Characterization of Anti-inflammatory and Immunomodulatory Properties of the Extract from the Leaves of *Eupatorium adenophorum.*

Tamal Mazumder

Thesis submitted for Degree of Doctor of Philosophy (Science)
of

University of North Bengal
2010

DEDICATED TO MY LATE PATERNAL AND MATERNAL GRANDPARENTS

UNIVERSITY OF NORTH BENGAL

DEPARTMENT OF ZOOLOGY

DST-FIST & UGC-SAP Sponsored

P.O. North Bengal University,
Raja Rammohunpur, Dt. Darjeeling,
West Bengal, India, PIN - 734 013

June 2, 2010

This is to certify that Mr. Tamal Mazumder, M.Sc. worked in my laboratory since September, 2003 under my supervision on the topic "Characterization of anti-inflammatory and immunomodulatory properties of the extract from the leaves of *Eupatorium adenophorum*" for fulfillment of the requirements of the degree of Doctor of Philosophy (Science) of the University of North Bengal. Mr. Mazumder was supported with the Junior Research Fellowship from Lady Tata Memorial Trust and then Project Fellow in UGC funded major research project.

Mr. Mazumder performed all the experiments thoroughly and meticulously. He is conversant with the technique and literature cited in the dissertation. It seems that the thesis is fit for submission for Ph.D. and it is worthy for award of the degree.

Prof. Ashim K Chakravarty, FNASc.
(Retd.)

Immunology and Cell Biology Lab.

Department of Zoology

University of North Bengal

ACKNOWLEDGEMENTS

I take this opportunity to express my heartfelt indebtedness and gratefulness to my supervisor, Prof. Ashim K. Chakravarty. I would like to thank him for giving me the opportunity to work under his valuable guidance. His vast knowledge and novel guidance has helped me immensely to pursue the work meticulously. I would like to thank him again for spending his extensive valuable time in giving suggestions while writing the thesis and for bearing with me and my shortcomings. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist want to be. I believe that I'm blessed with his guidance and will always be grateful to him for whatever he has done for me.

I am thankful to all the faculty members of the Dept. of Zoology, North Bengal University starting from Prof. B.C. Pal, Prof. D.C. Deb, Prof. J. Pal, Prof. A Mukhopadhyay, Prof. T.K. Chaudhuri, Prof. S. Barat, Dr. Min Bahadur and Dr. S. Bhattacharya for their constant encouragement.

I would like to thank all the non-teaching staffs of Dept. of Zoology and also Centre for Life Sciences and special thanks goes to Mr. S.B. Sunar, Mr. S Kundu and Mr. D Singha for rendering their helping hands to me.

I am very much thankful to Dr. Bholanath Paul, IITR, Lucknow for allowing me to carry out quantization of TNF-α in his laboratory and providing valuable chemicals for ELISA reading. I am also thankful to Dr. Nripendra Nath Mandal, Division of Molecular Medicine, Bose Institute, Kolkata and his scholars for their enormous help to carry out FACS reading in the Centre for Instrument Facilities, Bose Institute.

I am indebted to Prof. B. Basu, Dept. of Chemistry, NBU for his valuable guidance and interpreting spectroscopic data in carrying out chemical characterization of ethanolic leaf extract of Eupatorium adenophorum. Thanks also goes to Dr. A.K. Nanda, Dept of Chemistry, NBU for his on and off help in this regard. I am also thankful to Dr. Sajal Das, Dept of Chemistry, NBU and all the scholars of Organic Synthesis Laboratory of the department.

I gratefully acknowledge Dr. Shankar N Chatterjee, former Jt. Director, Seribiotechnology, Govt. of India, Bangalore for his advice, supervision and crucial contribution in carrying out the experiments for gene expression analysis.

I like to express my sincere gratitude to Dr. A. Sen, Dept. of Botany, NBU, for allowing me to access instrumental facilities, especially PCR, in his laboratory. I also like to thank Dr. Ranadhir Chakraborty, Dept. of Biotechnology, NBU to let me avail instrumental facilities in his laboratory whenever required. I must also thank Prof. A.P. Das for carrying out scientific identification of the plant material and his encouraging words.

I am sincerely grateful to Dr. (Mrs.) Alo Chakravarty for treating me as a member of the family and her motherly attitude to me all the time.

I am very much thankful to Dr. Hadida Yasmin for her immense help, valuable suggestions and encouragement through out the period.

I also like to thank all the research scholars of the Department of Zoology including Mr. Bikash Mitra, Dr. Arindam Das, Dr. Sumanta Bagchi and Dr. Prithwiraj Jha. I would like to specially thank Mr. Bikash Mitra for his untiring help that led to

submission of the thesis in due time. Special thanks also goes to Mr. Gautam Debnath and Mr. Rudra Prasad Roy for their brotherly support.

Where would I be without my family? My parents deserve special mention for their inseparable support and prayers. I am very much indebted to them for raising me with their caring and gentle love. I specially convey my acknowledgement to my sister, Tiyasha. This thesis also owes much to the love and encouragement I received from my other family members.

Words fail me to express my appreciation to my wife Dipanjana whose dedication, love and persistent confidence in me, has taken the load off my shoulder. I owe her for being unselfishly let her intelligence, passions, and ambitions collide with mine. Therefore, I would also thank my parents in law for letting me take her hand in marriage, and accepting me as a member of the family, warmly.

I am also sincerely thankful to the Lady Tata Memorial Trust and the University Grants Commission for financial assistance to complete this work.

Finally, I would like to thank everybody who was important to the successful realization of this thesis, as well as expressing my apology that I could not mention personally one by one.

Tamal Mazumder 2/6/2010

TAMAL MAZUMDER

Table of Contents

FIGURE INDEX

40 Immunopharmacological properties of EEA compared with certain other plant extracts. Indices represent the level of efficacy of the extracts as ratio over control; the indices presented at the top of the column for easy representation. Any index ratio of 1.2 or more has been considered to have positive effect. Data for the natural products from the plant species other than *E. adenophorum* compiled on the basis of data in published literature. 76

TABLE INDEX

INTRODUCTION

Inflammation is the body's way of dealing with infections and tissue damage, but there is a fine balance between the beneficial effects of inflammation cascades and their potential for long-term tissue destruction (Simmons, 2006; Saukkonen *et. al.*, 1990; Parenteau & Hardin-Young, 2007). If they are not controlled or resolved, inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis (Fabbri *et al.*, 1991; Stevens *et. al.*, 2005; Klareskog *et. al.*, 2006; Simka, 2009; Zisper, 1988; Drews & Ryser, 1997). Inflammation occurs as a defensive response which induces physiological adaptations to limit tissue damage and remove pathogenic infections. Both chronic and acute inflammation causes such diseases that may lead to morbidity and mortality in humans in many cases (Yilmaz, 2007; Oberg *et al.*, 2004).

Inflammation is mostly treated with synthetic non steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, meloxicam, naproxen etc. (Baron & Sandler, 2000; Simmons, 2006; Burton & Waddel,1998; Van Tuolder, Koes & Bouter, 1996); till date a very few anti-inflammatory drugs from herbal origin have been well characterized and a number of plants from ethno-medicinal databases are under laboratory investigation across the world. Herbal medicine to treat various ailments have been compiled in Ayurveda for last four thousand years, and still remains dominant in use in India compared to modern medicine. Ayurvedic preparations are prescribed out of 17,000 species of higher plants of India, 7,500 are known for medicinal uses (Shiva, 1996; Kala,

Dhyani and Sajwan 2006). Thus, Ayurveda is known to use highest number of plants for medicinal purpose in any country of the world.

The Eastern Himalayas, one of the two biodiversity hotspots in India, offers a vast resource of medicinal plants among which many are used by the local inhabitants but not yet validated scientifically. The local people of Terai belt of the Eastern Himalayas use leaves of *Eupatorium adenophorum* of Asteraceae (Compositae) family for treating mouth sores and sores of skin as well. Use of *E. adenophorum* for treating sores suggests its efficacy as an anti-inflammatory and immunomodulatory agent, which has not been scientifically explored so far. The plant grows profoundly in the range of 800-2050 meters of altitude in the Eastern Himalayas. There are two reports in the literature about analgesic property of the methanolic extract of leaves of *E. adenophorum* (Mandal *et al.*, 2005 & 2009). The present investigation analyses in detail the efficacy of the ethanolic extract of the leaves of the plant as an anti-inflammatory and immunomodulatory agent.

An extract from herbal source needs a screening for its toxicity before studying its effects in *in vivo* or *in vitro* situation. The toxicity, if any, of alcoholic extract of leaves of *E. adenophorum* (EEA) was judged in reference to hematological parameters, such as percentage of haemoglobin, RBC count and WBC count after injecting the leaf extract in mice.

The effect of EEA particularly on B and T lymphocytes, responsible for different types of immune functions was also studied. The blastoid transformation of the lymphocytes was considered as immunostimulation. The degree of stimulation of lymphocytes from spleen was then investigated by measuring DNA synthesis and cell cycle analysis with FACS.

Anti-inflammatory property of different plant extracts have been judged in many ways (Srimal & Dhawan, 1973; Srivastava & Srimal, 1985; Huang *et. al*, 1991; Goel, Boland, Chauhan, 2001). Here, anti-inflammatory property of EEA was tested by its ability to inhibit DTH induration induced in mouse paw by resensitization with 2,4 dinitro flurobenzene (DNFB).

A multifactorial network of chemical signals initiates and maintains a host response to heal the afflicted tissue undergoing inflammation (Marx, 2004). This initiates 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 was undertaken to understand the effect of EEA on the cells migrating to DTH reaction site.

DTH reaction is initiated by pre-sensitized $CD4^+$ T_{DTH} cells (Black 1998; Matsushima & Stohlman, 2005) and then other inflammatory cells and cytokines are involved at the site of reaction. Number of $CD4^+$ T cells in course of DTH reaction and treatment with EEA has been enumerated to understand the effect of EEA on these cells. TNF-α is the most important cytokine that plays major role in all the inflammation reactions. Serum TNF-α of DTH mice has also been investigated in the present study in course of inflammation.

Besides TNF-α many other cytokines play key role in orchestrating immune responses in inflammation (Bauel & Karim, 2001, Dinarello, 2000; Strieter, Kunkel & Bone, 1993;

Lukacs *et. al.* 1995; Tracey & Cerami, 1994; Schweizer *et. al.*, 1998; Gabay, 2006; Osmoigui, 2007 and Jutel *et. al.*, 2003). Here expression of certain cytokine genes such as TNF-α, IL-1β, IL-6, IL-10 and TGF-β in splenic T cells of DTH mice during inflammation has been studied at transcription level with and without intravenous application of the plant extract. Expression of inhibitory kappa kinase (*IKK*) gene has also been judged. The enzyme degrades IκB subunit to release active NF-κB (Alkalay *et. al.*, 1995; Baeuerle, 1998; Pande & Ramos, 2005) for activating genes involved in inflammatory responses as shown by Tak and Firestein (2001) and Yamamoto and Gaynor (2001).

The expression of COX 1 and COX 2 genes encoding two isozymes of cycloxygenase has been taken into account here. Cycloxygenase is known to play a significant role in induction of inflammation by producing inflammatory mediators like prostaglandins and leukotrienes from arachidonic acid (Turini & DuBois, 2002; Smith *et. al.*, 1998; Smith, Garavito & DeWitt, 1996; Mitchell, Larkin &Williams, 1995; Vane, Bakhlel & Botting, 1998; Chen *et. al.*, 1997; Devaux *et. al.*, 2001).

Wound healing of the inflamed tissue is an important index for successful recovery from inflammation reaction. Thus, inflammation reaction is accompanied with events necessary for wound healing. Inflammation causes blood vessels to become leaky releasing plasma and various leukocytes surrounding the wound (Wahl & Wahl, 1992). Cytokines and growth factors released are likely to play an important role in wound healing by inducing vascularization and other tissue growth. Thus, any effect of a plant

extract on inflammation may indicate its possible effect in wound healing. Wound healing property of EEA has been investigated separately by applying it to linear skin incision and to limited area of skin burnt in mice.

Coussens and Werb (2002) indicated probable participation of inflammatory cells in cancer promotion by secreting cytokines and growth factors that stimulate angiogenesis. Clinical studies by Bidwell *et al.* (1999) and Howell *et al.* (2002) showed that polymorphisms in IL-1β, TNF-α and IL-6 genes, encoding proinflammatory cytokine are associated with diverse diseases including cancer. Tu and coworkers (2008) indicated that overexpression of IL-1β could lead to gastric inflammation and cancer in IL-1β transgenic mice. These observations make one hopeful about the anti-cancer potential of an anti-inflammatory agent. In view of that, effect of EEA on solid subcutaneous tumor in mice, induced with Ehrlich's ascitic carcinoma, was tested for its possible anti-tumor activity in the present study.

Ability of EEA to stimulate blastogenesis of lymphocytes *in vitro* had been studied in the beginning. Blastogenesis is indicative of the first step of lymphocytes to be driven for functional differentiation. Therefore, the differentiation of cytotoxic T cells, the most effective cell type to combat malignancy, was studied in ^{51}Cr release assay after injecting EEA in mice. Several authors (Heinenger *et. al,* 1976; Waterfield, Waterfield & Moller, 1976; Waterfield, *et. al,* 1979; Chaudhury & Chakravarty 1983a) demonstrated that polyclonal stimulation with ConA or PHA could generate cytotoxic T cells with anti-tumor potential. It was found that lymphocytes activated with Con A

could restrict the tumor induced angiogenesis and growth of tumor in the anterior eye chamber of mice (Chakravarty and Maitra, 1983 & 1990).

Search for many other novel herbal products with immunostimulating activity is being continued across the globe. Cragg and Newman (2005) showed that several natural compounds, such as phenolic compounds, terpenoids, sulphur compounds, pigments like anthocyanins, xanthenes, and other natural antioxidants provide protection against cancer, as well cardiovascular diseases. Watery extracts of *Phyllanthus embilica* enhances 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 (DLA) 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). These works encouraged further to test efficacy of EEA in activation of T cell cytotoxic response against tumor target cells, using ^{51}Cr-release assay.

Cytotoxic response of T cell is mediated by synthesis and release of perforins and their polymerization on the tumor cells to form pores. Next, the expression of perforin gene along with certain other immunologically active genes such as IL-1β, IL-2, IL-6, IL-10,

TNF-α, TGF-β, iNOS, IKK and PKC-theta in splenic T cells of mice injected with EEA and alcohol (control) have been carried out.

Although the cell mediated response is primarily responsible for anti-tumor activity, the effect of EEA on primary and secondary antibody mediated immune response in terms of differentiation of antibody secreting cells by plaque forming cell (PFC) assay and measure of IgG by ELISA have been studied.

A study of inflammatory reaction and its control almost automatically includes involvement of free radicals. Many reports reveal that reactive oxygen species (ROS) play an important role in developing various pathophysiological conditions including inflammation, and potent anti-inflammatory agents can scavenge the free radicals to quench the biochemical fire (Winrow *et al.*, 1993; Garrido *et al.*, 2001; Weber *et al.*, 2002, Dedon & Tannebaum, 2004; Halliwell, 1`997; Halliwell *et al.*, 1998; Barnes 1990). Generation of free radicals by univalent reduction of molecular oxygen (O_2) occurs in any biochemical process and metabolism (Bandyopadhyay, Das, Banerjee, 1999). It becomes much higher during inflammation due to oxidative burst in the phagocytic leukocytes at the inflammatory site. The free radical generation aggravates the inflammation reaction including stimulation of biosynthesis of inflammatory mediators like prostaglandin and leukotriene (Garrido *et al.*, 2001; Weber *et al.*, 2002). Several workers (Winrow *et al.*, 1993; Nguemfol *et al.*, 2009; Abreu *et al.*, 2006; Joseph *et al.*, 2009) showed that generation of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot}$), hydrogen peroxide ($H_2O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide ($NO^{\cdot}$) also activate NF-κB signaling pathway to release secondary mediators that aggravate inflammation

reaction. Others (Simonian & Coyle, 1996; Chandel, *et.al,* 2000; Vafa, *et. al.*, 2001; Klaunig & Kamendulis, 2004) observed that these free radicals can also affect other signaling pathways such as inactivation of protease-1 inhibitor pathway that ultimately leads to increased protease-1 activity and destruction of inflammatory tissue. Naik (2003) reviewed anti-oxidant properties of different natural anti-oxidants such as vitamin E, vitamin C, *Gingko biloba* extract, spirulina, red wine, spices like garlic, pepper etc. Tiwari (2001) emphasized on inhibition of the generation of free radicals in the body by natural anti-oxidants and resistance to several diseases like atherosclerosis, hypertension, ischaemic diseases, Alzheimer's disease, parkinsonism, cancer and inflammatory conditions. In the present investigation, scavenging activity of EEA for the three deleterious ROS, $O_2^{\cdot}$, $H_2O_2^{\cdot}$ and $OH^{\cdot}$ have been tested. EEA's ability to induce $NO^{\cdot}$, another ROS, was also studied by measuring the level of nitric oxide synthase (iNOS) in murine lymphocytes.

Finally, chemical characterization of active component in the ethanolic leaf extract of *E. adenophorum* h as been carried out. So far, Shi and coworkers (Zhang et al., 2008) reported presence of a few sesquiterpenes in *E. adenophorum*, but the active active component is yet to be identified. EEA was initially fractionated by solvent partition and thin layered chromatography (TLC). A major band was found in the TLC and the fraction was isolated further by column chromatography and subjected for chemical characterization using UV-spectra, IR-spectra, NMR and Mass Spectra. Bioactivity of the fraction isolated, was tested in reference to blastogenesis of lymphocytes and quenching of reactive oxygen species, and compared with that of total extract.

MATERIALS AND METHODS

Animals

Inbred adult Swiss albino 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. Animals of approximately equal age and weight were used for experimental and control groups in an experiment. The experimental protocols used in the study have been approved by the Animal Ethical Committee (Regn. No. 840/ac/04/CPCSEA).

Preparation of ethanolic leaf extract of *Eupatorium adenophorum* (EEA)

Fresh leaves of the plant, *Eupatorium adenophorum* were collected from their natural habitat at about 1400 mt high slope of the Eastern Himalayas, mainly around Kurseong hill. The scientific identification of the plant has been checked by Prof. A. P. Das, Plant Taxonomy Lab., Dept. of Botany, Univ. of North Bengal.

Eupatorium adenophorum

Division – Magliophyta
Class – Magnoliopsida
Subclass– Asteridae
Order – Asterales
Family – Asteraceae
Genus – *Eupatorium*
Species – *E. adenophorum*
(According to Arthur Cronquist, 2002)

Local name – 'Banmara'
Nature – Herb
Flowering and fruiting time – February to June.

The leaves were cleaned thoroughly with water and allowed to air dry. Ten gms of leaves were crushed to a paste with a mortar and pestle. An amount of 10 ml of absolute alcohol (ethanol) was added to the paste and kept in refrigerator overnight for extraction. The alcoholic extract was then filtered first through Whatmann filter paper and the filtrate was refiltered again through cellulose acetate filter paper (0.2 μm porosity, Sartorius) for sterilization and finally stored in airtight sterilized vial at 4^0C for further use.

1 ml of EEA after each batch of extract preparation was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^0 C and the dry weight was in the range of 0.529±0.019 mg.

Separation of immunocompetent cells

Separation of Macrophages (Mϕs)

Spleen and lymph node were collected aseptically from mice and the cells were dissociated mechanically 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 with a Pasteur pipette 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 cells, 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 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. Nylon wool adherent B cells in the column were eluted out with an excess amount of chilled 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 and viability assay

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 µg/ml streptomycin, 50 µg/ml nystatin and 10% heat inactivated sterile goat serum (Chaudhuri & Chakravarty, 1983b). 25 µl of EEA was added to splenic lymphocytes in a glass culture tube (Borosil) of volume 5 ml at a density of $1x10^6$ cells in 2 ml of culture medium. Cell survival at different hours (10 mins, 1, 4, 8, 16, 24, 48 and 72 hr) of culture was judged by trypan blue dye exclusion test. Counting of the cells was made with haemocytometer.

Measure of *in vitro* blastogenesis

The transformation of T and B cells into blasts after *in vitro* activation by EEA was studied. The percentage of blast was enumerated with a haemocytometer in presence of trypan blue. Cells with diameter over 6 µm were considered as blast (Chakravarty & Maitra, 1983).

Measure of DNA synthesis

Blast transformation is usually accompanied by DNA synthesis and cell proliferation. DNA synthesis at different hours of EEA treatment was measured by incorporation of ^{3}H-thymidine (^{3}H-TdR) into DNA. Cells were obtained from mice injected (i.v.) earlier

with 25 µl EEA 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, BRIT, 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, spleen lymphocytes were treated *in vitro* with 25 µl EEA and alcohol (control) separately for 16, 24 and 48 hrs. After incubation cells were suspended in 1 ml PBS. 1 ml of 1% paraformaldehyde was then added to the suspension and the cells were fixed overnight at 4° C. Fixed cells were centrifuged, the supernatant was decanted off, and 0.5µl of 500 µg/ml RNAse A was added, and incubated for 45 mins at 37° C. The cells were then centrifuged and suspended in 0.5 ml of 69 mM ethidium bromide in 38 mM sodium citrate at room temperature for 30 min. Ethidium bromide is a fluorochrome which stains DNA. 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.

Induction of Delayed type hypersensitivity reaction with 2,4-DNFB and application of EEA

Delayed type hypersensitive (DTH) reaction was induced in mouse foot paw by subcutaneous application of 2,4-dinitrofluorobenzene (DNFB) (Chakravarty *et al.*, 2009; Izima & Katz, 1983). Primary sensitization was carried out by applying 0.0001% DNFB subcutaneously in the right foot pad. After 8 days, mice were resensitized with 0.000001% DNFB on the left foot pad. Two different volumes of percentage solutions of DNFB, 25 µl or 50 µl for both sensitization and resensitization, were used in separate experimental set ups. The day of resensitization was considered as '0' day for enumeration of DTH reaction. Size of the left paw before resensitization was considered as normal size for the paw. The degree of inflammatory swelling set in the resensitized left paw was measured by a slide caliper. Each reading is the average of two measurements of the left paw at right angle by slide caliper. The effect of EEA on DTH reaction set in by two different doses of DNFB, was judged after topical or intravenous application of the extract. For topical application 5µl of EEA was applied on the resensitized paw per day from 1st day of resensitization. For intravenous administration, 25 µl of EEA was used 1hr prior to resensitization. Percentage of inhibition of

inflammation by EEA (experimental) in reference to the ethanol treated control has been calculated by using the following formula:

$$\%\text{of Inhibition of DTH} = \frac{\text{Ethanol treated DTH paw size} - \text{Experimental paw size}}{\text{Ethanol treated DTH paw size}} \times 100$$

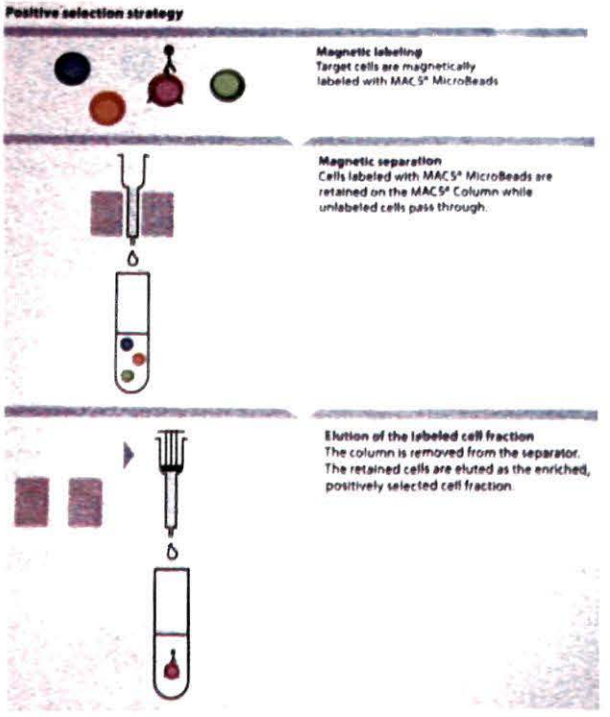
Differential leukocyte count from inflammation site of DTH mice

Differential count of the leukocytes at the inflammation site of control and experimental mice was made, to understand if there is any change in percentage of different WBCs with and without EEA treatment for 24 and 48 hrs. For this a thin and uniform film of oozing fluid from the inflammation site 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.

Isolation of $CD4^+$ T cells through Magnetic Assorted Cell Sorter (MACS)

The splenic lymphocytes were obtained from DTH mice untreated, and DTH mice separately injected intravenously with EEA and ethanol 1 hr prior to resentization. The spleen of mice were removed after 24, 48 and 72 hrs of resensitization. The protocol of Chakravarty and Maitra (1990) was followed to get the spleen cell suspension. Erythrocytes in the spleen cell suspension were lysed by exposure to tris-buffered ammonium chloride (0.83%, pH 7.2). Then, the suspension was incubated in a plastic petri dish at 37^0 C in humidified atmosphere for 30 mins, for depletion of adherent cells. Non-adherent lymphocyte population was collected and centrifuged and finally

resuspended at a concentration of 10^7 cells in 80 µl. To the aliquot of 80 µl cell suspension, 20µl of $CD4^+$ (L3TH) microbeads (130-049-201, Miltenyi Biotech, Germany) (Busch *et al.*, 2004; Matheu & Cahalan, 2007; Stanciu & Djukanovic, 2000) with magnetic probe was added in the test tube. The tubes were refrigerated at 4^0-6^0 C for attachment of the bead to the $CD4^+$ cells for 15 min. The mixture of cells and magnetic beads is then poured into the magnetic separation (MS) column fitted in the slot of the magnet of MACS. The unlabeled cells passed through the column and were collected in a tube. The MS column was removed from the separator and placed in a fresh collection tube. 1ml of PBS was pipetted onto the MS column and labeled $CD4^+$ cells were flushed out from the column by firmly pushing the designated plunger into the column. The magnetic labeled $CD4^+$ cells were then counted in a haemocytometer.



Digramatical representation of separation strategies with MACS

Estimation of serum TNF-α

Quantitation of serum TNF-α in DTH bearing mice and DTH mice treated (i.v.) with EEA and alcohol was performed by solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen, USA) following the protocol outlined by Drew & Chavis (2000) and Paul and his coworkers (Sirish Kumar *et al.*, 2003; Sureshkumar *et al.*, 2005).

Gene expression analysis

Expression of the inflammation associated genes viz., TNF-α, TGF-β, IL-1β, IL-6, IL-10, IKK, COX1, COX2 and PKC-theta has been carried out using single cell RNA phenotyping procedure as outlined by Rappolee *et. al.* (1988 a&b).

RNA isolation

RNA was isolated from splenic T cells of 9 mice from each group – untreated DTH, DTH treated with alcohol and with EEA using RNeasy Mini kit (74104, Qiagen, Valencia, U.S.A.), as per manufacturer's protocol. Briefly, 6x 10^6 T cells were homogenized with 300µl RLT buffer and passing them through a 2ml syringe fitted with a 27 gauge needle. 300 µl of 70% ethanol was added to the homogenate and transferred to a spin column fitted upon a collection tube. The spin columns and collection tubes were supplied by the manufacturer. After a brief centrifugation for 15s at 10,000 rpm the fluid passed into the collection tube which was then decanted and reattached to the spin column. With addition of 500 µl of buffer RW1 into the spin column centrifugation was made again for 15s at 10000 rpm. Following decantation of collection tube 500 µl of buffer RPE was added to the spin column and centrifuged similarly, and the step was repeated one more time. Finally, the spin column was fitted upon a fresh collection tube and washed twice with 15 µl of DEPC treated water by

centrifugation at 10,000 rpm to come up with a total of 30 µl volume containing RNA sample.

The concentration of RNA was measured spectrophotometrically at 400X dilution with Shimadzu UV-160, Japan. The extracted RNA was used for cDNA synthesis.

cDNA synthesis

The isolated RNA was used for First strand cDNA synthesis utilizing RevertAid^{TM} First strand cDNA synthesis kit # K1621 from Fermentas and the manufacturer's protocol was followed. For synthesis of first strand cDNA the primer used for PCR amplification was oligo(dT^{13}) synthesized by GMBH. cDNA constructed was stored at -20^{0}C for further use.

Primer utilized and amplification schedule

Primers were designed from various geneBank accession retrieved from PUBMED Data Bank as listed below, using primer program available in internet. The designed primers were synthesized by GMBH, Germany. Details of the primers are

Primers	Accession No.	Sense (5′-3′)	Antisense (3′-5′)	T_m(^{0}C)
IKK	NM_010546	CCAGACTCCAAGGTGGTGTT	TGCAGATCACAGGCAGAAAC	60.0
TNF-α	NM_013693	TGGCACAGCCAAG	GGGACCCCTGCTC	52.36
TGF-β	NM_011577	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC	59.99
IL-1β	NM_008361	GTGGCAGCTACCTGTGTCTT	GGAGCCTGTAGTGCAGTTGT	57.96
IL-6	NM_031168	GGGAAATCGTGGA	AGGTTTGCCGAGT	43.9
IL-10	NM_010548	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG	60.035
COX1	BC023322	AGAAACTGGTCTGCCTCA	AACCCACATCAAGGACTG	54.02
COX2	NM_011198	AGCACCATTCTCCTTGAA	GTAGGCTGTGGATCTTGC	54.0
PKC- theta	NM_008859	AAGTGAGAAACCCCGGCTAT	AGGCAAATCCCTTCCAGTCT	60.01
Perforin	NM_011073	ACCCTGAATGGGCTCACA	GCAGCAGTCCTGGTTGGT	57.0

PCR was performed using thermocycler (PeqLab, Germany) for 35 cycles in 30 µl reaction mixture containing Taq DNA polymerase buffer, all four dNTPs, oligonucleotide primers, Taq DNA polymerase and cDNA products. After amplification PCR products were analysed on 0.8% (w/v) agarose gel. The band density was quantified on the basis of the known concentration of lambda DNA (30 ng) through ImageAide, Spectronics Corporation, NY.

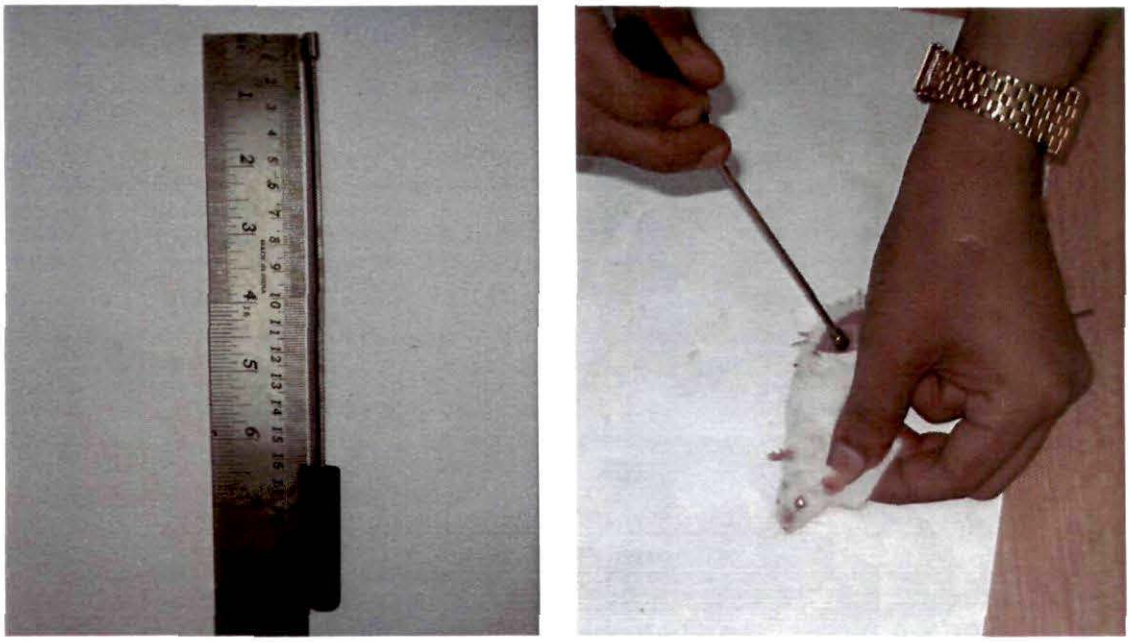
Linear skin incision and topical EEA treatment

Wound healing property of EEA was judged by applying it on linear incision on skin of mice; the incision was made at right dorsolateral side of the vertebral column towards lower half of the body. Hair on the skin of mice was removed with hair remover cream (fem, Fem Care Pharma/Marisu Marketing LTD., 210, Nariman Point, Mumbai, India) 1 day prior to make the incision on the skin. Mice were anaesthetized by intraperitoneal injection of sodium thiopentone (5mg/animal) and a linear incision of 1 cm length was made on the shaved skin with a sharp scalpel, placing a small 1 cm scale next to the incision. 5 µl EEA was applied topically on the wound after 1 hr of incision and the same treatment repeated once in a day for the period of the experiment.

Induction of burn wound and EEA treatment

Mice were shaved and anaesthetized following the same procedure as mentioned above. A circular area of 0.5 cm on skin was burnt with a steel device made in workshop. A steel rod of 16 cm mounted on a plastic handle and ending on other side into a knob of 1 cm x 0.5 cm diameter (Figure below) was heated in a bath of edible mustard oil at 110^0C. The

heated knob was pressed on the shaven area of the mice skin for 5 seconds to make the burn injury (Figure below). Topical application of 5 µl EEA on the circular burnt spot of experimental mice and equal volume of ethanol in control mice were made twice daily with an interval of 12 hours.



a) b)

Figure: a) Photograph of the device to make the burn injury; b) Photograph showing the process of making burn injury.

Tumor induction

Ehrlich ascitic carcinoma cell line was obtained from Chittaranjan National Cancer Research Institute, Kolkata and maintained in mice in our laboratory by serial passage.

Maintenance of ascitic tumor cell line by serial passage

Tumor cells were collected from peritoneal exudates of the mice bearing ascitic tumor by aspiration with a syringe fitted with 22 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 thigh region of the left leg of a normal mouse. The mean time for appearance of palpable tumor was 14 days at the site.

Effect of EEA on tumor growth

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.

^{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 a half hour at 37^0 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 injected earlier i.v. with 25 µl of EEA 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 at 37^0C in humidified atmosphere of 5% CO_2 in air.

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 at 1500 rpm for 10 min and 1 ml of supernatant was collected from each tube in a fresh 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:

$$\% \text{ of Cytotoxicity} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Immunization with SRBC and EEA treatment

Sheep's erythrocytes were used as model antigen for immunization. Blood from jugular vein of a sheep was collected in Elsevier'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). Primary immunization was carried out by injecting 0.1ml of 25% SRBC intravenously in lateral tail vein of mice and for secondary immunization, 0.05 ml of the 25% SRBC was injected through the same route after 7 days of primary immunization. Mice were treated by injecting (i.v.) 25 µl of EEA and alcohol 1 day prior to primary immunization. Effect of EEA on primary and secondary antibody mediated immune response was studied in separate experiments. Mice were sacrificed after 7 days of primary and secondary immunization to carry out the following studies:

Count of B and T lymphocytes and macrophages after primary and secondary immunization

The three categories of cells were obtained following the protocol outlined earlier. 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.

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). 0.1 ml suspension of spleen cells in PBS from mice immunized with SRBC 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 sides 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.

Immunoglobulin G (IgG) estimation by ELISA

Mouse IgG was assayed in micro wells coated with 100µl of diluted serum samples (8µl of serum sample and 1992µl of coating buffer) and incubated overnight at 4^0C. Certain other wells in the same micro plate were coated with different concentrations of mouse standard IgG (10pg/ml) (Genel, Bangalore) diluted in 100µl coating buffer for the purpose of generating the standard curve; and a few wells were incubated only with 100µl coating buffer and no serum to ascertain the reaction in other wells was antigen-antibody reacrion. After incubation, wells were aspirated and washed 3 times with wash buffer (300µl/well). Then the wells were blocked with 200µl of 10% bovine serum albumin (BSA) and kept at room temperature for 1 hr. Again the wells were washed 4 times with wash buffer and 100 µl of rabbit anti-mouse IgG primary antibody (Genel, Bangalore) was pipetted into each well and kept at room temperature for 2 hrs. Then the wells were washed 4 times with wash buffer and horseradish peroxidase linked polyclonal goat anti-rabbit IgG (Genel, Bangalore) was added to each well and incubated at room temperature for 1 hr. Following a wash cycle with wash buffer to remove any unbound antibody-enzyme reagent, 50 ml of substrate solution (hydrogen peroxide and

tetramethyl- benzidine) was added to each well and incubated for 30 mins in room temperature. The enzyme substrate reaction yielded a product of blue colour that turned yellow on addition of the 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 with the different dilutions of standard mouse IgG were used for determining the concentration of IgG on the basis of Beer Lambarts' Law.

