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Isolation and Biochemical Analysis of Leaf Protein Concentrates from the Leaves of *Shorea robusta*

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Abstract: Shortage of supply of good quality protein for meeting the requirements of increasing animal and human population has necessitated search for additional sources. Leaf protein concentrate (LPC), a concentrated form of proteins derived from the foliage of plants, is an inexpensive and most abundant source of available protein. Their protein value equals that of most animal products. Trees have been suggested as a potential source of LPC and the production of protein from tree leaves is advantageous over crops as they do not involve the recurring cost of cultivation. *Shorea robusta* is planted on large scale in India. The potential of its leaves as protein source has not been investigated. The present communication describes the isolation of LPCs, mineral elements from the leaves of *Shorea robusta* and its chemical evaluation. Fresh and matured leaves of *Shorea robusta* yield fairly good amount of LPC (5.96 g) per 100 g leaves. It was also found to contain very high amount of ash (9.24%), which consisted of calcium, Iron, Phosphorus, Potassium, Sulphur micronutriets. Comparing all the biochemical analysis, the LPCs recovered from *Shorea robusta* shows fairly good amount of protein-37.25%, fat-7.41%, nitrogen free extract-37.85%, total carbohydrates-45.5%, total soluble sugar-1.94% along with low amount of anti-nutritional factors such as total phenolics and total saponins. Thus it was inferred that the leaves of the *Shorea robusta* have potential for its use in production of LPCs. However, detailed studies regarding minerals, vitamins, *in-vitro* digestibility, toxicity testing and amino acid compositions are needed to be carried out to standardize the use of leaves as a source of LPCs.

Key words: *Shorea robusta*, leaf protein concentrate, micro-kjeldahl method, anti-nutritional

INTRODUCTION

A large section of the population of the under developing countries live under substandard conditions, including nutritionally inadequate diets (Maforah, 1994). In a survey conducted by the food security Index among South Asian countries, India ranked 69th, Pakistan ranked 77th, Sri Lanka 60th, Bangladesh 88th, Nepal 85th and Myanmar 86th (Dupont, 2014). In availability of food India was ahead of other South Asian countries while in the matter of quality of food consumed, Pakistan was ahead of these countries. India ranked even below Myanmar and Sri Lanka. India stood very low on the basis of essential amino acid present within the food stuff. In India, today protein deficiency has led to malnutrition and cases of kwashiorkor and marasmus are on an increase (Ahmed *et al*., 2009). The available food supplies are not sufficient to meet the demand of the ever growing population. The widening gap between the supply and demand can only be bridged by increasing the production of food and by supplementing the existing resources with novel foodstuffs (Brunger, 2007). So, research interest has been focused on such food sources which are cosmopolitan available, so easily procured and should posses least toxicity problem. The most important among all these food stuff that possess all the above characteristics are the different leaf meals which could serve as a potential protein sources in animal feeds.

The production of protein food from tree leaves appears to have a unique advantage as they do not involve recurring cost of cultivation (Pirie, 1966; Tangka, 2003) as in the case of agricultural crops. However, the trees have not yet been sufficiently studied, with respect to their leaf protein production. Leaf protein concentrate (LPC) is a concentrated form of the proteins found in the leaves of plants. It has been examined as a human or animal food source, because it is potentially the cheapest and most abundant source of available protein. Leaf protein is a good source of amino acids, with methionine being a limiting factor (Wallace *et al*., 1998). Nutritionists in different part of the world are investigating the possibilities of supplementing diet with novel source of protein. One such source is Leaf protein concentrate. Value of leaf protein concentrates lies in between that of soybean and milk (Belitz *et al*., 2009). Exhaustive study on plant leaf protein concentrate (LPS) has been undergone at the University of Wisconsin, Madison, where several Biochemists, agronomists,

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nutritionist were involved and significant results were published (Dale, 1974; Russel *et al*., 1974; Koegel and Bruhn, 1972; Stahmann, 1968). The product is green and presents no palatability problems when included in mixed feeds. The present investigation was therefore, undertaken to find out the extractability and chemical composition of leaf protein from *Shorea robusta* and findings are reported herein.

The tree has been described in 'Charaka Samhita' as well as in 'Vedanasthapana Mahakashya' the therapeutic potential of its leaves has been described in Susruta Samhita (Adlakha *et al*., 2013).

