

Optimization of Process Conditions for Isolation of Leaf Protein Concentrate from *Pongamia pinnata* and its Proximate Nutritional Composition

Lutful Haque Khan and V. K. Varshney

Chemistry Division, Forest Research Institute, DehraDun-248006, India

Abstract: Substantial amount of leaves as biomass residue is produced from *Pongamia pinnata* (karanj) plantations which has no commercial value and remains underutilized. Feasibility of utilization of this biomass residue for development of protein concentrate as a food/ feed supplement was examined. A process for isolation of leaf protein concentrate (LPC) was optimized through investigating the influence of various process parameters on the yield and protein content of the LPC. The parameters optimized were ratio of fresh leaves to water (1:9), coagulation temperature (90°C) and duration (11 minutes), and pH (4.0). The optimized process was applied for isolation of LPCs from lower, middle and upper canopy of the tree, and the LPCs containing protein 43.77-44.34% were recovered in 7.27-7.34% yield. The proximate nutritional composition (moisture, 6.49-6.58%; fat, 12.54-12.82%; crude fibre, 1.84-1.86%; ash, 3.66-3.97%; carbohydrates, 39.18-39.83%; organic matter, 96.02-96.34%; total free amino acids, 0.056-0.057 % E of leucine; Gross energy, 447.29-449.77 Kcal/100g LPC; pigments, % (chlorophyll, 0.37-0.38; total carotene, 0.025-0.026; xanthophylls, 0.82-0.83); minerals, mg/100g LPC (Na, 4.42-4.72; Ca, 1070-1087; P, 746.33-754; Fe, 53.20-55.05; Cu, 12.95-13.13; Zn, 7.57-8.05; Mn, 0.37-0.38; Mg, 26.62-27.66; K, 20.68-21.05); in vitro digestibility, 82.62-82.95%; total polyphenols, 0.120-0.123%GAE; total saponins, 0.501-0.513%; total alkaloids, not detected) of these LPCs were determined. ANOVA of these data revealed no significant difference with respect to canopy. It was inferred that the *Pongamia pinnata* leaves hold potential for development of LPC as a food/ feed supplement to combat nutrition deficiency.

Keywords: Leaf protein concentrate, *Pongamia pinnata*, Leaves, In vitro digestibility, Anti-nutritional factor, Food/feed supplement.

I. Introduction

The rapid population growth in most developing countries has led to serious food crises, especially among pre-school children, pregnant or nursing mothers, etc. They are particularly prone to dietary protein, minerals and vitamins inadequacies [1]. These dietary inadequacies are resulted in kwashiorkor, marasmus, infant blindness, mortality and morbidity [2, 3]. According to World Health Organization (WHO), half of the world's population life is threatened by serious nutritional deficiencies [4]. Further, shortage of supply of good quality protein is also a serious concern of the feed producers to produce animal protein. These considerations have necessitated the search for additional sources of protein. The unconventional sources of protein which include Fish Protein Concentrate (FPC), Single Cell Protein (SCP), Soybean Protein (SBP), insect protein (IP) and leaf proteins have tremendous scope for developing low cost protein foods [5-8]. Leaf protein appears to have better utilization in the light of excessive photosynthesis and availability of abundant green lush vegetation [9].

Leaf protein concentrate (LPC) is a concentrated form of protein derived from the foliage of plants. Their protein value equals that of most animal products and sometime surpasses them also [10]. Leaf proteins have been found to have greater nutritive value than most of the pulses, resembling skim milk in the diet of infants recovering from Kwashiorkor. It also helps avert certain diseases associated with malnutrition, in particular anaemia, diarrhea, respiratory infections and nutritional blindness [4]. LPCs have been assessed as food for children [11] and included in food formulation [12]. Use of LPCs as a protein supplement in animal feed has also been demonstrated [13]. It is also combined with a variety of inexpensive foods to make culturally acceptable dishes. In recognition of the viable role of LPCs in combating protein deficiency, several studies aimed at production of protein concentrates from green leaf material have been carried out [13-17].

Trees have also been suggested as a possible source of LPC and the production of protein from tree leaves is advantageous over crops as they do not involve recurring cost of cultivation [15,18]. *Pongamia pinnata* (Karanj) is planted in agro-forestry/social-forestry system at large scale in India. Karanj plantations produce a large amount of the leaves which are simply burnt or underutilized and thus may have serious environmental impact from their disposal. If appropriate technologies for their value addition are developed, the farmers may get another income from such plantations. As a part of our ongoing research programme aiming to

develop protein concentrates from the plantation by products (leaves), feasibility of utilization of karanj leaves for production of protein concentrates as a food supplement was examined and results of the study are reported herein. To the best of our knowledge, there is no work investigating the potential of karanj leaves for production of LPC.

II. Materials And Methods

2.1 Plant Materials

Fresh leaves of *Pongamia pinnata* were collected in the early morning from the trees grown in the campus of Forest Research Institute, Dehradun, India which lies between latitudes 29°58'N and 31°2'N and longitudes 77°34'E and 78°18'E. The plant material was authenticated at Systematic Botany Discipline, Botany Division, Forest Research Institute, Dehradun, India. The leaves were analyzed for moisture content using oven dry procedure. Based on the moisture content (60.69 ± 0.32 %), the average dry matter content of the leaves was 39.31 ± 0.32 %.

