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:

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

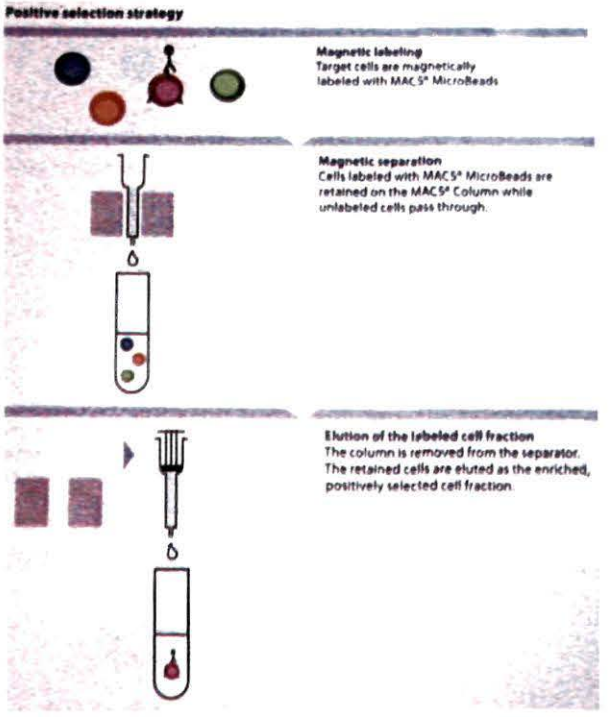
Differential leukocyte count from inflammation site of DTH mice

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.

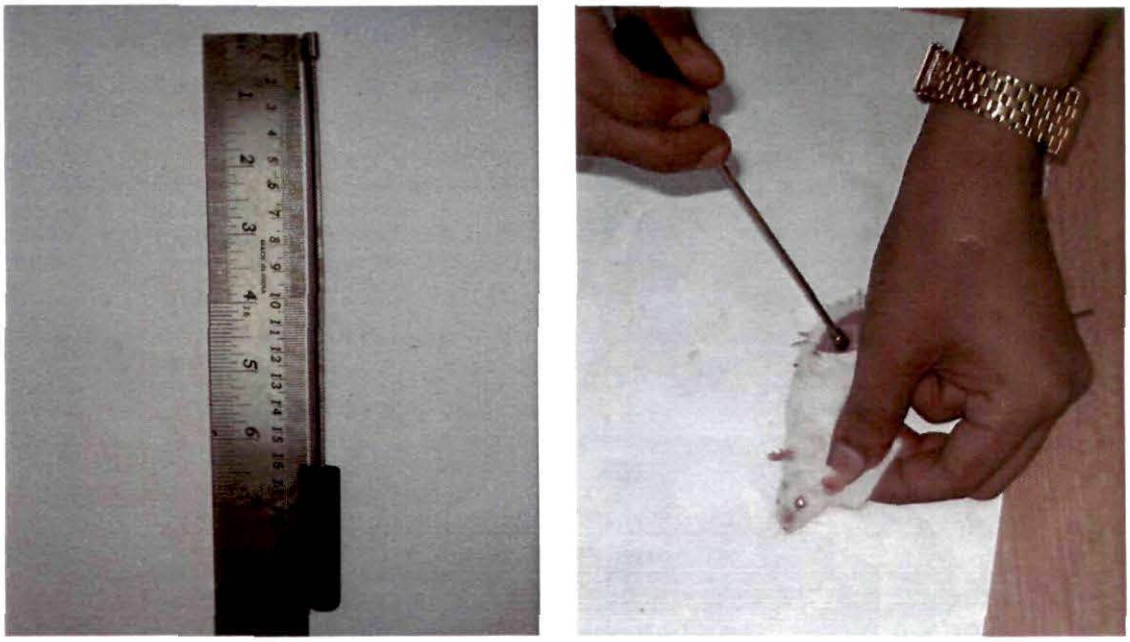
Linear skin incision and topical EEA treatment

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.