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Effect of processing on antinutrients and *in vitro* protein digestibility of the underutilized legume, *Vigna unguiculata* (L.) Walp subsp. *unguiculata*

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ABSTRACT

The effects of hydration, cooking, autoclaving, germination and their combination on the reduction/elimination of antinutrients and the improvement of *in vitro* protein digestibility of the tribal pulse, *Vigna unguiculata* subsp. *unguiculata* were investigated. Hydration and germination processes were less effective in reducing L-Dopa and trypsin inhibitor activity. Cooking and autoclaving of presoaked seeds drastically reduced the content of total free phenolics, tannins, phytic acid, hydrogen cyanide, trypsin inhibitors and oligosaccharides. Germination (96 h) process reduced the total free phenolics, tannins, hydrogen cyanide and phytic acid. The combination of germination followed by autoclaving completely eliminated the total free phenolics, tannins, hydrogen cyanide, phytic acid, trypsin inhibitor, oligosaccharides and phytoheamagglutinating activity. The combination process (germination 96 h + autoclaving) improved the *in vitro* protein digestibility. We concluded that, a prolonged period of germination followed by autoclaving completely destroyed some of the antinutritional factors.

1. Introduction

In India, legumes constitute an important food stuff and are chief economic sources of proteins in the diets of economically weaker sections of population. Now-a-days, research is being geared up, to exploit the protein source from underutilized grain legume seeds. Underutilized species (both plant and animal) are those with a potential, not yet fully exploited to contribute to food security and poverty alleviation (Bhat & Karim, 2009). The underutilized legumes/wild tribal pulses have tremendous potential for commercial exploitation but remain ignored. They offer a good scope to meet the ever-increasing demands for vegetable protein. Although they have high protein content and possess good nutritional value, their utilization is limited by the presence of some antinutritional/antiphysiological/toxic substances.

Nutritive value is the ability of food to provide a usable form of nutrients, proteins, carbohydrates, vitamins and minerals. The food processing methods including soaking, germination, decortication, fermentation and cooking greatly influence their nutritive values. Of these, cooking and germination play an important role, as they influence the bioavailability and utilization of nutrients and also improve palatability which incidentally may result in enhancing the digestibility and nutritive value of the pulses (Bakr, 1996; Oboh et al., 2000; Ramakrishna, Rani, & Rao, 2006). The presence of antinutritional factors in legumes is shown to be reduced at varying degrees based upon the food preparation method involved and the properties exhibited by various types of legumes. Therefore, more information is needed about the potential nutritional implications of legume based diets. Though extensive information is available on the nutritive value of many common legumes, the information available on the nutritive value of underutilized legumes is limited.

Vigna unguiculata subsp. *unguiculata* is a lesser-known legume, which has not received due attention by biochemists and nutritionists. Therefore, the present study was undertaken to study the changes of antinutritional factors like total free phenolics, tannins, L-Dopa, phytic acid, hydrogen cyanide, trypsin inhibitor activity, oligosaccharides and phytoheamagglutinating activity of processed and unprocessed *V. unguiculata* subsp. *unguiculata*. The efficiency of the different processing treatments individually and in combination to suppress the adverse effects of the antinutritional compounds in *V. unguiculata* subsp. *unguiculata* was also assessed.

2. Materials and methods

2.1. Samples

The seeds of *V. unguiculata* subsp. *unguiculata* were collected in wild conditions during the month of August 2011 from Anakodi, Krishnagiri district, Eastern Ghats, Tamil Nadu. Soon after collection

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the seeds were sun-dried for two days. After removal of immature seeds and unwanted materials, the seeds were stored in plastic containers at room temperature (25 °C).

2.2. Processing methods

The processing methods used for the reduction/elimination of antinutritional factors were soaking (hydration), germination, cooking, autoclaving and their combinations.

2.2.1. Soaking (hydration)

Whole seeds were soaked in distilled water and 0.05 g/100 mL sodium bicarbonate ($NaHCO_3$) solution (pH 8.6) for 12 h (overnight) at seed to water ratio of 1:10 g:mL. The water was drained off and then the seeds were dried at 55 °C in hot air oven.

2.2.2. Germination

The seeds were sterilized with (0.1 g/100 mL) mercuric chloride and washed, and then were soaked in distilled water for 12 h, the soaked seeds were placed on double layer of moist filter paper in sterile petridishes and incubated at 30 °C and allowed to germinate for 24, 48, 72 and 96 h. The seeds were moistened with distilled water at regular intervals of 12 h. The sprouts were rinsed with distilled water and freeze-dried.

2.2.3. Cooking

Separate batches of seeds were soaked in distilled water and 0.05 g/100 mL sodium bicarbonate solution (1:10 g:mL) for 12 h at room temperature (25 °C). The water was drained off, and then the seeds were cooked in distilled water (100 °C) in the ratio of 1:10 g:mL for 30 min. Similarly, unsoaked seeds were also cooked using seed to water ratio of 1:10 g:mL at 100 °C for 30 min. Cooked seeds were rinsed and dried at 55 °C in hot air oven.

