells (Fig. 44 A & B) and titre values (Table 8, Fig. 45 & 46).



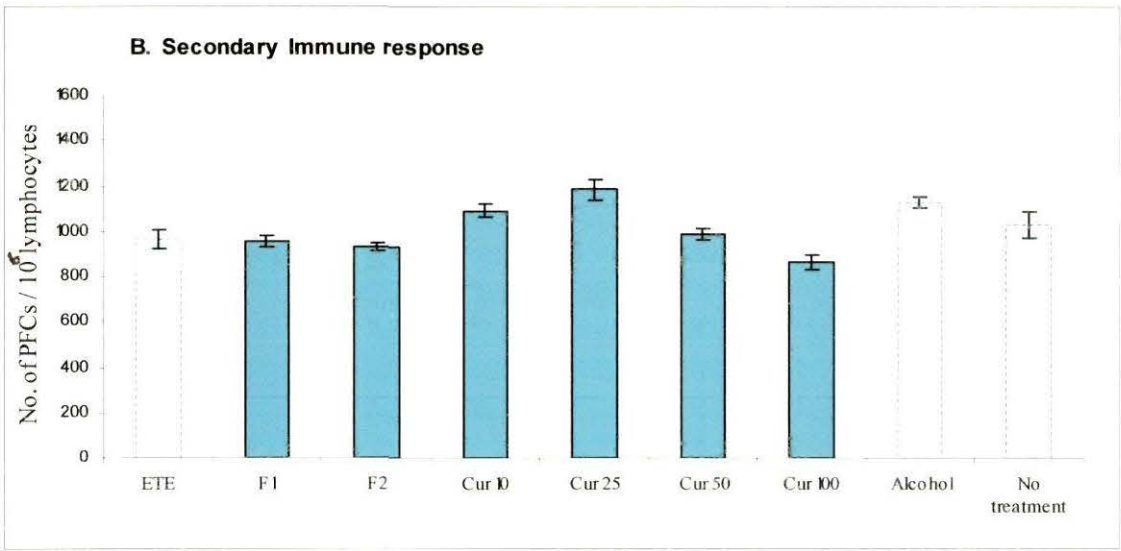


Fig: 44 No. of plaque forming cells (PFCs) from spleen of mice treated with ETE, F1, F2, Curcumin (10 to 100μM), alcohol and no treatment in : A) Primary and, B) Secondary immune response. [Values of ETE and control groups are quoted here from earlier data (Fig. 4)]

Primary HA Titre Response

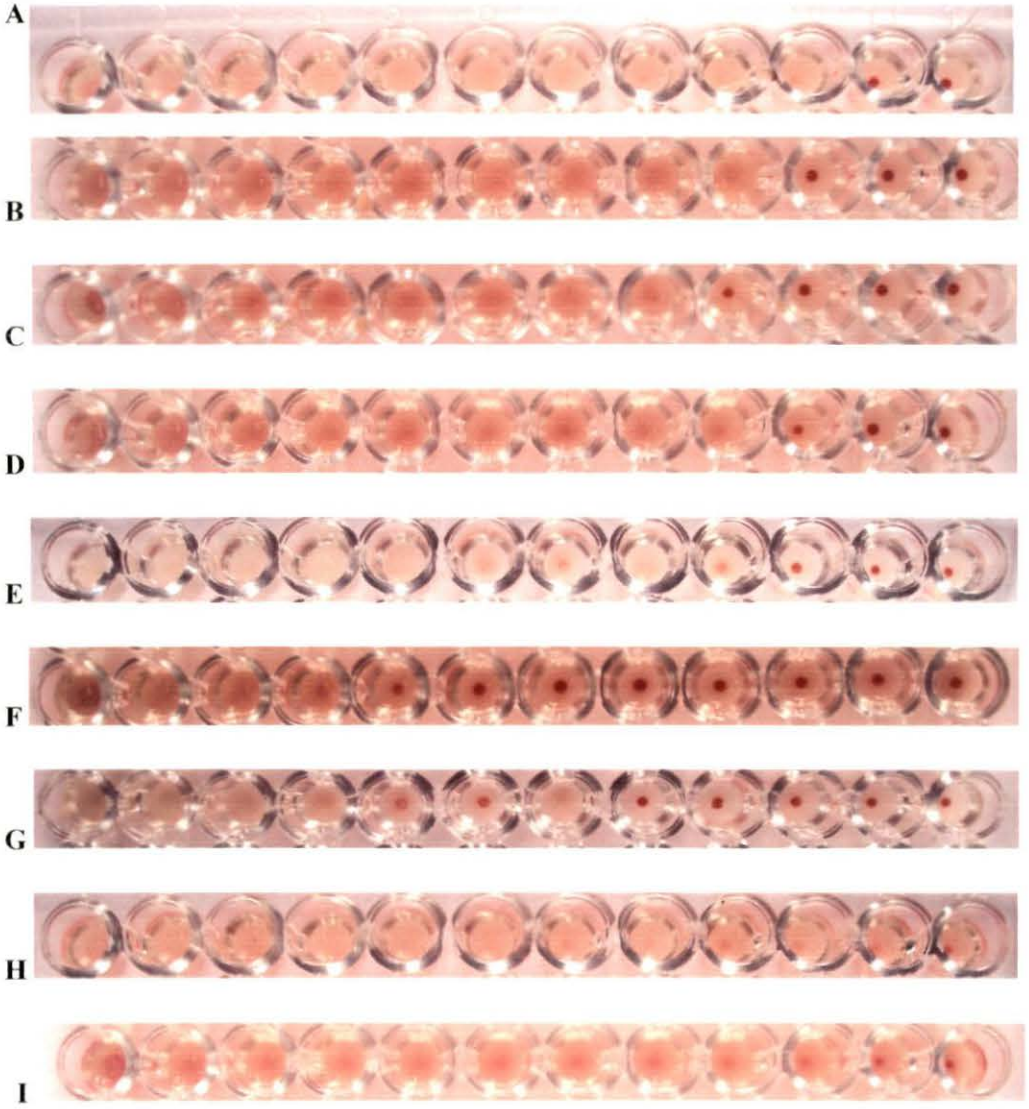


FIG: 45 Haemagglutination (Ab) titre with antiserum from mice injected with SRBCs after treatment with: A) ETE B) F1, C) F2, D) Curcumin 10 μM, E) -25μM, F) -50 μM, G) -100 μM, H) Alcohol (control) , I) No treatment. Wells with opaque look indicate agglutination of RBCs and well with distinct precipitation of RBCs at the bottom indicate no haemagglutination.

Secondary HA Titre Response

Plate 1

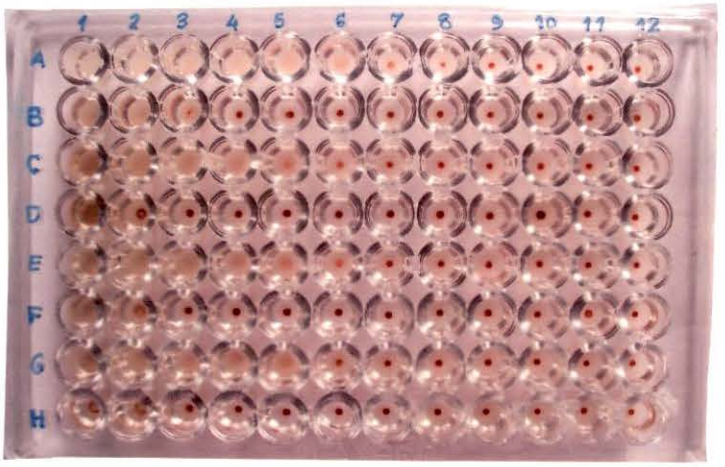


Plate 2

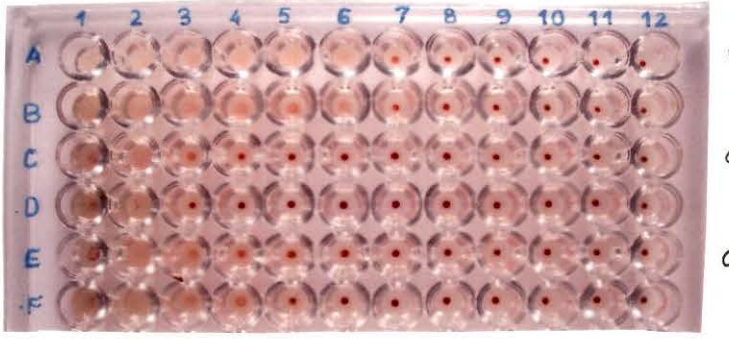


Plate 3

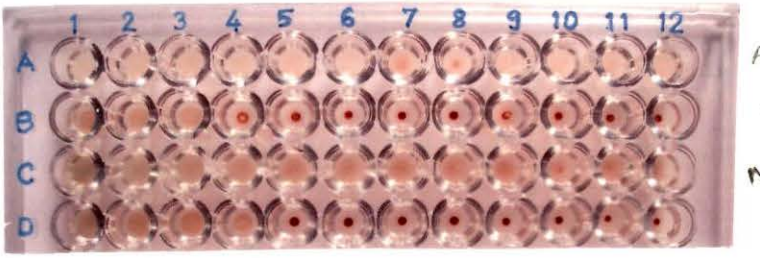


Fig. 46 Haemagglutination (Ab) titre of secondary immune response with antiserum from mice injected twice with SRBCs. [2- Merceptoethanol (ME) was added in the well to reduce IgM and thus to measure secondary (IgG) response only]

Table. 8 Haemagglutination (Ab) titre value from mice immunized with SRBC after treatment with ETE, F1, F2, Curcumin (10 to 100μM), alcohol (control) and no treatment in primary and secondary immune response.

