Materials and Methods
MATERIALS AND METHODS

3.1 Plant Materials

The mature seed materials of various tribal pulses were collected from different agro-climatic regions of Western and Eastern Ghats in South India. Botanical name of the tribal pulses and their place of collection, district, state, month and year are given below in tabular form.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Botanical name of the tribal pulse</th>
<th>Place of collection with Month and Year</th>
</tr>
</thead>
</table>

Contd....
<table>
<thead>
<tr>
<th>S. No</th>
<th>Botanical name of the tribal pulse</th>
<th>Place of collection with Month and Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td><em>Cassia obtusifolia</em> L.</td>
<td>Courtallum, Tirunelveli District, Tamil Nadu, (Western Ghats), March, 1993.</td>
</tr>
<tr>
<td>b</td>
<td><em>C. obtusifolia</em> L.</td>
<td>Topslip, Coimbatore District, Tamil Nadu (Western Ghats), March, 1993.</td>
</tr>
<tr>
<td>7a</td>
<td><em>M. pruriens</em> var <em>utilis</em> Wall ex Wight. (Black - coloured seed coat)</td>
<td>Keriparai, Kanyakumari District, Tamil Nadu (Western Ghats). March 1993.</td>
</tr>
<tr>
<td>c</td>
<td><em>M. pruriens</em> var <em>utilis</em> Wall ex Wight. (White - coloured seed coat)</td>
<td>Thachenmalai, Kanyakumari District, Tamil Nadu (Western Ghats). March, 1993.</td>
</tr>
</tbody>
</table>

### 3.2 Chemicals

The chemicals used were BDH (AR) or Sigma Chemical Company, St. Louis M.O., U.S.A., throughout the study unless and otherwise specified.
Canavalia ensiformis (1-4)
1. Valacode germplasm (Red-coloured seed coat)
2. Dasukuppam germplasm (Red-coloured seed coat)
3. Maananthavaadi germplasm (Maroon-coloured seed coat)
4. Pathanamthitta germplasm (Maroon-coloured seed coat)

Canavalia gladiata (5-8)
5. Courtallum germplasm (Maroon-coloured seed coat)
6. Paramangalam germplasm (Maroon-coloured seed coat)
7. Hogenekkal germplasm (White-coloured seed coat)
8. Kumlsa germplasm (White-colored seed coat)

Cassia floribunda (9-11)
9. Erattupattai germplasm
10. Kumlsa germplasm
11. Kargal germplasm

Cassia obtusifolia (12-14)
12. Courtallum germplasm
13. Topslip germplasm
14. Sidapura germplasm

Mucuna monosperma (15-17)
15. Chitoor germplasm
16. Bogalthode germplasm
17. Borra village germplasm

Mucuna pruriens (18-20)
18. Aliyar germplasm
19. Arunooli germplasm
20. Karwar germplasm

Mucuna pruriens var. utilis (21-23)
21. Keriparai germplasm (Black-coloured seed coat)
22. Valanad germplasm (Black-coloured seed coat)
23. Thachenmalai germplasm (White-coloured seed coat)
3.3 Preparation of seed flour

About 50g each of air-dried and oven-dried seeds were powdered in a Wiley Mill (Scientific Equipment Works, New Delhi, India) to 60 mesh size. Care was taken to clean the Wiley Mill thoroughly after powdering a particular sample and before starting to powder a new sample to avoid mixing up of samples. The fine seed powder, so obtained will be referred, hereafter, as air-dried or over-dried seed flour. The powdered samples were stored in screw-cap bottles until further use.

3.4 Proximate analysis

3.4.1 Determination of moisture content (Janardhanan, 1982)

The air-dried mature seeds (50 seeds at a time) were weighed, cut transversely with a sharp knife and incubated in a hot-air oven at 80°C for 24 h. Then the sample was cooled in a desiccator and weighed again. The loss in weight of the sample was calculated as moisture content and the average value of triplicate determinations expressed on percentage basis.

