Influence of ethanolic extract of *Tephrosia purpurea* Linn. on mast cells and erythrocytes membrane integrity

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The ethanolic extract of *T. purpurea* Linn. was studied for its *in vitro* effect on rat mast cell degranulation and erythrocyte membrane integrity *in vitro*. The extract in concentration of 25-200 µg/ml showed a dose-dependent inhibition of rat mast cell degranulation induced by compound 48/80 and egg albumin. *T. purpurea* extract was found to inhibit haemolysis of erythrocytes induced by hypotonic solution but accelerated haemolysis induced by heat at a concentration of 100 µg/ml. The studies reveal that the ethanolic extract of *T. purpurea* may inhibit degranulation of mast cells by a mechanism other than membrane stabilization.

A number of plants have been used since time immemorial for the treatment of allergic and inflammatory disorders.

Sharapunkha, *Tephrosia purpurea* Linn. (Family-Leguminosae) is a copiously branched, perennial herb found throughout India. Ayurvedic texts have reported the whole plant to be used to cure asthma and bronchitis. Clinical trials of entire dried plant conducted on patients with bronchitis have shown improvement in their condition.

The central event in type I hypersensitivity or allergic reaction is mast cell degranulation. Mast cell activation could occur due to an antigenic or a chemical stimulus which results in membrane stimulation of the cell after binding of the agent, with subsequent mobilization of calcium and degranulation of the cell. The degranulated cell releases a number of preformed and *de novo* synthesized mediators which include histamine, leukotrienes, prostaglandins and chemotactic factors. The present investigation was aimed at exploring the possibility that *T. purpurea* might be active in the Type I allergic conditions by its ability to inhibit the release of mediators from mast cells and basophils and thus influence the course of the disease by preventing the harmful effects of the released mediators.

**Plant Material**

The dry, aerial parts of *Tephrosia purpurea* Linn., obtained from Yucca Enterprises, Mumbai, India, were identified and authenticated by Dr. K. S. Laddha, Department of Pharmacognosy, University Department of Chemical Technology, Mumbai, India. A voucher specimen has been submitted to the Bombay College of Pharmacy, Mumbai.

**Preparation of plant extract**

The coarse powder (200 g) of the dried aerial parts of *T. purpurea* was exhaustively extracted using 85% ethanol (1000 ml) in a Soxhlet extractor. The extract was concentrated under reduced pressure at a bath temperature below 50°C to yield a syrupy mass (27 g). For dosing, it was suspended in distilled water using 0.1% sodium carboxymethylcellulose.

**In vitro mast cell degranulation studies**

**Isolation of total peritoneal cells**

Physiological salt solution (10 ml) of the following composition in mM - NaCl, 137; NaHCO₃, 12; NaH₂PO₄, 2H₂O, 0.3; KCl, 2.7; MgCl₂ 1.0; CaCl₂, 1.8 and dextrose 5.6 containing 5 units/ml of heparin was injected in the peritoneal cavity of male rats lightly anaesthetized with ether. After a gentle abdominal massage for about 30-45 sec, the peritoneal fluid containing mast cells was collected over ice and centrifuged at 2000 rpm for 5 min. The cells were washed twice with the physiological salt solution and resuspended in 1 ml of the salt solution. The peritoneal fluid was obtained from 10 rats, the final volume of the fluid was pooled and used for the *in vitro* studies.
Degranulation studies

Sensitized mast cells were obtained from animals sensitized with three doses of 350 μg of Egg albumin adsorbed on 60 mg of aluminium hydroxide gel, the doses being given on the first, third and fifth day subcutaneously. The sensitized mast cells were degranulated using Egg albumin (1 mg/ml) on the tenth day of sensitization. The normal mast cells were degranulated using Compound 48/80 (1 μg/ml).

