Final Project Completion Report (FPCR)

Development of value added food products from leaf protein concentrate of green leafy vegetables of Assam

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Submitted to

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Submitted by

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1.	Title of the project	Development of value added food products from leaf protein concentrate of green leafy vegetables of Assam
2.	Principal Investigator(s) and Co-Investigator(s):	Dr.Sankar Chandra Deka (PI) Dr.Nandan Sit (Co.PI)
3.	Implementing Institution(s) and other collaborating Institution(s):	Department of Food Engineering and Technology Tezpur University, Tezpur
4.	Date of commencement	01/04/2015
5.	Planned date of completion	12 June 2015
6.	Actual date of completion	01/04/2017
7.	Objectives as stated in the project proposal:	(i) To study the nutritional and antinutritional parameters of some important green leafy vegetables (GLV) of Assam. (ii) To study the extraction and optimization of leaf protein concentrate (LPC) from the GLV having highest protein content (iii) To study the chemical composition and functional properties of LPC (iv) To develop protein rich value added product from LPC (v) To study the shelf-life of the value added product and sensory studies
8.	Deviation made from original objectives if any, while implementing the project and reasons thereof	No

9.	Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams and photographs:	**Attached as Annexure - I**
10.	Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject:	**Attached as Annexure - I**
11.	Conclusions summarizing the achievements and indication of scope for future work:	There are plethora of green leafy vegetables whose nutritional and antinutritional profiles are yet to be documented and are lying hidden within mother nature. The documentation of these nutritional and antinutritional profiles will not only serve as a repository for the future research but will also educate mankind in their daily eating habits. Similar technologies can be utilized in the extraction of protein from other green leafy vegetables which are not yet documented. Innovative ways for formulation of recipes will encourage people to consume green leafy vegetable. Further studies on the preservation of these leafy vegetables can be taken up in order to prevent nutritional losses and for availability during lean seasons.
12.	S and T benefits accrued	The present study was undertaken and investigated under five clearly focused objectives. Eight locally available green leafy

		vegetables of Sonitpur district of Assam were selected and their nutritional and antinutritional parameters were analyzed and compared. Based on this study *Diplazium esculentum* was selected and its leaf protein concentrate was extractedusing ultrasound and optimized using response surface methodology. Physicochemical properties of leaf protein concentrate extracted using sonication and nonsonication were studied and compared and results showed that there was improvement in physicochemical properties of LPC extracted by sonication when compared to its nonsonicated counterpart.Further the leaf protein concentrate was examined for its cytotoxicity using three cell lines viz. HEK-293,Hep-G2 and PBMC and the results showed no cytotoxicity of the LPC. Then this LPC was used for the preparation of value added product (Pasta) and its biochemical, microbiological and cooking behavior and storage studies were also investigated. Storage upto three months did not significantly affect the sensory evaluation and cooking quality. Pasta samples stored for three months were safe to consume even from microbiological point of view

i. List of Research publications

a.	Saha, J. and Deka, S. C. (2013). Nutrient content of green leafy vegetables of Assam, India. Presented at the 7thInternational Food Convention, IFCON. Organized by AFSTI, Mysore, CSIR-CFTRI, Mysore, MoFPI, New Delhi, NIFTEM, Haryana, and DFRL, Mysore. (Venue CSIR-CFTRI, Mysore, India),
b.	Saha, J. and Deka, S. C. (2014). Mineral contents of some underutilized green leafy vegetables of Assam, India. Presented at the National Conference on Emerging Technology Trends in Agricultural Engineering (ETTAE). Organized by Department of Agricultural Engineering, North Eastern Regional Institute of Science and Technology, Nirjuli, Itanagar, Arunachal Pradesh (Poster in Proceedings Publication, pp 537, ISBN No. 9789383842797).(Venue NERIST,Nirjuli, Itanagar, Arunachal Pradesh)
c.	Saha, J., Biswal, A. K. and Deka, S. C. (2015). Sankar Chandra"Chemical composition of some underutilized green leafy vegetables of Sonitpur district of Assam, India." *International Food Research Journal* 22 (4): 1466-1473.
d.	Saha, J., Chakraborty S. and Deka, S. C. (2016). A Comparative Study between Response Surface Methodology and Genetic Algorithm in Optimization and Extraction of Leaf Protein Concentrate from *Diplazium esculentum* of Assam. *International Journal of Biotechnology for Wellness Industries* 5(3): 111-120.
e.	Saha, J. and Deka, S. C. (2017). Functional properties of sonicated and non-sonicated extracted leaf protein concentrate from *Diplazium esculentum*. *International Journal of Food Properties* 20(5): 1051-1061.
f.	Book Chapter Saha, J. and Deka, S. C. (2017).Green leaf protein concentrate and its application in extruded food products. Innovative food science and emerging technologies, Apple academic press(CRC Press, A Taylor and Francis Group) (Under review)

ii. Manpower trained on the project

a) Research Scientists or Research Associates: Nil

b) No. of Ph.D. produced: One

c) Other Technical Personnel trained: Nil

d) Patents taken, if any: N/A

13. Financial Position:

No	Financial Position/ Budget Head	Funds Sanctioned (Lac.)	Expenditure (Lac.)	% of Total cost
I	Salaries/ Manpower costs			
II	Equipment			
III	Supplies and Materials (Consumables)			
V	Travel			
VI	Overhead Expenses			
	Total			

14. Procurement/ Usage of Equipment: a)

Sr. No	Name of Equipment	Make/Model	Cost (FE/Rs)	Utilization Rate (%)	Remarks regarding maintenance/ breakdown
1	Weighing balance	Mettler, Toledo, Swizerland, Model No:MS205DU	1,90,076.00	> 80%	The instrument is annually maintained
2	Spectrophotometer	Eppendorf, Germany Eppendorf AG,22331, Hamburg	6,09,500.00	> 80%	The instrument is annually maintained
3	Hot air oven	Advantage Lab,Belgium Model No:AL01-05-115	2,00,000.00	> 90%	The instrument is annually maintained
Total			9,99,576.00		

b) Plans for utilizing the equipment facilities in future

1. Laboratory Class -Yes
2. Student Project work-Yes
3. Other sponsored research project-Yes

Name and Signature with Date

a.Dr.Sankar Chandra Deka
(Principal Investigator)

b.Dr. Nandan Sit
(Co-Investigator)

Annexure- I

Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams and photographs:

Objective (i): To study the nutritional and antinutritional parameters of some important green leafy vegetables (GLV) of Assam

Introduction

It has been recognized in the developing world that there is a serious gap between the demand and supply of food. Now a days researchers are focused in narrowing this gap by introducing lesser known edible plant that can uplift the nutritional security of the state and in turn the country.[1] In India there are 6000 species of plants for consumption, among them 1/3rd are green leafy vegetables which are rich sources of minerals and vitamins, which if consumed regularly can substantially improve the micronutrient status of a particular population.[2]

Assam, a state of India is one of the few places on earth with a unique biodiversity, comprising different climatic zones with a wide range of plant species. Assam is located between 24°44´ N to 27°45´ N Latitude and 89°41´ E to 96°02´ E Longitude, covering 2.4% of the total geographical area of the country i.e 78,438 sq.km. The annual rainfall ranges between 305 cm. max to 178 cm. min with an average of 211.76 cm. The temperature recorded in summer is 37°C max and 18°C min. In winter it is 26°C max and 7° C min with an average humidity of 83.00%. The land and the climatic conditions of Assam are very suitable for growth and survival of innumerable plant species. Most of these rare indigenous floras grow by natural propagation and

are a part of the diet of this region. Traditionally in Assam many of these green leafy vegetables are used as ethno medicines by different tribes of the state for the treatment of jaundice, cancer, hypertension, arthritis, neurological problems and many more. The leafy vegetables have been occupying an important position in the Assamese society due to their plethora of health benefits. Exhaustive literature survey on green leafy vegetables revealed clearly that very few works have been carried out in our country and such works can be considered to be negligible in Northeast India. It is worth to mention here that North east India has the richest sources of green leafy vegetables not only in the country but in the world as a whole.

As it is a well-known fact that traditional knowledge of ethnic groups around the globe forms the basis of modern plant based manufacturing especially allopathic medicine as well as food processing industry,[3] hence these green leafy vegetables can play an important role in uplifting the socio economic status of the people of Assam. The identification and detailed phytochemical evaluation of active components in these individual GLV's will help us learn and be more aware about the importance of the indigenous GLV's, which can further be exploited by the pharmaceutical industry for commercial purpose.

The presence of phytochemicals in addition to vitamins and provitamins, in fruits and vegetables has been recently considered to be crucial in the prevention of chronic diseases, such as cancer, cardiovascular diseases and diabetes. A significant inverse correlation has also been reported between total fruits and vegetable intake and cerebrovascular disease mortality. The complex mixture of phytochemicals in fruits and vegetables can provide a better protective effect on health than a single phytochemical.

Nutritional importance

From literature surveys it can be well predicted that green leafy vegetables are the powerhouse of important phytochemicals as well as possesses important health benefiting substances which can further be exploited for the development of value added products that can in turn elevate the health of a particular population.Sheela et al. [4] identified and analyzed various underutilized green leafy vegetables of southern Karnataka for their nutrient content. A total of 38 underutilized green leafy vegetables including *Colocasia esculenta* were selected. Out of these 38 green leafy vegetables it was found that moisture, protein, fat, fiber, carbohydrate and energy content of *Colocasia esculenta* was 85%, 0.7g, 2.0g, 0.9g, 1.4g, 17 kcal, respectively. Kubmarwa et al.[5] studied the proximate composition of two non-conventional leafy vegetables *Hibiscus cannabinus* and *Haematostaphis barteri* and found that protein content were relatively high (13.78 and 12.40%) respectively. Carbohydrates, lipids, ash and moisture content were within the range of values as expected for dry leafy vegetables more over it was found to have all the essential amino acids. Only cysteine and methionine was below 50% in comparison to the world health organization reference protein. Antinutritional factors were in low range as reported for other vegetables.

Barminas et al.[6] analyzed mineral content of 6 wild leafy vegetables commonly eaten in rural areas of Nigeria namely *Amaranthus spinosus, Adansonia digitata, Colocasia esculenta ,Corchorus tridens,* and *Moringa oleifera*. *Amaranthus spinosus,* and *Adansonia digitata* leaves contained the highest levels of iron (38.4 and 30.6 mg /100g dry wt., respectively). All these vegetables studied contained high levels of calcium, compared to common vegetables. Zinc content was highest in *Adansonia digitata ,Moringa oleifera*, and *Colocasia esculenta* leaves (25.5, 22.4 and 20.9mg/100g dry weight, respectively).

Medicinal importance

Since ages man has been using green leafy vegetables as medicine. Ethno botanist have been elucidating their over lapping role in context to nutrition and therapeutics. Plants used for medicinal attributes contain phytochemicals with pharmacological and physiological significance.[7]These are identified to be good sources of natural antioxidants such as tocopherols, vitamin C and polyphenols which play an important role in the sustenance of good health. Categorically GLV are the major sources of lutein and in developing countries where access to animal food is restricted, these green leafy vegetables contribute substantially in fighting out retinol deficiencies being rich sources of the provitamin A, and β-carotene.[7] The study conducted by Vaishali et al.[8] on 9 green leafy vegetables of Kolahpur district of Maharashtra, reported to contain active components useful for treating various ailments such as tooth ache, dysentery, diarrhea, urinary incontinence, headaches, stimulants for lactating mothers ,fever, anemia, antispasmodic etc. In another study conducted by Subhashreeet al.[9] on four green leafy vegetables of Coimbatore district of Tamilnadu concluded with their findings to contain antioxidant properties. A similar study conducted by Gupta and Prakash [10] on 4 green leafy vegetables of Mysore district of Karnataka also reported to find anti-oxidant properties. Similar studies were done in abroad by many researchers.[11,12,13,14]

Materials and methods

Materials

Eight green leafy vegetables (Table 1 and Fig. 1and 2) selected for the study, were collected from Sonitpur district of Assam and the samples were identified by a Taxonomist. After collecting the samples, the edible portions were separated and washed under running tap

water followed by distilled water and these leaves were further analysed for nutritional composition, antinutritional factors and minerals.

Basella rubra

Basella alba

Diplazium esculentum

Moringa oliefera

Fig. 1 Photographs of green leafy vegetables taken for study

Brassica juncea

Chenopodium album

Amaranthus viridis

Brassica nigra

Fig.2 Photographs of green leafy vegetables taken for study

Table 1 Common and scientific names of locally available green leafy vegetables and their importance

Botanical Name	Family	Common English name	Local vernacular Assamese name	Cultivation	Uses
Basellarubra	Basellaceae	Malabar night shade	Rongapuroi	Found in settled and cultivated areas in hedges	Habitual headaches and laxative [77,78]
Basella alba	Basellaceae	Vine spinach, red vine spinach, climbing spinach, creeping spinach, buffalo spinach, malabar spinach and ceylon spinach	Xeujiapuroi	It thrives in moist, fertile and well drained soils	Antimicrobial and anti- inflammatory effects.[79,80]
Diplazium esculentum	Athyriaceae	Edible fern	Dhekia	Open marshy areas, stream banks and canals from sea level to 2,300 m	Antibacterial properties [81]
Moringaoleifera	Moringaceae	Moringadrumstick tree	Sojina	Semiarid, tropical and subtropical areas	Antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic,diuretic, antihypertensive,cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities.[82]
Brassica juncea	Brassicaceae	Mustardgreens, Indian mustard, Chinese mustard	Lai	It is mostly found in Boreal Wet to Tropical Thorn through Tropical Wet Forest Life Zones.	Hepato protective effects, Antidiabetic properties [83,84]
Chenopodium album	Chenopodiaceae	White goosefoot	Zilmil	It thrives on all soil types.	Antibacterial effects[85]
Amaranthusviridis	Amaranthaceae	Slender amaranth	Khotura	It grows or are distributed in cultivated lands and it flowers and fruits throughout the year	Antinflammatory effects[86]
Brassica nigra	Brassicaceae	Black mustard	Sorio	Distributed in dry areas	Antioxidant and anti-inflammatory[87]

Nutritional analysis

The recommended methods of the Association of Official Analytical Chemists (AOAC.1990)[15] were used for the determination of moisture, ash, lipid, crude fiber, crude nitrogen and carbohydrate.

Ash content

Ash was determined in silica crucibles by incineration in a muffle furnace (Model No: MF03, KK Scientific Supplier, Korea.) at 600°C for 5 h. Clean crucibles were placed in muffle furnace at 600°C for 1h then the crucibles were transferred from furnace to a desiccator and cooled at room temperature. Sample (2g) was weighed by difference and incinerated in muffle furnace for 5 h and transferred to a desiccator and cooled at room temperature.

Calculation

Ash (%) on partial dry or as fed basis = weight of ash /weight of sample × 100

Lipid content

Lipid was extracted by continuous soxhlet method with petroleum ether (b.p.60°C).Dried sample (1g) was weighed and put in a thimble and placed in a pre-weighed solvent flask (‘a’, g). The soxhlet apparatus with the required volume of solvent was connected to condenser and adjusted to give a rate of condensation rate of 2-3 drops and extracted for 16 h, then the thimble was removed and the ether was retained from the apparatus. The excess of ether was evaporated from the flask at 105°C for 30 min. The flask was cooled in a desiccator and weighed (‘b’, g)

Calculation

Crude fat or oil content in sample (% dry wt. basis) = (b-a) ×100/Wt of sample(g)

Crude fibre

Crude fibre was estimated infibresocs (FES-08, Pellicanfibre plus, India) by acid-base digestion with 1.25% H_2SO_4 and 1.25% NaOH solution [16].The sample (2g) was grounded with petroleum ether(b.p 60°C) to remove the fat then 2 g of dried sample was boiled with 200 ml of H_2SO_4 for 30 min with bumping chips. After that the sample was filtered through muslin cloth until the washings were free from acid. The residue was again boiled with 200 ml of 1.25% NaOH for 30 min. Then again the residue was washed with boiling water followed by 25 ml of alcohol until the washings were free from water. After that the residues were transferred to preweighed ashing dish (W_1,g). After that the residue was dried for 2h at 130±2°C, in a desiccator and weighed ($W_{2,}$g) cooled at room temperature. Ignite for 30 min at 600±15°C then the sample was cooled in a desiccator and weighed ($W_{2,}$g). The sample was cooled in a desiccator and reweighed ($W_{3,}$g).

Calculation

Loss in weight×(W_2-W_1)- ($W_{3-}W_1$)

% Crude fibre content=on ignition/Weight of sample(g)×100.

Protein content

Nitrogen was estimated by Kjeldahl method inKjeldahl system (Model:Kel plus 20L, Pellican Equipment, India) with steam distillation and titrated with standard 0.01 M HCl solution.

Calculation

% Nitrogen = 14 × (Normality of acid) × (Titrant value burette reading) ×100/Sample weight×1000

For this crude protein was estimated by multiplying the crude nitrogen content by a factor of 6.25 (% Protein= % Nitrogen × 6.25).

Carbohydrate content

Carbohydrate content of the samples was estimated by anthronemethod. [17] The sample (0.5g) was homogenized with hot 80% ethanol to remove sugars and centrifuged until the washings did not givecolour with anthrone reagent. To the residue 5ml of water and 6.5ml of 52% perchloric acid was added. The standards were prepared by taking 0.2 to 1.0 ml of working standards and the volume was made up to 1ml in each tube with water. Then 4ml of anthrone reagent was added to each tube and heated in boiling water bath. Then it was cooled rapidly and the intensity was read at 630 nm.

Table 2 Proximate composition of the selected green leafy vegetables of Assam

Sl. No	Parameters	*Basella rubra*	*Basella alba*	*Diplazium esculentum*	*Moringa oleifera*	*Brassica juncea*	*Chenopodium album*	*Amaranthus viridis*	*Brassica nigra*
1.	Edible portions, (g/100g)	28±0.02[a]	40±0.04[b]	54±0.03[c]	70±0.06[d]	79±0.07[e]	54±0.02[c]	80±0.05[f]	87±0.04[g]
2.	Moisture content (%)	88.44±0.004[b]	88.46±0.029[b]	71.74±0.417[a]	98.20±0.046[d]	92.22±1.003[c]	88.55±0.009[b]	88.01±0.421[b]	89.95±0.476[b]
3.	Lipid content (%)	0.61±0.010[d]	0.66±0.010[e]	0.34±0.020[c]	3.01±0.010[g]	2.99±0.020[f]	0.19±0.011[a]	0.27±0.011[b]	4.19±0.005[h]
4.	Ash content (g/100g)	20.76±0.01[g]	8.23±0.01[a]	14.42±0.01[c]	8.93±0.01[b]	26.01±0.01[h]	20.44±0.01[f]	20.20±0.01[e]	18.41±0.05[d]
5.	Crude fibre (g/100g)	8.61±0.014[f]	2.34±0.017[d]	4.45±0.013[e]	0.25±0.011[a]	1.35±0.017[b]	1.70±0.005[c]	0.25±0.016[a]	1.34±0.017[b]
6.	Carbohydrate content(mg/100g)	8.36±0.05[c]	12.26±0.04[f]	6.12±0.08[a]	8.92±0.03[d]	7.95±0.06[b]	9.04±0.03[d]	9.40±0.03[e]	6.03±0.06[a]
7.	Protein content (g/100g)	5.28±0.03[b]	6.09±0.03[c]	7.05±0.04[d]	6.10±0.09[c]	3.59±0.89[a]	6.31±0.04[c]	6.89±0.03[c]	6.03±0.08[c]

(Results were expressed as mean of three replications ± standard deviation and values followed by different letters are significantly ($P \leq 0.05$) different from each other)

Energy content

Energy contents in the samples were calculated as described by Oshea and Maguire [18]. A composite 80 mesh sample weighing 0.125 g was oxidized with 20 ml of 1.5 N potassium dichromate and 40 ml of concentrated H_2SO_4 for one and half hour and the total volume was raised to 250 ml. Then to a 25 ml aliquot 10 ml of potassium iodide and sodium bicarbonate were added and kept in dark for 20 min. The contents were diluted with 15 ml of distilled water, and the iodine liberated was titrated against standard 0.15 N sodium thiosulphate using 2 to 3 drops of starch indicator. Duplicate blanks were run for each set. The amount of $K_2Cr_2O_7$ used for oxidizing the sample was calculated by subtracting the above reading from the blank.

The results were expressed in kcal/100 g of leaf sample.

The calorific value was calculated using the following relationship

$$\text{Calorific value(kcal)} = \frac{\text{ml of 0.15N } Na_2S_2\ O_3}{C}$$

Where C is the coefficient of oxidation. The coefficient varies with the protein content of the food material, and is obtained from either of the two equations.

$C= 23.39-0.069p+0.000226p^2$……………….. (i)

$C=24.02-0.1055+0.000621p^2$……………….. (ii)

Where P is the percentage protein of the substance oxidized. Equation (i) is applicable to substances whose fat content is above 10 percent. In the present investigation the equation (ii) was used and the calorific values of different leaf genotypes were calculated.

Antioxidant activity

Antioxidant activity was estimated by DPPH radical scavenging activity according toYen and Chen.[19]For DPPH scavenging activity assay, 4 ml of the methanolic extract was mixed with 1ml of 1mM of DPPH and kept at room temperature for 30 min. The absorbance was read at 517 nm. As, control, 1mMof DPPH scavenging activity was the quotient of the absorbance of the sample and that of the control expressed in percentage.

Ascorbic acid

Ascorbic acid content was estimated by titrimetric method.[20]Pipette out 5ml of the working standard solution into a 100 ml conical flask. Then add 10 ml of 4% oxalic acid and titrate against the dye (V_1 ml). End point is the appearance of pink colour which persists for few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. The sample was extracted in 4% oxalic acid and centrifuged. The volume was made upto100 ml. Pipette out 5 ml of this supernatant, add 10 ml of 4% oxalic acid and titrate against the dye (V_2 ml)

Calculation

Amount of ascorbic acid (mg/100g sample)

$$= \frac{0.5mg}{V_1} \times \frac{V_2}{15\,ml} \times \frac{100\,ml}{Wt of the sample} \times 100$$

β- carotenoid

β- carotenoid was determined spectrophotometrically according toJensen.[21]A known amount of the fresh material (2g) was grounded with 20 ml of distilledacetone and filtered through Whatman No 42 filter paper. The procedure was repeated until the tissues were free

from pigments. The filtrates were pooled thrice with equal volume of peroxide free ether using a separatory funnel, the combined ether layers were evaporated under reduced pressure at 35°C in a rotary evaporator and the residue were dissolved in minimum quantity of ethanol, to that 60% of aqueous KOH was added at a rate of 1ml for every 10 ml of the ethanol extract to saponify and the mixture was kept in dark at room temperature. Then equal volume of water was added and partitioned twice with ether. Then the combined ether layers were evaporated and the residues were dissolved in minimum volume of ethanol. After that the absorbance of the solution was calculated at 450 nm using a calibration curve prepared against a high purity β- carotene.

$$C=\frac{D\times V\times f\times 10}{2500}$$

Where, C = Total amount of carotenoids (mg)

D = Absorbance at 450 nm

V = Volume of the original extract in ml

f = Dilution factor and

2500 = Average extinction coefficient of the pigments.