3.4.2 Determination of crude protein content

a Digestion

The nitrogen content of the sample was determined by micro-kjeldahl method (Humphries, 1956). 100 mg of oven-dried seed flour was taken into a micro-kjeldahl digestion flask. To this 2 ml of 5% (w/v) salicylic acid dissolved in conc. H₂SO₄ was added and mixed well. After 20 min., 0.3 g of sodium thiosulphate was added and heated gently until fumes disappeared. After cooling the contents of the flask, 60 mg of catalyst (a mixture of 1g copper sulphate, 8g potassium sulphate and 1g selenium dioxide) followed by 1 ml of conc. H₂SO₄ were added. The contents of the flask were digested until they turned apple green in colour. The flask was cooled and the contents were made up to a known volume.

b Distillation

Ten ml aliquot from the volumetric flask was transferred to Paranas micro-kjeldahl distillation flask. To this 10 ml of 40% (w/v) NaOH solution along with 2 ml of glass distilled water were added. The contents were heated by a bunsen burner. The liberated ammonia was collected in 2% (w/v) boric acid solution containing a drop of double indicator (83.3 mg of bromocresol green + 16.6 mg of methyl red dissolved in 10 ml of 95% ethanol).

The contents were titrated against N/50 sulphuric acid. A blank was run simultaneously using all the reagents and the value of the blank was deducted from the value of the sample before calculation. One ml of N/50 H₂SO₄ corresponds 0.00028g of N, which forms the basis for calculation.
of N content in the sample. The crude protein content was calculated by multiplying the per cent nitrogen content of the sample with the factor 6.25.

3.4.3 Determination of ether extract (or) total crude lipid content (AOAC, 1970)

Two g of air-dried seed flour was extracted with ether in a Soxhlet apparatus for 16 h., according to the AOAC (1970). The ether was evaporated and the residue was weighed. The average value of triplicate experiments was expressed as percentage of ether extract or total crude lipid content on dry weight basis.

3.4.4 Determination of crude fibre content (AOAC, 1970)

After extraction with ether, the left-out residue was successively digested with 0.225N H2SO4 solution and 0.313N NaOH solution. After digestion, it was washed with boiling water followed by absolute ethanol in a Gooch crucible. The contents of the crucible were dried to constant weight, cooled, weighed and ignited in an electric muffle furnace for 30 min. at 600°C. After cooling, the contents were reweighed. The loss in weight was expressed as percentage of crude fibre on dry weight basis.

3.4.5 Determination of ash content (AOAC, 1970)

Two g of oven-dried seed flour was weighed into a preweighed porcelain crucible. The crucible with the seed flour was placed in an electric muffle furnace set at 600°C and maintained for 2h. The contents of the crucible were cooled in a desiccator and weighed immediately. The difference in weight of the crucible gives the ash content. The ash content was expressed as percentage on dry weight basis.

3.4.6 Determination of Nitrogen Free Extractives (NFE) or total crude carbohydrates content

(Muller and Tobin, 1980)

Percentage of NFE was calculated as given below:

\[ \% \text{NFE} = 100 - (\text{CP\%} + \text{EE\%} + \text{CF\%} + \text{Ash\%}) \]

where

- CP = Crude protein
- EE = Ether extract
- CF = Crude fibre

3.4.7 Determination of calorific value (Siddhuraju et al., 1992b)

The calorific values of the seeds were determined in kJ by multiplying the percentage of crude protein, crude lipid and NFE by the factors 16.7, 37.7 and 16.7, respectively.

* AOAC - Association of Official Agricultural Chemists, Washington, DC, USA.

