Materials and Methods
Materials and Methods

Material Source

TMB- Sigma Chemical Company, USA, $^3$H-Thymidine-Amersham, Human IL-2, IFN-γ, TNF-α, IL-4, TGFβ1 and IL-8 Duo-set kit from Genzyme Diagnostic, Cambridge, MA, USA, Duo-set kits-R&D System, Minneapolis, USA, Anti CD28 and IL-8R antibodies, R&D System, USA.
Collection of plant material

The *Tridax procumbens* plant was cultivated in a particular field (fig.1) and was identified by Dr. H.B.Singh, Head, Raw Materials Herbarium & Museum, voucher specimen was prepared (Field No.1907) and stored in Raw Material & Herbarium Division, National Institute of Science Communication and Information Resources (Council of Scientific and Industrial Research), Dr. K.S. Krishnan Marg, (Near Pusa Gate), New Delhi-110012.

Preparation of aqueous extract from fresh *Tridax procumbens* leaves

Materials
1. Freshly plucked leaves of *Tridax procumbens*
2. Liquid N2
3. Mortar and pestle
4. Phosphate buffered saline, pH 7.2 (PBS)
5. PMSF
6. Centrifuge
7. Whatman’s no-1 filter paper

Method
1. Fresh leaves (up to 5 g) of the plant *Tridax procumbens* were separated from the plants, cleaned and were grinded to a fine powder using a mortar and pestle under liquid nitrogen. Then chilled phosphate buffered saline, pH 7.2 (5 ml) per 1g of leaves and 100 µl of 10mM PMSF an isoamyl alcohol were added and mixed with pestle and poured into 50 ml centrifuge tube.

2. Then the slurry was centrifuged using SS34 rotor in a Sorvall Centrifuge (RC5C) at 12,000g for 30 minutes at 4°C and the supernatant was collected. The pellet was discarded.

3. Supernatant was filtered through Whatman’s filter paper No-1 and the filtrate was concentrated by lyophilization and plant extracts were kept at -20°C till further use.
Figure 1. A: *Tridax procumbens* L.
B: Flower of *Tridax procumbens* L.
Preparation of aqueous extract from dried *Tridax procumbens* leaves

**Materials**
1. Fresh, dried leaves of *Tridax procumbens*
2. Mortar and pestle
3. Phosphate buffer saline (PBS), pH 7.2
4. PMSF
5. Centrifuge
6. Whatman's No-1 filter paper

**Method**
1. Fresh leaves of the plant *Tridax procumbens* were plucked from the plants, cleaned and dried in the shade and were grinded to fine powder using mortar and pestle at room temperature.

2. The fine powder of *Tridax procumbens* leaves was extracted with phosphate buffered saline (pH 7.2) in the presence of PMSF (0.1mM) by shaking them for 24 hours at room temperature. Then the slurry was centrifuged using SS34 rotor in a Sorvall Centrifuge (RC5C) at 12,000g for 30 minutes at 4°C and the supernatant was collected.

3. The pellet was discarded. The supernatant was filtered through Whatman's filter paper No-1 and the filtrate was concentrated by using lyophilizer. The plant extracts was kept at -20°C till further use.

**Protein Estimation**
Protein concentrations in both the extracts were estimated by Lowry method (200).

**Trypsinization of aqueous extract and testing for activity**

**Materials**
1. Aqueous extract of *Tridax procumbens* leaves
2. Trypsin
3. Water bath
4. Ammonium bicarbonate buffer pH 8.0
5. Trypsin inhibitor
6. Test tubes

Method
1. Lyophilized *Tridax procumbens* aqueous extract powder 10mg was dissolved in 1ml ammonium bicarbonate buffer, pH 8.0.

2. Trypsin 0.2mg in 100\(\mu\)l of ammonium bicarbonate buffer was added and kept over night at 37°C.

3. Trypsin was inactivated by putting soyabean trypsin inhibitor and was kept at -20°C till further use.

Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of aqueous extracts of freshly plucked and dried *Tridax procumbens* leaves

SDS-PAGE analysis of both plant extracts (dry leaves and fresh leaves) was performed by using a 15% polyacrylamide gel according to the method described by Laemmli using discontinuous buffer system (198).

Materials

2X Sample Buffer-100 ml

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>1.52 gm</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>(\beta) - Mercaptoethanol</td>
<td>2.0 ml (for reducing condition)</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.002%</td>
</tr>
</tbody>
</table>

Electrophoresis buffer- 1 liters

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>3.02 gm</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 gm</td>
</tr>
</tbody>
</table>
SDS  \( \text{1.0 gm} \)

pH is autoadjusted to 8.3

Samples preparation

1. One volume of aqueous extracts 4 volumes of chilled acetone was added, mixed and was kept for over night at \(-20^\circ\text{C}\). The pellet was centrifuged for 15 min at 4°C in microcentrifuge tube at 12000 rpm.

2. Supernatant was discharged and the pellet was dried by inversion of tube on tissue paper. The pellet was resuspended in a minimal volume of SDS sample buffer.

Composition of SDS separating gel and resolving gel

Solution for 10% acryl amide gel (20 ml) – Separating gel

\[
\begin{align*}
30\% \text{ Acrylamide} & \quad 6.7 \text{ ml} \\
(29.2\% \text{ Acrylamide} & \quad 0.8\% \text{ Bis Acrylamide})
1.5\text{M Tris-Cl, pH 8.8} & \quad 5.0 \text{ ml}
10\% \text{ SDS} & \quad 0.2 \text{ ml}
10\% \text{ APS} & \quad 0.2 \text{ ml}
\text{TEMED} & \quad 0.01 \text{ ml}
\text{DDW} & \quad 7.9 \text{ ml}
\end{align*}
\]

Stacking gel – 5 ml

\[
\begin{align*}
30\% \text{ Acrylamide Mix} & \quad 0.83 \text{ ml}
1.0 \text{ M Tris –Cl pH 6.8} & \quad 0.63 \text{ ml}
10\% \text{ SDS} & \quad 0.05 \text{ ml}
10\% \text{ APS} & \quad 0.05 \text{ ml}
\text{TEMED} & \quad 0.005 \text{ ml}
\text{DDW} & \quad 3.4 \text{ ml}
\end{align*}
\]
Method
1. The solution of resolving gel was casted into slab and was overlayered with saturated butanol to remove air bubble and for making sharp interface during polymerization.