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 EEA. 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 the EEA extract over the control was calculated.

Hydroxyl radical scavenging assay

Hydroxyl radical (OH^-) was generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fentons' reaction), which attacks the deoxy D-ribose, and a series of reaction follows to form malonaldehyde (MDA) (Halliwell, Gutteridge & Aruoma, 1987). 20mM phosphate buffer, 2mM $FeCl_3$, 1mM EDTA, 2.8 mM 2-deoxy D-ribose, 1mM H_2O_2 and 1mM L-ascorbic acid were mixed to prepare the assay reaction mixture. 1 ml of the reaction mixture was aliquot in each tube of experimental, alcohol control and normal control sets and was incubated at 37^0C for 1 hr. Two different doses of EEA, 10 µl and 25 µl, were

tested *in vitro* to judge scavenging effect of the plant extract on generation of hydroxyl radical. In alcohol control sets, same volume of ethanol was added and for normal control, the volume adjusted with 10 µl and 25 µl distilled water. After incubation, 2 ml of TBA-TCA reagent was added in each tube and boiled for 15 mins for generation of MDA. MDA generated was measured at 552 nm in spectrophotometer. The effect of both EEA and alcohol on generation of hydroxyl radical has been expressed as % of inhibition in MDA generation over normal control sets. The formula used is given below:

$$\% \text{ of inhibition} = \left[100 - \frac{\text{MDA generated in experimental/alcohol control tubes}}{\text{MDA generated in normal control tubes}}\right] \%$$

Lipid Peroxidation Assay

Lipid peroxidation of lymphocytes under the influence of EEA extract and ethanol was estimated separately according to Miller and Aust, 1989. Lipid peroxidation was induced by copper - ascorbate system and estimated by OD value of 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 of TBARS absorbing at 535 nm.

The reaction mixture contained $1X10^6$ packed lymphocytes in 0.2M phosphate buffer pH (7.4), with 20mMTris-HCl, 2mM $CuCl_2$, 10mM ascorbic acid and 10 or 25 µl of EEA and was incubated for 1hour at 37^{O} C in humidified atmosphere containing 5%

CO_2 in air. Degree of lipid peroxidation was indexed by measuring the level of malonedialdehyde (MDA), generated 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 2 ml 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.

Nitric Oxide Synthase (NOS) Activity

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

Briefly, $1x10^6$ packed lymphocytes were incubated for 2 hr with 50 mM Tris-HCl buffer (pH 7.4), 10mM L-arginine, 64mM hemoglobin, with two different doses, 10 and 25µl of EEA at 37^{O} C in humidified atmosphere containing 5% CO_2 in air. After incubation reaction mixture was centrifuged at 1000 rpm for 5 min and the optical

density of supernatant was measured in UV- spectrophotometer (ELICO, S L164) at 535nm. Results of $NO^{\cdot}$ production were expressed as pmol of NO produced/hr.

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

Isolation and characterization of the different fractions of EEA

The total extract, EEA was first monitored on a fluorescent bound silica TLC plate (E Merck, Germany) with 16% ethyl acetate in petroleum ether solvent. The major band in TLC was then separated by column chromatography over silica gel (60-120 mesh, SRL, India) using the same percentage of solvent. The fraction separated was finally collected in conical flasks and dried under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^{0} C to ascertain its dry weight in the total extract. The fraction was then dissolved in ethanol maintaining the dry weight equivalence as in original EEA extract, and kept at 4^{0}C for further experimentations and chemical characterization. Chemical characterization of the fraction has been carried out using HPLC and IR, NMR and mass spectroscopic studies. The data have been interpreted with the help of expertise from the Department of Chemistry, Univ. of North Bengal.

Statistical analysis

In all the experiments, the effect of EEA was compared with two sets of control, one with equivalent amount of ethanol present in EEA and the other without any treatment. Triplicates in experimental and control set were maintained in each experiment. An experiment was repeated thrice or more. Results are expressed as Mean±SD of *n* observations. Statistical significance was analyzed using one way ANOVA software package.

RESULTS

Effect of ethanolic leaf extract of *Eupatorium adenophorum* (EEA) on Hematological parameters

The Blood is important for pulmonary and tissue respiration, as a medium of endocrine and neurohumoral transmissions, biotransformation and metabolic excretion (Adebayo *et. al.*, 2005), nutritional and immunological processes, as well as homeostatic responses (Oze, *et al.* 2008). This makes it imperative to study the effects of an extract on blood parameters before its use for treatment.

Effect on percentage of hemoglobin

Intravenous application of 25 µl EEA and alcohol in mice did not cause any marked variation in the percentage of hemoglobin in blood (Fig.1).

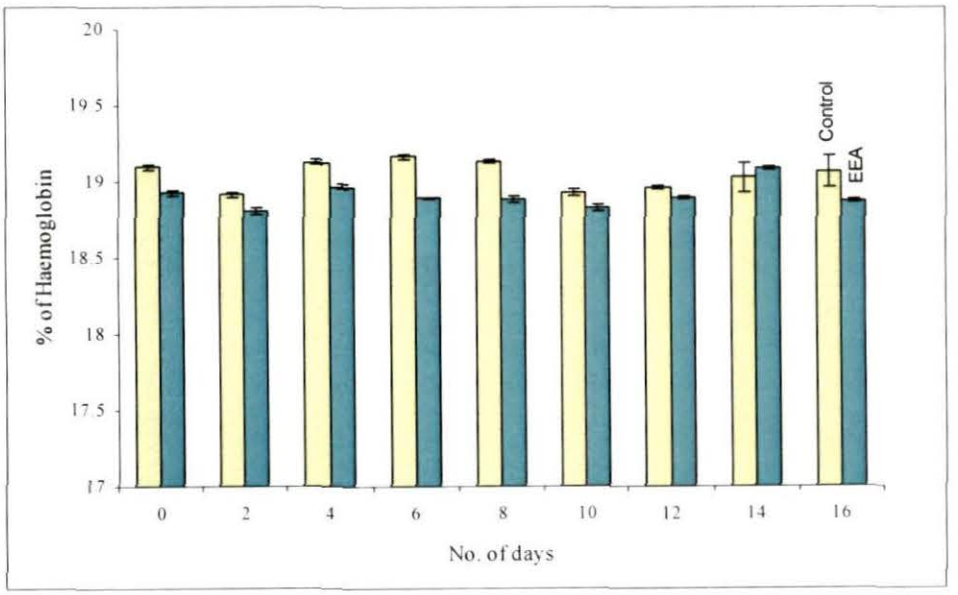


Fig.1: Percentage of hemoglobin in mouse blood after intravenous injection of EEA and ethanol (control). (Count on 0 day indicates the level of hemoglobin in normal mice in Fig. 1-3)

Effect on RBC count

RBC count of both experimental and alcohol control mice did not show any marked variation from the normal counts (Fig. 2).

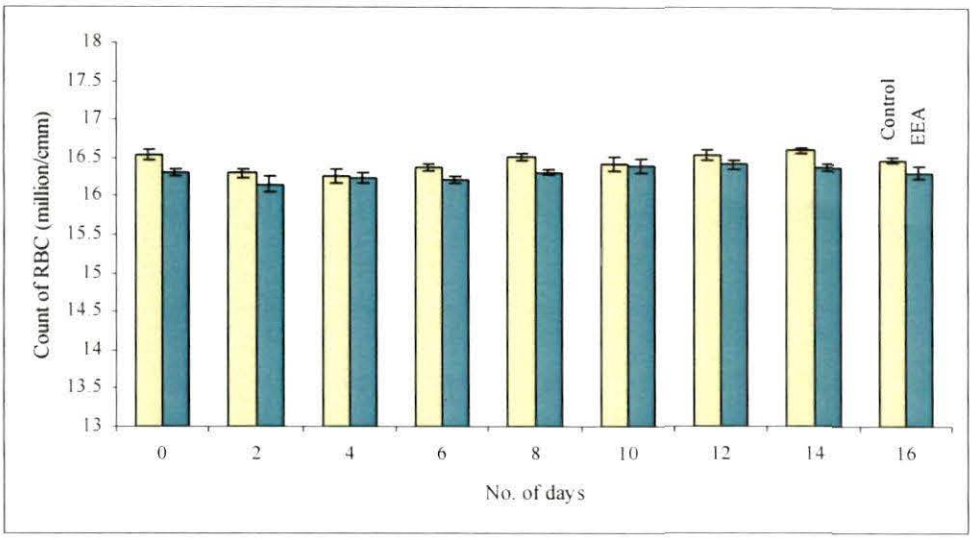


Fig. 2: Count of RBC in mouse blood after i.v. injection of EEA and ethanol.

Effect on WBC count

The total count of WBCs increased upto 8 days after intravenous treatment with EEA, whereas, no such response was found in alcohol treated control mice. Therefore, EEA seems to play a possible promotional role for WBCs (Fig. 3).

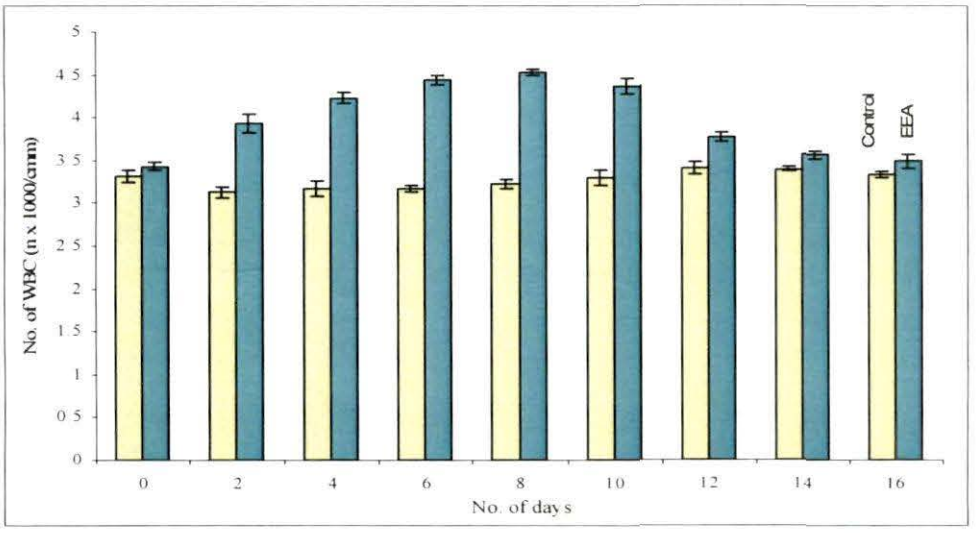


Fig. 3: Total count of WBC after intravenous injection of EEA and ethanol

Effect of EEA on viability of lymphocytes *in vitro*

Viability of lymphocytes

Lymphocytes treated with 25 µl of EEA showed better survivality at later hours in comparison to the control containing same amount of ethanol; 11.89% of lymphocytes were alive at 48 hrs of incubation in comparison to 2.23% in ethanol control (Fig.4).

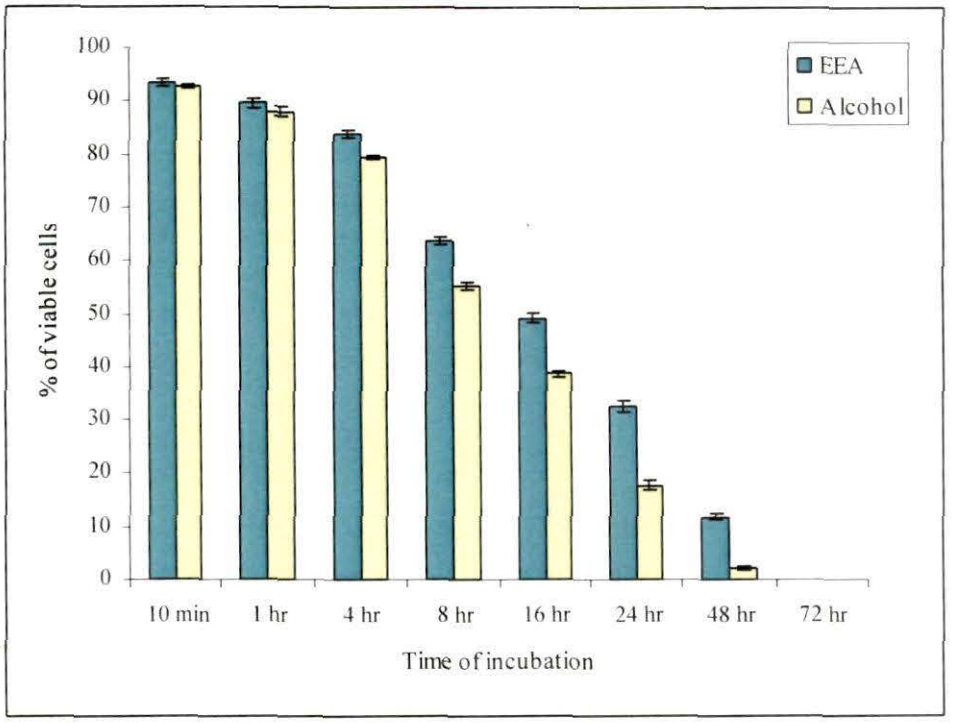


Fig. 4: Survivality of lymphocytes *in vitro* in presence and absence of EEA

***In vitro* blastogenic transformation of T and B cells in presence of EEA**

Blastogenic transformation of lymphocyte is normally indicative of stimulation of immunocompetent cells. Hence, the quantum of blasts after treatment with the extract is likely to reveal the immunostimulatory property of the extract. We studied blastogenic transformation of T and B cells *in vitro* with both 10 µl and 25 µl doses of EEA.

T cells

EEA treatment with both the doses caused blastogenic transformation of T cells better than B cells; the higher dose, 25 µl, was more effective at both 24 (36.84%) and 48 (57.89%) hrs (Fig.5).

B cells

Blastogenic transformation of B cells in response to EEA treatment was also higher in comparison to ethanol control sets. The maximum percentage of B cell blasts with treatment of 10 µl EEA were 28.57% and 36.36% at 24 and 48 hrs respectively (Fig.5). The corresponding percentage of blasts in control at 24 hrs and 48 hrs were 12% and 15% (Fig.5). The percentages of blast with 25 µl EEA treatment were comparable with the results of 10 µl EEA treatment at both the hours.

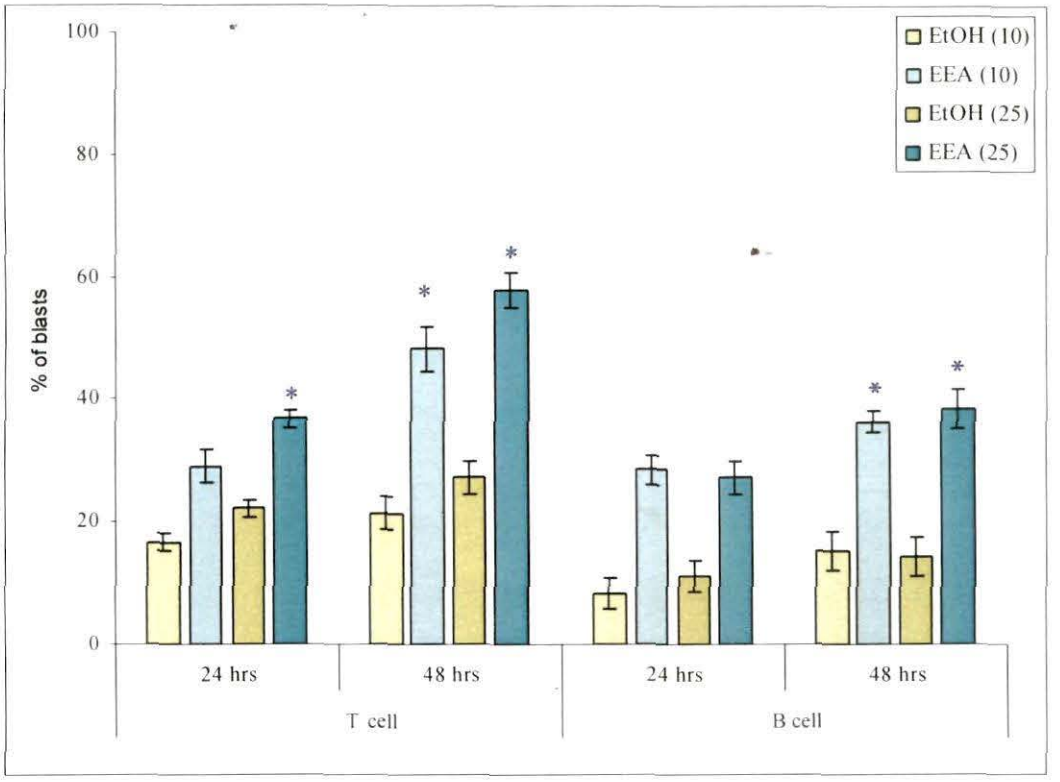


Fig. 5: *In vitro* blastogenic transformation of T and B cells in presence and absence of EEA. (Significance of results with EEA over control at *p<0.01)

Measure of DNA synthesis

The radioactive counts were higher with EEA treatment (i.v.) than the controls at all the points - 16, 24 and 48 hrs (Fig. 6). EEA induced maximum level of DNA synthesis even at 16 hrs. The level was more or less maintained upto 48 hrs. Alcohol as such seems to be stimulatory for ^{3}H-TdR incorporation to some extent but the level of incorporation was

far below than that stimulated by EEA. Furthermore, the response with alcohol was in declining phase from 24 hrs onward (Fig. 6). Alcohol could also induce marginally higher level of blastogenesis in case of T cells (Fig. 5).

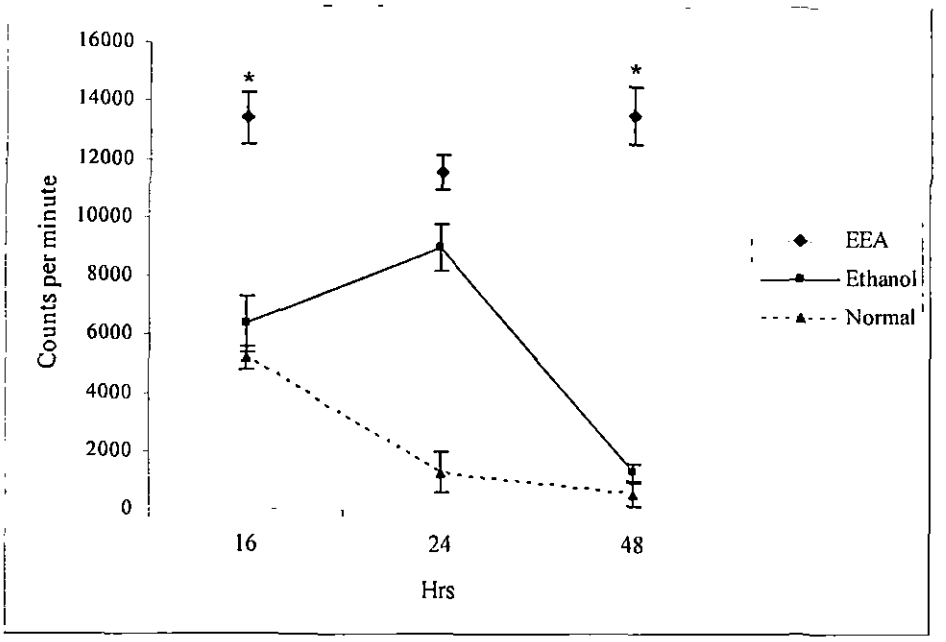


Fig. 6: Pattern of incorporation of ^{3}H-thymidine by lymphocytes treated for different hours with EEA. Results are expressed as mean ± SD, * $p<0.01$ compared to respective alcohol control.

Cell cycle analysis of lymphocytes with EEA treatment

The amount of nuclear DNA in course of cell cycle was studied by fluorescence activated cell sorter (FACS) after staining of DNA content in cells with fluorochrome ethidium bromide (EB) and plotted against cell numbers in the DNA histograms. DNA histograms of one experiment have been shown in Fig. 7; the indices refer to percentage of cells at a pertcular cell cycle stage represented by M1, M2, M3 and M4. Average data of all the triplicate experiments have been summarized in Table 1.

EEA could drive higher percentage (27.67%) of lymphocytes towards S phase (M3) from 16 hrs onward and maintained the stimulation upto 48 hrs. (Fig. 7, Table 1). This result has corroboration from our data on DNA synthesis (Fig. 6). The percentage with alcohol control was slightly higher than normal control, possibly reflecting a marginal

stimulatory activity of alcohol on blastogenesis and DNA synthesis. Further progression of the cells towards G2-M, represented by M4, continued with EEA treatment at 24 and 48 hrs. Notably, with EEA treatment percentage at G0 phase (M1) representing quiescent and apoptotic cells was comparatively lower at all the hours in comparison to the controls (Fig.7, Table 1).

Table 1: Percentage of cells in different stages of cell cycle after 16, 24 and 48 hrs of *in vitro* treatment with EEA and alcohol. The results are average of readings of all the triplicate experiments for cell cycle analysis.

Hrs	Phases	EEA	Alcohol	Normal
16	G0	38.17	52.6	40.84
	G1	21.46	17.09	19.92
	S	**27.67**	9.92	3.61
	G2-M	3.5	1.04	0.56
24	G0	42.97	60.06	40.08
	G1	20.84	21.74	17.99
	S	**25.73**	7.56	3.15
	G2-M	**5.82**	0.965	0.87
48	G0	38.26	56.13	62.18
	G1	19.64	19.2	14.98
	S	**30.24**	10.89	2.59
	G2-M	**8.27**	1.37	0.79

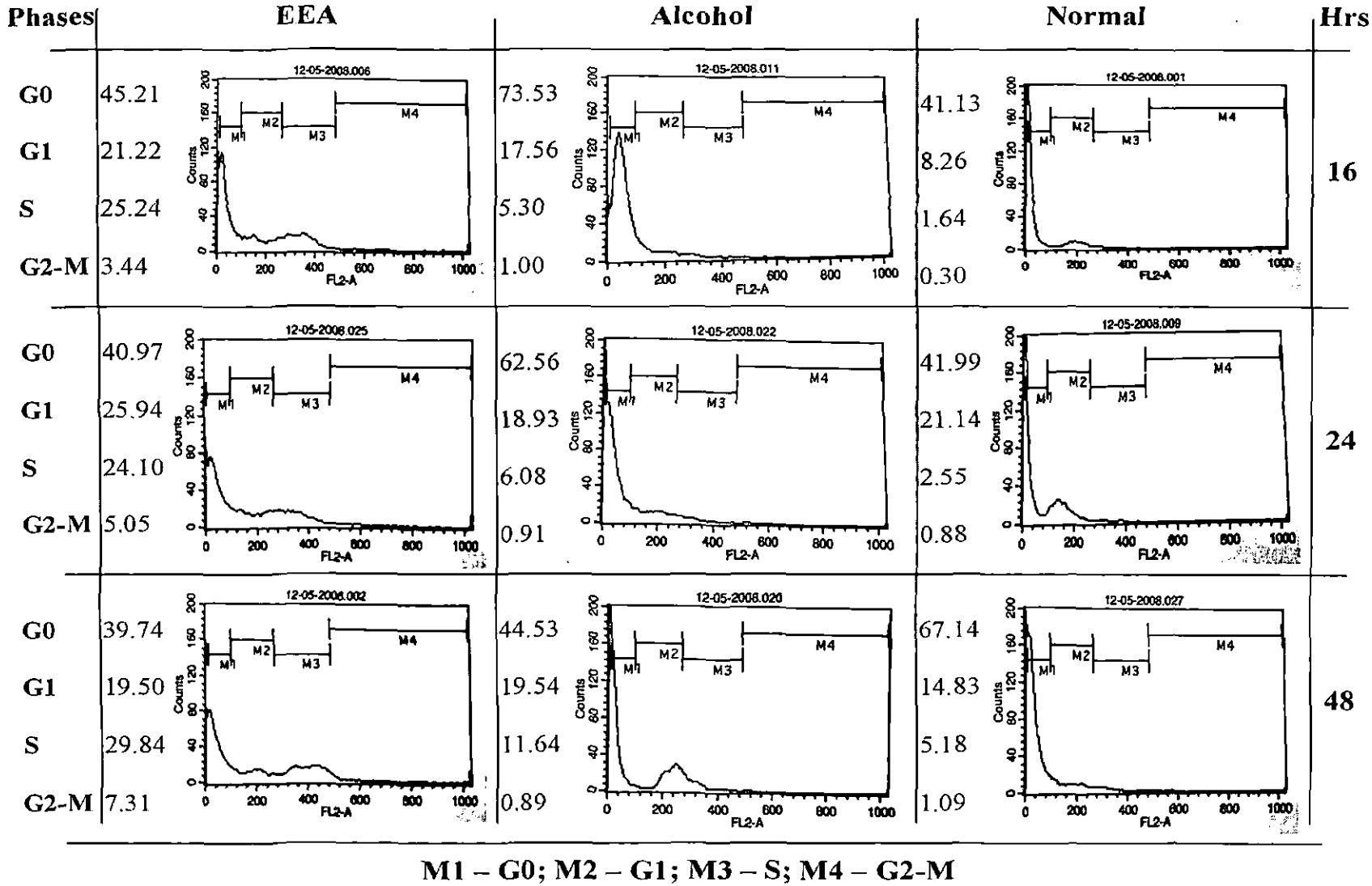


Fig. 7: DNA histograms representing data of one experiment of cell cycle analysis of splenic lymphocytes upon *in vitro* treatment with EEA and alcohol.

Effect of EEA on delayed type hypersensitive (DTH) reaction

With topical application

Figure 8 represents the data of delayed type hypersensitivity reaction induced with 25 µl DNFB and treatment with ethanolic leaf extract of *Eupatorium adenophorum* (EEA) (experimental) and ethanol in control. Swelling of resensitized paw in both control and experimental mice was maximum on 3rd day; with topical application of EEA, the maximum swelling was in the range of 0.4733 ± 0.0227 cm. and in ethanol treated control mice the maximum value was 0.5667 ± 0.173 cm. (Fig. 8). The paw size in EEA treated mice gets back to normal range by the 9th day of resensitization. Fig. 8 also represents the percentages of inhibition on different days with EEA treatment. The percentage of inhibition was calculated in reference to the alcohol control on the respective days as indicated in Materials and Methods.

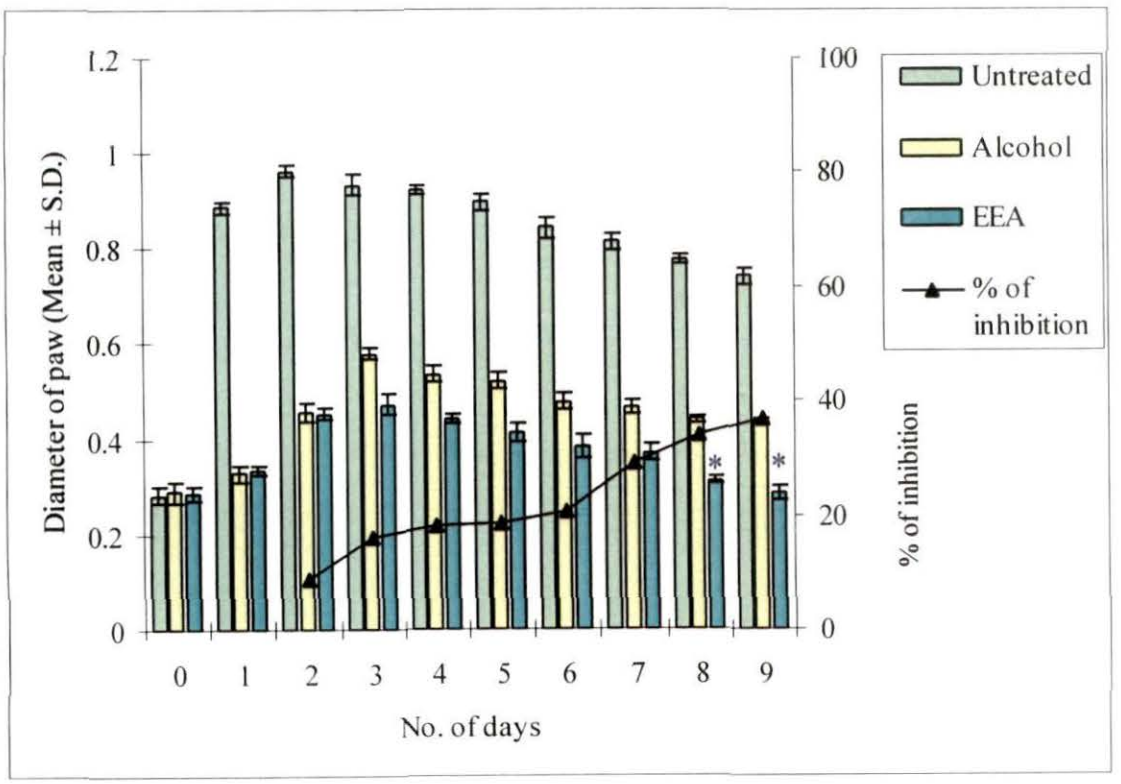


Fig. 8: Inhibitory effect of topical application of EEA on delayed type hypersensitive (DTH) reaction induced with 25 µl 2,4-dinitro-fluorobenzene (DNFB). (Significance of result with EEA over alcohol control at *p< 0.01).

Higher dose (50 µl) of DNFB caused the swelling to be more and the peak on 2nd day; however recovery with EEA treatment was on 9th day (Fig. 9). In fact EEA inhibited the swelling by 60% on the 9th day. Figure 10 is the photographic representation of DTH reaction induced with 50 µl DNFB and inhibition with EEA treatment.

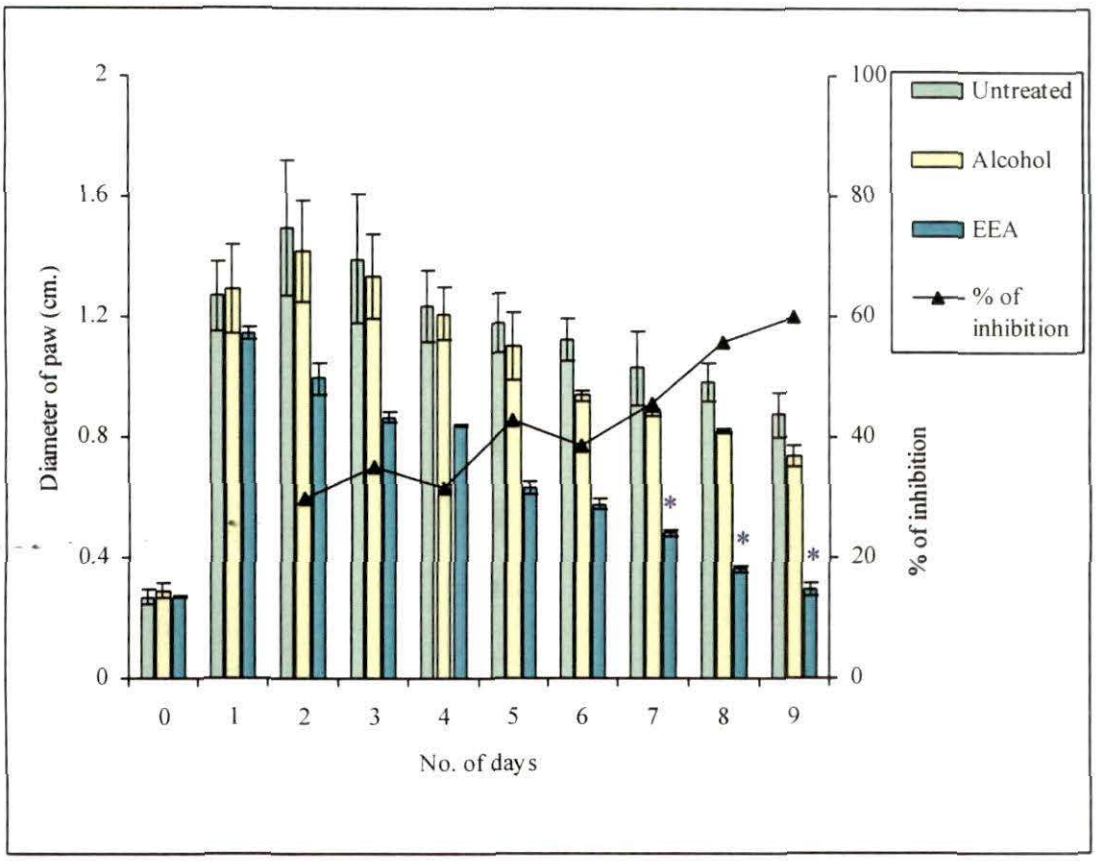


Fig. 9: Indurations of DTH swelling, induced with 50 µl DNFB, and inhibition after topical application of EEA and alcohol. (significance at *p< 0.01).

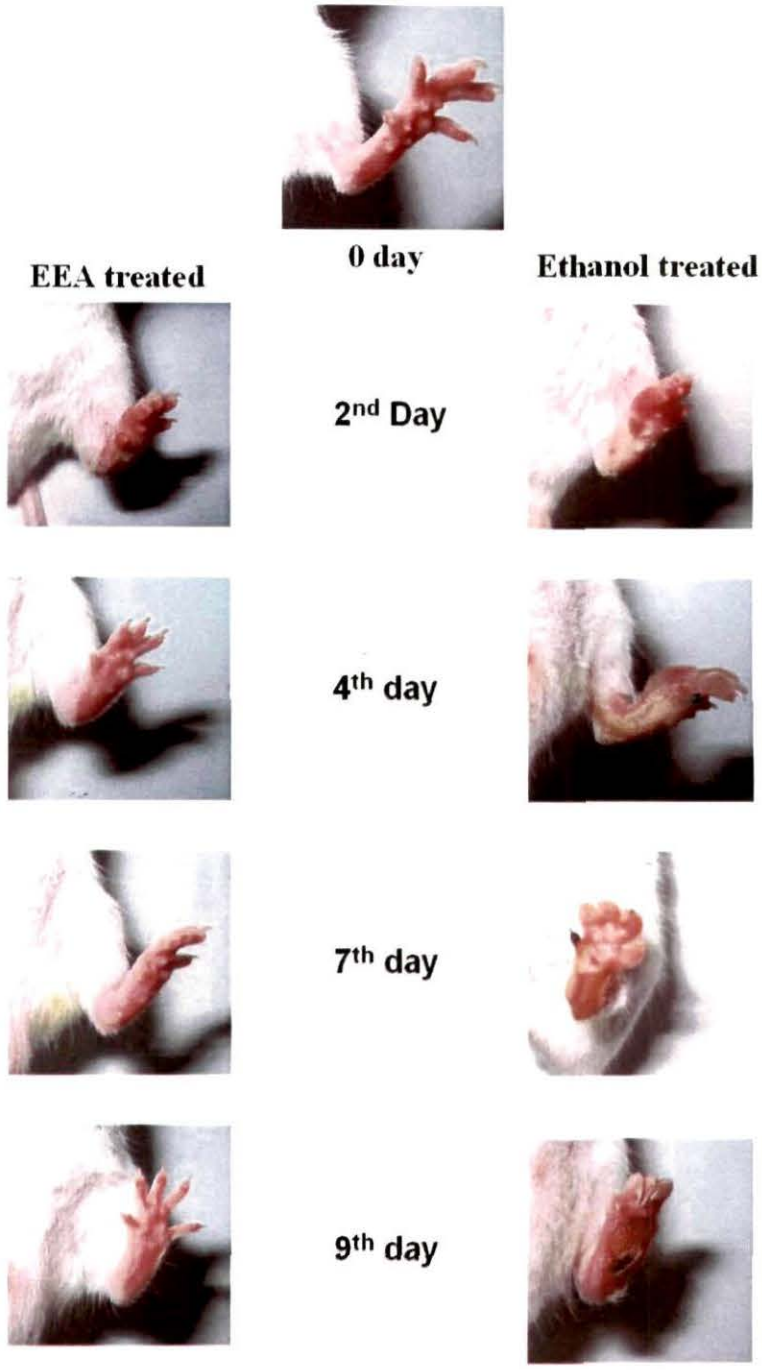


Fig. 10: Photographs showing effect of topical application of EEA and alcohol on resensitized paw of DTH mice, induced by 50 µl DNFB, during 9 day period of study.