Folic acid

The folic acid were estimated according to Schiaffino et.al [22] method In a 100ml volumetric flask 1 gm of sample is added with 50 ml of K_2HPO_4 solution and the mixture is heated in a temperature below 60°C with swirling till the mixture is completely dispersed. The mixture is then cooled at room temperature and the volume is made upto 100ml with K_2HPO_4 solution. The solution is then centrifuged and the supernatant is retained. In a series of test tubes a known amount of supernatant is added in the tubes ranging from (0.2ml-1.0ml). The volume in these test tubes is made up to 1 ml using K_2HPO_4 solution, in these tubes 1ml of $NaNO_2$ is added along with 1ml of 5N HCl and this mixture is allowed to stand for 2 min. In

this mixture Ammonium sulphamate solution ($H_6N_2O_3S$) is added with continuous swirling. After that 1ml of N-(1-napthyl) ethylenediaminedihydrochloride solution is added in all the tubes and allowed to stand for 10 min after that 1g of NaCl and 10 ml of isobutyl alcohol is added, the mixture is then vortexed vigorously for 2-3 min this mixture is then centrifuged and about 9ml of clear supernatant is separated. The colour of the supernatant is read in a spectrophotometer at 550nm using isobutyl alcohol as blank

Calculation

Folic acid present in the sample is calculated using the expression

$$0.4\ C = \frac{A_1 - A_3}{A_2 + A_3 - (A_1 + A_4)}$$

Where C=Concentration of working standard of folic acid in mg/ml and A_1,A_2,A_3 and A_4 are the absorbance of tubes 1, 2,3 and 4, respectively

Total flavonoid compounds

Total flavonoid content was determined using $AlCl_3$ method as described by Meda et al.[23]To 0.1 ml of extract, 0.4ml of distilled water was added, followed by 0.1 ml of 5% $NaNO_2$. After 5min, 0.1 ml of 10% $AlCl_3$ was added followed by 0.2 ml of 1 M NaOH and the volume was made up to 2.5 ml with distilled water. Absorbance was measured against reagent blank at 510 nm. For the standard solution a stock solution of quercetin 1 mg/ml was prepared. Then the aliquots of 0.2, 0.4, 0.6, 0.8, and 1 ml were taken and made up to a total of 2 ml. from this range of concentration, 0.1 ml was taken into different test tubes. The standard curves were prepared using 100, 200, 300, 400, 500 µg/ml solutions of quercetin equivalent (mg/g of sample), which is a common reference compound.

Total phenolic content(TPC)

Total phenolic content were estimated using Bray and Thorpe, [24]method. TPC reacts under alkaline condition with phosphomolybdate under alkaline condition resulting the formation of blue coloured complex the blue coloured complex so formed is measured calorimetrically at 650 nm. For this experiment 1 g of the sample is weighed and grinded with mortar and pestle with ten times the volume with 80% ethanol the homogenate obtained is centrifuged at 10,000 rpm for 20 min the supernatant is extracted and evaporated to dryness, the residue is dissolved with 5 ml of distilled water. Now from this test tube 0.2-2ml of the aliquots were pipetted out in different test tubes. The volume of all the tubes is made up to 3ml with distilled water and 0.5 ml of Folin-Coicalteu reagent is added. After 30 min 2ml of 20% Na_2CO_3 solution is added to each tube then each tube were thoroughly mixed and placed in boiling water bath for 1 min and cooled to room temperature and the absorbance is read at 650 nm against a reagent blank.

Calculation

From the standard curve the concentration of the unknown is found out and expressed in mg/100g.

Minerals analysis

All the green leafy vegetable samples were analyzed using atomic absorption spectrophotometer (Model: Thermo Scientific, ICE 3000 Series, Newington, USA) for determination of Na, K, Ca, Mg, Cu, Mn, Zn and Fe. The method was by direct sample digests using air and acetylene flame. The calculations of the minerals were based on the comparison

of absorption of samples against known concentration of standards and results were converted to mg /100g.

Antinutritional analysis

Oxalic acid was estimated by titration method.[25] Tannins were estimated spectrophotometrically (Model No Cecil CE 7400 series 7000, Aquarious double unit) by Folin-Denis method.[26]Phyticacid was estimated by Wheeler and Ferrel[27] method.

Oxalic acid

The green leafy vegetables were dried in 80°C in hot air oven until it achieved a constant weight the tissues were finely powdered in a mortar and pestle and from there 0.5 g of sample was weighed. The sample was mixed with 1g of asbestos and 1.5 ml of 4N, H_2SO_4 was added. Then the sample was mixed with these reagents and transferred to a soxhlet apparatus and extracted with 500ml of diethyl ether for 48 hours, 5ml of 1N NaOH and 7 ml of water was added to extract the oxalic acid. The extract was evaporated in a rotary evaporator, and the aqueous phase was transferred in centrifuge tubes 4ml of calcium- chloride acetate bufferwas added and allowed to stand overnight. Then the centrifuge tubes were centrifuged at 3000 rpm for 10 min. The supernatant was discarded and thepellet was saturated with 5ml of 5% acetic acid and calcium oxalic acid and centrifuged.The residue was dissolved in 5ml of 4N H_2SO_4 and heated at 80°C on a water bath filtered and titrated against standard 0.02N potassium permangate solutions.

Calculation

The amount of oxalic acid (mg/100g) present in the sample was calculated using the relationship: 1ml of 0.02 N Pottasium permanganate = 1.2653 mg of oxalic acid

Phytic acid

The sample was finely ground and the sample was estimated to contain 5to 30 mg phytate P into an Erlenmeyer flask. Extracted in 50 ml of 3% TCA for 30 min with mechanical shaking and occasional swirling with hand for 45 min. After mechanical shaking the suspension was centrifuged. $FeCl_3$ (4ml) solution was added by blowing rapidly from the pipette. The content was heated in boiling water bath for 45 min after that it was centrifuged for 10-15 min and the supernatant was decanted, the precipitate was washed twice by dispersing well in 25 ml of 3% TCA heated in boiling water bath for 5-10 min and centrifuged. The washings were repeated with water and the precipitate was dispersed in few ml of water and 3ml of 1.5 N NaOH with mixing. The volume was brought to about 30 ml with water and heated in boiling water bath for 30 min and filteredwith Whatman No 2 filter paper when it was moderately hot. The precipitate was washed with 60- 70 ml of hot water and the filtrate was discarded. The precipitate was dissolved in 40 ml hot 3.2 N HNO_3 into a 100 ml of volumetric flask. The paper was washed with several portions of water and collected in the same flask. A 5ml aliquot was transferred to another 100ml volumetric flask and diluted to approximately 70 ml and 20 ml of 1.5 M, KSCN was used to dilute the volume and the colour was read immediately at 480 nm.

Tannin content

A sample of 0.5g was powdered and transferred to a 250 ml of conical flask to that 75ml of water was added and heated for 30 min then the mixture was centrifuged at 2000 rpm for 20 min the supernatant was collected in a 100 ml volumetric flask and the volume was made up. From the volumetric flask 1ml of the sample was transferred to a volumetric flask containing 75 ml of water. To this 5ml of Folin-Denis reagent was added and 10 ml of sodium

carbonate was also added and diluted to 100 ml with water.The solution was shaken well and the absorbance was read at 700 nm after 30 min.

Saponin content

A sample of 0.5 g was added to 20 ml of 1N HCL and was boiled for 4 h. After cooling it was filtered and 50 ml of petroleum ether was added to the filtrate for ether layer and evaporated to dryness. 5ml of acetone was added to the residue and 0.4ml of each was taken into three different test tubes. 6ml of ferrous sulphate reagent was added into them followed by 2ml of conc. H_2SO_4. It was thoroughly mixed after 10 minutes and the absorbance was taken at 490 nm.[28]

Statistical analysis

All the experiments were conducted in triplicates and were evaluated by one-way ANOVA. Values of $P \leq 0.05$ were considered as statistically significant. Statistical evaluations were carried out using SPSS 16.0 software package for windows.

Results and discussion

Nutritional content

The edible portions, moisture, ash, lipid, crude protein, crude fibre, carbohydrates are reported in Table 3.2.The edible portions of the green leafy vegetables ranged within 28-87 g / 100 g of fresh vegetables. Moisture content of the vegetables ranged from 71.74-98.20 % wet basis, highest being in *Moringa oleifera* (98.20 g / 100 g) and lowest in *Diplazium esculentum* (71.74 g / 100 g).

Moisture content

Water is one of the most important nutrients which are abundantly available in the human body. Three fourth of human body comprises of water .Even water helps to separate (by a process called hydrolysis) phosphate group from adenosine triphosphate (ATP) or guanosine

triphosphate (GTP) to get energy.[29] It was also reported by Mepha et al.[30] that water is the solvent for electrolytes and all other ions throughout the human body. Badauet al.[31] reported that higher moisture content provides greater activity of water soluble enzyme and coenzyme, which is needed for metabolic activity of leaves.

The moisture content obtained in leafy vegetables was close to the values reported by Gupta and Prakash [32], where they studied to formulate micronutrient rich products with dried greens of *Amaranthus peniculatus* and *Peucedanum graveolens*.

Ash Content

Minerals are the constituents which remain as ash after combustion of plant and animal tissue. Mineral supply depends not only on the intake in food but primarily on the bioavailability, which is essentially related to the composition of food.The importance of minerals as food ingredients depends not only on their nutritional and physiological roles but they also contribute to food flavor and activate or inhibit enzyme-catalyzed other reactions, and they also affect the texture of food.[33]

The values of ash content ranged between 0.16-4.06 g /100g, highest in *Diplazium esculentum*(4.06 g / 100 g) and lowest in *Brassica juncea* (0.16 g / 100 g), which are in line with Aletoret al.[34], where they studied the chemical composition and functional properties of the leaf protein concentrates of commonly available leafy vegetables viz.*Vernonia amygdalina* (Bitter leaf), *Amaranthus hybridus* (Green tete) and *Telfaria occidentalis* (Fluted pumpkins).

Lipid content

Lipid content ranged between 0.19-4.19 g / 100 g, which is very low as compared to the leaves of *Anona senegalensis*(24.0%) reported by Yisaet al. [35]*Talinum triangulare*(5.90%), *Amaranthus hybridis*(4.80%) and *Basella alba* (8.71%) reported by Mzirayet al.[36],respectively.

Fibre content

Fibre assists and speeds up the excretion of waste and toxins from the body, preventing them from sitting in the intestine or bowel for too long, which could cause a build-up and lead to several diseases.[37]

Fibre consumption helps in softening stool and lowering of plasma cholesterols in the body. It also plays a physiological role in maintaining the internal distension for a normal peristaltic movement of intestinal tract. However, it is also reported that vegetables which has high fiber content may cause intestinal irritation and decrease the nutritional bioavailability .[38] *Amaranthus viridis* showed the highest crude fibre value(8.61 g/100g) and lowest was found to be in *Moringa oleifera* (0.25 g/100 g). The results were comparable with the works of Aletor et al.[34]

Protein content

Proteins are important constituents of food. They supply the required building blocks for protein biosynthesis. In addition, they directly contribute to the flavor of food and are precursors for aromatic compounds and colours formed during thermal or enzymatic reactions in production, processing and storage of food.[33] *Diplazium esculentum* was found to have the highest amount (7.05 g/ 100 g) and *Brassica juncea* was found to have lowest protein content of 3.59 g/100 g and are line with Agbaire.[39] where they studied the nutritional and antinutritional levels of some local vegetables viz. *Vernomia anaydalira, Manihot esculenta, Teiferia occidentalis, Talinum traingulare and Amaranthus spinosus* and Abidimi et al.[40] where they compared proximate composition of shea butter leaf with some raw Nigerian leaf.

Carbohydrate content

The high carbohydrate content in food means high energy content, which helps in digestion and assimilation of other foods. They are also responsible for carrying out daily activities in day to day life.[41,35]It is also responsible for break-down of fatty acids and preventing ketosis.[42]Carbohydrate contents of all green leafy vegetables ranged between 6.03 g/100g to 12.26 g/100 g. *Basella alba* had the highest 12.26 g/100g and lowest in *Brassica nigra* 6.03 g/100 g which can be compared with the works of Joshi and Mehta.[43]

Calorific value

The calorific value of green leafy vegetable ranged between 195.00 Kcal to 541.33 Kcal, (Table 3) and highest being in *Amaranthus viridis* and lowest in *Diplazium esculentum*. Similar values were found in *Vitexdoniane (*194.03 Kcal)*, Limosella aquatic* (152.00 Kcal)and *Corchorus olitorius* (184.00 kcal). [44]

Antioxidant activity

Nowadays, it is believed that regular consumption of dietary antioxidants reduces the risk of several serious diseases.[45] Regular consumption of fruits and vegetables has always been associated with health benefits, but their mechanism of action has become clear only in the recent decades. Researchers have estimated that every serving of fruits and vegetables consumption reduces the risk of cancer by 15%, cardiovascular disease by 30% and mortality of any cause by 20%. This has been confirmed by epidemiological studies.[46, 47] Antioxidant activity, ascorbic acid, β-carotenoid, and phenolic contents are presented in Table 3. All the green leafy vegetables showed appreciable free radical scavenging activity. Antioxidant activity was almost same for all the samples but *Brassica nigra* (98.30%) showed the highest free radical scavenging

activity and *Chenopodium album* showed the lowest (94.6%). Similar results were reported by Gupta and Prakash [32] in Indian green leafy vegetables for their antioxidant activity.

Ascorbic acid content

There was not much difference found in the ascorbic acid content of the green leafy vegetables (Table 3). *Diplazium esculentum* was found to be rich source of ascorbic acid, having 42.22 mg / 100 g and lowest in *Basella rubra* 24.32 mg / 100 g. The results of ascorbic acid content found in the present study are comparatively higher than the values reported by Dansi et al. [48]in their studies on *Vernonia amygdalina* (13.41 mg/100 g) and *Cleome gynandra*(14.00 mg/100g) but can be comparable with the works of Lawal.[49] It is also required for the synthesis of collagen.[50]

β-carotenoid content

Moringa oleifera had the highest content of β-carotenoid 26.90 mg / 100 g and lowest in *Brassica nigra* 13.72 mg/100g. The results are similar with the works of Negi and Roy [51] on fenugreek (15.0-26.5 mg/100g). Kaur et al.[52] reported similar results when they studied physicochemical properties of mustard, mint and spinach respectively.

Folic acid content

The Folic acid content estimated of the eight samples is presented in Table3. The highest Folic acid content estimated was found to be in *Diplazium esculentum* (0.25 mg/100g) and the lowest folic acid content was estimated in *Basella alba* (0.13 mg/100g). The present results can be corroborated with the findings of Igileet al.[53] in *Vernonia calvaona* a green leafy vegetable of Nigeria. Folic acid is important vitamins required in the blood plasma of women's

during pregnancy it maintains the neural tube defects during pregnancy it is also an important vitamin required during normal delivery of babies.

Total phenolic content (TPC)

Green leafy vegetables are good source of phenolic content (Table3). *Amaranthus viridis* had the highest phenolic content of 10.84 mg/g and *Moringa oleifera*(3.16 mg /g) recorded the lowest the results can be corroborated with the findings of Amin et al[54], where they studied the antioxidant activity and phenolic content of raw and blanched *Amaranthus* species. Total phenolic contents of the all leafy vegetables were almost similar, and are comparable with the works of Saxena et al.[55] and Ng et al.[56], where they worked on nutritional profile and antioxidative properties of selected tropical wild vegetables. Phenolic compounds are nonnutritive secondary metabolites which are beneficial to humans to prevent various diseases. It is reported that phenolic compounds are able to protect human cells against oxidative damage and also possesses anti carcinogenic properties.[57,58]

Flavonoid compounds

Flavonoids arethe plant pigments that are synthesized from phenylalanine[59]and generally exhibit colors in the flowering parts of the plant. As a dietary supplement they are thought to have health promoting properties as they are rich sources of antioxidants both invivo and *invitro* activities.[59]The flavonoid content of the sample (Table 3) ranged between 0.364 to 0.572 mg/100 g. The lowest flavonoid content was recorded in *Moringa oleifera* and the highest was recorded in *Brassica nigra*. The present results can be comparable with the works of Adesina[60] where he worked with three green leafy vegetable consumed in Ekiti State of Nigeria, Namely *Cucurbita maxima, Amaranthus viridis* and *Basella alba.*

Table 3: Nutritional parameters of selected green leafy vegetables of Assam

Sl No	Parameters	*Basella rubra*	*Basella alba*	*Diplazium esculentum*	*Moringa oleifera*	*Brassica juncea*	*Chenopodium album*	*Amaranthus viridis*	*Brassica nigra*
1.	Ascorbic acid (mg/100 g)	24.32±0.07^{a}	25.26±0.05^{b}	42.22±0.19^{g}	36.45±0.07^{e}	40.10±0.09^{e}	35.27±0.67^{d}	41.58±0.75^{f}	32.36±0.34^{c}
2.	Anti oxidant activity(%)	95.81±0.03^{b}	96.25±0.07	95.18±0.02^{b}	96.76±0.05^{c}	95.96±0.06^{b}	94.6±0.20^{a}	95.04±0.06^{b}	98.30±0.04^{d}
3.	Total Phenolic content(mg/ 100g)	6.26±0.05^{d}	6.71±0.04^{d}	5.31±0.03^{c}	3.16±0.04^{a}	7.02±0.05^{e}	5.88±0.05^{c}	10.84±0.04	4.15±0.05^{b}
4.	βCarotenoid (mg/100g)	19.02±0.03^{b}	19.09±0.06^{b}	19.31±0.03^{b}	26.90±0.28^{e}	20.12±0.03^{c}	22.60±0.07^{d}	19.60±0.08^{b}	13.72±0.05^{a}
5.	Folic acid content(mg/ 100g)	0.16±0.05^{c}	0.13±0.03^{a}	0.25±0.07^{h}	0.24±0.02^{g}	0.22±0.08^{f}	0.18±0.01^{d}	0.15±0.09^{b}	0.21±0.06^{e}
5.	Flavanoid content (mg/100g)	0.463±0.04^{c}	0.483±0.05^{d}	0.384±0.06^{b}	0.364±0.24^{a}	0.521±0.04^{e}	0.534±0.07^{f}	0.546±0.03^{g}	0.572±0.09^{h}
6.	Calorific value (kcal)	375.00±3.60^{d}	402.66±2.51^{e}	195.00±2.00^{a}	293.66±6.40^{c}	267.66±1.15^{b}	425.33±2.51^{g}	541.33±1.52^{h}	413.66±1.15^{f}

(Results were expressed as mean of three replications ± standard deviation and values followed by different letters are significantly ($P \leq 0.05$) different from each other)

Mineral content

The mineral contents of the green leafy vegetables were statistically significant ($P \leq 0.05$), and found to be the rich sources of minerals (Table 4). Iron content was found highest in the order *Brassica nigra* (241.20 mg/100 g), *Brassica juncea* (118.50 mg / 100 g), *Amaranthus viridis*(118.13 mg/100 g), *Basella alba* (90.80 mg / 100 g) and *Chenopodium album* (85.46 mg / 100 g) respectively. While the rest of the green leafy vegetables had iron content in the range of 29.40-57.37 mg / 100 g. Iron content analyzed in the present study were similar to the values as reported by Singh et al.[61], where they studied the nutritional compositions of selected green leafy vegetables, herbs and carrots of Haryana, India.

Of the eight green leafy vegetables analyzed, *Moringa oleifera* contained the highest amount of potassium (75.33 mg /100 g) and *Chenopodium album* had the lowest amount (70.70 mg / 100 g), the rest had the potassium contents in the range of 71.36-74.46 mg / 100 g. Nair et al.[62] reported that the high amount of potassium increases the iron utilization and is also beneficial to the people taking diuretics to control hypertension.

In all the samples studied the calcium content was found to be in the range of 36.60-67.93 mg/100 g. *Moringa oleifera* had the maximum amount and *Brassica juncea*recordedthe minimum amount. Calcium is an important mineral for human beings, which provides good strength of bones and teeth.[63]It plays an important role in blood clotting, muscles contraction, and neurological function and also helps in enzymatic metabolic processes.[64]

The sodium content of green leafy vegetables ranged between 29.00- 116.66 mg/100 g highest was found in *Basellarubra* and lowest in *Diplazium esculentum*. The results are comparable with the works of Gopalanet al.[65], where they studied the proximate nutritive contents of Indian foods. Alinnor and Oze[66] reported that sodium is an important mineral which assists in the

regulation of the body fluids and maintenance of electrolyte balance in the body. *Chenopodium album* (3.80 mg /100 g)*, Basella rubra* (2.80 mg / 100 g), *Basella alba* (2.60 mg/100g) *Brassica juncea* (2.60 mg/100 g), *Moringa oleifera* (2.40 mg/100 g)and *Amaranthus viridis* (2.40 mg/100 g) evinced the highest in the order of copper content and *Diplazium esculentum* (1.7 mg/100 g) recorded the lowest. The copper content of the green leafy vegetables in the present study is similar to the works done by Barminas et al.[67], where they studied the the mineral composition of non-conventional leafy vegetables like *Moringa oleifera, Adansoni adigitata, Colocasia esculenta* and *Cassiatora*. Alinnor and Oze[66] opined that copper is required for enzyme production in the body and helps in biological electron transport.

The magnesium level of the green leafy vegetables was found in between (15.30- 16.70 mg/100 g). Similar values were reported by Salazar et al.[65], on chemical composition and antinutritional factors of *Lycianthes synanthera* leaves (chomte). According to Alinnor and Oze.[66]Magnesium plays an important role in calcium metabolism in bones and also prevents circulatory diseases. It also helps in regulating blood pressure and insulin release.