2.2 Chemicals and Reagents

Citric acid, Potassium sulphate, Copper sulphate, Sulphuric acid, Bromocresol green, Methyl red, Ortho phosphoric acid, Diphenyl amine indicator, Ferrous ammonium sulphate, Ninhydrin, Stannous chloride, Sodium citrate, n-Propanol, Potassium hydroxide, Hexane, Toluene, Ammonium oxalate, Potassium permanganate, Ammonium molybdate, Potassium nitrate, Folin-Ciocalteu's Phenol reagent, Sodium carbonate, Ammonium acetate, Diethylene-triamine-penta acetic acid (DTPA), Calcium chloride, Triethanolamine and Sodium azide were purchased from Merck India. Acetic acid, Hydrochloric acid, Sodium Hydroxide, Boric acid, Petroleum ether, Acetone, Sodium sulphate, Ammonia solution Nitric acid, Chloroform and Ethyl acetate were from Rankem, India. Potassium dichromate, Methoxy ethanol, Methanol, Diethyl ether and Ethanol were supplied by Sd Fine Chem. Ltd. India. Dimethyl sulfoxide, Phenolphthalein were procured from Ranbaxy, India. Leucine, Pepsin, Trypsin, and Pancreatin were obtained from Himedia, India and Gallic acid from Sigma Chemicals Co. (St. Louis, MO, USA).

2.3 Isolation of Leaf protein concentrate (LPC)

Fresh leaves (100g) were washed with tap water, drained and minced with distilled water (1:1 to 1:11, w/v) in a locally purchased mixer followed by filtration through muslin cloth. The leaf protein from the separated juice was coagulated by 1N acetic acid at ambient temperature or by heating after adjusting the pH (acidic pH-4.0, basic pH-9.0 and bio pH-7.0) for the desired temperature (50°C to 100°C) for fixed duration (1-15 minutes). The coagulum was centrifuged at 10000 rpm for 10 minutes, washed with distilled water till neutral pH and oven dried until constant weight.

2.4 Isolation of LPCs from lower, middle and upper canopy of *Pongamia pinnata*

The LPCs (PPLPCL, PPLPCM, PPLPCU) were isolated from lower, middle and upper canopy, respectively of *Pongamia pinnata* using the optimized protocol.

2.5 Determination of proximate nutritional composition

The LPCs (PPLPCL, PPLPCM, PPLPCU) were analyzed for the contents of moisture, nitrogen, organic matter, crude protein (N X 6.25), fat, crude fiber, ash, total carbohydrates, total free amino acids, pigments (chlorophylls, total carotenes and xanthophylls), in vitro protein digestibility and anti-nutritional factors (total polyphenols detected as total phenolic content (TPCs) and expressed as % Gallic acid equivalents (%GAE), total saponins and total alkaloids) using the standard methods [19-23]. The minerals were analysed by dry ashing the LPCs at 550°C in a Muffle furnace. The content of Na and K were determined by Flame Photometer using the reported method [24]. Ca and P were estimated employing the AOAC volumetric method [19]. Other mineral elements (Fe, Cu, Zn, Mn, Mg and Co) were determined by Atomic Absorption Spectrophotometer (GBC 904, Electronic Corporation of India) using the DTPA-CaCl₂-TEA extraction method [24]. The gross energy was calculated based on the following formula [25].

Gross energy (Kcal/100g LPC) = (Protein X 4) + (Fat X 9) + (Carbohydrates X 4)

2.6 Statistical Analysis

The experiments were done in triplicate and the values determined, are presented as means \pm standard deviation. The data were subjected to one-way analysis of variance (ANOVA) using the SPSS version 16.00 (Statistical Package for Social Sciences Version 16.00). Statistical significance of the differences was performed by post-hoc analysis using Tukey test. Differences with $p < 0.05$ were considered significant. The critical difference (CD) or least significant difference (LSD) was done using Genstat version 3.2 statistical software.

III. Results And Discussion

3.1 Isolation of Leaf protein concentrate (LPC)

The procedure for isolation of LPC from *Pongamia pinnata* (PPLPC) was optimized with respect to the yield of LPC (expressed in g/100g leaves) and protein content (%) by varying the process parameters (Table 1). Each of these parameters was varied one by one keeping the remaining parameters constant. The yield of LPC and the protein content of the products obtained under varied process conditions were determined and their dependence on each of the variables was investigated.

3.1.1 Effect of fresh leaves to water (w/v)

The effect of the ratio of fresh leaves to water on the yield of LPC and protein content was studied and the results are shown in Fig. 1. It was observed that the LPC yield and protein content increased from 1.75 ± 0.12 to 7.35 ± 0.04 g and 33.44 ± 0.09 to 43.72 ± 0.12 %, respectively with increasing the ratio of fresh leaves to water from 1:1 to 1:9 and thereafter remained constant. The increase in the LPC yield and protein content up to 7.35 ± 0.04 g and 43.72 ± 0.12 %, respectively could be attributed to good flowability of the leaf slurry and softening of the cell wall resulted from the increased moisture content [16]. The influence of the fresh leaves to water ratio on LPC yield and protein content was found to be statistically significant ($P < 0.05$; $CD_{LPC} = 0.15$; $CD_{Protein} = 0.26$).

3.1.2 Effect of coagulation pH

With the ratio of fresh leaves to water 1:9, the effect of pH required to cause protein coagulation, on the LPC yield and protein content was examined. For this, protein was coagulated using 1N acetic acid at room temperature and at different pH viz, acidic pH-4.0 (adjusted by 1N HCl), basic pH-9.0 (adjusted by 1N NaOH) and bio pH-7.0 followed by heating in boiling water. The results are presented in Fig. 2. There was significant difference in the yield of LPC and protein content under above varied conditions ($P < 0.05$; $CD_{LPC} = 0.38$; $CD_{Protein} = 0.14$). Use of 1N HCl followed by heating in boiling water produced LPC with the highest yield (7.37 ± 0.07 g) and protein content (44.03 ± 0.06 %).