2.2.4. Autoclaving

Distilled water soaked seeds (overnight) and germinated seeds (24, 48, 72 and 96 h) were autoclaved at 103.4 kPa pressure for 30 min. Similar procedure were used for unsoaked seeds and then autoclaved for 30 min. Subsequently, the seeds were rinsed with distilled water and dried at 55 °C in hot air oven.

2.3. Preparation of seed flour

About 50 g of both processed and unprocessed (raw) seed samples were powdered in a Wiley mill (Scientific Equipment Works, New Delhi, India) to 60 mesh size (0.250 mm). To avoid mixing up of samples, care was taken to clean the Wiley mill thoroughly after powdering a particular sample and before starting to powder a new sample. The powdered samples were stored in screw-cap bottles until further use.

2.4. Biochemical analysis

2.4.1. Analysis of antinutritional compounds

The antinutritional compounds, total free phenolics (Bray & Thorne, 1954), tannins (Burns, 1971), the non-protein amino acid, L-Dopa (3, 4-dihydroxyphenylalanine) (Brain, 1976), phytic acid (Wheeler & Ferrel, 1971) and hydrogen cyanide (Jackson, 1967) were quantified. Trypsin inhibitor activity was determined by the enzyme assay of Kakade, Rackis, Ghce, and Puski (1974) using benzoil-DL-arginin-*p*-nitroanilide (BAPNA) as a substrate. One trypsin inhibitor unit (TIU) has been expressed as an increase of 0.01 Absorbance Units (AU)/10 mL of reaction mixture at 410 nm. Trypsin inhibitor activity has been defined in terms of trypsin units inhibited per mg protein.

2.4.2. Extraction, thin layer chromatography separation and estimation of oligosaccharides

Extraction of TLC separation and estimation of oligosaccharides were done following the method of Somiari and Balogh (1993) and Tanaka, Thanankul, Lee, and Chichester (1975).

2.4.3. Quantitative determination of phytohaemagglutinating (lectin) activity

Lectin activity was determined by the method of Almedia, Calderon de la Barca, and Valencia (1991) and Tan, Rahim, Khor, and Wong (1983).

2.4.4. Determination of in vitro protein digestibility (IVPD)

In vitro protein digestibility (IVPD) of unprocessed and processed seed samples was determined using the multi-enzyme techniques (Hsu, Vavak, Satterlee, & Miller, 1977).

2.5. Statistical analysis

The above said data were estimated on triplicate determinations. Analysis of variance (ANOVA) and Paired samples –'*t*' test were used for analysis (SPSS software for windows release 17.0; SPSS/Inc., Chicago IL, USA) of any significant difference in chemical compositions among the various processed legumes. Significance was accepted at $p < 0.05$ and $p < 0.01$.

3. Results and discussion

The influence of hydration, germination, cooking, autoclaving and their combinations on the levels of certain antinutrients (total free phenolics, tannins, L-Dopa, hydrogen cyanide, phytic acid, trypsin inhibitor activity, oligosaccharides and lectins) present in the seeds of *V. unguiculata* subsp. *unguiculata* and *in vitro* protein digestibility was studied.

3.1. Effect of hydration

The levels of total free phenolics, tannins, L-Dopa, hydrogen cyanide, phytic acid, trypsin inhibitor activity, oligosaccharides and lectins in the seed sample have been affected by hydration in distilled water and sodium bicarbonate solution as shown in Tables 1–4. Losses of total free phenolics and tannins were 27 g/100 g and 26 g/100 g respectively in water soaked seeds and 29 g/100 g each in sodium bicarbonate soaked seeds. During the hydration process, levels of total free phenolics and tannins were significantly ($p < 0.01$) reduced. The percentage loss of total free phenolics and tannins was little higher with sodium bicarbonate solution when compared to distilled water hydration. Several possible reasons have been suggested for reductions in polyphenol and tannin concentrations due to soaking. Losses may result simply from leaching into the soak water (Deshpande, Sathe, Salunkhe, & Cornforth, 1982; Igbedioh, Shaire, & Aderiye, 1995; Khandelwal, Udipi, & Ghugre, 2010). Losses may also be attributed to decreases in extractability, as lower molecular weight phenolic compounds polymerize, thus becoming insoluble in water (Deshpande et al., 1982). Bravo (1998) and Saharan, Khetarpaul, and Bishnoi (2002) have attributed the losses to binding of polyphenols with other organic substances such as carbohydrates or protein. Alternatively, during the period of soaking, the enzyme polyphenoloxidase may be activated, resulting in degradation and consequent losses of polyphenols (Jood, Bishnoi, & Segal, 1998; Saxena, Chadha, & Sharma, 2003). L-Dopa content was reduced by 21 g/100 g in seed samples soaked in sodium bicarbonate solution. Soaking in sodium bicarbonate solution was more effective in reducing the L-Dopa than soaking in water. This is in agreement

Table 1
Effect of different processing methods on the levels of total free phenolics, tannins and L-Dopa of *Vigna unguiculata* subsp. *unguiculata*.