With intravenous (i.v.) application

When EEA was administered intravenously in DTH bearing mice 1 hr prior to resensitization, inflammatory swelling of the resensitized paw persisted longer than in cases of topical application of EEA. The dose of 25 µl DNFB could induce maximum swelling 0.963 ±0.012 cm on 2nd day in untreated mice. Intravenous application of EEA caused the DTH reaction to slow down. In alcohol group the swelling was maximum of 0.7367± 0.0045 cm on 5th and 6th day. EEA treatment restricted the swelling to 0.5123±0.0112 cm on 5th day and brought back normalcy (0.2967±0.0103 cm) by 11th day; the inhibition over the ethanol control was found to be 45.39% (Fig. 11).

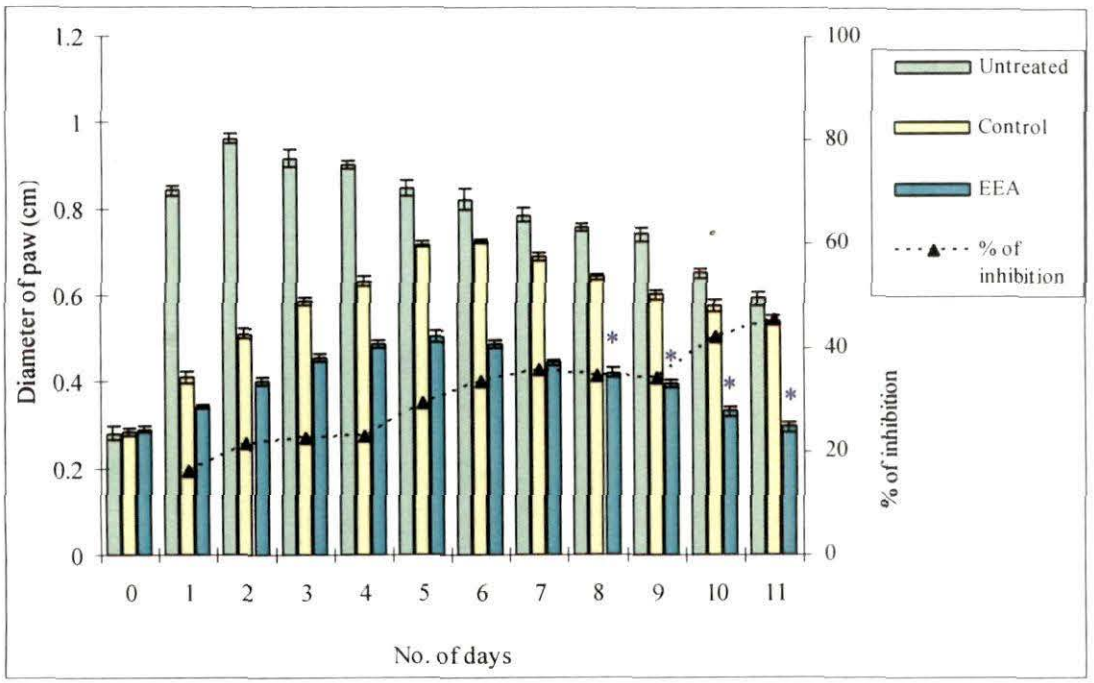


Fig. 11: Changes in paw size of DTH mice, induced with 25 µl DNFB, and prior i.v. application of EEA and alcohol. (significance of inhibition by EEA at *p< 0.01).

The degree of swelling was more at DTH site with higher dose (50 µl) of DNFB (Fig. 12). EEA (i.v.) inhibited the reaction more effectively than alcohol alone (control) and allowed regaining normalcy by 13 days (Fig. 12).

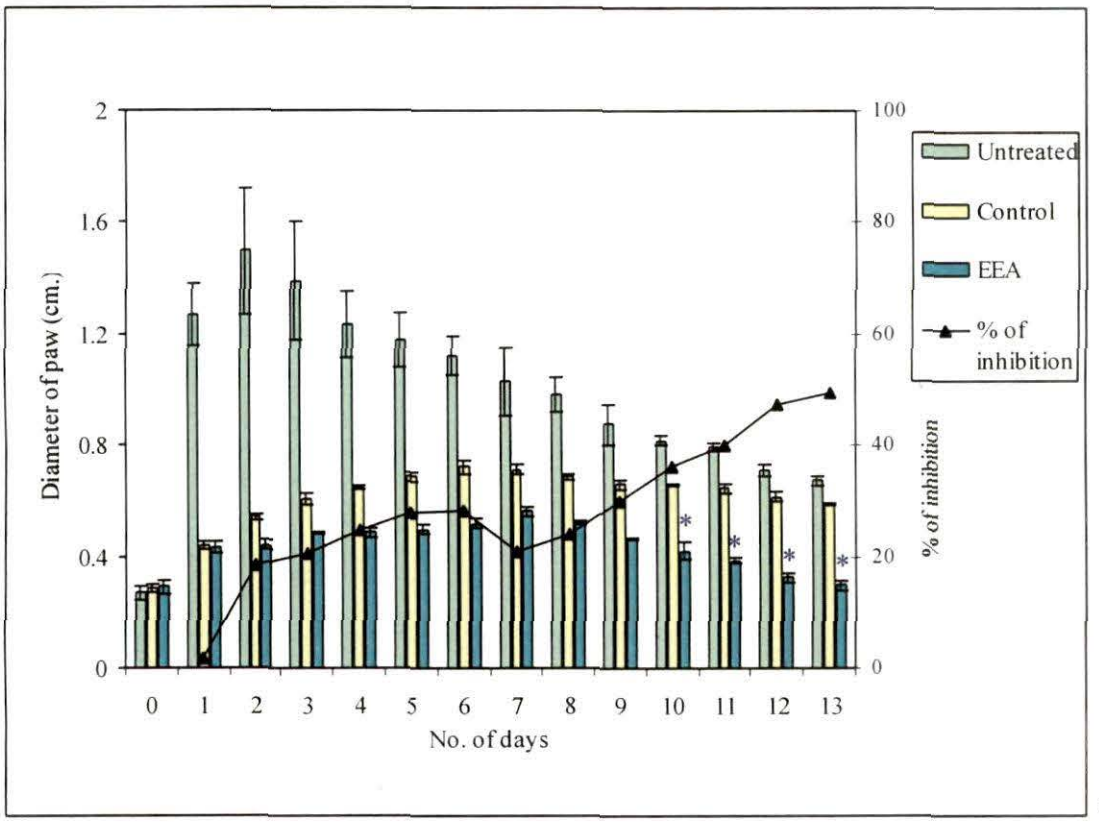


Fig. 12: Changes in paw size of DTH mice, induced with 50 µl DNFB, and after i.v. application of EEA and alcohol. (Significance of inhibition by EEA at *p< 0.01).

Differential count of leukocytes at the inflammation site of the DTH mouse after EEA treatment

DTH reaction is mainly carried out by infiltrating lymphocytes, macrophages and neutrophils 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 the effect of EEA treatment, differential count of leukocytes was taken into account.

Increase in the percentage of lymphocytes and vis-à-vis decrease in neutrophils were noted in case of EEA treatment than in the control. Although the monocyte counts remained comparable in experimental groups at 24, 48 h, an increase in the count at 48 h was observed in controls (Fig. 13). No appreciable change in the number of eosinophils and basophils occurred with EEA treatment when compared to controls (Fig. 13).

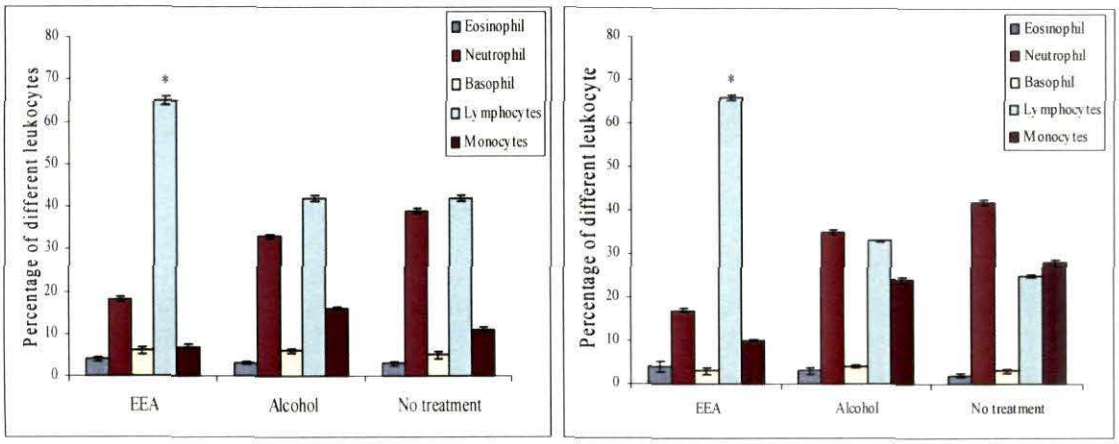


Fig. 13: Percentage of different leukocytes at the inflammation site (resensitized paw) with EEA treatment (1 hr prior to resensitization); A) 24 hrs and B) 48 hrs after resensitization. *Results are expressed as mean ± SD, *p< 0.01 EEA values compared to respective controls.*

Estimation of the number of $CD4^+$ cells in DTH mice

$CD4^+$ T cells from splenic lymphocyte population were isolated by labeling with microbeads containing paramagnetic probe and passing through MACS. Number of $CD4^+$ T lymphocytes in spleen of DTH mice treated with EEA was significantly more than the controls, about two and half times at 24 and 48 hrs and two times at 72 hrs (Fig. 14). A slight increase in the cell number in the alcohol control groups was noted (Fig. 14).

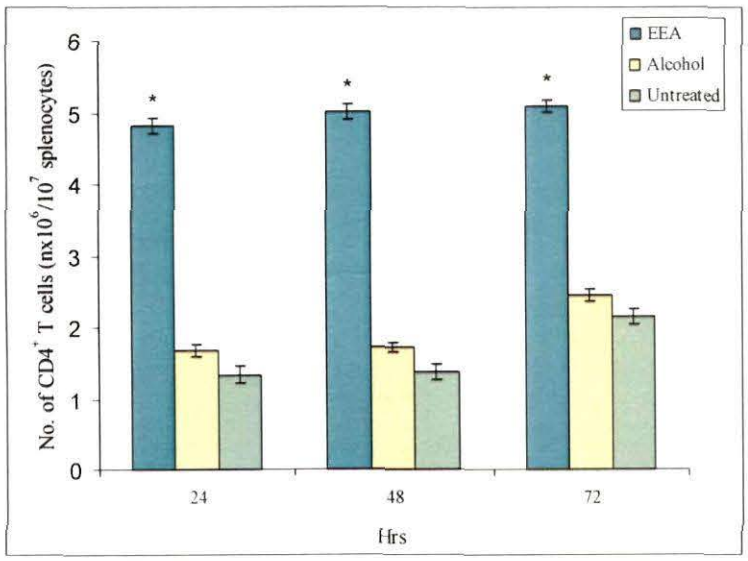


Fig. 14: Effect of intravenously administered EEA and alcohol on count of $CD4^+$ T cells from spleen of mice after 24, 48 and 72 hrs of induction of DTH with 25 µl DNFB. (significance of results with EEA over control at *p< 0.01).

Elevation of serum TNF-α of DTH bearing mice upon EEA treatment

Serum TNF-α in three groups of mice bearing DTH reaction – untreated, alcohol and EEA treated (i.v.) was measured by solid phase sandwich enzyme-linked immunosorbent assay (ELISA).

There was no appreciable difference in the level of serum TNF-α in the three groups of mice at 24 hrs (Fig. 15). By 48 hrs, EEA treatment caused increase (93.23 pg/ml) in TNF-α level in DTH mice when compared with other groups and the level was maintained upto 72 hrs (Fig. 15).

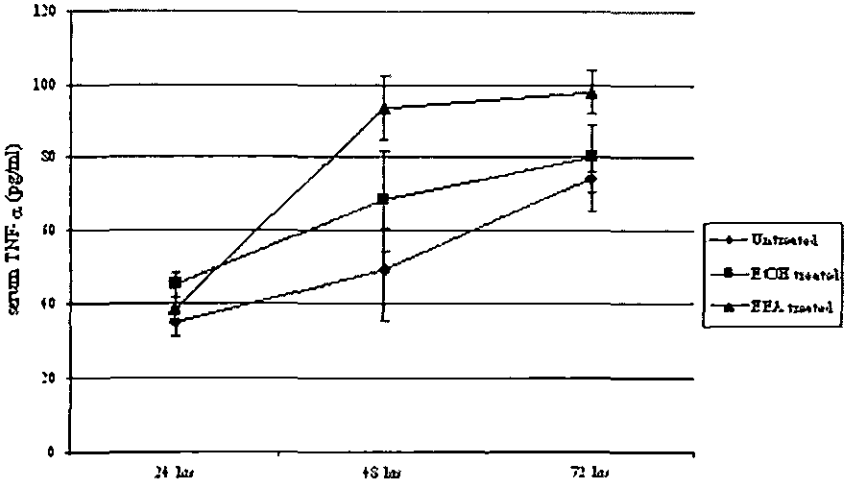


Fig. 15: Level of serum TNF-α from DTH mice (induced with 25 µl DNFB) at three different hours in presence and absence of EEA.

Expression of certain genes in splenic T cells of DTH mice with and without EEA treatment

The T cells actively participate in the progression of DTH reaction. Up or down regulation of certain genes in the cells must be related to the production of pro- and anti-inflammatory cytokines, transcription factors and mediators. The level of expression of some of these genes at transcription level in splenic T cells of DTH mice untreated and treated with EEA was judged by quantitating the cDNA PCR product amplified with specific primers. The quantitation was done against 30 ng of lambda DNA as standard using ImageAide, Spectronics Corporation, NY. The data presented in figure 16 & 17.

EEA caused an increment in the expression of *TNF-α*, a pro-inflammatory cytokine (Fig. 16 & 17). At the same time, EEA inhibited expression of pro-inflammatory cytokine *IL-1β* and showed no significant effect on *IL-6*, another pro-inflammatory cytokine.

EEA did not influence the expression of *IL-10*, an anti-inflammatory cytokine beyond alcohol control. The expression of *TGF-β* encoding a cytokine involved in regeneration, was induced by EEA at higher level than in the controls.

EEA apparently did not influence expression of *IKK* and *COX1* genes but down regulated the expression of *COX2* gene.

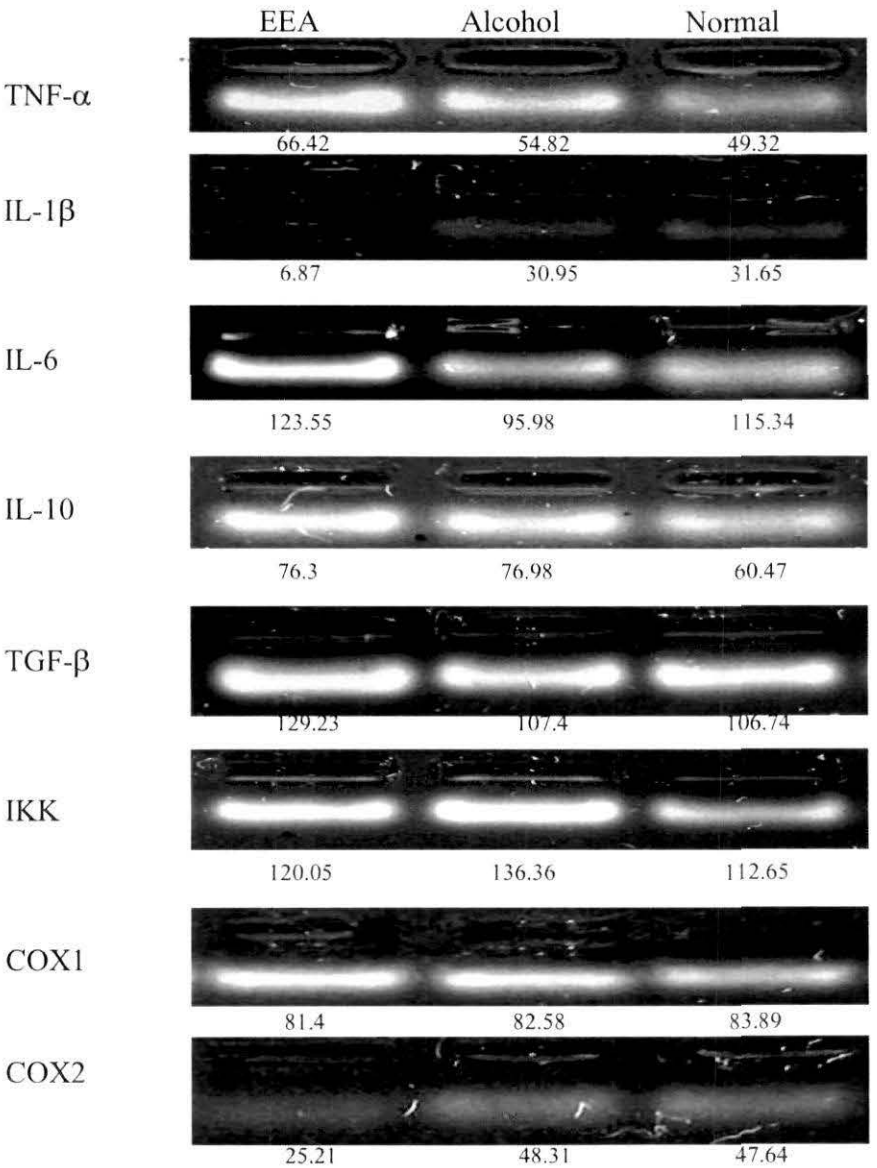


Fig. 16: Expression of certain inflammation related genes in T cells obtained from DTH mice, and with and without EEA treatment, containing 9 mice in each group. Agarose gel electrophoresis of cDNA PCR products amplified with different gene specific primers.

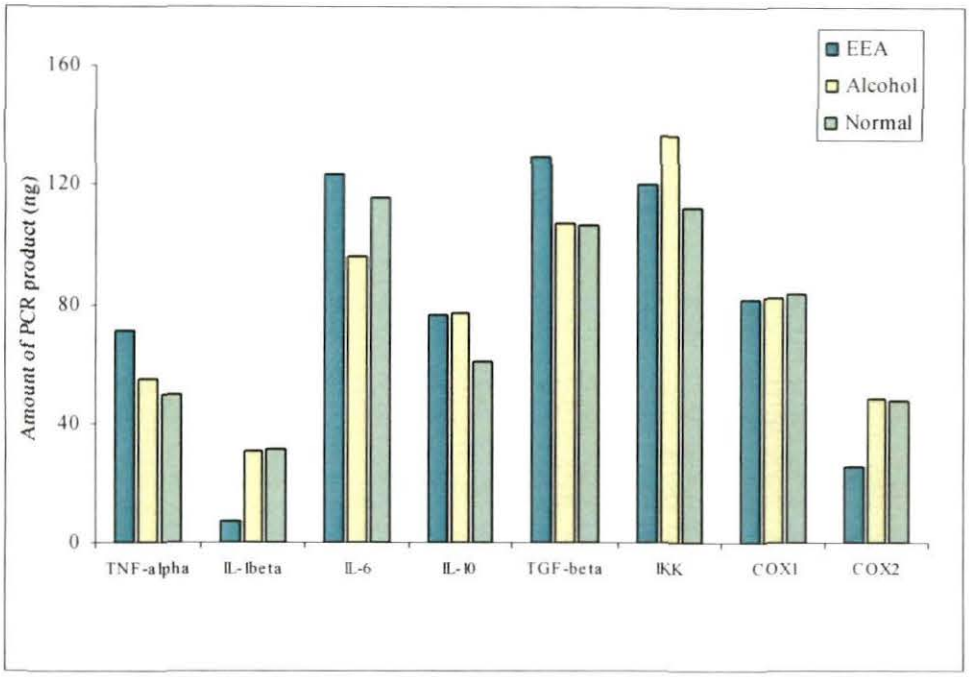


Fig. 17: Graphical representation of quantified PCR products for the expression of different genes in splenic T cells of DTH mice with EEA treatment compared to necessary controls (alcohol treated and untreated).

Wound healing property of EEA

Effect of EEA on skin incision

Inhibition of inflammation related injuries by EEA (Fig. 10) indicated a probable wound healing activity of the plant extract. Linear skin incision of 1 cm length in mice was treated topically with EEA and ethanol (control). Daily application of 5 µl EEA at the wound site caused a faster healing of the skin incision. The healing effect of EEA is evident from day 2 onward as the wound gap was sealed by this time in experimental mice (Fig. 18). With EEA treatment the wound healed up completely by day 5, whereas on the same day wounds were still present in control mice (Fig. 18).

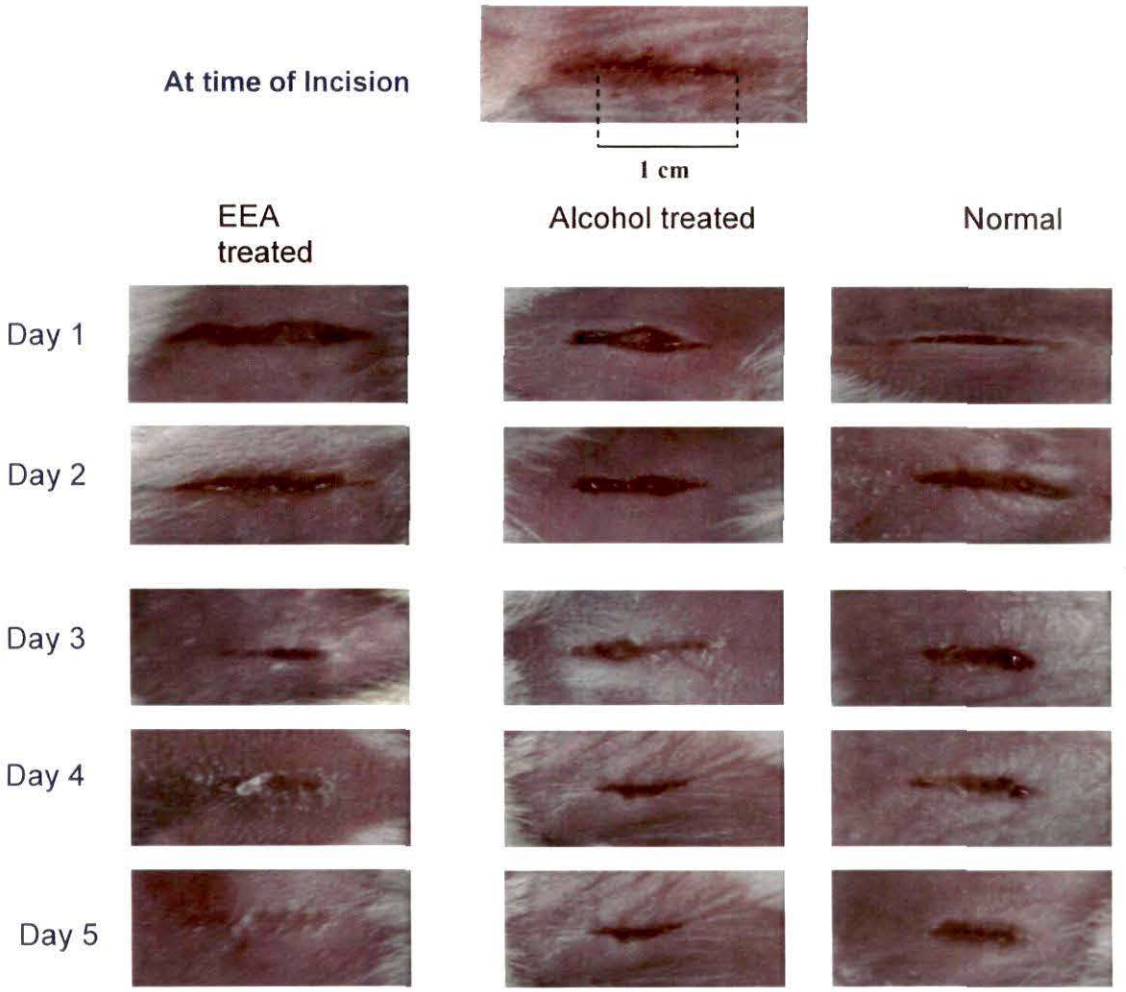


Fig. 18: Healing of linear skin incision in mice in presence and absence of EEA

Effect of EEA on burnt skin

Burnt skin of 0.5 cm diameter was treated topically with 5 µl EEA twice daily at an interval of 12 hrs. Treatment with EEA caused faster healing of the burnt site. The burnt wound seems to be better healed in EEA treated mice from day 3 onward (Fig. 19). With EEA treatment the wound healed up completely by day 10; on the same day wounds were still present in control mice (Fig. 19).

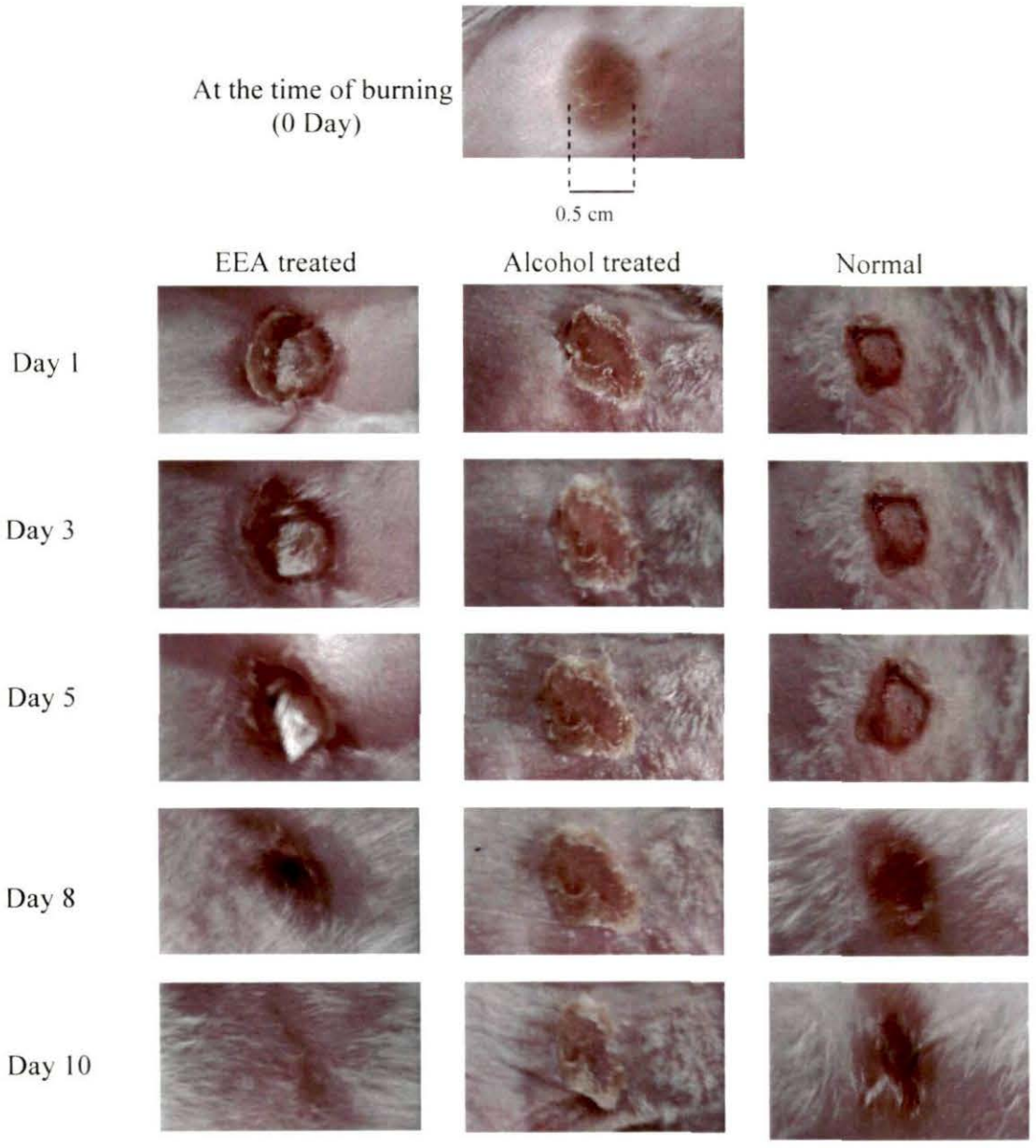


Fig. 19: Healing of burnt skin in mice with and without EEA treatment.

Tumor regression assay

Solid tumor was induced in mice subcutaneously by injecting 10^6 Ehrlich ascites carcinoma cells in 0.1 ml phosphate buffered saline (PBS). It took about 33 days for the

tumor to attain 1.365±0.0115 cm^2 size, measured by a slide caliper. Animals with tumor were then treated with 5 µl of the extract or ethanol topically everyday. The size of the tumor was measured each day but the data of 7 days interval has been presented in the figure. EEA seems to inhibit the tumor growth and thus increase the longevity of the tumor bearing mice by about 2 weeks in comparison to the alcohol control (Fig. 20).

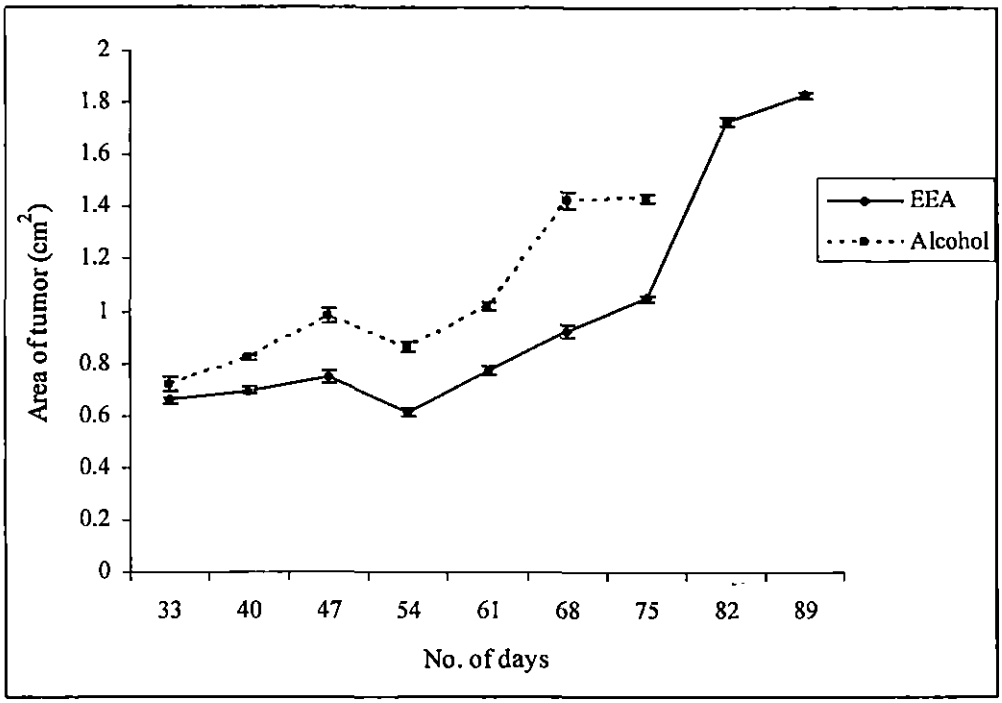


Fig. 20: EEA inhibits solid tumor growth and increases longevity of tumor bearing mice upon topical application of the extract.

Cytotoxicity to tumor cells measured by ^{51}Cr release assay

Lymphocytes from mice injected i.v. with 25 µl of EEA for 48 hrs, showed significantly higher percentage of cytotoxicity towards the tumor target cells than the alcohol treated and the normal lymphocytes. This was true with all the three different target: effector cell ratios such as 1:10, 1:50 and 1:100 (Fig. 21). The cytotoxicity indices with different ratios produced a graded response in experimental group. The data reveals that EEA is capable of activating cytotoxic effector T cells.

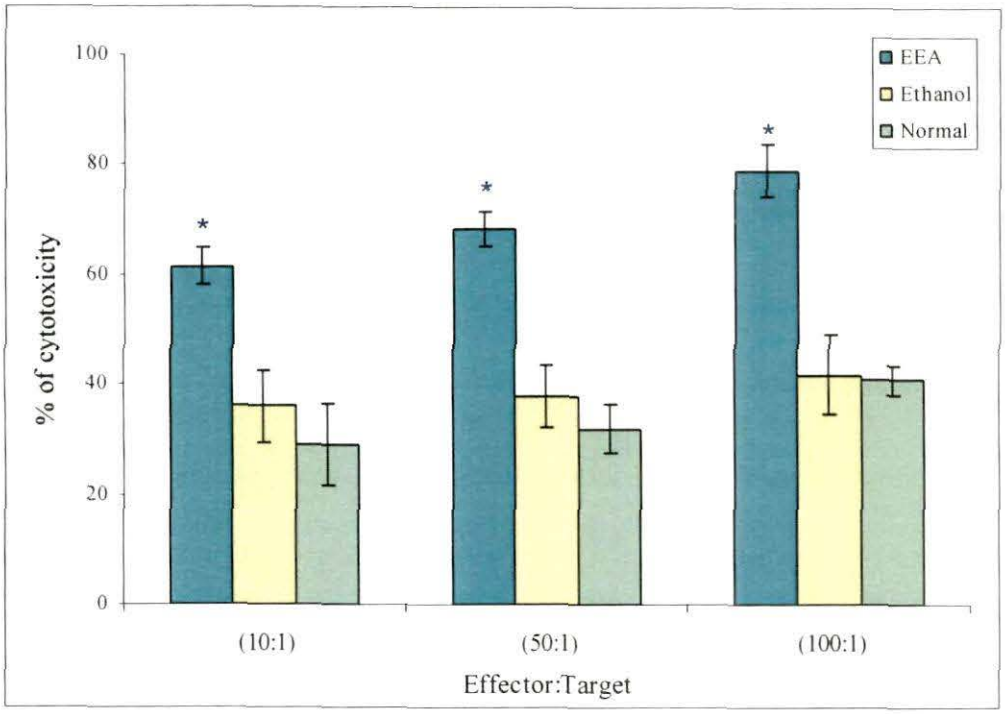


Fig. 21: Percentage of cytotoxic response by EEA treated lymphocytes after 6hrs incubation with labeled (^{51}Cr) tumor target cells. (significance of results with EEA over control at *p< 0.01).

Effect of EEA on expression of a few genes related to immune regulation

Expressions of certain immunologically active genes in splenic T cells of mice have been analyzed using the earlier protocol after 24 hrs of EEA or alcohol injection (i.v.).

EEA could effectively augment the expression of *perforin* gene in the T cells population (Fig.22 & 23). Perforin is the effector molecule for cell mediated killing of target cells. Thus, EEA seems to induce cytotoxic differentiation of T cells and corroborates with the finding of higher level of cytotoxicity with these cells against ^{51}Cr-labeled tumor target cells (Fig.21).

For the different genes encoding immunoregulatory cytokines, EEA did not show much effect on expression of *IL-2*, *IL-6* and *IL-10* (Fig. 22 & 23) but inhibited the expression of pro-inflammatory gene *IL-1β* (Fig.22 & 23). EEA induced higher expression of *TGF-β* gene that encodes a cytokine responsible for activation of cell mediated immunity. A marginal increment in expression of *iNOS* gene with EEA treatment was also recorded (Fig.22 & 23). Interestingly, EEA did not show much effect on expression of *IKK* and

PKC-θ molecules related to activation of cells (Fig.22 & 23). EEA could not influence the expression of *COX1* but inhibited *COX2* gene (Fig. 22 & 23).