3.1.3 Effect of temperature

Coagulating the protein at pH-4.0 using 1N HCl, effect of coagulation temperature varied from 50°C-100°C, on the yield of LPC and protein content was investigated and the results are illustrated in Fig. 3. On increasing the temperature from 50°C-90°C, LPC yield and protein content were found to increase from 3.73 ± 0.12 to 7.39 ± 0.26 g and 33.09 ± 0.08 to 43.90 ± 0.06 %, respectively. However, further increase in temperature to 100°C resulted in no appreciable change in the LPC yield and the protein content. This may probably be attributed to the non availability of the enough protein for coagulation. The increment in yield and protein content with increasing the temperature up to 90°C could be due to the favorable effect of temperature on protein coagulation. Coagulation of the chloroplast protein has been reported up to the temperature of 70°C beyond which cytoplasm protein is coagulated. Thus the coagulation temperature was optimized to 90°C. Above observation was also verified from the statistical analysis of the data which were found to be statistically significant ($P < 0.05$; $CD_{LPC} = 0.32$; $CD_{Protein} = 0.22$).

3.1.4 Effect of coagulation duration

Using the above optimized parameters (leaves to water ratio 1:9, coagulation pH-4.0 and temperature 90°C), the effect of coagulation duration varied from 1 to 15 minutes, was studied on the LPC yield and protein content. Results are shown in Fig. 4. As shown in Fig. 4, an increase in the LPC yield (2.19 ± 0.06 to 7.35 ± 0.05 g) and protein content (18.66 ± 0.06 to 43.79 ± 0.05 %) was observed with the increase in duration from 1 minutes to 11 minutes and thereafter, the yield of LPC and protein content remained almost constant. Statistical analysis of the data also confirmed the above findings ($P < 0.05$; $CD_{LPC} = 0.12$; $CD_{Protein} = 0.21$). Thus the coagulation duration was optimized to 11 minutes.

3.1.5 Effect of different acids on coagulation pH-4.0

Further experiments were carried out to bring the pH-4.0 of the juice using 1N acetic acid (111.67 ml), 1N HCl (11.33 ml) and 1N citric acid (25.67 ml) before heating it in boiling water. There was no significant difference in the yield of LPC and protein content ranged from 7.33 ± 0.24 - 7.38 ± 0.07 g and 43.91 ± 0.03 - 44.02 ± 0.07 %, respectively ($P > 0.05$; $CD_{LPC} = 0.30$; $CD_{Protein} = 0.13$). These three acids followed the order, in terms of the quantity required to coagulate protein at pH-4.0, Acetic acid > Citric acid > HCl. Although the quantity of 1N HCl was the lowest, application of the citric acid was found to be suitable in view of the environmental concerns associated with the use of HCl.

Based on the above experiments, the optimized protocol and set of conditions for isolation of LPC from *Pongamia pinnata*, is illustrated in Fig. 5.

Using the above optimized process conditions, the LPCs from lower, middle and upper canopy of *Pongamia pinnata* were isolated and yield was determined to be 7.27 ± 0.08 , 7.28 ± 0.04 and 7.34 ± 0.04 g, respectively which was statistically insignificant ($p>0.05$; $CD_{LPC}=0.11$).

3.2 Proximate nutritional composition

The proximate nutritional composition of PPLPCL, PPLPCM and PPLPCU is given in Table 2. The moisture content of PPLPCL, PPLPCM and PPLPCU was 6.58 ± 0.36 , 6.49 ± 0.10 and $6.51\pm 0.09\%$, respectively. Moisture in food determines the rate of food absorption and assimilation within the body. Moisture content of LPC also determines its keeping quality and less than 10% moisture content is recommended [15]. LPCs extracted from *Moringa oleifera*, *Telfeira occidentalis*, *Manihot esculenta*, *Cnidioscolus aconitifolius*, *Salvia hispanica*, *Amarathus hybridus* and *Medicago sativa* have been reported to contain moisture content in the range 5.44-9.0% [13,15-17]. Our findings are in good agreement with the reported ones.

PPLPCL, PPLPCM and PPLPCU contained appreciable amount of crude protein (43.77 ± 0.23 , 43.95 ± 0.34 and $44.34\pm 0.53\%$, respectively). It is generally recommended that a leaf suitable for the preparation of LPC should contain at least 20% protein [26]. The proteins taken in as food are indispensable to ensure the renewal of the amino-acids required for the synthesis of the structural and functional cells of the body. The recommended dietary allowance (RDA) of protein for children, adult males and females are 28, 63 and 50 gm, respectively [15]. LPCs extracted from *Salvia hispanica*, *Telfeira occidentalis*, *Amarathus hybridus*, *Manihot esculenta*, and *Cnidioscolus aconitifolius* have been reported to contain crude protein content in the range 24.97-44.62% [13-17]. The aforesaid facts indicate that PPLPC could be used as nutritionally valuable healthy ingredient to improve protein deficiency of human diet.

The fats are main source of energy in body but should not exceed the daily recommended dose of not more than 30 calories so as to avoid obesity and other related diseases [26]. They are also involved in the making of hormones, prostaglandins and are also necessary for the absorption of some fat soluble vitamins (A, D, E and K), permeability of cell membranes, the transmission of nerve impulses, the metabolism of adrenal hormones and male fertility [4]. They are, therefore, of great importance physiologically, both to animals and man. The fat contents in PPLPCL, PPLPCM and PPLPCU were 12.54 ± 0.31 , 12.72 ± 0.50 and $12.82\pm 0.49\%$, respectively. This value is greater than that reported in literature for the LPCs derived from *Leucaena leucocephala* (7.4%), *Vernonia amygdalina* (9.20 %), *Gliricidia sepium* (11.85%), water hyacinth (10.21%), spinach (0.3%), *Amaranthus hybrids* (1.60%) and *Medicago sativa* (8 – 12 %) [4, 13-17].

Crude fibre contents of the PPLPCL, PPLPCM and PPLPCU were determined to be 1.84 ± 0.05 , 1.86 ± 0.06 and 1.85 ± 0.10 %, respectively. These contents of crude fiber are lower than those reported in the LPCs recovered from *Vernonia amygdalina* (10.46 %), sweet potato (7.20 %), *Amaranth hybridus* (6.4%), *Manihot esculenta* (9.0%), *Gliricidia sepium* (4.37%), *Leucaena leucocephala* (10.60%), and *Salvia hispanica* (11.48%) [13-17, 27] but comparable to *Medicago sativa* (Lucerne) LPC (< 2 %) [4] and water hyacinth LPC (1.15%) [28]. The low fibre level both allows the concentration of useful components such as minerals and improves their assimilation in the digestive tract. It also prevents the absorption of excess cholesterol. The fibre is also necessary for the good functioning of the bowel [4]. This indicates that PPLPC can be a good contributor of dietary fiber.