2. Stacking gel was layered on the top of resolving gel after removing butanol and washing with water.

3. Samples were prepared by boiling in SDS sample buffer for 5 minutes (one volume of sample and one volume of 2X sample buffer) and loaded into the gel.

4. Samples were stacked at 80 volts and resolved at 100 volts (constant voltage) along with standard molecular weight markers.

5. After the dye reached the end of the slab, the gel was taken out and stained in Commassie brilliant blue250 R.

Commassie staining of the gel
1. The gel was stained in 0.05% commassie brilliant blue (R250) in 50% methanol & 5% acetic acid kept for overnight.

2. The stain was removed and the gel was destained using destaining solution containing 7% acetic acid and 5% methanol till the background was reduced.

Fractionation of aqueous extract by G-100 sephadex chromatography for identification of fraction with immunomodulatory activity

The aqueous extract of freshly plucked as well as dried *Tridax procumbens* leaves were fractionated by G-100 sephadex column chromatography.

Materials
1. Aqueous extracted samples of *Tridax procumbens*
2. Glass column suitable size
3. Glass wool
4. Sephadex G-100 for column chromatography
Method
1. A suitable size glass column was chosen and small piece of glass wool were placed at the bottom.

2. The column was clamped in vertical position and bed volume of the glass column has been determined by filing the column with water and measuring with a graduated cylinder.

3. Sephadex G-100 gel was prepared. The gel has a fractionation range for proteins of 4000-150,000 Daltons. Sephadex G-100 is supplied as a dry powder and was hydrated by keeping in PBS pH 7.2 for 24 hours and resuspending in the buffer several times.

4. The buffer was decanted and the gel was equilibrated with PBS pH 7.2.

5. For packing the column, the outlet of the column was closed. The gel was stirred to create a slurry and was filled in the column without creating areas of different densities. As the gel was packed, the outlet of the column was opened and buffer was added.

6. The column was equilibrated by eluting 20-30 bed volume of PBS buffer pH 7.2 at a flow rate of 1ml/min.

7. The void volume was determined by using blue dextran.

8. The aqueous extract (1 mg/ml concentration), 1-2% of the column volume was added carefully to the top of the column.

9. The column was eluted at a rate of 0.5 ml/min. one ml fractions were collected by using fraction collector.

10. The absorbance of each fraction was measured at 280 nm.

11. The protein concentration of each fraction was estimated by Lowry method.

12. A graph of concentration of protein versus fraction number and absorbance at 280nm versus fraction number was plotted.
13. Different fractions were stored at \(-20^\circ C\) for testing their immunomodulatory activity.

**Concanavalin A sepharose 4B chromatography**

**Materials**
1. Concanavalin A sepharose 4B
2. Glass column suitable size
3. Glass wool
4. Con. A binding buffer (40Mm Tris-HCl, 1mM CaCl₂, 1mM MgCl₂, 1MnCl₂ and 0.5M NaCl, pH7.2)
5. \(\alpha\) Methyl D-mannoside
6. Test tubes
7. Fraction collector

**Method**
1. A suitable size glass column size was chosen and small piece of glass wool was placed at the bottom.

2. 10 ml slurry of con. A sepharose 4B in con. A binding buffer was loaded into the column.

3. Then the column was equilibrated with con. A binding buffer.

4. The fraction was applied and con. A unbound fraction was washed with three bed volumes of con. A buffer.

5. The flow rate was maintained at rate of 10 ml/h and 2.5 ml fractions were collected.

6. Con A bound fraction was eluted with con. A binding buffer containing 0.5 M \(\alpha\)-methyl D-mannoside.

7. The eluted fraction was desalted by dialysis against distilled water and the lyophilized and stored at \(-20^\circ C\) for testing the immunomodulatory activity.
Dialysis of aqueous extract for retention of activity

Materials
1. Aqueous extract of *Tridax procumbens* leaves
2. Dialysis tubing (12kDa cut off size)
3. Magnetic stirrer
4. Normal saline

Method
1. The aqueous extracts of both dried leaves and freshly plucked *Tridax procumbens* leaves was lyophilized and redissolved in minimum volume of normal saline and was dialyzed overnight against normal saline by using a 12-15 kDa cut off dialysis tubing to remove small molecules.

2. The samples after dialysis were stored at -20°C till further testing the immunomodulatory activity.

Boiling of aqueous extract at 98°C to test for retention of activity

Materials
1. Aqueous extract of *Tridax procumbens* leaves
2. Water bath
3. Test tubes

Method
1. 10 ml the aqueous extract 10ml was heated at 98°C in water bath for 15 minutes and stored at -20°C till further testing the immunomodulatory activity.

Organic extraction from *Tridax procumbens* leaves at room temperature (23°C)

Materials
1. Fresh, dried leaves of *Tridax procumbens*
2. Flat bottom flask
3. Acetonitrile (CDH, bp-81°C)
4. Ethyl acetate (Qualigens, bp-77°C)
5. Hexane (CDH, bp-69°C)
6. Methanol (Merck/Qualigens, bp-65°C)
7. Methylene chloride (Qualigens, bp-39°C)
Method

1. The fine powder of *Tridax procumbens* leaves obtained as described before was extracted with different solvents like hexane, ethyl acetate, methylene chloride, acetonitrile and methanol. The leaf powder was kept in each flat bottom flask and different organic solvents viz. methanol, hexane, and ethyl acetate were added (1:25 w/v) respectively.

2. The extraction was done by continuously shaking the suspension made of 1gm of the leaf powder with 25 ml of organic solvent on a shaker at 125 rpm at room temperature for 24 hours.

3. Then the slurry was centrifuged at 12,000g for 30 minutes at room temperature and the supernatant was collected. The pellet was discarded.

4. Supernatant was filtered through Whatman’s filter paper No-1 and the filtrate was concentrated under reduced pressure by using a Rotary Evaporator.

5. The solvents present in the concentrated extracts were removed in a vacuum dessicator and the plant extracts were kept for further studies.

Extraction at boiling temperature of the solvent, using Soxhlet apparatus

Materials

1. Fresh, dried leaves of *Tridax procumbens*
2. Acetonitrile (CDH, bp-81°C)
3. Ethyl acetate (Qualigens, bp-77°C)
4. Hexane (CDH, bp-69°C)
5. Methanol (Merck/Qualigens, bp-65°C)
6. Methylene chloride (Qualigens, bp-39°C)
7. Soxhlet Extraction Apparatus
8. Rotary evaporator
Method
1. The leaf powder was kept in extractor flask of Soxhlet Extraction Apparatus and different organic solvents viz. hexane, ethyl acetate, acetonitrile, methylene chloride and methanol were added (1:25 w/v) respectively.