	Ab. Titre value [a]		
	Primary immune response	**Secondary immune response**	
	Without ME	Without ME	With ME
ETE	5120	320	40
F1	2560	160	20
F2	1280	160	20
Cur 10	2560	160	10
Cur 25	1280	320	80
Cur 50	80	20	20
Cur 100	80	20	20
Alcohol	10240	> [b]	40
No treatment	2560	> [b]	80

[a] indicating last dilution of serum capable of agglutinating antigen. Higher value indicates better antibody response.

[b] sign to indicate titre value more than the dilution present in the 12th or last well of the row in the titre plate.

Furthermore, ELISA analysis for secondary (IgG) immune response showed that there was no significant difference in the data of different groups (Fig. 47). In other words, ETE, fractions and curcumin could not influence much the IgG response.

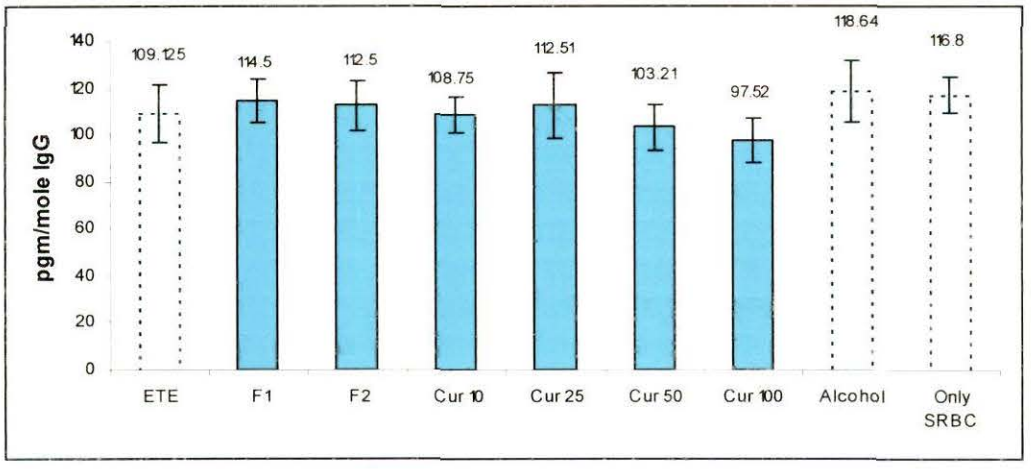


Fig: 47 Estimation of serum IgG level (pgm/ml) by ELISA in secondary immune response with ETE F1, F2, Curcumin (100, 50, 25 and 10 μM), alcohol (control) and no treatment (only SRBC).

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.

CONCLUSION

Research works with turmeric or curcumin till date were carried out mostly to show their inhibitory property towards tumor growth, viruses etc. The present investigation was framed to look into detail the effect of turmeric and curcumin on immunocompetent cells, which did not get much attention so far. The effects of malignant cells were also taken into account.

The effect of turmeric was judged at cellular level for both lymphocytes and malignant cells. Blastogenesis, DNA synthesis, cell cycle study and set in of apoptosis and cytotoxicity towards tumor cells were investigated. By all these counts turmeric seems to be stimulatory for the lymphocytes and causing cytotoxic differentiation of T cells. Ethanolic turmeric extract (ETE) itself was capable of setting apoptosis in the malignant cells.

Thus, turmeric plays diabolically opposite role on lymphocytes and malignant cells in murine model. The present investigation showed in detail these opposite effects, in conjunction promotes the role of turmeric as a strong immunotherapeutic agent for cancer. Then investigation was carried out by intravenous injection and oral administration of ETE to find out its effectiveness in curbing tumor induction and tumor growth. Turmeric was effective in delaying the appearance of tumor, growth of tumor and thus increasing the life span of the tumor bearing host. The tumor growth was affected but tumor could not be totally eliminated in present experimental set up. Devising further experiments including combination of some other agents with turmeric will be attempted in future.

Furthermore we documented the anti- inflammatory role of turmeric in 2,4 DNFB induced DTH reaction in mouse paw. Inhibition of neutrophil and macrophages at the site seems to affect lesser secretion of proinflammatory cytokine which might allow faster wound healing.

Generation of free radicals by univalent reduction of O_2 is fundamental to any biochemical process and involved in cellular metabolism. Simultaneously cellular antioxidant quenches the free radicals to maintain a balance and not allow them to affect the system deleteriously. We observed a strong antioxidant role of turmeric inhibiting generation of $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$. This explains maintenance of integrity of cell membrane especially of the lymphocytes. This feature allows turmeric to be a therapeutic agent to fight cancer.

ETE stimulates NO production in both lymphocyte & tumor cell significantly, allowing the first to be activated and induction of apoptosis for the tumor cells.

Upon chromatographic separation of ETE two fractions (F1 & F2) were found and F2 was more comparable with commercially available curcumin. When the effect of ETE, two fractions and curcumin was judged in the cell, ETE was found better in overall performance.

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Two-way Efficacy of Alcoholic Turmeric Extract: Stimulatory for Murine Lymphocytes and Inhibitory for Fibrosarcoma Cells

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Abstract

The ethanol turmeric extract has been found to be stimulatory for murine lymphocytes and inhibitory for ascitic fibrosarcoma cells which have been investigated on the basis of *in vitro* viability assay, cell cycle study by fluorescence activated cell sorter (FACS), and DNA synthesis by ^{3}H-thymidine incorporation. Viability of lymphocytes is better with turmeric; DNA synthesis increases and the majority of the lymphocytes are driven toward mitotic stage as observed by FACS. Whereas turmeric causes a significant level of death in ascitic fibrosarcoma cells *in vitro* and possibly causes arrest of the cell cycle at S-phase, it induces programmed cell death.

Keywords: Curcumin, DNA synthesis, FACS, fibrosarcoma inhibition, lymphocyte stimulation, turmeric, viability.

Introduction

Curcumin is a constituent of the yellow-colored Indian spice turmeric obtained from the rhizome of *Curcuma longa* Linn. (Zingiberaceae). It is used for the preparation of many Indian dishes. Its medicinal value was mentioned in the great Ayurvedic medicinal literature of India, which is several millennia old. Recently, much interest has been generated for varieties of pharmacological effects of curcumin, including anti-tumor (Huang et al., 1994; Ruby et al., 1995), anti-infectious (Mazumdar et al., 1995), and antibacterial and anti-inflammatory (Srivastava, 1989; Brouet & Ohshima, 1995) activities. Several authors (Mehta & Moon, 1991; Huang et al., 1992, 1994; Tanaka et al., 1994) have demonstrated anti-carcinogenic properties of curcumin in animal models. Our laboratory also became interested in the anticarcinogenic effects of curcumin several years ago (Choudhury, 1998; Das, 1999, 2000; Yasmin, 2000). In this paper, we report our findings that an alcohol extract of turmeric is inhibitory for murine fibrosarcoma, while it stimulates the growth of murine lymphocytes. The analysis was made on the basis of the viability of cells, cell cycle analysis, and ^{3}H-thymidine incorporation.

Materials and Methods

Tumor induction

Inbred adult Swiss mice of both sexes, 8–16 weeks of age, were used for all experiments. Ascitic fibrosarcoma cell line was obtained from Chittaranjan National Cancer Research Institute, Calcutta, and maintained in our laboratory by serial passages. To continue with the cell line, adult mice were injected intraperitoneally with 10^{6} ascitic fibrosarcoma cells per mouse in 0.1 ml of PBS for induction of ascitic tumor. A cell suspension was prepared in phosphate-buffered saline (PBS) following a standard protocol. After 10 to 15 days, tumors would develop.

Ethanol turmeric extract preparation

For the preparation of turmeric extract, fresh rhizomes of turmeric (*Curcuma longa* Linn.) were obtained from a local market, and after cleaning, 10 g of sample was crushed to a paste with a mortar and pestle. To this paste, 10 ml of absolute alcohol were added and kept in a refrigerator at

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4 °C. After 12 h, the mixture was taken out and filtered through Whatman filter paper no. 1; the filtrate was refiltered through Millipore filter paper, and the final solution obtained was stored at 4 °C for further use.

Lymphocyte preparation

Spleen was collected aseptically from mice, and the cells were dissociated in PBS, pH 7.2, with the help of stainless steel wire mesh by passing through a syringe fitted with a 27-gauge needle. The splenocytes were washed twice in cold PBS, and the suspension was then kept in cold, sterilized ammonium chloride solution (0.83%) for 10 min in order to lyse the red cells completely. Then, the cell suspension was washed twice with cold PBS. Whenever it was necessary to remove debris, the cell suspension was layered on Ficoll and Hypaque solution (Sigma Chemical Co., St. Louis, MO, USA) and spun down at 3000 rpm for 15 min. The lymphocytes were collected from the interface and then washed twice with PBS. Finally, the cells were suspended in minimum essential medium (MEM, Hi Media).

In vitro viability assay

Splenic lymphocytes and ascitic fibrosarcoma cells were transferred to culture plates at a density of 1×10^6 cells in 0.2 ml of minimum essential medium (MEM, Hi-Media) supplemented with 10% heat-inactivated sterile goat serum (Chaudhuri & Chakravarty, 1983), 10 µl or other doses of ethanol turmeric extract, and 200 U of penicillin/ml at 37 °C. As the turmeric extract was made in ethyl alcohol, an equivalent amount of ethanol for a particular dose of turmeric was used for control. Cell survival studies were performed at different times (4, 6, 8, 16, 24, 48 h) of cell culture by the Trypan blue dye exclusion test. The total number of viable cells were counted in a hemocytometer.