The suspension of the peritoneal cells was treated as follows -

To 0.1 ml of the total peritoneal cell suspension, 0.1 ml of the test agent in saline was added and incubated in a constant-temperature water bath (37°C) for 15 min. Then 0.1 ml of the degranulating agent (Egg albumin-1 mg/ml or Compound 48/80-10 μg/ml) was added and the suspension was further incubated for a period of 10 min. The cells were then stained with 0.1% alcoholic solution of neutral red and observed under the high power of a light microscope. The percent protection of the mast cells in the control group and the treated groups was calculated by counting the number of mast cells. A minimum of 100 cells were counted.

T. purpurea extract was studied in the range of 25 - 200 μg/ml. Ketotifen (10 μg/ml) was used as a reference standard for comparison. The groups also consisted of a positive control in which either compound 48/80 or egg albumin was added without the addition of test agent and a negative control group which was used to correct for spontaneous degranulation of mast cells occurring in absence of egg albumin or compound 48/80.

Studies on erythrocyte membrane integrity

Collection of erythrocytes

Whole blood was collected from healthy human volunteers by vein puncture. Heparin was used as an anticoagulant. The collected blood was washed three times with 0.9% saline. The volume of the erythrocytes was measured and reconstituted as a 40% v/v suspension with isotonic buffer solution which contained in 1 litre of distilled water: NaH₂PO₄·2H₂O, 0.26 g; Na₂HPO₄·2H₂O, 1.15 g; NaCl, 9 g (10 mM sodium phosphate buffer).

Hypotonic solution-induced haemolysis

The isotonic buffer solution was composed of 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) and the hypotonic solution contained 56 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4). The experiments were carried out in duplicate pairs. Stock erythrocyte suspension (30 μl) was mixed with 5 ml of the hypotonic solution containing different concentrations of T. purpurea extract, viz. 25, 50 and 100 μg/ml while the control samples were mixed with drug free solution. The mixtures were incubated for 10 min at room temperature and centrifuged at 1000 rpm for 3 min. The absorbance (A) of the supernatant was measured at 540 nm using Shimadzu UV-160A spectrophotometer.

Heat-induced haemolysis

Portions (5 ml) of isotonic buffer solution containing different concentrations of T. purpurea extract, viz. 25, 50, 100 and 200 μg/ml, cooled to 0° - 5° C in an ice bath, were added to each of two duplicate sets of centrifuge tubes. The solution containing the extract was added to another tube in each of the sets as a control. Erythrocyte suspension (30 μl) was added to each tube and mixed gently by inversion. One pair of tubes was incubated for 20 min at 54° C in a constant temperature water bath. The other pair was maintained at 0 - 5° C in an ice bath. The absorbance (A) of the supernatant was measured at 540 nm using Shimadzu UV-160A spectrophotometer.

Calculation

The percent inhibition or acceleration of haemolysis by either of the above methods was calculated according to the equation:

\[ \% \text{ inhibition or acceleration of haemolysis} = 100 - 100 \times \frac{A_1 - A_2}{A_3 - A_4} \]

where,

- \( A_1 \) = Absorbance of test sample heated or in hypotonic solution
- \( A_2 \) = Absorbance of test sample unheated or in isotonic solution
- \( A_3 \) = Absorbance of control sample heated or in hypotonic solution
- \( A_4 \) = Absorbance of control sample unheated or in isotonic solution

Drugs and animals

Drugs used were compound 48/80 (Sigma Chemical Co., USA), Ketotifen fumarate (FDC Ltd., Mumbai, India), Egg albumin (Hi-Media Laboratories,
Mumbai) and Heparin (Biological E. Ltd., Mumbai). All other reagents used were of a high grade of purity. Compound 48/80 was made in 0.9% NaCl solution. Ketotifen fumarate was dissolved in 0.9% NaCl solution.

Inbred Wistar rats weighing between 175-200 g were used. The animals were housed under standard conditions of temperature (23°C ± 1°C), relative humidity (55 ± 10%), 12 hr/12 hr light/dark cycles and fed with standard pellet diet (Lipton India, India) and tap water ad libitum.