2. The extraction was done by continuously boiling the suspension made of 1gm of the leaf powder with 25 ml of organic solvent in an extractor flask at boiling points for each organic solvent for 4 hours.

3. Then the slurry was centrifuged at 12,000g for 30 minutes at room temperature and the supernatant was collected. The pellet was discarded.

4. Supernatant was filtered through Whatman's filter paper No-1 and the filtrate was concentrated under reduced pressure by using a Rotary Evaporator.

5. The solvents present in the concentrated extracts were removed in a vacuum dessicator and the plant extracts were kept for further studies.

Thin layer chromatography of organic extracts of *Tridax procumbens* leaves to visualize the extracted molecules

Extracts prepared by using different organic solvents were separated by thin layer chromatography using different solvent systems indicated below for selection of an appropriate organic solvent system which can give the best separation of extracted compounds.

Materials
1. Extracts of *Tridax procumbens*
2. Silica gel-G for TLC
3. Applicator
4. TLC chamber
5. TLC glass plates
6. Hexane
7. Acetone
8. Iodine (I$_2$)
9. UV lamp
10. Heating oven
11. Distilled H$_2$O
12. Solvent systems
   i. Hexane: ethylacetate (70:30)
   ii. Hexane: ethylacetate (80:20)
   iii.
   methanol:water:acetone:ethylacetate:chloroform(10:8:30:40:12),
   iv. ethylacetate:methanol:water (10:1.7:1.4),
   v. hexane:acetone (60:40),
   vi. ethylacetate: glacial acetic acid:formic acid:water (10:0.1:0.1:2.6)
   vii. chloroform: acetone:formic acid (7.5:1.7:0.9)

**Method**

1. Silica gel (3 grams) was mixed with 6 ml double distilled H$_2$O. The slurry was made and applied uniformly on the surface of the glass plate (12.5 cm x 10.2 cm) using applicator and was allowed to dry air.

2. The plate was kept in an oven at 130°C for activation for 2 hours. After 2 hours plate was taken out from the oven.

3. Different solvent systems like hexane: ethylacetate (70:30, 80:20), methanol:water:acetone:ethylacetate:chloroform(10:8:30:40:12), ethyl acetate:methanol:water (10:1.7:1.4), hexane:acetone (60:40), ethylacetate: glacial acetic acid:formic acid:water (10:0.1:0.1:2.6) and chloroform: acetone:formic acid (7.5:1.7:0.9) were used. 150 ml of a particular solvent system was added to a glass chromatography tank (20 cm x 15 cm x 10 cm) and covered tightly and was allowed to equilibrate for 2 hours.

4. A very light pencil line was made at 1.5 cm parallel to the bottom of the plate, while taking care of not scratching the silica gel.

5. Different extracted samples were slowly loaded on the TLC plate by using a glass microsyringe and allowed to dry.
6. Using a pair of long forceps, the top of the plate was grasped and placed in the tank; maintaining the spotted sample just above the level of the solvent.

7. The tank was covered tightly and the plate was allowed to remain undisturbed until the ascending solvent line reached the top of the plate (approx. 30-45 minutes).

8. The TLC plate was removed by grasping the top edge with forceps and was allowed to dry thoroughly.

9. The spots in the TLC plate were visualised by putting the plate in I\(^2\) containing chamber or by UV light.

Silica gel Column chromatography of the organic solvent extracts of *Tridax procumbens* leaves

**Materials**
1. Organic solvent extracts of *Tridax procumbens* leaves
2. Glass column of suitable size
3. Hexane 100%
4. Acetone 100%
5. Organic solvent system (hexane:acetone 60:40)
6. Glass wool
7. Silica gel (60-120 mesh) for column chromatography
8. Glass vials
9. Rotary Evaporator

**Method**
1. A suitable size glass column was chosen and small piece of glass wool were placed at the bottom.

2. The column was clamped in vertical position and enough silica gel slurry in hexane was poured to prepare a column of approximately 50cm length and 2cm in width.

3. Excess hexane was drained until it just reached the top level of the silica gel matrix in the column.
4. Ethyl acetate extracted sample of the plant (1ml) was added to the top of the column and was allowed to slowly drain through the column and collected in vials.

5. After the extracted sample had drained to just above the silica gel, an additional 5 ml of hexane was added and was allowed to drain to just above the silica gel.

6. Then several portions of 20 ml of the 60:40 hexane: acetone solution was added to the column and these were allowed to drain and fractions were collected in the vials.

7. A yellow band of pigments was seen to pass through the column (These were the carotenoids).

8. Just before the yellow band was drained out of the column, the vial was replaced, one continued to add the solvent hexane/acetone and the yellow band was collected into same vial.

9. Just as the eluting solvent became completely cleared, the vial was replaced.

10. Pure acetone was added to the column and the green band started to move slowly.

11. The eluting clear solvent was collected in the vial until the green band started to elute.

12. Pure acetone was added to the column and the entire green band was collected into the same vial.

13. Collected fractions were concentrated by Rotary Evaporator under reduced pressure.

14. The concentrated fractions were collected in the glass viol and the organic solvent was allowed to evaporate to completely dry the fractions and these were kept for further analysis.
H.P.L.C. analysis of the organic solvent extracts of *Tridax procumbens* leaves

HPLC analysis of the crude as well as silica gel fractionated samples was done by using Shimadzu HPLC Apparatus.

**Materials**
1. Organic extracts samples of *Tridax procumbens* leaves (crude)
2. Silica gel column fractionated samples of *Tridax procumbens* leaves
3. Solvent (mobile phase) A-water, B- acetonitrile
4. Hamilton syringe
5. Shimadzu HPLC Apparatus
6. Pump binary LC10 AT Vp
7. Rheodyne injector
8. HPLC column (particle size 5μ, column size 250x4.6mm)
9. SPD 10A Vp (UV-VIS)

**Method**
1. 50 μl of 100% acetone was taken in a Hamilton syringe and injected into the HPLC injection port. Turned the handle down, this started the run.

2. The flow rate was 1.0 ml/minute.

3. The acetone blank was run to clean the HPLC column and one can tell when the system is ready as there are no peaks on the paper readout.

4. The sample was dissolved in acetonitrile:methanol (90:10),

5. 20 μl of sample was taken in Hamilton syringe and was injected into the sample port.