Cell cycle analysis by fluorescence activated cell sorter

For cell cycle analysis, mice were injected intravenously with turmeric extract, and at 16 and 24 h, spleen cells and ascitic tumor cell suspensions were prepared in PBS separately. To 1 ml of cell suspension, 1 ml of 80% ethanol was added, and the cells were fixed overnight at 4 °C. Fixed cells were centrifuged, the supernatant was decanted off, and 0.5 µl of 500 µg/ml (Standard 250 µg/ml) RNAse A were added, followed by incubation for 45 min at 37 °C. Ethidium bromide (69 mM) was prepared in 38 mM sodium citrate. The cells were centrifuged and suspended in 0.5 ml of 69 mM ethidium bromide (a fluorochrome that stains nuclear DNA) at room temperature at least for 30 min. Finally, the cell cycle analysis was done with a fluorescence activated cell sorter (FACS; Caliber, Becton Dickinson).

Measure of DNA synthesis

Proliferation of lymphocytes was determined by measuring the rate of ^{3}H-thymidine (^{3}H-TdR) incorporation into DNA. Cells were obtained from mice injected (i.v.) earlier with turmeric extract and suspended at a concentration of 2×10^6 cells/ml in culture medium (RPMI-1640), of which 200 µl of cell suspension was transferred to each well of a 96-well

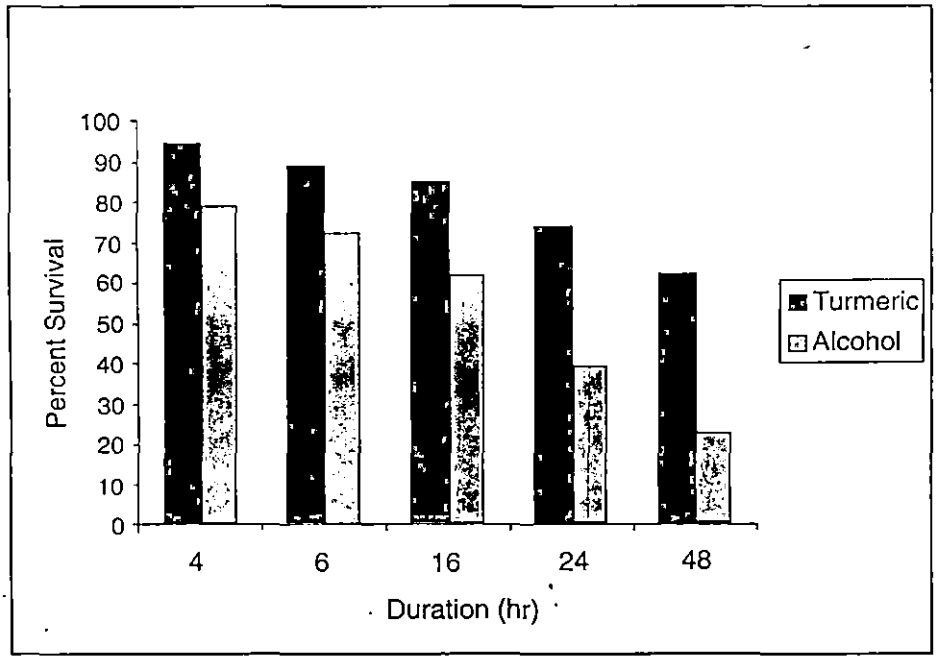


Figure 1. Diagram showing the comparative survival of splenic lymphocytes at different hours of turmeric treatment *in vitro*.

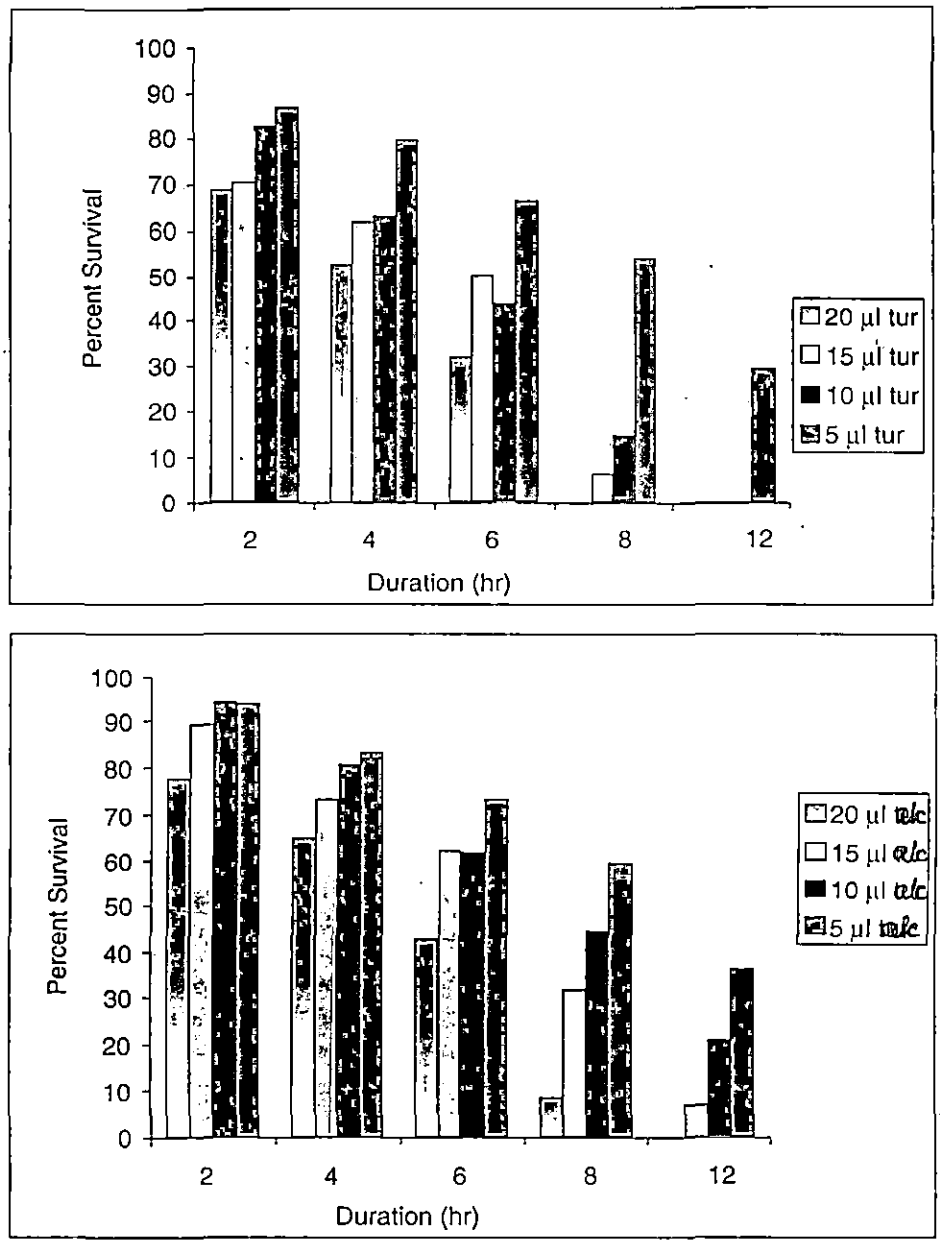


Figure 2. The comparative survival of ascitic fibrosarcoma cells at different hours of *in vitro* treatment with various doses of ethanolic turmeric extract.

microculture plate. The microculture plate was incubated for 8 h at 37 °C in a humidified atmosphere containing 5% CO_2 in air in the presence of 1 µCi of ^{3}H-thymidine (Bhaba Atomic Research Centre) per well. At the end of the culture period, cells were harvested with a PHD Cell Harvester (Cambridge, MA, USA) onto glass fiber filters, washed with methanol, dried, and kept in standard scintillation vials (Beckman). At the time of radioactivity count, 5 ml of scintillation fluid (6 gm PPO, 0.5 gm POPOP/1 of toluene) were added into each vial. All assays were done in triplicate and the level of ^{3}H-TdR incorporation was expressed as counts per minute.

Results

In vitro viability studies

For *in vitro* studies of the effect of turmeric on cell growth and viability, the splenic lymphocytes and ascitic tumor cells were cultured separately. After treatment with 10 µl of ethanol turmeric extract for 4 h, 94.37% of the lymphocytes were viable, whereas a survival of 79.21% was observed in the control (Fig. 1). Thus, the viability of lymphocytes was better with turmeric. This trend was also observed at the end of 24 h, when the percentage of live lymphocytes was 73.57% in the treatment group *versus* 38.84% in control. However,