Taxonomic classification:

Kingdom : Plantae
Unranked : Angiosperms
Unranked : Eudicots
Unranked : Rosids
Order : Malvales
Family : Dipterocarpaceae
Genus : *Shorea*
Species : *Robusta*

MATERIALS AND METHODS

In plants there are two types of Phytochemical compound which are present:

A: Inorganic Compounds
B: Organic Compounds

Biochemical tests were performed to determine the presence of inorganic and organic compounds in the leaves of *Shoreo robusta*. Inorganic compound are the one that lacks carbon atom within them and so are solely made up of inorganic ions. These include micronutrients in the form of metal ions. Micronutrients are the nutrients required by humans and other organisms throughout their life span in smaller quantities. These include inorganic metal ions. Organic compounds are the one which are made up of carbon and its compounds. These are chemical elements that animals consume in large quantity and include Proteins, Carbohydrates and Fats (Prentice, 2005).

Qualitative determination of inorganic compounds: Qualitative determination refers to the estimation of inorganic ions present within the sample. All these inorganic ions form an important constituent of enzymes in the form of prosthetic group or forms complex compounds with organic compounds.

Preparation of sample for analysis: Fully matured and fresh leaves of *Shorea robusta* was collected from the Forest Research Institute (FRI) campus. The leaves were collected in early morning and processed immediately. Fresh and mature leaves were taken and washed with tap water followed by distilled water so as to remove the surface contaminants.

Determination of moisture content: Moisture content refers to the amount of water content within the sample. Sun dried; finely grinded 5 gm of the leaf powder was taken in a pre weighed Petri dish (W_1) and was weighted again to a constant weight (W_2). It was kept in a hot-air oven for 24 h at 100±5°C cooled in desiccators and weighted (W_3). The loss in weight was considered as the moisture percentage and was calculated using the following formula:

$$\text{Percentage of Moisture} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where,

W_1 = Wt. of empty Petri dish
W_2 = Wt. of Petri dish with sample before hot-air oven dry
W_3 = Wt. of Petri dish with dried sample

Determination of ash content: The dried crushed leaf powder was weighed to 5, 10 and 15 gm and kept in three different crucibles which had been well dried and weighted before. These crucibles were heated at a temperature of 550°C on direct flame, till the organic material was completely burned and only ash remained. The crucible containing the ash was then allowed to cool down in desiccators and later on weighed to determine the ash content using the following formula:

$$\text{Ash of (\%)} = \frac{(\text{Wt. of Crucible} + \text{Ash Wt. of Empty crucible})}{(\text{Sample wt.} \times \text{dry matter coefficient})}$$

The ash was dissolved in double distilled water and was used for the determination of micro and macronutrients. Biochemical tests for Micronutrients and Macronutrients were performed as per the chemical tests advocated by Adlakha *et al*. (2013).

Tests for micronutrients: The following micronutrients were analyzed:

1: **Test for iron:** 0.5 mL of the test sample was taken and to it 5 drops of Potassium thiocyanate (KSCN) were added. Appearance of red colour indicates the presence.
2: **Test for calcium:** 0.5 mL of the test sample was taken and to it 2-3 drops of sulfuric acid (H_2SO_4) were added. Appearance of white precipitate indicates the presence.
3: **Test for phosphorus:** 0.5 mL of the test sample was taken and to it 2-3 drops of Ammonium orthomolybdate [$(NH_4)_2MoO_4$] were added. Appearance of yellow color indicates the presence.