Treatment	Total free phenolics (g/100 g)	Reduction (%)	Tannins (g/100 g)	Reduction (%)	L-Dopa (g/100 g)	Reduction (%)
Raw	1.21 ± 0.06	–	0.38 ± 0.05	–	2.46 ± 0.11	–
Soaking in dist H_2O for 12 h	0.88 ± 0.04[a]	27	0.28 ± 0.03[1]	26	2.00 ± 0.08[a]	19
Soaking in 0.05 g/100 mL sodium bicarbonate solution for 12 h	0.86 ± 0.03[a]	28	0.27 ± 0.03[a]	29	1.94 ± 0.03[a]	21
Germination for 24 h	0.54 ± 0.03[a–c]	55	0.26 ± 0.02[1,2]	32	2.11 ± 0.04[a]	14
Germination for 48 h	0.36 ± 0.01[a–d]	70	0.13 ± 0.01[a–d]	66	2.01 ± 0.03[a,d]	18
Germination for 72 h	0.34 ± 0.02[a–d,5]	72	0.11 ± 0.01[a–d]	71	1.82 ± 0.01[a,2,c–e]	26
Germination for 96 h	0.32 ± 0.01[a–d,6]	74	0.09 ± 0.01[a–d]	76	1.74 ± 0.04 [a–c,e,f]	29
Cooking of unsoaked seeds	0.84 ± 0.04[a,c]	31	0.24 ± 0.02[a–c]	37	1.84 ± 0.02[a–e]	25
Soaking in dist H_2O for 12 h followed by cooking (30 min)	0.38 ± 0.04[a–d]	68	0.08 ± 0.01[a–d,h]	79	1.73 ± 0.04 [a–c,e,f,h]	29
Soaking in 0.05 g/100mL sodium bicarbonate for 12 h followed by cooking (30 min)	0.37 ± 0.03[a–c,h]	69	0.09 ± 0.01[a–d,h]	76	1.72 ± 0.03[a,b,d,f,7,h]	30
Autoclaving unsoaked seeds	0.35 ± 0.04[a–d,10]	71	0.07 ± 0.01[a–d,h]	82	1.62 ± 0.02 [a–g,i,j]	34
Soaking in dist H_2O for 12 h followed by autoclaving	0.34 ± 0.03[a–c,h,i]	12	0.06 ± 0.01 [a–d,h]	84	1.51 ± 0.04 [a–c,e,f,h,j,k]	39
Germination for 24 h + autoclaving	0.18 ± 0.01[a–d,f,h–l]	85	0.05 ± 0.01 [a–d]	87	1.42 ± 0.08[a,d,e,f,h,j,k]	42
Germination for 48 h + autoclaving	0.06 ± 0.02[a–e,g–m]	95	ND	100	1.36 ± 0.04 [a–c,e,f,h,j,k]	45
Germination for 72 h + autoclaving	ND	100	ND	100	1.21 ± 0.06 [a–n]	51
Germination for 96 h + autoclaving	ND	100	ND	100	1.10 ± 0.07 [a–o]	55

[a ,1]– Significance at $p < 0.01$ $p<0.05$% between treated & untreated. [b–o] – Significance at $p < 0.01$ within treated,[2–15] – Significance at $p < 0.05$ within treated, ND– Not determined.

with the results of Vijayakumari, Siddhuraju, and Janardhanan (1996) and Siddhuraju and Becker (2001).

The content of HCN level in the raw seeds of *V. unguiculata* subsp. *unguiculata* is far below the lethal level, i.e. 36 mg/100 g (Oke, 1969). Significant reduction of HCN content has been observed when subjected to both distilled water as well as sodium bicarbonate solution soaking. Endogenous and autolytic enzymes were inactive, but they were activated by hydrolysis (Panasuik & Bill, 1984). The HCN produced during hydrolysis is water soluble and this accounts for the decrease in cyanide content during soaking. Similar results have been obtained earlier in *V. unguiculata*, *Phaseolus aureus* (Okolie & Ugochukwu, 1989) and *Dolichos lablab* var. *vulgaris* (Vijayakumari, Siddhuraju, & Janardhanan, 1995).

Hydration process significantly ($p < 0.01$) reduced the phytic acid content of presently investigated *V. unguiculata* subsp. *unguiculata* seed. The percentage loss of phytic acid is higher with distilled water hydration compared to salt water hydration. This agrees with previous research conducted with white variety of *Cicer arietinum* (Khan, Zaman, & Elahi, 1988) and *Phaseolus vulgaris* (Shimelis & Rakshit, 2007). Hydration induced reduction in phytate content in legumes may be attributed to the activity of phytase and diffusion of the products. An increase in the phytase activity with a decrease in the level of phytate as a result of hydration in faba bean had been shown in an earlier study of Eskin and Wiebe (1983).

