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NUTRITIONAL POTENTIAL OF FIVE ACCESSIONS OF A SOUTH INDIAN TRIBAL PULSE *Mucuna pruriens* var *utilis:* II. Investigations on total free phenolics, tannins, trypsin and chymotrypsin inhibitors, phytohaemagglutinins, and *in vitro* protein digestibility

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SUMMARY

Five different accessions of an indigenous tribal pulse, *Mucuna pruriens* var. *utilis* (three white-coloured accessions and two black-coloured accessions), were gathered from Western Ghats, South India. The levels of the anti-nutrients, total free phenolics, tannins, trypsin and chymotrypsin inhibitors, phytohaemagglutinins (lectins) along with *in vitro* protein digestibility (IVPD) in the investigated seed samples revealed that the accession "Thachenmalai (black)" contained the highest levels of all the investigated anti-nutrients. Lower levels of phytohaemagglutinating activity for human erythrocytes of 'O' blood group than for 'A' and 'B' blood groups were found in all accessions. *In vitro* protein digestibility (IVPD) ranged between 72.41 and 76.92 %. IVPD of both the black-coloured accessions (Thachenmalai and Valanad) registered lower values of 72.41 and 72.86 %, respectively.

Key words: *Mucuna* beans, accessions, anti-nutrients, phenols, tannins, IVPD, Western Ghats, South India.

INTRODUCTION

As reviewed in Part I of this series by Janardhanan *et al*. (this volume), *Mucuna* is used as a source of medicine and food in several regions of South Asia. Several of its accessions have been characterized by researchers and have been shown to contain diverse anti-nutritional factors. Part I characterized the content of L-Dopa, phytic acid, and oligosaccharides in five accessions collected in Western Ghats, South India. Other common anti-nutrients in beans are protease inhibitors, lectins, phenols and tannins. These anti-nutrients have important physiological effects:

- *Protease inhibitors:* The presence of protease inhibitors in the diet leads to the formation of irreversible trypsin enzyme-trypsin inhibitor complexes, causing a decrease in trypsin in the intestine and decrease in the digestibility of dietary protein, thus leading to slower animal growth. As a result, the secretory activity of the pancreas increases, which could cause pancreatic hypertrophy and hyperplasia (Liener, 1994).

- *Lectins (Heamagglutinins):* Lectins are sugar-binding proteins derived from plants which do not change chemically the recognized carbohydrate structures and do not exert antibody function. Lectins show specificity for terminal and/or subterminal carbohydrate residues and have at least two sugar-combining sites. Therefore, lectins will specifically recognize and bind to carbohydrate residues on the cell surface (Sharon and Lis, 1989). Phytolectins are toxic factors which interact with glycoprotein on the surface of the red blood cells and cause agglutination (Grant *et al.,* 1983).

- *Phenols and tannins:* Tannins may be broadly defined as polyphenolic substances having a molecular weight greater than 500 kd (Liener, 1994). Phenols are known to decrease the digestibility of proteins, carbohydrates, and minerals. In addition, they may lower the activity of digestive enzymes and may cause damage to the mucosa of the digestive tract (Bressani *et al.,* 1982; Deshpande and Salunkhe, 1982; Mangan, 1988; Salunkhe *et al.,* 1990; Garcia-Lopez *et al.,* 1990; Liener, 1994). Most of the tannins are located in the seed coat, with only traces in cotyledons (Ravindran and Ravindran, 1988; Mary Josephine and Janardhanan, 1992). Condensed tannins appear to bind proteins very strongly and decrease *in vitro* protein digestibility in cowpea (Laurena *et al.,* 1984; Sathe and Salunkhe, 1984).

Additionally, an important determinant of the nutritional quality of pulses is the digestibility of proteins (Reddy and Gowramma, 1987). Digestibility is one of the most important factors influencing the bioavailability of amino acids. Hence, it is important to assess the grain legumes for digestibility (Liener, 1962; 1973).

This study characterized the content of protease inhibitors, lectins, phenols and tannins in the five accessions of *Mucuna pruriens* var *utilis*, collected in Western Ghats. Additionally, the *in vitro* digestibility of these accessions was determined.

MATERIALS AND METHODS

Background information on the four accessions is given in Part I of this series (Janardhanan *et al*., this volume) which also includes information on the preparation of the samples to be analyzed.