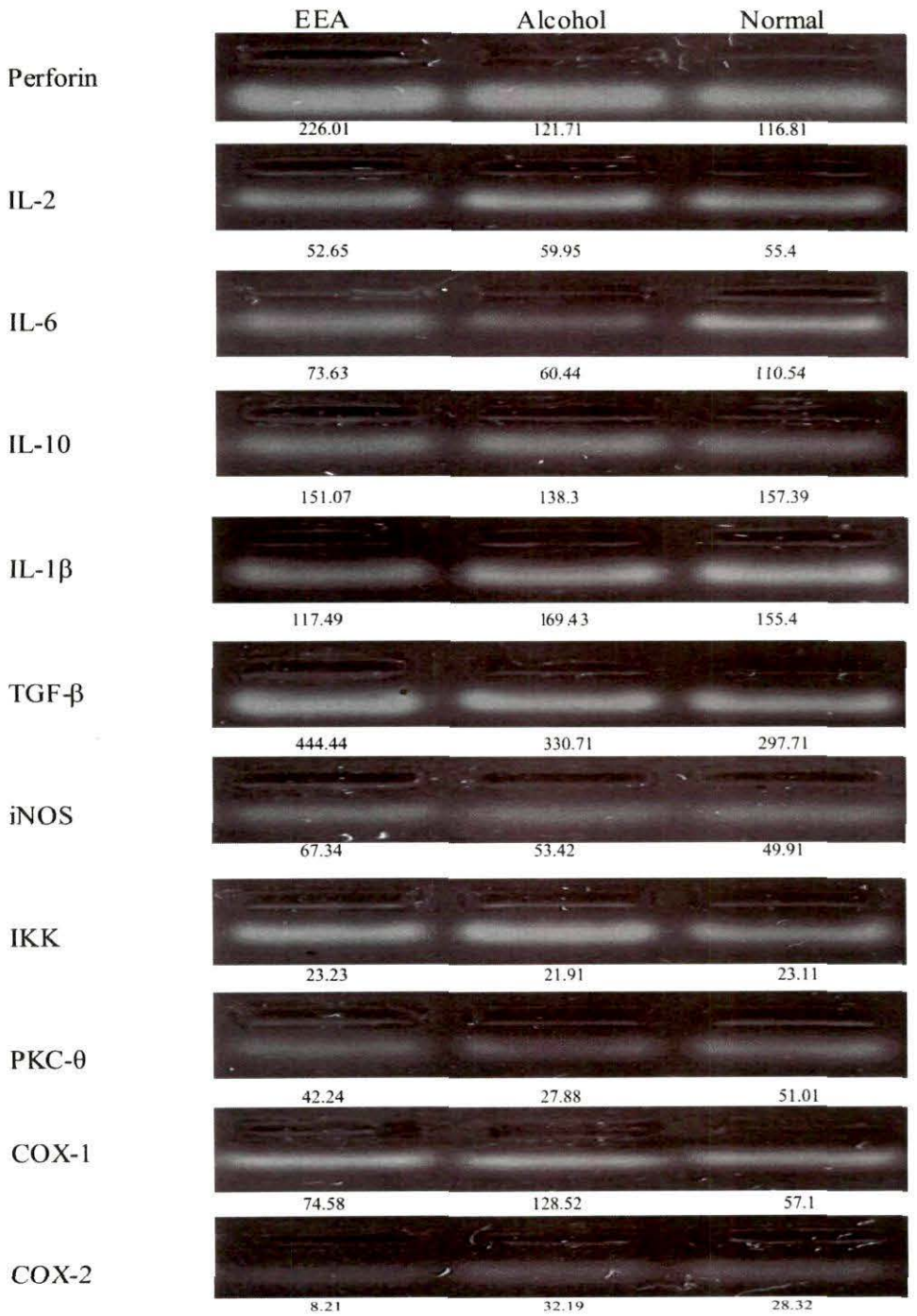


Fig 22: Expression of certain immunologically active genes in T cells from mice with and without EEA treatment, containing 9 mice in each group. Agarose gel electrophoresis of cDNA PCR products amplified with different gene specific primers.

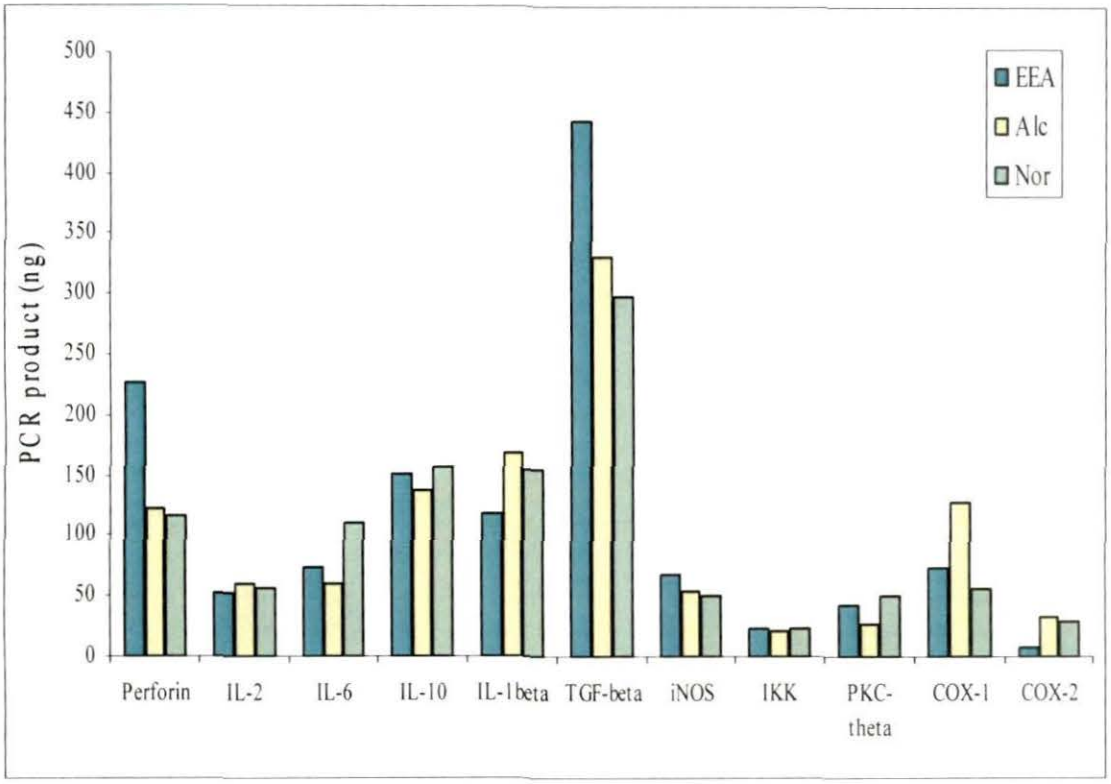


Fig. 23: Graphical presentation of quantification of the expression of the immunologically active genes in T cells from mice with and without EEA treatment, containing 9 mice in each group. The DNA bands were quantified against 30 ng of lambda DNA through ImageAide, Spectronics Corporation, NY.

Effect of EEA on primary and secondary immunization

Increment in total lymphocyte and macrophage count with EEA treatment

EEA treatment caused higher total lymphocyte count than in the control groups both in primary and secondary immunization. EEA treatment could also increase macrophage count and this was much higher in secondary immune response (Table 2).

Table 2: Increment in total lymphocyte and macrophage count in SRBC immunized mice with EEA 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 +	Lymphocyte count x 10^7	Macrophage count x 10^7	Lymphocyte count x 10^7	Macrophage count x 10^7
EEA	4.710±0.0873*	2.3568±0.674*	4.320±0.053*	5.118±0.93**
Alcohol	2.418±0.105	1.428±0.108	3.031±0.024	1.983±0.056
No Treatment	3.476±0.113	1.983±0.370	3.06±0.032	2.24±0.037

PFC response during primary and secondary response

Splenic lymphocytes from mice immunized with 25% SRBC and treated (i.v.) with EEA and alcohol (control) 1 day prior to primary immunization, were taken to enumerate the number of plaque forming cells (PFCs) in the cell population. The number of PFCs or antibody secreting cells can be taken as index for degree of antibody mediated immune response. Higher number of plaque forming cells was enumerated in control groups injected with SRBC only than in the EEA plus SRBC treated ones in primary and also secondary immune response (Fig. 24).

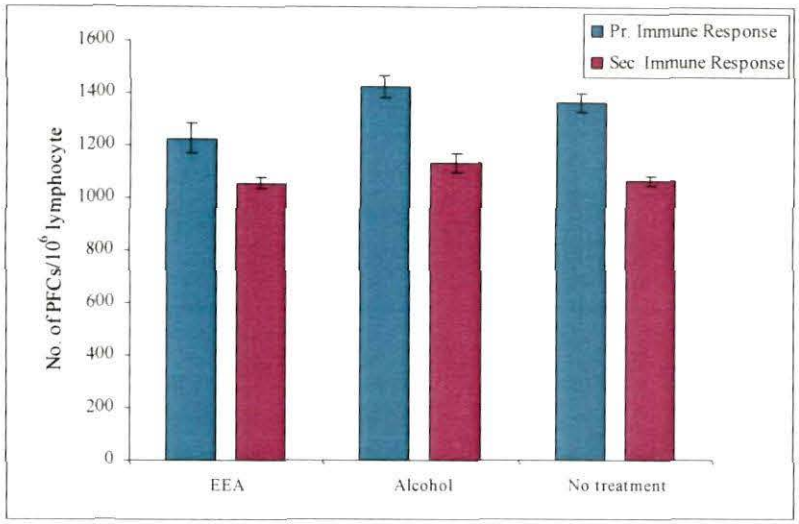


Fig. 24: No. of plaque forming cells (PFCs) in 10^6 lymphocytes from spleen of mice treated with EEA in Primary and Secondary immune response. Results are expressed as mean ± SD.

Amount of IgG in serum of mice after secondary immunization

The level of serum antibody is a direct evidence to judge the degree of stimulation of antibody mediated immune response. The amount of IgG in serum of mice immunized primarily with 0.1 ml 25% SRBC and secondarily boosted with 0.05 ml 25% SRBC, has been quantified by ELISA. The amount of serum IgG in mice after secondary immunization was comparable in all the three groups, EEA treated, alcohol treated and untreated (Fig. 25).

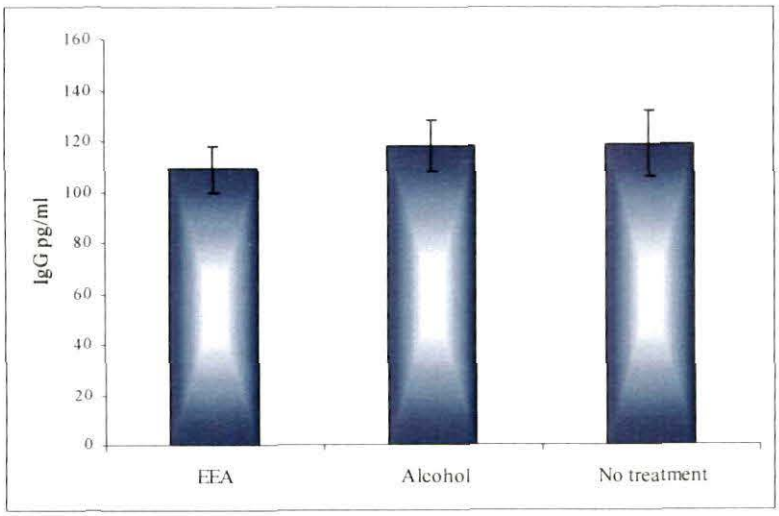


Fig. 25: Estimation of serum IgG level (pg/ml) by ELISA in secondary immune response with EEA treatment, alcohol (control) and no treatment (only SRBC).

Anti-oxidant property of EEA

Inhibition of superoxide generation

The generation of free radicals affects the cellular metabolism in an extensive way. Thus, EEA's role in quenching the generation of free radicals was studied here. EEA could inhibit generation of superoxide radical ($O_2^{\cdot}$) as assayed by autoxidation of hematoxylin. EEA could effectively quench the radical; 25 µl dose of EEA seems to be optimal for the purpose (Fig. 26).

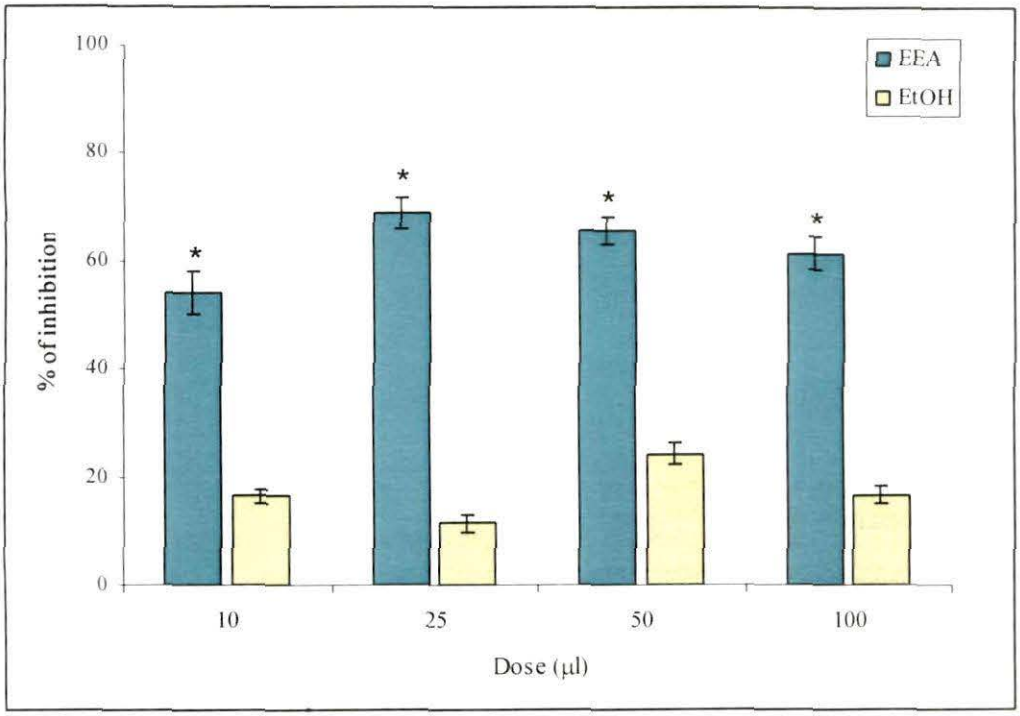


Fig. 26: Inhibition of superoxide radical generation by EEA. (Significance of results with EEA over alcohol control at *$p<0.01$)

Inhibition of lipid peroxidation in murine lymphocytes

$H_2O_2^{\cdot}$ generation induces lipid peroxidation in the cell membrane which in turn produces malonaldehyde (MDA). The ability of EEA to perform as inhibitor for generation of the free radical in murine lymphocytes was tested using copper-ascorbate induced lipid peroxidation assay system. EEA inhibited generation of $H_2O_2^{\cdot}$ in lymphocytes possibly in

a dose dependent manner. Thus, EEA is likely to buy more protection for the immunocompetent cells by inhibiting lipid peroxidation (Fig. 27).

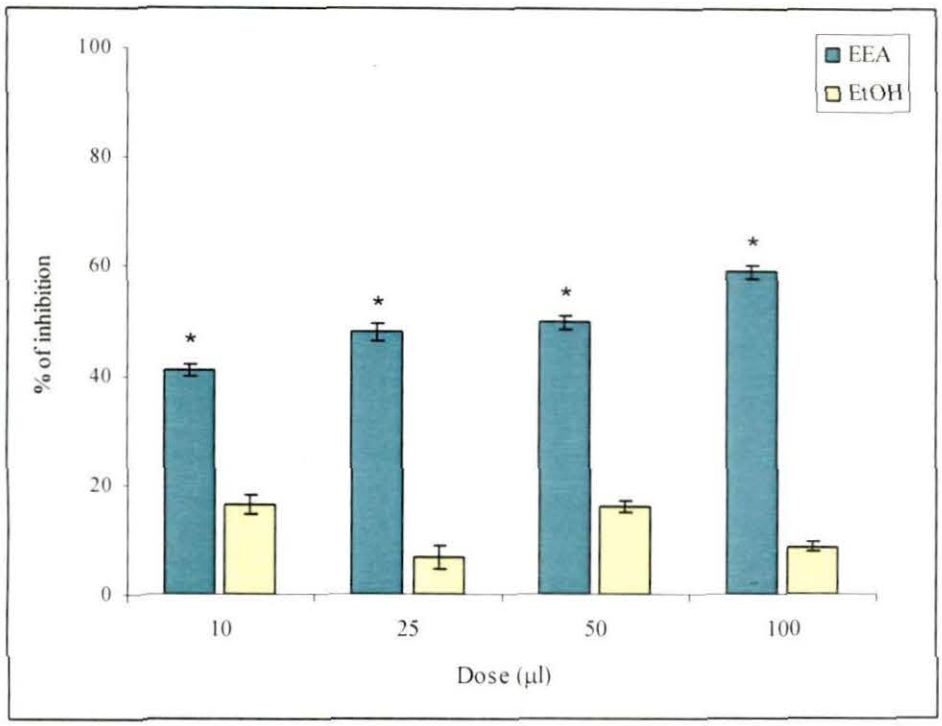


Fig. 27: EEA inhibits lipid peroxidation in mouse splenocytes. (The inhibition was significant over alcohol control at *p<0.01)

Inhibition of hydroxyl radical generation

Hydroxyl radical ($OH^{\cdot}$) is potentially harmful for the cellular macromolecules and is implicated in pathophysiology of inflammation. EEA's ability to inhibit $OH^{\cdot}$ radical generation has been assessed by measuring the final product, malonaldehyde (MDA) from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fentons' reaction). EEA was found also to inhibit hydroxyl radical generation in a dose dependent manner (Fig. 28).

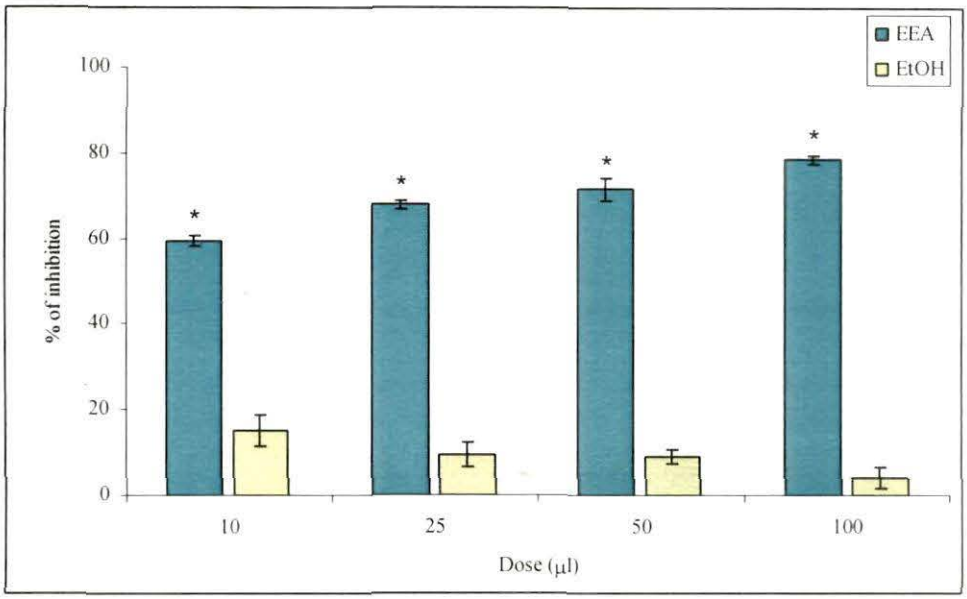


Fig. 28: Inhibition of hydroxyl radical generation by EEA. (significance of inhibition by EEA at *p< 0.01)

Induction of NO· generation in murine lymphocytes by EEA

NO· is one of the smallest molecular products of mammalian cells, it functions in the regulation of many cellular events and in defense against various pathogens and tumorigenic transformation. Here, the level of activity of cellular enzyme nitric oxide synthase (NOS) responsible for generation of NO· from L-arginine was measured in lymphocytes. EEA treatment could increase the NOS activity more than 2 fold with 10 µl dose and about 3 fold with 25 µl one in comparison to the ethanol control (Fig. 29). The higher doses of EEA such as 50 µl and 100 µl could not elicit NOS activity further (Fig. 29).

In all the experiments a particular set of tubes were maintained in each of which 10 µM N^{G}methyl-L-arginine acetate ester (NAME), a competitive inhibitor of NOS, was added to confirm the generation of NO· was due to NOS activity. The addition of the inhibitor in each case did not allow the formation of met-hemoglobin and hence no change in photometric reading.

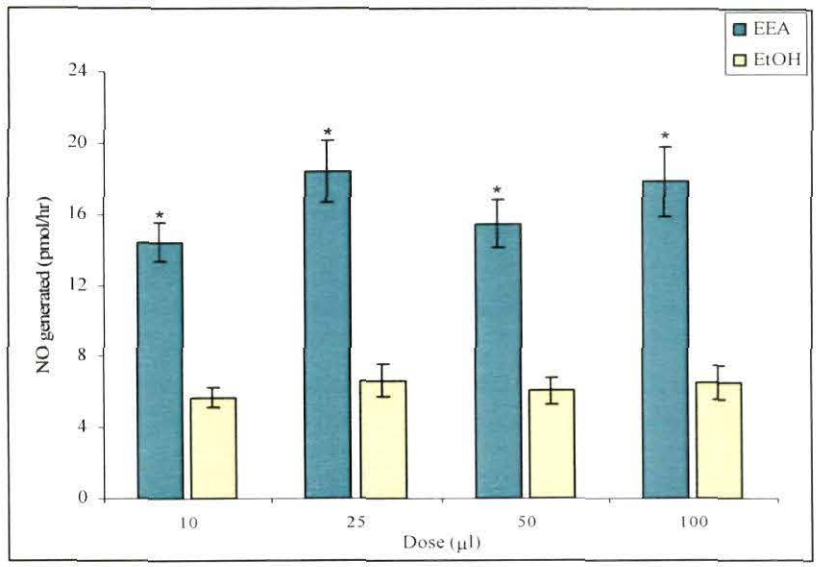


Fig. 29: Generation of nitric oxide in murine lymphocytes in presence of EEA and alcohol. (significance of results with EEA over control at *p< 0.01).

Isolation and chemical characterization of the major fraction from total EEA extract

Thin layered chromatographic (TLC) separation of the total EEA extract with 16% ethyl acetate in petroleum ether showed a thick UV-active band (Rf. 0.54), which seemed to be the major constituent of the total extract. The fraction was isolated further in column chromatography using same percentage of solvent. In addition, high performance liquid chromatography (HPLC) analysis of the total extract also showed highest peak for the fraction at 268 nm, having the highest percentage of the chemical component (Fig. 30).

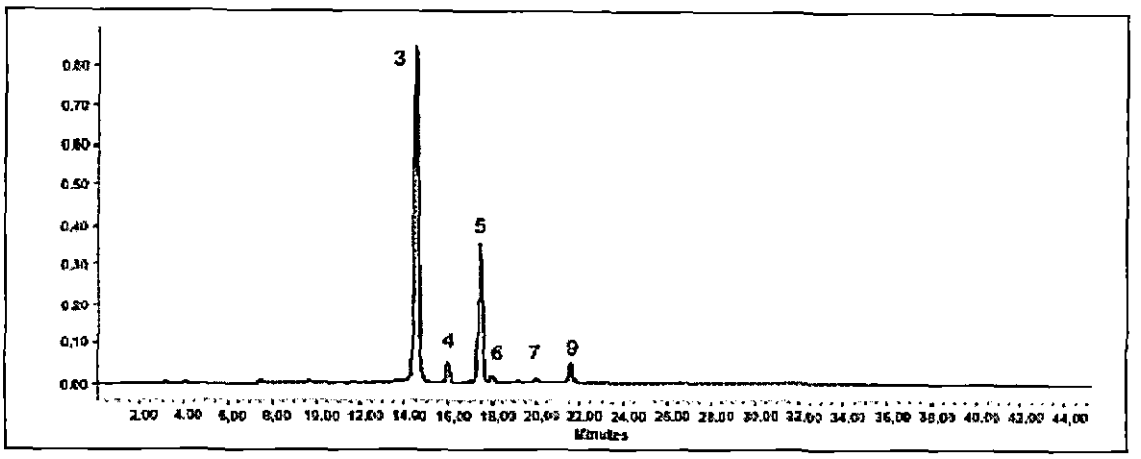


Fig 30: HPLC analysis of total EEA extract showing a major peak at 468 nm.

The IR spectrum of the compound showed absorption bands at 3440, 1720 and 1640 cm^{-1} indicating the presence of hydroxyl, carbonyl and unsaturated moieties in the molecule (Fig. 31). Its ^1H NMR spectrum (Table 3) exhibited five methyl signals at δ 0.69 (s), 0.79 (t), 0.92 (d), 1.16 (s) and 1.56 (s). A pair of olefinic proton signals at δ 4.63 and 4.72 and an olefinic proton signal at δ 5.44 as a doublet (J = 5 Hz) were observed. The latter signal is characteristic for olefinic H-6 in steroid compounds while the pair of olefinic protons was assigned to C-6 methylene protons. The observation of a proton signal at δ 4.02 as a multiplet indicated the presence of a hydroxyl group. The correlation of this proton signal to a methine at δ 68.9 in the HETCOR spectrum showed the presence of a secondary hydroxyl group. Its location and stereochemistry were determined as C-11 and to be in α position based on multiplicity and Dreiding model studies as well as comparison of its spectral data with literature values of similar compounds. The ^{13}C NMR spectrum (by APT technique) revealed 29 carbon signals originating from five methyl, twelve methylene, seven methine and five quaternary carbon atoms (Table 3). The olefinic methylene signal was observed at δ 109.4, the olefinic methine carbon signal was at δ 122.3 and the hydroxymethine carbon signal at δ 68.9. Furthermore, two olefinic quaternary carbon signals were observed at δ 148.6 and 139.4. The presence of a carbonyl group was supported by a 13C NMR signal at δ 210.6 which should not be conjugated to the double bond, based on the olefinic proton chemical shift and multiplicity. Based on the downfield chemical shift of Me-19, which was observed at δ

1.16, indicated the location of the carbonyl group to be at C-1. The HRMS spectrum of the compound gave a molecular ion peak at *m/z* 426.3483, accounting for a molecular composition C29H49O2 (Fig. 32). The stereochemistry at C-14 asymmetric center was investigated by a careful comparison with the 1H NMR spectra of both epimers at C-14 in similar sterols, and deduced to be 24S = 24 configuration. Thus, the spectral analyses suggested the compound to be a terpenoid one.

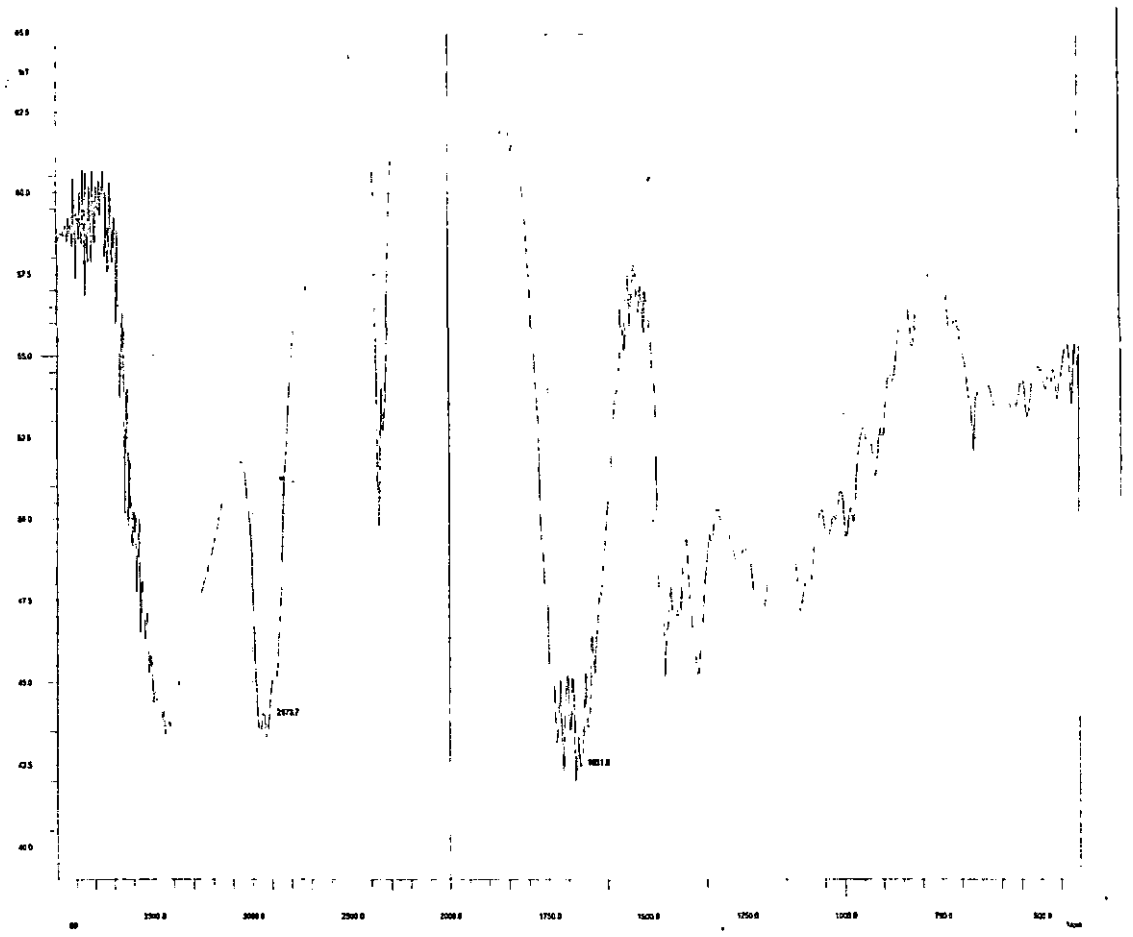


Fig 31: IR spectroscopic analysis of the major fraction from EEA.

Table 3. ^{1}H and ^{13}C NMR data for the fraction isolated from EEA

Pos.	δ_C(DEPT)	δ_H	HMBC	^{1}H-^{1}H COSY	NOESY
1	29.8(CH_2)	1.89 *m* Hα 2.33 *dd* 2.8, 15.5 Hβ		H-1β, H-2, H-10 H-1α, H-2, H-10	H-1β, H-2, H-5, H-10 H-1α, H-2, H-10, H-14
2	68.0(CH)	5.22 br*d* 5.0	H-1β, H-4, H-15	H-1α, H-1β, H-15	H-1α, H-1β, H-15
3	133.8(C)		H-1β, H-2, H-15		
4	125.4(CH)	5.32 *s*	H-2, H-15	H-5, H-15	H-5, H-7, H-13, H-15
5	38.1(CH)	3.54 br*s*	H-1α, H-1β, H-4	H-4, H-10, H-15	H-1α, H-4, H-10, H-13
6	164.2(C)		H-4, H-13		
7	78.5(CH)	4.71 *m*	H-8α, H-13	H-8α, H-8β, H-13	H-8β, H-9
8	41.9(CH2)	0.98 *m* Hα 2.34 *m* Hβ	H-14	H-7, H-8β, H-9 H-7, H-8α,	H-8β, H-10 H-7, H-8α, H-9, H-14
9	27.4(CH)	2.13 *m*	H-8α, H-14	H-8α, H-10, H-14	H-7, H-8β, H-10, H-14
10	39.6(CH)	1.47 *m*	H-1α, H-2, H-4, H-8α, H-14	H-1α, H-1β, H-5 H-9	H-1α, H-1β,H-5, H-14
11	120.3(C)		H-13		
12	174.6(C)		H-13		
13	8.2(CH3)	1.84 *s*		H-7	H-5
14	19.1 (CH3)	0.96 *d* 6.5	H-8α	H-9	H-1β, H-9, H-10
15	20.3(CH3)	1.71 *s*	H-4	H-4, H-5	H-2, H-4
COCH3	170.7(C)		H-2, COCH3		
COCH3	21.1(CH3)	2.09 *s*			

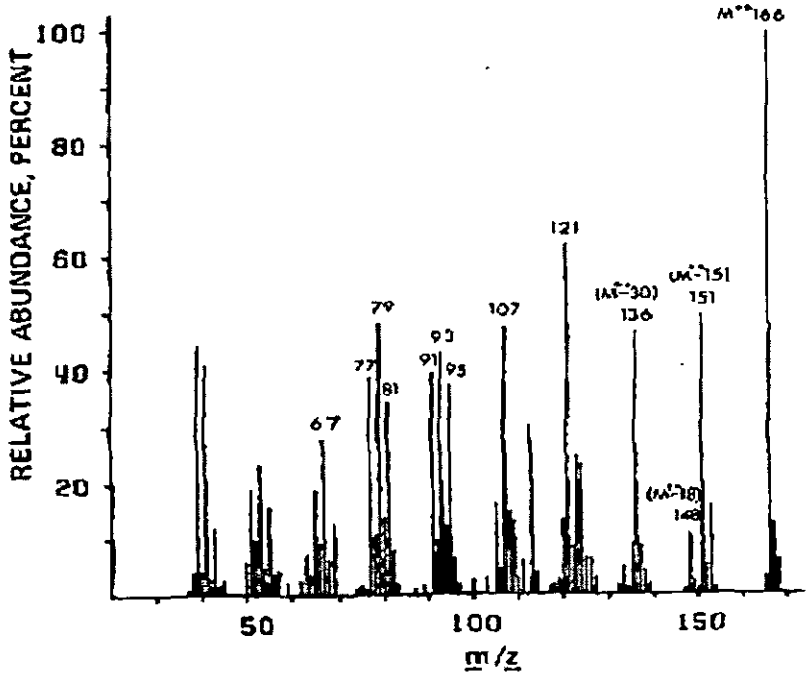


Fig 32: Mass spectroscopic analysis of the major constituent isolated from EEA

Effects of the major fraction of EEA isolated in chromatography on blastogenesis and generation of ROS

The major fraction from total EEA extract was isolated by column chromatography and tested for its effect on blastoid transformation of T and B cells, and on generation of free radicals, $O_2^{\cdot}$, $H_2O_2^{\cdot}$, $OH^{\cdot}$ and $NO^{\cdot}$. For column chromatography 16% ethyl acetate solvent was used. The fraction was dried and dissolved in ethanol maintaining the dry weight equivalence as in original EEA extract. 25 µl of the fraction was used for each experiment and results were compared with that of equal amount of EEA and ethanol (control).

Effect on blastoid transformation of T and B cells

The fraction from EEA could not elicit blastoid transformation of T and B cells beyond alcohol control (Fig. 33) *in vitro* at 24 and 48 hrs.

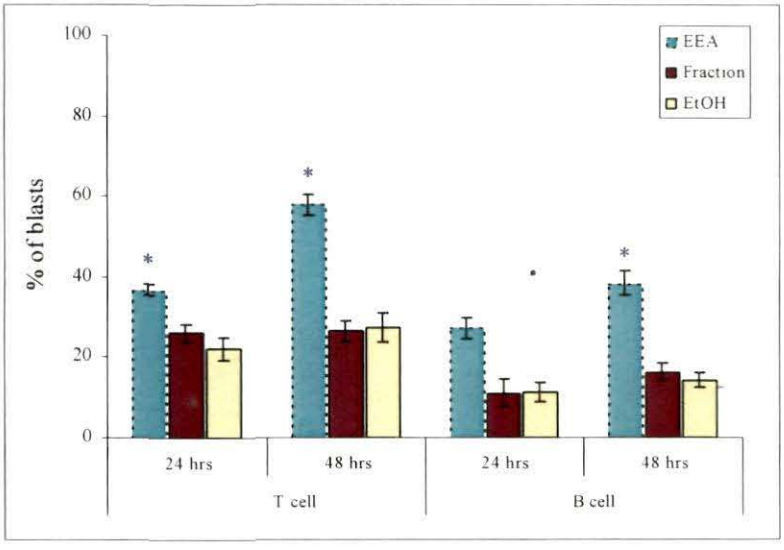


Fig. 33: *In vitro* blastogenic transformation of T and B cells after treatment with the major fraction from EEA and compared to that of EEA total extract. (Significance of results at *p< 0.01).

Effect on inhibition of superoxide radical generartion

The fraction of EEA could significantly inhibit autoxidation of hematoxylin and generation of superoxide radical (Fig. 34); however the EEA extract seemed to be slightly better than the fraction.