6. In the mobile phase, the solvent gradient was allowed to pass (at time zero minute - solvent B was 30% after 25 –it was 90%)

7. After loading the sample, H.P.L.C. apparatus system was run at wavelength (215nm, 254nm, and 410nm).
termination of antibacterial activity of aqueous as well as
ganic extracts of *Tridax procumbens*

**Broth culture Method**

**Materials**
1. Extracts of *Tridax procumbens*
2. Bacterial Strains (see below)
4. Mueller Hinton broth
5. Laminar flow
6. Tetracycline
7. Test tubes
8. Spectrophotometer

<table>
<thead>
<tr>
<th><strong>Bacterial Strains</strong></th>
<th>Gram stain status</th>
<th>Growth condition</th>
<th>Sensitivity to Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>-ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
<tr>
<td><em>Enterobacter aerogens</em></td>
<td>-ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>-ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>-ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>+ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>-ve</td>
<td>Mueller Hinton Agar/Broth</td>
<td>+</td>
</tr>
</tbody>
</table>

**Method**
1. Broth culture media was prepared by suspending 21 grams of the powder of Mueller Hinton Broth in 1 litre of distilled water and warmed gently to dissolve. Then it was autoclaved at 121°C for 15 minutes.
2. Bacteria were grown in liquid culture in several test tubes, which were incubated at 37°C.

3. During log phase of the bacterial growth (different for different bacteria), crude aqueous as well as crude ethyl acetate extracts of *Tridax procumbens* leaves and their fractions (1mg/ml) were added into each test tube and test tubes were incubated overnight at 37°C.

4. Growth inhibition was monitored by measuring the turbidity of bacterial culture by spectrophotometer at 600nm

**Determination of anti-proliferative activity**

Anti-proliferative activity of crude organic as well as aqueous extracts *Tridax procumbens* leaves and their fractions were determined using different tumor cell lines.

**Cell Culture**

**Preparation of cell culture medium**

**Materials**
(For 1 liter)
1. RPMI 1640 (Rosewell Park Memorial Institute1640) 10.4 g/L
2. FCS (fetal calf serum)-10%
3. Hepes buffer-2.83 g/L
4. Sodium pyruvate-0.22 g/L
5. Mercapto-ethanol-3.5 µL/L
6. Sodium carbonate-2.0 g/L
7. Glutamine-0.2922 g/L
8. Antibiotic Antimycotic 10X (10,000 units’ penicillin, 25 µg streptomycin in 0.9% NaCl).

**Method**
1. All the above mentioned compounds were added to triple distilled H₂O. This is incomplete medium to which 10% FCS (Fetal calf serum) was added to make it complete. It was sterilized by passing it through pre-sterilized 0.2µm Cellulose Acetate filter (Sartorius).
2. Inactivation of FCS was done by keeping it at 56° C for 30 minutes, after which it was filtered and added to incomplete-RPMI to make complete medium.

**Cell thawing**

**Materials**
1. Cell lines:
   a) A549 (lung carcinoma, adherent)
   b) KB (oral carcinoma, adherent) originally obtained from the Cancer Research Institute, Parel, Mumbai.
2. RPMI 1640
3. T25 culture flask
4. CO₂ incubator (Hera Incubator, 37° C, 5% CO₂)

**Method**
1. RPMI was thawed to 37° C in a water bath, and added to T25 culture flask.

2. Cell lines were removed from liquid nitrogen and placed in water bath. Then the outside of the vial was cleaned with 70% ethanol.

3. Thawed cell suspension was then added to the culture flask containing the pre-warmed media.

4. The cell lines were incubated at 37° C, 5% CO₂ (in Hera incubator) and the media replaced the next day.

**Trypsinizing cells**
Trypsinization is a technique that uses the proteolytic enzyme trypsin to detach adherent cells from the surface of a cell culture vessel. This procedure was performed whenever the cells were required to be harvested (e.g., for passaging, counting, or for nucleic acid isolation).

**Materials**
1. Trypsin solution (0.25% w/v Trypsin, 0.53 mM EDTA in 1X PBS buffer)
2. Incomplete RPMI 1640, i.e., without FCS
3. FCS (fetal calf serum)
4.1X PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄)

**Method**

1. The cells were washed with RPMI after discarding the original media, to remove the dead cells.

2. Added trypsin–EDTA solution to cover the monolayer, and rocked the flask 4–5 times to coat the monolayer.

3. The flask was then placed in CO₂ incubator at 37°C for 30-60 seconds.

4. Then it was removed from the incubator and 10 ml of complete RPMI medium was added to the flask.

5. Aspirated the media so as to completely remove the adherent cells.

6. Proceeded as required (e.g., passaging, freezing etc.)

**Passaging cells**

Many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of cell culture vessel), and some will die if they are left in a confluent state for too long. Adherent cell cultures therefore need to be routinely passaged, that is, once the cells are confluent, a fraction of the cells need to be transferred to a new cell culture vessel. For adherent cells, enough cells were added so that the culture took approximately one week to reach confluence again. This minimized the number of times the cells were trypsinized as well as the handling time required to maintain the culture.

**Method**

1. After detaching the cells by Trypsinization, complete RPMI medium was added to the cell culture flask and centrifuged. The supernatant was removed. The complete RPMI 1640 medium was added and the cells were resuspended.

2. An appropriate volume of the cells was transferred to a fresh cell culture vessel containing growth medium.
3. Incubated the cells under their usual growth conditions.

$^3$H Thymidine Incorporation Assay

Materials
1. Extracts of *Tridax procumbens* leaves
2. Cell lines that were used: (a)-549 (Lung carcinoma, adherent) (b) KB- (Oral carcinoma, loosely adherent)
3. Tissue culture plate (96 well plate)
4. SDS solution (1%)
5. Scintillation vials
6. Cyclophosphamide (positive control)
7. RPMI 1640 medium only
8. Scintillation fluid (Cocktail T)
9. Liquid Scintillation Counter (Packard, Model No 1600, Serial No.104699, made in USA).
10. $^3$H Thymidine
11. 96 well cell culture plate

Method
1. Appropriate number of cells were seeded into a 96 well culture plate in RPMI1640 complete medium in a total volume of 100µl and incubated in a CO$_2$ incubator.

2. After 24 hours, different concentrations of the aqueous extracts and their fractions, ethyl acetate extracts and their fractions in DMSO were added to the wells. DMSO was added as a control.

2. Cells were cultured in the presence of different extracts for 48 hours (time standardization was done previously) in CO$_2$ incubator at 37°C.