Amaranthus viridis contained the highest amount of manganese (224.46 mg/100 g) and the rest of the green leafy vegetables varied within 21.11 mg /100 g to 132.27 mg/100 g). *Diplazium esculentum* had the least content (21.11 mg/100g), which are comparable with the works of Odhava*et al.*[69] on *Bidens pilosa* (21 mg / 100 g) and *Centella asiatica* (23 mg / 100 g), *Chenopodium album* (27 mg / 100 g), *Portulaca oleracea* (24 mg /100 g), respectively.

Table 4: Mineral contents of the selected green leafy vegetables of Assam

Green leafy vegetables	Iron (mg/100g)	Zinc (mg/100g)	Copper (mg/100g)	Manganese (mg/100g)	Sodium (mg/100g)	Potassium (mg/100g)	Calcium (mg/100g)	Magnesium (mg/100g)
Basella rubra	57.37±0.02^{c}	3.13±0.30^{b}	2.80±0.025^{d}	132.27±1.05^{g}	116.66±1.50^{g}	71.36±0.665^{b}	65.30±0.45^{e}	16.13±0.15^{c}
Basella alba	90.80±0.06^{e}	3.50±0.26^{b}	2.60±0.045^{c}	74.20±1.73^{d}	52.33±0.64^{c}	73.30±1.00^{c}	62.00±0.08^{c}	16.20±0.20^{b}
Diplazium esculentum	38.20±0.07^{b}	4.30±0.26^{c}	1.70± 0.20^{a}	21.11±0.87^{a}	29.00±1.38^{a}	74.46±1.36^{e}	52.66±0.49^{b}	15.30±0.20^{a}
Moringa oleifera	29.40±0.07^{a}	1.50±0.25^{a}	2.40±0.25^{b}	43.27±0.88^{b}	43.33±1.15^{b}	75.33±1.15^{f}	67.93±0.16^{g}	16.03±0.15^{b}
Brassica juncea	118.50±0.07^{g}	7.50±0.30^{f}	2.60±0.15^{c}	45.97±1.28^{c}	51.20±0.85^{c}	73.47±1.05^{d}	36.60±0.24^{a}	16.33±0.20^{d}
Chenopodium album	85.46±0.06^{d}	7.50±0.25^{f}	3.80±0.26^{e}	94.44±1.21^{e}	78.30±2.45^{e}	70.70±0.36^{a}	64.10±0.16^{d}	16.23±0.20^{d}
Amaranthus viridis	118.13±0.05^{f}	6.10±0.26^{e}	2.40±0.20^{b}	224.46±1.50^{h}	94.66±1.42^{f}	73.08±1.00^{a}	66.70±0.49^{f}	16.70±0.20^{e}
Brassica nigra	241.20±0.03^{g}	5.50±0.26^{d}	1.90±0.15^{a}	102.73±0.58^{f}	62.06±1.67^{d}	73.83±0.76^{d,e}	67.13±0.22^{f}	15.96±0.15^{b}

(Results were expressed as mean of three replications ± standard deviation and values followed by different letters are significantly ($P \leq 0.05$) different from each other)

Highest zinc content was recorded in *Brassica juncea* and *Chenopodium album* (7.50 mg/100g) and lowest was found in *Moringa oleifera* (1.50 mg /100 g). Zinc content present in the study can be supported with the works done by Singh et al.[61]Shankar and Prasad[70] reported that zinc plays an important role in cellular and humoral immunity.

Antinutritional content

Green leafy vegetables play an important role in the diet of humans as they provide the necessary vitamins, and minerals.[71]However, they also contain antinutritional components which reduces the bioavailability of these nutrients.[72,73]

Oxalic acid

Oxalic acid, tannin and phytic acid are antinutritional factors, which are widely found in plant foods. Comparatively *Amaranthus viridis* (10.73 mg/100 g), *Chenopodium album*(10.34 mg/100g) *Brassica junceae* (7.93 mg/100 g)and *Brassica nigra* (6.35 mg/100 g) revealed more oxalic acid contents and *Diplaziume sculentum* (3.56 mg/100 g) was found to have the lowest oxalic acid content (Table 5). The results are in line with the works of Gupta *et al.* (2005)[71], where they analyzed the nutrient and antinutrient contents of underutilized green leafy vegetables.

Table 5 Antinutritional parameters of selected green leafy vegetables of Assam

Sl No	Parameters	*Basella rubra*	*Basella alba*	*Diplazium esculentum*	*Moringa oleifera*	*Brassica juncea*	*Chenopodium album*	*Amaranthus viridis*	*Brassica nigra*
1.	Phytic acid content(mg/100g)	368 ± 3.00^{c}	378 ± 4.00^{d}	289 ± 2.00^{a}	568 ± 3.60^{e}	349.3 ± 6.11^{b}	397.6 ± 5.5^{d}	344 ± 3.00^{b}	489.6 ± 3.05^{e}
2.	Tanin content(mg/100g)	1.24 ± 0.03^{b}	1.28 ± 0.03^{b}	1.21 ± 0.04^{a}	1.34 ± 0.03^{b}	1.30 ± 0.02^{b}	6.33 ± 0.02^{d}	4.41 ± 0.04^{c}	6.18 ± 0.02^{d}
3.	Oxalic acid content(mg/100g)	6.30 ± 0.02^{c}	6.18 ± 0.03^{c}	3.56 ± 0.05^{a}	5.48 ± 0.03^{b}	7.93 ± 0.03^{d}	10.34 ± 0.03^{e}	10.78 ± 0.03^{e}	6.35 ± 0.03^{c}
4.	Saponin content(mg/100g)	267 ± 0.04^{c}	258 ± 0.06^{b}	186 ± 0.07^{a}	325 ± 0.03^{d}	367 ± 0.09^{e}	385 ± 0.02^{f}	421 ± 0.08^{g}	433 ± 0.02^{h}

(Results were expressed as mean of three replications ± standard deviation and values followed by different letters are significantly ($P \leq 0.05$) different from each other)

Tannin content

Tannin content was highest in *Chenopodium album* 6.33 mg/100 g and lowest in *Diplazium esculentum*1.21 mg/100 g. The results are in line with the works of Gupta et al .[71]

Phytic acid

Phytic acid is a naturally occurring antinutritional factor mostly present in plant foods and it is also phosphorus storage compound present in green leafy vegetables. The advantage of phytic acid is on its effect in digestion and absorption of minerals. *Brassica nigra* evinced highest phytic acid content (489.6 mg /100 g) and *Diplazium esculentum* had the lowest phytic acid content (289 mg / 100 g). However, Yadav and Sehgal [72] reported 234.50 mg/100 g phytic acid content in *Chenopodium album* but there is a wide variation in the present study and the variation may be attributed to agro climatic condition.

Saponin content

Saponins are a group of low molecular weight secondary metabolites that are widely present in the plant kingdom they are glucosides with foaming characteristics that have been used as a surface active agents like detergents, pesticides, molluscicides, antiviral and

antifungal components. They also play an important role in immune system by fighting against cancer,arrest the formation of dental caries and also possesshypolipidemic activities they also have been used in the treatment of hypercalciuria and as an antidote against acute lead poisoning. The highest saponin content was observed in *Brassica nigra* (433 mg/100g) and lowest was observed in *Diplazium esculentum* (186 mg /100g). Similar results were reported in *Manihot esculenta* and *Basella rubra* leaves by Odufuwa et al.[73]

Conclusions

Table 6 Selection of *Diplazium esculentum* for further experiments based on protein content, nutritional and antinutritional parameters

Name of the plant	**Nutritional parameters (g/100g)**	**Antinutritional parameters (mg/100g)**
Diplazium esculentum	Protein content (7.05±0.04) Folic acid (0.25±0.07) Ascorbic acid (42.22±0.19)	Phytic acid (289±2.00) Tannin content (1.21±0.04) Saponin content (186±0.07) Oxalic acid (3.56±0.05)

Based on the analysis performed in this study we can conclude that the green leafy vegetables, which are mostly neglected, have a good potential in terms of food value and can serve as an easily accessible food resources. Among the eight green leafy vegetables selected *Diplazium esculentum*(Table 6) was found to be best based on the nutritional and anti nutritional screening, this plant was found to contain highest protein content, folic acid and ascorbic acid concentration among the selected GLV's also it had the lowest antinutritional contents such as oxalic acid, phytic acid tannin and saponin content. So based on this

profiling it was selected for further experiments .Green leafy vegetables are rich sources of proteins and minerals, but their utilization is limited. Variations in the chemical constituents may be attributed to species differences and different climatic conditions, and age of the plants. There are many green leafy vegetables available in this part of the country, whose nutritional profile are yet to be documented. More systematic study on these green leafy vegetables is required to exploit it industrially.

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Objective (ii): To study the extraction and optimization of leaf protein concentrate (LPC) from the GLV having highest protein content

Introduction

Protein deficiency

Protein deficiency is one of the major problems in the developing world.[1,2]Scientists across the world are facing a grim challenge to shorten this gap of demand and supply of protein deficiency by finding out new resources of plant proteins whichare most abundant in the world. Studies of plant proteins, as a non-conventional source has been on the increase, due to the new challenges of providing adequate protein for an expanding world population [3].A number of vegetable proteins have been tried for incorporation in various food products as functional and nutritional ingredients[4]. About 36 million people die every year as a result of hunger on contrary it has been reported by Gerloff et al.[5]that amino acid composition of leaf protein concentrate (LPC) is as good or as better than that of many common food stuffs. Many of the local vegetable materials are under exploited because of inadequate scientific knowledge of their nutritional potentials. Though several works reporting compositional evaluation and functional properties of various types of edible wild plants arein use in the developing countries but they are abound in literature, and much is still need to be done. Ekop[6]has reported the compositional evaluation and functional properties of various types of edible wild plants in use in the developing countries. *Diplazium esculentum* is an edible fern found throughout Asia and Oceania. It is perhaps the most commonly consumed fern.[7]It is known as *Pucuk paku* in Malaysia, *Paco* in Philippines, *Dhekia*in Assam, *Dhenkir shaak* in Bengali and *Linguda* in Northern India, referring to the

curled fronds [8]. In Thailand it is known as *Phakkhut*. It is reported to have mild amounts of fern toxins but no major toxic effects were recorded as reported by Gangwar,[9]. This plant has higher amount of bioactive compounds such as antioxidants, vitamins, proteins, carbohydrates and lipids Jovail et al. [10]

Ultrasound

Now a day's application of ultrasound is gaining much popularity in food industry because it has the ability to change some food properties which may have some technological advantage. Ultrasound is a type of sound wave that has the frequency above the threshold of human hearing (18 kHz).[11]Low intensity (20-100kHz) ultrasound has various applications in food industry such as it has lethal effect on microorganism so it is used in food preservation Entezari et al.[12] High intensity ultrasound has many applications in food industry namely emulsifying, sterilizing, extracting, degassing, filtrating, drying, and enhancing oxidation. [13,11]

Principle of ultrasound

High intensity ultrasound generates high alteration of pressure in the fluid medium leading in the formation of rapidly growing bubbles (cavities)[11] this expands during negative pressure excursion and implode violently during the positive execution which generates high temperature pressure this phenomenon is known as cavitation.[14]During this phenomenon the temperature reaches around 5500 K and pressure around 50 MPa.[11,14]

Extraction of protein

The amount of protein that is available from a particular source of food or non-food is called extractable proteins. One of the important factors which determine whether or not a protein source can be adopted commercially is by using the protein extraction efficiency of such protein.[15]In traditional optimization the process was achieved by the one-factor-at-a-time approach, which was time-consuming and could ignore the interactions between the variables. In the conventional extraction procedure, many variables such as the extraction time, temperature, and solid-liquid ratio (S/L) significantly influence the extraction efficiency. Ultrasonic-assisted extraction (UAE) has been widely employed in the extraction of valuable compounds from bio-mass since it has many advantages such as working at (or close to) ambient temperature, a higher efficiency than the conventional extraction methods, and at lower cost.[16] Ultrasonic assisted extraction has been studied in the extraction of biological compounds for different plant materials.[17,18,19] It has also been used as a low cost alternative for solvent reflux extraction of phenolic compounds from coconut shell.[20]

Response surface methodology

Response surface methodology is an effective statistical technique for optimizing complex processes it is one of the commonly used statistical techniques for designining of experiments for food processes and food formulations. Response surface methodology (RSM) has been shown to be an effective tool for optimizing processes by different workers.[21,22,23,24] Basically RSM relates product properties by using regression equations that describes

interrelations between input parameters and product properties. Colonna et al. [25]; Onwulata et al, [26] used RSM to optimize the conditions for an extruder, producing an amaranth-based snack food. Vatsala et al.[23] used RSM to analyze the effect of corn flour, green gram flour, xanthan, guar gum, arabic gum and carboxy methyl cellulose (CMC) on the sensory attributes (expansion ratio) of an extruded snack food and found that the responses were affected mostly by changing the levels of corn flour, green gram flour and guar gum and to a lesser extent by changing the levels of xanthan, arabic gum and CMC. Onwulata et al.[26] optimized the ingredients and process conditions for preparing puri using RSM. Some good examples of the appropriate applications of this technique were tried on textured products for the optimization of complex products, properties and many process variables[27,28,29] response surface methodology.

Materials and methods

Diplazium esculentum were collected from the local markets of Sonitpur district of Assam. The leaves were washed with running tap water followed by distilled water and weighed prior to pulping. Leaf protein concentrate wasobtained according to Fellows [30] with slight modification using sonication. Whereas for nonsonicated extraction the procedure of Fellows[30] was followed (Fig. 3).The leaf protein concentrate obtained was then hot air oven dried at 40°C for future chemical and functional property studies.

Protein extraction

The leaves were subjected to grinding with distilled water. The slurry derived after grinding was then subjected to filtration with muslin cloth, the filtrate obtained was subjected to pH adjustment with 1N, HCL and 1N, NaOH which was then subjected to ultrasound in bath

sonicator (BandelinSonorex, Z659800, Berlin) inbuilt with temperature adjustment. After this process it was centrifuged at 5000 RPM for 30 min the residue obtained was the leaf protein concentrate.

Experimental design

A central –composite experimental design with 4 variables was used to study the response pattern and to determine the optimum combination of variables. The variables were X_1(Ultrasonic time), X_2(Ultrasonic temperature), X_3(Solvent volume) and X_4 (pH) on the response of protein yield(Y). The process variables and their corresponding levels are shown in Table 6 and Table 7 represents RCCD experimental design with process variables. The variables were coded according to the following equation using a computer generated nonlinear quadratic equation which is as follows

$$Y = \beta_O + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} {X_i}^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j$$

Where Y is measured response associated with each factor level combination, β_0 is an intercept and β_1 is regression coefficient computed from the experimental values of Y and Xi,Xj is the coded levels of independent variables the terms $Xi.X_j$ and X_i^2 represents the interaction and quadratic terms respectively.

Table 7 : Processes variables and their corresponding levels

Independent variable	Codified	Uncodified	Level				
			-2	**-1**	**0**	**1**	**2**
Ultrasonic time (min)	x_1	X_1	2.5	15	27.5	40	52.5
Temperature (°C)	x_2	X_2	25	40	55	70	85
pH	x_3	X_3	1	4	7.5	11	14
Solvent concentration (ml)	x_4	X_4	15	40	65	90	115

Table 8: RCCD experimental design with process variables for sonicated treatment on protein yield

S/No	Time (x_1)	Temp (x_2)	pH (x_3)	Solvent volume(x_4)	Protein yield (%)
1.	-1	-1	-1	-1	10.61
2.	1	-1	-1	-1	10.84
3.	-1	1	-1	-1	13.85
4.	1	1	-1	-1	10.75

5.	-1	-1	1	-1	13.51
6.	1	-1	1	-1	20.16
7.	-1	1	1	-1	15.13
8.	1	1	1	-1	18.44
9.	-1	-1	-1	1	8.17
10.	1	-1	-1	1	9.11
11.	-1	1	-1	1	11.36
12.	1	1	-1	1	8.96
13.	-1	-1	1	1	11.64
14.	1	-1	1	1	18.99
15.	-1	1	1	1	15.61
16.	1	1	1	1	18.22
17.	-2	0	0	0	6.04
18.	2	0	0	0	10.29
19.	0	-2	0	0	20.75
20.	0	2	0	0	23.22
21.	0	0	-2	0	9.87
22.	0	0	2	0	20.78
23.	0	0	0	-2	14.98
24.	0	0	0	2	13.32

25.	0	0	0	0	31
26.	0	0	0	0	35
27.	0	0	0	0	34
28.	0	0	0	0	32.8
29.	0	0	0	0	35.56
30.	0	0	0	0	33.24

Protein determination

The protein content of the leaf protein concentrate was determined using the Kjeldahl method and multiplying the nitrogen content with protein conversion factor of 6.25 (Fig 3)

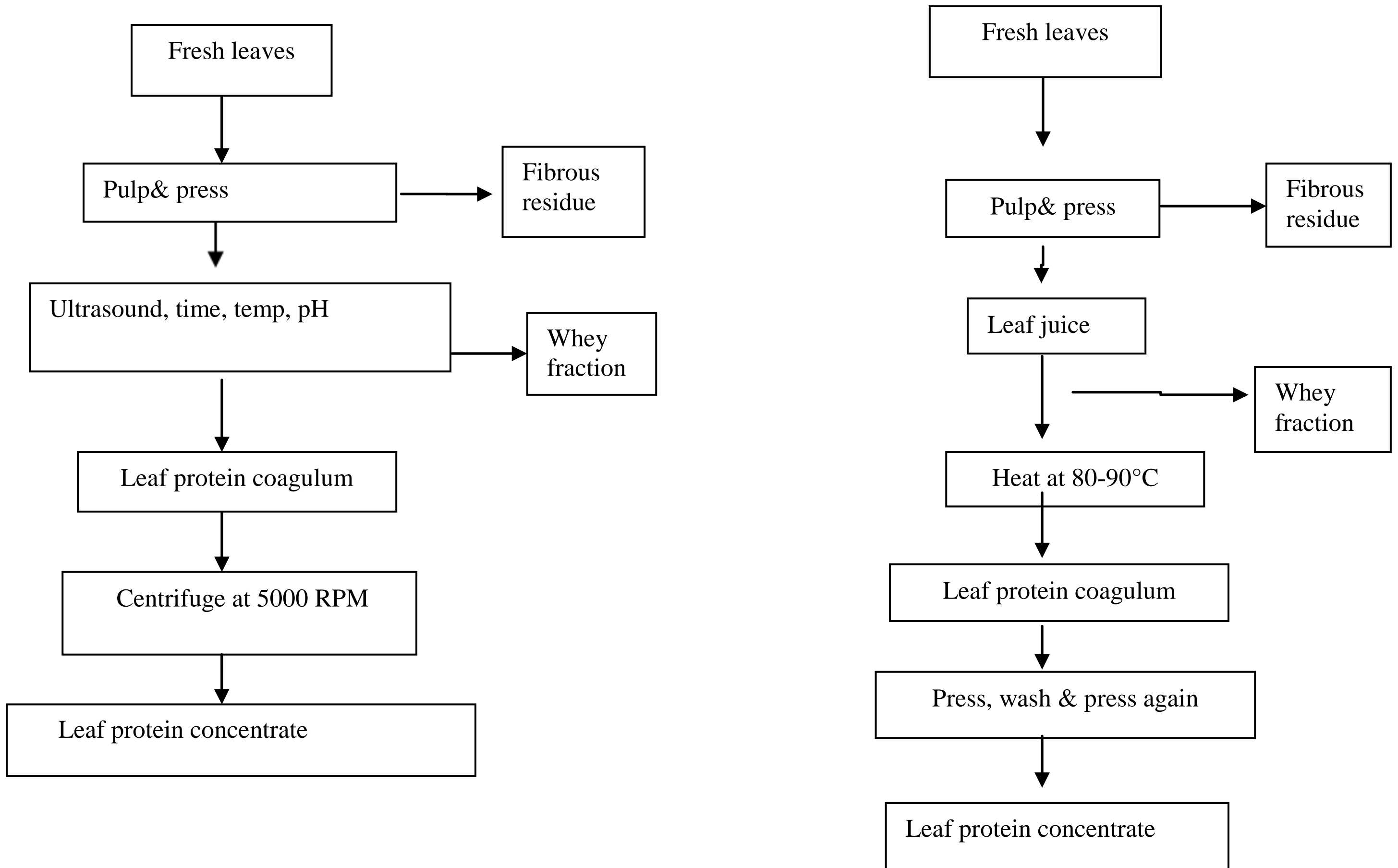

Fig. 3 Flowchart of leaf protein concentrates (LPC) production (A) sonicated and (B) nonsonicated (adapted from Fellows, 1987).

Data analysis

Data's were represented as means of 3 replicated determinations. The responses obtained from each set of experimental design were subjected to multiple non-linear regressions using design expert version 6.0.11 (Stat-ease, Minneapolis, MN, USA). The fit of nonlinear regression equation and significances of regression coefficient were evaluated by the coefficient of determination (R^2) p-value, respectively. Model verification for checking the predicted response was done using two-tailed, one sample t- test using MS Excel 2007.

Results and discussions:

t-test between the sonicated and non-sonicated extracted sample was done and the null hypothesis was rejected at 5% level of significance that means there is a significant difference between sonicated and nonsonicated extraction (Table 8). Even from the graph (Fig. 4 and 5) the difference can be observed. Hence the extraction done by using sonication was superior compared to nonsonicated extraction procedure.

Table 9: t-Test: Two-sample assuming equal variances

Mean	9.516	5.565
Variance	14.368	6.549
Observation	26	26
Pooled variance	10.458	
Hypothesised mean difference	0	
df	50	
T stat	4.405	
P(T≤t)one- tail	2.80×10^{-5}	
t-Critical one-tail	1.676	

*Significant at p≤0.05
**Significant at p≤0.01
***Significant at p≤0.0001

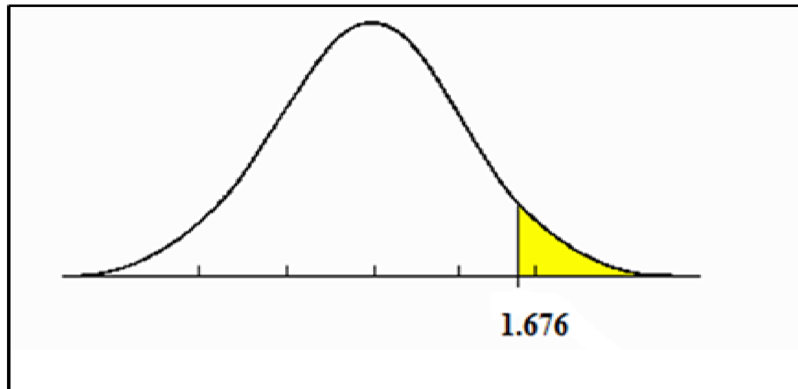

Fig. 4 One tail t-test showing the significance between sonicated and non sonicated protein extraction.