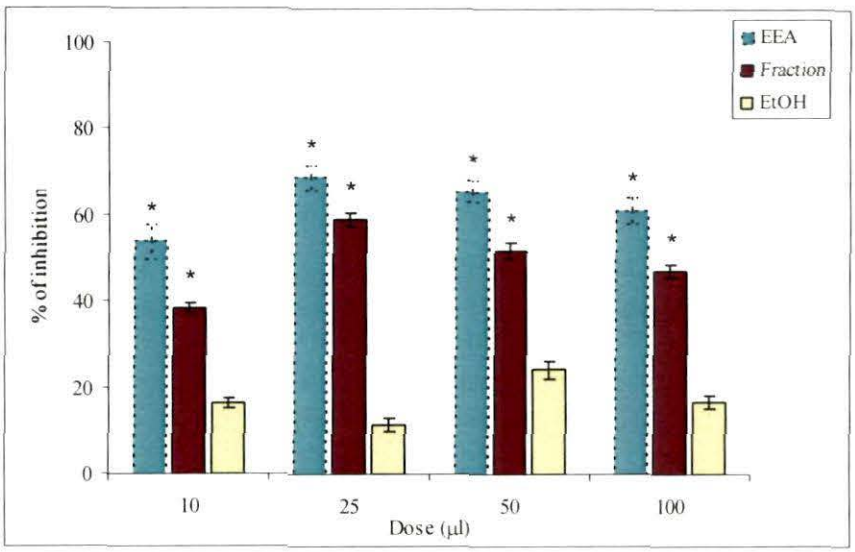


Fig. 34: Inhibition of superoxide radical by the fraction of EEA and compared to that of EEA total extract. (Significance of results at *p< 0.01).

Effect on inhibition of lipid peroxidation in mouse splenocytes

Inhibition of lipid peroxidation, measured using copper-ascorbate system, with the fraction from EEA was comparable to that of total EEA extract and both were significantly high when compared to alcohol control (Fig. 35).

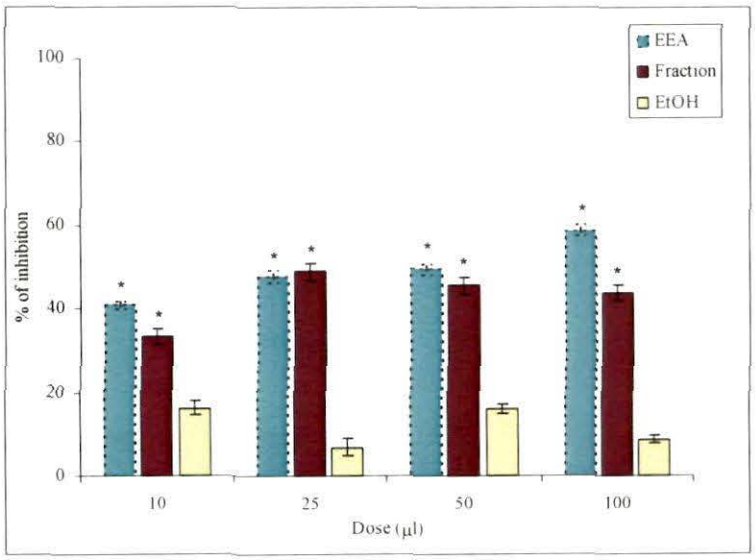


Fig. 35: Inhibition of lipid peroxidation by fraction from EEA and compared to total EEA extract. (Significance of results at *p< 0.01).

Effect on generation of hydroxyl radical

The Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fentons' reaction) revealed that the fraction from EEA was equally effective as total EEA in inhibiting hydroxyl radical with all the four doses (Fig. 36).

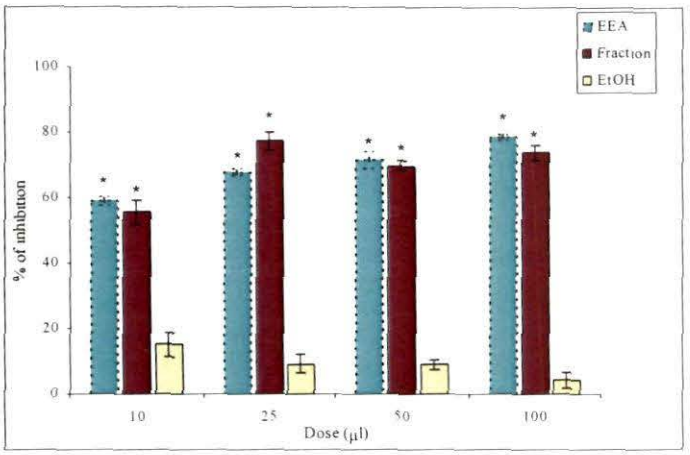


Fig. 36: Inhibition of hydroxyl radical generation by fraction from EEA and total EEA. (Significance of results at *p< 0.01).

Effect on generation of NO· in mouse splenocytes

Treatment with fraction from EEA could not induce NO generation as effectively as the total EEA extract (Fig. 37). Rather the level generated with the fraction was comparable to that of control.

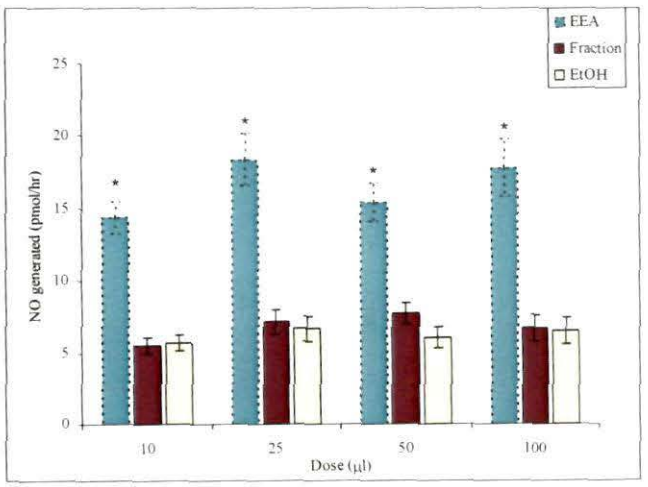


Fig. 37: Generation of NO· in mouse splenocytes treated with total EEA and fraction from EEA. (Significance of results at *p< 0.01).

DISCUSSION

Herbal medicines with their potency and less side effects are getting much attention nowadays for treating various diseases including inflammation. The ethnomedicinal use of *Eupatorium adenophorum* for treating sores indicated its possible anti-inflammatory and immunomodulatory properties. To screen the toxicity is a foremost requirement for introducing any use of an extract from herbal source. Irrespective of their target organs, the extracts are carried to their sites of action via the blood stream. Therefore, any natural product or plant extract is subjected to test for its any toxic effect towards the hematological parameters prior to investigating any pharmacological properties (Gupta, Mazumder & Das, 1994; Iranloye, 2002; Senthilkumar, 2008). To begin with the hematological parameters, such as percentage of haemoglobin, RBC count and WBC count were considered after injecting the ethanolic leaf extract of *E. adenophorum* (EEA) in mice. The haematological assays apparently indicated that EEA doesn't have any deleterious effect on any of the parameters rather possibly plays some promotional role for the leukocytes (Fig. 1-3). The viability assay of lymphocytes also indicated better survivality for lymphocytes with EEA (Fig. 4) and therefore no inimical effect on the immunocompetent cells.

The promotional effect of EEA for leukocytes advocated probable immunostimulatory activity of the extract. Indeed, EEA could induce blastogenesis in lymphocytes in general and T cells more efficiently, *in vitro* (Fig. 5). This transformation is likely to contribute to the increased number of leukocytes (Fig. 3). This was again supported by DNA synthesis data (Fig. 6). The cell cycle analysis by FACS showed that EEA could drive more cells

towards S and G2-M phases (Fig. 7), leading to cell division. The cell division was shown to be prerequisite for functional differentiation of the lymphocytes (Chakravarty & Clark, 1977; Das & Chakravarty, 1998).

Anti-inflammatory activity of EEA was judged in reference to its ability to inhibit inflammatory delayed type hypersensitive (DTH) reaction set in mouse foot paw by injecting two different doses of 2,4-dinitrofluorobenzene (DNFB). Both topical and intravenous application of EEA could effectively suppress the inflammatory reaction. Topical application of EEA was more effective in inhibition of the swelling of foot paw and gaining normalcy faster than its i.v. application (Fig. 8-12).

Different leukocytes infiltrate at the site of inflammation and play a crucial role in regulating the reaction. After infiltration, monocytes (turned into macrophages), neutrophils and eosinophils release cytokines, lytic enzymes, and phagocytose the inflammatory tissues and thus lead to aggravation of the inflammation reaction. EEA could inhibit recruitment of phagocytic inflammatory cells like neutrophils and monocytes at the DTH site (Fig. 13). The lower level in recruitment of the phagocytic cells is likely to provide relief from ongoing inflammation reaction at DTH site. However, the percentage of lymphocyte at the DTH site was significantly high with EEA treatment (Fig. 13). In fact EEA proved its worthiness in induction of blastogenesis and cell division of lymphocytes as indicated by DNA synthesis and FACS analysis (Fig. 5-7). In lymphocyte population EEA treatment particularly stimulates the T cell better (Fig. 5). The increase was notable with the $CD4^+$ T cells (Fig. 14), the lymphocyte type known to play pivotal role in inflammation. The $CD4^+$ T cells secrete all different kinds of

cytokines to regulate participation of other cells types in inflammation (Ehlers, Mielke & Hahn, 1994; Matsushima & Stohlman, 2005; Poulter *et al.*, 1982).

A special category of T_{DTH} cells in $CD4^+$ T cell population is considered to play major role in DTH reaction (Black, 1998). The T_{DTH} cells secrete various cytokines including the pro-inflammatory and anti-inflammatory ones to regulate the DTH reaction. TNF-α is one of the most important cytokine involved in the process (Dinarello, 2000; Strieter, Kunkel & Bone, 1993; Lukacs *et. al.*, 1995; Tracey & Cerami, 1994). EEA induced higher level of serum TNF-α, surpassing the level in mice undergoing DTH reaction or in mice treated with alcohol (Fig. 15). It is worthwhile to note EEA capable of inhibiting DTH reaction is inducing a pro-inflammatory cytokine like TNF-α. Banno *et al.* (2004) showed TNF-α promotes tissue repair of damage skin by inducing basement membrane components and collagen degrading proteases to participate actively in reconstruction of extracellular matrix. Yoshida and his coworkers (1997) found TNF-α can also induce growth promoting event like angiogenesis by increasing mRNA level of IL-8, vascular endothelial growth factor, fibroblast growth factors in endothelial cells. The present study suggests similar participation of TNF-α in tissue repair and regeneration to bring back normalcy aftermath of DTH reaction. Thus, TNF-α plays double role in DTH reaction – pro-inflammatory cum restoring agent.

The ability of EEA to induce higher level of the cytokine TNF-α made it imperative to study the effect of the plant extract on expression of *TNF-α* gene encoding the cytokine. Indeed, EEA induced higher level of expression of the gene (Fig. 16 & 17). Thus the inducing ability of EEA for *TNF-a* gene could be correlated with higher level of the cytokine in serum of DTH mice treated with EEA. Hence, the effect of EEA on certain

other cytokines and inflammatory mediators could also be carried out only by studying expression analysis of these genes. EEA did not necessarily affect the expression of gene for other pro-inflammatory cytokines such as *IL-1β* and *IL-6* in similar fashion. EEA inhibited *IL-1β* expression and did not influence the expression of *IL-6* gene (Fig. 10 & 11). It seems that inhibition of IL-1β, the cytokine without any known function in repair mechanism, manifests more of inhibitory effect of EEA on inflammation. Stimmeder and his co-workers (Berg *et al.*, 1999) observed that lornoxicam and other non steroidal anti-inflammatory drugs inhibit *IL-1β* expression as well as inflammation. Kohli *et al.* (2005) reported curcumin, the active component in the rhizome of *Curcuma longa* Linn., demonstrates its anti-inflammatory activity by inhibiting production of IL-1β in lung inflammatory cells.

An anti-inflammatory agent seems not to regulate all the anti-inflammatory cytokine genes always; in the present study did EEA did not influence anti-inflammatory cytokine gene *IL-10* (Fig. 16 & 17).

EEA up regulated expression of *TGF-β* (Fig. 16 & 17). TGF-β performs as a growth factor in all different kinds of events of collagen production and extracellular matrix reorganization as shown by Barcellos- Hoff (1993). This cytokine might function here to restore normalcy along with TNF-α in repair mode as discussed earlier. Simultaneous up regulation of these two genes have also been observed by Chao *et al.* (1995) in microglial cell culture. Sullivan *et al.* (2005) also reported similar trend in expression of these two cytokines in interstitial pulmonary fibrosis affected lung fibroblasts.

Tak and Firestein (2001) and Yamamoto and Gaynor (2001) elucidated involvement of NF-κB pathway for induction of inflammation. Activation of NF-κB is mediated by

action of inhibitory kappa kinase (IKK) degrading inhibitory IκB subunit. Thus measuring the expression of *IKK*, one can derive the involvement of NF-κB pathway in a reaction. EEA could not influence much the expression of *IKK* gene in relation to the controls (Fig. 16 & 17) suggesting inability of EEA in activation of NF-κB pathway for DTH reaction. Much of induction of the pathway is likely to aggravate the inflammatory reaction (Ghoshm May & Kopp, 1998; Sethi, Sung & Aggarwal, 2008).

COX1 and COX2 gene products are two isoforms of cycloxygenase enzyme which metabolizes arachidonic acid into the inflammatory mediators like leukotrienes and prostaglandins respectively (Turini & DuBois, 2002; Smith *et. al.*, 1998; Smith, Garavito & DeWitt, 1996; Mitchell, Larkin &Williams, 1995; Vane, Bakhlel & Botting, 1998; Chen *et. al.*, 1997 and Devaux *et. al.*, 2001). Notably EEA only influences expression of *COX2* gene by way of inhibition (Fig. 16 & 17). This may be another way of execution of anti-inflammatory activity by EEA. Salvioli *et al.* (2007) reviewed curcumin, a potent anti-inflammatory agent, that also inhibits COX2 in abetting inflammation.

Wahl and Wahl (1992) showed the beneficial aspect of inflammatory events like release of cytokines and growth factors to induce vascularization and other tissue growth in wound healing. Bodekar and Hughes (1996) demonstrated that wound healing involves a variety of processes such as acute inflammation, cell proliferation etc. EEA besides inhibiting inflammatory swelling and inflammation related injuries could also induce faster healing of linear skin incision (Fig. 18) and burnt wounds (Fig. 19) in mice. Therefore it seems that the upregulation of TNF-α and TGF-β genes with EEA treatment may contribute in the process of faster wound healing by the plant extract.

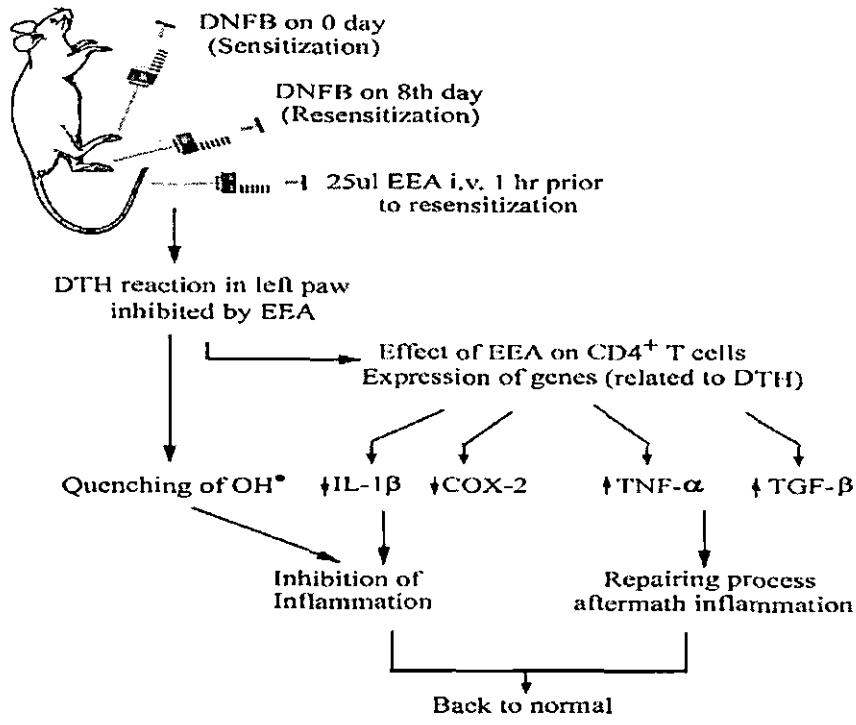


Fig. 38: Effect of EEA on inhibition of DTH reaction and repair mechanism presented schematically.

The cytokine and growth factor mediated angiogenesis and other tissue growth promotional events in the aftermath of inflammation, have also been observed in malignant growth. The association between inflammation and cancer has been noted in nineteenth century when Rudolph Virchow (1863) first observed the presence of leukocytes in neoplastic tissues (Balkwill and Mantovani, 2001). In recent time, scientists have shown that a number of cancers are linked to inflammatory origins (Farrow and Evers, 2002; Ben-Baruch, 2006; Schwartsburd, 2003). Farrow and Evers (2002) showed that inflammatory cytokines, mediators and reactive oxygen species during pancreatitis could increase cell cycling, cause loss of tumor suppression and stimulate oncogene expression, and lead to pancreatic malignancy. Schwartsburd (2003) proposed a model

showing chronic inflammation is capable of generating a potentially 'vicious self-sustaining loop(s)' resulting in the procancer microenvironment favourable for survival of tumor cells and their growth. Thus, chronic inflammation leads to an environment that fosters genomic lesions and tumor initiation. Ben-Baruch (2003) reviewed how tumor associated macrophages (TAM) secrete various chemokines which provide signal for angiogenesis during development of breast cancer. In addition, the tumor microenvironment in many cases was also found to resemble an inflammatory site (Coussens and Werb, 2002; Arias *et al.*, 2007). The inflammatory response leads to enhanced production of AP-1, survivin etc which in all probablity provide survival and proliferative signals to the transformed mutated cells, thereby leading to tumor promotion. Involvement of activation factor NF-kB (Chen et al., 2003, Pikarsky et al., 2004), pro-inflammatory cytokines (Zlotnik, 2006), cycloxygenases (Wang & DuBois, 2006) and angiogenic growth factors (Coussens *et al*, 1999) have been suggested to play key role in development of tumor. Geetha *et al.* (1998) and Sarkar *et al.* (2005) reported the ROS mediated damage in coonective tissues leading to aggravation of inflammation. Enhanced generation of free radicals such as $O_2^{\cdot}$, $OH^{\cdot}$, $NO^{\cdot}$ and peroxynitrite ($ONOO^{\cdot}$) during inflammation reaction is known to be contributor toward inflammation reaction as well to the microenvironment necessary for induction of tumors, likely affecting genomic DNA. Events involved in inflammation reaction and tumor induction have been summarized in figure 39.

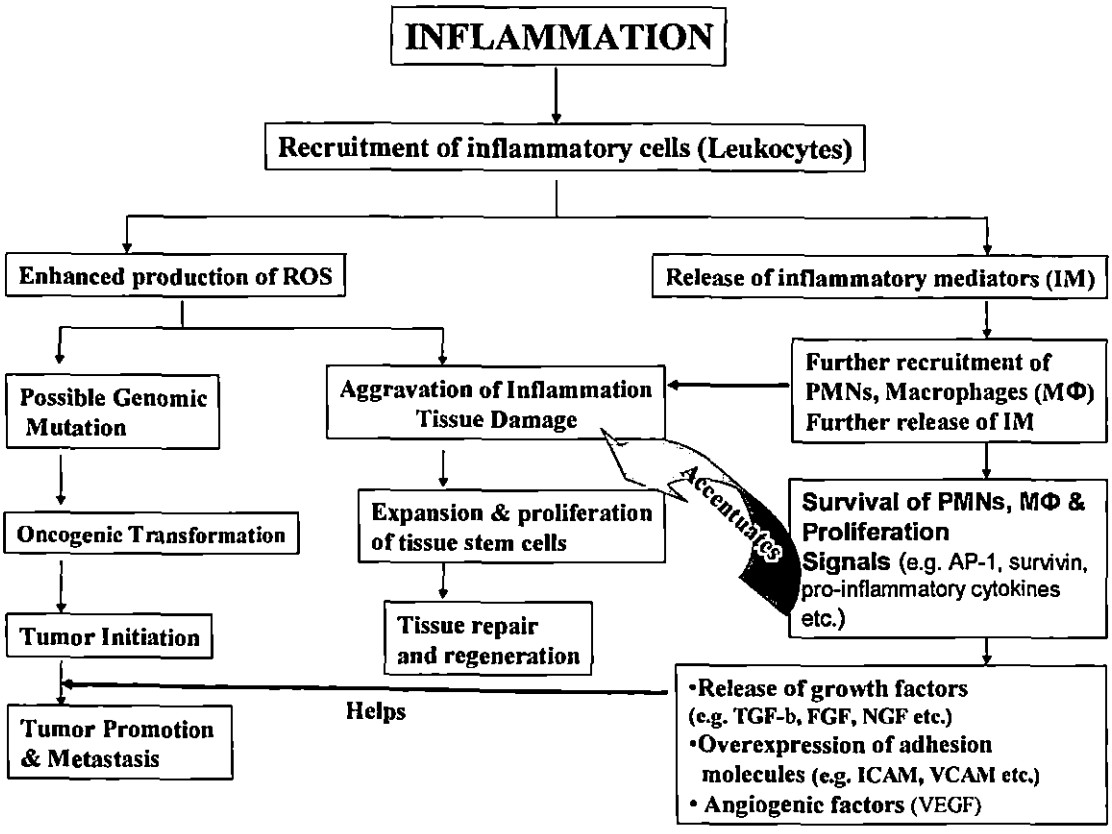


Fig.39: Summation of inflammation related events leading to tissue repair or promotion of tumor.

In view of involvement of similar factors in development of inflammation and malignant growth, it is logical to use an inhibiting agent to one for the other. That is why EEA with inhibitory property for inflammation was topically applied on solid tumors induced subcutaneously with cell line. EEA could retard the growth of tumor to some extend at initial phase and increased the longevity of tumor bearing mice about 15 to 20 days in comparison to control (Fig. 20). It is possible that inhibition of proinflammatory mediators such as IL-1β and COX-2 by EEA (Fig. 16 & 17) could also affect retardation of the tumor growth.

Although EEA has been found here as an effective anti-inflammatory agent, its anti-tumor effect is recognizable but not all curing. Rather ethanolic extract of turmeric,

Curcuma longa Linn.was found to be anti-inflammatory as well inhibitory for Ehrlich ascites carcinoma (Yasmin and Chakravarty 2010).

EEA could activate T cells for blast transformation (Fig. 5) as first step towards their functional differentiation, as well it has some minimal anti-tumor effect. Thus, it was necessary to judge cytotoxicity level of EEA activated T cells towards ^{51}Cr labeled tumor cells. The percentages of cytotoxicity were at significant level (Fig. 21). Chakravarty and coworkers showed earlier (Chakravarty & Maitra, 1983, 1990; Charavarty & Jha, 1997) that polyclonal stimulation of murine T cells with Con A could generate effective cytotoxic response against fibrosarcoma. Knuth *et al* (1984) and Krackhardt *et al* (2002) could generate similar response by activation with PHA.

As EEA was reasonably effective to raise cytotoxic T cells against tumor target cells, its ability to activate *perforin* gene in the T cells was studied. Perforin molecules polymerize to form pores on target cell membrane to mount cytotoxic damage to the target cells. Clarke and his coworkers (Walsh, 1994) demonstrated that mice lacking *perforin* gene could not mount immune response against lymphocytic choriomeningitis virus (LCMV). We observed that EEA could upregulate the expression of *perforin* gene in T cells obtained from mouse injected i.v. with EEA (Fig. 22 & 23). Besides activation of *perforin*, EEA induced expression of the cytokine *TGF-β* (Fig. 22 & 23), which contributes towards T cell activation. TGF-β also acts as growth factor at the end of DTH reaction (Fig. 16 & 17).

Although EEA could activate T cells and cell mediated immune response, but could not induce B cell mediated immunity beyond the control, as measured by plaque forming cell assay (Fig. 24) and measure of serum IgG (Fig. 25) in mice.

Both inflammatory DTH reaction and tumor growth are accompanied with enhanced production of reactive oxygen species (ROS) (Fig. 39). Haddad and coworkers (Haddad, Olver & Land, 2000; Haddad, 2002a, 2002b & 2004) demonstrated that ROS can regulate a variety of signaling pathways and induce inflammation by production of pro-inflammatory cytokines. MacNee and Rahaman (1999) and MacNee (2001) also showed that generation of ROS play a crucial role in developing airway inflammation in lung. Halliwell (1997) and Thannekal and Fanburg (2002) discussed ability of ROS to alter various cell signaling pathways and effect on human diseases like inflammation and cancer. The association of ROS and carcinogenesis has also been documented by others (Klaunig & Kamendulis, 2004; Vafa *et al.*, 2001). Bandyopadhyay, Das and Banerjee (1999) reported that about 5% or more of the inhaled oxygen is converted to ROS such as superoxide ($O_2^{\cdot}$), hydrogen peroxide ($H_2O_2^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$) by univalent reduction of O_2. EEA has been found effective in inhibition of the inflammatory DTH reaction and to some extent tumor growth, at the same time it effectively quenches the generation of these three deleterious ROS – $O_2^{\cdot}$, $H_2O_2^{\cdot}$ and $OH^{\cdot}$ (Fig. 26-28). $O_2^{\cdot}$ and $OH^{\cdot}$ radicals are the highly reactive ones that attack cellular macromolecules like proteins and nucleic acids and cause damage to them; and $H_2O_2^{\cdot}$ attacks membrane lipids resulting in increased leakyness and disintegration of the plasma membrane. Thus, quenching of these three free radicals by EEA probably contributes in better survivality of lymphocytes in later hours of viability assay (Fig. 4). EEA could also induce $NO^{\cdot}$ generation in mouse lymphocytes from L-arginine by activating inducible nitric oxide synthase (Fig. 29). Bredt & Snyder (1994), Gladwin, Crawford & Patel (2004) and Koncz *et al* (2007) designated $NO^{\cdot}$ as a messenger molecule in different biological functions. Chakravarty &

Yasmin (2008) showed the $NO^{\cdot}$ generation in T cells increases with cytotoxic differentiation of the cells. Thus, quenching of the three deleterious ROS and induction in $NO^{\cdot}$ generation seem to inhibit the deleterious effect of the free radicals and activation of the cells to function better.

EEA, without any hemotoxic effect, could effectively inhibit DTH reaction, downregulate pro-inflammatory mediators, induce growth promoting cytokines to bring back normalcy in the aftermath of DTH reaction, and quench free radicals like $O_2^{\cdot}$, $H_2O_2^{\cdot}$ and $OH^{\cdot}$; and therefore seems to be a potent anti-inflammatory agent. The number of such anti-inflammatory agents isolated from herbal sources is very few till date. The natural products of herbal origin offer a vast structural diversity and are small molecules (<1000 daltons) with existing drug like properties (Harvey, 1999; Wagner, 1993). Most plant products with potent anti-inflammatory and immunomodulatory activities explored so far belong to terpenoids, flavonoids, isoflavonoids, alkaloids, quinones, isobutylamides and simple phenolic compounds (Wagner, 1990; Atal, 1986). Majority of the above mentioned compounds bear phenolic hydroxyl groups in their structure. Phenolic compounds are proton donor and e^{-} receiver, and thus they can quench free radicals (Tiwari, 2001) and inhibit their generation by neutralization of $O_2^{\cdot}$ radical, the first generated reactive oxygen species (ROS). Inhibition of ROS by these compounds is likely to play a central role in regulating the downstream cascade reaction and various signaling pathways (Arch & Thompson, 1999; Naik, 2003; Simmons, 2006). Wegener and Fintelmann (1999) proposed a similar mechanism for different pharmacological activities of flavonoids, carried out by their anti-oxidant and enzyme modifying actions. Similarly, anti-oxidant effects of terpenoids like curcumin account for their ability to

protect against degenative diseases (Cohly *et al.*, 1998; Conney *et al*, 1997) and inflammation (Ireson *et al.*, 2001; Kohli *et al*, 2005).

Isoflavones have phenolic OH as functional group in A and B ring and are effective in inhibiting lipid peroxidation. Toda and Shirataki (2002) showed that among the three different isoflavones from *Sophora moorocrotiana*, licoisoflavone B was more effective to inhibit lipid peroxidation, with IC_{50} (50% inhibitory concentration) value of 2.7 μM. The other two, licoisoflavone A and sophoraisoflavone A could inhibit lipid peroxidation in less effective manner. The authors also demonstrated that the presence of 2□ hydroxyl group in B ring of licoisiflavone B is responsible for its stronger inhibitory role in lipid peroxidation than the two other isoflavones. Hu *et. al.* (1995) had already demonstrated that superoxide anion scavenging activities of isoflavones increase with an increasing number of hydroxyl radicals in the B ring. Wagner (1993) showed phenolic hydroxyl groups at 'ortho' position in ring A and B of flavonoids and catechins are responsible for anti-oxidant activities and subsequent inhibition of cycloxygenases. In our case, EEA with significant anti-oxidant activity could also inhibit expression of *COX-2* (Fig. 16 & 17). Thus, the different activities of natural compounds belonging to different classes like alkaloids, terpenoids, flavonoids, etc, or sometimes even in the same class as in case of isoflavones, might depend on the presence of elctrophile functional groups such as phenolic '-OH' rather than their chemical nature.

The fraction of EEA under major peak obtained from HPLC was analysed further by IR spectroscopy. IR spectroscopy suggested the substance in the main peak to have phenolic 'OH' group (Fig. 31). It seems that phenolic OH of EEA might have played a role in inhibition of ROS. Many researchers have shown different plant extract containing such

phenolic compounds like alkaloids, flavonoids, terpenoids, etc. to possess anti-inflammatory and anti-tumor activities as we found with EEA. Efficacy of the extract from these plants in inhibition of inflammation, ROS generation and tumor growth, as evidenced from published literature, have been compared here with EEA (Fig. 40). To obtain the index in each case the vehicle control values, which is alcohol in our case were devided by experimental values and any index ratio of 1.2 or more has been considered to have positive effect. EEA, with maximum index value for inhibition of inflammation seems to be the most effective anti-inflammatory agent (Fig. 40). Whereas *Curcuma longa* (turmeric) rhizome extract acted better for anti-tumor activity supassing EEA. Interestingly, the extracts from these plants have comparable anti-oxidant activity. That does not necessarily qualify all to be anti-inflammatory to the same tune.

EEA also could inhibit expression of genes for pro-inflammatory mediators, *IL-1β*, and *COX-2* to inhibit inflammation reaction (Fig. 16 & 17), upregulate growth promoting cytokines like *TNF-α* and *TGF-β* to bring back normalcy of the inflammation site (Fig. 16 & 17) and induce *perforin* expression to mount cytotoxic function of T cells against tumor cells (Fig. 22 & 23) at RNA level as discussed earlier. Several researchers have documented that different plant products with anti-inflammatory and anti-tumor activities could also alter expression of molecules like NF-κB, AP-1, pro-inflammatory cytokines etc. and that have been listed in Appendix 2. From the appendix 2, and the present investigation some correlation can be drawn between anti-inflammatory and anti-tumor activities of an agent and expression of certain genes related to these phenomena. The effectiveness of some new agents for these functions might be screened on the basis of

gene expression rather than elaborate functional analysis. Probably that will facilitate faster screening of bioactive agents from the vast reservoir of nature.

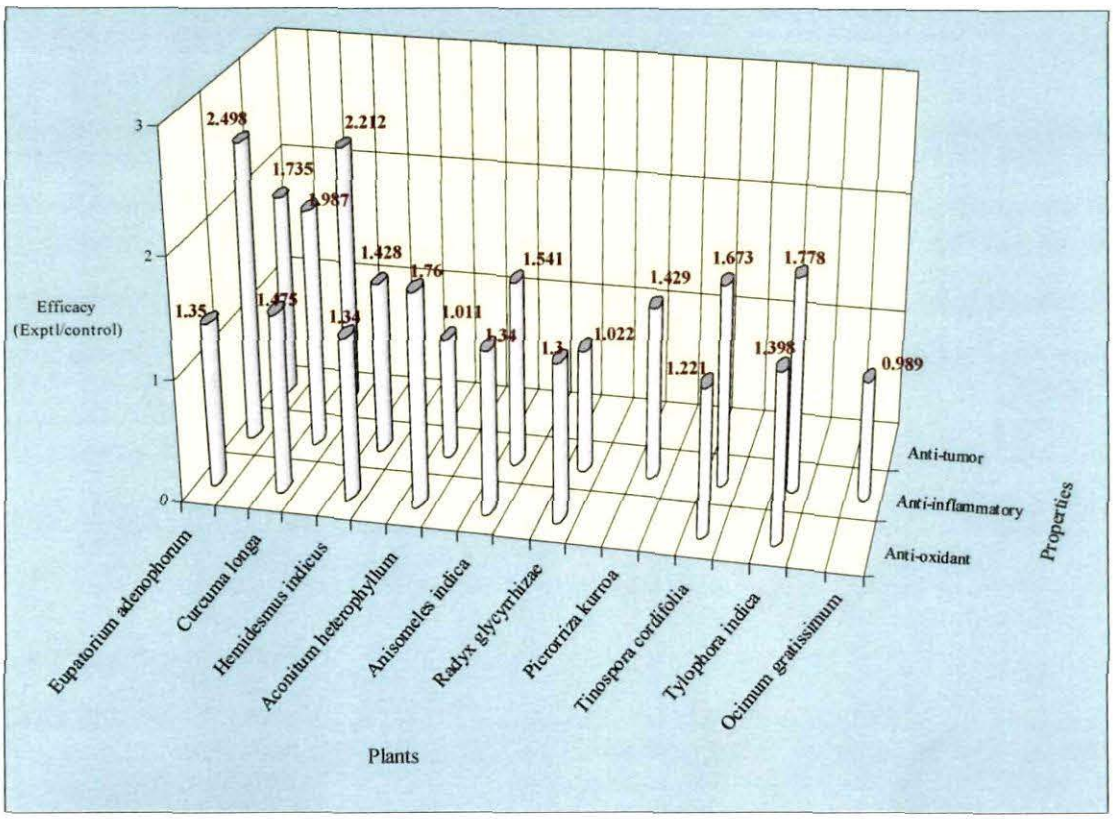


Fig 40: Immunopharmacological properties of EEA compared with certain other plant extracts. Indices represent the level of efficacy of the extracts as ratio over control; the indices presented at the top of the column for easy representation. Any index ratio of 1.2 or more has been considered to have positive effect. Data for the natural products from the plant species other than *E. adenophorum* compiled on the basis of data in published literature. The chemical nature of the active substance is in Appendix 1.

Chemical characterization of an extract like EEA was necessary to find out the active component(s). The major fraction of EEA extract isolated in column chromatography, and identified under HPLC peak at 268 nm (Fig. 30) was subjected for IR spectral analysis. It was found to have phenolic '-OH' and '›C=O' functional groups (Fig. 31). The presence of phenolic '-OH' is likely to contribute in quenching of ROS by EEA (Fig.

26-28) and its isolated major fraction (Fig. 34-36). NMR (Table 3) and mass spectroscopy (Fig. 32) revealed further that the major fraction of EEA is a terpenoid compound.

Comparison of biological efficacies of the total EEA extract and its major fraction containing terpenoid revealed that the total extract is better to some extent in inducing blastogenesis of lymphocytes (Fig. 33) and generation of $NO^{\cdot}$ in the cells (Fig. 37). However, total extract and the major fraction are more or less equally effective in quenching of ROS like $O_2^{\cdot}$, $H_2O_2^{\cdot}$ and $OH^{\cdot}$ radicals (Fig. 34-36). At times, better efficacy of total EEA could be due to the presence of certain other components got lost in course of purification of the fraction.