3. Cells were then visually monitored and pulsed with $^3$H thymidine (1 µCi/well/100 µl) for 18 hours.

4. After 18 hours of incubations, cells were harvested after trypsinization in 1.5 ml micro centrifuge tube.
5. Cells were then pelleted at 7000 rpm for 10 minutes, followed by washing with PBS.

6. 100 μl of 1% SDS was added to lyse the cells.

7. Then, 900μl of scintillation (Cocktail-T) fluid was added and mixed by vortexing and a count of radioactioactivity was recorded by a Liquid Scintillation Counter.

**Isolation of peripheral blood mononuclear cells (PBMC) from human blood**

Peripheral blood mononuclear cells were purified from human blood by sedimentation on Histopaque as described by Boyum (204).

**Materials**
1. Heparinized human blood (10 IU heparin per ml) from healthy individual
2. RPMI-1640 medium containing 10mM HEPES 10% FCS, 100μg/ml streptomycin, 100units/ml penicillin and 0.25μg/ml amphotericin B,
3. RPMI-1640 medium without FCS
4. Histopaque
5. 96 well culture plates,
6. *Tridax procumbens* leaves extracts and their fractions
7. DMSO

**Method**
1. Heparinized blood was diluted 1:1 in RPMI-1640 without FCS and layered slowly on Histopaque in a ratio of 1:1 (one volumes of diluted blood and one volume of Histopaque) and centrifuged at 1500 rpm in swing out rotor for 30 minutes at room temperature.

2. The cells at the interface of plasma and Histopaque were taken out. This contained the PBMC population.

3. These cells were then washed thee times with medium and pellet was finally resuspended in RPMI-1640 complete medium.
In vitro stimulation of PBMC for proliferation and cytokine secretion

1. PBMC at 2 x 10^5 cells/well were cultured in 200μl of C-RPMI medium in 96 well tissue culture plates (Nunc) at 37°C in an atmosphere of 5% CO2.

2. Aqueous extracts of *Tridax procumbens* leaves and their different fractions were added to the wells. The ethyl acetate extracts and its different fractions were added dissolved in DMSO. DMSO was also used as a control.

3. PBMC culture supernatants were assayed at a time found to be optimal for the secretion of that cytokine which was 24 hours for IL-2, IL-8, TNF-α and 48 hours for IFN-γ, IL-4, TGF-β1.

4. Cytokines levels in culture supernatant were measured by sandwich ELISA using commercially available kits as described below.

Immunoassay of human IL-2

Materials (Genzyme Kit)

Capture antibody - Monoclonal mouse anti-human IL-2 (stock 700μg/ml, working dilution concentration 4μg/ml)

Second antibody - Biotinylated polyclonal rabbit anti-human IL-2 (stock 9μg/ml, working concentration 50ng/ml)

Standard - Recombinant IL-2 (stock 25μg/ml)

Detection Reagents - Horseradish peroxidase conjugated Streptavidin (Strept-HRP)

Buffers, diluents and solutions

Coating buffer - 0.01M phosphate buffered saline (PBS), pH 7.3

Blocking buffer - 0.01M Phosphate buffered saline (PBS), pH 7.3 with 4%BSA

Standard diluent - cell culture medium or serum depending on sample matrix to be tested
Biotinylated Antibody: PBS with 0.05% Tween-20 and 1% BSA and Strep-HRP diluent
Wash buffer: PBS with 0.05% Tween-20
Substrate: Tetramethylbenzidine (TMB) and H$_2$O$_2$
Stop solution: 2N H$_2$SO$_4$

**Method**

1. A 96 well microtitre plate was coated with 100μl per well of capture antibody (2.5μg/ml) diluted in coating buffer and incubated O/N at 4°C or 2-8°C.

2. The captured antibody was aspirated and the wells were washed three times with wash buffer.

3. The plate was blocked with 250μl per well of blocking buffer, sealed with plate cover and incubated for 2 hours at 37°C.

4. Blocking buffer was aspirated from wells and blot dried.

5. 100μl per well of diluted standards (0-1000 pg/ml) and samples were added to different wells of microtitre plate, sealed and incubated for 1 hour at 37°C.

6. Standards and samples were aspirated and the wells were washed three times with wash buffer.

7. 100μl per well of diluted second antibody (1.25μg/ml) was added to all wells, sealed and incubated for 1 hour at 37°C.

8. Second antibody was aspirated and wells were washed three times with wash buffer.

9. 100μl per well of diluted detection reagent (1/1000 dilution) was added, plate was sealed and incubated for 20 minutes at 37°C.

10. Detection reagent was aspirated and wells were washed thrice with wash buffer.
11. 100μl per well of working TMB substrate solution (0.5mg TMB and 1μl of 30% H₂O₂ per ml of citrate phosphate buffer, pH 5.0) was added, and incubated for 10-15 minutes at room temperature.

12. 100μl per well of stop solution was added.

13. Absorbance was read at 450nm within 30 minutes.

14. A standard curve was constructed by plotting the mean absorbance for each standard on the vertical axis (Y-axis) versus the corresponding standard concentration on the horizontal axis (X-axis).

Immunoadsay of human IFN-γ

Materials (Genzyme Kit)
Capture antibody - Anti-human IFN-γ (working dilution 1:800)
Second antibody - Anti-human IFN-γ conjugated to HRPO
(Stock 250μl/ml, working concentration 0.625μg/ml)
Standard - Recombinant IFN-γ (14pg/ml)

Buffers, diluents and solutions
Coating buffer - 0.1M Carbonate buffer/bicarbonate, pH 9.5
Blocking buffer - 0.01M Phosphate buffered saline (PBS), pH 7.3 with 4%BSA
Standard diluent - Cell culture medium or blocking buffer depending on sample matrix to be tested
Antibody diluent - PBS with 0.05% Tween-20 and 1% BSA (used for second antibody)
For testing serum or plasma samples, 5% normal goat serum was added to antibody diluent
Wash buffer - PBS with 0.05% Tween-20
Substrate - Tetramethylbenzidine (TMB) and H₂O₂
Stop solution - 2N H₂SO₄

Method
1. A 96 well microtitre plate was coated with 100μl per well of capture antibody (1/800 dilution) diluted in coating buffer and incubated O/N at 2-8°C.
2. The captured antibody was aspirated and the wells were washed three times with wash buffer.

3. The plate was blocked with 250μl per well of blocking buffer and sealed with plate cover and incubated for 2 hours at 37°C.

4. Blocking buffer was aspirated from wells and blot dried.

5. 100μl per well of diluted standards (0-1000 pg/ml) and samples were added to different wells of microtiter plate, sealed and incubated for 2 hours at 37°C.