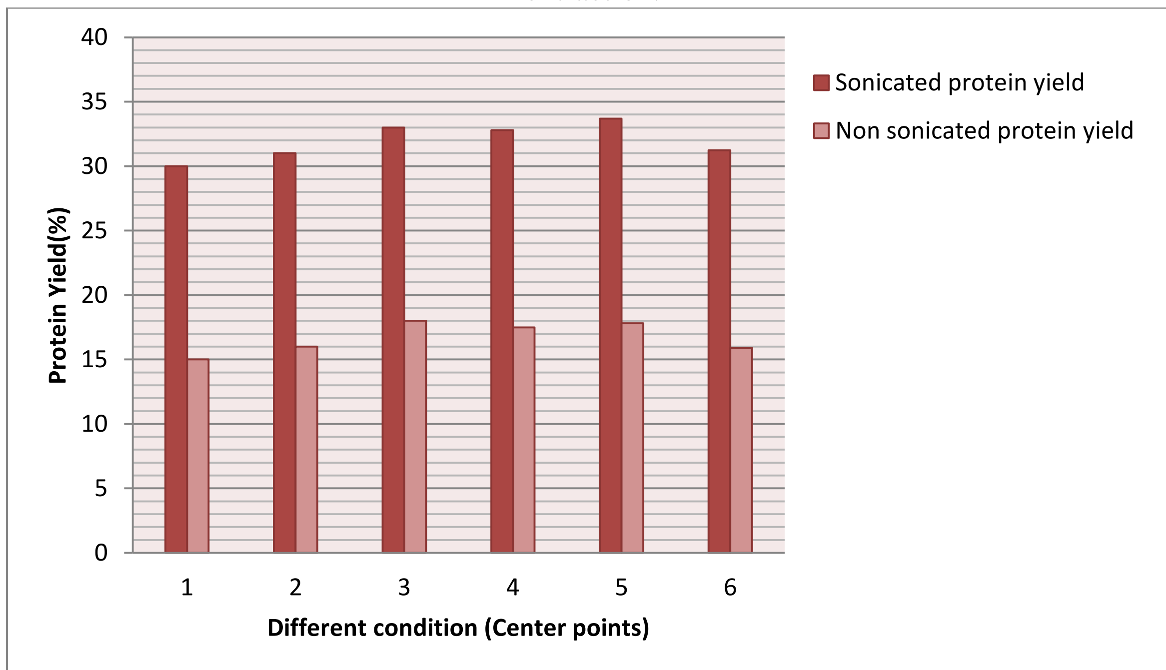

Fig. 5 Bar diagram for showing difference of protein yield between two extraction processes with the centre points.

For finding the significant difference between sonicated and non-sonicated protein yield, one tail and two tail t test were used. In both cases two types of hypothesis namely null and alternative hypothesis were assumed. The hypothesis is given as follows:

H0: X=Y

H1: $X \neq Y$

Where, X and Y are sonicated and non sonicated protein yield respectively.

In case of one tail t test, calculated value of t was 4.405 and critical value of t for 50 degree of freedom at 5% significance level was 1.676 (Table 8). From this result, it can be observed that calculated value of t is larger than tabulated value of t. So, based on the result null hypothesis is rejected at 5% significance level. The same result is shown in Fig. 4 From the figure, it can be observed that null hypothesis is in the rejection region due to the greater value of t calculated than t critical. Hence it can be concluded that there is significant difference between sonicated and non sonicated protein yield.

Based on the significant difference between the sonicated and non sonicated protein yield in terms of t test obtained null hypothesis rejection, bar diagram has been plotted (Fig. 5). For plotting the bar diagram, center points of the experimental design for both the cases, were used. From the bar diagram, it can be observed that there is a greater yield of protein in case of sonicated extraction compared to non sonicated counterpart. Hence, it can be concluded that sonicated extraction is superior over non sonicated extraction in terms higher protein yield.

Table 6 shows process variables and their corresponding levels. For determining the significance of the independent parameters (Ultrasonic time, Ultrasonic Temperature, Solid-liquid ratio and pH) on the response (Protein Yield) statistical analysis were performed. Using multiple regression analysis, evaluation of mathematical model utilized for response, was done. For estimating the behavior of the response as a function of independent variables, multiple regression coefficients were calculated. For determining the significance terms of the

experimental data, ANOVA on each response variable was performed followed by the judgment of experimental data with the calculated F-value.[32](Table 9) Adequacy of the model was checked and tested by lack of fit test, considering statistical parameters like fitted R^2, predicted R^2, PRESS and adequacy precision. On the basis of non- significant lack of fit ($p>0.05$), higher R^2 value (closer to 1), low PRESS and higher adequacy precision value, the fitted model can be considered as the adequate one for predicting the mentioned response.[33,34]

Table 10 Analysis of variance showing the linear, quadratic, interaction and lack of fit of the response variable

Source of variation	df	Response variables		
		Protein yield		
		Sequential sum of squares	F-value	p-value
Model	14	2078.6	104.23	< 0.0001**
x_1	1	27.09	19.02	0.0006**
x_2	1	6.85	4.81	0.0445*
x_3	1	209.81	147.29	< 0.0001**
x_4	1	10.61	7.45	0.0155*
x_1^2	1	1046.65	734.77	< 0.0001**
x_2^2	1	203.27	142.7	< 0.0001**
x_3^2	1	566.23	397.51	< 0.0001**
x_4^2	1	601.02	421.93	< 0.0001**
x_1*x_2	1	11.12	7.81	0.0136*
x_1*x_3	1	41.15	28.89	< 0.0001**
x_1*x_4	1	0.5	0.35	0.5635
x_2*x_3	1	1.27	0.89	0.3608
x_2*x_4	1	0.2	0.14	0.7145
x_3*x_4	1	1.13	0.8	0.3863
Residual	15	21.37		
Lack of Fit	10	11.31	0.56	0.7953
Pure Error	5	10.06	104.23	< 0.0001**
Correlation Total	29	2099.97	19.02	0.0006**
R^2	0.9898			

Adjusted R^2	0.9803			
Predicted R^2	0.9621			
Adequate precision	31.797			
PRESS	79.61			
*Significant at	$p \leq 0.05$			
**Significant at	$p \leq 0.01$			
***Significant at	$p \leq 0.0001$			

Effect of interaction of various factors on protein yield

From Table 9 it can be observed that all the statistical parameters like probability (p) values of the model, 4 independent variables, quadratic terms involved in the model and interaction of $X_1 \times X_2$, $X_1 \times X_3$, and $X_3 \times X$ for the response were significant. The lack of fit ($p>0.05$) value of the model was non-significant with p-value of 0.7953, which implies accuracy of the model. The R^2value of the model was 0.9898, whereas the adjusted $R^2$and predicted R^2were 0.9803 and 0.9621 respectively. On the basis of the above results, it can be conferred that the model was adequate and accurate enough for predicting the mentioned response.

The final equation in terms of coded factors was as follows:

$$\text{Protein Yield} = +31.95 + 1.06 * X_1 + 0.53 * X_2 + 2.96 * X_3 - 0.67 * X_4 - 6.18 * X_1^2 - 2.72 * X_2^2 - 4.54 * X_3^2 - 4.68 * X_4^2 - 0.83 * X_1 * X_2 + 1.60 * X_1 * X_3$$

Fig. 6 A and B shows the effect time, temperature and pH on protein yield. It can be observed that the protein yield increases with sonication temperature and time. The same result of positive increasing effect of time and temperature can be observed from the equation also. Similar findings were observed on sonication time for the extraction of phenolic compounds from coconut shell[18]. Duy et al. [35] reported similar findings for extraction temperature in the extraction

of phytic acid from peanut seeds. The increase in the protein yield with the increase in sonication time might be accredited due to the increase in mass transfer process with the rising trend in

sonication time, whereas increase in temperature leads to breakdown of cell wall of the *Diplazium esculentum* plant resulting in maximum yield of protein.[36,37]

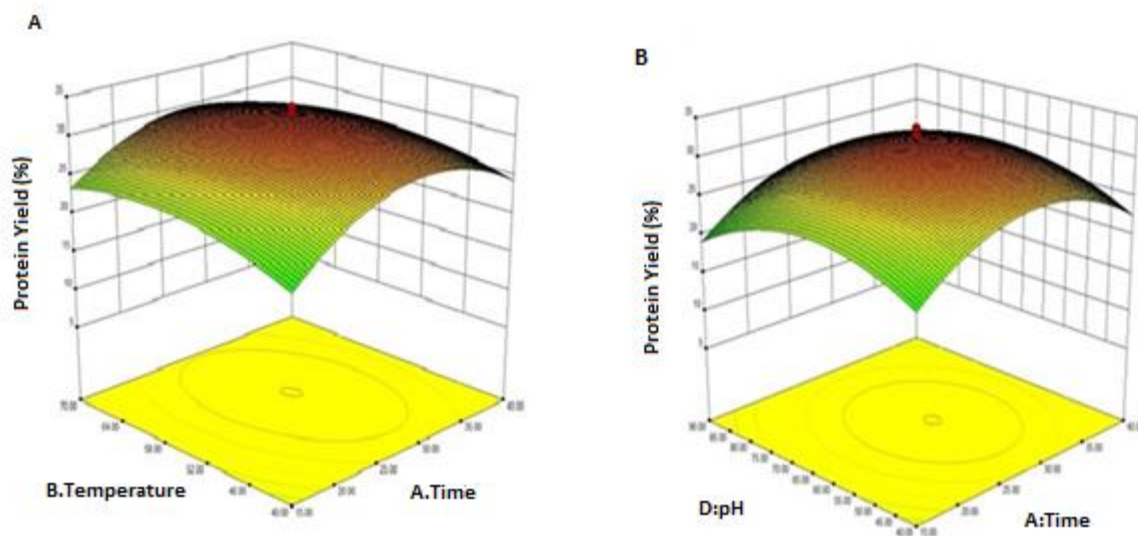

Fig. 6 Variation of protein yield against different processing factors Interaction effect of temperature, time and pH on protein yield

Fig.7 C and D shows the effect of, time , temperature and solvent volume on protein yield. It can be observed that solvent volume with the increase of sonication time or temperature increases up to a certain point and then decreases. The increase in protein yield with the increasing concentration of solvent up to a certain point might be due to effective swelling of the leaves, resulting in increased surface area for solute–solvent contact. But after certain period of time, as the bound water is released from the swelled leaves, the concentration of the solution starts decreasing. As a result of which there is no significant effect of solvent volume on the protein yield. Similar findings were observed by Mohammad etal.[39] on the effect of solvent volume for the extraction of rebaudioside A from stevia leaves.

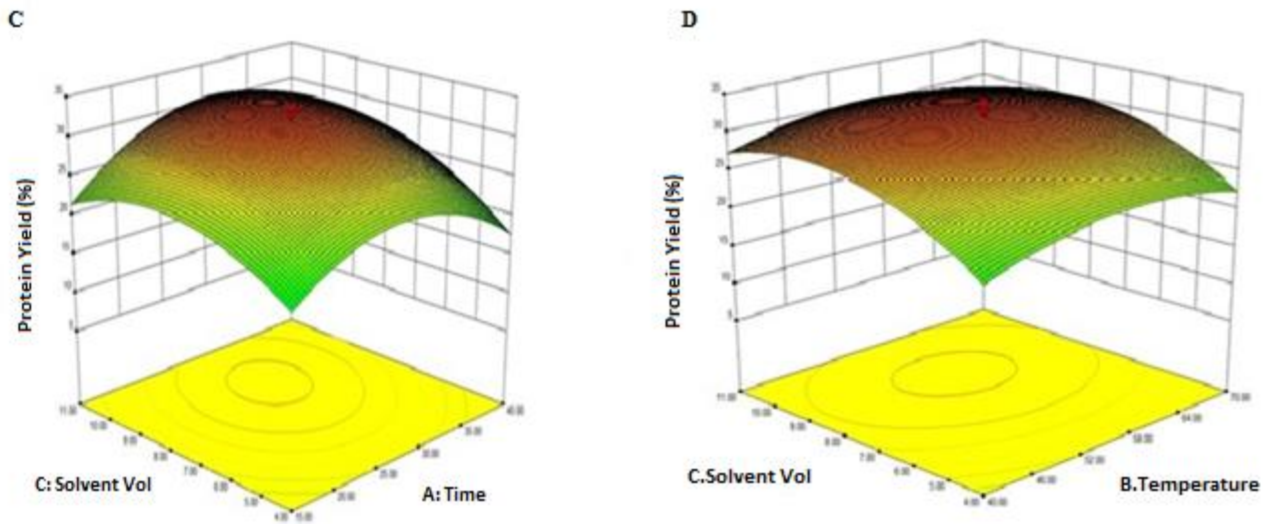

Fig. 7 Variation of protein yield against different processing factors solvent volume, time and temperature on protein yield

In Fig. 8 E and F, effect of p H, temperature and solvent volume on protein yield can be observed on the basis of sonication time and temperature respectively.Fig.8 F shows the effect of pH and solvent volume on protein yield. Protein yield clearly increases with the increase in pH as mentioned earlier, but solvent volume shows rising effect on the protein yield up to a certain point followed by final decrease in the protein yield up on further increase of its concentration. In this case protein yield increases with the increase of both pH and sonication time. From the equation also, the positive effect of linear and quadratic terms on the protein yield can be observed. This work can be explained with the works of Irakoze and Sindayigaya[38] where they worked on the optimization of Malted Sorghum. The increase in protein yield with the increase of pH is due to the fact that protein denaturation decreases with the rise in pH value which results in higher amount of protein yield.

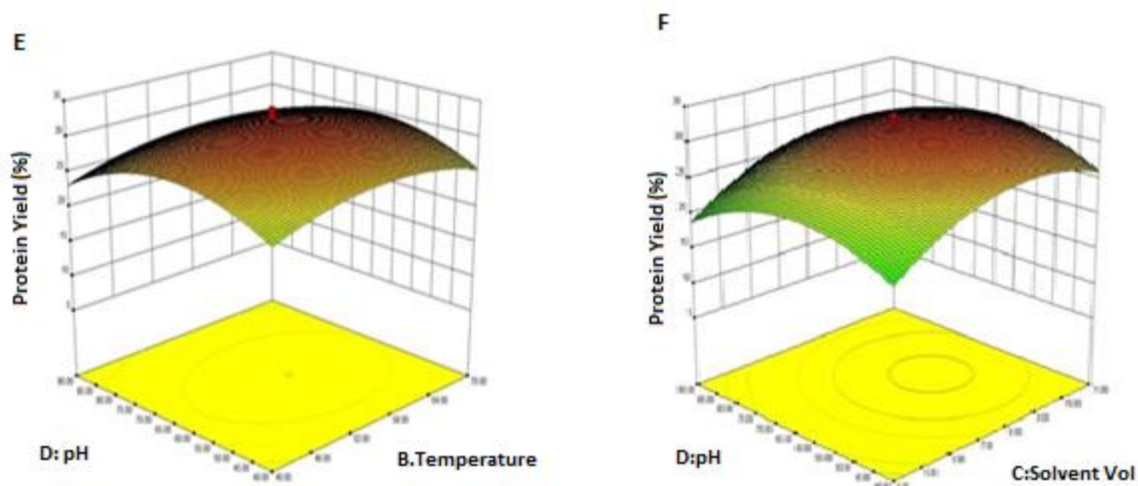

Figure 8 Variation of protein yield against different processing factorspH, temperature and solvent volume on protein yield

Table 11 Validation of the optimized result

	Response obtained from RSM
	Protein yield
Predicted value (%)	34.28
Actual value[a](%)	32.96±0.18
% Variation	3.85
Mean difference	1.32

[a]Values are mean of three replicates±SD

Conclusion

In conclusion Sonication had a positive effect in the protein yield when compared to its nonsonicated extraction. Temperature had a positive effect in protein yield. Effect of sonication time had a positive effect on protein yield which increased with the increase of sonication time. pH had positive effect on protein yield. Solvent volume did not have any significant effect on protein yield. The optimized condition was observed to be 29.07 min of sonication time, 55.8 °C temperature, 8.70 pH and 63.53 ml of solvent with a desirability value of 0.959 the optimized result obtained from RSM in protein yield was 34.28%.

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Objective (iii): To study the chemical composition and functional properties of LPC

Introduction

Importance of protein in human diet

Proteins are formed by amino acids which serve as the building blocks of major structural component of muscle and other tissues in the body. In addition, they are used to synthesize enzymes, hormones, and haemoglobin.[1]Proteins are also alternative sources of energy. For proteins to be used up by the body they are first needed to be metabolized into their simplest form, amino acids. There are 20 amino acids identified that are needed for human growth and metabolism. Twelve of these amino acids (eleven in children) are termed nonessential, i.e they can be synthesized by our body and need not be consumed in the diet. The remaining amino acids cannot be synthesized in the body described as essential meaning that they need to beconsumed in our diets. The absence of any of these amino acids will compromise the ability of tissue to grow, be repaired or be maintained.[2]

Comparative study between animal and plant sources of proteins

Although protein from animal sources provide highest quality of protein because of their completeness of the proteins from these sources but serious questions have arisen regarding the health concerns of these proteins and the primary health risk associated are the cardiovascular disorder (due to the high saturated fat and cholesterol consumption) bone health (from bone resorption due to sulfur-containing amino acids associated with animal protein) and other physiological system disease.[2]On the other hand plant proteins, when combined provide for all these essential amino acids, considering that they will likely result in a reduction in the intake of saturated fat and cholesterol. Popular sources include legumes, nuts and soy. Apart from these products, vegetable protein can also be found in a fibrous form called Textured Vegetable Protein (TVP). TVP is mainly a meat alternative and functions as a meat analog in vegetarian hot dogs, hamburgers, chicken patties, etc. It is also a low calorie and low fat source of vegetable protein. Vegetable sources of protein also provide numerous other nutrients such as phytochemicals and fiber that are also highly regarded in the diet.[2]

Functional properties of proteinsand factors affecting properties of proteins in food systems

Functionality of food protein has been defined as those physical and chemical properties which affect the behavior of proteins in food systems during processing, storage, preparation and consumption.[3]Intrinsic factors, extrinsic factors, processing treatments and other intentional modifications can all influence the chemical and functional properties of proteins including moisture, temperature, pH, enzyme concentration, reaction time, chemical additives, mechanical processing, ionic strength and amount, sequence, rate and time of additives.[4] In food systems, intrinsic factors include the basic chemical and physical properties of the amino acids comprising a particular protein. The distribution of nonpolar and polar or charged side chains on the surface protein plays a key role in the chemical properties and ultimately the functions of the proteins. It is well known that proteins are of prime importanceto health, but they are deficient in diets of most peoplein the developing countries. Available literature clearly indicate that, apart from lower methionine content, the amino acid profiles of leaf protein from most species compare favorably well with those of soybean, meat, fish and egg and generally surpass the FAO essential amino acid pattern.[5,6,7]

Protein and their interactions with other food constituents

Major food constituents such as water, other proteins, lipids and carbohydrates, as well as minor constituents such as salts, metal ions, acidulants, flavour components and phenolic compounds interact with proteins to shape chemical and functions of proteins.[8]Water molecules that are hydrogen bonded to protein molecules play a critical role in the structural stability of the protein.[9] The balance between protein-water interactions versus protein-protein interactions are important in functional properties such as swelling, water binding capacity and solubility of protein ingredients, as well as their ability to establish networking structures such as gels or films including those surrounding foam bubbles. Salts may promote either solubilization (salting-in) or precipitation (salting-out) of proteins, depending on the concentration and nature of the salt involved.[10] The increased β-sheet content that has been observed in the salt induced aggregated state may be attributed to the relatively large surface area and opportunities for hydrogen bonding provided by the β-sheet structure.[11] Furthermore, the weaker strength of water hydration to β-sheet than to α-helix structures, due to different geometry of the water-carbonyl group interactions in these secondary structure conformations, may influence the changes in water-

protein and protein-protein interactions that favour aggregate or network formation.[12]Acidulants alter the net charge and isoelectric point of the protein molecule and may also affect the local distribution of positive or negative charges on the protein surface, again with possible alterations of the protein-solvent, protein-protein balance and associated properties. The specific binding of metal ions can also affect stability of proteins.[8]Carbohydrates, with their multiple hydroxyl groups, may contribute to the structural stability of proteins, either by exclusion of carbohydrate from the protein surface resulting in preferential hydration of the protein, or by interaction of carbohydrate with hydroxyl or ionic functional groups of the protein molecule.[13] These carbohydrate-protein interactions affect stability of food proteins to processes such as thermal treatment, dehydration or frozen storage, and are the basis for addition of ingredients such as sucrose or sorbitol to stabilize fish muscle proteins during frozen storage or drying.[13] Attractive (complexation) or repulsive (segregation) interactions may occur between proteins and anionic polysaccharides such as carrageenan or pectin, leading to precipitate or gel formation, depending on conditions such as biopolymer concentration, pH and ionic strength.[14]Nonpolar residues of proteins are primarily responsible for hydrophobic interactions with lipid molecules at oil water interfaces such as emulsions.[15] although electrostatic, covalent, hydrogen and hydrophobic forces may all contribute to protein-lipid interactions.[16] Protein-lipid interactions may also be implicated in protein-protein interactions for example, protein-lipid complexes in wheat gluten have been associated with the lipid-mediated aggregation of high and low molecular weight polypeptides in the gliadin fraction.[17]

As green leafy vegetables are acknowledged to be the cheapest and the most potential source of protein because of its capability to form amino acid from readily available sources of primary material such as sunlight, carbon dioxide and atmospheric nitrogen,[18,19,20] more over leaves are reported to be rich sources of proteins (20-30%), minerals (micro and macro) and vitamins.[20,21,22]Leaf protein concentrate of *Diplazium esculentum*, may also be affected by all these intrinsic and extrinsic factors on sonication extraction. The objective of this work was to find out in what ways these factors affect the functional properties of leaf protein concentrate on sonication extraction.