4: **Test for potassium:** 0.5 mL of the test sample was taken and to it 2-3 drops of 15% hypochlorous acid ($HClO_4$) solution was added. Appearance of $KClO_4$ crystals indicates the presence.
5: **Test for sulphur:** 0.5 mL of the test sample was taken and to it 2-3 drops of Barium Chloride ($BaCl_2$) was added. Appearance of white precipitate indicates the presence.
6: **Test for zinc:** 10 mg of ash was dissolved in 1 mL of the double distilled water. To the solution obtained 0.2 mL of 10 M sodium hydroxide (NaOH) was added. A white precipitate was obtained which was dissolved in 2 mL of 10 M sodium hydroxide solution. To it 5 mL of 2 M Ammonium Chloride (NH_4Cl) was added followed by addition of 0.1 mL of sodium sulphate (Na_2SO_4). Appearance of a flocculent white precipitate indicates the presence.
7: **Test for nickel:** 10 mg of the ash was dissolved in 1 mL of the double distilled water, acidified with 1 or 2 drops of dilute hydrochloric acid (HCl) and then a drop of dilute sodium hydroxide were added. Appearance of a blue precipitate which turns green on warming indicates the presence.
8: **Test for copper:** 10 mg of the ash was dissolved in 1 mL of the double distilled water to it dil ammonia solution was added drop wise up till a clear blue color appears. With continuous heating alcoholic solution of α-benzoinoxime was added drop wise. Appearance of green precipitate indicates the presence.

Quantitative estimation of organic matter: The amount of weight lost while determining the ash content of the sample is the amount of Organic matter present within the sample:

Amount of Org. matter = Wt. of Dried Sample-Wt. of Ash

Tests for macronutrients: The following macronutrients were analyzed within the organic matter:

Determination of fiber content: The fiber content of the sample was determined through the method as advocated by Jeraci and Van Soest (1990).

Recovery of fiber: The fat sample (W_1) obtained after ether extraction was transferred from the thimble to a 500 mL of round bottom condenser flask. To it 200 mL of 1.25% Sulphuric acid solution was poured into the flask and heated in a heating mantle. After boiling for 30 min, the content of the flask was filtered through the muslin cloth. The residue was washed with distilled water to remove the acid. It was transferred to a crucible and kept in a hot-air oven at 100±5°C for drying. The crucible was cooled in a desiccators and weighted to a constant weight (W_2).

The content was ashed in a muffle furnace at 600°C, cooled in desiccators and weighed (W_3). The loss in weight during ash formation was the percentage of crude fiber and was calculated as follows:

$$\text{Percentage of Fiber} = \frac{W_2 - W_3}{W_1} \times 100$$

Determination of anti-nutritional factors: The anti-nutritional factors such Polyphenols and saponins were determined through method as advocated by Association of Official Analytical Chemistry (AOAC, 1980) as described below.

Recovery of saponins: A modified Morales and Curl method (1983) was used for Saponin determination. Three gram of the air-dried powered sample was taken in a 250 mL round bottom flask and extracted with 25 mL of methanol (90% v/v) by refluxing it for half an hour. The same extraction process was repeated thrice and the solvent was distilled off. The soft extract left over was treated with 25 mL of petroleum ether (60-80°C) by refluxing it for half an hour.
Solvent was cooled, removed by decantation. Soft extract left was then treated with a mixture of 25 mL chloroform and 25 mL ethyl acetate. The solvent were poured off after cooling, keeping the soft extract in the same flask. It was again dissolved in 25 mL methanol, filtered (Whatman no. 42) and concentrated to 5 mL. Methanolic extract was then added to 25 mL of acetone to precipitate glycosides. The ppt was then filtered, collected and dried to a constant weight and percentage of saponin was calculated by subtracting the weight of the beaker (B) from the weight of beaker with dried precipitate (P+B) and dividing with the weight of sample taken:

$$\text{Total Saponin} = \frac{\{(P+B)-(B)\}}{\text{Wt.of sample (ingm)}} \times 100$$

Recovery of polyphenols: Total polyphenolic content was estimated using the Folin Ciocalteus colorimetric method (Negi *et al*., 2012).

Determining protein content: The protein content of the leaf was determined by firstly determining the nitrogen content through the Micro-Kjeldahl method described by Pearson (1976) and the percentage nitrogen was converted to crude protein by multiplying with a factor of 6.25 (Omotoso, 2005).

Recovery of LPC: The procedure for extraction of Leaf Protein Concentrate (LPC) suggested by Pirie (Morrison and Pirie, 1961; Pirie, 1971) was followed. Fresh leaves (100 g) were taken and wash with tap water and grinded

in a mixer using sufficient amount of triple distilled water (900 mL) and squeezed by hand using muslin cloth. The above procedure was repeated 4-5 times in order to achieve the complete extraction. The extracts was coagulated by heating the filtrate at 75-80°C in water bath for 8-10 min then cooled at room temp, centrifuged at 1000 rpm for 10 min, washed with acidified water followed by acetone and dried in hot air oven at 60°C for 30 min. All the samples were stored at 4°C, until analyzed.