Extraction and estimation of total free phenolics and tannins

Total free phenolics were extracted by the method of Maxon and Rooney, (1972). One gram of air-dried seed flour was placed in a 100 mL flask, with 50 mL of 1 % (v/v) HCl in methanol. The samples were shaken on a reciprocating shaker for 24 hours 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.

The extracted free phenolics were estimated following the method of Sadasivam and Manickam (1992). One mL aliquots of the above extract were pipetted into different test tubes to which 1 mL of folin-phenol reagent and 2 mL of 20 % (w/v) Na_2CO_3 solution were added. The tubes were shaken and placed in a boiling water bath for exactly 1 min and then 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 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 values of triplicate estimations were expressed as g 100 g^{-1} of the seed flour on a dry weight basis.

The tannin content of seed samples of *Mucuna* beans was estimated by the method of 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 500 nm with a Spectronic 20 D spectrophotometer. The average values of triplicate estimations of all samples were expressed as g 100 g^{-1} seed flour on dry weight basis.

Measurement of trypsin inhibitor activity

The trypsin inhibitor activity was measured indirectly by inhibiting the activity of trypsin (Sadasivam and Manickam, 1992). A synthetic substrate, benzoyl-DL-arginine-paranitranilide (BAPNA), was subjected to hydrolysis by trypsin to produce yellow-coloured p-nitoanilide. The degree of inhibition by the extract (yellow colour production) was measured at 410 nm. Thirty percent glacial acetic acid (v/v) was used for trypsin source; 6.25 mg of lyophilized trypsin was dissolved and made up to 25 mL with 0.001 M HCl. Two mL of this solution was diluted to 25 mL for assay; 40 mg of BAPNA was completely dissolved in 0.5 mL of dimethyl sulphoxide and made up to 100 mL with HCl buffer, pH 8.2 and used as substrat. Tris-HCl buffer at pH 8.2 was prepared by dissolving 6.05 g of tris (hydroxymethyl aminomethane) and 2.94 g of $CaCl_2H_2O$ in 900 mL of distilled water and the pH was adjusted with dilute HCl and made up to 1000 mL with distilled water.

A 0.5 g of sample was extracted in 25 mL of water by grinding in a pre-chilled mortar and pestle. The ground sample was extracted in a refrigerator for 2-3 hours with occasional shaking for complete extraction of TI. The homogenate was centrifuged at 12, 000 rpm for 20 min. at 4-6 °C. One mL of the supernatant was diluted to 10 mL with distilled H_2O and used as the TI source. The consequent procedure included the following steps:

1. Zero to one mL of the extract in duplicate sets was pipetted into test tubes, one to serve as endogenous (E) and the other as test (T).
2. The volume was made up to 2 mL with buffer in the endogenous set.
3. The volume was made up to 1 mL in the test set.
4. One mL of trypsin solution (20 μg) was added to each tube in the test set. Into a separate test tube, 1 mL of buffer and 1 mL of trypsin solution were pipetted to serve as control.
5. All the tubes were incubated in a water bath at 37°C.
6. After a few minutes, 20 mL of substrate (1 mg BAPNA) was added to each tube.
7. The reaction was allowed to proceed for 30 min at 37°C.
8. The reaction was stopped by adding 0.5 mL of 30% glacial acetic acid.
9. The absorbance was read at 410 nm in a spectrophotometer.
10. The protein content was determined in the extract by the method of Lowry *et al.* (1951).

The absorbance was plotted against the volume of extract. The aliquot size of the extract required to inhibit 50 % of the trypsin activity was determined (S/2). That aliquot size was considered to be one unit of trypsin inhibitor. One unit of activity corresponds to that amount of TI in μg protein, which gives 50 % inhibition of enzyme activity under experimental conditions. The TI activity was expressed as trypsin

inhibitor units (TIU) per mg protein (the dilutions of TI source were made in such a way that 0.5 mL produced 50 % inhibition).

