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.

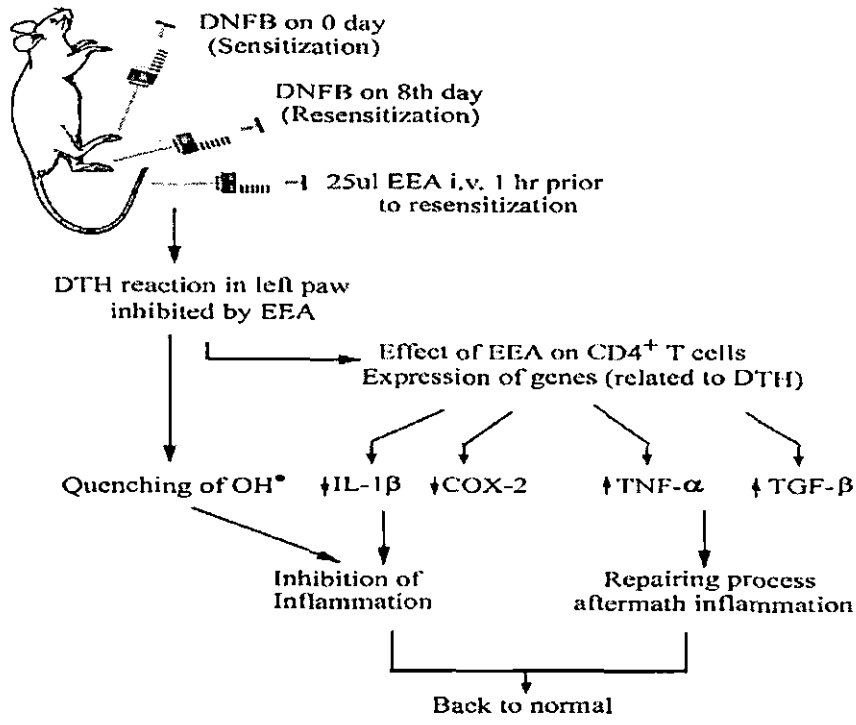

Fig. 38: Effect of EEA on inhibition of DTH reaction and repair mechanism presented schematically.

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.