Estimation of protein nitrogen: Investigation on protein content are of immense nutritional value for animals and humans as they provide valuable information about the total protein content as well as about the essential amino acids present in proteins. The nitrogen content was determined using the Micro-Kjeldahl method.

One gram of the finely powdered sample was taken in a digestion flask; to it 9.5 gm K_2SO_4, 0.5 gm $CuSO_4.5H_2O$ and 10 mL of Conc. H_2SO_4 was added. The flask was heated at 440°C on Micro-Kjeldahl heating unit till the solution become clear. 25 mL of boric acid solution was taken within the conical flask and the receiving flask was placed in such a way that outlet of the condenser of Micro-Kjeldahl's distillation apparatus dips into the boric acid solution. The acid digested sample was transferred to the steam chamber of Micro-Kjeldahl's apparatus. Later on 40 mL of water and 40 mL of 40% NaOH was added to the aliquot of the digested sample. The stopcock was closed immediately and steam was passed through the steam chamber to distill ammonia till about 30-40 mL of distillate was collected in the receiving boric acid containing flask. The receiving flask was removed and the condenser outlet tip was rinsed with water into the receiving flask. The content of receiving flask was titrated against 0.1 NH_2SO_4 till bluish green color changes to grayish silver. A blank preparation was also simultaneously made to run in parallel experiments which was identically prepared except it does not contain the sample itself. The titration volume of H_2SO_4 was recorded for both the sample and that of the blank and calculations were performed using the formula as under.

Calculation of nitrogen percentage: Percentage Nitrogen content in the sample was calculated by using the following formula:

$$\text{Nitrogen (\%)} = \frac{14.01 \times (\text{Reading of Sample} - \text{Reading of Blank}) \times 0.1}{\text{Weight of sample} \times 10}$$

(Pearson, 1976):

$$\text{Protein (\%)} = \text{Percentage Nitrogen} \times 6.25$$

(Omotoso, 2005).

Determining fat content: The fat content in the LPC was determined through Soxhlet extraction method using petroleum ether.

Recovery of fat: In three sets of experiments performed 500, 250 and 100 mg of the dried, crushed leaf samples were taken in a dried thimble and place it in three different soxhlet apparatus which were made to run simultaneously for 6 h at 50°C using 150 mL of the petroleum ether.

After the extraction was completed the solvent was removed using a flask rotator evaporator, the fat left in the flask is cooled at room temperature and weighed. The percentage fat was calculated using the following formula.

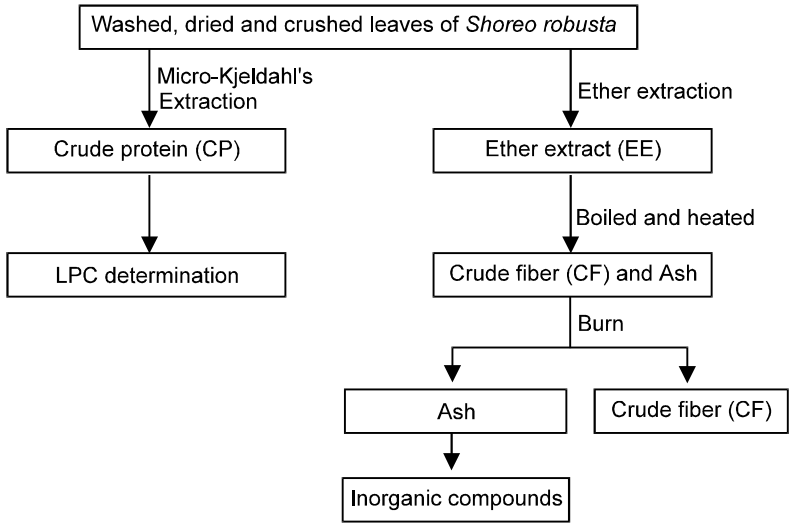

Fig. 1: Flowchart of Biochemical analysis of different components present in leaves of Shorea robusta

Table 1: Recovery of Mineral elements from the ash of leaves of *Shorea robusta* through biochemical tests