The ethanolic leaf extract of *Eupatorium adenophorum* thus seems to be a potent anti-inflammatory agent with anti-tumor activity to some extent and effective immunostimulatory and anti-oxidant activities. The present study provides scientific rationale for the ethnomedicinal use of the plant to treat sores. It also reveals the anti-inflammatory property of the plant extract and its underlying cellular and molecular mechanisms for the first time.

SUMMARY

1. The Eastern Himalayas, one of the two biodiversity hotspots in India, harbours a vast resource of medicinal plants which are often used by local inhabitants of their own experience. The people of Terai belt of the Eastern Himalayas use the leaf of one such plant *Eupatorium adenophorum* of Astaraceae (Compositae) family for treating mouth and skin sores. The healing property of *E. adenophorum* suggested to us the possible role of this plant could be anti-inflammatory and immunomodulatory.

2. *E. adenophorum*, a shrub was collected mainly from Kurseong hill at 1400 mt high slope of the Esatern Himalayas. Ten gms of freshly collected leaves were cleaned thoroughly with water, air dried, crushed and extracted in 10 ml absolute ethanol overnight. After sterilization through 0.22 μm cellulose acetate filter the ethanolic leaf extract of *E. adenophorum* (EEA) was stored at 4^0C and used at different doses for different experiments.

3. Toxicity of EEA, if any, was tested in reference to hematological parameters after injecting (i.v.) the extract in mice and *in vitro* culture of lymphocytes with EEA. There was no marked effect on hemoglobin percentage and RBC count. Rather it was promotional for WBCs and without any inimical effect for the lymphocytes.

4. EEA was in fact found to induce blastogenesis in both the B and T cell populations, and more efficiently for T cells. The stimulatory effect of EEA for lymphocytes was also reflected in DNA synthesis, assayed by ^{3}H-incorporation.

5. The cell cycle analysis by FACS showed that EEA could drive a notable percentage of lymphocytes towards S phase at 16 hrs and maintained the stimulation upto 48 hrs. Progression of the cells towards G_2-M phase is evident at 24 and 48 hrs. Thus, EEA can set the lymphocytes for cell division. The cell division is a prerequisite for the lymphocytes for functional differentiation.

6. Anti-inflammatory activity of EEA was judged by ability to inhibit delayed type hypersensitive (DTH) reaction set in mouse paw by subcutaneous injection of 25 or 50 μl 2,4-dinitrofluorobenzene (DNFB). Both topical and intravenous application of

EEA could inhibit the DTH induration and brought back normalcy of the paw much earlier than controls. Topical application was more effective in both the counts.

7. Differential counts of leukocytes in the oozing fluid from the DTH inflammation site in experimental and control mice revealed that EEA could restrict infiltration of inflammatory cells like neutrophils and monocytes in the afflicted site (paw). Inhibiting the number of these phagocytic cells likely lessen the ongoing inflammation reaction.

8. $CD4^+T$ cells participate in DTH reaction by secreting all different kinds of cytokines. Enumeration of $CD4^+T$ cells by labeling with paramagnetic probe and then separation in MACS revealed that treatment with EEA raised the number of $CD4^+T$ cells in splenic lymphocytes of DTH mice.

9. Serum level of the pro-inflammatory cytokine TNF-α increases with the progression of inflammation. It was apparently a paradox to find that EEA having anti-inflammatory effect, augmented TNF-α lev el. It was resolved that T NF-α li kely participates in tissue repair and growth in the aftermath of DTH reaction. This worthwhile finding has been discussed in view of others work. Subsequently, the effect of EEA on expression of *TNF-α* gene was studied by mRNA phenotyping; indeed, EEA induced higher level of expression of the gene.

   TGF-β encodes a growth factor and was found to be upregulated by EEA.This afctor might contribute in the growth process following inflammatory damage to the tissues.

10. Besides TNF-α, the regulatory role of EEA in expression of certain other pro- and anti-inflammatory cytokine genes was also analysed. Interestingly, EEA inhibited the expression of a pro-inflammatory cytokine gene *IL-1β*, but did not influence *IL-6* gene known to encode another pro-inflammatory cytokine. Neither EEA influenced the expression of *IL-10* gene known to participate in anti-inflammatory event. Again EEA acted differentially for expression of *COX1* and *COX2* genes encoding isoforms of cycloxygenase enzyme responsible for producing secondary mediators, leukotrienes and prostaglandins respectively for inflammatory reaction. EEA downregulated only the *COX2* gene.

Thus, genes encoding pro- and anti-inflammatory cytokines and mediators might not function or triggered all at a time.

11. EEA could not influence much the expression of *IKK* gene in relation to the controls indicating inability of EEA in activation of NF-κB pathway for contributing towards inflammation.

12. Topical application of EEA could heal up linear incisions and limited burnt area in skin of mice faster. Upregulation of *TNF-α* and *TGF-β* by EEA has been suggested to be likely reason for faster healing as it was found with repair of tissue following DTH reaction. The finding corroborates with the practice for ethnomedicinal use of the plant to treat skin and mouth sores.

13. Tumor initiation and progression bears similarities with the process of inflammation reaction in certain aspects, such as cytokine production, growth factor medited vascularization and induction of pro-inflammatory mediators that provide favourable environment for proliferation of cells. That is why EEA having anti-inflammatory properties was tested for its effect on tumor growth. EEA infact retarded growth of the solid tumor induced with Ehrlich ascitic carcinoma cells, to some extend at the initial phase and increased the longevity of the tumor bearing mice.

    In addition, EEA also induced cytotoxic differentiation of T lymphocytes capable of mounting lysis of ^{51}Cr-labeled tumor target cells. EEA in fact effectively induced expression of *perforin* gene, encoding the protein monomers in the cytotoxic T cells. Being secreted by cytotoxic T cells, perforin monomers polymerize on the membrane of the tumor target cells to create pores, and leading to the target cell death.

14. Enhanced production of reactive oxygen species (ROS) is hallmark to inflammation and tumor growth. EEA, capable of inhibiting DTH reaction and tumor growth to some extend, effectively quenches the generation of three deleterious ROS – superoxide ($O_2^{\cdot}$), hydrogen peroxide ($H_2O_2^{\cdot}$) and hydroxyl ($OH^{\cdot}$) radicals.

15. EEA induced generation of $NO^{\cdot}$, a messenger molecule, from L-arginine in lymphocytes by activating the enzyme inducible nitric oxide synthase (iNOS). $NO^{\cdot}$ generation was found earlier to be conducive for cytotoxic differentiation of T cells.

The ability of EEA to quench deleterious ROS and induce $NO^{\cdot}$ generation seem to promote lymphocytes for function including cytotoxicity.

16. The anti-oxidant activity of an agent might depend on the presence of electrophile functional groups such as phenolic '-OH' on its structure. Chemical characterization and identification of active component(s) from an extract like EEA is likely to provide better understanding of its functional mechanism. Initial fractionation and TLC separation of the total extract revealed a major UV-active band and was identified by HPLC peak at 268 nm. IR spectroscopic analysis revealed the fraction to have hydroxyl and carbonyl functional groups and further analyses with NMR and mass spectroscopy suggested terpenoid nature of the fraction.

17. Comparison of biological effects such as blastogenesis and quenching of free radicals revealed the total EEA extract perform better than the major fraction (isolated through column chromatography). This could due to the presence of certain other minor components got lost in course of the purification of the major fraction.

18. Expression of genes related to anti-inflammatory and anti-tumor function under the influence of a herbal agent can be taken into consideration to screen for an effective agent from the herbl reservoir.

APPENDICES

Appendix 1: Immunopharmacological activities of certain plant extracts.

Name of plant	Part used	Active constituent (Chemical nature)	Activity	Experiment conducted	Reference
Eupatorium adenophorum	Leaves	Terpenoid	Anti-inflammatory Anti-tumor Anti-oxidant	Inhibition of DTH reaction Tumor regression assay Inhbition of ROS	
Curcuma longa	Rhizome	Terpenoid	Anti-tumor Anti-inflammatory Anti-oxidant	Tumor regression assay Inhibition of DTH reaction Inhbition of ROS	Chakravarty & Yasmin, 2003 and 2005. Kim JM, *et. al.* 1998. Khar A *et. al.* 1999. Srimal & Dhawan, 1973 Brouet & Ohshima, 1995 Cohly, *et al.*, 1998 Kunchandy & Rao, 1989 &1990 Ruby, et al., 1995

Hemidesmus indicus	Roots	□	Anti-inflammatory Anti-oxidant	Carrageenan induced mice paw edema Inhbition of ROS	Atal *et al.*, 1986 Karnick, 1977;
Aconium heterophyllum	Rhizome	Steroidal alkaloid	Anti-inflammatory Anti-oxidant	Carrageenan induced mice paw edema Inhbition of ROS	Atal *et al.*, 1986
Anisomeles indica		Flavone	Anti-inflammatory Anti-oxidant	Carrageenan induced mice paw edema Inhbition of ROS	Dharmasiri *et al.*, 2002 Atal *et al.*, 1986
Radyx glycyrrhizae		□	Anti-inflammatory Anti-oxidant	Carrageenan induced mice paw edema Inhbition of ROS	Atal *et al.*, 1986
Tinospora cordifolia	Stem	Irriboid glycosides	Anti-inflammatory Anti-oxidant	Carrageenan induced mice paw edema Inhbition of ROS	Rai and Gupta, 1966 Singh, *et al.*,1984 Singh *et. al.*, 2003
Tylophora indica	Leaves	Alkaloid	Anti-inflammatory Anti-oxidant	Carrageenan induced mice paw edema Inhbition of ROS	Atal *et al.*, 1986 Shivpuri, Singhal & Prkash, 1972.

Ocimum gratissimum	Leaves	Pinenes, limonene	Anti-inflammatory Anti-oxidant	Carrageenan induced mice paw edema Inhbition of ROS	Atal *et al.*, 1986

Appendix 2: Some natural compounds and their active functional groups with probable mechanism of their activities

Natural compound	Functional group/ Chemical nature	Immunopharma-cological properties	Probable machanism	References
Curcumin	Phenolic OH and β-diketone/ Terpenoid	Anti-tumor	• Suppresses NF-κB regulated gene products • activates caspases • downregulates p53 • inhibits angiogenesis • suppresses AP-1	Aggarwal, Kumar & Bharti, 2003 Mukhopadhyay *et al.*, 2001 Soudamini & Kuttan, 1989 Bharti, *et al.* 2003 Lin *et al.*, 2007 Shishodia, *et al.*, 2005
		Anti-inflammatory	• Inhibits COX • Downregulates TNF	Chen *et al.*, 1996 Shankar & Srivastava, 2007 Kakar & Roy, 1994 Ireson, *et. al.* 2001 Liu *et al*, 1993 Lu *et al*, 1994

Resveratr-ol	Phenolic OH/ Stibene	Anti-tumor Anti-inflammatory	• Suppresses NF-kB • Suppresses AP-1 • Activates caspases • Inhibits COX-2 and lipoxygenase	Baur *et al.*, 2006 Manna *et al.*, 2000 Aggarwal *et al.*, 2004 Tseng *et al.*, 2004
Celastrol	Phenolic OH/ Terpenoid	Anti-tumor	• Inhibits NF-kB and related gene products	Sethi *et al.*, 2007
Fisetin	Phenolic OH/ Flavone	Anti-tumor	• Inhibits NF-kB and related genes	Sung, Pandey & Aggarwal, 2007
Genistein	Phenolic OH/ Isoflavone	Anti-tumor	• Inhibits NF-κB • Inhibits Akt	Wang *et al.*, 2007 Gong *et al.*, 2003
Quercetin	Phenolic OH/ Flavonol	Anti-inflammatory Anti-oxidant	• Inhibits NF-κB • Scavenges ROS	Ruiz *et al.*, 2003 Boots *et al.*, 2008
Kaemferol	Phenolic OH/ Flavonol	Anti-cancer	• Inhibits NF-κB	Li *et al.* 2009

REFERENCES

Abreu P, Matthew S, Gonza´lez T, Costa D, Segundo MA, Fernandes E. 2006. Anti-inflammatory and antioxidant activity of a medicinal tincture from *Pedilanthus tithymaloides*. *Life Sciences*. 78:1578 – 1585.

Adebayo JO, Adesokan AA, Olatuni LA, Buoro DO, Soladoye AO. 2005. Effect of ethanolic extract of Bougainvillea spectabilis leaves on haematological and serum lipid variables in rats. *Biokemistri*. 17: 45-50.

Aggarwal BB, *et al.* 2004. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res.* 24:2783-2840.

Aggarwal BB, Kumar A, Bharti AC. 2003. Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res.* 23:363-398.

Alkalay I, Yaron A., Hatzubai A, *et al.* 1995. Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway, *Proc Natl Acad Sci USA.* 92:10599–603.

Arch RH, Thompson CB. 1999. Lymphocyte survival – the struggle against death. *Annu. Rev. Cell. Dev. Biol.* 15:113-140.

Arias JI, Aller MA, Arias J. 2007. Cancer cell: using inflammation to invade the host. *Molecular Cancer*. 6:29-38.

Arthur Cronquist. 2002. Class Magnoliopsida in An Integral System of Classification of Flowering Plants. Bishan Singh Mahendra Pal Singh, 23-A, New Connaught Place, Dehra Dun-248001,India.

Atal CK, Sharma ML, Kaul A, Khajuria A. 1986. Immunomodulatory agents of plant origin. I: Preliminary screening. *Journal of Ethnopharmacology*. 18:133-141.

Baeuerle PA. 1998. IkB-NFkB structures: at the interface of inflammation control. *Cell.* 95:729-731.

Balkwill F, Mantovani A. 2001. Inflammation and cancer: back to Virchow? *Lancet.* 357: 539-545.

Bandyopadhyay U, Das D, Banerjee RK. 1999. Reactive oxygen species: oxidative damage and pathogenesis. *Curr Sc.* 77:658-666.

Banno T, Azel A, Blumenberg M. 2004. Effects of tumor necrosis factor-a (TNF-a) in epidermal keratinocytes revealed using global transcription profiling. *J. Biol. Chem.* 279:32633-32542.

Barcellos-Hoff MH. 1993. Radiation induced transforming growth factor beta and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res.* 53:3880-3886.

Barnes PJ. 1990. Reactive oxygen species and airway inflammation. *Free Rad Biol Med.* 9:235– 243.

Baron JA, Sandler RS. 2000. Non steroidal anti-inflammatory drugs and cancer prevention. *Annu. Rev. Med.* 51:511-523.

Bauel V and M Karim. 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* 11:372-377.

Baur JA, Pearson KJ, Price NL, Jamieson HA, *et al.* 2006. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 444:337-342.

Ben-Baruch A. 2006. Inflammation associated immune suppression in cancer: The roles played by cytokines, chemokines and additional mediators. *Semin. Cancer Biol.* 16:38-52.

Berg J, Fellier H, Christoph T, Grarup J, Stimmeder D. 1999. The analgesic NSAID lornoxicam inhibits cycloxygenase (COX)-1/-2, inducuble nitric oxide synthase and the formation of IL-6 in vitro. *Inflammation Res.* 48:369-379.

Bharti AC, Donato N, Singh S, Aggarwal BB. 2003. Curcumin (diferuloylmethane) downregulates the constitutive activation of NF-kB and IkBa kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. *Blood.* 101:1053-1062.

Bidwell J, Keen L, Gallagher G, Kimberly R, *et al.* 1999. Cytokine gene polymorphism in human disease: on-line databases. *Genes Immun.* 1:3-19.

Black CA. 1998. Delayed Type Hypersensitivity: Current Theories with an Historic Perspective. *Dermatology Online journal.* 5:123-138.

Bodekar G, Hughes MA. 1998. Wound healing traditional treatments and research policy. In: Prendergast HDV, Etkin NL, Harris DR, Houghton PJ ed., Plants for food and medicine. 1st Edn., Royal Botanical Gardens Kew, London, pp. 345-359.

Boots AW, Haenen GR, Bast A. 2008. Health effects of quercetin: from antioxidant to nutraceutical. *Eur J Pharmacol.* 582:325-37.

Bredt DS, Snyder SH. 1994. Nitric oxide: a physiologic messenger molecule. *Annu. Rev. Biochem.* 63:175-195.

Brouet I, Ohshima H. 1995. Curcumin, an anti-tumor promoter and anti-inflammatory agents, inhibits induction of nitric oxide synthase in activated macrophages. *Biochem. Biophys Res. Commun.* 206:533-540.

Burton AK, Waddel G. 1998. Clinical guidelines in the management of low back pain. *Bailliere's Clin Rheumatol.* 12:17-35.

Busch R, Cesar D, Higuera-Alhino D, Gee T, et. al. 2004. Isolation of peripheral blood $CD4^+$ T cells using RosetteSep™ and MACS™ for studies of DNA turnove by deuterium labeling. *Journal of Immunological Methods.* 286: 97-109.

Chakraborty A, Chakravarty AK. 1983. Plaque forming cell assay for antibody mediated response in *Pteropus giganteus. Ind. J. Exptl. Biol.* 21:5-7.

Chakravarty AK, Chatterjee SN, Yasmin H, Mazumder T. 2009 Comparison of efficacy of turmeric and commercial curcumin in immunological functions and gene regulation. *Intl. J. Pharmacol.* 5:333-345.

Chakravarty AK, Chaudhuri TK. 1983. Correlation of blastogenesis and DNA synthesis in murine lymphocytes during *in vivo* activation with concanavalin A. *Jap. J of Med Sci and Biol.* 36:43-46.

Chakravarty AK, Clark WR. 1977. Lectin driven maturation of cytotoxic effector cells: The nature of effector memory. *J. Exp. Med;* 146:230-240.

Chakravarty AK, Jha S. 1997. Homing of polyclonally activated syngeneic T cells at tumor site and their efficacy in post operative immunotherapy of malignancy in mouse. *Curr. Sc.* 73:201-203.

Chakravarty AK, Maitra UK. 1983. Inhibition of tumor induced angiogenesis and tumor growth by activated lymphocytes. *Experientia.* 39:542-544.

Chakravarty AK, Maitra UK. 1990 Polyclonally stimulated murine T cells in control fibrosarcoma *in situ. J Indian Inst Sci.* 70:503-516.

Chakravarty AK, Maitra UK. 1990. Polyclonal stimulated murine T cells in control of fibrosarcoma in-situ. *J.Ind.Is.Sci.* 70:503-516.

Chakravarty AK, Yas min H . 2003. Two way efficacy of alcoholic turmeric extract: stimulatory for murine lymphocytes and inhibitory for ascitic fibrosarcoma. *Pharmaceutical Biology*; 42:217-224.

Chakravarty AK, Yasmin H. 2005. Alcoholic turmeric extract simultaneously activating murine lymphocytes and inducing apoptosis of Ehrlich's Carcinoma cells.. *International Immunopharmacology;* 5:1574-1581.

Chakravarty AK, Yasmin H. 2008. Free radical scavenging and nitric oxide synthase activation in murine lymphocytes and ehrlich ascitic carcinoma cells treated with ethanolic extract of turmeric. *Proc. Nat. Acad. Sci. India*; 78(B)I:49.

Chandel NS, *et. al.* 2000. Redox regulation of p53 during hypoxia. *Oncogene.* 19:3840-3848.

Chao CC, Hu S, Sheng WS, Tsang M, Peterson PK. 1995. Tumor necrosis factor-α mediates the release of bioactive transforming growth factor-ß in murine microglial cell cultures. *Clin Immunol Immunopathol.* 77:358–365.

Chaudhuri TK, Chakravarty AK. 1983a. Study of murine lymphoid cells in-situ after stimulation with concanavalin A. *Japan J.Med .Sci and Biol.* 36:97-103.

Chaudhuri TK, Chakravarty AK. 1983b. Goat serum as a substitute for fetal calf serum in *in vitro* culture of murine lymphocytes. *Indian J Exptl. Biol.* 21:494-496.

Chen F, Sun S, Kuhn DC, Gaydos LJ, Shi X, Lu Y, et al. 1997. Involvement of NF-kappaB in silica-induced cyclooxygenase II gene expression in rat alveolar macrophages. *Am J Physiol* 272:L779-L786.

Chen LW, *et al.* 2003. The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nat Med.* 9:575-581.

Chen YC, Kuo TC, Lin-Shiau SY, Lin JK. 1996. Induction of HSP70 gene expression by modulation of Ca^{2+} ion and cellular P53 protein by curcumin in colorectal carcinoma cells. *Mol. Carcinogenesis.* 17:224-234.

Cohly HHP, Taylor A, Angel MF, Salahudeen AK. 1998. Effect of turmeric, turmerin and curcumin on H_2O_2- induced renal epithelial (LLC-PK_1) cell injury. *Free Rad Biol Med.* 24:49-54.

Conney AH, *et al.* 1997. Some perspectives on dietary inhibition of carcinogenesis: studies with curcumin and tea. *Experimental Biology and Medicine.* 216:234-245.

Coussens LM, Raymond W, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey G, Hanahan D. 1999. Inflammatory mast cells upregulate angiogenesis during squamous carcinogenesis. *Genes & Development.* 13:1382-1397.

Coussens LM, Werb Z. 2002. Inflammation and cancer. *Nature.* 420:860-867.

Cragg MC, Newman DJ. 2005. Plants as a source of anti-cancer agents. *J Immunopharmacology.* 100:72-79.

Cunningham AJ, Szenberg A. 1968. Further improvement in the plaque technique for detecting single antibody forming cell. *Immunology.* 14:599-601.

Das SK, Chakravarty AK. 1998. Tumor infiltrating lymphocytes from murine sarcoma; enrichment and proliferative response. *Science and Culture.* 64:50-53.

Dedon PC, Tannenbaum SR. 2004. Reactive nitrogen species in the chemical biology of inflammation. *Arch Biochem Biophys.* 423:12– 22.

Devaux Y, Seguin C, Grosjean S, de Talance N, Camaeti V, Burlet A, *et al.* 2001. Lipopolysaccharide-induced increase of prostaglandin E(2) is mediated by inducible nitric oxide synthase activation of the constitutive cyclooxygenase and induction of membrane-associated prostaglandin E synthase. *J Immunol.*167:3962-3971.

Dharmasiri MG, Ratnasooriya WD, Thabrew MI. 2002. Anti-inflammatory activity of decoctions of leaves and stems of *Anisomeles indica* at preflowering and flowering stages. *Pharmaceutical Biology.* 40:433-439.

Dinarello CA. 2000. Proinflammatory cytokines. *Chest.* 118:503-508.

Drew PD, Chavis J. 2000. Female Sex steroids: Effects upon microglial cell activation. *J Neuroimmunology.* 111:77-85.

Drews J, Ryser S. 1997. Classic drug targets. *Nat. Biotechnol.* 15:1318-1319.

Dureja H, Kaushik D, Kumar V. 2003. Developments in neutraceuticals. *Indian J Pharmacology.* 35:363-372.

Ehlers S, Mielke ME, Hahn H. 1994. CD4+ T cell associated cytokine gene expression during experimental infection with Listeria monocytogenes: the mRNA phenotype of granuloma formation. *Int Immunol.* 6:1727-1737.

Fabbri LM, Saetta M, Picotti G, Mapp CE. 1991. Late asthmatic reactions, airway inflammation AND chronic asthma IN toluene-diisocyanate-sensitized subjects. *Respiration.* 58:18-21.

Farrow B, Evers BM. 2002. Inflammation and the development of pancreatic cancer. *Surg. Oncol.* 10:153-169.

Gabay C. 2006. Interleukin-6 and chronic inflammation. *Arthritis Res Ther.* 8(Suppl 2): S3-S8.

Garrido G, Gonzalez D, Delporte C, Backhouse N, Quintero G, Nunez-Selles AJ, Morales MA. 2001. Analgesic and anti-inflammatory effects of *Mangifera indica* L. extract (Vimang). *Phytother. Res.* 15:18-21.

Geetha T, Varalakshmi P, Mary LR. 1998. Effect of triterpenes from *Crateava nurvata* stem bark on lipid peroxidation in adjuvant induced arthritis in rats. *Pharmacol. Res.* 97:191-195.

Geetha VS, Viswanathan S, Kameswaran L. 1981. Comparison of total alkaloids of *Tylophora indica* and disodium cromoglycate on mast cell stabilization. *Indian Journal of Pharmacology.* 13:199-202.

Ghosh S, May MJ, Kopp EB. 1998. NF-κB and Rel Proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16:225-260.

Gladwin MT, Crawford JH, Patel RP. 2004. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. *Free Rad. Biol. Med.* 6:707-717.

Goel A, Boland C, Chauhan D. 2001. Specific inhibition of cylooxygenase -2 (COX-2) expression by dietary Curcumin in HT- Human colon cancer cells. *Cancer Letters* 172:111-118.

Gong L, Li Y, Kurepa AN, Sarkar FH. 2003. Inactivation of NF-kB by genistein is mediated via Akt signaling pathway in breast cancer cells. *Oncogene.* 22:4702-4709.

Gupta M, Mazumder UK, Das S. 1994. Effect of alkaloidal extract from *Clerodendron colebrookianum* on hematological parameters and hepatorenal functions in mice. *Indian J Exp Biol.* 32:189-91.

Haddad JJ, RE Olver and SC Land. 2000. Antioxidant pro-oxidant equilibrium regulates HIF-1□ and NF-□B redox sensitivity: evidence for inhibition by glutathione oxidation in alveolar epithelial cells. *J Biol Chem.* 275:21130-9.

Haddad JJ. 2002a. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cellular Signaling.* 14:879-897.

Haddad JJ. 2002b. The involvement of L-□-glutamyl-L-cysteinyl-glycine(glutathione GSH) in the mechanism of redox signaling mediated $MAPK^{p38}$ – dependent regulation of pro-inflammatory cytokine production. *Biochem Pharmacol.* 63:305-20.

Haddad JJ. 2004. Oxygen sensing and oxidant/redox-related pathways. *Biochem. Biophys. Res. Commun.* 316:969-977.

Halliwell B, Gutteridge JMC, Aruoma OI. 1987. The deoxyribose method: A simple "test-Tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry.* 165: 215-219.

Halliwell B, Hoult JR, Blake DR. 1998. Oxidants, inflammation, and anti-inflammatory drugs. *The FASEB Journal* 2:2867–2873.

Halliwell B. 1997. Antioxidants and human disease: a general introduction. *Nutrition Reviews*. 5:544–552.

Harvey AL. 1999. Medicines from nature: are natural products still relevant to drug discovery? *Trends Pharmacol Sci.* 20:196-198.

Heinenger D, Touton M, Chakravarty AK, Clark WR. 1976. Activation of cytotoxic function in T lymphocytes. *J. Immunol.* 117:2175-2180.

Howell WM, Galder PC, Grimble RF. 2002. Gene polymorphisms, inflammatory diseases and cancer. *Proc. Nutr. Soc.* 61:447-456.

Hu JP, *et al.* 1995. Structure-activity relationship of flavonoids with superoxide scavenging activity. *Biol. Trace Element Res.* 47:327-331.

Huang MT, Lysz T, Ferraro T. Laskin JD, Conney AH. 1991. Inhibitory effects of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Res.* 51:813-819.

Iizima M, Katz SI. 1983. Specific immunologic tolerance to dinitrofluorobenzene following topical application of dinitrothyocyanobenzene: mulation by suppressor T cells. *Journal of Investigative Dermatology.* 81:325-330.

Iranloye BO. 2002. Effect of chronic garlic feeding on some hematological parameters. *African Journal of Biomedical Research.* 5:81-82.

Ireson C, *et. al.* 2001. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. *Cancer Res.* 61:1058-1064.

Jia L, Bonaventua C, Bonaventura J, Stamler SJ. 1996. S-nitrosohemoglobulin: a dynamic activity of blood involved in vascular control. *Nature*. 380:221-226.

Joseph S, Sabulal B, George V, Smina TP, Janardhanan KK. 2009. Antioxidative and Antiinflammatory Activities of the Chloroform Extract of *Ganoderma lucidum* Found in South India. *Sci Pharm*. 77:111–121.

Julius M, E Simpson and AL Herzenberg. 1973. A rapid method for isolation of functional thymus derived murine lymphocytes. *Eur. J. Immuno.* 3:645-657.

Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. 2003. IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 33:1205-1214.

Kakar SS, Roy D. 1994. Curcumin inhibit TPA induced expression of c-fos, c-jun and c-myc proto-oncogene messenger RNAs in mouse skin. *Cancer Lett.* 87:85-89.

Kala CP, Dhyani PP, Sajwan BS. 2006. Developing the medicinal plants sector in northern India: challenges and opportunities. *Journal of Ethnobiology and Ethnomedicine*. 2:32-46.

Karnick CR. 1977. Ethnobotanical pharmacognostical and cultivation studies of *Hemidesmus indicus* R. Br., Indian sarsaparilla. *Herba Hungarcia*. 16:7-16.

Khar A *et. al.* 1999. Antitumour activity of curcumin is mediated through the induction of apoptosis in AK-5 tumour cells. *FEBS Lett*. 445:165-168.

Kim JM, *et. al.* 1998. Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis*. 19:81-85.

Klareskog L, Padyukov L, Ronnelid J, Alfredsson L. 2006. Genes, environment and immunity in the development of rheumatoid arthritis. *Current Opinion in Immunology*. 18:650–655.

Klaunig JE, Kamendulis LM. 2004. The role of oxidative stress in carcinogenesis. *Annu Rev Pharmacol Toxicol.* 44:239-267.

Klein C, Sato, T., Meguid, M.M., Miyata, G.2000. From food to nutritional support to specific nutraceuticals: a journey across time in the treatment of disease. *J. Gastroenterol*: 35:1-6.

Knuth A, Danowski B, Ottgen HF, Old LJ. 1984. T.-cell-mediated cytotoxicity against autologous malignant melanoma: Analysis with interleukin 2-dependent T-cell cultures. *Proc. Natl. Acad. Sci. USA.* 81:3511-3515.

Kohli K, Ali J, Ansari MJ, Raheman Z. 2005. Curcumin: A natural anti-iflammatory agent. *Ind J Pharmacol.* 37:141-147.

Koncz A, *et al.* 2007. Nitric oxide mediates T cell cytokine production and signal transduction in histidine decarboxylase knockout mice. *J. Immunol.* 179:6613-6619.

Kong DX, Li XJ, Zhang HY. Where is the hope for drug discovery? Let history tell the future, *Drug Discov Today* 2008, doi:10.1016/j.drudis.2008.07.002

Krackhardt A M, Sabine H, Mathias W, Ryan B, Patrick B, John GG. 2002. T-cell responses against chronic lymphocytic leukemia cells: implications for immunotherapy. *Blood* 100:167-173.

Kunchandy E, Rao MNA. 1989. Effect of curcumin on hydroxyl radical generation through Fenton reaction. *International Journal of Pharmaceutics.* 57:173-176.

Kunchandy E, Rao MNA. 1990. Oxygen radical scavenging activity of curcumin. *International Journal of Pharmaceutics.* 58:230-240.

Li W, Du B, Wang T, Wang S, Zhang J. 2009. Kaempferol induces apoptosis in human HCT116 colon cancer cells via the Ataxia-Telangiectasia Mutated-p53 pathway with the involvement of p53 Upregulated Modulator of Apoptosis. *Chemico-Biological Interactions.* 177:121-127.

Lin YG, *et al.* 2007. Curcumin inhibits tumor growth and angiogenesis in ovarian carcinoma by targeting the nuclear factor-kB pathway. *Clin. Cancer Res.* 13:3423-3433.

Liu JY, Lin SJ, Lin JK. 1993.Inhibitory effects of curcumin on protein kinase C activity induced by 12-O-tetradecanoyl phorbol-13-acetate in NIH 3T3 cells, *Carcinogenesis*, 14:857-861.

Lu YP, *et. al.* 1994. Effect of curcumin on 12-*O*-tetradecanoylphorbol-13-acetate-and ultraviolet B light induced expression of c-Jun and c-Fos in JB6 cells in mouse epidermis. *Carcinogenesis*. 15:2363-2370.

Lukacs NW, Strieter RM, Chensue SW, Widmer M, Kunkel SL. 1995. TNF-alpha mediates recruitment of neutrophils and eosinophils during airway inflammation. *J Immunol* 154:5411-5417.

MacNee W, Rahman I. 1999. Oxidants and oxidants as therapeutic targets in chronic obstructive pulmonary disease. *Am J Respire Crit Care Med.* 160:S58-65.

MacNee W. 2001. Oxidative stress and lung inflammation in airway disease. *Eur J Pharmacol.* 429:195-207.

Mandal SK, Boominathan R, Parimaladevi B, Dewanjee S, Mandal S. 2005. Analgesic activity of methanol extract of *Eupatorium adenophorum* Spreng. leaves. *Ind. J. Exp. Biol.* 43:262-263.

Manna SK, Mukhopadhyay A, Aggarwal BB. 2000. Resveratrol suppresses TNF-induced activation of nuclear transcription factor NF-kB, activator protein-1 and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J. Immunol.* 164:6509-6519.

Martin JP, Daiby M, Sugrman E. 1987. Negative and positive assays of superoxide dismutase based on hematoxilin autoxidation. *Archives of Biochemistry and Biophysics*. 208:329-336.

Marx J. 2004. Inflammation and cancer: The link grows stronger. *Science*. 306: 966.

Matheu MP, Cahalan MD. 2007. Isolation of CD4+ T cells from mouse lymph nodes using miltenyi MACS purification. *Journal of Visualized Experiments* 9. DOI: 10.3791/409

Matsushima GK, Stohlman SA. 2005. Maturation of the delayed-type hypersensitivity response in SJL mice: absence of effector cell induction. *Eur J Immunol* 18:1411-1416.

Miller DM, Aust SD. 1989. Studies of ascorbate- dependent, iron-catalyzed lipid peroxidation. *Arch. Biochem. Biophysics*. 271:113-119.

Mitchell JA, Larkin S, Williams TJ. 1995. Cyclooxygenase 2: regulation and relevance in inflammation. *Biochem Pharmacol* 50:1535-1542.

Mukhopadhyay A, Ramos CB, Chatterjee D, Pantazis P, Aggarwal BB. 2001. Curcumin downregulates cell survival mechanisms in human prostate cancer cell lines. *Oncogene*. 20:7597-7609.

Naik SR. 2003. Antioxidants and their role in biological functions: an overview. *Indian Drugs*. 40:501-16.

Nguemfol EL, Dimol T, Dongmo AB, Azebaze AGB, Alaoui K, Asongalem AE, *et al.* 2009. Anti-oxidative and anti-inflammatory activities of some isolated constituents from the stem bark of *Allanblackia monticola* Staner L.C (Guttiferae). *Inflammopharmacology* 17:37–41.

Oberg BP, McMenamin E, Lucas FL, *et al.* 2004. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int.* 65:1009-1016.

Omoigui S. 2007. The interleukin-6 inflammation pathway from cholesterol to aging–Role of statins, bisphosphonates and plant polyphenols in aging and age-related diseases. *Immunity & Ageing.* 4:1-22.

Oze G, Harrision N, Okey O, Ukoha A . 2008. Changes In Lipid And Haematological Profile Of Aqueous Ethanolic Extract Of Alstonia Boonei In Rats: *The Internet Journal of Hematology.* Volume 4, Number 1.

Pande V, Ramos MJ. 2005. NF-κB in Human Disease: Current Inhibitors and Prospects for *De Novo* Structure Based Design of Inhibitors. *Curr Med Chem.* 12:357-374.

Parenteau NL, Hardin-Young J. 2007. The biological mechanisms behind injury and inflammation: how they can affect treatment strategy, product performance, and healing. *Wounds.* 19:87-96.

Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. 2004. NF-□B functions as a tumour promoter in inflammation-associated cancer. *Nature* 431:461–466.

Poulter LW, Seymour GJ, Duke O, Janossy G, Panayi G. 1982. Immunohistological analysis of delayed-type hypersensitivity in man. *Cell Immunol* 74:358-69.

Rai M, Gupta SS. 1966. The deposition of secondary salts over the five pellets in rats was inhibited by aqueous extract of *T. cordifolia. J. Res. Ind. Med.* 10:113-116.

Rappolee DA, Brenner CA, Schultz R, Mark D, Werb Z. 1988b. Developmental expression of PDGF, TGF-alpha, and TGF-beta genes in preimplantation mouse embryos. *Science* 241:1823-1825.

Rappolee DA, Mark D, Banda MJ, Werb Z. 1988a. Wound macrophags express TGF-β and other growth factors in vivo: Analysis by mRNA phenotyping. *Science* 241:708-712.