** NFE - Nitrogen Free Extractives - a standard abbreviation in Plant Foods for Nutrition. 16.7, 33.7, and 16.7 are tested constants.
3.5 Extraction and estimation of total proteins (true proteins)

a Extractions (Rajaram and Janardhanan, 1990)

One g of air-dried seed flour was defatted by macerating with petroleum ether (1:10 w/v) for 6h. The petroleum ether extract was removed by centrifugation at 5000 x g for 10 min. The pellet was washed with petroleum ether twice and the defatted meal was washed with 100 ml of cold 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 20,000 x g for 15 min. The procedure was repeated, the resulting TCA-washed pellet was suspended in 50 ml of 1N NaOH solution and incubated at 45°C for 16h. The resulting hydrolysate was centrifuged at 20,000 x g for 20 min. The pellet was re-extracted with 20 ml of 1N NaOH solution and centrifuged. The supernatants were pooled together.

b Protein determination

The protein from 1 ml of the pooled supernatants was precipitated with equal volume of cold 20% (w/v) TCA for 30 min. at 4°C. After centrifugation, the protein pellet was dissolved in 0.1N NaOH and from suitable aliquots, the protein content was measured by the method of Lowry et al. (1951) using bovine serum albumin fraction V (Sigma Chemical) as a standard in a Spectronic 20 D spectrophotometer at 750 nm. Average value of triplicate estimations was calculated and the content of true protein was expressed on dry weight basis.

3.6 Fractionation and estimation of different solubility classes of seed proteins

a Extraction of albumins and globulins

Albumin and globulin fractions of seed proteins were extracted following the method of Murray (1979). Two g of air-dried seed flour was extracted with 30 ml of 5% (w/v) K$_2$SO$_4$ in 0.1M sodium phosphate buffer (pH 7.0) along with 600 mg of insoluble polyvinylpolypyrrolidone (PVPP) at 4°C for 24h with constant magnetic stirring and centrifuged in 20,000 x g at 4°C for 20 min. The superantant was removed and further 600mg of PVPP was mixed with the pellet in 15 ml of the same buffer and stirred once more for 30 min. Then the suspension was clarified by centrifugation as before. The pellet after centrifugation was saved for the extraction of prolamin and glutelins in sequence. The superantants were pooled and dialysed against running tape water for 18h. with two changes in distilled water. The dialysates were centrifuged in 20,000 x g at 4°C for 20 min. The superantant was designated as albumins and pellet as globulins. The pellet was redissolved in the same extraction buffer.
b. Extraction of prolams and glutelins (Rajaram and Janardhanan, 1990)

The pellet saved from the above experiment after the extraction of albumins and globulins was extracted with 80% (v/v) ethanol 1:5 (w/v) overnight at room temperature. The contents were centrifuged at 20,000 x g for 20 min. The supernatant was air-dried at room temperature, ground to a fine powder, dissolved in 0.1 N NaOH solution and designated as prolams. The left out pellet, after extraction of prolams, was extracted with 0.02% (w/v) NaOH 1:10 (w/v) overnight at room temperature. The contents were centrifuged at 20,000 x g for 20 min. The supernatant thus obtained was designated as glutelins.

c. Protein determination

The protein contents of different solubility classes of proteins separated were estimated following the method of Lowry et al. (1951) after TCA precipitation, as has been described earlier. Average value of triplicate determinations was calculated and expressed as percentage on dry weight basis.

3.7 Amino acid analysis of purified total seed proteins

The total seed proteins from the seed flour were extracted as has been described earlier. After extraction the proteins were precipitated with cold 10% (w/v) TCA and centrifuged. 100 mg of purified seed proteins thus obtained was hydrolysed with 6 N HCl at 110°C for 24 h. in sealed ampoules under vacuum. Then the contents were centrifuged. The clear supernatant was dried in a rotary vacuum evaporator to remove the traces of acid. After washing with glass distilled water and drying, the dried residues were once again dissolved in glass distilled water. The amino acid analyses, were performed using an automated pre-column derivatization method with O-phthaldialdehyde (OPA) using reverse-phase HPLC, Model 23250 with spherisorp C18 column and ISCO - Dual pump, by the method of Rajendra (1987). The flow rate was 1.5 ml/min. with fluorescence detector. The detected amino acids were presented as g 100g⁻¹ proteins and were compared with FAO / WHO (1991) reference pattern.