Materials and methods

Diplazium esculentum was procured from the local markets of Tezpur, Sonitpur district of Assam and thereafter brought to laboratory where the vegetable stalks were removed and the leaves were thoroughly washed with tap water followed by rinsing with distilled water. The leaves were then allowed to drain the excess amount of water. Thereafter it was used for extraction of leaf protein concentrate and studies of functional, thermal and structural properties.

Leaf protein concentrate (LPC) production

The leaf protein concentrate(LPC) production has been done as described in Fig 3(Objective 2) and a comparison of the physicochemical properties has been done between two extraction procedures i.e sonication and non sonication

Determination of total protein content

Nitrogen content was determined by micro-Kjeldahl method [23] using a (Kel Plus-Kes, 201 Digestion unit attached to a Kel plus-classic DX Distillation unit Make: Pelican Equipment, Chennai, India) and the percentage of nitrogen was converted to crude protein by multiplying it with a factor of 6.25.

Water absorption capacity (WAC)

Water absorption capacity was determined according to the method of Rodriguez., et al.[24] with some modifications. Sample (1.0 g) was dissolved in 10 ml distilled water and after mixing it was allowed to stand for 30 min and then protein was centrifuged at 3000 rpm for 20 min. The supernatant was decanted and amount of sediment present in tube was weighed. Water absorption capacity as per gram was calculated by the following formula:

WAC (g H_2O) = $(W_2-W_1) / W_0 \times 100$

Where,

W_0= weight of the dry sample

W_1=weight of the tube + dry sample

W_2=weight of the tube + sediment

Oil absorption capacity (OAC)

Using the method of Lin and Zayas.[25]sample (1.0 g) was taken in a pre-weighed centrifuge tube and thoroughly mixed with 5 ml sunflower oil. The mixture was then centrifuged at 3000 rpm for 30 min at 25 °C. Then supernatant was removed and the tube was reweighed. OAC (g of oil per g of protein) was calculated as follows:

OAC (g Oil) = F_2- F_1/F_0 ×100

Where

F_0= weight of the dry sample

F_1= weight of the tube + dry sample

F_2= weight of the tube + sediment

Emulsifying properties

Emulsifying activity and stability was determined as described by Pedroche.,et al.[26] with slight modification. Sample (1.0 g) was added with 30 ml distilled water and homogenized at 10,000 rpm for 5 min. The protein solution was then mixed with 30 ml sunflower oil and homogenized at 10,000 rpm for 5 min. Finally emulsion was centrifuged at 2000 rpm for 5 min. Emulsion activity was calculated as follows:

Emulsion activity (EA) % = (Height of the emulsion)/ (Total volume of the mixture) ×100

Emulsion stability (ES) was determined by the heating of emulsion, as prepared before, 20 min at 85 °C, followed by re-centrifugation at 3000 rpm for 10 min.

Then ES was expressed as follows:

Emulsion stability (ES) %=(Height of the remaining emulsion layer) / (Height of the emulsion layer) ×100

Foaming properties

The Foaming capacity and stability was determined based on the methods described by Adebiyi and Aluko[27] with slight modification. Sample (5.0 g) was dispersed in 50 ml distilled water. The solution was stirred at 10,000 rpm for 5 min and the blend was immediately transferred to 100 ml measuring cylinder. The Foaming capacity was expressed in volume percentage due to stirring. Foaming stability was determined by measuring the foam volume after the standing condition of 30 min of storage. The foaming capacity and stability was calculated as follows

Foaming capacity (%) = (Volume after homogenization-volume before homogenization ml) / (Volume before homogenization) ml ×100

Foaming stability (%) = (Volume after standing-Volume before homogenization ml) /(Volume before homogenization ml) ×100

Differential scanning calorimetric (DSC) analysis and thermal gravimetric analysis (TGA)

Thermal denaturation of LPC of *Diplazium esculentum* was performed in DSC- 60 Thermal analyzer (Shimdzu, Japan), according to the methods of Guerrero et al (2010)[28] with some modification. 2 mg samples were weighed into aluminum pans and heated from 30-200 °C at a rate of 10 °C/min. Denaturation temperature (T_d), and enthalpy (ΔH) were measured from thermograms by TA-60WS software (Shimdzu). Thermal stability was studied using thermogravimetric analyzer (TGA, Perkin Elmer STA 6000, USA). TGA measurements were performed in the range from 50-900°C with nitrogen atmosphere keeping the nitrogen flow rate of 20 ml min^{-1} using heating rate of 20 °C min^{-1}.

Contact angle

Static contact angle measurements were carried out using the sessile drop technique on gradients formed on flat silicon wafers. A small droplet (approximately100 μL) of high-purity Milli-Q water, (supplied by an Elga UHQ water system), was deposited on a given position of the surface using a motorised syringe. The silhouette of the droplet was captured and imaged with a progressive scan CCD camera attached in the contact angle measurement set up (Krusseasydrop, OCA 15EC). The contact angle was determined by drawing a tangent close to the edge of the droplet at the three-phase point (using Image J software, Drop-Snake). Experiments were conducted at 22 °C in a class-100 clean room.

FT-IR Spectroscopy

Leaf protein concentrate was grounded with KBr at a (1:100) ratio. The protein isolate was pressed at a high pressure into KBr pellet. The spectral analysis was carried out using a FT-IR spectrometer (Perkin Elmer Spectrum- model 100, USA). The FT-IR spectra of the sample were recorded in the range of 4000-400 cm^{-1} region at room temperature.

Amino acid analysis by UHPLC

For amino acid composition analysis, leaf protein concentrate (1g) of *Diplazium esculentum* was hydrolyzed with 6 N HCl at 110 °C for 24 h under vacuum. The hydrolyzate (25 µl) was mixed with 25 µl of ortho- pthaldialdehyde (Sigma), 3-merceptopropionic acid and 9-fluorenylmethyl chloroformate (Sigma) reagents in borate buffer. This was followed by addition of 8µl of 1M

acetic acid and contents were mixed well. The 3 µl of the resulting mixture was injected in Acclaim RSLC 120, C_{18} RP-UPLC column (2.1 X 150 mm, 3 µm, 300 A°) coupled to ultimate 3000RSLC (Dionex) UHPLC system. After washing the column by eluent A (10 m mol/L Na_2HPO_4, $Na_2B_4O_7$ containing 0.5mmol/L NaN_3), the amino acid derivatives were separated by eluent B (CH_3CN /MeOH/H_20 in 45:45:10) at a flow rate of 0.722 ml/min. The elution was monitored initially at 338 nm for 0-7.2 min and then at 262 nm for the next 7.2 -10.5 min.[31]The concentration of individual amino acid of the leaf protein concentrate was determined against a calibration curve of standard amino acids derivatives run in the same RP-UPLC column under the identical conditions.

Results and discussions

Total protein content

The total protein content of LPC of *Diplazium esculentum* extracted through sonication and nonsonication was found to be 34.28% and 9.89% (Table 12). The enhancement in extractability of protein for sonicated extraction might be attributed to the propagation of ultrasonic pressure resulting in cavitation phenomena. The energy released during the collapse of cavitation bubbles might have promoted higher penetration of the solvent into the cellular material which might have improved the mass transfer to and from the interfaces.[30,31.32]

Table 12. Functional properties parameters of sonicated and nonsonicated LPC of *Diplazium esculentum*

Sl. No	Name of the parameter	Sonicated LPC of *Diplazium esculentum*	Nonsonicated LPC of *Diplazium esculentum*
1.	Total protein content (%)	34.28±0.64	9.89±0.12
2.	Water absorption capacity (g of water/g of LPC)	8.36±0.14	7.65±0.15
3.	Oil absorption capacity (g of oil/g of LPC)	7.55±0.20	7.41±0.06
4.	Emulsion activity (%)	36.36±0.14	31.10±0.28
5.	Emulsion stability (%)	30 ±0.02	25±0.04
6.	Foaming capacity (%)	7.27±0.14	6.99±0.01
7.	Foaming stability (%)	5.21±0.03	4.26±0.06

Water absorption capacity

The ability of a protein matrix, such as protein particles, protein gels or muscle to imbibe and retain water against gravity is known as Water Holding Capacity (WHC).In food systems interactions with water and proteins are very important because it affects the flavor and texture of foods.[33] Water absorption capacity is dependent on various parameters such as size, configuration, conformational characteristics, hydrophobic and hydrophilic balance of the protein.[34] Water holding capacity is an important processing parameter and has implications for viscosity, bulking and consistency of products, as well as in baking applications. The water absorption capacity was found to be (8.36 g water / g of protein concentrate) for sonicated LPC of *Diplazium esculentum* whereas for nonsonicated LPC it was found to be (7.65 g water / g of protein concentrate), these results are comparatively high with the results of Essuman,[1] and Suresh, et al. [35] in *Arthropteris orientalis* (2.39 g water / g of protein concentrate) , *Nephrolepis biserrata* (2.13 g water / g protein) and *Kappaphycus alvarezii* (Doty) an edible seaweed where they found the WAC to be 2.22 g water / g of protein. According to Kinsella,[36] an increase in the water holding capacity is because of the ability of a protein to isolate, swell and unfold, exposing additional binding sites, whereas the carbohydrate and other components of the protein concentrate may impair it.[37]. The pH of a system markedly influences the water absorption capacity due to changes in the surface charges of protein. When pH is altered from isoelectric point it results in increase of water absorption capacity by creating charge imbalance.

Oil absorption capacity

Oil absorption capacity was found to be 7.55 g of oil / g of leaf protein concentrate extracted with sonication where as it was 7.41 g of oil / g of protein for nonsonicated extraction. The results of the present study are high compared to the works of Essuman,[1] with two ferns namely *Arthropteri sorientalis* (2.93 ml / g of protein) and *Nephrolepis biserrata* (2.73 ml / g of protein), but are comparatively low with the works of Aletor., et al.[18] Fasuyi,[22] where they worked on the chemical compositions of common leafy vegetables and functional properties with their protein concentrates, oil absorption capacity being one of the parameters of their study, on plants namely *Vernonia amygdalina*, *Solanum africana, Amaranthus hybridus ,Telfaria*

occidentalis, Talinum triangulare, and *Amaranthus cruentus*. The possible reasons of the increase in oil absorption capacity of the LPC extracted by sonication could be decrease in droplet size which increased the percentage of adsorbed proteins with the ultrasound treatment. [40]Under unstable conditions ultrasound treatment (like homogenization) might have favored the adsorption of proteins and formation of aggregates.[39]

Emulsion activity and emulsion stability

The emulsion activity of sonicated and nonsonicated leaf protein concentrate of *Diplazium esculentum* was found to be 36.36% and 31.10%, which are comparable with the works of Aletor., et al.[18] where they worked on four plant species namely *Vernonia amygdalina, Solanum africana, Amaranthus hybridus ,Telfaria occidentalis,* although the result of the present study is lower than the works of Aletor., et al.[18], but is higher than the value of 7-11% reported for wheat flour.[42] The emulsion stability of the present study was found to be 30±0.02% for sonicated LPC whereas for nonsonicated LPC it was found to be 25±0.04%. The increase in the emulsion activity of the sonicated sample was due to the influence of turbulent behavior produced by the ultrasound that resulted in the aggregation of oil bubbles in the emulsion.[41] Over all the results are comparatively low than that of the works of Aletor., et al.[18] It can be suggested from the present study that leaf protein concentrate of *Diplazium esculentum* can be used as an alternative for the stabilization of emulsions.

Foaming properties

Foams are formed because of rapid diffusion of molecules in the interface followed by the molecular rearrangement which allows these films to entrap air.[1] The foaming capacity of sonicated LPC of *Diplazium esculentum* was found to be 7.27±0.14%. Whereas for nonsonicated LPC it was 6.99±0.01%. The increase in foaming capacity might be due to the homogenizing effect of ultrasound.[42] The foaming stability of the nonsonicated LPC of *Diplazium esculentum* was found to be 5.21±0.03 % for sonicated LPC of *Diplazium esculentum* after 30 min and 4.26±0.06 % for nonsonicated LPC the results were comparatively low with the works of Oshodi and Adeladun.[43] Hence the LPC of *Diplazium esculentum* would not be good choice for product like cakes, or whipping products where foaming is an important character.[36]

Thermal properties

DSC properties

The protein sample was heated from 30 to 200 °C at a rate of 10°C/min. The LPC showed two observable denaturation temperatures at about 55.58 and 95.95°C for sonicated LPC whereas it was found to be 46.75 and 75.60°C for nonsonicated LPC as shown in the Fig 9 The enthalpy change reflects the status of ordered confirmations of food proteins.[44] Therefore the net enthalpy change (ΔH) indicates cumulative effects of endothermic events such as breakdown of hydrogen bonds and exothermic phenomenon such as aggregation of food proteins.[45]The DSC of the present study depicts that the sonicated LPC shows more stability compared to nonsonicated one this may presumably due to prolonged sonication increased the enthalpy of denaturation due to protein aggregation. [48]

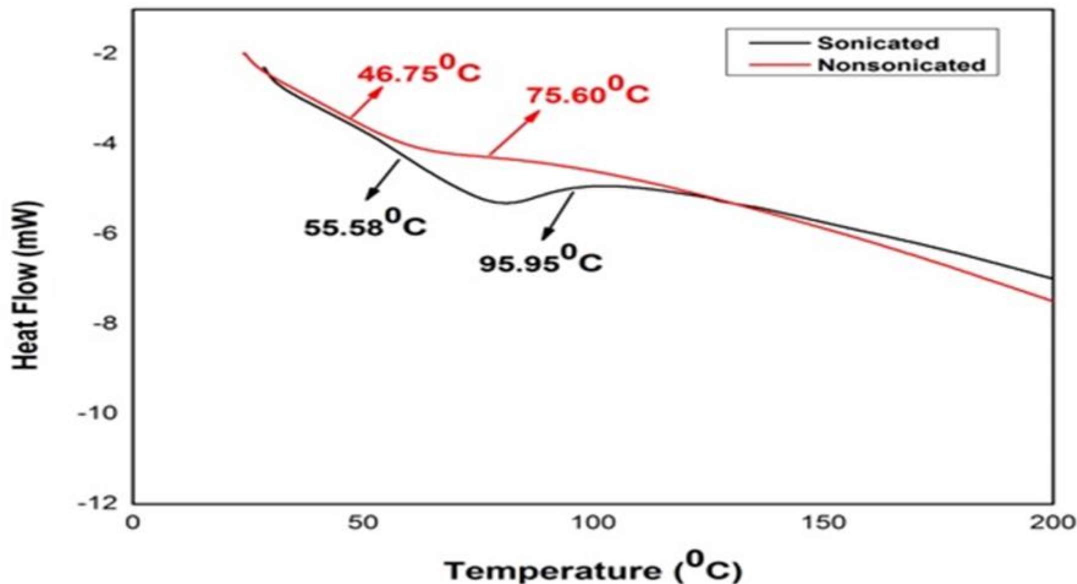

Fig. 9 DSC graph of sonicated and nonsonicated LPC of *Diplazium esculentum*

TGA properties

TGA of both sonicated and nonsonicated leaf protein concentrate are represented in Fig. 10 In this study the initial degradation temperature of sonicated and nonsonicated LPC are 173°C and 167°C respectively. However, the final decomposition temperature of both the extraction method is 318°C and 315°C respectively. In this present study the decomposition temperature of LPC can be correlated with the TGA of soy protein whose initial and final decomposition temperature was found to be 260.86 (9.69% weight loss) and 356.68°C.[49]The percentage weight

loss observed was 6% there is a small weight loss at temperatures between 167 to173°C, which is due to the loss of moisture however the total weight loss occurred with the degradation of major protein component. This is well supported by Suhaimibin and Yasir[50] who worked on cross linking of soy flour texture. This observation clearly shows that sonicated LPC has higher thermal stability compared to non-sonicated LPC.

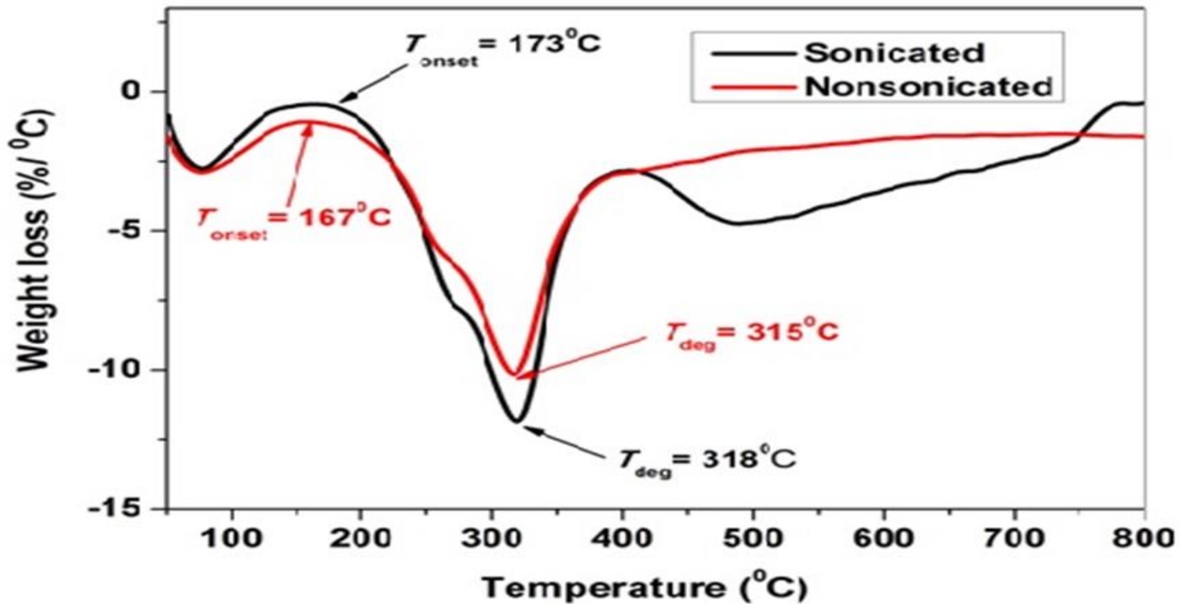

Fig. 10 TGA graphs of sonicated and nonsonicated LPC of *Diplazium esculentum*

Contact angle explanation

Contact angle measurements reveal whether a surface is hydrophilic or hydrophobic. The measured values of contact angles of the samples before and after sonication shows that both the angles are less than 90° (as in Fig. 11) which proves that the samples are hydrophilic in nature. It is emphasized that the system becomes conventionally hydrophilic, if the contact angle is less than 90°.[49] From the results it could be seen that ultrasound increased the surface area which in turn increased the wettability as a result there was increase even in the other physicochemical behavior such as water holding capacity, oil holding capacity, foaming property, and emulsifying property there largest increase in specific surface area for leaf protein concentrate samples are after ultrasound treatment.[41]

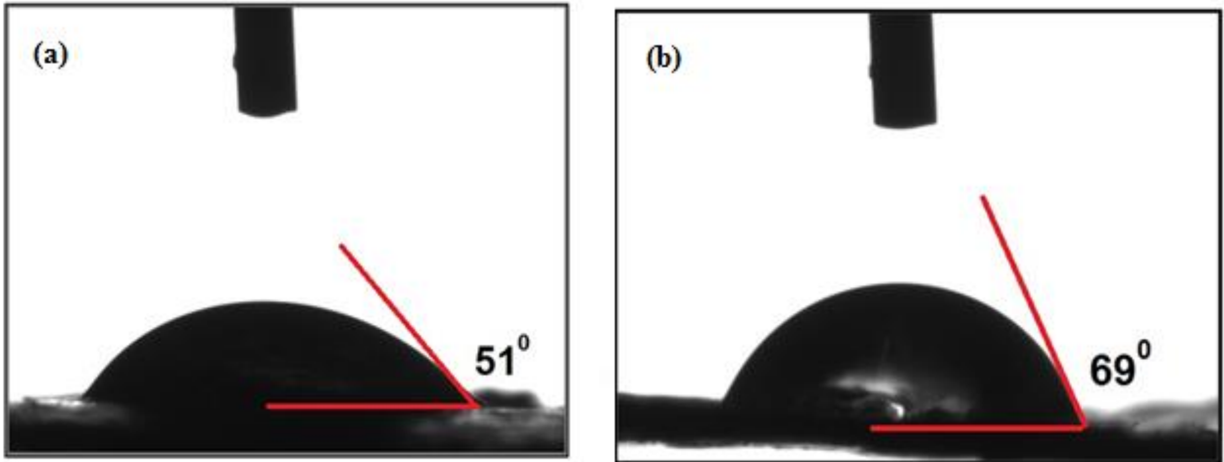

Fig. 11 Contact-angle of sonicated and nonsonicated LPC of *Diplazium esculentum*

FT-IR analysis

FT-IR analyses were performed with the leaf protein concentrate of *Diplazium esulentum* which were extracted with sonication and without sonication. The measurements were recorded between 4000 to 400 cm^{-1}. Determination of secondary structure of protein is one of the major applications of the FT-IR methods.[50]The IR spectral band are assigned and described in Table 5.2.and Fig 5.5 Proteins are generally known to have different fraction of structural components (α-helix, β-sheet, random coil etc.); however, the secondary structural composition is most important information for the structural elucidation of unknown protein. The spectrum of sonicated LPC and nonsonicated LPC of *Diplazium esculentum* presented in Table 12 showed the bands at 2990 shifted to 2995 and 2864 shifted to 2866 cm^{-1}respectively,(Fig 12) which could be due to CH_2 and CH_3 asymmetric stretching and are found in aliphatic side chain of the protein. The five major peaks at 1654, 1637, 1525, 1565, 1292 and 1655, 1636, 1522, 1554, 1288 cm^{-1} are assigned to be as Amide I, Amide II and Amide III, respectively, which are found both in the sonicated LPC and non-sonicated LPC.[50] These three peaks corresponds to C=O Stretching, C-N Stretching NH-Bending and CH_3 bending vibration of the protein. Similar bands were also reported in soy protein using aqueous extraction methods.[51]The bands at 1350, 1391, 1637, 1655 and 1714 cm^{-1} for sonicated LPC has shifted to 1349, 1390, 1636, 1654 and 1714 cm^{-1} for nonsonicated LPC. There was no significant difference in their position. Apart from that, the bands at 890, 1090, 1184, 1292, 1434, 1484, 1525, 2866 and 2995 cm^{-1} for sonicated LPC had shifted to lower frequency of 883, 1087, 1180, 1288, 1427, 1479, 1422, 2864 and 2990 cm^{-1} for

nonsonicated LPC, which are assigned to be as Out of plane/ N-H bending, C-O stretch of α-anomer, OH and CO group stretching vibration, CN stretching and NH bending, CH_2 bending vibration, CH_3 asymmetric bending vibration of protein, CH_3 asymmetric bending vibration of protein, CH_2 asymmetric stretching and CH_3 asymmetric stretching. So it indicates that, there is hydrostatic interaction and electrostatic interaction between sonicated LPC and non-sonicated LPC,[52] which may affect the structure of the protein.