Ruby AJ, Kuttan G, Babu KD, Rajasekharan KN, Kuttan R. 1995. Anti-tumour and antioxidant activity of natural curcuminoids. *Cancer Lett.* 94:79-83.

Ruiz PA, Braune A, Holzlwimmer G, Quintanilla-Fend L, Haller D. 2007. Quercetin inhibits TNF-induced NF-kappaB transcription factor recruitment to proinflammatory gene promoters in murine intestinal epithelial cells. *J Nutr.* 137:1208-1215.

Salvioli S, Sikora E, Cooper EL, Franceschi C. 2007. Curcumin in cell death processes: a challenge for CAM of age related pathologies. *Evid Based Complement Alternat Med* 4:181-190.

Sarkar D, Dutta A, Das M, *et al.* 2005. Effect of *Aloe vera* on nitric oxide production by macrophages during inflammation. *Ind. J. Pharmacol.* 36:371-375.

Saukkonen K, Sande S, Cioffe C, Wolpe S, Sherry B, Cerami A, et al. 1990. The role of cytokines in the generation of inflammation and tissue damage in experimental gram-positive meningitis. *J. Exp. Med.* 171:439-448.

Schwartsburd PM. 2003. Chronic inflammation as inductor of procancer microenvironment: pathogenesis of dysregulated feed back control. *Cancer Metastasis Rev.* 22:95-102.

Schweizer A, Feige U, Fontana A, et al. 1998. Interleukin-1 enhances pain reflexes: mediation through increased prostaglandin E2 levels. *Agents Actions* 25:246–251.

Senthilkumar R, *et al.* 2008. Effects of ethanol extract of *Pisonia aculeata* Linn. on ehrlich ascites carcinoma tumor bearing mice. *Int J Green Pharm.* 2:50-53.

Sethi G, Ahn KS, Pandey MK, Aggarwal BB. 2007. Celastrol, a novel triterpene, potentiates TNF-induced apoptosis and suppresses invasion of tumor cell by inhibiting NF-κB regulated gene products and TAK1-mediated NF-κB activation. *Blood.* 109:2727-2735.

Sethi G, Sung B, Aggarwal BB. 2008 Nuclear factor-kappaB activation: from bench to bedside. *Exp Biol Med.* 233:21-31.

Shankar S, Srivastava RK. 2007. Involvement of Bcl-2 family members, phosphatidylinositol 3-kinase/Akt and mitochondrial p53 in curcumin (diferuloylmethane) –induced apoptosis in prostate cancer. *Int. J. Oncol.* 30:905-918.

Shishodia S, Amin HM, Lai R, Aggarwal BB. 2005. Curcumin (diferuloylmethane) inhibits constitutive NF-kB activation, induces G_1/S arrest, suppresses proliferation and induces apoptosis in mantle cell lymphoma. *Biochem Pharmacol.* 70:700-713.

Shiva MP. 1996. *Inventory of Forestry Resources for Sustainable Management and Biodiversity Conservation.* New Delhi: Indus Publishing Company, India.

Shivpuri DN, Singhal SC , Prakash D. 1972. Treatment of asthma with an alcoholic extract of *Tylophora indica.* A cross-over double-blind study. *Annals of Allergy.* 30:407-412.

Simka M. 2009. Blood brain barrier compromise with endothelial inflammation may lead to autoimmune loss of myelin during multiple sclerosis. *Curr Neurovasc Res* 6:132-139.

Simmons DL. 2006. What makes a good anti-inflammatory drug target? *Drug Discovery Today* 11:210-219.

Simonian NA, Coyle JT. 1996. Oxidative stress in neurodegenerative diseases. *Annu Rev Pharmacol Toxicol.* 36:83-106.

Singh B, Sharma ML, Gupta DK, Atal CK, Arya RK. 1984. Protective effect of *Tinospora cordifolia* Miers. on carbon tetrachloride induced hepatotoxicity. *Indian Journal of Pharmacology.* 16:139-142.

Singh SS, *et al.* 2003. Chemistry and medicinal properties of *Tionospora cordifolia* (Guduchi). *Indian Journal of Pharmacology.* 35:83-91.

Sirish Kumar IVMLR, Paul BN, et. al. 2003. *Swertia chirayita* mediated modulation of interleukin-1β, interleukin-6, interleukin-10, interferon-γ and tumor necrosis factor-α□ in arthritic mice. *Immunopharmacol Immunotoxicol* 25:573-583.

Smith CJ, Zhang Y, Koboldt CM, Muhammad J, et. al. 1998. Pharmacological analysis of cycloxygenase 1 in inflammation. *Proc Natl Acad Sci USA* 95:13313-13318.

Smith WL, Garavito RM, DeWitt DL. 1996. Prostaglandin endoperoxide H synthase (cyclooxygenases)-1 and -2. *J Biol Chem* 271:33157-33160.

Soudamini KK, Kuttan R. 1989. Inhibition of chemical carcinogenesis by curcumin. *J. Ethnopharmacology.* 27:227-233.

Srimal RC, Dhawan BN. 1973. Pharmacology of diferuloyl methane (curcumin), a non-steriodal anti-inflammatory agent. *J. Pharma Pharmacol.* 25:447-452.

Srivastava R, Srimal RC. 1985. Modification of certain inflammation-induced biochemical changes by curcumin. *Indian J Med Res.,* 81:215-223.

Stanciu LA, Djukanovic R. 2000. Isolation of T cell subsets by magnetic cell sorting. *Methods in Molecular Biology* 134: 133. DOI: 10.1385/1-59259-682-7:133-141.

Stevens RJ, Douglas KMJ, Saratzis A.N, Kitas GD. 2005. Inflammation and atherosclerosis in rheumatoid arthritis. *Expert Reviews in Molecular Medicine.* 7:1-24.

Strieter R, Kunkel S, Bone R. 1993. Role of Tumor Necrosis Factor-alpha in disease states and inflammation. *Crit Care Med* 21:S447-S463.

Sullivan DE, Ferris M, Pociask D, Brody AR. 2005. Tumor Necrosis Factor-α induces Transforming Growth Factor-ß$_1$ expression in lung fibroblasts through the extracellular signal–regulated kinase pathway. *Am J Respir Cell Mol Biol* 32:342-349.

Sung B, Pandey MK, Aggarwal BB. 2007. Fisetin, an i8nhibitor of cyclin dependent kinase 6, downregulates nuclear factor-κB regulated cell proliferation, antiapoptotic and matastatic gene products through the suppression of TAK-1 and receptor interacting protein regulated IκBα kinase activation. *Mol. Pharmacol.* 71:1703-1714.

Sunila ES, Kuttan G. 2004. Immunomodulatory and antitumor activity of Piper longum Linn. and piperine. *J. Ethnopharmacology.* 90:339-346.

Suresh K, Vasudevan DM. 1994. Augmentation of murine natural killer cell and antibody dependent cellular cytotoxicity activities by *Phyllanthus emblica*, a new immunomodulator. *J. Ethnopharmacol* 44:55-60.

Sureshkumar V, Paul B, et al. 2005. Pro-inflammatory and anti-inflammatory cytokine balance in gasoline exhaust induced pulmonary injury in mice. *Inhalation Toxicology* 17:161-168.

Tak PP, Firestein GS. 2001. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 107:7-11.

Thannekal VJ, Fanburg BL. 2002. Reactive oxygen species in cell signaling. *Am J Physiol. Lung Cell Mol Physiol.* 279:L1005-1028.

Tiwari AK. 2001. Imbalance in antioxidant defense and human diseases: Multiple approach of natural antioxidants therapy. *Current Science.* 81:1179-1186.

Toda S, Shirataki Y. 2002. Inhibitory effects of isoflavones in *Sophora Mooracrotiana* on lipid peroxidation by superoxide. *Pharmaceutical Biology.* 40:422-424.

Tracey K, Cerami A. 1994. Tumor Necrosis Factor: A pleiotropic cytokine and therapeutic target. *Annu Rev Med* 45:491-503.

Tseng SH, Lin SM, Chen JC, Su YH, Huang HY, Chen CK, Lin PY, Chen Y. 2004. Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. *Clin Cancer Res* 10: 2190–*2202.*

Tu S, Bhagat G, Cui G, Takaishi S, *et al.* 2008. Overexpression of interleukin-1b induces gastric inflammation and cancer and mobilizes myeloid derived suppressor cells in mice. *Cancer Cell.* 14:408-419.

Turini ME, DuBois RN. 2002. Cyclooxygenase-2: A Therapeutic Target. *Annu Rev Med* 53:35–57.

Vafa O, et. al. 2001. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p^{53} function; a mechanism for oncogene induced genetic instability. *Mol. Cell.* 9:1031-1044.

Van Tulder MW, Koes BW, Bouter LM. 1996. Conservative treatment of acute and chronic nonspecific low back pain. *Spine* 22: 2128-2156.

Vane JR, Bakhle1 YS, Botting RM. 1998. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 38:97–120.

Wagner H. 1990. Search for plant derived natural products with immunostimulatory activity (recent advances). *Pure & Appl. Chem.* 62:1217-1222.

Wagner H. 1993. Leading structures from plant origin. *Journal of Ethnopharmacology.* 38:105-112.

Wahl LM, Wahl SM. 1992. Inflammation, in Cohen IK, Diegelman RF, Lindblad WJ (eds): *Wound Healing: Biochemical and Clinical Aspects.* Philadelphia, PA, W.B. Saunders, pp 40-62.

Walsh CM, Matloubian M, Liu CC, Ueda R, *et al.* 1994. Immune function in mice lacking the perforin gene. *Proc. Natl. Acad. Sci. USA* 91: 10854-10858.

and fibroblast growth factors in endothelial cells. We think of similar participation of TNF-α in tissue repair and regeneration to bring back normalcy aftermath of DTH reaction. Thus, TNF-α plays a double role in DTH reaction—pro-inflammatory cum restoring agent.

The ability of EEA for induction of *TNF-α* gene was studied next. Indeed, it induced higher level of expression of the gene (Figs 10 and 11). EEA does not necessarily affect the expression of gene for other pro-inflammatory cytokines such as *IL-1β* and IL-6 in a similar fashion. EEA inhibited *IL-1β* expression and did not influence the expression of *IL-6* gene (Figs 10 and 11). It seems that IL-1β without any known function in repair mechanism manifests more inhibitory effect of EEA on inflammation. Stimmeder and his co-workers (67) observed that lornoxicam and other non-steroidal anti-inflammatory drugs inhibit *IL-1β* expression as well as inflammation. Kohli *et al.* (68) reported that curcumin, the active component in the rhizome of *Curcuma longa* Linn., demonstrates its anti-inflammatory activity by inhibiting production of IL-1β in lung inflammatory cells.

An anti-inflammatory agent does not necessarily always regulate all the anti-inflammatory cytokine genes as we find that in the present study EEA does not influence the anti-inflammatory cytokine gene *IL-10* (Figs 10 and 11).

EEA upregulates expression of *TGF-β* (Figs 10 and 11). TGF-β performs as a growth factor in all different kinds of events of collagen production and extracellular matrix reorganization as shown by Barcellos–Hoff (69). This cytokine might function here to restore normalcy along with TNF-α in repair mode as discussed earlier. Simultaneous upregulation of these two genes have also been observed by Chao *et al.* (70) in microglial cell culture. Sullivan *et al.* (71) also reported similar trend in expression of these two cytokines in interstitial pulmonary fibrosis-affected lung fibroblasts.

Tak and Firestein (39) and Yamamoto and Gaynor (40) elucidated involvement of NF-κB pathway for induction of inflammation. Activation of NF-κB is mediated by the action of Inhibitory kappa kinase (IKK) degrading inhibitory IκB subunit. Thus, measuring the expression of *IKK*, one can derive the involvement of NF-κB pathway in a reaction. EEA could not induce the expression of *IKK* gene beyond the controls (Figs 10 and 11) indicating non-involvement of NF-κB activation pathway for DTH reaction induced with DNFB.

COX1 and COX2 gene products are two isoforms of the cycloxygenase enzyme that metabolizes arachidonic acid into the inflammatory mediators like prostaglandins and leukotrienes (41–47). Notably EEA only influences expression of *COX2* gene by way of inhibition (Figs 10 and 11). This may be another way of execution of anti-inflammatory activity by EEA. Salvioli *et al.* (72) reviewed curcumin, a potent anti-inflammatory agent, also inhibits COX2 in abetting inflammation.

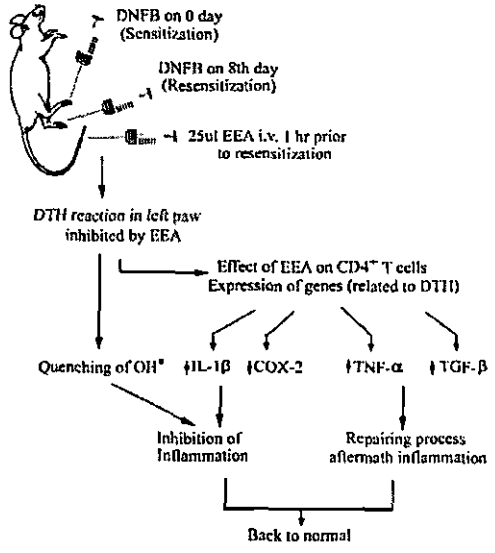


Figure 12. Effect of EEA on inhibition of DTH reaction and repair mechanism presented schematically.

It is known that reactive oxygen species play an important role in development of inflammation (22–25). In the present study, the ability of EEA in quenching the generation of hydroxyl radical has been tested and found to be effective (Fig. 9).

In summary, ethanolic leaf extract of *E. adenophorum* (EEA) exerts anti-inflammatory activity (Fig. 12), likely through inhibition of *IL-1β*, *COX2* genes and quenching ROS like hydroxyl radical. Simultaneously EEA induces production of TNF-α, a pro-inflammatory cytokine. This paradox can only be resolved in the light of participation of TNF-α in tissue repair in the aftermath of inflammation. Interestingly, the expression of *TGF-β* gene encoding the cytokine responsible for growth and repair mechanism is also inducible by EEA. *E. adenophorum* as a source of anti-inflammatory substance seems worthy to report. The active compound from the extract is yet to be identified. Isolation of that compound will allow understanding molecular mechanism of the activity of the substance. So far, Zhang *et al.* (73) reported presence of a few flavonones and sesquiterpene lactones in *E. adenophorum*.

The present investigation also intends that any herbal agent having anti-inflammatory property might be screened faster by estimating its ability to induce or inhibit the genes encoding substances participating in inflammation.

Acknowledgements

Authors are thankful to the University Grants Commission (UGC), India, for providing financial

assistance. They also express their heartfelt gratitude to Dr. Arnab Sen, Department of Botany, NBU, and Dr. Ranadhir Chakraborty, Department of Biotechnology, NBU, to let us avail instrumental facilities in their laboratories. They are also thankful to Dr. Bholanath Paul, IITR, Lucknow, India for providing valuable chemicals to carry out ELISA in his laboratory. They would like to extend their sincere thanks to Professor A. P. Das, Department of Botany, NBU, for identification of the plant.

Funding

University Grants Commission (UGC), India.

References

1. Simmons DL. What makes a good anti-inflammatory drug target? *Drug Discov Today* 2006;11:210–19.
2. Saukkonen K, Sande S, Cioffe C, Wolpe S, Sherry B, Cerami A, et al. The role of cytokines in the generation of inflammation and tissue damage in experimental gram-positive meningitis. *J Exp Med* 1990;171:439–48.
3. Parenteau NL, Hardin-Young J. The biological mechanisms behind injury and inflammation: how they can affect treatment strategy, product performance, and healing. *Wounds* 2007;19:87–96.
4. Fabbri LM, Saetta M, Picotti G, Mapp CE. Late asthmatic reactions, airway inflammation and chronic asthma in toluene-diisocyanate-sensitized subjects. *Respiration* 1991;58:18–21.
5. Stevens RJ, Douglas KMJ, Saratzis AN, Kitas GD. Inflammation and atherosclerosis in rheumatoid arthritis. *Exp Rev Mol Med* 2005;7:1–24.
6. Klareskog L, Padyukov L, Ronnelid J, Alfredsson L. Genes, environment and immunity in the development of rheumatoid arthritis. *Curr Opin Immunol* 2006;18:650–5.
7. Simka M. Blood brain barrier compromise with endothelial inflammation may lead to autoimmune loss of myelin during multiple sclerosis. *Curr Neurovasc Res* 2009;6:132–9.
8. Zipser RD. Mediators of inflammation in inflammatory bowel disease. *Dig Dis Sci* 1988;33:4S–5S.
9. Drews J, Ryser S. Classic drug targets. *Nat Biotechnol* 1997;15:1318–9.
10. Kong DX, Li XJ, Zhang HY. Where is the hope for drug discovery? Let history tell the future. *Drug Discov Today* 2008; doi:10.1016/j.drudis.2008.07.002.
11. AuLD BA, Martins PM. The autecology of *Eupatorium adenophorum* Spreng. In Australia. *Weed Res* 1975;15:27–31.
12. Muschietti L, Derita M, Sulsen V, Dios Munoz JD, Ferraro G, Zacchino S, et al. In vitro antifungal assay of traditional Argentine medicinal plants. *J Ethnopharmacol* 2005;102:233–8.
13. Ahmed E, Arshad M, Ahmad M, Saeed M, Ishaque M. Ethnopharmacological survey of some medicinally important plkants of Galliyat areas of NWFP, Pakistan. *Asian J Plant Sci* 2004;3:410–5.
14. Castillo-España P, Cisneros-Estrada A, Garduño-Ramirez ML, Hernández-Abreu O, Ramirez R, Estrada-Soto S. Preliminary ethnopharmacological survey of plants used in Mexico for the treatment of hypertension. *Pharmacognosy Rev* 2009;3:41–65.
15. Pieroni A, Giusti ME, de Pasquale C, Lenzarini C, et al. Circum-Mediterranean cultural heritage and medicinal plant uses in traditional animal healthcare: a field survey in eight selected areas within the RUBIA project. *J Ethnobiol Ethnomed* 2006;2:16–27.
16. Heinrich M, Robles M, West JE, Ortiz de Montellano BR, Rodriguez E. Ethnopharmacology of Mexican Astaraceae (*Compositae*). *Annu Rev Pharmacol Toxicol* 1998;38:539–65.
17. Monks NR, Ferraz A, et al. In vitro cytotoxicity of extracts from Brazilian Asteraceae. *Pharm Biol* 2002;40:494–500.
18. Akkol EK, Koca U, Pesin I, Yilmazer D. Evaluation of the wound healing potential of *Achillea biebersteinii* Afan. (Asteraceae) by in vivo excision and incision models. *eCAM* 2009; doi:10.1093/ecam/nep039.
19. Uddin SJ, Grice ID, Tiralongo E. Cytotoxic effects of Bangladeshi plant extracts. *eCAM* 2009; doi:10.1093/ecam/nep111.
20. Mandal SK, Boominathan R, Parimaladevi B, Dewanjee S, Mandal S. Analgesic activity of methanol extract of *Eupatorium adenophorum* Spreng. leaves. *Ind J Exp Biol* 2005;43:262–3.
21. Matsushima GK, Stohlman SA. Maturation of the delayed-type hypersensitivity response in SJL mice: absence of effector cell induction. *Eur J Immunol* 2005;18:1411–6.
22. Bandyopadhyay U, Das D, Banerjee RK. Reactive oxygen species: oxidative damage and pathogenesis. *Curr Sci* 1999;77:658–66.
23. Dedon PC, Tannenbaum SR. Reactive nitrogen species in the chemical biology of inflammation. *Arch Biochem Biophys* 2004;423:12–22.
24. Halliwell B, Hoult JR, Blake DR. Oxidants, inflammation, and anti-inflammatory drugs. *FASEB J* 1998;2:2867–73.
25. Barnes PJ. Reactive oxygen species and airway inflammation. *Free Rad Biol Med* 1990;9:235–43.
26. Nguemfol EL, Dimol T, Dongmo AB, Azebaze AGB, Alaoui K, Asongalem AE, et al. Anti-oxidative and anti-inflammatory activities of some isolated constituents from the stem bark of *Allanblackia monticola* Staner L.C (Guttiferae). *Inflammopharmacology* 2009;17:37–41.
27. Abreu P, Matthew S, Gonza'lez T, Costa D, Segundo MA, Fernandes E. Anti-inflammatory and antioxidant activity of a medicinal tincture from *Pedilanthus tithymaloides*. *Life Sci* 2006;78:1578–85.
28. Joseph S, Sabulal B, George V, Smina TP, Janardhanan KK. Antioxidative and anti-inflammatory activities of the chloroform extract of *Ganoderma lucidum* found in South India. *Sci Pharm* 2009;77:111–21.
29. Dinarello CA. Proinflammatory cytokines. *Chest* 2000;118:503–8.
30. Strieter R, Kunkel S, Bone R. Role of tumor necrosis factor-alpha in disease states and inflammation. *Crit Care Med* 1993;21:S447–63.
31. Lukacs NW, Strieter RM, Chensue SW, Widmer M, Kunkel SL. TNF-alpha mediates recruitment of neutrophils and eosinophils during airway inflammation. *J Immunol* 1995;154:5411–7.
32. Tracey K, Cerami A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu Rev Med* 1994;45:491–503.
33. Schweizer A, Feige U, Fontana A, et al. Interleukin-1 enhances pain reflexes: mediation through increased prostaglandin E2 levels. *Agents Actions* 1998;25:246–51.
34. Gabay C. Interleukin-6 and chronic inflammation. *Arthritis Res Ther* 2006;8(Suppl 2):S3–8.
35. Omoigui S. The interleukin-6 inflammation pathway from cholesterol to aging—role of statins, bisphosphonates and plant polyphenols in aging and age-related diseases. *Immun Ageing* 2007;4:1–22.
36. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003;33:1205–14.
37. Alkalay I, Yaron A, Hatzubai A, et al. Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 1995;92:10599–603.
38. Pande V, Ramos MJ. NF-κB in human disease: current inhibitors and prospects for *de novo* structure-based design of inhibitors. *Curr Med Chem* 2005;12:357–74.
39. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 2001;107:7–11.
40. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* 2001;107:135–42.
41. Turini ME, DuBois RN. Cyclooxygenase-2: a therapeutic target. *Annu Rev Med* 2002;53:35–57.
42. Smith CJ, Zhang Y, Koboldt CM, Muhammad J, et al. Pharmacological analysis of cyclooxygenase 1 in inflammation. *Proc Natl Acad Sci USA* 1998;95:13313–8.
43. Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthase (cyclooxygenases)-1 and -2. *J Biol Chem* 1996;271:33157–60.

K:/oup/ecam/neq033.3d [6.4.2010–6:57pm] [1–10] Paper: neq033
Copyedited by: NK MANUSCRIPT CATEGORY: ORIGINAL ARTICLE

eCAM 2010;Page 1 of 10
doi:10.1093/ecam/neq033

Original Article

Anti-inflammatory Potential of Ethanolic Leaf Extract of *Eupatorium adenophorum* Spreng. Through Alteration in Production of TNF-α, ROS and Expression of Certain Genes

Ashim K. Chakravarty[1,*], Tamal Mazumder[1] and Shankar N. Chatterjee[2]

[1]Immunology and Cell Biology Laboratory, Department of Zoology, University of North Bengal, Siliguri and [2]Former Joint Director, SeriBiotech, Ministry of Textiles, Govt. of India, Kolkata, India

*Corresponding author: Ashim K. Chakravarty, Immunology and Cell Biology Laboratory, Department of Zoology, School of life Sciences, University of North Bengal, Siliguri 734013, India.
E-mail: prof_ashim_chakravarty@rediffmail.com

Search for a novel anti-inflammatory agent from a herbal source, such as *Eupatorium adenophorum* Spreng., a plant from the Eastern Himalayas, is of prime interest in the present investigation. Inflammation causes tissue destruction and development of diseases such as asthma, rheumatoid arthritis, etc. The ethanolic leaf extract of *E. adenophorum* (EEA) was administered intravenously and in other cases topically at the site of delayed type hypersensitivity (DTH) reaction in mouse foot paw induced with dinitrofluorobenzene. EEA can effectively inhibit DTH reaction and bring back normalcy to the paw much earlier than the controls. Efficacy of EEA on regulatory mechanisms for inflammation has also been considered. Intravenous administration of EEA increased the number of $CD4^+$ T cells in spleen and tumor necrosis factor (TNF)-α in serum of DTH mice. Initially it was difficult to reconcile with the anti-inflammatory role of EEA and simultaneous induction of TNF-α, an established pro-inflammatory cytokine. EEA induces higher expression of *TNF-α* gene and amount of the cytokine in serum. We discussed the other role of TNF-α, its involvement in repairing tissue damage incurred in course of inflammatory reaction. EEA also induces TGF-β encoding a cytokine involved in tissue repair mechanism. EEA inhibits expression of another pro-inflammatory cytokine gene *IL-1β* and downregulates cycloxygenase 2 (*COX2*) gene responsible for metabolism of inflammatory mediators like prostaglandins. Furthermore, anti-inflammatory role of EEA is also revealed through its inhibition of hydroxyl radical generation. Notably EEA does not necessarily affect the expression of other inflammation-related genes such as *IL-6*, *IL-10* and *IKK*. The present study reports and analyzes for the first time the anti-inflammatory property of the leaf extract of *E. adenophorum*.

Keywords: anti-inflammatory activity – delayed type hypersensitivity – ethanolic leaf extract of *Eupatorium adenophorum* Spreng – tumor necrosis factor-α – gene expression

Introduction

Inflammation is the body's way of dealing with infections, maintaining a subtle balance between the beneficial effects of inflammation cascades to restrict the infection and potential for long-term tissue destruction (1–3). If not controlled, inflammation can lead to development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, etc. (4–9). Till date a very few anti-inflammatory drugs from herbal origin have been found, and a number of plants from

Figure 1. Photograph of *E. adenophorum* Spreng. in its natural habitat.

ethno-medicinal databases are under laboratory investigation across the world (10).

The plant *Eupatorium adenophorum* Spreng. (Fig. 1) belongs to the family Asteraceae (Compositae) (11). A number of plants of this family are commonly used in folklore medicine in different parts of the world (12–19). In Kurseong and Darjeeling hill region of the Eastern Himalayas, local people use leaves of *E. adenophorum* Spreng., growing at an altitude of 800–2050 m, for remedial purposes against oral and skin sores. These observations suggested a probable anti-inflammatory and immunomodulatory activity of the plant's leaf extract. Earlier Mandal *et al.* (20) reported analgesic property of methanolic extract of the leaves. The present investigation intends to explore the anti-inflammatory property of ethanolic leaf extract of *E. adenophorum* Spreng. (EEA) in delayed type hypersensitivity (DTH) induced by 2,4-dinitrofluorobenzene (DNFB) in mouse model.

DTH reaction is initiated by pre-sensitized $CD4^+$ T_{DTH} cells (21) and then other inflammatory cells and cytokines are involved at the site of reaction. The number of CD4 T cells in course of DTH reaction and treatment with EEA has been enumerated to understand the effect of EEA on these cells. TNF-α is the most important cytokine that plays a major role in all the inflammation reactions. Serum TNF-α of DTH mice has also been investigated in the present study in course of inflammation.

Many reports reveal that reactive oxygen species (ROS) play an important role in developing various pathophysiological conditions including inflammation (22–25) and potent anti-inflammatory agents can scavenge the free radicals to quench the biochemical fire (26–28). The hydroxyl radical (OH^-) especially plays a crucial role in developing inflammation. Scavenging of hydroxyl radical (OH^-) by EEA has been studied.

Besides TNF-α, many other cytokines play a key role in orchestrating immune responses in inflammation (29–36). Here, expression of some cytokine genes such as TNF-α, IL-1β, IL-6, IL-10 and TGF-β in splenic T cells of DTH mice has been studied at transcription level with and without intravenous (i.v.) application of the plant extract. Expression of inhibitory kappa kinase (*IKK*) gene has also been judged. The enzyme degrades IκB subunit to release active NF-κB (37,38) that is involved in activating inflammatory responses as shown by Tak and Firestein (39) and Yamamoto and Gaynor (40).

The expression of COX1 and COX2 genes encoding two isozymes of cycloxygenase has been taken into account here. Cycloxygenase is known to play a significant role in induction of inflammation by producing inflammatory mediators like prostaglandins and leukotrienes from arachidonic acid (41–47).

Methods

Animal

Inbred adult Swiss Albino mice of both sexes, 12–16 weeks old have been used. They are maintained in our animal house with food and water *ad libitum*. Animals of approximately equal age and weight were used for experimental and control groups in the experiment. The experimental protocols used in the study have been approved by the Animal Ethical Committee (Regn No. 840/ac/04/CPCSEA).

Extract Preparation

Extract was prepared from fresh leaves of the plant *E. adenophorum* using the protocol outlined earlier (48–51). Leaves were collected from their natural habitat at about 1400 m high slope of the Eastern Himalayas, mainly around Kurseong hill. The scientific identification of the plant has been checked by Professor A. P. Das, Plant Taxonomy Lab., Department of Botany, University of North Bengal. The leaves were cleaned thoroughly with water and allowed to air dry. In total, 10 g of leaves were crushed to a paste with a mortar and pestle. An amount of 10 ml of absolute alcohol (ethanol) was added to the paste and kept in a refrigerator overnight for extraction. The alcoholic extract was then filtered first through Whatmann filter paper and the filtrate was refiltered again through cellulose acetate filter paper (0.2 μm porosity, Sartorius) for sterilization

and finally stored in airtight sterilized vial at 4°C. The extract was used as such for different experiments.

Experimental and Control Sets and Statistical Analysis

In all the experiments, the effect of EEA was compared with two sets of control, one with equivalent amount of ethanol present in EEA and the other without any treatment. Each experiment was done on the basis of triplicate readings and such an experiment was repeated thrice or more. Results are expressed as Mean ± SD of at least nine observations. Statistical significance was analyzed using one-way ANOVA software package.

Induction of Delayed Type Hypersensitivity Reaction with 2,4-DNFB and Application of EEA

Delayed type hypersensitive (DTH) reaction was induced in mouse foot paw by subcutaneous application of 2,4-DNFB (48,52). Primary sensitization was carried out by applying 0.0001% DNFB subcutaneously in the right foot pad. After 8 days, mice were resensitized with 0.000001% DNFB on the left foot pad. Two different volumes of percentage solutions of DNFB, 25 or 50 µl for both sensitization and resensitization, were used in separate experimental setups. The day of resensitization was considered as '0' day for enumeration of DTH reaction. The size of the left paw before resensitization was considered as normal size for the paw. The degree of inflammatory swelling set in the resensitized left paw was measured by a slide caliper. The effect of EEA on DTH reaction set in by two different doses of DNFB was judged after topical or i.v. application of the extract. For topical application, 5 µl of EEA was applied on the resensitized paw per day from first day of resensitization. For i.v. administration, 25 µl of EEA was used 1 h prior to resensitization. The percentage of inhibition of inflammation by EEA in reference to the ethanol control has been calculated by using the following formula:

$$\text{Inhibition of DTH (\%)} = \frac{\text{Untreated DTH paw size} - \text{Experimental paw size}}{\text{Untreated DTH paw size}} \times 100$$

Isolation of $CD4^+$ T Cells through Magnetic Assorted Cell Sorter (MACS)

The splenic lymphocytes were obtained from untreated DTH mice and mice intravenously injected with EEA and ethanol after 24, 48 and 72 h of resensitization following the protocol of Chakravarty and Maitra (53). Erythrocytes in the spleen cell suspension were lysed by exposure to Tris-buffered ammonium chloride (0.83%, pH 7.2). For depletion of adherent cells, the suspension was incubated in a plastic petri dish at 37°C in humidified atmosphere for 30 min. Non-adherent lymphocyte population was collected and centrifuged and finally resuspended at a concentration of 10^7 cells in 80 µl. To the aliquot of 80 µl cell suspension, 20 µl of $CD4^+$ (L3TH) microbeads (130-049-201. Miltenyi Biotech, Germany) (54–56) with a magnetic probe was added in the test tube. The tubes were refrigerated at 4–6°C for attachment of the bead to the $CD4^+$ cells for 15 min. The mixture of cells and magnetic beads is then poured into the magnetic separation (MS) column fitted in the slot of the magnet of MACS. The unlabeled cells passed through the column and were collected in a tube. The MS column was removed from the separator and placed in a fresh collection tube. Of PBS, 1 ml was pipetted onto the MS column and labeled $CD4^+$ cells were flushed out from the column by firmly pushing the designated plunger into the column. The magnetic labeled $CD4^+$ cells were then counted in a hemocytometer.

Estimation of Serum TNF-α

The quantitation of serum TNF-α of DTH bearing mice treated (i.v.) with EEA and control mice was performed by solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen, USA) following the protocol outlined by Paul *et al.* (57–59).

Hydroxyl Radical Scavenging Assay

The hydroxyl radical (OH^-) was generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fentons' reaction). Assay reaction mixture was prepared by mixing a 20 mM phosphate buffer, 2 mM $FeCl_3$, 1 mM EDTA, 2.8 mM 2-deoxy D-ribose, 1 mM H_2O_2 and 1 mM L-ascorbic acid. OH^- reacts with the deoxy D-ribose and a series of reaction follows to form malonaldehyde (MDA) (60). An aliquot of 1 ml of the reaction mixture was added in each tube of experimental, alcohol control and normal control sets and incubated at 37°C for 1 h. Two different doses of EEA, 10 and 25 µl, were tested for scavenging hydroxyl radical. In alcohol and normal control, same volume of ethanol and water was added respectively. After incubation, 2 ml of TBA–TCA reagent was added in each tube and boiled for 15 min for generation of MDA. MDA generated was measured at 552 nm in a spectrophotometer. The effect of both EEA and alcohol on generation of hydroxyl radical has been expressed as percentage of inhibition in MDA generation over normal control sets. The formula used is given below:

$$\text{Percentage of inhibition} = \left[100 - \frac{\text{MDA generated in experimental/alcohol control tubes}}{\text{MDA generated in normal control tubes}}\right]\%$$

Gene Expression Analysis

Expression of the genes of interest in this study has been carried out using single-cell RNA phenotyping procedure as outlined by Rappolee *et al.* (47,61,62).

RNA Isolation

RNA was isolated from splenic T cells of nine mice in each group using RNeasy Mini kit (74104, Qiagen, Valencia, CA, USA), as per manufacturer's protocol. Briefly, 6×10^6 T cells were homogenized with a 300 µl RLT buffer and passing them through a 2 ml syringe fitted with a 27-gauge needle. Of 70% ethanol, 300 µl was added to the homogenate and collected in a spin column fitted upon a collection tube. The spin columns and collection tubes were supplied by the manufacturer. After a brief centrifugation for 15 s at 10 000 rpm, the fluid was passed into the collection tube that was then decanted and reattached to the spin column. With addition of 500 µl of buffer RW1 into the spin column centrifugation was made again for 15 s at 10 000 rpm. Following decantation of collection tube, 500 µl of buffer RPE was added to the spin column and centrifuged similarly, and the step was repeated one more time. Finally, the spin column was fitted upon a fresh collection tube and washed twice with 15 µl of DEPC-treated water to come up with a total of 30 µl volume containing the RNA sample.

The concentration of RNA was measured spectrophotometrically at 400× dilution with Shimadzu UV-160, Japan. The extracted RNA was used for cDNA synthesis.

cDNA Synthesis

The isolated RNA was used for First strand cDNA synthesis utilizing the RevertAid™ First strand cDNA synthesis kit # K1621 from Fermentas and the manufacturer's protocol was followed. For synthesis of first strand cDNA, the primer used for PCR amplification was oligo(dT^{13}) synthesized by GMBH. cDNA constructed was stored at −20°C for further use.

Primer Utilized and Amplification Schedule

Primers were designed from various geneBank accession retrieved from PUBMED Data Bank as listed in Table 1, using the primer program available on the internet. The designed primers were synthesized by GMBH, Germany. Details of the primers are given in Table 1.

PCR was performed using a thermocycler (PeqLab, Germany) for 35 cycles in a 30 µl reaction mixture containing Taq DNA polymerase buffer, all four dNTPs, oligonucleotide primers, Taq DNA polymerase and cDNA products. After amplification, PCR products were analyzed on a 0.8% (w/v) agarose gel. The band density was quantified on the basis of the known concentration of lambda DNA (30 ng) through ImageAide, Spectronics Corporation, NY.