6. Standards and samples were aspirated and wells were washed four times with wash buffer.

7. 100μl per well of diluted second antibody (0.625μg/ml) was added to all wells, sealed and incubated for 45 minutes at 37°C.

8. Second antibody was aspirated and wells were washed four times with wash buffer.

9. 100μl per well of TMB substrate solution was added and incubated for 30 minutes at room temperature (20-26°C).

10. 100μl of stop solution was added.

11. Absorbance was read at 450nm within 60 minutes.

12. A standard curve was constructed by plotting the mean absorbance for each standard on the vertical axis (Y-axis) versus the corresponding standard concentration on the horizontal axis (X-axis).

**Immunoassay of human IL-4**

**Materials (Genzyme Kit)**

Capture antibody - Monoclonal mouse anti-human IL-4 (stock 500μg/ml, working concentration 2μg/ml)
Second antibody  - Biotinylated polyclonal sheep anti-human IL-4 (stock 250μg/ml, working concentration 0.5μg/ml)
Standard  - Recombinant IL-4 (stock 299pg/ml)
Detection Reagents  - Streptavidin-HRP

Buffers, diluents and solutions
Coating buffer  - 0.01M phosphate buffered saline (PBS), pH 7.3
Blocking buffer  - 0.01M Phosphate buffered saline (PBS), pH 7.3 with 4%BSA
Standard diluent  - cell culture medium or serum depending on sample matrix to be tested
Biotinylated Antibody  - PBS with 0.05%Tween-20 and 1% BSA and Strept-HRP diluent
Wash buffer  - PBS with 0.05%Tween-20
Substrate  - Tetramethylbenzidine (TMB) and H₂O₂
Stop solution  - 2N H₂SO₄

Method
1. A 96 well microtitre plate was coated with 100μl per well of capture antibody (2.5μg/ml) diluted in coating buffer and incubated O/N at 4°C or 2-8°C.
2. The captured antibody was aspirated and the wells were washed three times with wash buffer.
3. The plate was blocked with 250μl per well of blocking buffer, sealed with plate cover and incubated for 2 hours at 37°C.
4. Blocking buffer was aspirated from wells and blot dried.
5. 100μl per well of diluted standards (0-1000 pg/ml) and samples were added to different wells of microtitre plate, sealed and incubated for 1 hour at 37°C.
6. Standards and samples were aspirated and the wells were washed three times with wash buffer.
7. 100µl per well of diluted second antibody (2µg/ml) was added to all wells, sealed and incubated for 1 hour at 37°C.

8. Second antibody was aspirated and wells were washed three times with wash buffer.

9. 100µl per well of diluted detection reagent (1/1000 dilution) was added, plate was sealed and incubated for 20 minutes at 37°C.

10. Detection reagent was aspirated and wells were washed three times with wash buffer.

11. 100µl per well of working TMB substrate solution (0.5mg TMB and 1µl of 30% H₂O₂ per ml of citrate phosphate buffer, pH 5.0) was added, and incubated for 10-15 minutes at room temperature.

12. 100µl per well of stop solution was added.

13. Absorbance was read at 450nm within 30 minutes.

13. A standard curve was constructed by plotting the mean absorbance for each standard on the vertical axis (Y-axis) versus the corresponding standard concentration on the horizontal axis (X-axis).

**Immunoassay of human IL-8**

**Materials (Genzyme Kit)**

- Capture antibody: Monoclonal mouse anti-human IL-8 (stock 500µg/ml, working concentration 2µg/ml)
- Second antibody: Biotinylated polyclonal sheep anti-human IL-8 (stock 250µg/ml, working concentration 0.5µg/ml)
- Standard: Recombinant IL-8 (stock 299pg/ml)
- Detection Reagents: Streptavidin-HRP

**Buffers, diluents and solutions**
Coating buffer -0.01M phosphate buffered saline (PBS), pH 7.3
Blocking buffer -0.01M Phosphate buffered saline (PBS), pH 7.3 with 4%BSA
Standard diluent - cell culture medium or serum depending on sample matrix to be tested
Biotinylated Antibody -PBS with 0.05%Tween-20 and 1% BSA and Strept-HRP diluent
Wash buffer -PBS with 0.05%Tween-20
Substrate -Tetramethylbenzidine (TMB) and H₂O₂
Stop solution -2N H₂SO₄

**Method**
1. A 96 well microtitre plate was coated with 100μl per well of capture antibody (2.5μg/ml) diluted in coating buffer and incubated O/N at 4°C or 2-8°C.

2. The captured antibody was aspirated and the wells were washed three times with wash buffer.

3. The plate was blocked with 250μl per well of blocking buffer, sealed with plate cover and incubated for 2 hours at 37°C.

4. Blocking buffer was aspirated from wells and blot dried.

5. 100μl per well of diluted standards (0-1000 pg/ml) and samples were added to different wells of microtitre plate, sealed and incubated for 1 hour at 37°C.

6. Standards and samples were aspirated and the wells were washed three times with wash buffer.

7. 100μl per well of diluted second antibody (2μg/ml) was added to all wells, sealed and incubated for 1hour at 37°C.

8. Second antibody was aspirated and wells were washed three times with wash buffer.

9. 100μl per well of diluted detection reagent (1/1000dilution) was added, plate was sealed and incubated for 20minutes at 37°C.
10. Detection reagent was aspirated and wells were washed three times with wash buffer.

11. 100μl per well of working TMB substrate solution (0.5mg TMB and 1μl of 30% H₂O₂ per ml of citrate phosphate buffer, pH 5.0) was added, and incubated for 10-15 minutes at room temperature.

12. 100μl per well of stop solution was added.

13. Absorbance was read at 450nm within 30 minutes.

14. A standard curve was constructed by plotting the mean absorbance for each standard on the vertical axis (Y-axis) versus the corresponding standard concentration on the horizontal axis (X-axis).

**Immunooassay of human TNF-α**

**Materials (Genzyme Kit)**

**Capture Antibody** – Monoclonal mouse anti-human TNF-α  
(stock 720 μg/ml, working concentration 4.0 μg/ml)

**Detection Antibody** - Biotinylated goat anti-human TNF-α (stock 54 μg/ml, working concentration. 300 ng/mL)

**Standard** - Recombinant human TNF-α (0.5 μg/ml)

**Streptavidin** - Horseradish peroxidase conjugated streptavidin (Strept-HRP)

**Buffers, diluents and solutions**

Coating buffer -0.01M phosphate buffered saline (PBS), pH 7.2

Blocking buffer -0.01M Phosphate buffered saline (PBS), pH 7.2 with 4%BSA with 0.05% NaN3.