The essential amino acid score was calculated as follows:

\[
\text{Essential amino acid score} = \frac{\text{g of essential amino acid in 100g of the test protein}}{\text{g of essential amino acid in 100 g FAO / WHO (1991) reference pattern}} \times 100
\]
3.8 Mineral analysis

3.8.1 Sample digestion

Five hundred mg of air-dried seed flour was mixed with 10 ml of conc. HNO$_3$ and 4 ml of 60% perchloric acid and 1 ml of conc. H$_2$SO$_4$ and the contents were kept undisturbed overnight.

After that it was heated on a hot plate containing conc. H$_2$SO$_4$ in a beaker until the brown fumes ceased coming out and then allowed to cool. After cooling it was filtered through Whatman No. 42 filter paper. After filtration the filtrate was made up to 100ml with glass distilled water.

3.8.2 Estimation of sodium and potassium

Sodium and potassium were estimated by using Flame Photometer Model - EEL. The sodium and potassium contents were calculated by referring to the calibration curves of sodium and potassium, respectively and expressed as mg 100 g$^{-1}$ of seed flour.

3.8.3 Estimation of calcium and magnesium (Jackson, 1967)

a Calcium

Five ml of triple acid digested extract was taken in a China dish, to this 10 ml of 10% (w/v) NaOH and 0.1 g of Murexide indicator powder [40g of potassium sulphate or potassium chloride was ground with 10 g ammonium chloride and 0.2 g of Murexide (ammonium purpurate)] were added and titrated against 0.02N versenate (19g of EDTA was dissolved in 5 litres of distilled water) and standardized against 0.2 N Na$_2$Co$_3$ solution and adjusted until the colour changes from red to violet.

b Calcium and magnesium

Five ml of triple acid digested extract was taken in China dish, to this 10 ml of ammonium chloride - ammonium hydroxide buffer, pH 10 and few drops of Erichrome black T indicator (0.1 g of Erichrome black T was dissolved in 25 ml of methanol containing 1g of hydroxylamine hydrochloride) were added and titrated against 0.02N versenate solution until the colour changes from red to blue.

c Calculation

Percentage of calcium in the sample = Titre value of calcium $\times$ 100/5 $\times$ 100/0.5 $\times$ 0.0004

Percentage of magnesium = Titre value of calcium + magnesium - titre value of calcium

or

titre value of calcium + magnesium $\times$ 0.96

Calcium and magnesium contents were expressed as mg 100g$^{-1}$ of seed flour.
3.8.4 Estimation of phosphorus (Dickman and Bray, 1940)

One ml of triple acid digested extract was pipetted into 100 ml volumetric flask. To this 50 ml glass distilled water was added, followed by 5 ml ammonium molybdate-sulphuric acid reagent (Solution A: 25 g of ammonium molybdate was dissolved in 100 ml of distilled water. Solution B: 280 ml of conc. H$_2$SO$_4$ was diluted to 800 ml. Solution A was added slowly with constant stirring to solution B and the volume was made upto 1000 ml with glass distilled water). Blue colour was developed by adding six drops of 2.5% (w/v) stannous chloride solution. The total volume was made upto 100 ml. The intensity of the blue colour was measured at 650 nm in a spectrophotometer. The phosphorus content present in the sample was calculated by referring to a standard graph of phosphorus using potassium dihydrogen phosphate (KH$_2$PO$_4$) as standard and expressed as mg 100 g$^{-1}$ of seed flour.

3.8.5 Estimation of micronutrients by Atomic Absorption Spectrophotometer (Issac and Johnson, 1975)

By feeding the sample on an Atomic Absorption Spectrophotometer (PERKIN - ELMER - Model, 5000) the following elements were estimated with appropriate wavelengths.

<table>
<thead>
<tr>
<th>Name of the mineral</th>
<th>Wavelength used for estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>246.8 nm</td>
</tr>
<tr>
<td>Copper</td>
<td>324.6 nm</td>
</tr>
<tr>
<td>Zinc</td>
<td>213.7 nm</td>
</tr>
<tr>
<td>Manganese</td>
<td>279.4 nm</td>
</tr>
</tbody>
</table>

The mineral contents were expressed as mg 100 g$^{-1}$ seed flour.