Species	Part of plant	Mineral element	Presence(+)/Absence(-)
Shorea robusta	Leaf	Iron	++
		Calcium	+
		Phosphorous	+
		Potassium	++
		Sulfur	+
		Zinc	-
		Nickel	-
		Copper	-

+ve: Indicates presence of a mineral
-ve: Indicates absence of a mineral

Table 2: Recovery of LPCs from *Shorea robusta* through redox titration

Species	Weight of sample (g)	Reading of sample (mL)	Reading of blank (mL)	Nitrogen (%)
Shorea robusta	1.0045	43.3	0.5	5.969
	1.0072	43.5	0.5	5.981
	1.0021	43.0	0.5	5.941
	0.0	0.5	0.5	0.0
	Average percentage of Nitrogen present		5.96%	

-ive control: Was taken to validate our results

Table 3: Yield of L.P.S and nitrogen content of leaf protein concentrates isolated from *Shorea robusta*

Species	Nitrogen (%) in dry LPC (per 100 gm)	Protein (%) in dry LPC (per 100 gm)
Shorea robusta	5.96±0.2	37.25±0.2

Data are represented as+standard deviation

Table 4(a): Biochemical composition of Leaf Protein concentrates isolated from *Shorea robusta* species

Ingredients	Percentage
Organic matter (%)	90.76±0.2
Ash (%)	9.24±0.2

Data are represented as±standard deviation

Table 4(b): Biochemical composition of organic matter present in leaf protein concentrates

Ingredients	Percentage
Protein (%)	37.25±0.2
Fiber (%)	8.25±0.2
Lipid (%)	7.41±0.2
Carbohydrate (%) (NFE)	37.85±0.2

Data are represented as±standard deviation

Table 5: Anti-nutritional factors recovery from LPCs of *Shorea robusta*

Species	Total phenolic content (%)	Total saponins (%)
Shorea robusta	0.123±0.001	0.087±0.001

Data are represented as±standard deviation

Determining nitrogen free extract: The nitrogen free extract or carbohydrate of the sample was calculated by using the following formula:

NFE (%) = Organic matter (%)-(Protein+Fat+Crude fiber+Ash (%)

Determining the total soluble sugar: The estimation of total sugar in LPC was carried out by ferricyanide method (Khanna and Sen, 1946).

RESULTS AND DISCUSSION

Qualitative determination of inorganic matter: High moisture content of 10.81% was determined in the dried, crushed leaf powder of *Shorea robusta*.

The qualitative estimation of Iron, Calcium, Phosphorus, Potassium, Sulphur, Nickel, Copper and Zinc was performed and the results are summarized below.

Quantitative estimation of organic matter: As the ash content of the extract was found to be 9.24% so the organic matter present within the sample was found to be 90.24%. Among this organic matter using the Micro-Kjeldahl's method it has been shown that *Shorea robusta* contained fairly good amount of LPC which is nutritionally important as a protein source. The percentage nitrogen was calculated through redox titration method and the data recorded is presented in the Table 2 as shown below. The percentage nitrogen and percentage protein was calculated by using the above formula.

Statistical analysis: Data obtained above was subjected to statistical analysis using one way analysis of variance using ANOVA (Steel and Torrie, 1980) and the significance of result was determined. The data on the recovery of LPC and nitrogen (Percentage Nitrogen) content of the LPC are presented in Table 3. The results from Table 2 confirmed that the leaves of *Shorea robusta* yielded 5.9 g of dry LPC/100 gm of fresh pulp.
The Leaf Protein Concentrates recovery of dry LPC from *Shorea robusta* had been shown to contain good amount of protein percentage (37.25%). Their fat content revealed that the LPC from the *Shorea robusta* was containing relatively low amount of fat (7.41%). The amount of the Ash was found to be 9.24%. High ash

Fig. 2: Shorea robusta leaves and crushed dried leaf powder

Fig. 3: Images of (A) Digester, (B) Micro Kjeldahl's apparatus, (C) Soxhlet extractor (D) Rotary Evaporator

content in LPC is a reflection of the amount of the mineral contained in the sample, *Shorea robusta* has been shown to possess calcium, Iron, Phosphorus, Potassium, Sulphur minerals so could be useful for pregnant and lactating women and children (Omotoso, 2005). However, extractability of the LPC from *Shorea robusta* was also found to contain fiber, lipid, carbohydrate and ash. Table 4 (a) and 4 (b) summarizes the results of biochemical analysis of the LPCs.