During hydration with distilled water and sodium bicarbonate solution, significant ($p < 0.01$) reduction had been observed in the levels of stachyose followed by verbascose contents in the presently investigated seed sample (Table 3). Upadhyay and Garcia (1988) demonstrated that, differential solubility of the individual oligosaccharides and their diffusion rates are two factors that could influence the sugar losses during hydration. Hydration in 0.05 g/100 mL sodium bicarbonate solution increased softening of the testa and cotyledons that resulted in the increase of sugar extraction. The lectins of *V. unguiculata* subsp. *unguiculata* exhibited a high level of agglutination activity specifically in 'B' group compared to other two blood groups 'A' and 'O'. The percentage loss of lectin activity in the blood group 'B' when subjected to both distilled water as well as sodium bicarbonate soaking is in line with the earlier reports in different cultivars of *C. arietinum* (Bansal, Dhindsa, & Batra, 1988), *Lens culinaris* (Batra, 1978) and *D. lablab* var. *vulgaris* (Vijayakumari et al., 1995). The incomplete destruction of haemagglutinating activity might be due to the presence of high levels of other antinutritional factors which may interfere with the lectin destruction.

Table 2
Effect of different processing methods on the levels of hydrogen cyanide, phytic acid and trypsin inhibitor activity of *Vigna unguiculata* subsp. *unguiculata*.

Treatment	Hydrogen cyanide mg 100 g^{-1}	Reduction (%)	Phytic acid mg 100 g^{-1}	Reduction (%)	Trypsin inhibitor TIU mg^{-1} protein	Reduction (%)
Raw	0.22 ± 0.02	–	398.28 ± 1.12	–	26.48 ± 0.11	–
Soaking in dist H_2O for 12 h	0.10 ± 0.01[a]	55	275.63 ± 1.08[a]	31	24.28 ± 0.08[a]	8
Soaking in 0.05 g/100 mL sodium bicarbonate solution for 12 h	0.10 ± 0.01	55	291.53 ± 0.78[a]	27	23.10 ± 0.06[a,b]	13
Germination for 24 h	0.14 ± 0.02	36	245.58 ± 0.56[a–c]	38	23.68 ± 0.14[a,b]	11
Germination for 48 h	0.12 ± 0.01[a,4]	45	82.50 ± 0.11[a–d]	79	22.04 ± 0.28 [a–d]	16
Germination for 72 h	0.08 ± 0.01[a,d]	64	56.24 ± 0.14[a–e]	86	21.94 ± 0.36[a–d]	17
Germination for 96 h	0.06 ± 0.01[a,d]	73	18.20 ± 0.08[a–f]	95	21.56 ± 0.58 [a–d,5]	19
Cooking of unsoaked seeds	0.14 ± 0.02	36	288.24 ± 1.14[a,c]	28	17.60 ± 0.36 [a–e,g]	34
Soaking in dist H_2O for 12 h followed by cooking (30 min)	0.11 ± 0.01[a,d,h]	50	128.18 ± 0.68[a–d,h]	68	15.28 ± 0.78 [a–h]	42
Soaking in 0.05 g/100 mL sodium bicarbonate for 12 h followed by cooking (30 min)	0.10 ± 0.01[a,d,h]	55	131.20 ± 0.054[a–d,h]	67	16.02 ± 0.18 [a–h]	40
Autoclaving unsoaked seeds	0.08 ± 0.01[a,d,h]	64	118.40 ± 0.36[a–d,h–j]	70	2.21 ± 0.03 [a–i]	92
Soaking in dist H_2O for 12 h followed by autoclaving	0.06 ± 0.01[a,d,h]	73	115.20 ± 1.04[a–d,h–k]	71	ND	100
Germination for 24 h + autoclaving	0.08 ± 0.01[a,d,h]	64	14.20 ± 0.09[a–l]	96	ND	100
Germination for 48 h + autoclaving	0.04 ± 0.01[a,d,h]	82	6.81 ± 0.18[a–m]	98	ND	100
Germination for 72h + autoclaving	0.02 ± 0.01[a,d,h]	91	ND	100	ND	100
Germination for 96 h + autoclaving	ND	100	ND	100	ND	100

[a, 1]– Significance at $p < 0.01$ $p < 0.05$ between treated & untreated. [b–o] – Significance at $p < 0.01$ within treated. [2–15] – Significance at $p < 0.05$ within treated, ND– Not determined.

Table 3
Effect of different processing methods on the levels of raffinose, stachyose and verbascose of *Vigna unguiculata* subsp. *unguiculata*.