Chymotrypsin inhibitor activity

Inhibition of the esterolytic activity of chymotrypsin was determined by mixing acetyl-L-tyrosine ethyl ester, 0.01 M in a reaction mixture at pH 8.1, 25 °C in UV/Visible spectrophotometer (Smirnoff *et al.,* 1976). The reaction mixture was composed of 30 µL of enzyme solution (containing 3µg of chymotrypsin), plus 5-20 µL aliquot of 0.1 % solution of inhibitor, which was preincubated for 5 min. at room temperature in 1 mL of 46 mM - Tris-HCl_2 buffer 8.1. The reaction was started by the addition of 100 µl of the enzyme plus inhibitor solution to 3 mL of the substrate solution. Inhibition of esterolysis was expressed in inhibition units, which were defined as µmol of substrate hydrolysed by 1 mg of enzyme per minute of reaction. Protein content was estimated by E 280 measurements. A protein solution giving EI Cm = 1.00 was defined as possessing one absorbance unit mL^{-1}. Specific inhibitory activity was defined as the number of chymotrypsin inhibition units per absorbance unit.

Quantitative determination of phytoheamagglutinating (lectin) activity

Lectin activity was determined by the method of Almedia *et al*. (1991). One g of air-dried seed flour was stirred with 10 mL of 0.15N sodium chloride solution for 2 hours and the pH was adjusted to 4.0. The contents were centrifuged at 10,000 * g for 20 min. and the supernatants were collected separately. The protein content was estimated by the Lowry *et al.* (1951) method. Human blood (blood groups A, B and O) was procured from the blood bank of Ray Vijay Clinical Laboratory, Coimbatore.

Blood erythrocyte suspensions were prepared by washing the blood samples separately with phosphate-buffered saline and centrifuged for 3 min at low speed. Supernatants were removed with Pasteur pipettes. The washing procedure was repeated three times. The washed cells were diluted by one drop of cells with 24 drops of phosphate-buffered saline.

The determination of lectin was done by the method of Tan *et al*. (1983). Clear supernatant (50µl) was poured into the depression (pit) on a microtitration plate and serially diluted 1:2 with normal saline. The human blood erythrocyte (A, B and O blood groups) suspensions (25µl) were added to each of the twenty depressions. The plates were incubated for 3 hours at room temperature. After the incubation period, the titer values were recorded. One heamagglutinating unit (HU) is defined as the least amount of heamagglutinin that will produce positive evidence of agglutination of 25µl of a blood group erythrocyte after 3 hours incubation at room temperature. The phytoheamagglutinating activity was expressed as heamagglutinating units (HU)/mg protein.

***In vitro* protein digestibility (IVPD)**

The IVPD of seed samples was measured according to the multienzyme technique (Satterlee *et al.,* 1979). Samples containing 62.2 mg of protein were suspended in 10 mL of distilled water, and the pH was adjusted to 8.0 with 0.1 N HCl or NaOH. Samples were stirred in a 37 °C water bath for 15 min. The multienzyme solution, consisting of 1.6 mg of trypsin, 3.1 mg of chymotrypsin and 1.3 mg of peptidase mL^{-1}, was maintained in an ice bath and adjusted to pH 8.0 with 0.1 N HCl (or NaOH). One milliliter of the above said multienzyme solution was added to the protein suspension while stirring at a constant temperature of 37 °C. After exactly 10 min from the time of the addition of the three enzyme solutions (still stirring) 1 mL of bacterial protease (type xiv *Streptomyses grieses*) solution (7.95 mg of enzyme mL of H_2O) was added to the sample. Immediately, the solution was transferred to a 55 °C water bath. Nine minutes after the bacterial protease solution had been added to the sample, the sample was transferred to a 37 °C water bath. Ten minutes after the addition of the bacterial protease (in total 19 min after addition of the three enzyme solution), the pH of the enzyme hydrolysate was recorded and the *in vitro* protein digestibility of the sample was calculated by using the following regression equation:

$$y = 234.84 - 22.56x,$$

where: y is the present protein digestibility and x is the pH of the protein suspension after 30 min digestion with four enzyme solutions.

Statistical analysis

Anti-nutritional factors, total free phenolics and tannins were estimated on triplicate determinations. Estimates of mean and standard error for the aforesaid parameters were calculated.

RESULTS AND DISCUSSION

Total free phenolics occurred in the range of 3.26 - 4.88%, but tannins ranged from 0.16 to 0.24% (Table 1). The content of both total free phenolics and tannins was higher in the black-coloured accessions (4.88 and 0.24%, respectively) than in the white-coloured accessions. These values are equal to those reported by Vadivel and Janardhanan (2000) but higher than those reported in a recent report by Siddhuraju *et al.* (2000). The values of tannins are found to be lower than those

of earlier reports with the *Mucuna* beans (Vijayakumari *et al.*, 1996; Siddhuraju *et al*., 1996).