3.9 Determination of in vitro protein digestibility (Ekpenyong and Borchers, 1979)

Fifty ml of glass distilled water was added to the seed flour (amount of sample was adjusted so as to contain 6.25 mg/ml). The sample was allowed to hydrate for 1 h. at 5°C. The sample suspension was adjusted to pH 8.0 with 0.1N HCl and / or 0.1N NaOH while stirring in a water bath maintained at 37°C for 15 min. The multienzyme solution, consisting of 1.6 mg trypsin, 3.1 mg chymotrypsin and 1.3 mg peptidase/ml, was maintained in an ice bath and adjusted to pH 8.0 with 0.1N HCl and / or NaOH. Five ml of this solution was added to the protein suspension while stirring at a constant temperature of 37°C. The pH of the hydrolysate was measured exactly 10 min. after the addition of multienzyme solution. The percentage of in vitro protein digestibility was calculated following the formula given below (Hsu et al., 1977).
\[ Y = 210.464 - 18.103 X \]

where \( X \) = pH of protein suspension after 10 min. digestion with multienzyme solution and

\( Y \) = Percentage of digestibility.

3.10 Antinutritional factors of the seed samples

3.10.1 Extraction and estimation of total free phenolics and tannins

a) Extraction (Maxon and Rooney, 1972).

One g of air-dried seed flour was taken in a 100 ml of flask, to which was added 50 ml of
1%(v/v) HCl in methanol. The samples were shaken on a reciprocating shaker for 24h. at room
temperature. The contents were centrifuged at 10,000 x g for 5 min. The supernatant was collected
separately and used for further analysis.

b) Estimation of total free phenolics (Sadasivam and Manickam, 1992a)

One ml aliquots of the above extract were pipetted into different test tubes to which 1 ml of
folin-ciocalteu's reagent followed by 2 ml of 20% (w/v) \( \text{Na}_2\text{CO}_3 \) solution were added and the tubes
were shaken and placed in a boiling water bath for exactly 1 min. The test tubes were cooled under
running tap water. The resulting blue solution was diluted to 25 ml with distilled water and the
absorbance was measured at 650 nm with a help of a Spectronic 20D spectrophotometer. If
precipitation occurred, it was removed by centrifugation at 5000 x g for 10 min., before measuring the
absorbance. The amount of phenolics present in the sample was determined from a standard curve
prepared with catechol. A blank containing all the reagents minus plant extract was used to adjust the
absorbance to zero. Average value of triplicate estimations was expressed as g 100g\(^{-1}\) of the seed
flour on dry weight basis.

c) Estimation of tannins (Burns, 1971)

From suitable aliquots of the above extract tannin content was quantified by the Vanillin
HCl method of Burns (1971) using phloroglucinol as a standard at 500nm with a Spectronic 20
D spectrophotometer. The average values of triplicate estimations of all samples were expressed as
\( g 100g\(^{-1}\) seed flour on dry weight basis.

3.10.2 Extraction and estimation of L-DOPA (3,4 - dihydroxyphenylalanine) (Brain, 1976)

One g of air-dried seed flour was extracted with 5 ml of 0.1 N HCl over a boiling water bath for 5
min. After cooling, an equal volume of ethanol was added. The mixture were shaken mechanically for
10 min. The contents were centrifuged at 5000 x g for 10 min. The supernatant was retained. The pellet
was re-extracted with an equal volume of ethanol and the extract was clarified by centrifugation. Both the supernatants were combined and made up to a known volume with ethanol.

L-DOPA content in the extract was quantified by measuring the ultra-violet light absorption at 282 nm in a Wibro-Backman spectrophotometer, Model-26 after correction for background absorption, using L-DOPA (Sigma Chemical) as a standard. The contents of L-DOPA in seed flour of all the samples were calculated and expressed as percentage on dry weight basis.

3.10.3 Trypsin inhibitor activity

a Source of Trypsin Inhibitor (TI) (Sadasivam and Manickam, 1992b)

The TI was extracted from 0.5 g of the powdered sample with 25 ml of distilled water, by grinding in a prechilled mortar and pestle. The ground sample was kept in a refrigerated for 3h, with frequent shaking, for the complete extraction of TI. Clear extract was obtained by centrifuging the sample at 15,000 x g for 20 min. at 0-4°C. One ml of the clear supernatant was made up to 10 ml with distilled water and used as TI source.