Treatment	Oligosaccharides (g/100 g)					
	Raffinose	Reduction (%)	Stachyose	Reduction (%)	Verbascose	Reduction (%)
Raw	0.68 ± 0.04	–	1.94 ± 0.07	–	1.24 ± 0.06	–
Soaking in dist H_2O for 12 h	0.54 ± 0.01[a]	21	1.04 ± 0.06[a]	46	0.78 ± 0.06	37
Soaking in 0.05 g/100 mL sodium bicarbonate solution for 12 h	0.51 ± 0.02[a,b]	25	1.00 ± 0.04[a,2]	48	0.71 ± 0.0[a,b]	43
Germination for 24 h	0.42 ± 0.01[a,c]	38	0.94 ± 0.03[a–c]	52	0.58 ± 0.02[a,b,c]	53
Germination for 48 h	0.31 ± 0.01[a,c]	54	0.21 ± 0.01[a–d]	89	0.16 ± 0.01[a–d]	87
Germination for 72 h	0.24 ± 0.01[a,c]	65	0.11 ± 0.01[a–d]	94	ND	100
Germination for 96 h	ND	100	ND	100	ND	100
Cooking of unsoaked seeds	0.38 ± 0.04[b,c]	44	0.91 ± 0.06[a,c,d]	53	0.66 ± 0.04[a,b]	47
Soaking in dist H_2O for 12 h followed by cooking (30 min)	0.29 ± 0.03[a,d,h]	57	0.64 ± 0.06[a,c,d]	67	0.52 ± 0.01[a–d,h]	58
Soaking in 0.05 g/100 mL sodium bicarbonate for 12 h followed by cooking (30 min)	0.24 ± 0.01[a,c,h,9]	65	0.61 ± 0.04[a,b,d,h]	69	0.50 ± 0.01 [a–d,h]	60
Autoclaving unsoaked seeds	0.21 ± 0.01[a,c,h,i]	69	0.58 ± 0.05[a–d,h–j]	70	0.44 ± 0.04[a,b,d,i,10]	65
Soaking in dist H_2O for 12 h followed by autoclaving	0.18 ± 0.01[a,c,h,i]	74	0.44 ± 0.03[a–c,h–k]	77	0.33 ± 0.05[a–d,h–k]	73
Germination for 24 h + autoclaving	0.32 ± 0.03 [a–d,h]	53	0.71 ± 0.06[a,c,d]	63	0.16 ± 0.02[a–c,h–l]	87
Germination for 48 h + autoclaving	0.24 ± 0.01[a,c,h,9,m]	65	0.11 ± 0.01[a–d,h–m]	94	0.08 ± 0.01[a–d,h,k–m]	94
Germination for 72 h + autoclaving	0.08 ± 0.01[a,c,h,i,m]	88	ND	100	ND	100
Germination for 96 h + autoclaving	ND	100	ND	100	ND	100

[a, 1]– Significance at $p < 0.01$ $p < 0.05$ between treated & untreated. [b–o] – Significance at $p < 0.01$ within treated. [2–15] – Significance at $p < 0.05$ within treated, ND– Not determined.

3.2. Effect of germination

The results in Table 1 show that, germination process causes a reduction in total free phenolic compounds which ranged from 55 g/100 g after 24 h of germination to 74 g/100 g after 96 h of germination in *V. unguiculata* subsp. *unguiculata*. Longer period of germination caused significant greater losses in total free phenolic compounds. A decrease in polyphenol content was observed by Giami, Akusu, and Emelike (2001) for germinated cowpea, Indian pulses by Khandelwal et al. (2010), soybean, mung bean and kidney bean by Mohamed, Gibriet, Ramy, Abu-Salem, and Abou-Arab (2011). The reduction of total free phenolic compounds during germination may be attributed to the presence of polyphenoloxidase and enzymatic hydrolysis (Rao & Deosthale, 1982). A significant reduction was observed in the tannin content of *V. unguiculata* subsp. *unguiculata* due to germination, the levels of reduction were 32, 66, 71 and 76 g/100 g after 24, 48, 72 and 96 h germination respectively. Decreases in the tannin content of pulses following germination have been reported by several authors (Khandelwal et al., 2010; Sangronis & Machado, 2007). These decreases may be attributed to the increased activity of polyphenoloxidase and other catabolic enzymes. During germination, enzymes are activated, resulting in the hydrolysis of various components, including carbohydrates, protein, fibre and lipid as well as phenolic compounds (Deshpande, Cheryan, & Salunkhe, 1986). Maximum reduction in the content of L-Dopa was noticed in the seed germinated for 96 h and the reduction in the content was 29 g/100 g. This reduction in L-Dopa was comparable to *Mucuna pruriens* (Mugendi et al., 2010). In general, as the period of germination increased there was a concomitant decrease in the level of L-Dopa in the present study.

Table 4
Effect of different processing methods on the levels of phytohaemagglutinating activity of *Vigna unguiculata* subsp. *unguiculata*.

Treatment	Phytoheamagglutinating activity (HU mg^{-1} protein)					
	A	Reduction (%)	B	Reduction (%)	O	Reduction (%)
Raw	38	–	148	–	16	–
Soaking in dist H_2O for 12 h	34	11	70	53	14	13
Soaking in 0.05 g/100 mL sodium bicarbonate solution for 12 h	32	16	70	53	12	25
Germination for 24 h	31	18	65	56	11	31
Germination for 48 h	30	21	63	57	11	31
Germination for 72 h	29	24	60	59	10	38
Germination for 96 h	28	26	60	59	10	38
Cooking of unsoaked seeds	6	84	24	84	3	81
Soaking in dist H_2O for 12 h followed by cooking (30 min)	4	89	20	86	2	87
Soaking in 0.05 g/100 mL sodium bicarbonate for 12 h followed by cooking (30 min)	4	89	19	87	ND	100
Autoclaving unsoaked seeds	ND	100	6	96		100
Soaking in dist H_2O for 12 h followed by autoclaving	ND	100	ND	100	ND	100
Germination for 24 h + autoclaving	ND	100	ND	100	ND	100
Germination for 48 h + autoclaving	ND	100	ND	100	ND	100
Germination for 72 h + autoclaving	ND	100	ND	100	ND	100
Germination for 96 h + autoclaving	ND	100	ND	100	ND	100

[a, 1]– Significance at $p < 0.01$, $p < 0.05$ between treated & untreated. [b–o] – Significance at $p < 0.01$ within treated. [2–15] – Significance at $p < 0.05$ within treated, ND– Not determined.