Ash content is a measure of minerals found in the food. The ash contents of the PPLPCL, PPLPCM and PPLPCU were found to be 3.85 ± 0.08 , 3.97 ± 0.69 and $3.66\pm 0.19\%$, respectively. These contents are lower than the LPCs from *Allium cepa* (12.48 %), *Laurea taxifolia* (27.14 %), *Crassocephalum crepidiodes* (11.43%) but higher than *Talium triangulare* (0.62%), and *Telfeira occidentalis* (0.68%) [14-17]. For the use as food the recommended ash contents of the LPC are 2-5% [27]. Our findings are in accordance to the recommended levels.

Total carbohydrates contents of the PPLPCL, PPLPCM and PPLPCU were 39.83 ± 0.38 , 39.35 ± 0.66 and 39.18 ± 0.83 %, respectively. These are much lower than those reported in the LPCs from *Tribulus terrestris* (55.67%), water spinach (54.20%) and higher than *Gliricidia sepium* (26.90%), *Amaranthus hybridus* (29.0%), *Telfeira occidentalis* (33.4%), *Salvia hispanica* (34.53%), *Vernonia amygdalina* (23.58%), *Moringa oleifera* protein concentrate (3.82) % [14-17,27]. Carbohydrates are the body's principle and most economical energy source. It also contributes to the sweetness, appearance and textural characteristics of many food substrates. There is no report on daily requirements of carbohydrate but 50 gm of carbohydrates should be enough to prevent breakdown of the body's store of protein and fats [15]. Lucerne LPC contains 6% sugars and the recommended dosage of LPC is 6-15 g/day which can help to stabilize glycaemia [4]. Our findings show that PPLPC can provide sufficient amount of carbohydrates.

PPLPCL, PPLPCM and PPLPCU were found to contain very good amount of organic matter 96.15 ± 0.08 , 96.02 ± 0.69 and $96.34\pm 0.20\%$, respectively. It is very important in the movement of nutrients and plays a role in water retention.

Total free amino acids (TFAAs) contents of the PPLPCL, PPLPCM and PPLPCU were determined to be 0.056 ± 0.01 , 0.057 ± 0.01 and 0.057 ± 0.0 % Equivalence of Leucine, respectively. These low values are indicative of the healthy status of the PP leaves used for recovery of LPC as the production of the TFAAs is associated with the physiological changes resulted from the disease in the leaves [21].

The gross energy calculated for PPLPCL, PPLPCM and PPLPCU was 447.29 ± 1.26 , 447.71 ± 3.30 and 449.77 ± 3.40 Kcal/100g, respectively and was higher than the LPCs from *Amaranthus hybridus* (344.34 Kcal/100g) and *Telfairia occidentalis* (373.55 Kcal/100g) [17,27] but lower than Lucerne LPC (470 Kcal/100g) [4]. Foods differ in their potential energy content. Bread, meat, potatoes and cabbage contain 262.73, 310.49-429.92, 95.54, 23.88 Kcal energy / 100g, respectively [17].

Chlorophyll contents were 0.38 ± 0.007 , 0.37 ± 0.007 and 0.37 ± 0.009 %, respectively. Chlorophyll estimation is one of the important biochemical parameters which is used as the index of production capacity. It promotes and stimulates the availability of red blood cells to the body. It also increases function of the heart, lungs, and vascular system. This can also help the immune system because it improves the circulation and oxygenation of the cells and body [4].

Total carotene, another important component of leaf protein, is also nutritionally important biochemical parameters. As shown in Table 2, the PPLPCL, PPLPCM and PPLPCU contained total carotene content 0.025 ± 0.001 , 0.025 ± 0.001 and 0.026 ± 0.001 %, respectively. Amongst the carotenes, the most important one is β -carotene which is important in lessening asthma symptoms, preventing certain cancers, heart disease, cataracts and age related macular degeneration and treating AIDS, alcoholism, Alzheimer's disease, Parkinson's disease and skin disorders such as psoriasis. β -carotene is also used in malnourished (underfed) women to reduce the chance of death and night blindness during pregnancy as well as diarrhea and fever after giving birth [4]. The recommended dose of β -carotene for children is 0.38 mg per kg body weight [29].

Xanthophylls concentration in the PPLPCL, PPLPCM and PPLPCU was 0.82 ± 0.01 , 0.83 ± 0.03 and 0.83 ± 0.02 %, respectively. In recent years, a great deal of attention has been focused on biological activities of dietary xanthophylls such as lutein, zeaxanthin, β -cryptoxanthin, capsanthin, astaxanthin, and fucoxanthin [30]. Xanthophylls have been thought to work as antioxidants and as blue light filters to protect the eyes from oxidative stresses [31]. Numerous studies have reported that xanthophylls have the potential to prevent cancers, diabetes, and inflammatory and cardiovascular diseases [32].

No significant difference in the contents of moisture, crude protein, fat, ash, crude fiber, total carbohydrates, organic matter, total free amino acids, gross energy, chlorophyll, total carotene and xanthophylls determined in the PPLPCL, PPLPCM and PPLPCU, as revealed by ANOVA analysis of these data ($p>0.05$), was observed. It was inferred that PPLPC irrespective of the canopy can be a good source of the above proximate nutritional composition.