Trypsin inhibitor activity was assessed by the method of Kakade et al. (1974).

b Preparation of reagents

Trypsin solution

Four mg of accurately weighed trypsin (Sigma Chemical) was dissolved in 200 ml of 0.001 M HCl.

Substrate solution

Forty mg of benzoyl-DL-arginine-paranitroanilide (BAPNA) (Sigma Chemical) was dissolved in 1 ml of dimethyl sulfoxide and diluted to 100 ml with tris-buffer prewarmed to 37°C.

Tris-buffer (0.05M, pH 8.2) containing 0.02 M CaCl$_2$

6.05 g tris (hydroxymethylaminomethane) and 2.94 g CaCl$_2$·2H$_2$O were dissolved in 900 ml of water. The pH was adjusted to 8.2 and the volume brought to 1000 ml with distilled water.

c Estimation

Portions (0, 0.6, 1.0, 1.4 and 1.8 ml) of extract were pipetted into duplicate sets of test tubes and adjusted to 2 ml with distilled water. After 2 ml of trypsin solution was added to each test tube, the tubes were placed in a water bath at 37°C. To each tube, 5 ml of BAPNA solution previously warmed to 37°C was added; exactly 10 min. later the reaction was terminated by adding 1 ml of 30% (v/v) acetic acid. After thorough mixing, read the absorbance at 410 nm with a help of Spectronic 20D spectrophotometer against a reagent blank. The reagent blank was prepared by adding 1 ml of 30%
(v/v) acetic acid to a test tube containing trypsin and water (2 ml each) before the 5 ml of BAPNA solution was added.

After determining the protein content in the extract by the method of Lowry et al. (1951), the trypsin inhibitor activity in Trypsin inhibitor Units or TIU/mg protein. One trypsin unit was expressed as an increase of 0.01 absorbance unit per 10 ml of reaction mixture at 410 nm.

3.10.4 Assay for haemagglutinating activity (Linear, 1976c)

Albumin and globulin protein fractions (as obtained under fractionation of different solubility classes of seed proteins) were employed as protein samples for determining haemagglutinating activity. Human blood from Blood bank of Ray Vijay Clinical Laboratory, Coimbatore was procured.

Blood erythrocyte suspension was prepared by washing the blood samples (A, B and O) separately, with phosphate - buffered - saline and centrifuged for 30 min. at low speed 1000 rpm. Supernatants were removed with Pasteur pipette. The washing procedure was repeated for three times. The washed cells were diluted by mixing 12 drops of cells with 24 drops of phosphate - buffered - saline.

Extracts (5 drops) of albumin and globulin protein fractions were mixed, separately, with an equal volume of different groups of blood erythrocyte suspensions and allowed to stand for 20 min. at room temperature. Then spinned for 3 min. gently at 1000 rpm. After centrifugation the tubes were gently shaken, the presence or absence of haemagglutinating activity was reported as below.

- No clumping, pellet dispersed easily
+ Clumping, pellet partially dispersed
++ Clumping, no dispersion of pellet

Analysis were performed independently twice.

3.11 Statistical analysis

Proximate analysis, protein fractionation, mineral analysis and antinutritional factors like total free phenolics, tannins and L-DOPA were estimated on triplicate determinations. Estimates of mean and standard error for the above stated parameters were calculated with help of a calculator.

Mean was calculated by using the formula.

\[
\bar{X} = \frac{\sum x}{N}
\]

where

\[
\bar{X} = \text{Mean}
\]
\[
\Sigma = \text{Summation}
\]
\[ x = \text{Observations} \]
\[ N = \text{Number of observations} \]

Standard error was calculated by using the formula.

\[ \text{S.E.} = \frac{SD}{\sqrt{N}} \]

where

\[ S.D. = \text{Estimates of Standard Deviation} \]
\[ N = \text{Number of observations}. \]