During the 96 h germination period, phytic acid content of *V. unguiculata* subsp. *unguiculata* showed decrement from 398.28 mg 100 g^{-1} to 18.20 mg 100 g^{-1} (Table 2). Earlier reports on the effects of germination on phytic acid in beans indicated that, as a result of increased phytase enzyme activity, more of the phytic acid is hydrolysed during germination (Deshpande, 1985). Similarly, Shimelis and Rakshit (2007) also reported that, a notable reduction (over 70 g/100 g) in the phytic acid content of *P. vulgaris* varieties after 96 h of germination was observed. The reduction (over 75 g/100 g) of phytic acid in *V. unguiculata* subsp. *unguiculata* indicated that, an increase in hydrolysis of phytates during germination led to the liberation of inorganic phosphates for plant growth from organic phosphorus containing compound (phytate). The breakdown of phytate during germination is attributed to the increased activity of the endogenous phytase (enzyme activity). Since phytic acid has been considered to be one of the factors responsible for

reducing minerals bioavailability, its reduction during germination may have enhanced the nutritional quality of beans.

During germination process, levels of oligosaccharides, raffinose, stachyose and verbascose were significantly ($p < 0.01$) reduced. The highest reduction in raffinose, stachyose and verbascose concentrations reaching its undetectable level was obtained at the end of 96 h of germination. This reduction in oligosaccharides was comparable to its maximum reduction in black eye bean and pink bean samples (Labaneiah & Luh, 1981) and Roba, Awash and Beshbesh varieties of *P. vulgaris* during 96 h germination (Shimelis & Rakshit, 2007). However, it was higher as compared with navy beans (Snauwaret & Markakis, 1976) at 96 h germination. These differences can be attributed to the origin and variety differences as well as the distinction in the levels of endogenous α-galactosidase activity in different beans. Furthermore, during the 72 h germination time, maximum reduction was observed in the levels of verbascose followed by stachyose levels. This may be attributed to germination process wherein, α-galactosidase first attacks verbascose and then stachyose. A number of studies have indicated that, the content of raffinose family oligosaccharides in legumes decreases during germination due to the action of α-galactosidase, which selectively cleaves the galactose from raffinose, stachyose and verbascose leaving behind sucrose (Shimelis & Rakshit, 2007; Siddhuraju, Becker, & Makkar, 2000; Vijayakumari, Siddhuraju, & Janardhanan, 1997).

The haemagglutinating activity also exhibits a decreasing tendency towards germination. It was accounted to a loss of upto only 26, 59 and 38 g/100 g in *V. unguiculata* subsp. *unguiculata* against A, B and O blood groups respectively after 96 h germination, Gurumoorthi and Uma (2011) reported about 54, 38 and 77 g/100 g loss in haemagglutinating activity in Thachenmalai germplasm of *M. pruriens* var. *utilis* against A, B and O blood groups respectively.

3.3. Effects of cooking and autoclaving

Total free phenolic content decreased in cooking (Table 1). The order of reduction was cooking after soaking in sodium bicarbonate solution (69 g/100 g) > cooking after soaking in distilled water (68 g/100 g) > cooking unsoaked seeds (31 g/100 g). It is in agreement with the earlier reports in *P. vulgaris* (Yasmin, Zeb, Khalil, Paracha, & Khattak, 2008). It was observed that, increasing the period of cooking caused more losses in total phenolic compounds in soybean, mung bean and kidney bean (Mohamed, Gibriet, Ramy, Abu-Salem, & Abou-Arab, 2011). However, a slight increase in the reduction of total free phenolic compounds was noticed after autoclaving than the reduction in total free phenolics compounds after cooking. Similar results were obtained by Ramakrishna et al. (2006), Xu and Chang (2008a,b) for peas and Kalogeropoulos et al. (2010) for some dry legumes. Xu and Chang (2008a,b) reported that, the reduction of total free phenolic compounds during cooking is not fully understood. However, this could be attributed to chemical transformation, decomposition of phenolics and formation of phenolic–protein complex under thermal and pressure conditions.