3.3 Minerals

Contents of the minerals Na, Ca, P, Fe, K, Cu, Zn, Mn, Mg and Co estimated in PPLPCL, PPLPCM and PPLPCU are shown in Table 2. These are largely water soluble. Since they are not synthesised by the body, must be taken in food [4]. Sodium concentration in the PPLPCL, PPLPCM and PPLPCU was 4.72 ± 0.17 , 4.56 ± 0.19 and 4.42 ± 0.47 mg/100g LPC, respectively. Sodium is the principal extracellular cation in the body. It also acts as an antagonistic and complementary to potassium. The daily allowance of sodium is 500mg for adult [26]. Calcium is required by children, pregnant and lactating woman for formation and growth of bones and teeth. It also acts as a catalyst in blood coagulation in the body. The concentration of calcium in the PPLPCL, PPLPCM and PPLPCU was 1079 ± 18.72 , 1087 ± 23.02 and 1070 ± 12.12 mg/100g LPC, respectively. The recommended daily allowance of calcium is 600-700 mg, 900 mg and 1 200 mg for child (1-9 yrs), adult and adolescent, elderly, lactating women, respectively [4]. This indicates that PPLPC can be a good contributor of calcium. Phosphorus is another important mineral required by children, pregnant and lactating woman for formation and growth of bones, teeth and cell membranes. It is also a key player in fat metabolism. It also acts as a catalyst in phosphorylation reaction. The concentration of phosphorus in the PPLPCL, PPLPCM and PPLPCU was 746.33 ± 10.21 , 747.33 ± 21.50 and 754 ± 13 mg/100g LPC, respectively. The recommended daily allowance of phosphorus is 500-600 mg, 800 mg and 1 000 mg for child (1-9 yrs), adult and adolescent, elderly, lactating women, respectively [4]. This indicates that PPLPC can be a good source of phosphorus. The iron concentration in the PPLPCL, PPLPCM and PPLPCU was 53.20 ± 0.49 , 53.42 ± 0.29 and 55.05 ± 1.31 mg/100gm LPC, respectively. Iron is required for haemoglobin synthesis and its deficiency leads to anaemia. Iron is also essential ion for respiration and anti-infective. The daily requirement of iron is 10 mg and 10-18 mg for child (1-9 yrs) and adult, adolescent, elderly, lactating women, respectively [4]. These values of iron indicate that PPLPC can provide the daily requirement of iron. The potassium concentration in the PPLPCL, PPLPCM and PPLPCU was 21.0 ± 0.19 , 20.68 ± 0.28 and 21.05 ± 0.08 mg/100g LPC, respectively. It is the principal intracellular cation in the body. High amount of potassium in the body has been reported to increase iron utilization. The recommended daily allowance of potassium is 2000 mg for adults [4]. The concentration of copper in PPLPCL,

PPLPCM and PPLPCU was 13.12 ± 0.17 , 13.13 ± 0.18 and 12.95 ± 0.23 mg/100g LPC, respectively. Copper is required in body for enzyme production and biological transfer of electron within the body. The daily requirement of copper is 1-1.5 mg and 2-3 mg for Child (1-9 yrs) and adolescent, adult, elderly, lactating women, respectively [4]. The concentration of zinc in PPLPCL, PPLPCM and PPLPCU was 7.57 ± 0.25 , 7.84 ± 0.09 and 8.05 ± 0.23 mg/100g LPC, respectively. Zinc plays a vital role in gene expression, regulation of cellular growth and acts as a coenzyme for various metabolism processes. The daily recommended allowance for zinc is 10-19 mg [4]. The concentration of manganese (Mn) in PPLPCL, PPLPCM and PPLPCU was 0.38 ± 0.01 , 0.37 ± 0.01 and 0.38 ± 0.01 mg/100g LPC, respectively. Manganese is required for building immune system, regulation of blood sugar level and production of energy. The daily recommended level of manganese is 1-4 mg [4]. The magnesium (Mg) concentration in PPLPCL, PPLPCM and PPLPCU was 27.66 ± 0.35 , 26.62 ± 0.80 and 27.32 ± 0.30 mg/100 g LPC, respectively. Mg is required for formation & growth of bones & muscles, activates ATP in the energy cycle and also activates numerous enzymes [4]. The cobalt (Co) was not detected in the LPCs isolated from PPL, PPM and PPU.

The contents of Na and P of the PPLPCs were comparable while those of Zn and Cu were higher than the LPC isolated from Lucerne [4]. There was no significant difference in the mineral contents of the PPLPCL, PPLPCM and PPLPCU, as revealed by ANOVA analysis of these data ($p > 0.05$). It was inferred that PPLPC irrespective of the canopy can be a good source of Na, Ca, P, Zn, Cu, and Fe.

3.4 In vitro protein digestibility and anti-nutritional factors

The in vitro protein digestibility of the PPLPCL, PPLPCM and PPLPCU was determined using the Pepsin-Pancreatin and Pepsin- Trypsin methods, and the results are shown in Table 3. The protein digestibility for PPLPCL, PPLPCM and PPLPCU, determined using both the methods was about 82% and statistically insignificant ($p > 0.05$). The actual protein digestibility obviously has an important effect on its overall nutritive value. In vitro methods are useful for nutritive evaluation of protein quality or digestibility because of their rapidity and sensitivity [33]. The in vitro digestibility measured here for PP protein concentrates are very encouraging. The digestibility of leaf protein concentrate has been reported to range from 65-85% [22]. The digestibility values determined for PPLPC were higher than those reported for wheat leaf protein concentrate and similar to values found for lupin and rape leaf protein concentrates and slightly lower than alfalfa protein concentrates [22]. Thus, PPLPC possesses good in vitro digestibility.