Statistical analysis
The results are presented as mean ± SD. Statistical significance between the groups was analysed by Student's t test.

Compound 48/80 induced mast cell degranulation
Compound 48/80 (10 µg/ml) was found to induce mast cell degranulation to the extent of 86.0%. Ketotifen (10 µg/ml) as a reference standard produced an inhibition of 79.08%. T. purpurea extract (25-200 µg/ml) produced dose-dependent inhibition of mast cell degranulation (Table 1).

Egg albumin induced mast cell degranulation
Egg albumin (1 mg/ml) was found to induce mast cell degranulation to the extent of 92.0%. Ketotifen (10 µg/ml) as a reference standard produced an inhibition of 82.34%. T. purpurea extract (25-200 µg/ml) produced dose-dependent inhibition of mast cell degranulation (Table 2).

Hypotonic solution-induced haemolysis of erythrocytes
T. purpurea extract at concentrations ranging from 25-100 µg/ml inhibited hypotonicity-induced haemolysis as evident by a decrease in the absorbance of the supernatant containing the cell suspension (Table 3).

Heat-induced haemolysis of erythrocytes
T. purpurea extract at concentrations ranging from 25-100 µg/ml was not found to inhibit haemolysis induced by hypotonic solution. It accelerated the

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<tr>
<th>Table 1—Effect of T. purpurea ethanolic extract on Compound 48/80 induced rat mast cell degranulation</th>
<th>[Values are mean ± SD of four observations]</th>
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* P<0.001

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<th>Table 2—Effect of T. purpurea ethanolic extract on egg albumin induced rat mast cell degranulation</th>
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* P<0.001

| Table 3—Effect of T. purpurea ethanolic extract on erythrocyte membrane Integrity |
|---------------------------------------------------|---------------------------------------------|
| Treatment                                         | Concentration (µg/ml) | % Inhibition or acceleration of haemolysis |
| Hypotonicity-induced haemolysis                   | —                        | —                                      |
| Heat-induced haemolysis                           | 25                       | +69.45                                 |
| T. purpurea extract                               | 50                       | +73.89                                 |
| T. purpurea extract                               | 100                      | +85.56                                 |

*'+' sign indicates inhibition of haemolysis
*'-' sign indicates acceleration of haemolysis
process of haemolysis at the given concentration to a great extent (Table 3).

In the present study, the *T. purpurea* ethanolic extract was found to inhibit the degranulation of mast cells induced by a non-immunological and an immunological stimulus.

It is known that the physiological stimulus for the release of histamine from mast cells is provided by a combination of antigen with specific antibody fixed on the cell surface. This combination is believed to transiently increase the permeability of membrane to calcium ions showing an absolute requirement for calcium ions for the secretory process to occur. Anaphylactic and compound 48/80 induced secretion from mast cells share a common requirement as far as the presence of calcium ions is concerned. However, compound 48/80 can utilize intracellular calcium stores to initiate the release process, even in the absence of calcium in the extracellular medium. On the other hand, anaphylactic release requires the presence of calcium in the extracellular medium which moves into the cell via calcium gates in the membrane.

*T. purpurea* extract was found to inhibit haemolysis of erythrocytes induced by hypotonic solution but could not prevent that induced by heat. Protection against heat-induced haemolysis is thought to be caused by stabilization of proteins in the membrane. Hence it may be suggested that the extract may interact but not stabilize membrane proteins. A possible explanation for inhibition of hypotonicity-induced haemolysis is an increase in surface area to volume ratio of the cells brought about by an expansion of the membrane, a shrinkage of the cell or both, and thus making them osmotically less fragile.

It has also been shown that the deformability and cell volume of erythrocytes is closely related to intracellular content of calcium. Hence it may be assumed that the cytoprotective effect induced by *T. purpurea* on mast cell surface could be due to its ability to alter the influx of calcium ions.

References