Standard diluent – cell culture medium or serum depending on sample matrix to be tested

Biotinylated Antibody –PBS with 0.05%Tween-20 and 1% BSA and Strept-HRP diluent

Wash buffer -PBS with 0.05%Tween-20
Substrate - Tetramethylbenzidine (TMB) and H₂O₂
Stop solution - 2N H₂SO₄

Method
1. A 96 well microtitre plate was coated with 100μl per well of capture antibody (2.5μg/ml) diluted in coating buffer and incubated O/N at 4°C or 2-8°C.

2. The captured antibody was aspirated and the wells were washed three times with wash buffer.

3. The plate was blocked with 250μl per well of blocking buffer, sealed with plate cover and incubated for 2 hours at 37°C.

4. Blocking buffer was aspirated from wells and blot dried.

5. 100μl per well of diluted standards (0-1000 pg/ml) and samples were added to different wells of microtitre plate, sealed and incubated for 1 hour at 37°C.

6. Standards and samples were aspirated and the wells were washed three times with wash buffer.

7. 100μl per well of diluted second antibody (2μg/ml) was added to all wells, sealed and incubated for 1 hour at 37°C.

8. Second antibody was aspirated and wells were washed three times with wash buffer.

9. 100μl per well of diluted detection reagent (1/1000 dilution) was added, plate was sealed and incubated for 20 minutes at 37°C.

10. Detection reagent was aspirated and wells were washed three times with wash buffer.

11. 100μl per well of working TMB substrate solution (0.5mg TMB and 1μl of 30% H₂O₂ per ml of citrate phosphate buffer, pH 5.0) was added, and incubated for 10-15 minutes at room temperature.
12. 100µl per well of stop solution was added.

13. Absorbance was read at 450nm within 30 minutes.

14. A standard curve was constructed by plotting the mean absorbance for each standard on the vertical axis (Y-axis) versus the corresponding standard concentration on the horizontal axis (X-axis).

**Immunoassay of human TGFβ1**

**Materials (Genzyme Kit)**
- Capture antibody - Monoclonal mouse anti-human TGF-β1 (stock conc. 360 µg/ml, working concentration 2µg/ml)
- Second antibody - Biotinylated polyclonal sheep anti-human IL-4 (stock conc. 54 µg/ml, working concentration 300 ng/ml)
- Standard - Recombinant human TGF-β1 (stock 100 ng/ml)
- Detection Reagents - Streptavidin-HRP

**Buffers, diluents and solutions**
- Coating buffer - 0.01M phosphate buffered saline (PBS), pH 7.3
- Blocking buffer - 0.01M Phosphate buffered saline (PBS), pH 7.3 with 4%BSA
- Standard diluent - Cell culture medium or serum depending on sample matrix to be tested
- Biotinylated Antibody - PBS with 0.05%Tween-20 and 1% BSA and Strept-HRP diluent
- Wash buffer - PBS with 0.05%Tween-20
- Substrate - Tetramethylbenzidine (TMB) and H₂O₂
- Stop solution - 2N H₂SO₄

**Method**
1. A 96 well microtitre plate was coated with 100µl per well of capture antibody (2µg/ml) diluted in coating buffer and incubated overnight at room temperature.
2. The captured antibody was aspirated and the wells were washed three times with wash buffer.

3. The plate was blocked with 250μl per well of blocking buffer, sealed with plate cover and incubated for 2 hours at 37°C.

4. Blocking buffer was aspirated from wells and blot dried.

5. 100μl per well of diluted standards (0-1000 pg/ml) and samples were added to different wells of microtitre plate, sealed and incubated for 1 hour at 37°C.

6. Standards and samples were aspirated and the wells were washed three times with wash buffer.

7. 100μl per well of diluted second antibody (2μg/ml) was added to all wells, sealed and incubated for 1 hour at 37°C.

8. Second antibody was aspirated and wells were washed three times with wash buffer.

9. 100μl per well of diluted detection reagent (1/1000 dilution) was added, plate was sealed and incubated for 20 minutes at 37°C.

10. Detection reagent was aspirated and wells were washed three times with wash buffer.

11. 100μl per well of working TMB substrate solution (0.5mg TMB and 1μl of 30% H₂O₂ per ml of citrate phosphate buffer, pH 5.0) was added, and incubated for 10-15 minutes at room temperature.

12. 100μl per well of stop solution was added.

13. Absorbance was read at 450nm within 30 minutes.

14. A standard curve was constructed by plotting the mean absorbance for each standard on the vertical axis (Y-axis) versus the corresponding standard concentration on the horizontal axis (X-axis).
**In vitro** stimulation of THP-1 cells by *Tridax procumbens* leaves extracts and their different fractions

**Materials**
1. THP-1 Cell line obtained from NCCS, Pune, India
2. RPMI-1640 complete medium (2mM L-glutamine, 10mM HEPES, 10% FCS, 100μg/ml streptomycin, 100units/ml penicillin and 0.25μg/ml amphotericin B.
3. RPMI-1640 medium without FCS
4. T25 culture flask, 24 well plate
5. CO₂ incubator (Hera Incubator, 37° C, 5% CO₂)
6. *Tridax procumbens* leaves aqueous extract and their fractions and ethyl acetate extract
7. DMSO

**Method**
1. THP-1 cells were seeded into 24-well plates at 2 x10⁵ cells/well in 1ml complete RPMI medium and different dosages of aqueous *Tridax procumbens* leaves extracts and their fractions in RPMI 1640 medium were added. The ethyl acetate extract and their fractions were added in DMSO. DMSO also in medium was used as a control.

3. The cell line, at each time point had unchallenged control wells (no LPS) and LPS- challenged wells at 1 ug/ml. Cell supernatants were removed at the various time points and frozen at -70° C.

4. Analysis for TNF-α (48hrs) and IL-8 (48hrs) in the supernatant was performed using R&D Systems ELISA kits as described previously.

**Isolation of neutrophils from human blood**

**Materials**
1. Heparinized human blood (10 IU heparin per ml) from healthy individual
2. RPMI-1640 complete medium (10mM HEPES 10% FCS, 100μg/ml streptomycin, 100units/ml penicillin and 0.25μg/ml amphotericin B, 2mM L-glutamine)
3. RPMI-1640 medium without FCS
4. Polymorph prep
5. *Tridax procumbens* leaves extracts and their fractions
6. DMSO

**Method**
1. Venous blood was collected from twenty five to thirty five years old healthy volunteers using sterile syringes with 10 U/ml heparin.