Plant leaves contain some naturally occurring compounds termed as anti-nutritional factors (ANFs) which limit their biological utilization on account of their nutritional implications, particularly the digestion and assimilation of some nutrients as well as the immune system of the animals [34]. The adverse effects of the ingestion of ANFs have extensively been reported [35]. It is believed that the most ANFs would be removed during the processing of leaf protein concentrate [36]. The contents of the ANFs such as total polyphenols, total saponins and total alkaloids were determined in the PPLPCL, PPLPCM, and PPLPCU, and shown in Table 3. Total polyphenol contents (TPCs) of the PPLPCL, PPLPCM and PPLPCU were 0.122 ± 0.006 , 0.123 ± 0.006 and 0.120 ± 0.004 % GAE, respectively. Statistically, phenolic content in all the three LPCs were insignificant. Polyphenols are chemically highly active compounds that can be at the root of several undesirable effects such as diminution of the absorption of Fe, oestrogenic effect of coumesterol or of isoflavones and diminution of protein digestibility. Polyphenols are well known to react with and form strong covalent bonds with the ϵ -amino groups of lysine [4]. Phenolic content has also been listed as a factor most often responsible for small yields of leaf protein [37]. TPCs in leaf protein concentrate from 1.12 to 5.62% have been reported in *Allmania nodiflora* and *Hygrophila spinosa* [37]. TPCs of leaf protein concentrate from vegetable and legume crops were reported to be varied from 1.4 to 2.2% [38]. It is also reported that the ratio of TPCs and nitrogen of leaf protein concentrate has an inverse relationship to protein quality [38]. Thus, the low value of TPCs in the PPLPCL, PPLPCM, PPLPCU are indicative of the good quality of PPLPC. Total saponins contents in PPLPCL, PPLPCM and PPLPCU were 0.510 ± 0.01 , 0.513 ± 0.02 and $0.501 \pm 0.01\%$, respectively. Some saponins sometimes slow down the growth of young animals by forming a complex with cholesterol which inhibits its assimilation. This has been seen in rats and poultry but not in pigs [4]. The total alkaloids were found to be absent in these LPCs. There was no significant difference in the content of TPC and total saponins of the PPLPCL, PPLPCM and PPLPCU, as revealed by ANOVA analysis of these data ($p > 0.05$).

IV. Conclusions

Possibility of utilization of *Pongamia pinnata* (karanj) plantations generated biomass residue (leaves) for production of LPC was investigated. The process parameters optimized for isolation of the LPC consisted of ratio of fresh leaves to water (1:9), coagulation temperature (90°C) and duration (11 minutes), and pH (4.0). Application of the optimized process enabled to isolate the LPCs containing protein 43.77 ± 0.23 , 43.95 ± 0.34 and $44.34 \pm 0.53\%$ in 7.27 ± 0.08 , 7.28 ± 0.04 and $7.34 \pm 0.04\%$ yield from lower, middle and upper canopy of the tree, respectively. The proximate nutritional composition, in vitro digestibility and anti nutritional factor determined

in these LPCs indicated their suitability as a good protein source. Based on statistical analysis of these data, no significant difference in the yield and nutritional characteristics of the LPCs isolated from different canopy of the tree was observed. It was shown that the biomass residue (leaves) generated from the karanj plantations could be utilized for production of LPC as a food supplement. To sum up, a new chemical utilization approach of the karanj leaves, the underutilized biomass residue from these plantations, was shown. This is in tune with the emerging biorefinery concept which has been attracting increasing interest in recent years from the perspective of the upgrading the agro forest biomass residues for value added utilization. The findings of the study are of benefit to the farmers in getting another income from karanj plantations.

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Table 1: Process conditions for recovery of LPC from *Pongamia pinnata*.

Leaves : Water ratio (w/v)	1:1-1:11
Coagulation temperature (°C)	50 ⁰ C-100 ⁰ C
Coagulation duration (Minutes)	1-15
Coagulation factor	pH-4.0 and Heating at 100 ⁰ C pH-9.0 and Heating at 100 ⁰ C Bio-pH and Heating at 100 ⁰ C 1N Acetic acid coagulation at ambient temperature
Optimization of different acids to bring pH-4.0	1N Acetic acid, 1N Citric acid and 1N HCl

Table 2: Proximate nutritional compositions and mineral content (mg/100 g LPC) of the LPCs isolated from lower, middle and upper canopy of *Pongamia pinnata*.

Biochemical Parameters	PPLPCL*	PPLPCM*	PPLPCU*	Significance level**	CD***
Moisture (%)	6.58±0.36	6.49±0.10	6.51±0.09	NS	0.44
Protein (%)	43.77±0.23	43.95±0.34	44.34±0.53	NS	0.77
Fat (%)	12.54±0.31	12.72±0.50	12.82±0.49	NS	0.89
Crude Fibre (%)	1.84±0.05	1.86±0.06	1.85±0.10	NS	0.15
Ash (%)	3.85±0.08	3.97±0.69	3.66±0.19	NS	0.84
Total Carbohydrates (%)	39.83±0.38	39.35±0.66	39.18±0.83	NS	1.30
Organic Matter (%)	96.15±0.08	96.02±0.69	96.34±0.20	NS	0.84
Total free amino acids (% E of Leucine)	0.056±0.01	0.057±0.01	0.057±0.00	NS	0.002
Gross energy (Kcal/100g LPC)	447.29±1.26	447.71±3.30	449.77±3.40	NS	5.66
Pigments					
Chlorophyll (%)	0.38±0.007	0.37±0.007	0.37±0.009	NS	0.015
Carotene (%)	0.025±0.001	0.025±0.001	0.026±0.001	NS	0.0015
Xanthophylls (%)	0.82±0.01	0.83±0.03	0.83±0.02	NS	0.04
Minerals					
Sodium	4.72±0.17	4.56±0.19	4.42±0.47	NS	0.57
Calcium	1079±18.72	1087±23.02	1070±12.12	NS	36.98
Phosphorus	746.33±10.21	747.33±21.50	754±13	NS	31.29
Iron	53.20±0.49	53.42±0.29	55.05±1.31	NS	1.26
Copper	13.12±0.17	13.13±0.18	12.95±0.23	NS	0.39
Zinc	7.57±0.25	7.84±0.09	8.05±0.23	NS	0.36
Manganese	0.38±0.01	0.37±0.01	0.38±0.01	NS	0.015
Magnesium	27.66±0.35	26.62±0.80	27.32±0.30	NS	1.18
Potassium	21.0±0.19	20.68±0.28	21.05±0.08	NS	0.62
Cobalt	N.D	N.D	N.D		

*The values are mean of 3 replicates ± S.D., **N.S= Not Significant at p>0.05., ***CD= Critical difference at 5% level of significance., ND=Not detected.