The trypsin and chymotrypsin inhibitor activities of both white-coloured and black-coloured accessions of *Mucuna* beans are found to range from 45.2 to 49.6 units mg^{-1} of protein and 26.2-30.1 units mg^{-1} of protein, respectively. Thachenmalai black-coloured accession exhibits the highest values both for trypsin and chymotrypsin inhibitor activities. These values are found to be equal to those in an earlier report (Vadivel and Janardhanan, 2000) and higher compared to those in a recent study in two varieties of *Mucuna* beans (Siddhuraju and Becker, 2001) and certain cultivars of *Cajanus cajan* (Mulimani and Paramjyothi, 1995). Nonetheless, both trypsin and chymotrypsin inhibitors are heat labile and can easily be eliminated by ordinary cooking.

Regarding phytohaemagglutinating activity, Thachenmalai and Kailasanadu (white-coloured) accessions register higher haemagglutinating activity with respect to 'A' blood group of human erythrocytes. All accessions have low levels of phyto-haemagglutinating activity with respect to erythrocytes of 'O' blood group. This is in good agreement with earlier reports in the same species (Vijayakumari *et al.,* 1996; Siddhuraju *et al.,* 1996). The *in vitro* protein digestibility of velvet beans observed in the present study (Table 2) is much less than that reported for casein (99.7%) (Acton *et al*., 1982). Among the five accessions of *Mucuna* beans, Thachenmalai white-coloured accession exhibits slightly higher level of *in vitro* protein digestibility compared to the other accessions of the same species. These values appear to be slightly higher than that of a recent study in *Mucuna* beans (Siddhuraju and Becker, 2001). The IVPD values of the present study are comparable with the values of some of the conventional pulses like *Vigna umbellata, Dolichos biflorus,* and *Glycine max*, but lower than *Vigna radiata* and higher compared to *Vicia faba* (Table 3).

Table 1. Content of total free phenolics, tannin, TIU, CIU and phytoheamagglutinating activity in raw seeds of accessions of *Mucuna* bean. The data are means and standard errors of triplicate determinations.

Component	Thachenmalai (black)	Thachenmalai (white)	Mundandurai (white)	Kailasanadu (white)	Valanad (black)
Total free phenolics (%)	4.88 ± 0.02	3.58 ± .0.04	3.69 ± 0.03	3.26 ± 0.02	3.88 ± 0.04
Tannins (%)	0.24 ± 0.01	0.16 ± 0.03	0.19 ± 0.03	0.14 ± 0.02	0.22 ± 0.02
Trypsin inhibitor activity (TIU / mg protein)	49.60	45.20	45.40	46.10	48.20
Chymotrypsin inhibitor Activity (CIU/mg protein)	30.1	27.1	26.2	26.2	28.7
HU (mg/ protein erythrocytes from human blood group):					
A	160	156	160	156	162
B	80	76	78	74	80
O	12	10	10	9	14

Table 2. *In vitro* protein digestibility (IVPD) of raw seeds of five accessions of *Mucuna* bean. Values are expressed on percentage basis.

Component	Thachenmalai (black)	Thachenmalai (white)	Mundandurai (white)	Kailasanadu (white)	Valanad (black)
IVPD	72.41	76.92	74.88	75.81	72.86

Table 3. *In vitro* protein digestibility (IVPD) of some conventional pulses.

Legume	IVPD (%)	Reference
Dolichos biflorus	71	Rajyalakshmi and Geervani (1990)
Phaseolus lunatus	70 - 71	Laurena *et al.* (1991)
Vigna radiata	80.05 - 85.33	Reddy and Gowramma (1987)
Vigna aconitifolia	71	Satwadhar *et al.* (1981)
Vicia faba	57.20 - 72.07	Moneam (1990)
Glycine max	71.10	Gross (1982)
Vigna umbellata	73.48 - 74.30	Laurena *et al.* (1991)

CONCLUSION

All the investigated five accessions of *Mucuna* beans exhibited variations in the levels of total free phenolics, tannins, trypsin and chymotrypsin inhibitors and phytoheamagglutinins. Except for phenolics and tannins, these anti-nutrients can be inactivated by moist heat treatment. *In vitro* protein digestibility of these accessions is comparable to that of such conventional pulses as soybean, *Vigna umbellata* and *Phaseolus lunatus.*

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