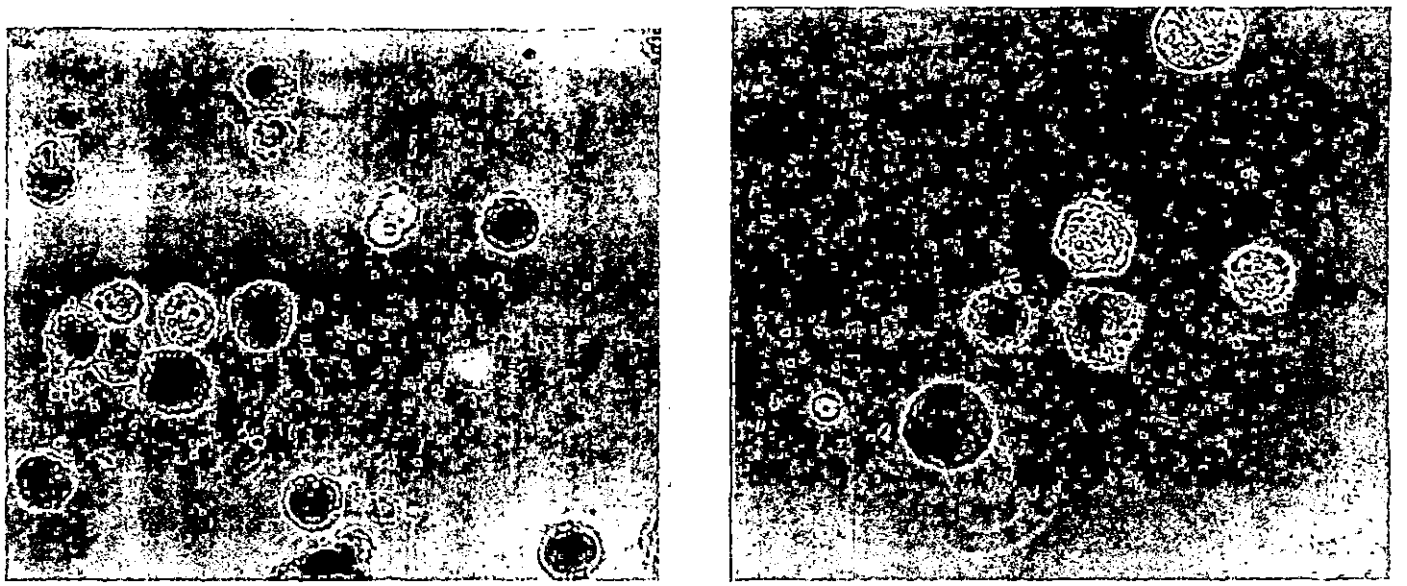


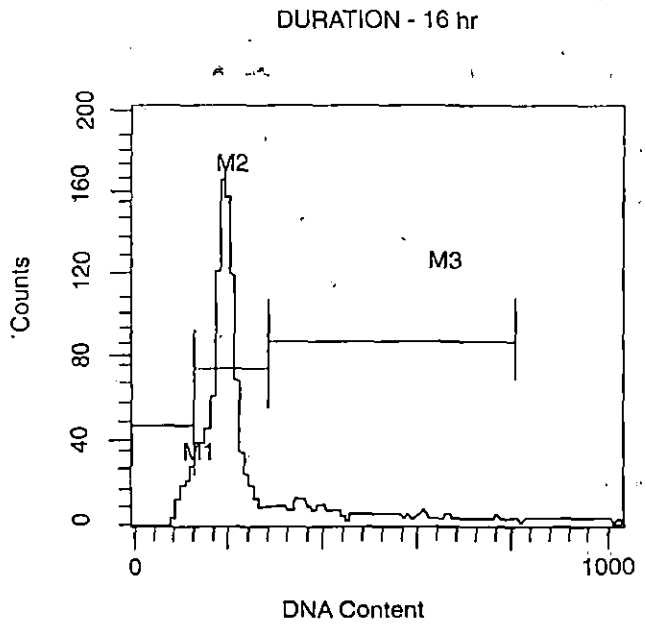
Figure 3. Trypan blue dye exclusion test for the ascitic fibrosarcoma cells at 4 h of *in vitro* treatment with (a) ethanolic extract of turmeric, (b) ethanol only (control). Turmeric treatment causes a higher rate of death to the tumor cells as evidenced from the majority of the cells turning blue in the photomicrograph (magnification ×1600).

the same dose of turmeric causes a significant level of death in ascitic fibrosarcoma cells. At 4 h, only 62.5% of viable cells could be recovered, whereas in control, 80.50% cells remained alive. Viability of tumor cells with turmeric fell dramatically after 6 h and continued to diminish with a longer incubation period in comparison with the control (Fig. 2). After 12 h of incubation, all turmeric extract – treated tumor cells were dead, whereas 21.23% of tumor cells were alive in the control. Besides 10 µl dose, a few other doses were tried (Fig. 2). The trends with other doses such as 5, 15, and 20 µl were similar to those with 10 µl, but in a graded fashion. At 12 h, total cell death was observed with 10, 15, and 20 µl doses, but not with 5 µl. A photomicrograph showing the results of the Trypan blue dye exclusion test for the ascitic fibrosarcoma cells at 4 h of *in vitro* treatment clearly indicates that in comparison to the control, the majority of tumor cells turned blue when treated with turmeric (Fig. 3). Thus, the survival indices for lymphocytes were better in alcohol turmeric extract, whereas the extract seemed to be inhibitory to murine fibrosarcoma cells.

Cell cycle analysis

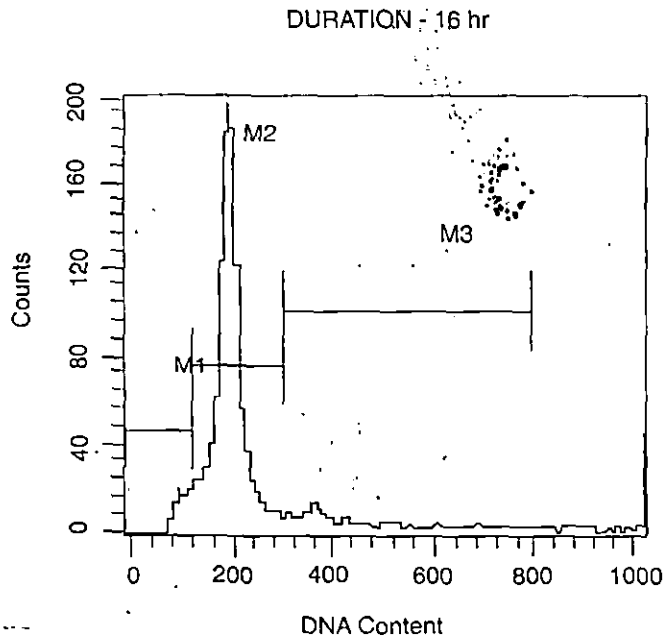
In cell cycle analysis by FACS, DNA replication was studied by tagging the fluorochrome ethidium bromide (EB). EB staining of permeabilized cells (DNA content) was plotted against cell numbers in the DNA histograms. In addition to dot-plot display and histogram display, the software of FACS also displays a table showing the percentage of cells at each cell cycle stage. In this paper, "index" refers to the percentage of cells at a particular cell cycle stage. Lymphocytes, after 16 h *in vivo* turmeric treatment, did not show any appreciable differences, relative to the control. The difference became apparent after 24 h. After 24 h of turmeric treatment, the M1 peak index was at 0.96 for lymphocytes relative to an index of 2.36 of the control (Fig. 4, Table 1). The M1 peak indicated the number of cells entered in the apoptotic state at sub G_0-G_1 phase. The observation that the index with turmeric-treated lymphocytes was lower at the M1 peak explains the higher count of viable cells with turmeric treatment as shown in Figure 1. The M2 peak showing the G_0-G_1 phase was also lower in the turmeric group in comparison to the control, suggesting that turmeric possibly has driven the cells quickly into the next phase (i.e., S-phase). At G_2-M phase (i.e. the M4 peak), the turmeric-treated lymphocytes showed an index of 12.63 in comparison with 3.34 in the control (Table 2). This indicated that turmeric has driven the majority of the lymphocytes toward mitotic stage.

However, the turmeric treatment of ascitic fibrosarcoma produced results of a reverse trend. At 16 h of treatment with turmeric, the indices for the M1 peak were 6.39, and 0.00 in control (Fig. 5, Table 1). Probably, the indices showed that the majority of the tumor cells had entered into the apoptotic state with turmeric treatment. The turmeric treatment, up to 24 h, possibly caused arrest of the cell cycle at the S-phase, as represented by the M3 peak. Thus, a fall in the M4 peak at the G_2-M phase has been observed, suggesting that the tumor cells have not entered into the mitotic stage. In summary, FACS analysis reveals that turmeric is promotional for murine lymphocytes by activating the cell cycle stages and, on the other hand, it is inhibitory for cell division and induces programmed cell death in murine ascitic fibrosarcoma cells.



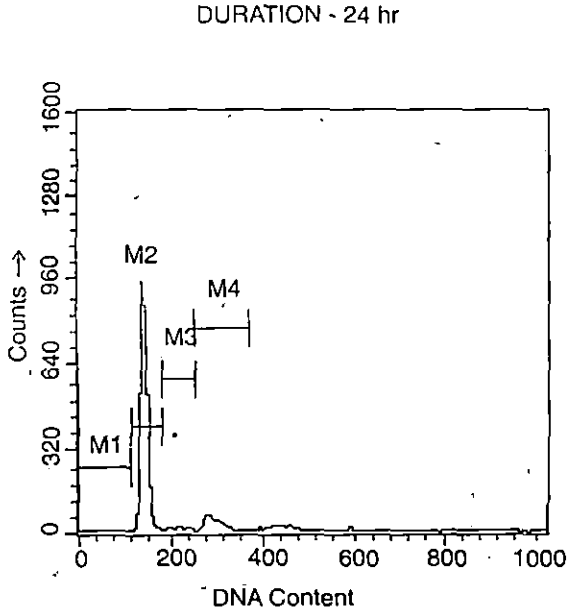
Splenic lymphocytes treated with ethanolic termeric extract

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S-G_2 - M



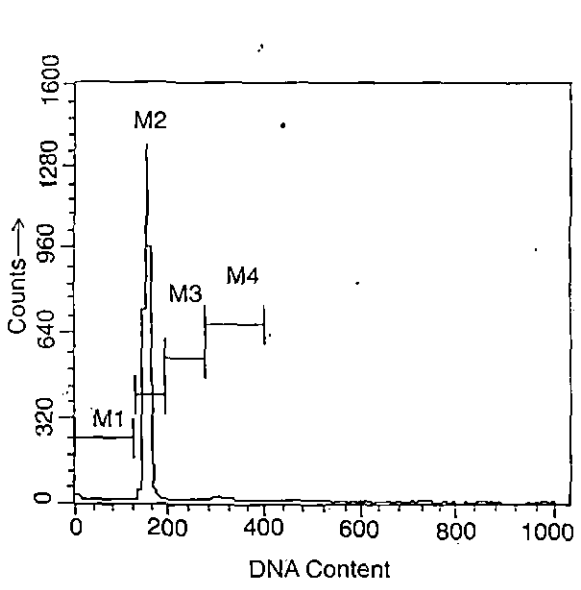
Splenic lymphocytes treated with alcohol

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S-G_2 - M



Splenic lymphocytes treated with ethanolic termeric extract

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S; M4, G_2 - M



Splenic lymphocytes treated with alcohol

M1, sub G_0 - G_1; M2, G_0 - G_1; M3, S; M4, G_2 - M

Figure 4. DNA histograms by FACS for cell cycle analysis of splenic lymphocytes.