Cooking and autoclaving processes significantly reduced tannins concentration on presoaked seeds, but their percentage reduction remained similar for these processes. The results of this study were consistent with those mentioned by Shimelis and Rakshit (2007), that cooking and autoclaving processes reduced tannins by 63–70 g/100 g and 61–75 g/100 g respectively. Khattab and Arntfield (2009) reported highest reduction of tannin content with boiling followed by autoclaving and microwave cooking in some legumes such as cowpea, pea and kidney bean. The reduction of tannins after soaking, boiling, microwave cooking and autoclaving is mainly due to the fact that, those compounds, in addition to their predominance in seed coats, are water soluble and consequently leach into the liquid medium (Kumar, Reddy, & Rao, 1979; Reddy & Pierson, 1994). This decrease could also be related to the fact that, these compounds are heat labile and degrade upon heat treatment (Rakic et al., 2007). Loss of tannins may also be due to heat degradation of tannin molecules or formation of water soluble complexes (Uzogara, Morton, & Daniel, 1990).

The results pertaining to the effect of differential cooking on the levels of L-Dopa are depicted in Table 2. The reduction in the content of L-Dopa by soaking followed by cooking is 30 g/100 g. However, soaking followed by autoclaving has reduced the contents of the same to the extent of 39 g/100 g. In general, among these five processing methods in the present study, soaking followed by autoclaving appears to be more efficient than cooking in reduction of the content of L-Dopa. Similar reduction in L-Dopa content is noticed in seven accessions of *M. pruriens* var. *utiilis* (Gurumoorthi, Janardhanan, & Myhrman, 2008).

Except unsoaked seed cooking, all other cooking and autoclaving processing techniques significantly ($p < 0.01$) reduced the hydrogen cyanide contents. The highest reduction (73 g/100 g) was achieved with autoclaving after soaking in distilled water. Vijayakumari et al. (1995) noted 87 g/100 g loss in hydrogen cyanide content of Indian tribal pulse only with autoclaving treatment, whereas, reduction with other treatments like water and $NaHCO_3$ solution etc. were less. Cooking is a safe method for the elimination of toxicity in legume seeds because cooking destroys the enzyme linamarase at 72 °C (Joachim & Panditteseker, 1944) but not the glucoside. Montgomery (1980) has reported that, most of the liberated HCN is lost by volatilization during cooking and cyanide is rapidly converted to thiocyanates or other compounds.

Furthermore, cooking significantly ($p < 0.05$) reduced the phytic acid contents in *V. unguiculata* subsp. *unguiculata*. Distilled water soaking followed by cooking and autoclaving of *V. unguiculata* subsp. *unguiculata* seeds resulted in the reduction of phytic acid content due to hydrolysis (Table 2). It was noted that, over 99 g/100 g of the total phytic acid in a water-soluble form which could serve as a means of removing or lowering phytic acid levels in *P. vulgaris* (Shimelis & Rakshit, 2007). Cooking and autoclaving processes significantly reduced phytic acid concentration on presoaked seeds. The apparent decrease in the content of phytic acid during autoclaving might be partly due to the leaching into the soaking medium or degradation of inositol hexaphosphate into pentatetraphosphate by heat under pressure (Vijayakumari, Pugalendi, & Vadivel, 2007).

Trypsin inhibitor activity was significantly ($p < 0.01$) decreased by different cooking treatments (Table 2). Reduction in the content of trypsin inhibitor by soaking followed by cooking is 42 g/100 g, on the other hand, a complete inactivation was achieved in trypsin inhibitor activity for *V. unguiculata* subsp. *unguiculata* after autoclaving the presoaked seeds at 121 °C for 30 min. Similar results were obtained by Shimelis and Rakshit (2007) in *P. vulgaris* varieties. Alajaji and El-Adawy (2006) reported that, the highest reduction was noted after autoclaving (83.67 g/100 g) followed by boiling (82.27 g/100 g) in *C. arietinum*. Khattab and Arntfield (2009) stated that, boiling, roasting, microwave cooking and autoclaving brought a total removal of trypsin inhibitor in cowpea, pea and kidney bean. The loss of trypsin inhibitor activity during cooking may be due to destroying by high temperature due to their heat sensitive nature to undetectable amounts when heating processes (cooking and autoclaving) were employed (Shimelis & Rakshit, 2007).

Amount of raffinose, stachyose and verbascose oligosaccharides were significantly ($p < 0.01$) reduced during heat processing (cooking, autoclaving) and their combinations (Table 3). Higher losses of raffinose, stachyose and verbascose were found when

soaked seeds were autoclaved. Autoclaving caused a decrease of all the investigated oligosaccharides on *V. unguiculata* subsp. *unguiculata*. These observations are in agreement with those reported by Alajaji and El-Adawy (2006) for *C. arietinum* and Shimelis and Rakshit (2007) for *P. vulgaris*. This was due to the decomposition of fructose which is a part of raffinose, stachyose and verbascose sugars that decomposes at 103–105 °C (Stecher, 1968, p. 471). Decrease in contents of raffinose, stachyose and verbascose due to cooking might be attributed to heat hydrolysis of the oligosaccharides to simple disaccharides and monosaccharides or to the formation of other compounds (Onigbinde & Akinyele, 1983).