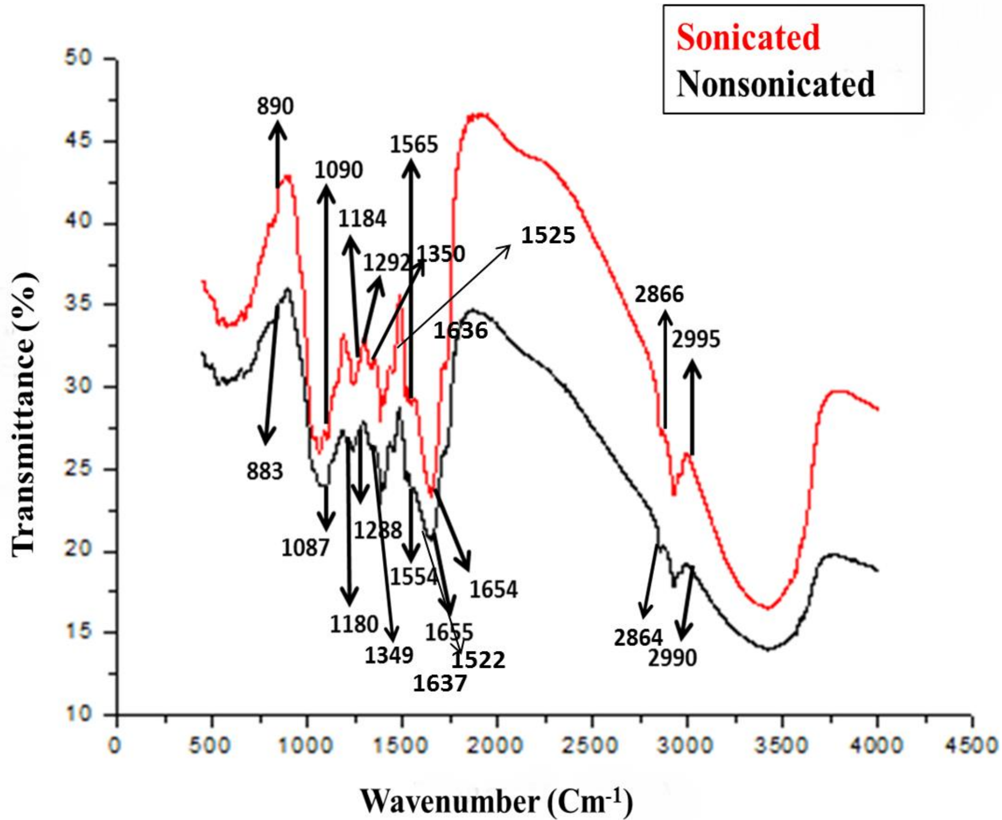

Fig. 12 FT-IR graphs of sonicated and nonsonicated LPC of *Diplazium esculentum*

Table 13: FT-IR analysis of sonicated and nonsonicated LPC of *Diplazium esculentum*

Sonicated LPC of *Diplazium esculentum* (cm^{-1})	Nonsonicated LPC of *Diplazium esculentum* (cm^{-1})	Function of Bands
890	883	Out of plane/ N-H bending
1090	1087	C-O stretch of α- anomer
1184	1180	OH and CO group stretching vibration
1292	1288	Amide III (CN stretching, NH bending)
1350	1349	CH_2 vibration of α- anomer
1391	1390	CH_3 bending vibration
1434	1427	CH_2 bending vibration
1483	1479	CH_3 asymmetric bending vibration of protein
1525	1522	Amide II (CN- Stretching and NH-Bending)
1565	1554	Amide II (C-N Stretching and NH-Bending)
1637	1636	Amide I (C=O Stretch)
1655	1654	Amide I (C=O Stretch)
2866	2864	CH_2 asymmetric stretching
2995	2990	CH_3 asymmetric stretching

The deconvoluted spectra of *Diplazium esculentum* protein concentrate using sonication and non-sonication method are done shown in Fig. 13 (A) and (B). In the present work, the spectral assignment and calculation are performed by second derivative infrared spectra (peak fit method). The frequencies of seven bands shifted in sonication method as compared to the frequency in non-sonication extraction procedure. As These results indicate that, the lower or higher wave numbers reflect the secondary structure of the protein, which may be because of the stronger interaction with alkali or aqueous medium of the protein molecule which in turn increased the effects of H-bonds, and decreased the frequency of amide I band by shifting of

bands, which effected the strong or weak H-bonds, easily formed between C=O and N-H stretch of amide I bands. If the shifting of bands take place towards the higher side, the hydrogen bonds get weakened.[53,54,55]

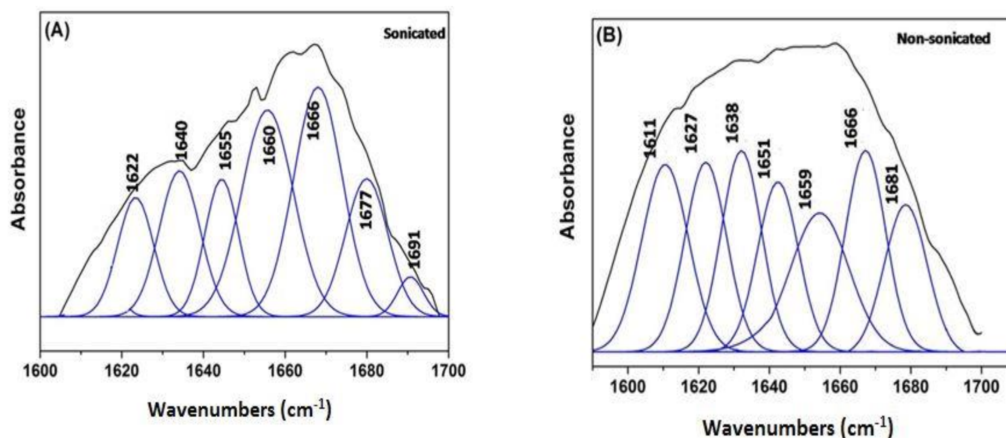

Fig. 13 FT-IR and deconvoluted FT-IR graphs of sonicated (A) and nonsonicated (B) LPC of *Diplazium esculentum*

According to Bayer and Susi [56], the bands at 1650-1660 cm^{-1} are assigned to α-helix. The bands at 1655 and 1660 cm^{-1} and 1651, 1659 cm^{-1} is found in both the extraction methods. The shifting of higher wave numbers may be due to addition of H-bond formed in sonicated LPC. The frequency regions of 1610-1640 cm^{-1} and 1670-1680 cm^{-1} have been assigned to β-sheet structure.[58]Three bands are observed (Fig. 13 A and B) in both the extraction process. In sonicated LPC of *Diplazium esculentum* the bands at 1622, 1640, 1677 cm^{-1} had shifted towards higher frequency then nonsonicated one, increment in band intensity may be due to protein unfolding, which increased the protein-protein interaction.[57] Similarly, in turn structure (1660-1700 cm^{-1}) bands of sonicated was also in higher frequency end compared to nonsonicated one. Interestingly the bands at 1666 cm-1 was not changing its position in both the extraction methods shown in Fig 13 A and B. As shown in Fig 13 A and B, no unordered structure was found in both the cases, which may be due to changes in the H-bond.[58]

Using second-derivative analysis of IR-SD (Infrared-standard deviation) direct quantitative analysis of the secondary structure of the *Diplazium esculentum* proteins has been done. The areas assigned as amide I bands in second derivative spectra has been corresponding to the

amount of different types of secondary structure in the protein. Quantitative analysis of secondary structure of protein has been summarized in Table 13

Table 14 Quantification of secondary structure of protein

Extraction Method	α-helix (%)	β-sheet (%)	Unordered	Turn (%)
Sonicated	49.06	33.59	N/A	42.02
Nonsonicated	69.16	17.02	N/A	35.68

By comparing the structure of LPC of *Diplazium esculentum* proteins extracted using nonsonicated and sonicated methods, the percentage of α-helix decreased on sonication (49.06 %) method, while the percentage of β-sheet (33.59 %) and turn (42.02 %) structure increased. The changes of secondary structure of *Diplazium esculentum* protein α-helix, β-sheet and turn might affect the structural conformation and functional properties of *Diplazium esculentum* protein.[57]

Evaluation of amino acid composition

The amino acid compositions in the leaves of *Diplazium esculentum* is presented in Table 15 and Fig.14 The total amino acid present in *Diplazium esculentum* is 210.81 mg/g which are comparatively high as reported by Adeyeye and Omolayo[59] in *Telferia occidentalis* and *Amaranthus hybridus*. In the present study total amount of essential amino acid of *Diplazium esculentum* is 48.36 mg/g which is high compared when compared with the works of Adeyeye and Omolayo[59] in *Telferia occidentalis* and *Amaranthus hybridus*. The total amount of sulphur containing amino acid (Cysteine and Methionine) was found to be 62.56 mg/g which are also when high compared to 58 mg/g recommended for infants by FAO/WHO/UNU.[60] Cysteine and methionine residue function in the catalytic cycle of many enzymes by forming disulphide bonds that contribute to the protein structure however the specific function of methionine is not known but a variety of oxidants reacts with methionine to form methionine sulphoxide which in turn serves as an efficient oxidant scavenger.[61]Amino acids such as glutamine and arginine are also present in high concentration in the particular leaf protein concentrate of *Diplazium esculentum*, which in turn are reported to have health benefitting effects. Glutamine is reported to be the free amino acid in the circulation and in the intracellular pools, it acts as a precursor for the synthesis of amino acids, proteins, nucleotides and many biologically important molecules and also in the

degradation of skeletal muscles and stimulating glycogen *synthesis* in the liver.[62] On the other hand arginine improves blood circulation, strengthens the immune system, accelerates the rate of healing of wounds, improves the burning of excess of fats, reduce the cholesterol levels of blood and acts as a biological precursor of nitrous oxide(NO).[63]

Table 15 Amino acid compositions in the LPC of *Diplazium esculentum*

Sl No	Name of amino acid	Concentration of amino acids in (mg/g)
1.	Glutamine	46
2.	Histidine	1.8
3.	Glycine	1.27
4.	Threonine	6.08
5.	Cysteine	41.71
6.	Arginine	53.6
7.	Alanine	3.9
8.	Tyrosine	6.18
9.	Valine	2.4
10.	Methionine	20.85
11.	Phenylalanine	8.32
12.	Isoleucine	6.18
13.	Leucine	7.05
14.	Lysine	2.73
15.	Asparagine	2.73

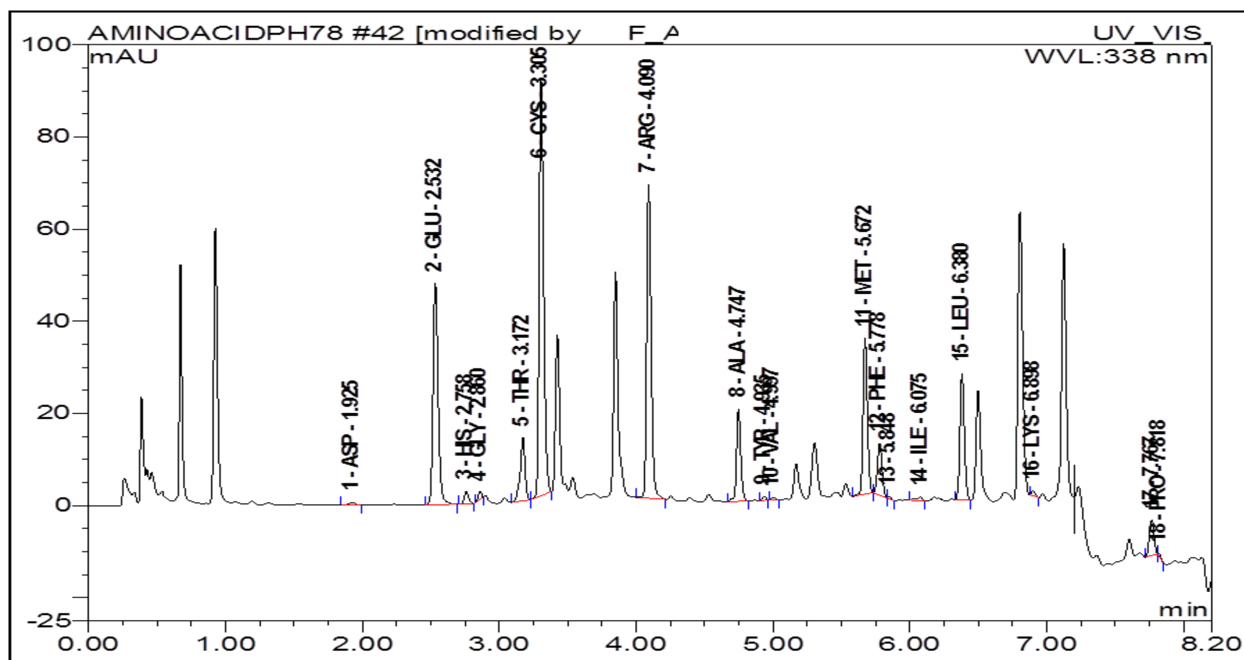

Fig. 14 UHPLC chromatograms for amino acid analysis of leaf protein concentrate of *Diplazium esculentum*

Conclusions

In this study sonicated extraction had significant improvement over nonsonicated extraction in protein content, functional properties such as water holding capacity, oil holding capacity, foaming property, and emulsifying property of leaf protein concentrate. The deconvoluted FT-IR graph reveled improvement in secondary structures, even there was improvement in functional properties of sonicated LPC compared to its nonsonicated counterpart. Contact angle experiments also proved an increase in hydrophilicity of sonicated LPC compared to its nonsonicated counterpart. Even there was an improvement of thermal behavior of the sonicated extraction compared to nonsonicated extraction, which was verified by TGA and DSC experiments. UHPLC results proved *Diplazium esculentum* to be good candidature of amino acids. Hence fourth it can be concluded that sonicated extraction of protein has modifying ability in improving the structure of proteins which in turn improves the structural, functional and thermal behavior, hence fourth it can be suggested that this procedure of extraction can be taken up by the industry as it is low cost and less time consuming.

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The Objectives (iv) and (v) are combined as it is related to development of product, shelf-life and its sensory studies.

Before developing the product the safety of leaf protein concentrates of edible fern was evaluated by cytotoxicity studies.

Cytotoxicitystudies of leaf protein concentrates of edible fern

Introduction

Vegetables play an important role in food security at the times of drought or poor harvest and are also vital for income generation. Vegetables apart from serving as food also are sources of medicines, and are important from the point of view of ecological, agronomic and cultural values.[1,2,3]Despite all these advantages, several studies have established that some vegetable species can be toxic to humans and animals. Chemical compounds of plants which are toxic to humans and livestockare part of the plant's defense system against pests and herbivores.[4]The philosophy behind toxicity testing is not only to check the safety of a test substance but also to characterize the potential toxic effects it can produce. The importance of toxicity testing was given much attention in early 1960s during thalidomide catastrophe where thousands of children were born worldwide with severe birth defects.[5] Cell lines have been used extensively to study the cytotoxicity of substances to animal cells. [6,7] Cell lines are the only regularly available source of biological material for experimentation. Evaluating general cytotoxicity can be done in a variety of ways, which can be referred to as cell viability assays. Aim of the present study was to evaluate leaf protein concentrate (*Diplazium esculentum)* cytotoxicity on HEK 293, Hep G2 and PBMC cell lines.

Materials and methods

Leaf protein concentrates (LPC) production

The leaf protein concentrate (LPC) production has been done as described in Fig 3 (Objective 2) and a comparision of the physicochemical properties has been done between two extraction procedures i.e sonication and non sonication (Objective 3) then LPC of sonication method was taken for the cytotoxicity analysis.

LPC was dissolved in 100% DMSO with stock concentration 100mg/ml in such a way so that final concentration of the DMSO in the cells would be 0.1% which is nontoxic to the cells. From the stock concentration 25,50 and 100µg/ml of LPC was used for treatment condition.

Isolation, culture, and treatment of lymphocytes

The cytotoxic effect of LPC (*Diplazium esculentum*) was investigated in human lymphocytes. Isolation performed according to the method described by Borah et al.[8]where 3 x 10^3 cells in 200 µl of isolated cells were seeded in RPMI-1640 supplemented with 10% FBS in 96 well plate. Initially, cells were incubated at 37 °C in 5% CO_2 for 8 h in RPMI-1640 without FBS. The cells were then treated as per experimental requirement and maintained with the inclusion of FBS for 12 h.

MTT assay

The cytotoxicity assay was performed by measuring the viability of cells according to the method described by Denizot and Lang[9] with slight modification. The key component 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) is yellowish in color and the mitochondrial dehydrogenase of viable cells dissolved in a suitable solvent cleave the tetrazolium ring, yielding purple insoluble formazan crystals according to Borah et al.[8] Then, 20 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diph-enyltetrazo-liumbromide) solution (5 mg/ml in PBS) was added to each well and the plate was incubated for an additional 4 h at 37ºC then the formazan crystals were dissolved in DMSO. The optical density at 570 nm was determined using a microplate reader to assess cell viability. The cell viability inhibitory ratio was calculated by the formula

$$\text{Inhibitory ratio } \% = [(\text{A control- A treated}) / \text{A control}] \times 100$$

Cell viability assays

Cell culture

The human embryonic kidney cell line HEK293 and HepG2 cells were purchased from the National Centre for Cell Science (NCCS), Pune, India. HEK293 cells were cultured in MEM medium (Himedia, India) containing 10% fetal bovine serum (Himedia, India,), 5% Penstrap Antibiotic solution (Himedia, India) ; HepG2 cells were cultured in DMEM medium (Himedia, India) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/L streptomycin. The cells were grown in a stable environment with 5% CO_2 at 37 ºC.

Cytotoxicity assay

In vitro cytotoxicity of LPC (*Diplazium esculentum*) was assessed using a standard MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide) assay with two cell lines (HEK293 and HepG2) 2 X 103 Cells were seeded in a 96-well plate and treated with increasing

concentrations 25, 50 and 100 µg/ml of LPC for 48 h at 37 °C, 5% CO_2 . Cytotoxicity was determined by adding 10 µl of MTT (0.5 mg/ml in PBS) to each well and incubated for 4 h. The medium was removed and 200 µl DMSO was added to each well and after 10 min of mechanical shaking, the optical density was measured at 570 nm in plate reader. The viability was determined in relation to control cells cultured in drug-free media. All experiments were repeated at least three times. SEM values were less than 10%.

Alamar Blue® viability assay: To determine cell viability, Alamar Blue® was added to cells in a 96-well plate for 2 h at 37 °C, 5 % CO_2. The cytotoxicity of the tested samples was performed by resazurin reduction assay as described O'Brien et al.[10]

Cell viability

The cell viability was determined using the trypan blue exclusion assay according to Lee et al.[11] were it was seeded in 25mm petri dish and treated with 25, 50 and 100 µg/ml of LPC for 48 h at 37 °C, 5% CO_2. The cells were trypsinised and stained with trypan blue (0.4% in PBS), and counted under a light microscope by hemocytometer according to the number of viable cells that excluded trypan blue. All experiments were repeated at least three times. SEM values were less than 10%. The viability ratio was calculated as:

Viability ratio % = [(The number of viable cells)/ (The total number of viable cells)] ×100

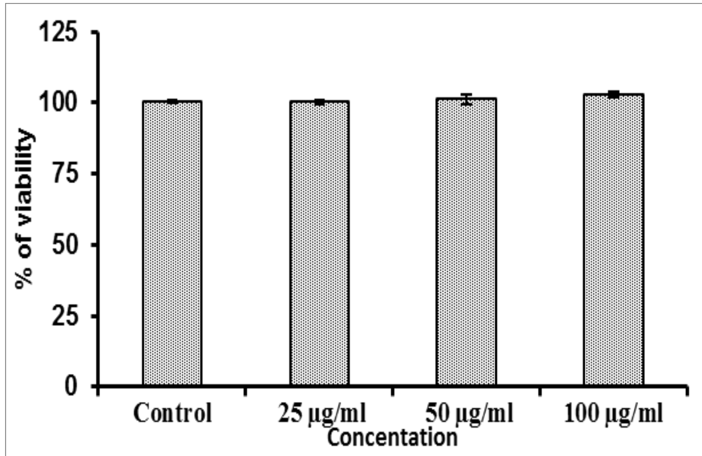
Results and discussions

Cytotoxicity study

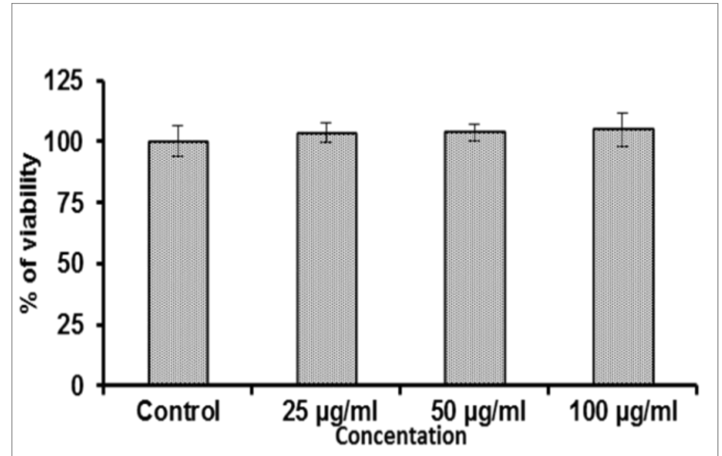
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Alamar blue and trypan blue exclusion assays were used to evaluate the viability of PBMC, HEK293 and HepG2 cells after treatment with LPC As shown in Fig. 15 (a),(b),(c), 16 and 17 there was no such significant difference observed upto 100 µg/ml of LPC for 48h in all three experiment. Therefore from the present data it can be observed that the plant sample is nontoxic to hepatocytes, kidney and lymphocytes.