2. Neutrophils were isolated using polymorph prep. 5ml of blood, diluted 1:1 with phosphate buffer saline, layered over 5 ml of polymorph prep in a 15 ml centrifuge tube and centrifuged at 400g for 30 minutes.

3. The Neutrophils band was collected and washed with RPMI medium without FCS.

4. The contaminating RBCs were lysed by hypotonic shock.

5. The isolated cells were characterized morphologically by Giemsa staining.

6. It is observed that >98% of the isolated cells were neutrophils. Lymphocytes contamination was less than 0.1%. Cells were cultured in RPMI 1640 with 10% autologus, heat inactivated serum and autologus adherent cells.

**Activation of neutrophils with *Tridax procumbens* leaves extracts**

1. Neutrophils (2 x10⁵ cells/well) were cultured in 200μl of RPMI 1640 complete medium in 96 well tissue culture plates (Nunc) at 37°C in an atmosphere of 5% CO₂ in presence of 10⁵ autologous adherent cells

2. Aqueous extracts of *Tridax procumbens* leaves and their different fractions were added to the wells. The ethyl acetate extracts and its different fractions were added dissolved in DMSO. DMSO was also used as a control.
3. Neutrophils culture supernatants were assayed at a time found to be optimal for the secretion of that cytokine which was 24 hours for IL-8 & TNF-α and 48 hours for IFN-γ.

4. Cytokine levels in culture supernatants were measured by sandwich ELISA using commercially available kits as described before (R&D System, USA)

**Imunoassay of human TNF-α**

Immunoassay of human TNF-α was done as described previously.

**Imunoassay of human IFN-γ**

Immunoassay of human IFN-γ was done as described previously.

**Imunoassay of human IL-8**

Immunoassay of human IL-8 was done as described previously.

**Staining of cultured neutrophils for CD28, BP55 and CD3**

**Materials**

1. Staining buffer- PBS containing 0.5% BSA and 0.1% sodium azide, 0.2μm filtered, pH 7.2-7.4, at 4°C.
2. Purified human immunoglobulins for Fc receptor blocking
3. Biotinylated mouse anti-human anti-CD28 monoclonal antibody (mouse IgG1, monoclonal antibody against BP55 and monoclonal antibody against CD3
4. streptavidin-FITC or streptavidin-PE conjugate

5. Appropriate Isotype control antibodies having identical fluorochrome label.
6. 4% Paraformaldehyde in staining buffers.
7. Sheath buffer- PBS containing 0.1% sodium azide, 0.2μm filtered.
8. Flow cytometry apparatus

**Method**

For each staining 10⁶ cells were used. The entire procedure was done over ice and in dim light. Cells were washed by centrifugation not exceeding 200g.
1. Neutrophils were harvested and washed twice in 1.0ml of staining buffer.

2. The cells were then incubated in 50μl of staining buffer containing 1μg of purified human immunoglobulins for Fc receptor blocking and incubated over ice for 30 minutes.

3. The cells were then incubated in 50μl of staining buffer containing 1μg biotinylated mouse anti-human CD28 monoclonal antibody(s) (IgG1) (fluorochrome labeled) and monoclonal antibody against BP55 and monoclonal antibody against CD3 and incubated over ice for 30 minutes. Similarly an equal number of cells were also incubated separately with the isotype control antibody(s).

4. Visualization of biotinylated Ab binding was performed using a streptavidin-FITC or streptavidin-PE conjugate.

5. Each incubation with antibody was followed by two washes in the staining buffer (1% BSA and 0.1% sodium azide in PBS).

6. Cells were resuspended in staining buffer containing 4% paraformaldehyde and kept over ice in dark till flow cytometry was done.

**Immunoprecipitation with anti-CD28 and anti-IL-8 receptor antibodies**

**Materials**
1. Polymorphonuclear Neutrophils (3x10⁷)
2. Lysis buffer: containing triton-x100, 1mM PMSF, aprotinin (1μg/ml), pepstatin (1μg/ml), and leupeptin (1μg/ml)
3. Centrifuge
4. Protein A- Agarose beads (Gibco-BRL, USA)
5. Monoclonal anti-CD28 antibody
6. Monoclonal anti-IL-8RA
7. Laemmli sample buffer.
8. 10% SDS-PAGE materials as taken previously)
10. TBS (Tris-buffered saline: 100mM Tris- HCl, Ph 7.5, 0.9% NaCl)
12. Blocking buffer: containing 5% BSA and 0.1% Tween-20
13. Goat anti-CD28 polyclonal antibody
14. HRPO conjugated anti-goat antibody
15. 3,3 Diaminobenzidine tetrahydrochloride (DAB)- 6mg in 10ml
   50mM Tris-Cl, pH7.6
16. 30% H₂O₂ solution (1µl/ml prepared fresh)

Method
1. Polymorphoneuclear neutrophils (3x10⁷) with or without
   stimulation with Tridax procumbens leaves extracts were lysed with
   lysis buffer for one hour at 4°C.

2. The lysates were centrifuged at 1000xg for 30 minutes.

3. Supernatant were sequentially pre-cleared with the 5µg and 20µl of
   Protein A- Agarose beads for one hour at 4°C followed by incubation
   with 5µg of monoclonal anti-CD28 or anti-IL-8 receptor antibodies
   overnight at 4°C.

4. The beads were washed four times with the lysis buffer and the
   immune complexes were resuspended in reducing Laemmli sample
   buffer.

5. The samples were heated to 95°C for five minutes and run on 10%
   SDS-PAGE.

6. The separated proteins were transferred onto nitrocellulose
   membrane.

7. The membrane was incubated with the TBS blocking buffer for one
   hour at 4°C.

8. The membrane was then incubated with goat anti-CD28 polyclonal
   antibody at 1:500 dilution in the same buffer containing 1% BSA and
   0.1% Tween-20 for one hour.
9. The blot was washed, and incubated for one hour with HRPO conjugated anti-goat antibody at 1:10000 dilution.

10. After washing thrice with PBS-Tween substrate solution (H$_2$O$_2$-1μl/ml & DAB-0.6μg/ml of 50mM Tris-Cl, pH 7.6) was put and kept for shaking till colour bands appeared.

11. The reaction was stopped by pouring off substrate solution and washing NC paper with distilled water.