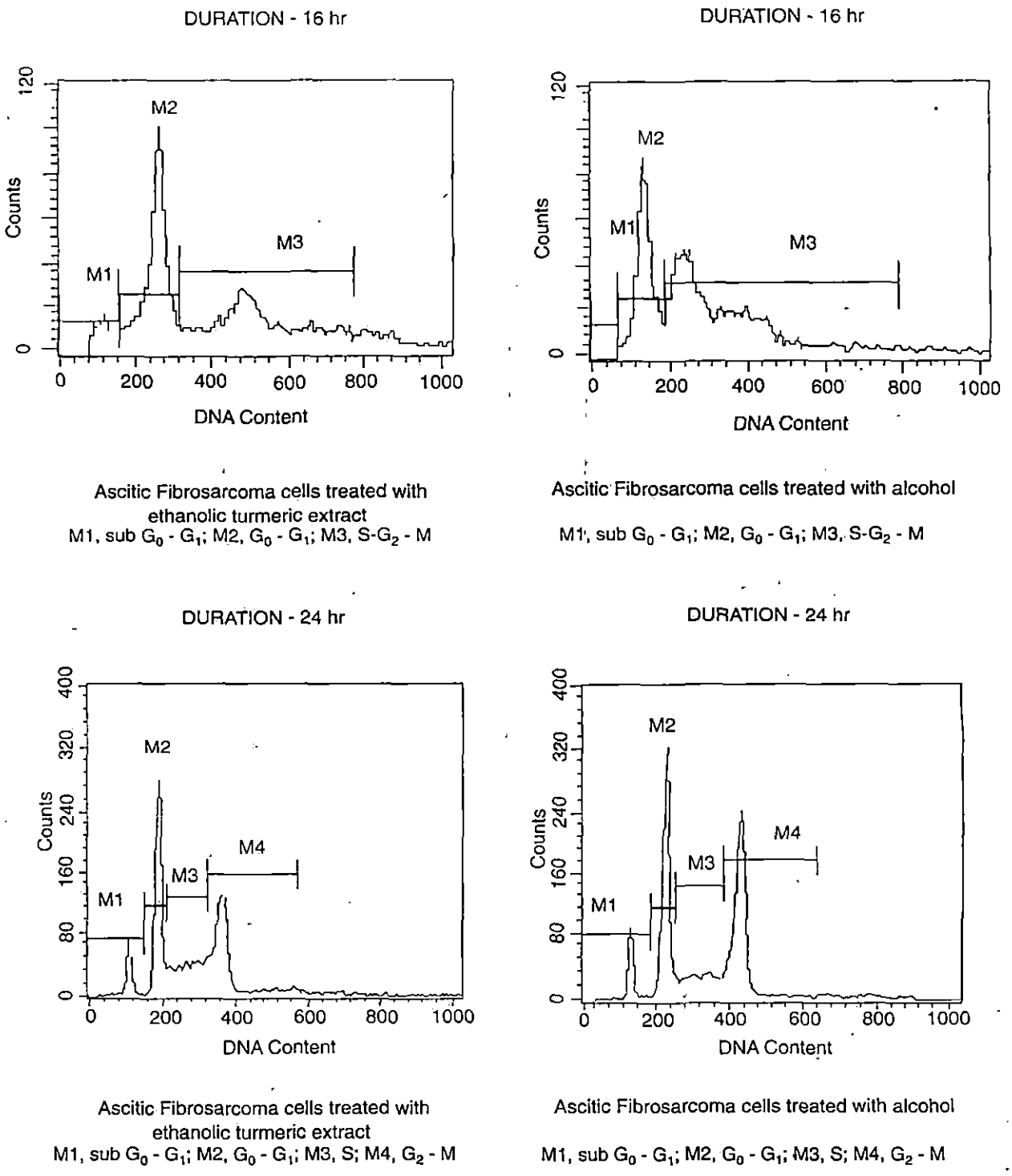


Figure 5. DNA histograms by FACS for cell cycle analysis of ascitic fibrosarcoma cells.

DNA synthesis

The inference from FACS analysis was further strengthened by the DNA synthesis data. At 24 h, ^{3}H-TdR incorporation was highest in lymphocytes with the 25 µl dose of turmeric treatment, and significantly higher than the control. The 50 µl dose of turmeric also seemed to be stimulatory, but the level of stimulation was lower than the 25 µl dose (Fig. 6). The data of ^{3}H-TdR incorporation was obtained in the course of another study using higher doses of turmeric and mentioned here for proper analysis of other observations.

Table 1. Percentage of cells at different cell cycle stages by FACS for 16 hours.

	Lymphocytes (%)		Tumor cells (%)	
Cell cycle stages	Turmeric	Control	Turmeric	Control
Sub G_0-G_1 (M1 peak)	1.89	2.72	6.39	0.00
G_0-G_1 (M2 peak)	84.10	88.92	46.41	34.27
S-G_2-M (M3 peak)	12.17	8.23	41.57	63.92

Table 2. Percentage of cells at different cell cycle stages by FACS for 24 hours.

	Lymphocytes (%)		Tumor cells (%)	
Cell cycle stages	Turmeric	Control	Turmeric	Control
Sub G_0-G_1 (M1 peak)	0.96	2.36	5.59	5.32
G_0-G_1 (M2 peak)	67.83	73.88	33.6	31.97
S (M3 peak)	2.39	1.25	22.2	15.92
G_2-M (M4 peak)	12.63	3.34	33.21	41.60

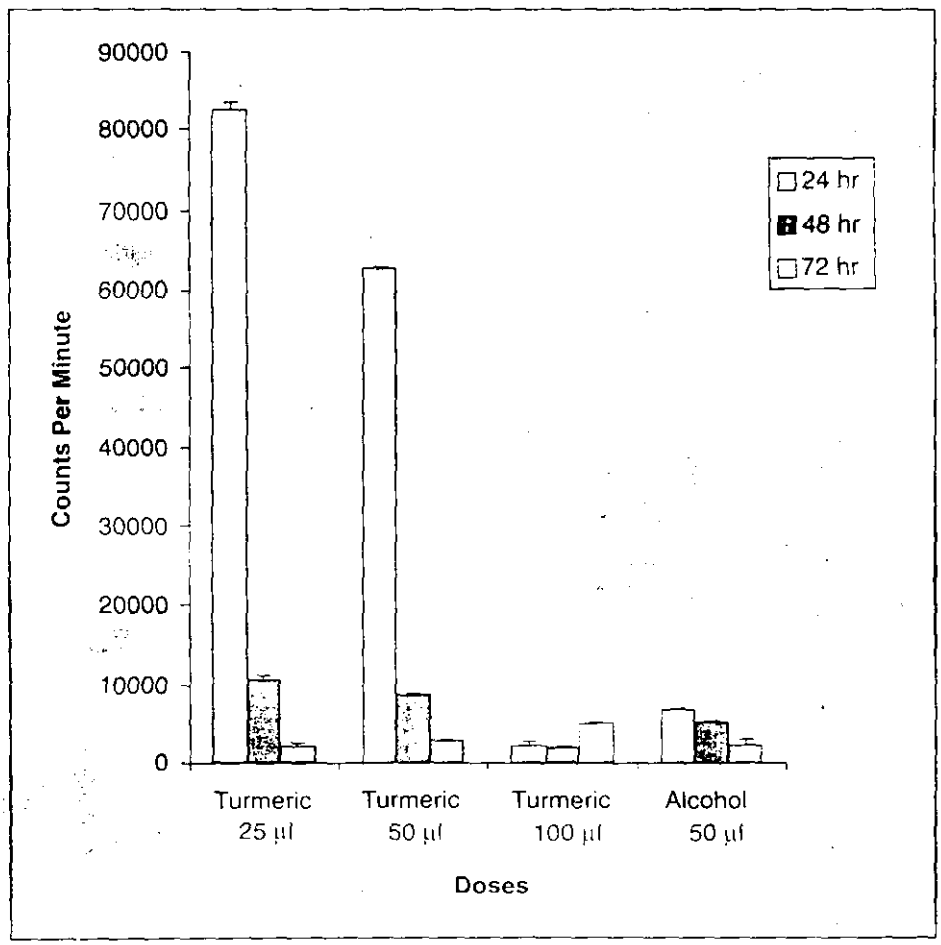


Figure 6. The level of incorporation of ^{3}H-thymidine by lymphocytes treated for different hours with different doses of ethanolic extract of turmeric, indicating the highest level of DNA synthesis is achieved with the dose of 25 µl. The control values with ethanol are shown in the extreme right.