Conclusion

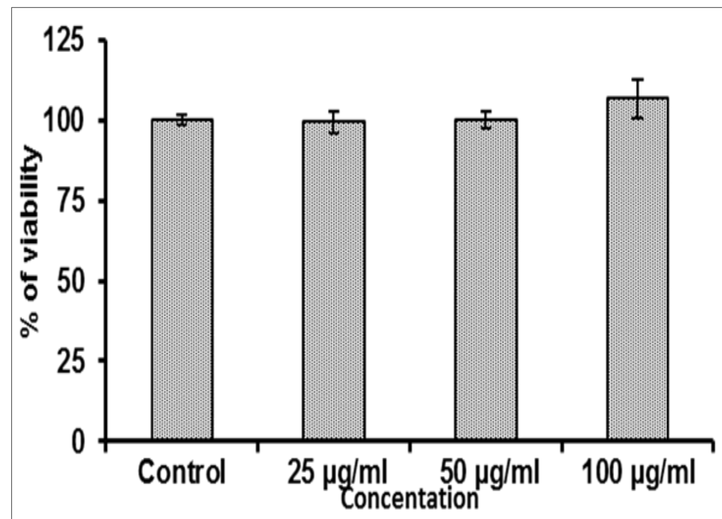
Therefore from the present data it can be observed that the plant sample is nontoxic to hepatocytes, kidney and lymphocytes



a. **PBMC**

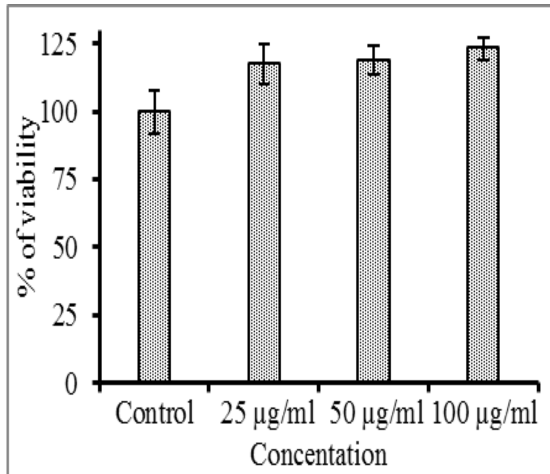


b. **HEK293**

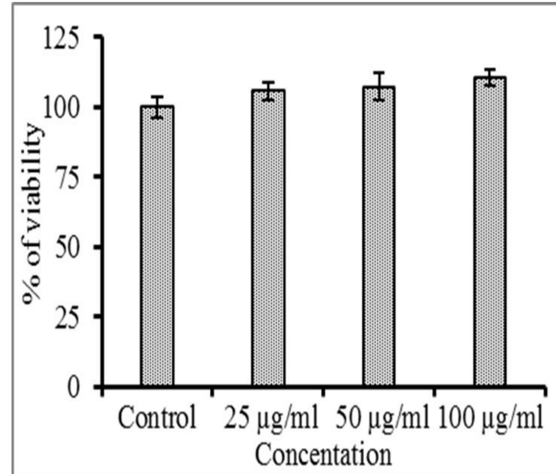


c. **HepG2**

Fig. 15 Results of cytotoxicity assay of Human lymphocyte (PBMC), HEK293, HepG2 against *Diplazium esculentum* measured by MTT based method. The viability is calculated as % of control (0%) and finally expressed as Mean ± SD, n=4. aP≤0.001 compared to control (DMSO); bP≤0.01 compared to control (DMSO); cP≤0.05 compared to control (DMSO).

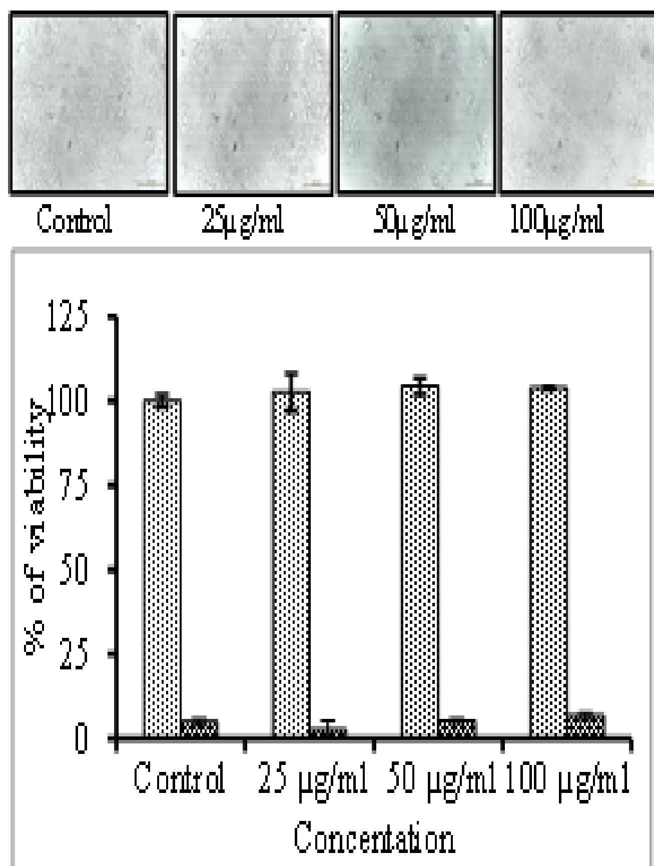


a. HEK293



b. HepG2

Fig. 16 Cell proliferation assay of Leaf Protein Concentrate of *Diplazium esculentum* at 48 hrs on **HEK293** and **HepG2** cell line measured by Alamar blue based method . The graph displays the Mean ± SEM percentage of total & dead cells of the three independent sets. Values are expressed as Mean of three independent observations. aP≤0.0001 compared to control plate; bP ≤ 0.001 compared to control plates; cP ≤ 0.01 and dP ≤ 0.05 compared to control plates



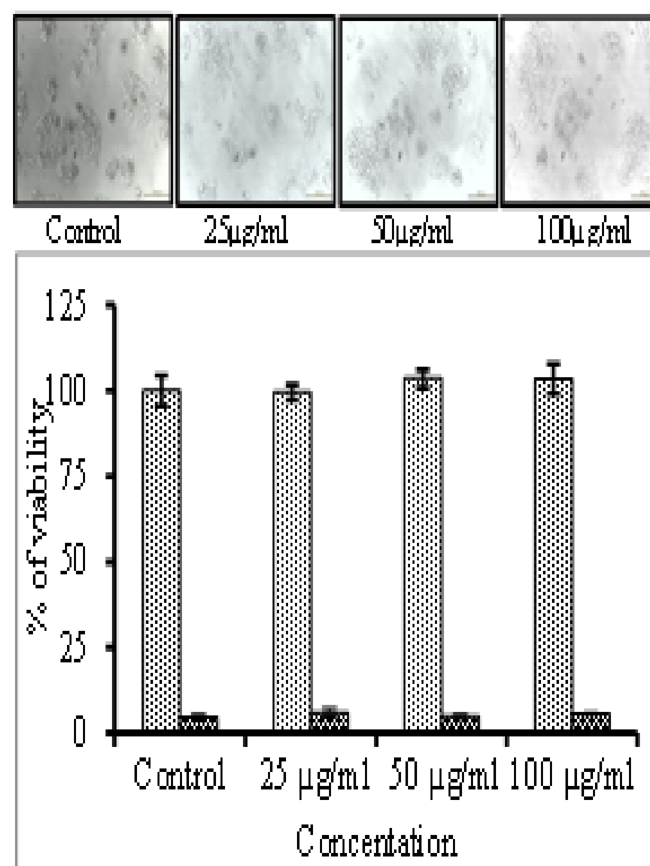


Fig 16 Cell proliferation assay of Leaf Protein Concentrate of ***Diplazium esculentum*** at 48 hrs on **HEK293** and **HepG2** cell line. The graph displays the Mean ± SD percentage of total & dead cells of the three independent sets. Values are expressed as Mean of three independent observations. [a]$P \leq 0.0001$ compared to control plate; [b]$P \leq 0.001$ compared to control plates; [c]$P \leq 0.01$ and [d]$P \leq 0.05$

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Objective (iv): To develop protein rich value added product from LPC and
Objective (v): To study the shelf-life of the value added product and sensory studies

Introduction

Health and convenience are the two major criteria for the development of snack and variety of food products. Now a day's fast life has created a necessity for the development of convenient and nutritious food products moreover poverty, malnutrition, low income problems among the underprivileged population are also among some of the compelling factors for the upgradation of nutrition related problems.[1] Pasta is a traditional extruded food which has its origins from first century BC.[2]It is very popular food because it is easy to cook and has high nutritional qualities. It is widely accepted around the globe because of its low cost, easy preparation, versatility, sensory qualities and long shelf life. Pasta is traditionally made from semolina including other nontraditional ingredients to create culinary diversity for improving nutritional quality.[3]The importance and need of additives in many foods is well understood by the food technologists and is essential in the fabrication of new products. Food additives hence are to provide a desired beneficial result. The major function of food additives is to provide

important properties such as texture, flavor, nutrition, colour, preservation and convenience. Additives therefore play an important role in fabricating and designing food. According to La Chance [4], proteins of any origins, if optimized guarantee better health and conserve the most critical and expensive resources. Based on this latter statement the most important sources of proteins for fabricating food products are vegetable proteins moreover rising meat prices all around the world and shortage of proteins are leading the growing acceptance of meat analogues and textured vegetable meat extenders. The nutritive value of LPC has been evaluated by amino acid analyses, enzymatic and dietary studies. The amino acid profile of LPC indicated that it is nutritionally superior to most cereal and legume seed proteins including cottonseed and soybeans. It also compares favorably well with most animal proteins except milk and eggs.[5,6,7]Invitro studies using pepsin- pancreatein showed that LPC was superior to beef and casein, but equivalent to milk and lactalbumin and inferior to egg protein.[8] LPC showed to be an effective protein supplement for diets consisting mainly of rice, raggi, wheat or groundnuts.[9,10,11,12]LPC has also been used as milk extender for infants and young children.[13]Many studies were performed on the enrichment of pasta by different nutritious ingredients nontraditional ingredients generally creates changes in physical and cooking behavior of pasta products.[14,15]Petiotet al.,[16] studied the structural behavior of pasta on the addition of (35% w/w) legume flour and showed that it decreased the pasta quality attributes such as higher cooking loss, lower breaking energy which could be attributed to non gluten protein and insoluble fiber that weakened the overall structure of pasta.

Electron microscopy is an important technique which allows one to observe the arrangements and organization of different components present in a particular micro organization. This technique was utilized here in this present study to observe the microstructural changes that occurred during the preparation and partial cooking of pasta as some amount of heat was generated during preparation of pasta

The objective of this research was to prepare pasta enriched with LPC in different percentage and check the effects of enrichment on pasta composition by texture analysis before and after cooking and also by SEM prior to cooking and study the proximate analysis, microbiological parameters, cooking quality, of the prepared product during the storage for 3 months at room temperature.

Materials and method

Leaf protein concentrate (LPC) production

The leaf protein concentrate(LPC) production has been done as described in Fig 3 (Objective 2)

Preparation of pasta

LPC were hydrated with warm distilled water (40°C) and mixed with semolina thereby extruded as pasta through an 66-strand Teflon coated die with 1.57 mm apertures using semi commercial laboratory extruder (FLORIDA 30, ITALY).

Extrusion occurred under the following conditions: Extrusion temperature, 45°C; mixing chamber vacuum, 46 cm of Hg an auger extrusion speed 25 r.p.m. The extrusion auger had a length diameter ratio of 8.1:1, a constant root diameter and a uniform pitch at the entire length of the auger. Extrusion pressure and dough temperature at the die, extrusion rate and mechanical energy were measured during extrusion.

Pasta was dried in a laboratory pasta drier (Standard Industries, Fargo, ND, USA) using high temperature (70°C) drying profile [19] that ran for 10 h. Dry pasta was stored in the dark at room temperature until needed.

Preparation of blends

LPC were blended with semolina at (5, 10, 15, 20) % levels and a control was prepared without adding any LPC as depicted in Fig.18 using extruder as depicted in Fig. 17

Fig. 17 Photograph of the extruder used for the preparation of pasta

Fig. 18 Photographs of pasta prepared with different percentage of LPC (*Diplazium esculentum*)

Proximate composition

Moisture, ash, protein, fat, carbohydrate, and fiber were estimated using standard procedure.[18]

SEM analysis

The microstructure of the pasta was analyzed by SEM (JEOL JSM-6390 LV,SEM,USA).Prepared pasta were fixed in the copper stub and then coated in gold. Appropriate areas were visualized under SEM and micro graphed (Fig.7.3)[19]

Texture analysis

The hardness (g) of the cooked pasta samples was measured by compression of 75% by the texture analyzer(TA-HD Plus,Stable Microsystem,UK) using a 35mm cylindrical probe of aluminum.The test was conducted at a pretest, test and posttest speed of 0.5, 0.5 and 5.0 mm/s, the test was carried with a trigger force of 3 g.

Titratable acidity

A mixture of 10g of pasta was used for pH determination as described in AOAC.[20]Total titratable acidity (TTA) was determined by titrating 20ml of the same sample against 0.1M NaOH.

Cooking quality of pasta

The cooking quality of pasta was determined by measuring the minimum cooking time, percent water absorption, volume expansion, gruel solid loss.[23]

Microbiological analysis

Enumeration of microbial load in Pasta: Microbes are ubiquitous on earth. Food products are not an exception because it is rich in macro and micro nutrients. Microbiological examination of the pasta was performed to enumerate the microbial density i.e. bacterial, fungal and yeast load under a defined laboratory conditions. Standard Plate Count (SPC) method was followed for the above mentioned purpose. Microbial count of the pasta enriched with different concentration of LPC were examined using Aerobic plate count agar (PCA; Merck Germany) according to BAM (1998)[21].LPC treated pasta was finely ground and serial dilution technique was performed followed by pour plate technique. Standard plate count was estimated by decimal dilution technique followed by pour plate method. Aerobic plate count agar (PCA;Merck Germany) was used for the analysis. 0.1 ml from the each dilution was transferred into a corresponding labeled plate and spreaded over the Agar surface. For the fungal colony enumeration, media was supplemented with Ampicillin antibiotic to suppress the bacterial growth (Robert *et al.,* 1981)[25]. After inoculation, the plates were incubated at 35- 37°C for 24-48 hours for yeast and bacterial count, 25- 27°C for 2 -3 days for the mold count. Colony counts for the plates ranged from 30-300 colonies. The numbers of colony forming units were counted and the microbial load was determined. Colony Forming Units (CFU) of the pasta samples could be determined using the formula:

CFU/g = number of colonies per g plated /Total dilution factor

Sensory analysis

Sensory evaluation: Sensory evaluation of pasta was conducted using a nine point hedonic scale with score ranging from like extremely (9) to dislike extremely (1). The evaluated parameters were flavor, taste, aroma, texture, appearance, and overall acceptability.

Statistical analysis

The data were statistically evaluated by a one way analysis of variance procedure using SPSS 14.0 software. Differences in pasta samples due to enrichment of raw and treated wheat germ were tested for significance using analysis of variance techniques (ANOVA). Duncan's multiple range tests were used when the analysis of variance indicated significant difference in means. A level of significance of $P \leq 0.05$ was used throughout the analysis.

Results and discussions

Texture analysis

Texture is an important parameter for pasta cooking quality. The hardness value of pasta in control was higher compared to the pasta incorporated with leaf protein concentrate (LPC) of *Diplazium esculentum* for both uncooked and cooked pasta (Table 17). Differences in hardness values mainly arise from the differences in leaf protein concentrate. On increasing the amount of leaf protein concentrate in turn reduces the amount of cooking water and increases the force required to produce a given extension in cooked spaghetti.[23]

Cooking loss is the amount of dry matter lost into the cooking water of optimally cooked pasta Cooking loss was highest in pasta enriched with 20% LPC and lowest for control pasta . The increase in cooking loss with the increase of leaf protein concentrate may be due to weakening of protein network. [24]

Surface ultrastructure studies using scanning electron microscope of various combinations of pasta prepared with LPC

Cooking of pasta strongly affects the characteristics and texture because of the presence of proteins, which varies both in qualities and quantities well supported by various researchers.[25,26,27,28,29]. It also affects starch by gelatinization, swelling and coagulation of proteins. In this present study, pasta is prepared by adding LPC of *Diplazium esculentum* in different concentration viz. 5%, 10%, 15% and 20% with semolina ,0% serves as a control .

When visualized under SEM a continuous film, probably of proteic nature is enveloping and hiding the starch granules which is similar as reported by Dexter and Matsuo[29] ; Matsuo et al., [30]; Resmini & Pagani[31] who worked on spaghetti. In the present study the protein appears to be fibrillary (Fig. 19) similar to as reported by Dexter & Matsuo[29]; Dexter et al.[32], which even appears to be organized in a fibrillary network that surrounds the starch granules where they stated that hydration of uncooked spaghetti did not cause changes in starch ultrastructure but promoted a compact protein structure in the good quality spaghetti. Similar interpretation can be drawn from the structures derived from this pasta incorporated with LPC of *Diplazium esculentum*. A network consisting of coagulated proteins surrounds the swollen starch granules. Same commentary on spaghetti ultrastructure's observed after cooking by the different techniques are reported by Resmini&Pagani[31] on sphaghetti which can be juxtaposed here with the morphological and behavioral changes of the present study. According to Resmini

&Pagani,[31] starch granules swelled because of water absorption and swelling which is clear in their study with SEM, and promoted a compact protein structure in the good quality spaghetti and porous protein structure in the poor quality one. A protein matrix showing the characteristics of more compactness promoted quicker water absorption at the beginning of cooking Resmini& Pagani.[31]Here in the present study the compactness in protein structure can be seen with the gradual increase of LPC in the pasta which varies in different percentage as seen in Fig. represents the structure of only LPC of *Diplazium esculentum*.

Proximate compositions of the pasta are presented in Table 15 Factors namely moisture, protein, crude oil, carbohydrate content, fibre and ash content possessed statistically significant variation with respect to the blank sample. The blank sample i.e the control sample had the lowest moisture content 9.44 %. When the moisture content is low, the energy and time requirement for the process of drying is low hence the risk of microbial contamination is also low which is a very important factor from industrial point of view. With the addition of LPC the moisture content increased with gradual supplementation. Statistical analysis even showed no significant increase in moisture content for three months. The lipid content in the pasta supplemented with different concentration of protein content showed gradual increase.

Table 16 Proximate composition of control and supplemented pasta with different concentration of LPC (*Diplazium esculentum*)

Level of supplementation in pasta (%)	Moisture (%)	Ash (%)	Crude protein (%)	Lipid (%)	Crude fiber (%)	Carbohydrate (%)
0 (Control as samolina)	9.44±0.01[a]	0.84±0.003[a]	11.57±0.03[a]	1.18±0.02[a]	0.26±0.01[a]	82.98±1.77[e]
5	9.60±0.02[b]	1.36±0.03[b]	15.66±0.04[b]	1.28±0.02[b]	0.35±0.03[b]	61.89±0.68[d]
10	9.64±0.02[c]	1.56±0.01[c]	21.33±0.09[c]	1.36±0.02[c]	0.41±0.02[c]	56.35±0.05[c]
15	9.71±0.01[c]	1.65±0.02[c]	27.38±0.03[d]	1.41±0.03[d]	0.45±0.02[c]	53.22±0.03[b]
20	9.74±0.02[d]	1.70±0.02[d]	34.30±0.02[e]	1.50±0.04[e]	0.51±0.03[d]	48.47±0.05[a]

Values in each column followed by different letters are significantly different ($p \leq 0.05$).
Values are mean±standard deviation of three measurements.