Results

Effect of EEA on Delayed Type Hypersensitive Reaction

With topical Application

Figure 2 represents the data of delayed type hypersensitivity reaction induced with 25 µl DNFB and treatment with ethanolic leaf extract of *E. adenophorum* (EEA) (experimental) and ethanol in control. Swelling of resensitized paw in both control and experimental mice was maximum on third day; with topical application of EEA, the maximum swelling was in the range of 0.4733 ± 0.0227 cm, and in ethanol-treated control mice the maximum value was 0.5667 ± 0.173 cm (Fig. 2). The paw size in EEA-treated mice gets back to normal range by the ninth day of resensitization. Inhibition with EEA is a continuous process throughout the experiment. Figure 2 also represents the percentages of inhibition on different days.

A higher dose (50 µl) of DNFB causes the swelling to be more and the peak on the second day; however, recovery with EEA treatment is on the ninth day (Fig. 3). In fact EEA inhibited the swelling by 60% on the ninth day. Figure 4 is the photographic

Table 1. Primers

Primers	Accession number	Sense (5′–3′)	Antisense (3′–5′)	T_m(°C)
IKK	NM_010546	CCAGACTCCAAGGTGGTGTT	TGCAGATCACAGGCAGAAAC	60.0
TNF-α	NM_013693	TGGCACAGCCAAG	GGGACCCCTGCTC	52.36
TGF-β	NM_011577	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC	59.99
IL-1β	NM_008361	GTGGCAGCTACCTGTGTCTT	GGAGCCTGTAGTGCAGTTGT	57.96
IL-6	NM_031168	GGGAAATCGTGGA	AGGTTTGCCGAGT	43.9
IL-10	NM_010548	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG	60.035
COX1	BC023322	AGAAACTGGTCTGCCTCA	AACCCACATCAAGGACTG	54.02
COX2	NM_011198	AGCACCATTCTCCTTGAA	GTAGGCTGTGGATCTTGC	54.0
PKC- θ	NM_008859	AAGTGAGAAACCCCGGCTAT	AGGCAAATCCCTTCCAGTCT	60.01
Perforin	NM_011073	ACCCTGAATGGGCTCACA	GCAGCAGTCCTGGTTGGT	57.0

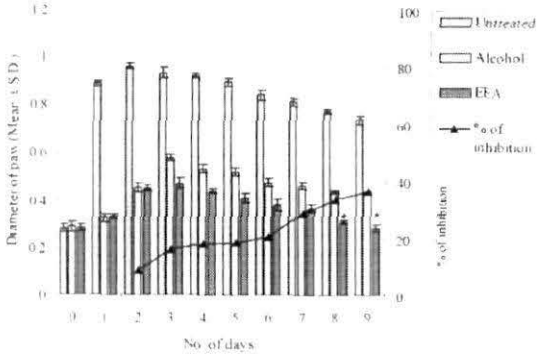


Figure 2. Inhibitory effect of topical application of EEA on delayed type hypersensitive (DTH) reaction induced with 25 µl 2,4-DNFB (significance at $P < 0.01$).

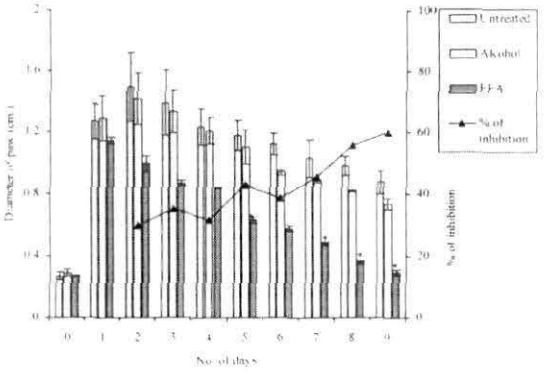


Figure 3. Indurations of DTH swelling, induced with 50 µl DNFB, and inhibition after topical application of EEA and alcohol (significance at $P < 0.01$).

representation of DTH reaction induced with 50 µl DNFB and inhibition with EEA treatment.

With i.v. Application

When EEA was administered intravenously in DTH bearing mice, inflammatory swelling of the resensitized paw persisted longer than in cases of topical application of EEA. The dose of 25 µl DNFB could induce maximum swelling of 0.963 ± 0.012 cm on the second day in untreated mice. Intravenous application of EEA caused the DTH reaction to slow down. In alcohol group the swelling was maximum, 0.7367 ± 0.0045 cm, on fifth day (also on sixth). EEA treatment restricted the swelling to 0.5123 ± 0.0112 cm on fifth day and brought back normalcy (0.2967 ± 0.0103 cm) by 11th day; the inhibition over the ethanol control was found to be 45.39% (Fig. 5).

The degree of swelling was more at DTH site with higher dose (50 µl) of DNFB (Fig. 6). EEA (i.v.) inhibited the reaction more effectively than alcohol alone (control) and allowed regaining normalcy by 13 days (Fig. 6).

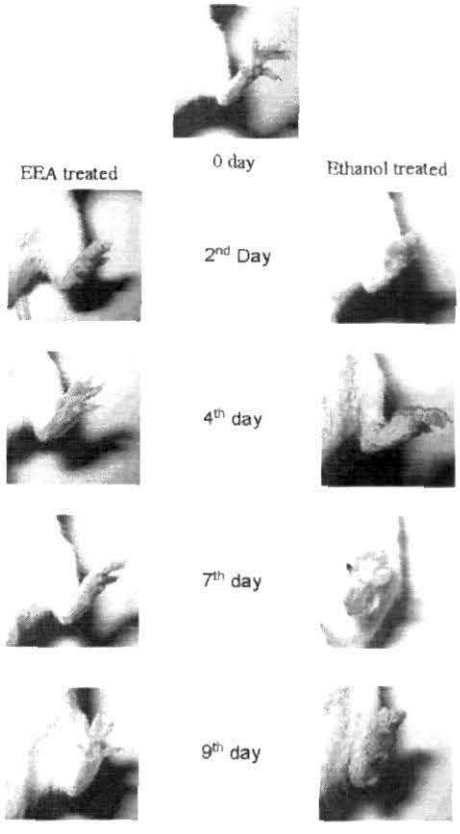


Figure 4. Photographs showing effect of topical application of EEA and alcohol on resensitized paw of DTH mice, induced by 50 µl DNFB, during 9-day period of the study.

Estimation of the Number of CD4$^+$ T Cells in DTH Mice

CD4$^+$ T cells from splenic lymphocyte population were isolated by labeling with microbeads containing a paramagnetic probe and passing through MACS. The number of CD4$^+$ T lymphocytes in the spleen of DTH mice treated with EEA was significantly more than the controls, about two and a half times at 24 and 48 h and two times at 72 h (Fig. 7). A slight increase in the cell number in the alcohol control group was noted (Fig. 7).

K:/oup/ecam/neq033.3d [6.4.2010–6:57pm] [1–10] Paper: neq033
Copyedited by: NK MANUSCRIPT CATEGORY: ORIGINAL ARTICLE

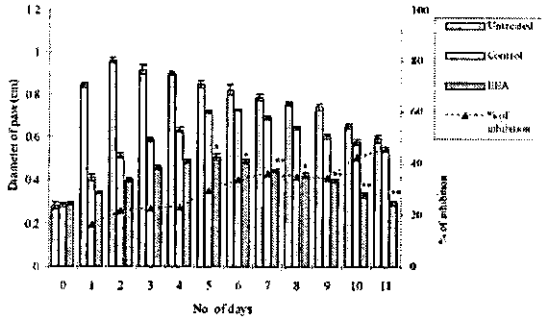


Figure 5. Changes in paw size of DTH mice, induced with 25 µl DNFB, and after i.v. application of EEA and alcohol (significance of inhibition by EEA at $P < 0.01$).

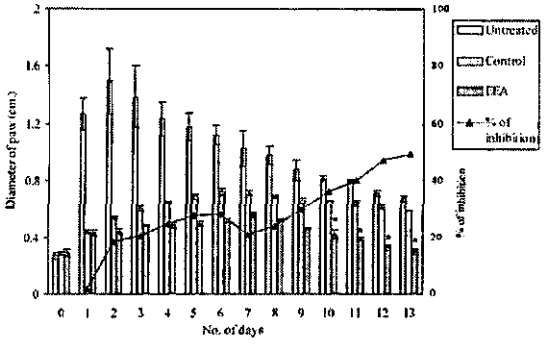


Figure 6. Changes in paw size of DTH mice, induced with 50 µl DNFB, and after i.v. application of EEA and alcohol (significance of inhibition by EEA at $P < 0.01$).

Serum TNF-α of DTH-bearing Mice and Upon EEA Treatment

Serum TNF-α in three groups of mice bearing DTH reaction—untreated, alcohol and EEA treated (i.v.)—was measured by a solid phase sandwich enzyme-linked immunosorbent assay (ELISA).

There was no appreciable difference in the level of serum TNF-α in the three groups of mice at 24 h (Fig. 8). The TNF-α level in DTH mice treated with EEA (i.v.) was more than that in other groups by 48 h (93.23 pg ml^{-1}) and the level was maintained upto 72 h (Fig. 8).

Generation of Hydroxyl Radical and Effect of EEA

The hydroxyl radical (OH·) is potentially harmful for the cellular macromolecules and is implicated in pathophysiology of inflammation. EEA inhibits hydroxyl radical generation up to 57.98% at a dose of 25 µl. The same

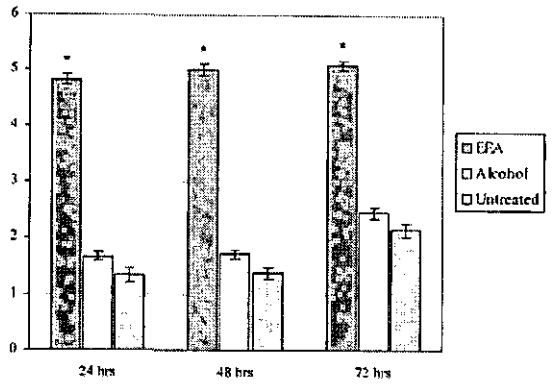


Figure 7. Effect of intravenously administered EEA and alcohol on count of CD4$^+$ T cells from spleen of mice after 24, 48 and 72 h of induction of DTH with 25 µl DNFB (significance of results with EEA over control at $P < 0.01$).

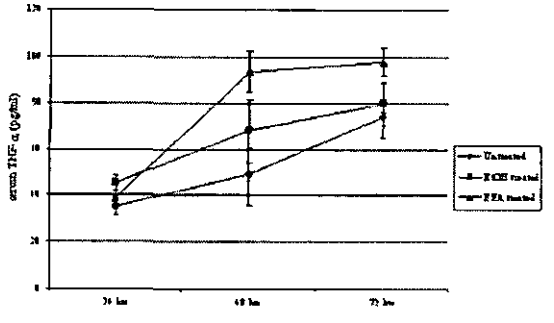


Figure 8. Level of serum TNF-α from DTH mice (induced with 25 µl DNFB) at three different hours in presence and absence of EEA (significance compared to controls at $P < 0.01$).

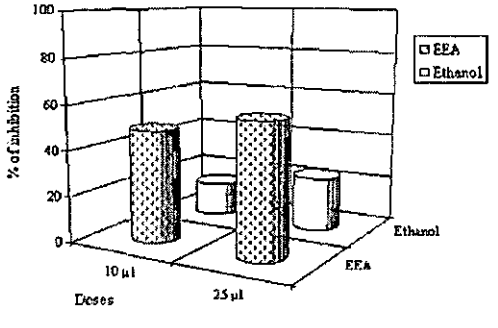


Figure 9. The effect of two different doses of EEA on inhibition of generation of hydroxyl radical (OH·).

dose of ethanol (control) showed 23.62% inhibition. At a lower dose of 10 µl, the degree of inhibition was about 49.25% in experimental ones and 14.88% in case of alcohol control (Fig. 9).

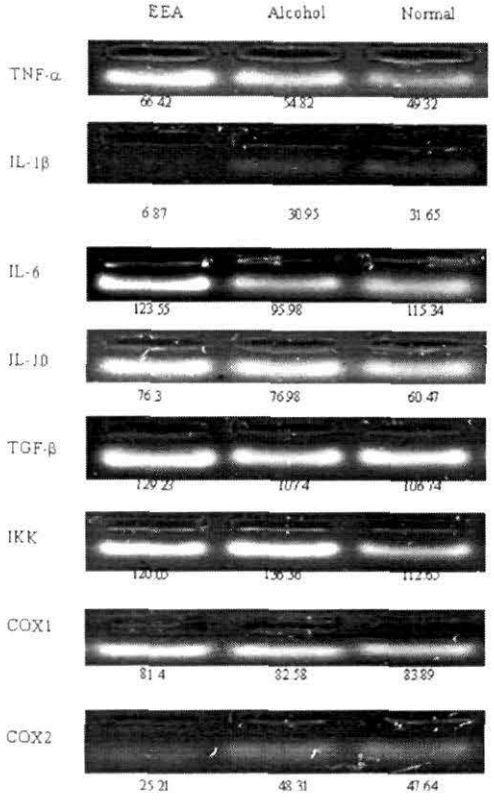


Figure 10. Expression of certain inflammation-related genes in T cells obtained from *DTH* mice, and with and without EEA treatment, containing nine mice in each group. Agarose gel electrophoresis of cDNA PCR products amplified with different gene-specific primers.

Expression of Certain Genes in Splenic T Cells of DTH Mice with and without EEA Treatment

The T cells actively participate in the progression of DTH reaction. Up- or downregulation of certain genes in the cells must be related to the production of pro- and anti-inflammatory cytokines, transcription factors and mediators. The level of expression of some of these genes at transcription level in splenic T cells of DTH mice untreated and treated with EEA was judged, by quantitating the cDNA PCR product amplified with specific primers. The quantitation was done against 30 ng of lambda DNA as a standard using ImageAide, Spectronics Corporation, NY. The data are presented in Figs 10 and 11.

EEA caused an increment in the expression of *TNF-α*, a pro-inflammatory cytokine (Figs 10 and 11). At the same time, EEA inhibited expression of pro-inflammatory cytokine *IL-1β* and showed no effect on *IL-6*, another pro-inflammatory cytokine.

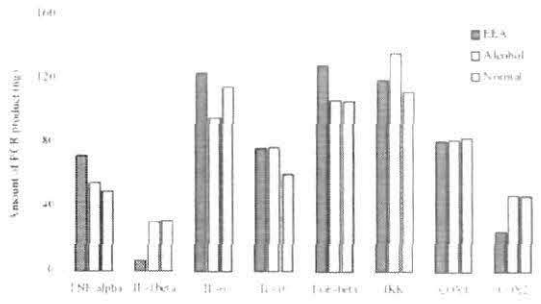


Figure 11. Graphical presentation of quantification of the DNA bands presented in Fig. 10 showing expression of the inflammation-related genes in T cells of DTH mice with and without EEA treatment, containing nine mice in each group. The DNA bands were quantified against 30 ng of lambda DNA through ImageAide, Spectronics Corporation, NY.

EEA did not influence the expression of *IL-10*, an anti-inflammatory cytokine beyond alcohol control. The expression of *TGF-β* encoding a cytokine involved in regeneration was induced by EEA beyond the level in the controls.

EEA apparently did not influence expression of *IKK* and *COX1* genes but downregulated the expression of *COX2* gene.

Discussion

The ethanolic leaf extract of *E. adenophorum* Spreng. (EEA) could effectively suppress the inflammatory reaction set in foot paw by injecting two different doses of 2,4-DNFB (Fig. 4). The topical application of EEA was more effective in inhibition of the swelling of foot paw and gaining normaley faster than its i.v. application (Figs 2, 3, 5 and 6).

EEA treatment caused significant increase in the number of the $CD4^+$ T cell population in DTH mice (Fig. 7). These cells are known to play a central role in inflammatory reactions by secreting all different kinds of cytokines that regulate participation of other kinds of cells (21,63,64).

TNF-α is a major cytokine involved in DTH reaction. EEA induced higher level of serum TNF-α, surpassing the level in mice undergoing DTH reaction or in mice treated with alcohol (Fig. 8). It is worthwhile to note EEA capable of inhibiting DTH reaction is inducing a pro-inflammatory cytokine like TNF-α. Banno *et al.* (65) showed that TNF-α promotes tissue repair of damage skin by inducing basement membrane components and collagen degrading proteases to participate actively in reconstruction of extracellular matrix. Kuwano and his coworkers (66) found that TNF-α can also induce growth-promoting event like angiogenesis by increasing mRNA level of IL-8, vascular endothelial growth factor

Wang D, DuBois RN. 2006. Prostaglandins and cancer. *Gut* 55:115-122.

Wang J, Elterun IE, Lamartiniere CA. 2007. Genistein chemoprevention of prostate cancer in TRAMP mice. *Journal of Cercinogenesis.* 6:3-12.

Waterfield JD, Dennert G, Swain SL, Dutton RW. 1979. Continuously proliferating allospecific T cells. I. Specificity of Cooperation with Allogeneic B Cells in the Humoral Antibody Response to Sheep Erythroeytes. *J. Exp. Med.* 149:808-814.

Waterfield JD, Waterfield EM, Moller G. 1976. Lymphocyte mediated cytotoxicity against tumor cells. II. Characteristics and tissue distribution of Concanavalin A activated cytotoxic effector cell. *Eur.J. Immunol.* 6:309-316.

Weber V, Coudert P, Rubat C, Duroux E, *et al.* 2002. Novel 4,5-Diaryl-3-hydroxy-2(5*H*)-furanones as Anti-Oxidants and Anti-Inflammatory Agents. *Bioorg. Med. Chem.* 10:1647-1658.

Wegener T, Fintelmann V. 1999. Flavonoids and Bioactivity. *Wein Med Wochem Schr.* 149:241-247.

Winrow VR, Winyard PG, Morris CJ, Blake DR. 1993. Free radicals in inflammation: second messengers and mediators of tissue destruction. *British Medical Bulletin* 49:506-522.

Yamamoto Y, Gaynor RB. 2001. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* 107:135-42.

Yasmin H, Chakravarty AK. 2010. Turmeric accentuates cell mediated cytotoxicity towards tumor target cells and activates nitric oxide synthase in lymphocytes. (Communicated)

Yilmaz MI. 2007. The causes of inflammation and possible therapeutic options in dialysis patients. *Gulhane Tip. Dergisi.* 49:271-276.

Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, *et al.* 1997. Involvement of interleukin-8, vascular endothelial growth factor, basic fibroblast growth factor in tumor necrosis factor alpha-dependant angiogenesis. *Mol Cell Biol.* 17:4015-4023.

Zhang ML, Wu M, Zhang JJ, Irwin D, Gu YC, Shi QW. 2008. Chemical Constituents of Plants from the Genus Eupatorium. *Chemistry and Biodiversity* 5:40-45.

Zipser RD. 1998. Mediators of inflammation in inflammatory bowel disease. *Digestive Diseases and Sciences* 33:4S-5S.

Zlotnik A. 2006. Chemokines and cancer. *Int. J. Cancer.* 119:2026-2029.

List of Publications

1. Chakravarty A.K., **Mazumder T.** and Chatterjee S.N. (2010). Anti-inflammatory potential of ethanolic leaf extract of *Eupatorium adenophorum* Spreng. through alteration in production of TNF-α, ROS and expression of certain genes. *Evidence Based Complementary and Alternative Medicine*. (In Press)

2. Chakravarty A.K., **Mazumder T.** and Yasmin H. (2009). Bioactive agents from herbal sources with immunopharmacological properties abating inflammation and malignancy. In "*Compendium of Bioactive Natural Products*", Studium Press LLC, USA, Ch. 9, pp. 216-263.

3. Chakravarty A.K., Chatterjee S.N., Yasmin H. and **Mazumder T.** (2009) Comparison of efficacy of turmeric and commercial curcumin in immunological functions and gene regulation. *Intl. J. Pharmacol.* 5:333-345.

4. Chakravarty A.K. and **Mazumder T.** (2009). *Leucas aspera* as a Source of Immunostimulatory Substance for Lymphocyte Activation and Inhibition of Delayed Type Hypersensitive (DTH) Reaction. *NBU J Anim Sci* 3:10-17

5. Yasmin H, **Mazumder T**, Mittal P and Chakravarty AK. (2005). Turmeric Medicated Activations of lymphocytes and Induction of Apoptosis of Tumor Cells. *Indian Journal of Medical Research.* 121: 61.

Corrigendum

1. Initially survivality of splenic lymphocytes in presence of three doses (5, 10 & 25 µl) of ethanolic leaf extract of *Eupatorium adenophorum* (EEA) was observed *in vitro* at several hours as they are in Fig. 4. At each hour for different doses three cultures were maintained in an experiment and the experiment repeated thrice. Thus, in the final figure, each point represents nine readings. The data are now appended herewith (marked as Fig. 4A as continuation of Fig. 4 in the thesis). The percentage of cell viability was found to be at reasonable level with 25 µl dose. In view of EEA's efficacy at 25 µl dose and to keep the amount of vehicle on the lower side, further higher doses were not preferred. That is why the 25 µl dose was used in subsequent experiments.

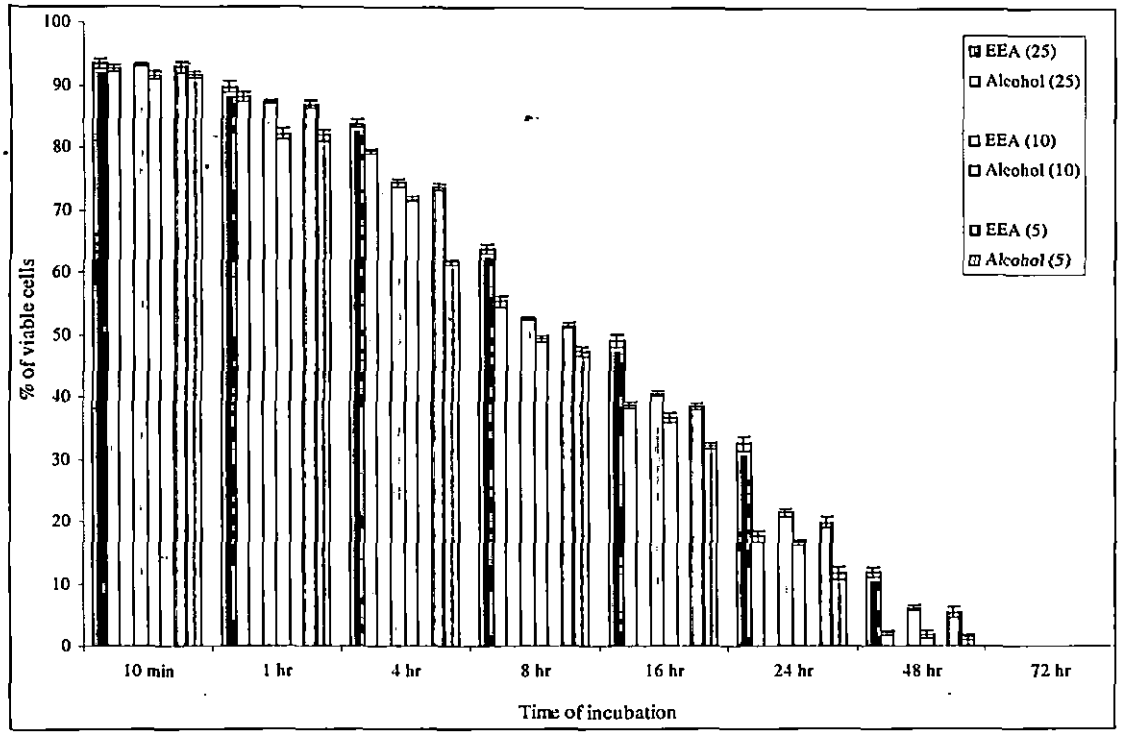


Fig. 4A: Survivality of lymphocytes *in vitro* in presence and absence of EEA. Results are expressed as mean ± SD. Numbers in parenthesis indicate doses in µl.

As the 25 µl dose was found effective *in vitro* the same was maintained for *in vivo* experiment, and it was found to be non-toxic for the animals and also activate

lymphocytes *in vivo*. Two different doses for *in vivo* and *in vitro* experiment might not allow discussion of *in vivo* and *in vitro* studies at a time.

It is not possible to carry out all the experiments over the years with the same batch of extract. It is expected that batch to batch there would be certain minimal variations in the constituents. We tried to maintain a dry weight equivalence for different batches of EEA extract. 1 ml of EEA after each batch of extract preparation was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^0 C and the dry weight was in the range of 0.529±0.019 mg. This is mentioned under Preparation of ethanolic leaf extract of *Eupatorium adenophorum* (EEA) in Materials and Methods in Page 10. Dry weight equivalence indicates amount of bulk material in different batches of extract and there might be subtle variation in principal compounds. However, there were not much significant differences in the results from experiment to experiment and the final data represents average of all the experiments.

2. In the present thesis, the figures and tables have been formatted with running text in the result section. The explanatory note for each figure and table were in the text. That was not included in the legends just not to be repetitive.

3. Fig. 3 in page 30 denotes total count of WBCs in blood. It is mentioned in Page 29 that the effects of the extract on blood parameters have been studied in this section.

The proliferative response of the total WBCs on treatment with EEA seems to reflect promotional effect of the extract towards the leukocytes. This has been mentioned in Page 30 of the result section, and this proliferative activity of WBC advocated probable immunostimulatory activity of the extract and encouraged further evaluation of EEA as immunomodulatory agent (mentioned in Discussion, Page 63, Para 2, Line 1).

The effect of EEA on hematological parameters was studied on different days after injecting (i.v.) 25 µl EEA or ethanol in mice only once on '0' day (Fig. 1-3).

lymphocytes *in vivo*. Two different doses for *in vivo* and *in vitro* experiment might not allow discussion of *in vivo* and *in vitro* studies at a time.

It is not possible to carry out all the experiments over the years with the same batch of extract. It is expected that batch to batch there would be certain minimal variations in the constituents. We tried to maintain a dry weight equivalence for different batches of EEA extract. 1 ml of EEA after each batch of extract preparation was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55^0 C and the dry weight was in the range of 0.529±0.019 mg. This is mentioned under Preparation of ethanolic leaf extract of *Eupatorium adenophorum* (EEA) in Materials and Methods in Page 10. Dry weight equivalence indicates amount of bulk material in different batches of extract and there might be subtle variation in principal compounds. However, there were not much significant differences in the results from experiment to experiment and the final data represents average of all the experiments.

2. In the present thesis, the figures and tables have been formatted with running text in the result section. The explanatory note for each figure and table were in the text. That was not included in the legends just not to be repetitive.

3. Fig. 3 in page 30 denotes total count of WBCs in blood. It is mentioned in Page 29 that the effects of the extract on blood parameters have been studied in this section.

The proliferative response of the total WBCs on treatment with EEA seems to reflect promotional effect of the extract towards the leukocytes. This has been mentioned in Page 30 of the result section, and this proliferative activity of WBC advocated probable immunostimulatory activity of the extract and encouraged further evaluation of EEA as immunomodulatory agent (mentioned in Discussion, Page 63, Para 2, Line 1).

The effect of EEA on hematological parameters was studied on different days after injecting (i.v.) 25 µl EEA or ethanol in mice only once on '0' day (Fig. 1-3).

4. The information about number of cultures or animals used in each experiment has been stated under 'Statistical analysis' in Materials and Methods, in page 28 – "Triplicates in experimental and control set were maintained (for each point) in each experiment. An experiment was repeated thrice or more. Results are expressed as Mean±SD of *n* observations". Thus, for both *in vitro* and *in vivo* experiments, data at each point represent at least 9 readings from cultures or animals.

5. The cells in culture medium only (untreated cells) gradually die off in course of culture. Alcohol control was always maintained as alcohol was the vehicle for leaf extract of *E. adenophorum*. So the data of untreated cells were not included in Fig. 4, page 31 to keep the diagram focussed on the effect of the extract. However the results of untreated cells at 16, 24 and 48 hrs are included here in Fig. 4B. In our experience, the percentage of survival of unactivated (without any treatment) splenic lymphocytes is always poor.

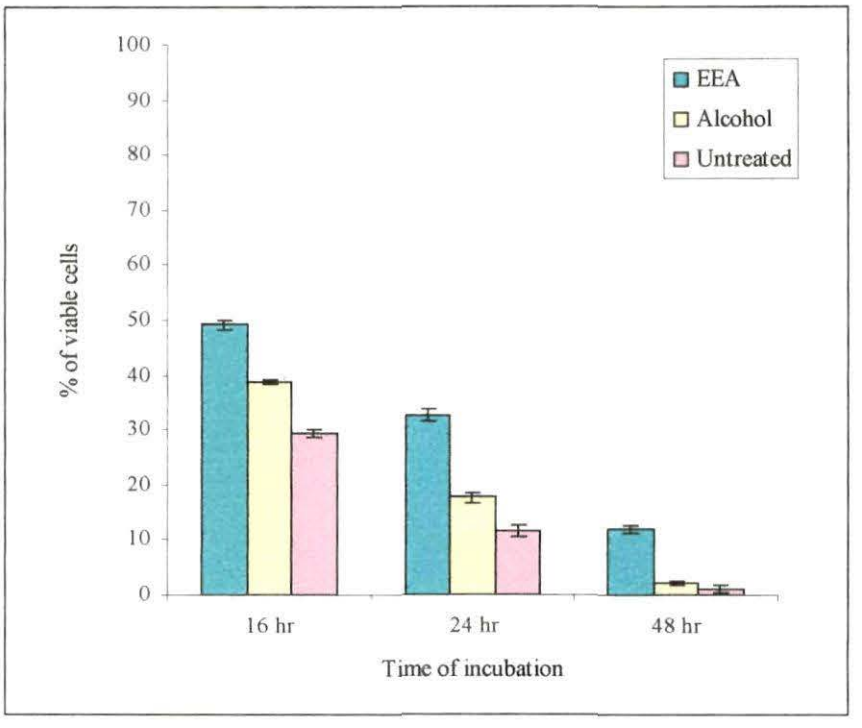


Fig. 4B: Survivality of lymphocytes *in vitro* treated with EEA, alcohol and without any treatment in medium only. Results are expressed as mean ± SD.

6. Page 32, Fig. 5: One of the main objectives of the present study was to judge the immunomodulatory property of EEA, and significantly higher percentage of blastogenic transformation of both T and B lymphocytes with EEA over ethanol control was found. Experiments with several conventional mitogenic polyclonal stimulators for murine lymphocytes were carried out in our laboratory earlier (see in Reference Chakravarty and Chaudhuri, 1983; Chakravarty and Maitra, 1983; Chaudhuri and Chakravarty, 1983a; Chaudhuri T.K. and A.K. Chakravarty. (1981). *J. Ind. Inst. Sci.* 63(C): 149-156; Maitra U.K. and A.K. Chakravarty (1982). *Current Sci.* 51: 1012-1015.). The stimulatory property of any mitogenic substance is usually measured in reference to the vehicle control. There is a note about untreated lymphocytes (i.e. in culture medium only) in point 5. The data of untreated splenocytes is not provided because lymphocytes without stimulation are not supposed to undergo blastogenic-transformation.

7. Page 33, Fig. 6: Our laboratory has remained busy for last one decade to characterize immunotherapeutic activities of bioactive natural products such as turmeric (*Curcuma longa* Linn.), *Eupatorium adenophorum* and *Leucas aspera*. The efficacies of ethanolic extracts from these agents were always compared with that of ethanol as vehicle control. Many a times, ethanol was found to have some stimulatory effect compared to untreated control at initial hours only and the effect did not continue too long: That is also reflected in the measure of DNA synthesis in lymphocytes in the present study.

It seems that the maximum level of stimulation for DNA synthesis with EEA treatment was achieved by 16 hrs and the proliferation index continued further. This has been written in Result section, page32, para 2.

8. Page 34, Table 1: Cell cycle analysis by FACS reading was carried out at Central Regional Instrument Facilities, Bose Institute, Centenary Building, Kolkata. The software

of this machine (FACS, Caliber, Beckton Dickinson) has counted the cells in four different phases – G_0, G_1, S and G_2-M (denoted as M1, M2, M3 and M4 in Fig. 7, Page 35). The G_0 phase signifies the cells in quiescent or apoptotic stages as stated in Page 34, Para 1.

9. Page 35, Fig. 7: The data in Fig. 7 were automatically generated by the FACS machine. At 16, 24 and 48 hrs the cells were pooled from respective cultures and adjusted to 10^6 living cells per ml for FACS reading. It is likely even after two washes some of the dead cells from the culture got included in the counting sample. The first peak includes dead cells and quiescent cells as indicated in point 8.

10. Page 36, Fig. 8: The inhibitory effect of topical application of EEA and alcohol on DTH is comparable for first two days. From third day onward EEA was slightly better in inhibition over control and reached a significant level by 8^{th} and 9^{th} day.

11. Fig. 9: Higher dosage (50 µl) of DNFB induced a much higher level of DTH swelling in comparison to that of lower dosage (25 µl) of DNFB (Fig. 8). Perhaps inhibitory effect of alcohol was not discernable with a much bigger DTH swelling. But EEA could show once more its effectiveness in inhibition of DTH.

12. Page 41, Fig. 14: EEA's ability to stimulate $CD4^+T$ lymphocytes significantly, is mentioned in result section of page 41 and in Discussion in page 64, para 2, line 9. It is

fairly indicated that by property EEA is acting as a mitogen but this has not been mentioned overtly.

13. Page 41, Fig. 13: The differential count of WBCs at DTH site shows EEA could effectively inhibit recruitment of monocytes, neutrophils and eosinophils besides promoting lymphocyte number. The possible explanation for these events have been discussed in page 64, para 3. Further, the discussion is continued in page 65 to tell about regulation of participatory cytokines in inflammation.

14. Page 47, 48, Fig. 21: The results of blastogenic transformation (Fig. 5) showed that EEA activates T cells. It is known that polyclonal activation of T cells can generate cytotoxic cells toward tumor target cells (mentioned in Introduction, page 5, para 3, line 5 and Discussion, page 71, para 2, line 4).

In our experience not necessarily the percentage of cytotoxicity is always proportional to the increment of ratio of effector to target cells. The data were presented the way we observed in Fig. 21. We did not want to enter into many suppositions to explain the results.

15. Page 47, Fig. 20: A sudden increase in tumor size from 75 to 89 days in EEA treated mice is notable indeed. The trend of increment actually starts from 61 days onward. Possibly regular dose of 5 µl EEA for topical application on the growing tumor is not sufficient to restrict the tumor growth for all the time, although it was effective in initial phase.

The focus in this study was to analyze the effect of EEA on tumor growth in reference to alcohol control. We did not keep the set without any treatment in the present experiment as we are aware about the growth of tumor bearing mice in the similar set maintained in connection of experiments testing efficacy of turmeric performed earlier in our laboratory, and not to kill the animals unnecessarily this time. However, the mice without any treatment survived for 60 to 65 days bearing tumor of 2.198 to 2.322 sq.cm.

16. Page 49, Figs. 22 and 23: PCR protocol followed here is a basic one adopting the mRNA phenotyping procedure and the principle of Real Time PCR is also based on it. We had the set up and chemicals in our laboratory to continue with the usual PCR. It is true that Real Time PCR would possibly provide quantitation of gene expression on the Ct value. (Kindly refer to the galley proof of the paper accepted for publication in "Evidence Based Complementary and Alternative Medicine" (eCAM), Oxford University Press, at the end of the thesis.)

17. Page 52, Fig. 24: PFC assay has been carried out with splenocytes from mouse injected with EEA or ethanol 1 day prior to primary immunization (Materials and Methods, page 23). There were no significant variations between the sets in both primary and secondary immune response; 'p' values calculated are greater than 0.5 in all cases.

18. TLC separation of the total extract showed a major UV active band at Rf 0.54, indicating the major constituent of the total EEA extract. This particular band was further analyzed for chemical characterization and the band was found to be terpenoid. It is true that there are certain other components in the total extract and characterization of those will initiate a new study in future.

Tamal Mazumder 13.5.2011.
.....
Signature of the candidate
DEPT. OF ZOOLOGY
UNIV. OF NORTH BENGAL.

.....Chakravarty.....
13.5.2011
Signature of the supervisor
PROF. A. K. CHAKRAVARTY
Professor
Dept. Of Zoology
North Bengal University
Pin-734013
Siliguri, District (W.B.)