Conclusions

The various modes of tumor-suppressive activity of turmeric have been analyzed by different authors. Curcumin, the active component of turmeric, shows antiproliferative effects with tumor cells by inhibiting protein tyrosine kinase and protein kinase C activity, as well as c-myc and bcl-2 mRNA expression (Chen & Huang, 1998). Khar et al. (1999) demonstrated that curcumin inhibited AK5 tumor growth and induced apoptosis through the activation of caspase-3 and the generation of reactive oxygen intermediates. It has also been found to inhibit the expression of several proto-oncogenes: c-Fos, c-Jun, and c-Myc in the mouse (Kakar & Roy, 1999) and c-Jun and c-Fos in JB6 cells and mouse epidermis (Lu et al., 1994).

Thus, although the inhibitory mechanisms of turmeric on tumor cells have been studied to some extent, there is not much work on the stimulatory role of curcumin for lymphocytes, as has been observed in the current investigation. This opposite effect of turmeric on ascitic fibrosarcoma cells murine lymphocytes might be additive for the effective destruction of the tumor. Thus, turmeric can be suggested for the treatment of malignancy.

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We are highly indebted to Dr. Santu Bandhopadhay, Immunology Laboratory, Indian Institute of Chemical Biology, Calcutta, for his help in taking the readings with FACS. This paper incorporates certain readings from the M.Sc. thesis carried out by different students under the supervision of the first author in our laboratory. We acknowledge Mr. A. Pal Chaudhury, Mr. A. Das, and Mr. M. Das in this connection.

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Preliminary report

Alcoholic turmeric extract simultaneously activating murine lymphocytes and inducing apoptosis of Ehlrich ascitic carcinoma cells

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Abstract

In the present investigation ethanolic turmeric extract has been found to play diabolically opposite role on murine lymphocytes and on Ehlrich ascitic carcinoma cells. Turmeric stimulates the lymphocytes into the effector pathway as studied through in vitro viability, blastogenesis and ^{3}H-TdR incorporation and also seems to be healthy under scanning electron microscopy (SEM). SEM revealed the formation of cytoplasmic blebs and plasma membrane disintegration of tumor cells with ethanolic turmeric extract treatment, suggesting turmeric to be initiating apoptosis of tumor cells. Thus, in the present work viability of the cells, blastogenesis, DNA synthesis and SEM study establish the fact that turmeric is a conducive agent for lymphocytes and inhibitory as well as apoptotic for tumor cells.

Keywords: Curcumin; Lymphocytes; Activation; Tumor cells; Apoptosis

1. Introduction

The rhizome of *Curcuma longa* Linn. (turmeric) is being widely used in different food preparations and external application in India for its medicinal value since ancient times [1]. Curcumin, 1,7-6-bis [4-hydroxy 3-methoxy phenyl]-1,6-heptadiene-3,5-dione, the major pigment and phenol compound in turmeric has been shown to possess both anti-inflammatory [2,3] and antioxidant properties [4–6] and has also been found to be inhibitory against chemical induced carcinoma in several experimental models [7–9].

In the present study we have found ethanolic turmeric extract (ETE), playing simultaneously opposite role on murine lymphocytes and Ehlrich ascitic carcinoma cells as judged through in vitro viability assay, blastogenesis, DNA synthesis and scanning electron microscopy (SEM). SEM was employed to study the changes on the cell surface topography of both murine lymphocytes and Ehlrich ascitic carcinoma cells treated with ETE.

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2. Materials and methods

2.1. Animals

Inbred adult Swiss mice of both sexes, 8–12 weeks of age, were used for all experiments.

2.2. Chemicals

Ficoll (type 400), RPMI 1640 medium, HEPES buffer, nystatin, phosphate buffer saline (PBS), toluene, POPOP and PPO for scintillation fluids were purchased from SIGMA, USA. ^{3}H-thymidine, obtained from Bhaba Atomic Research Centre (BARC), Mumbai, India and Nylon wool from Robins' Scientific Corporation, USA.

2.3. Tumor induction

Ehlrich ascitic carcinoma cell line was obtained from Chittaranjan National Cancer Research Institute, Calcutta and maintained in our laboratory by serial passages. To continue with the cell line, adult mice were injected intraperitoneally with 10^6 ascitic fibrosarcoma cells per mouse in 0.1 ml PBS for induction as ascitic tumor. After 10–15 days, a full-fledged tumor would develop.

2.4. Ethanol turmeric extract (ETE) preparation

For the preparation of turmeric extract, fresh rhizomes of turmeric (*Curcuma longa* Linn.) were obtained from local market, and after cleaning; 10 g of sample was crushed to a paste with mortar and pestle. To this paste 10 ml of absolute alcohol was added and kept in a refrigerator at 4 °C. After 12 h the mixture was taken out and filtered through Whatman filter paper 1; the filtrate was refiltered through Millipore filter paper, and the final solution obtained was stored at 4 °C for further use. One milliliter of ETE contain 0.435 mg dry weight of turmeric. For each experiment, an equivalent amount of ethanol for a particular dose of turmeric was used for control.

2.5. Cell suspension

Spleen and lymph nodes were collected aseptically and cells were dissociated in phosphate buffered saline (PBS, pH 7.2) separately with the help of stainless steel wire mesh and by repeated passage through a 27-gauge needle. Cell suspensions were separately layered on Ficoll Hypaque gradient (Sigma USA) and spun at 3000 rpm for 30 min. The band of lymphocytes at the junction of Ficoll Hypaque and PBS was taken out. Lymphocytes were finally washed twice with PBS.

2.6. Separation of B and T cells

B and T cells were separated by nylon wool fiber column technique from the lymphocyte suspension obtained from spleen or lymph nodes, as outlined by Julius et al. [10]. Briefly, a 0.1 g teased and sterilized nylon wool (Robins' Scientific Corporation, USA), soaked in RPMI was gently packed in 1 ml syringes. The columns were loaded with cell suspensions and incubated at 37 °C for 45 min. Non adherent T cells were eluted out with an excess amount of warm RPMI and re-suspended in fresh medium. The column was then filled up with chilled RPMI and further incubated in ice for 10 min. Nylon wool adherent B cells were eluted out with an excess amount of cold RPMI by vigorous agitation of the wool and then re-suspended in fresh medium. T and B cells were counted with the help of haemocytometer.

2.7. Culture medium

For in vitro culture, lymphocyte suspensions were aliquot separately in round bottom glass culture tube (vol. capacity—5 ml) at a concentration of 1×10^6 cells in 2 ml of RPMI, supplemented with glutamine, HEPES buffer, 200 mg $NaHCO_3$/100 ml, 100 U of penicillin/ml, 100 mg/ml streptomycin, 50 mg/ml nystatin and 10% heat inactivated goat serum. Finally cells were cultured at 37 °C in humidified atmosphere containing 5% CO_2 in air.

2.8. Cell viability assay

Our earlier findings [9] showed good results with a 25 μl dose of ETE and hence this dose of ETE was used for all the experiments. Viability of splenocytes in each culture tube was counted at 8, 16, 24, 48 and 72 h with a 25 μl dose of ETE. As the turmeric extract was made in ethyl alcohol, the equivalent

amount of ethanol for a particular dose of turmeric was used for control in all the experiments. Percentages of viable cells were counted in a haemocytometer in the presence of trypan blue, considering the number of viable cells at the beginning as a hundred.

2.9. Measure of blastogenesis

Blastogenic activity of turmeric was compared with that of ConA, a polyclonal activator. ConA at a concentration of 5 μg ConA/ml was used. The percentage of blast cells was counted by a haemocytometer in the presence of trypan blue. Cells with a diameter over 6 μm and ranging more than 10 μm were considered as blast. All the experiments were repeated more than thrice and triplicate sets were kept for individual experiments.

2.10. Measure of DNA synthesis

Proliferation of lymphocytes was determined by measuring the rate of ^{3}H-thymidine (^{3}H-TdR) incorporation in vitro. Cells were suspended at a concentration of 2×10^{6} cells/ml in culture medium, from which 200 μl of cell suspension was aliquot in each well of a 96-well micro-culture plate. In each of the experimental wells 25 μl ETE and in control, an equivalent amount of alcohol was added. After 24, 48 and 72 h of incubation at 37 °C in humidified atmosphere containing 5% CO_2 in air, the cells were labeled for 8 h at the same atmosphere in the presence of 1 μCi of ^{3}H-thymidine (specific activity—15.8 Ci/mM) per well. At the end of labeling cultures were terminated by washing with cold PBS and precipitated with cold 10% trichloroacetic acid (TCA). The TCA precipitate was then filtered onto glass fiber filters; washed with methanol and dried with a PHD cell harvester (Cambridge, MA). The discs from filter paper containing individual samples were placed in standard scintillation vials (Beckman, USA). At the time of radioactivity count, 5 ml of scintillation fluid (6 g PPO, 0.5 g POPOP/1 l of toluene) was added into each vial. Radioactivity was counted in β-scintillation counter (LS 1800 BECKMAN, USA). All assays were done in triplicate, ^{3}H-TdR incorporation was expressed as counts per minute.

2.11. Scanning electron microscopy (SEM)

Murine splenocytes treated earlier with turmeric extract in vitro were fixed in Karnovsky fixative for 3–4 h at 4 °C. After fixation, cells were washed in PBS twice and then dehydrated. After dehydration cells were dried by critical point drying method and finally gold plating was done. Cells were examined and photographed under a scanning electron microscope (EDAX-9900) at AIIMS, New Delhi.

3. Results

3.1. In vitro viability of murine lymphocytes

At different hours of assay, percentage of the viable lymphocytes cultured with ETE was much higher than the alcohol treated lymphocytes (Fig. 1). At the end of 74 h, 42.89% of lymphocytes were alive with turmeric treatment, whereas in the case of alcohol treated control, the viability was zero. Hence, lymphocytes survived better with turmeric than control.