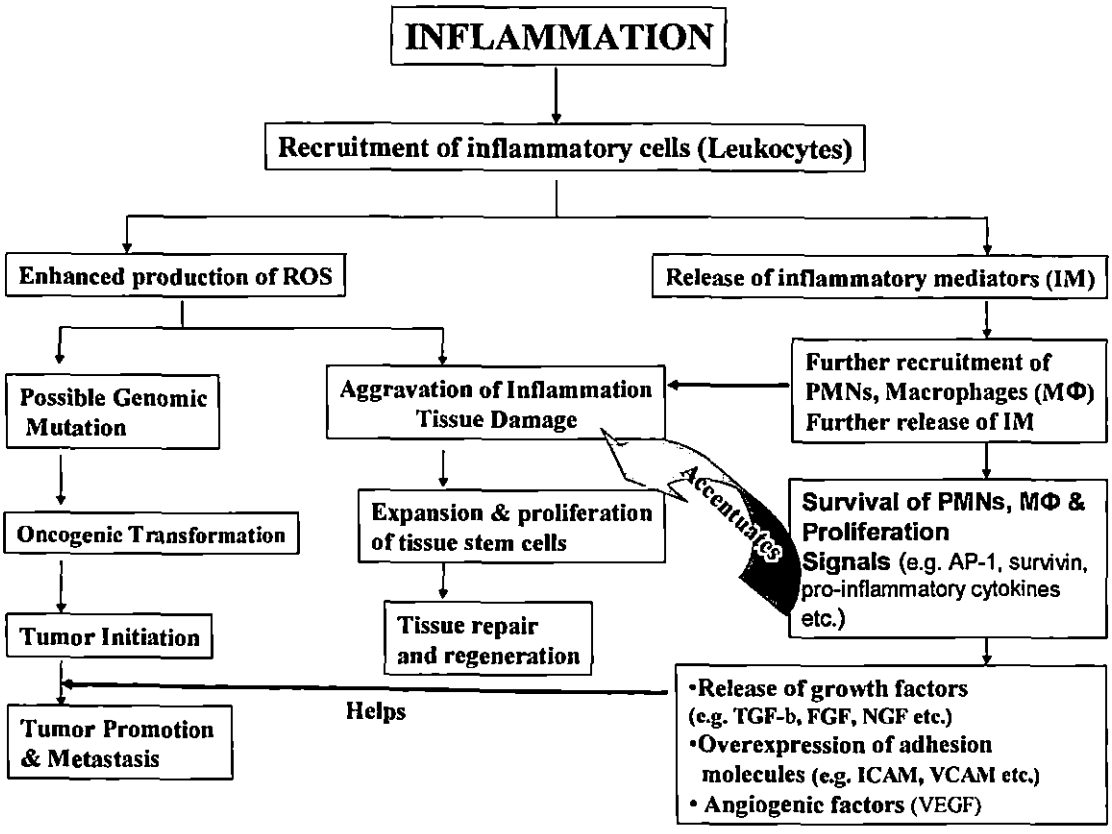

Fig.39: Summation of inflammation related events leading to tissue repair or promotion of tumor.

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.

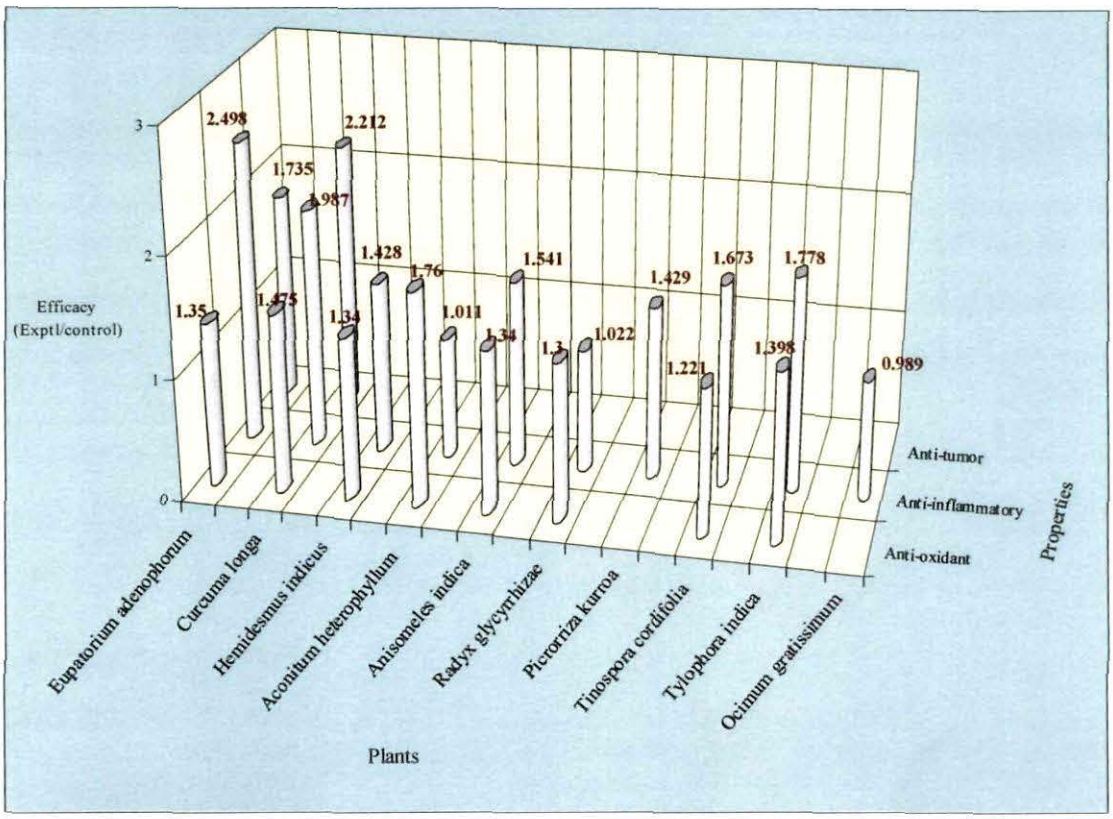

Fig 40: Immunopharmacological properties of EEA compared with certain other plant extracts. Indices represent the level of efficacy of the extracts as ratio over control; the indices presented at the top of the column for easy representation. Any index ratio of 1.2 or more has been considered to have 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.