The crude fiber content in the LPCs of *Shorea robusta* is 8.25%. The nitrogen free extract (NFE) or carbohydrate percentage, organic matter (OM) and total carbohydrate in dry LPCs was determined by different phytochemical tests. It was observed that LPC recovered from *Shorea robusta* contain the good amount of nitrogen free extract (NFE/Carbohydrate) (37.85%), organic matter (OM) (90.76%). Finally the biochemical evaluation had also shown the percentage of total soluble sugar (1.94%) to be present in dry LPCs.

The LPCs from *Shorea robusta* was also evaluated for anti-nutritional factors such as total phenolic contents (TPC) and total saponins. Data regarding anti-nutritional factors was presented in Table 5. The TPCs and total Saponin contents are 0.123 and 0.087%, respectively.

Conclusion: The phytochemical analysis results shows that Leaf Protein concentrates isolated from *Shorea robusta* species could serve as an important source of Protein, Carbohydrates simultaneously the low lipid content could facilitate its use as an important tests for cardiovascular patients. The presence of anti-nutritional factors such as phenolic and saponin compounds as well as the presence of micronutrients also enhances its utility as well as the absence of heavy metal ions like zinc, nickel and copper provides a positive sign and indicates about the non toxicity of the leaf extract, also making it much safer to be consumed.

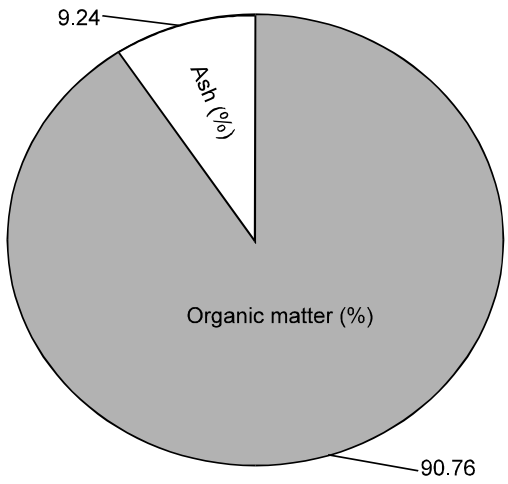

Fig. 4: Compositin of Leaf Protein Concentrate of Shorea robusta

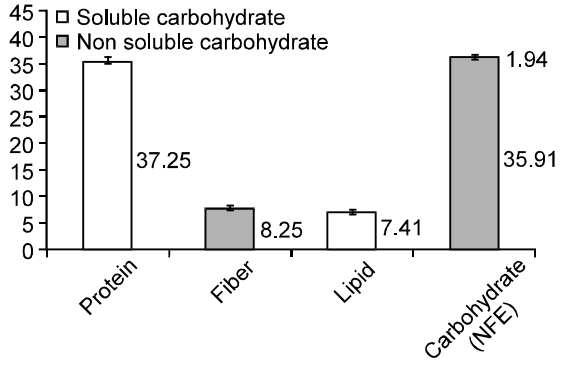

Fig. 5: Percentage of Organic Components within L.P.C.

The high moisture content as determined to be 10.81% may provide favorable environmental condition for fungal growth in LPCs (Roy and Chourasia, 1989; Halt, 1998; Rawat *et al*., 2014) so suitable antifungal agents should be used during its storage.

Although undoubtedly it is one of the best protein source for animals moreover as ruminants have been grazing on the leaves on *Shorea robusta* so it provoke no oral or hepatic toxicity in animals.

Further mineral, vitamins, digestibility, oral and hepatic toxicity testing should be performed before the leaf protein extract could be used as an important constituent of food material for human population.

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REFERENCES

Adlakha, M.K., R. Kapoor, A.K. Sharma and M. Kotecha, 2013. Phytochemical study of *Shora robusta* (SHALA). Int. J. Pharma. and Bio. Arch., 4: 663-671.

Ahmed, T., S. Rahman and A. Cravioto, 2009. Oedematous malnutrition. Ind. J. Med. Res., 130: 651-654.