Table 3: In vitro protein digestibility and anti-nutritional factors of LPCs isolated from lower, middle and upper canopy of *Pongamia pinnata*.

Standard Enzymatic System	PPLPCL*	PPLPCM*	PPLPCU*	Significance level**	CD***
Pepsin-Pancreatin (%)	82.72±0.30	82.83±0.26	82.62±0.09	NS	0.47
Pepsin-Trypsin (%)	82.95±0.19	82.83±0.24	82.81±0.43	NS	0.61
Anti-nutritional factor					
Total Polyphenols Contents (% GAE)	0.122±0.006	0.123±0.006	0.120±0.004	NS	0.012
Total Saponins (%)	0.510±0.01	0.513±0.02	0.501±0.01	NS	0.03
Alkaloids	ND	ND	ND		

*The values are mean of 3 replicates ± S.D., **N.S= Not Significant at p>0.05., ***CD= Critical difference at 5% level of significance., ND=Not detected.

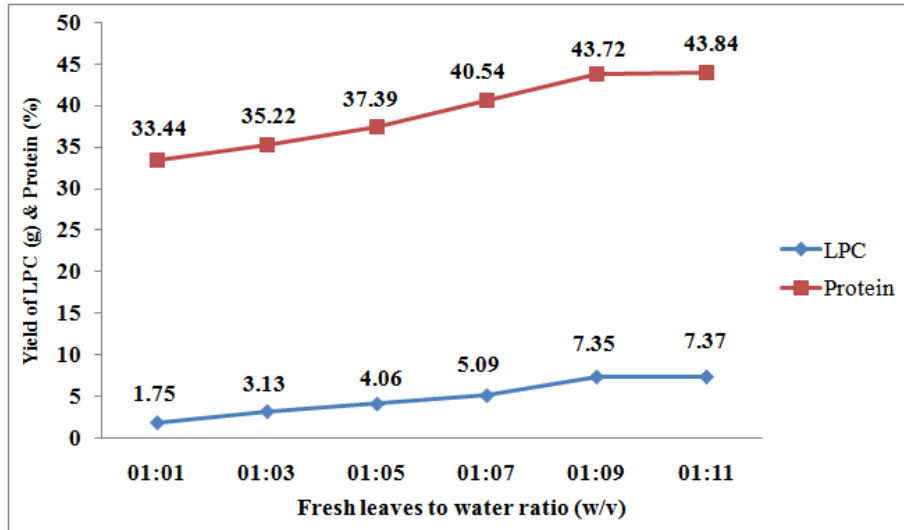


Figure 1: Effect of fresh leaves to water (w/v) ratio on the yield of LPC and corresponding protein content of *Pongamia pinnata*.

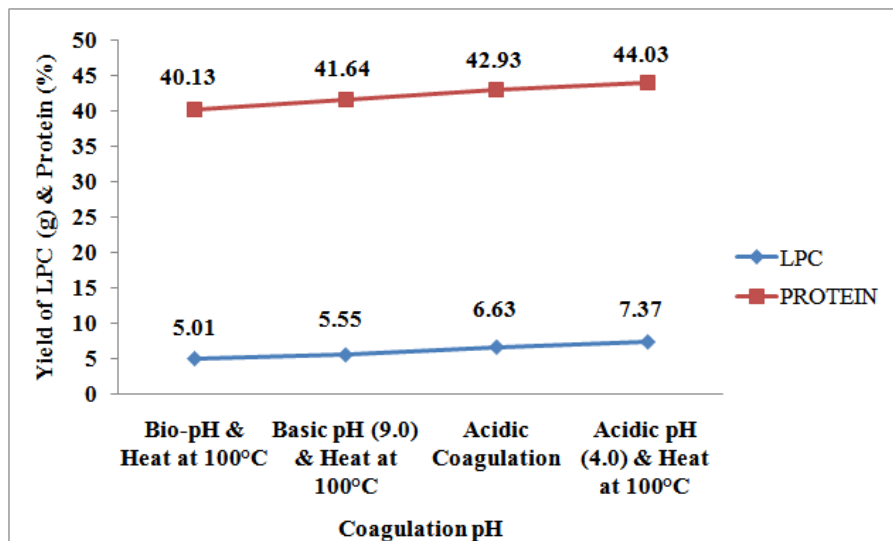


Figure 2: Effect of different coagulation pH on the yield of LPC and corresponding protein content of *Pongamia pinnata*.

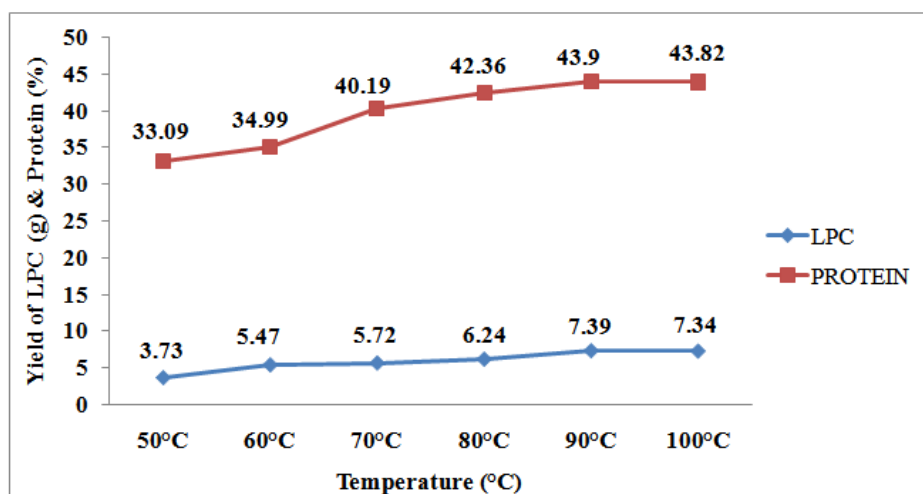


Figure 3: Effect of coagulation temperature on the yield of LPC and corresponding protein content of *Pongamia pinnata*.

