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.