3.2. In vitro blastogenic response of lymphoid cell types in the presence of ETE

Blastogenic response has been observed in B and T cells obtained from the spleen and lymph node.

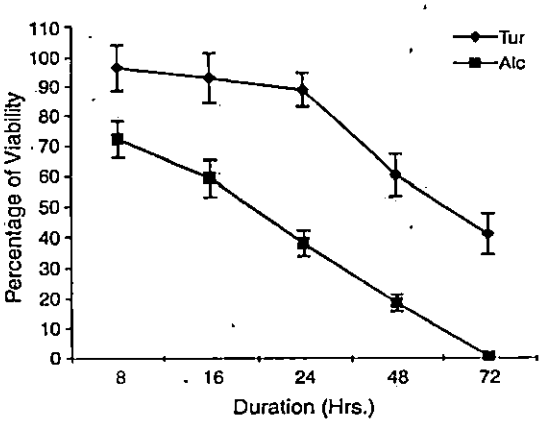

Fig. 1. Percentage of viable lymphocytes at 8, 16, 24, 48 and 72 h after treatment with ethanolic extract treatment (ETE).

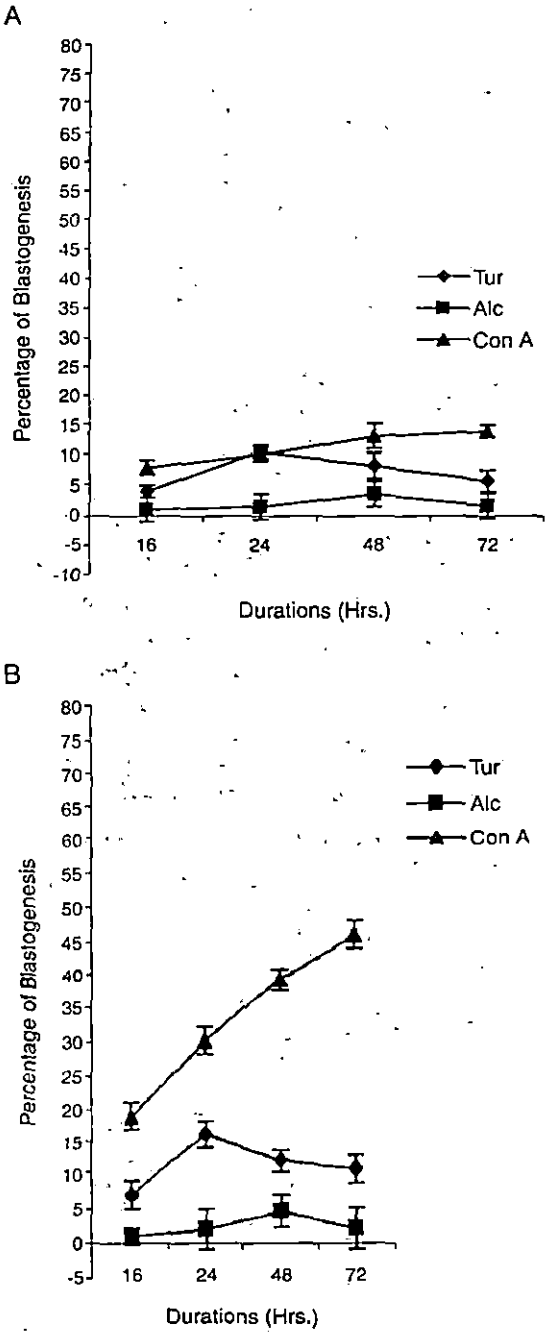

Fig. 2. Histogram showing blastogenic response of lymphocytes from spleen treated with ETE, alcohol and ConA; A) B lymphocytes, B) T lymphocytes.

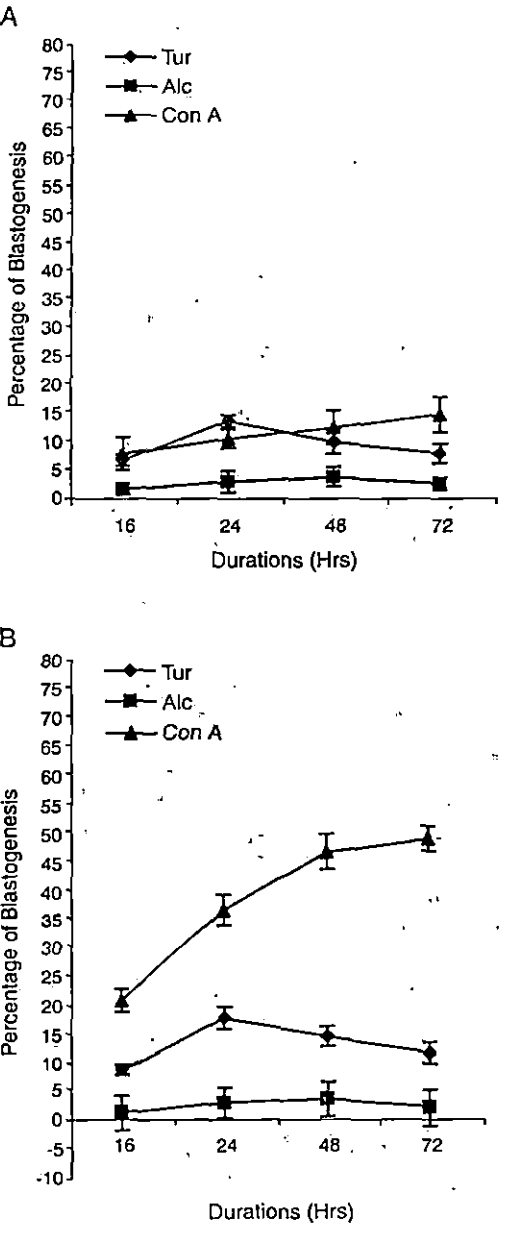

Fig. 3. Histogram showing blastogenic response of lymphocytes from lymph node treated with ETE, alcohol and ConA; A) B lymphocytes, B) T lymphocytes.

Normal lymphocytes measure about 5 μm in diameter. Blast transformation with turmeric treatment results in increment of cell size from 6 to 10 μm or more; as observed under phase contrast microscope.

The treatment of ETE caused an increase in number of blasts over the control (alcohol). Blastogenic response of T cells (Figs. 2B and 3B) was better than B cells (Figs. 2A and 3A). With turmeric treatment, the peak of blastogenesis was effectively reached by 24 h. Percentage of blasts was more in the lymph node cells (Fig. 3A and B) than splenocytes (Fig. 2A and B). When blastogenesis of T cells with ConA, an established polyclonal stimulator for murine T cells, was compared, ConA was found to be a better stimulator than ETE (Figs. 2B and 3B).

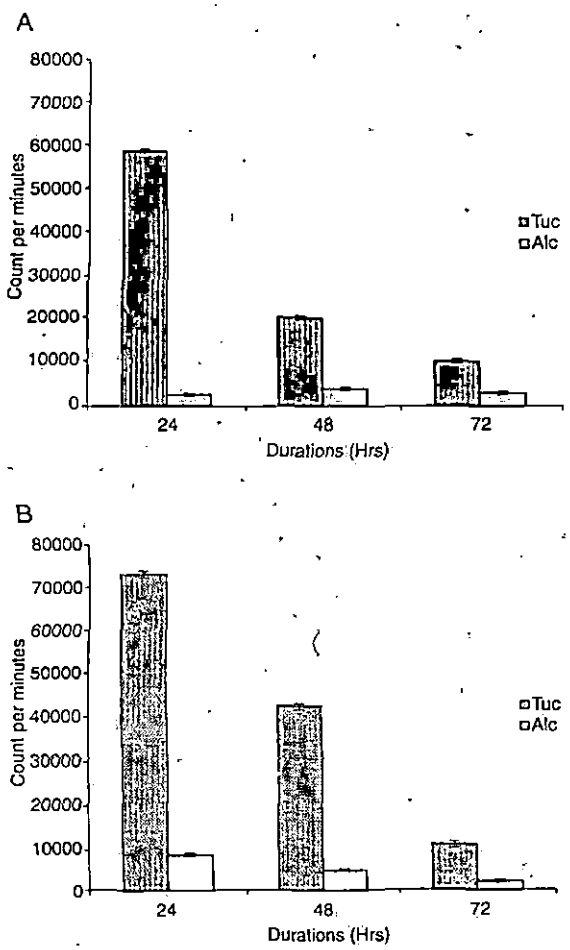

Fig. 4. Pattern of incorporation of ^{3}H-thymidine by lymphocytes treated for different hours with ETE; A) lymphocytes from spleen, B) lymphocytes from lymph node.