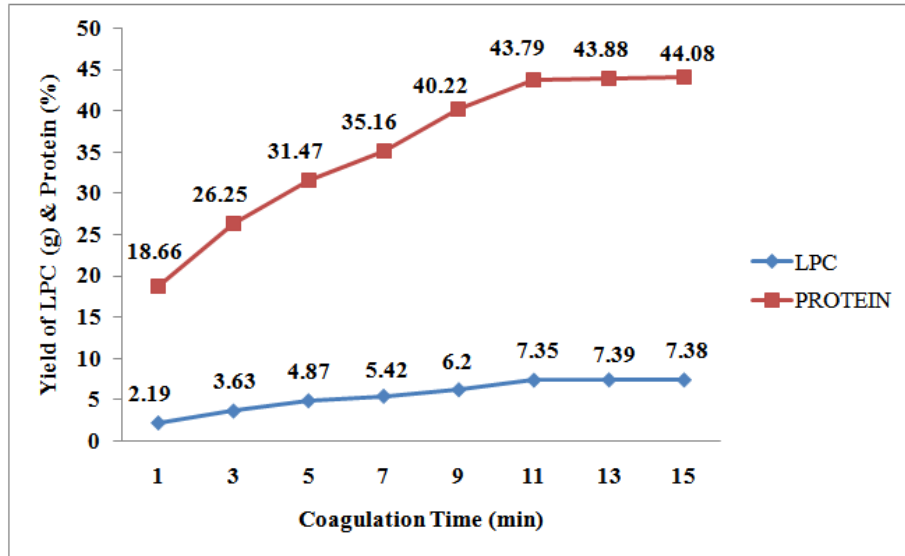


Figure 4: Effect of coagulation duration on the yield of LPC and corresponding protein content of *Pongamia pinnata*.

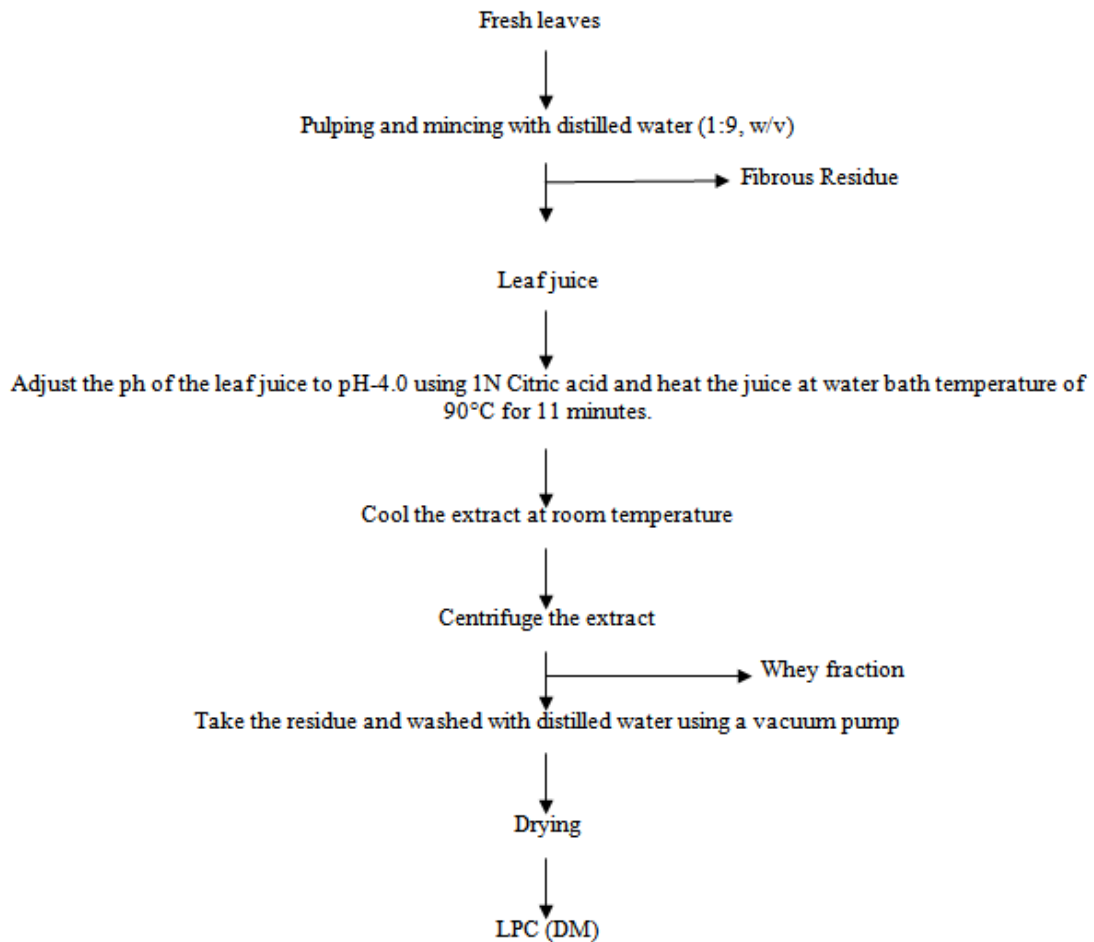


Figure 5: The protocol for recovery of the LPC from *Pongamia pinnata*, based on the optimized set of conditions.