AOAC (Association of official Analytical Chemists), 1980. Official methods of analysis of the Association of Official Analytical Chemists. 13th ed. Washington, DC.

Belitz, H.D., W. Grosch and P. Schieberle, 2009. Food Chemistry, 4th ed. Springer Book, Heidelberg, Germany.

Brunger, G., 2007. Improving economic access to local foods for those in need in waterloo region. Project Report, p: 18. University of waterloo, Ontario.

Dale, S., 1974. Experiments with the potassium fertilizer of alfalfa for maximum production, p: 42-50. In: Proceedings of the 4th Annual Alfaalfa Symposium, University of Wisconsin, Madison.

Dupont, 2014. An annual measure of the state of global food security. Global food security Index, 3rd ed. Economist Intelligence Unit Study, 13-16.

Halt, M., 1998. Moulds and mycotoxins in herb tea and medicinal plants. Eur. J. Epidermiol., 14: 269-274.

Jeraci, J.L. and P.J. Van Soest, 1990. Improved methods for analysis and biological characterization of fiber. Adv. Exp. Med. Biol., 270: 245-263.

Khanna, K.L. and S.C. Sen, 1946. Further application of potassium ferricyanide method in the estimation of organic carbon in soils p. 75-79. In: Proceed. Ind. Acad. Sci. Section B., 24: 0370-0097.

Koegel, R.G. and H.D. Bruhn, 1972. Pressure fractionation characteristics of Alfalfa. Transaction of ASAE, 15: 856-860.

Maforah, F., 1994. The Impact of poverty on health in urbanizing communities. J. Soc. Dev. in Afr., 9: 87-89.

Morales, P.P. and C. Curl, 1983. A physicochemical method for total saponin determination in quinoa samples. Rev. Bolivia Quim, 6: 13-9.

Morrison, J.E. and N.W. Pirie, 1961. The large scale production of protein from leaf extracts. J. Sci. Food Agric., 12: 1-15.

Negi, A., N. Sharma, R. Pant and M.F. Singh, 2012. Determination of total Phenolic content of the stem bark of *Bauhinia variegate* linn.; An approach to standardization. Pharma J., 7: 16-22.

Omotoso, O.T., 2005. Chemical composition and nutritive significance of the land crab, *Cardisoma armatum* (Decapoda), 7: 68-72.

Pearson, D., 1976. The chemical analysis of foods. 7th ed. London, Churchill Livingstone Edinburgh, London.

Pirie, N.W., 1966. Leaf protein as a human food. Sci., 152: 1701-1705.

Pirie, N.W., 1971. Equipment and methods for extracting and separating protein. In: Leaf protein: Its agronomy, preparation, quality and use (Ed. N.W. Pirie), p: 53-62. Blackwell Scientific Publication, Oxford.

Prentice, A.M., 2005. Macronutrients as sources of food energy. Pub. Health Nutr., 8: 932-939.

Rawat, A., S. Mahajan, A. Gupta, R.K. Agnihotri, N. Wahi and R. Sharma, 2014. Detection of toxigenic fungi and mycotoxins in some stored medicinal plant samples. Int. J. Appl. Sci. and Biotech., 2: 211-216.

Roy, A.K. and H.K. Chourasia, 1989. Aflatoxin problem in some medicinal plants under storage. Int. J. Crude Drug Res., 27: 156-160.

Russel, J.R., N.A. Jorgensen and G.P. Barrington, 1974. Progress report and potential for use of residue and protein concentrate of alfalfa in feeding dairy cattle p: 42-50. In: Proceedings of the 4th Annual Alfaalfa Symposium, University of Wisconsin, Madison.

Stahmann, M.A., 1968. The potential for protein production from green plants. Econ. Bot., 22: 73-79.

Steel, R.G.D and J.H. Torrie, 1980. Principles and procedures of Statistics. A Biometrical approach, 2nd ed. McGraw Hill Book Co., New York.

Tangka, J.K., 2003. Analysis of the thermal energy requirements for the extraction of leaf protein concentrate from some green plants. Biosystems Eng., 86: 473-479.

Wallace, P.A., E.K. Marfo and W.A. Plahar, 1998. Nutritional quality and antinutritional composition of four non-conventional leafy vegetables. Food Chem., 61: 287-291.