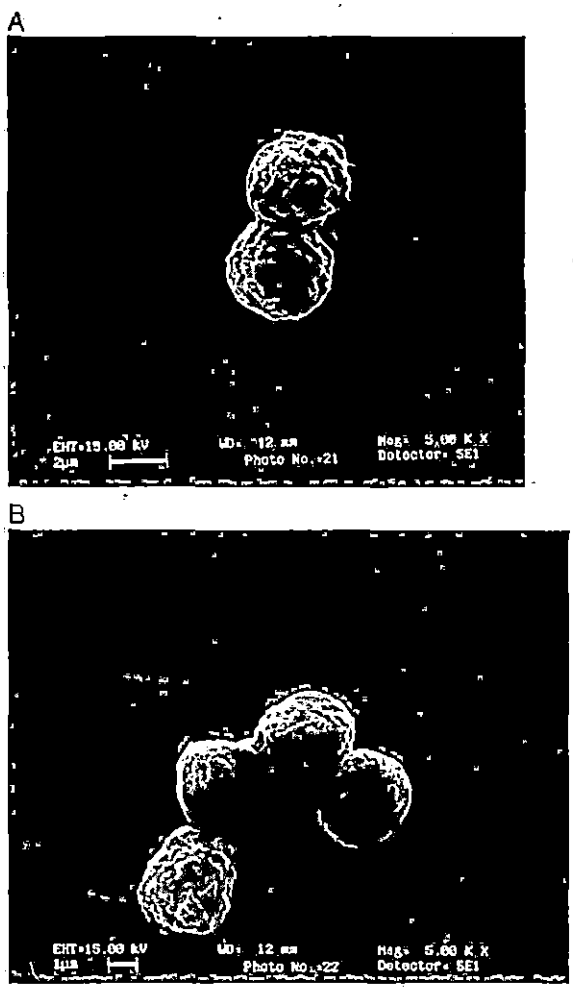

Fig. 5. Scanning electron micrographs of murine lymphocytes from spleen. A) After 16 h of turmeric treatment, lymphocytes showing no significant changes on its surface topography and seem to be healthy. B) Control, lymphocytes at 16 h.

3.3. ^{3}H-TdR incorporation in lymphocytes with ETE treatment

Blast transformation usually is a way indicative for DNA synthesis in stimulator cells. At 24 h, ^{3}H-thymidine incorporation was highest in ETE treated lymphocytes (Fig. 4A and B). Thus, the kinetics of DNA synthesis is in agreement with the blastogenesis (Figs. 2A and B and 3A and B). Lymphocytes from lymph nodes showed better response in both the counts.

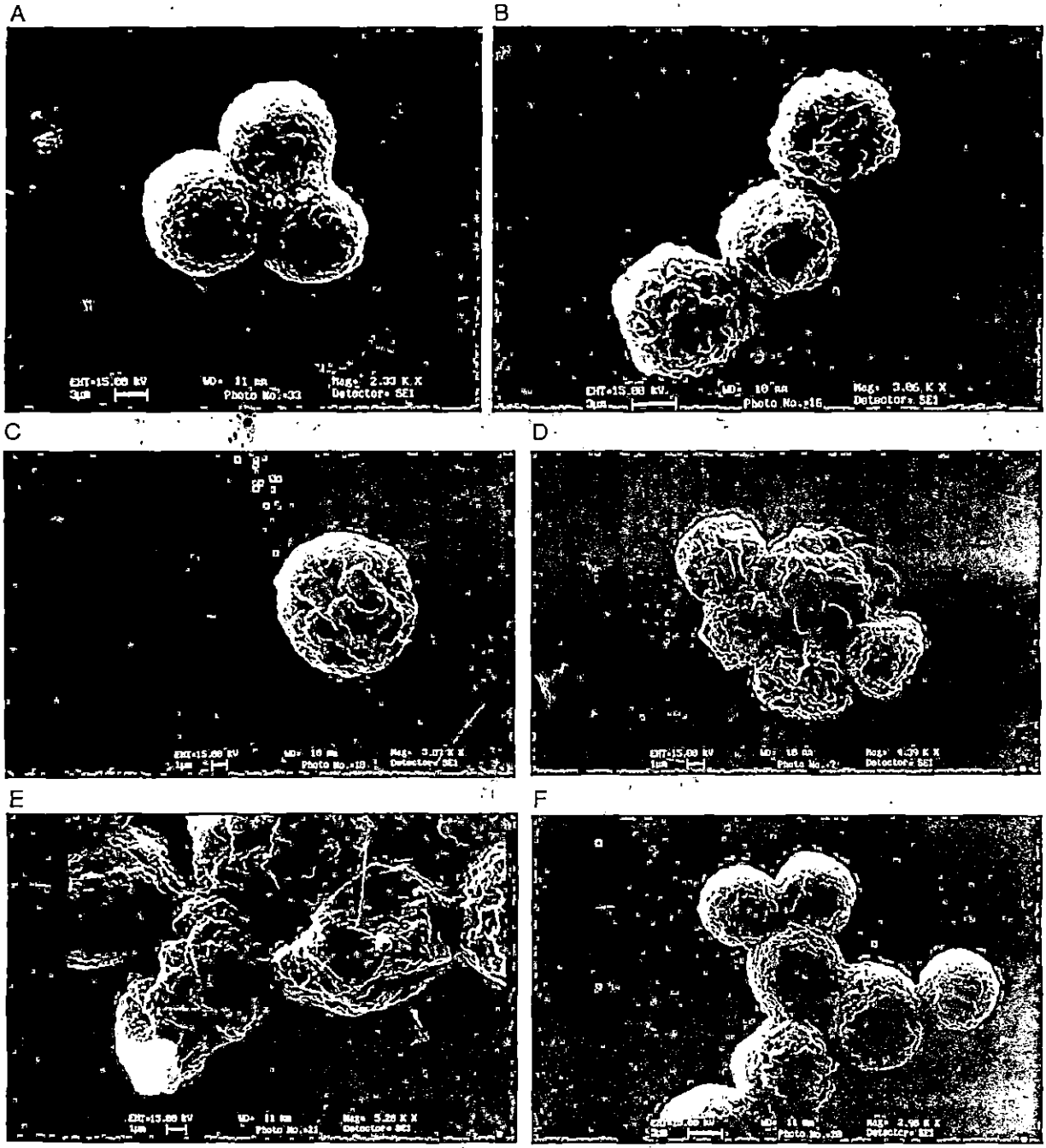

Fig. 6. Scanning electron micrographs of Ehlrich ascitic carcinoma cells. A) Tumor cells prior to turmeric treatment, showing ruffles distributed evenly all over the surface; B) after 10 min of turmeric treatment, cells showing formation of cytoplasmic blebs; C) after 4 h of turmeric treatment, cytoplasmic blebs were broader and conspicuous; D) after 8 h of treatment, cytoplasmic blebs, became numerous, cell volume tends to decrease; E) after 16 h of turmeric treatment, loss of cellular organization with disintegration of plasma membrane; F) control, after 16 h of culture tumor cells remain unchanged.

3.4. Scanning electron microscopic study

Murine lymphocytes with in vitro turmeric treatment did not show any significant changes on its surface topography from that of control after 16 h (Fig. 5A). But an increment in the size of ETE treated lymphocytes over the control was observed; this is possibly indicative for blastogenesis of treated cells. On the other hand, turmeric treatment caused progressive changes in cell surface of Ehlrich ascitic carcinoma cells (Fig. 6B–E). Ruffles on cell surface were distributed evenly over its surface prior to treatment (Fig. 6A). Within 10 min of in vitro turmeric treatment tumor cells starts showing formation of cytoplasm blebs over its surface (Fig. 6B). These changes accentuated within 4 h of treatment; particularly the cytoplasm blebs were broader and conspicuous (Fig. 6C). After 8 h of turmeric treatment, the blebs became numerous and the cell volume tends to decrease, this was indicative of initiation of disintegration of cell structure (Fig. 6D). Loss of cellular organization and disintegration of plasma membrane was more pronounced at 16 h of treatment (Fig. 6E). These changes clearly showed the onset of apoptosis in treated tumor cells, when tumor cells treated with alcohol (control) remain unchanged (Fig. 6F).

4. Discussion

The ETE has been found to stimulate the murine lymphocytes in general, causing blastoid differentiation of lymphocytes in vitro, more particularly T lymphocytes (Figs. 2B and 3B). The peak of blastogenic response was attained by 24 h, much earlier than with a polyclonal stimulator like ConA. The percentage of blasts with ETE treatment was lower than the percentage of blasts obtained with ConA. ^{3}H-TdR incorporation by the lymphocytes treated with ETE also show the peak at 24 h (Fig. 4A and B).

Comparison of the blastogenesis with ETE and ConA indicates that ETE provides blastogenic stimulus for T cells but not to the extent of a polyclonal stimulator like ConA.

Viability, blastogenic response, DNA synthesis and SEM results (Fig. 5A) in our study show the promotional effect of turmeric on murine lymphocytes. Whereas, ETE treatment of Ehlrich ascitic carcinoma cells caused pronounced cytoplasm blebing and plasma membrane disintegration leading to apoptosis. Several authors [11–14] observed apoptotic effect of turmeric on various transformed cells. Kim et al. [11] suggests curcumin-induced apoptosis was mainly due to prominent downregulation of Bcl-2 and upregulation of Bax expressions. Khar et al. [12], demonstrated that curcumin induced apoptosis through the activation of caspase-3 and by the generation of reactive oxygen intermediates. And according to some it can even inhibit the expression of several proto-oncogenes, such as c-*jun*, c-*fos* and c-*myc* in mouse skin [13] and transcription factors such as NF-κB[14].

The present investigation reports the novelty of turmeric in setting apoptosis to tumor cells and on the other hand activating lymphocytes. Our earlier studies of lymphocytes from mice treated in vivo with ETE clearly showed DNA synthesis and cells entering in mitotic cycle, whereas the treatment arrest the tumor cells at S-phase [15]. FACS performed the analysis of cell cycle.

Most of the natural antioxidants possess either a phenolic group or a β-diketone group. Curcumin and its analogues are unique, having both phenolic and β-diketone functional groups on the same molecule [16]. Like Vitamin E, curcumin is a lipid soluble antioxidant and believed to be localized within the membranous sub cellular fraction of cells [17]. The presence of curcumin in the cell membrane might have some implication for initiating the process of activation for lymphocytes and apoptosis in malignant cells.

Molecular mechanism of action of curcumin on cell physiology for two-way efficacy on two types of cells is in the agenda for our current investigation.

Acknowledgement

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