Haemagglutinin activity was completely destroyed by unsoaked and soaked seeds were autoclaved. Khalil and Mansour (1995) reported that, boiling and autoclaving of faba bean seeds completely eliminated haemagglutinin activity. Alajaji and El-Adawy (2006) reported that, boiling and autoclaving of *C. arietinum* seeds completely eliminated haemagglutinin activity.

3.4. Combination of hydration, germination and heat processing

It was observed that, hydration and germination lead to a reduction in total phenolics, tannins, hydrogen cyanide, phytic acid, raffinose, stachyose and verbascose. The heat processing methods on the other hand lead to a reduction in heat sensitive antinutrients like lectins and trypsin inhibitors. Hence, a combination of these processes was attempted by heating the *V. unguiculata* subsp. *unguiculata* seeds just after they have germinated.

The combinations of presoaked germinated and autoclaved seeds were able to eliminate/reduce heat-stable and heat sensitive antinutrients. Phytic acid located on cotyledon of the seeds which was retained during germination processes was attacked by autoclaving process. Presoaked germinated (96 h) and autoclaved seeds were found to have reduced the L-Dopa content nearly 50 g/100 g. The combined processes significantly eliminated the antinutrients such as total free phenolics, tannins, hydrogen cyanide, phytic acid, trypsin inhibitors, raffinose, stachyose, verbascose and phytohaemagglutinating activity. The only problem with the combined process was the extended time required for the process of germination.

3.5. In vitro protein digestibility

In vitro protein digestibility of raw and processed seeds of *V. unguiculata* subsp. *unguiculata* is given in Table 5. *V. unguiculata* subsp. *unguiculata* showed significant ($p < 0.01$) increase in protein digestibility after treatments (hydration, cooking, autoclaving and germination) and thus improved its protein quality. The improvement of *in vitro* protein digestibility of *V. unguiculata* subsp. *unguiculata* in all the processed seeds may be attributed not only to the removal/reduction of antinutrients, but also to the structural disintegration of the native protein, including enzyme inhibitors and lectins, differential solubility of the individual oligosaccharides and their diffusion rates, phytase activity to breakdown phytic acid in the seeds and the development of endogenous α-galactosidase activity to diminish oligosaccharides. Raw seeds of *V. unguiculata* subsp. *unguiculata* exhibited *in vitro* protein digestibility of 71.28 g/100 g. Furthermore, protein digestibility of *V. unguiculata* subsp. *unguiculata* after processing reached 84.86 g/100 g. Autoclaving of germinated seeds had significant effects on protein digestibility of seed samples compared to that of the seeds subjected to simple processes like hydration, cooking, autoclaving and germination. From the results obtained of this study, it can be concluded that, only one processing method cannot produce desired removal of all antinutrients and required improvement in the *in vitro* protein digestibility of the seeds. Germination of presoaked seeds and autoclaving can be used to obtain enhanced protein quality of seed flour.

Table 5
Effect of different processing methods on the levels of *in vitro* protein digestibility (IVPD) of *Vigna unguiculata* subsp. *unguiculata*.

Treatment	IVPD (%)	Increment (%)
Raw	71.28 ± 0.12	–
Soaking in dist H_2O for 12 h	73.16 ± 0.28	3
Soaking in 0.05 g/100 mL sodium bicarbonate solution for 12 h	75.28 ± 0.36	5
Germination for 24 h	78.10 ± 0.18	9
Germination for 48 h	79.46 ± 0.31	11
Germination for 72 h	75.30 ± 0.40[d,e]	6
Germination for 96 h	73.54 ± 0.37[c–f]	3
Cooking of unsoaked seeds	78.12 ± 0.20[e]	10
Soaking in dist H_2O for 12 h followed by cooking (30 min)	78.48 ± 0.19[e]	10
Soaking in 0.05 g/100 mL sodium bicarbonate for 12 h followed by cooking (30 min)	78.68 ± 0.11[e]	10
Autoclaving unsoaked seeds	77.08 ± 0.39[e,h,i,j]	8
Soaking in dist H_2O for 12 h followed by autoclaving	80.12 ± 0.54	12
Germination for 24 h + autoclaving	81.26 ± 0.62[d]	14
Germination for 48 h + autoclaving	83.24 ± 0.59	17
Germination for 72 h + autoclaving	84.56 ± 0.30	19
Germination for 96 h + autoclaving	84.86 ± 0.27	19

[a, 1]– Significance at $p < 0.01$, $p < 0.05$ between treated & untreated. [b–o] – Significance at $p < 0.01$ within treated. [2–15]– Significance at $p < 0.05$ within treated, ND– Not determined.

The results obtained showed that, germination significantly reduced certain heat stable antinutritional factors such as phytic acid, tannins and oligosaccharides. On the other hand, cooking and autoclaving of presoaked seeds in water appeared as an adequate method for reducing heat sensitive antinutrients i.e. trypsin inhibitors and phytoheamagglutinating activity. Based on the results of this study, it can be concluded that, no single method can remove or eliminate most of the antinutrients and toxic factors. A combination of germination and autoclaving brought about all the desirable changes in *V. unguiculata* subsp. *unguiculata* seeds.

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