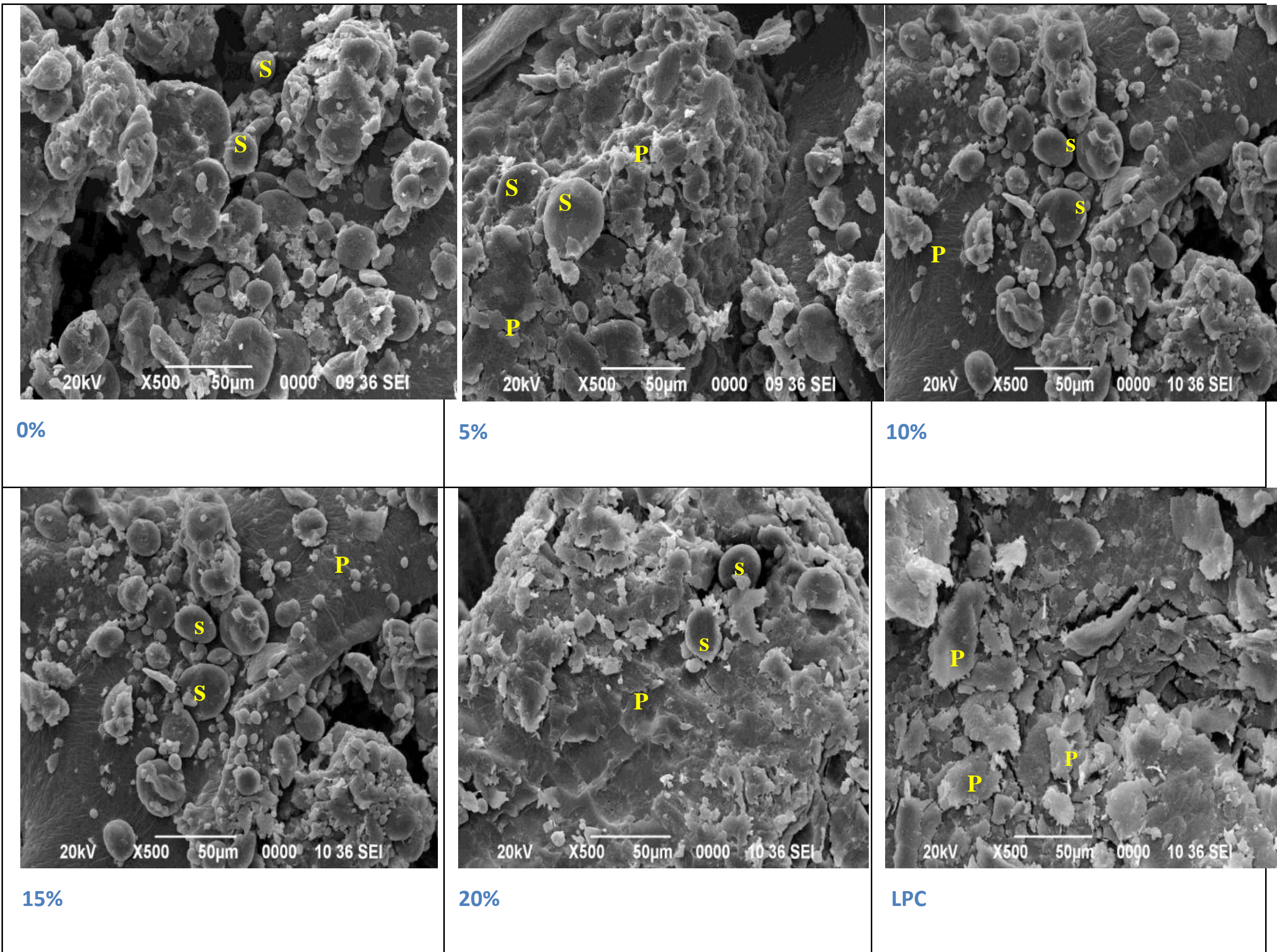

Fig. 19 SEM images of pasta prepared with different percentage of LPC (*Diplazium esculentum*)

Table 17: Proximate analysis of supplemented pasta samples with different concentration of LPC of *Diplazium esculentum* for 90 days

Level of Supplementation in pasta	0 Day	15 days	30 days	45 days	60 days	75 days	90 days
Moisture content (%)							
0% (Control)	9.44±0.01^{aA}	9.48±0.06^{aA}	9.46±0.04^{aA}	9.52±0.03^{aA}	9.53±0.02^{aA}	9.52±0.03^{aA}	9.57±0.01^{aA}
5%	9.60±0.04^{bB}	9.62±0.02^{bB}	9.67±0.05^{bB}	9.64±0.03^{bB}	9.66±0.06^{bB}	9.64±0.04^{bB}	9.65±0.01^{bB}
10%	9.64±0.03^{bB}	9.65±0.04^{bB}	9.63±0.03^{aB}	9.66±0.04^{bB}	9.68±0.04^{cB}	9.63±0.03^{aB}	9.67±0.01^{cB}
15%	9.71±0.01^{cC}	9.67±0.03^{aB}	9.66±0.04^{aB}	9.65±0.04^{aB}	9.63±0.04^{aB}	9.68±0.05^{aB}	9.71±0.03^{aC}
20%	9.74±0.02^{cC}	9.77±0.05^{bC}	9.76±0.04^{bC}	9.78±0.05^{bC}	9.77±0.04^{bC}	9.79±0.08^{bC}	9.81±0.02^{cD}
Ash content (%)							
0% (Control)	0.84±0.003^{aA}	0.87±0.03^{bA}	0.88±0.05^{cA}	0.86±0.06^{aA}	0.88±0.06^{cA}	0.87±0.07^{bA}	0.86±0.01^{aA}
5%	1.36±0.03^{bB}	1.33±0.05^{aB}	1.37±0.06^{bB}	1.38±0.04^{bB}	1.36±0.04^{bB}	1.38±0.05^{bB}	1.39±0.04^{bB}
10%	1.56±0.01^{cC}	1.58±0.04^{bC}	1.53±0.05^{aC}	1.58±0.07^{bC}	1.54±0.06^{aC}	1.58±0.03^{bC}	1.55±0.03^{aC}
15%	1.65±0.02^{dD}	1.64±0.06^{aD}	1.68±0.06^{bD}	1.66±0.07^{bD}	1.68±0.05^{bD}	1.69±0.07^{bD}	1.63±0.03^{aD}
20%	1.70±0.02^{dE}	1.73±0.05^{bE}	1.75±0.05^{bE}	1.74±0.05^{bE}	1.77±0.03^{cE}	1.75±0.04^{bE}	1.76±0.02^{cE}
Crude protein (%)							
0% (Control)	11.57±0.03^{aA}	11.59±0.04^{bA}	11.58±0.04^{bA}	11.53±0.06^{aA}	11.55±0.04^{aA}	11.59±0.04^{bA}	11.56±0.04^{aA}
5%	15.66±0.05^{bB}	15.69±0.05^{bB}	15.63±0.07^{aB}	15.71±0.04^{bB}	15.79±0.03^{bB}	15.81±0.05^{bB}	15.65±0.03^{aB}
10%	21.33±0.03^{cC}	21.38±0.04^{aC}	21.41±0.05^{aC}	21.36±0.05^{aC}	21.37±0.04^{aC}	21.35±0.06^{aC}	21.37±0.06^{aC}
15%	27.38±0.03^{dD}	27.36±0.05^{aD}	27.64±0.03^{bD}	27.71±0.05^{bD}	27.66±0.04^{bD}	27.69±0.04^{bD}	27.64±0.03^{bD}
20%	34.02±0.02^{eE}	34.06±0.05^{aE}	35.07±0.05^{bE}	35.04±0.06^{bE}	35.06±0.05^{bE}	35.08±0.06^{bE}	35.08±0.06^{bE}
Lipid content (%)							
0% (Control)	1.18±0.02^{cA}	1.17±0.05^{bA}	1.19±0.04^{cA}	1.18±0.03^{cA}	1.17±0.03^{bA}	1.15±0.03^{aA}	1.16±0.05^{aA}
5%	1.28±0.02^{aB}	1.31±0.04^{aB}	1.32±0.03^{aB}	1.29±0.04^{aB}	1.29±0.04^{aB}	1.30±0.04^{aB}	1.27±0.06^{aB}
10%	1.36±0.02^{aC}	1.34±0.03^{aB}	1.37±0.02^{bB}	1.36±0.03^{aC}	1.37±0.04^{bC}	1.35±0.03^{aB}	1.34±0.07^{aC}
15%	1.41±0.03^{aD}	1.44±0.05^{aC}	1.42±0.01^{aC}	1.43±0.05^{aD}	1.46±0.03^{bD}	1.43±0.02^{aC}	1.47±0.07^{bD}
20%	1.50±0.04^{aE}	1.52±0.03^{aD}	1.49±0.03^{aC}	1.51±0.03^{aE}	1.54±0.04^{bE}	1.55±0.04^{cD}	1.56±0.04^{cE}
Fibre content (%)							
0% (Control)	0.26±0.01^{bA}	0.27±0.02^{cA}	0.26±0.03^{bA}	0.28±0.04^{dA}	0.27±0.05^{cA}	0.25±0.06^{aA}	0.25±0.07^{aA}
5%	0.35±0.03^{aB}	0.37±0.05^{cB}	0.39±0.04^{eB}	0.36±0.06^{bB}	0.37±0.03^{cB}	0.38±0.04^{dB}	0.34±0.07^{aB}

10%	0.41±0.02^{aC}	0.43±0.04^{bC}	0.46±0.03^{bC}	0.46±0.02^{bC}	0.44±0.03^{bC}	0.44±0.02^{bC}	0.46±0.05^{bC}
15%	0.45±0.02^{bC}	0.47±0.07^{bC}	0.44±0.02^{aC}	0.46±0.05^{bC}	0.44±0.06^{aC}	0.49±0.03^{cC}	0.47±0.06^{bC}
20%	0.51±0.03^{aD}	0.53±0.05^{bD}	0.52±0.03^{bD}	0.54±0.03^{bD}	0.55±0.04^{bD}	0.56±0.02^{bD}	0.57±0.05^{bD}
Carbohydrate content (%)							
0%(Control)	82.98±1.77^{bD}	82.94±0.08^{bD}	81.83±0.06^{aD}	82.87±0.06^{bD}	82.83±0.09^{bD}	83.67±0.07^{cD}	83.69±0.05^{cD}
5%	61.89±0.69^{aC}	62.21±0.04^{bC}	62.34±0.04^{bC}	62.46±0.06^{bC}	61.67±0.05^{aC}	62.76±0.08^{bC}	62.71±0.04^{bC}
10%	56.35±0.05^{aB}	56.44±0.07^{aB}	56.63±0.06^{aB}	56.88±0.09^{aB}	56.92±0.04^{aB}	56.55±0.03^{aB}	56.78±0.03^{aB}
15%	53.22±0.03^{aB}	53.44±0.06^{aB}	53.47±0.04^{aB}	53.56±0.04^{aB}	53.61±0.07^{aB}	53.66±0.03^{aB}	53.69±0.04^{aB}
20%	48.47±0.03^{aA}	48.68±0.07^{aA}	48.71±0.06^{aA}	48.76±0.03^{aA}	48.82±0.05^{aA}	48.88±0.06^{aA}	48.77±0.06^{aA}

Values in each column followed by different letters are significantly different ($p \leq 0.05$).
Values are mean±standard deviation of three measurements.

According to Savita et al.,[1] the lipid content of semolina was found to be 1.10%.The present result of the lipid content in control pasta was found to be 1.18% made with pure semolina is even comparable with the works of Savita et al.[1] where they worked on the pasta supplemented with with various flours such as cow pea, mung bean, pigeon pea, casein, skim milk powder, whey protein concentrate, egg whole and egg albumin. Highest lipid content in their study was recorded in egg whole in contrast the highest lipid content was found to be in the pasta supplemented with LPC of 20 %. Highest fibre content was found in pasta supplemented with 20% LPC whereas the lowest fibre content was found to be in the control pasta made with pure semolina similarly ash content, carbohydrate content also increased as the percentage of supplementation increased the present study can be comparable with the works of other research workers, [33,34,35,36]who reported the incorporation of plant proteins like oyster, oak, mushroom and bengal gram flour increased the protein content, fiber content, moisture content and ash content of the final products. The proximate analysis of pasta when stored for 90 days in high density polyethylene package at ambient temperature did not show any significant changes as depicted in Table 16 and the results of the present investigation can be corroborated with the findings of various workers.[43]

Table 18 Cooking and sensory evaluation of control and supplemented pasta with different concentration of LPC of *Diplazium esculentum*

Level of Supplementation in pasta, (%)	Minimum cooking time (min)	Water absorption, (%)	Volume expansion (ml/g)	Gruel solid loss (%)	Overall acceptability score out of 9.0	Textural property Hardness/g Uncooked	Cooked
0 (control)	7.13±0.02^{a}	177.2±1.58^{a}	1.25±0.03^{d}	1.76±0.04^{a}	7.83±0.23^{c}	2884±18^{e}	182±19^{a}
5	7.17±0.03^{b}	187.03±0.69^{b}	1.16±0.04^{b}	2.12±0.02^{b}	6.83±0.62^{a}	2283±19^{a}	236±16^{b}
10	7.18±0.01^{b}	188.3±0.45^{c}	1.21±0.02^{c}	2.16±0.04^{c}	6.93±0.73^{b}	2371±10^{b}	348±14^{c}
15	7.21±0.02^{c}	193.1±1.36^{d}	1.05±0.03^{a}	3.17±0.01^{d}	8.20±0.16^{d}	2448±16^{c}	682±18^{d}
20	7.30±0.02^{d}	197.1±1.21^{e}	1.06±0.02^{a}	3.43±0.03^{e}	8.67±0.20^{e}	2662±12^{d}	889±15^{e}

Values are mean±standard deviation of three measurements
Values in each column followed by different letters are significantly different ($p \leq 0.05$).

Table 19 Cooking behavior of pasta prepared with different levels of LPC (*Diplazium esculentum*) and their storage study

Level of Supplementation	0 day	15 Days	30 days	45 days	60 days	75 days	90 days
Minimum cooking time(min)							
0% (Control)	7.13±0.02^{aA}	7.14±0.03^{bA}	7.18±0.06^{dA}	7.21±0.05^{eA}	7.16±0.06^{cA}	7.18±0.05^{dA}	7.16±0.05^{cA}
5%	7.17±0.03^{aA}	7.19±0.06^{bA}	7.20±0.05^{bB}	7.22±0.03^{cA}	7.19±0.05^{bA}	7.20±0.04^{bB}	7.22±0.05^{cB}
10%	7.18±0.01^{aA}	7.19±0.04^{aA}	7.22±0.03^{bB}	7.25±0.06^{bB}	7.17±0.03^{aA}	7.21±0.03^{bB}	7.23±0.06^{bB}
15%	7.21±0.02^{aB}	7.25±0.03^{bB}	7.24±0.03^{bB}	7.26±0.04^{bB}	7.25±0.06^{bB}	7.26±0.04^{bC}	7.29±0.04^{cC}
20%	7.30±0.02^{aB}	7.33±0.05^{bC}	7.34±0.03^{bC}	7.36±0.02^{bC}	7.36±0.02^{bC}	7.37±0.03^{bD}	7.33±0.07^{bD}
Water absorbtion(%)							
0%(Control)	177.2±1.58^{cA}	176.43±1.53^{bA}	178.53±1.27^{dA}	175.64±1.45^{aA}	178.42±1.44^{dA}	176.62±1.38^{bA}	178.55±1.54^{dA}
5%	187.03±0.69^{dB}	186.54±0.32^{cB}	185.64±0.65^{bB}	188.31±0.63^{dB}	184.43±0.53^{aB}	186.42±0.46^{cB}	185.76±0.49^{bB}
10%	188.3±0.45^{dB}	187.48±0.48^{cB}	186.86±0.74^{bB}	187.85±0.77^{cB}	185.49±0.55^{aB}	187.42±0.64^{cB}	188.84±0.53^{dB}
15%	193.1±1.36^{aC}	194.5±1.34^{bC}	196.7±1.29^{cC}	194.62±1.28^{bC}	195.6±1.21^{cC}	194.2±1.36^{bC}	197.62±1.42^{cC}
20%	197.1±1.21^{cD}	197.34±1.34^{cD}	198.31±1.24^{cD}	196.76±1.74^{bD}	195.65±1.55^{bD}	196.23±1.54^{bD}	194.54±1.54^{aD}
Volume of expansion(ml/g)							
0%(Control)	1.25±0.03^{bD}	1.27±0.06^{bC}	1.23±0.04^{aC}	1.22±0.08^{aD}	1.29±0.05^{bC}	1.27±0.08^{bC}	1.28±0.04^{bC}
5%	1.16±0.04^{bB}	1.13±0.05^{aB}	1.18±0.07^{bB}	1.17±0.06^{bC}	1.19±0.03^{cB}	1.14±0.03^{aB}	1.19±0.08^{cB}
10%	1.21±0.02^{aC}	1.26±0.06^{bC}	1.26±0.04^{bC}	1.29±0.09^{cE}	1.27±0.01^{bC}	1.25±0.06^{bC}	1.22±0.05^{aC}
15%	1.05±0.03^{aA}	1.09±0.04^{bA}	1.14±0.01^{cB}	1.11±0.07^{bA}	1.17±0.06^{cB}	1.13±0.03^{cB}	1.11±0.07^{bA}
20%	1.06±0.02^{aA}	1.09±0.05^{dA}	1.11±0.04^{eA}	1.13±0.04^{fB}	1.07±0.04^{bA}	1.08±0.04^{cA}	1.12±0.05^{eA}
Gruel solid loss(%)							
0%(Control)	1.76±0.04^{bA}	1.71±0.03^{aA}	1.74±0.07^{bA}	1.75±0.05^{bA}	1.77±0.08^{bA}	1.75±0.02^{bA}	1.78±0.03^{bA}
5%	2.12±0.02^{aB}	2.17±0.07^{bB}	2.13±0.04^{aB}	2.18±0.05^{bB}	2.14±0.04^{aB}	2.19±0.05^{bB}	2.16±0.06^{bB}
10%	2.16±0.04^{aB}	2.15±0.03^{aB}	2.14±0.03^{aB}	2.19±0.08^{aB}	2.16±0.05^{aB}	2.17±0.04^{aB}	2.18±0.03^{aB}
15%	3.17±0.01^{aC}	3.19±0.04^{aC}	3.14±0.02^{aB}	3.18±0.06^{aC}	3.15±0.04^{aC}	3.16±0.04^{aC}	3.14±0.05^{aC}
20%	3.43±0.03^{aD}	3.42±0.04^{aD}	3.41±0.04^{aC}	3.44±0.03^{aD}	3.47±0.07^{bD}	3.48±0.05^{cD}	3.44±0.06^{aD}
Titratable cidity(%)							
0%(Control)	3.86±0.96^{bA}	3.81±0.89^{aA}	3.88±0.93^{cA}	3.86±0.95^{bA}	3.88±0.94^{cA}	3.89±0.82^{cA}	3.85±0.97^{bA}
5%	4.93±0.66^{fB}	4.91±0.63^{eB}	4.88±0.66^{cB}	4.82±0.64^{aB}	4.85±0.67^{bB}	4.86±0.67^{bB}	4.92±0.62^{eB}
10%	5.10±0.54^{aC}	5.18±0.55^{dC}	5.17±0.57^{cC}	5.18±0.54^{dC}	5.17±0.53^{cC}	5.13±0.54^{bC}	5.19±0.58^{eC}
15%	5.40±0.57^{aC}	5.52±0.54^{bD}	5.53±0.57^{bD}	5.57±0.58^{bD}	5.47±0.57^{aD}	5.47±0.53^{aD}	5.44±0.59^{aD}
20%	5.70±0.37^{aD}	5.74±0.33^{bE}	5.74±0.34^{bE}	5.79±0.38^{cE}	5.71±0.38^{aE}	5.74±0.66^{bE}	5.69±0.05^{aE}

Values are mean±standard deviation of three measurements
Values in each column followed by different letters are significantly different ($p\leq0.05$).

Effect of supplementation on cooking quality of the prepared pasta

Table 18 presents the data on the effect of cooking quality characters of pasta supplemented with different concentration of LPC. Supplementation of pasta with LPC enhanced the quality of pasta. Supplementation of pasta increased the time taken by the pasta to gelatinize compared to control by a statistical non-significant variation. Higher water absorption was observed with the gradual increase of supplementation. Even with the enrichment of pasta with different concentration of LPC there was increase in cooking loss when compared with blank or control. The present data can be corroborated with the studies of various authors.[36,37,38,39.].The enrichment of pasta with wheat germ which might be related to gluten dilution and the protein solubility fraction of wheat germ[39]. In all the cases with the increase of cooking time there was an increase in cooking loss. The effect of time on cooking loss can be explained by the thermal effect of excessive heating of protein matrix. There might be some modification in protein solubility which might have arisen and changed the degree of association between polypeptide chains, hydrogen bonds, hydrophobic interactions and salt linkages.[40]The higher cooking loss for enriched pasta samples could be attributed to the disruptive effect of germ composition on the protein matrix, which allowed the starch granules to rupture during cooking, hence releasing higher levels of amylose into the water. On storage (Table 18) there was no significant change in the cooking behavior of the pasta.

Titratable acidity

Titratable acidity is directly related to the presence of organic acids. Organic acids exists as free acids or in combined form in the pasta On the basis of pH and total titratable acidity of five samples there was a significant increase in total titratable acidity of the pasta samples compared to the control or blank (Table 18) which might be due to metabolic changes that occurred during the storage [41] in contrast high titratable acidity has been reported to reduce the incidence of diarrhea in infants for consuming fermented legumes. In the present study the LPC blended pasta might add two dimensions in the study such as antimicrobial properties and high protein contents with the gradual increase of LPC in the pasta. There was no significant increase in titratability of acid when kept for storage study for three months.

Microbiological count

It is well known that pasta has relatively low moisture content and it is recommended as safe food product microbiologically. LPC is an alternative source of protein from plant kingdom which may be contaminated in the extraction procedure by various pathogens and then can survive for extended periods. Therefore in this study aerobic plate counts as well as mould and yeast counts were performed for blank and enriched pasta with LPC for three months.

Table 20 Aerobic plate count, mould and yeast count of supplemented pasta samples with different concentration of LPC of *Diplazium esculentum*

Microbial Test	Levels of supplementation in pasta(%)	After production (cfu/g)	15 days after production (cfu/g)	1 month after production (cfu/g)	45 days after production (cfu/g)	60 days after production (cfu/g)	75 days after production (cfu/g)	90 days after production (cfu/g)
Aerobic plate count	0(control)	16 ± 2.0^{aA}	21 ± 8.0^{bA}	24 ± 12.0^{cA}	27 ± 14.0^{dA}	34 ± 9.0^{eA}	37 ± 8.0^{fA}	41 ± 7.0^{gA}
	5	21 ± 3.0^{aB}	32 ± 9.0^{cB}	39 ± 13.0^{dB}	54 ± 13.0^{bB}	59 ± 13.0^{dB}	63 ± 7.0^{dB}	67 ± 9.0^{eB}
	10	25 ± 25^{aC}	39 ± 5.0^{bC}	42 ± 10.0^{cC}	47 ± 9.0^{dC}	51 ± 11.0^{dC}	59 ± 8.0^{eC}	68 ± 7.0^{fC}
	15	32 ± 31^{aD}	41 ± 21.0^{bD}	47 ± 22.0^{cD}	58 ± 6.0^{cD}	63 ± 15.0^{dD}	71 ± 7.0^{dD}	82 ± 8.0^{eD}
	20	35 ± 5.0^{aE}	48 ± 16.0^{bE}	58 ± 18.0^{cE}	64 ± 12.0^{bE}	71 ± 14.0^{dE}	77 ± 7.0^{eE}	81 ± 8.0^{fD}
Mould and yeast count	0(control)	-	-	58 ± 13.0^{aA}	61 ± 9.0^{bA}	67 ± 8.0^{cA}	71 ± 15.0^{dA}	75 ± 7.0^{eA}
	5	-	-	62 ± 9.0^{aB}	69 ± 15.0^{bB}	75 ± 8.0^{cB}	81 ± 9.0^{dB}	89 ± 9.0^{eB}
	10	-	-	69 ± 28.0^{aC}	71 ± 21.0^{bC}	75 ± 12.0^{bC}	78 ± 8.0^{cC}	81 ± 8.0^{dC}
	15	-	-	75 ± 21.0^{aD}	77 ± 27.0^{bD}	81 ± 31.0^{bD}	89 ± 12.0^{bD}	93 ± 9.0^{bD}
	20	-	-	77 ± 14.0^{aE}	84 ± 22.0^{bE}	88 ± 19.0^{bE}	93 ± 8.0^{cE}	98 ± 7.0^{dE}

Values in each column followed by different letters are significantly different ($p\leq0.05$)

Values are mean±standard deviation of three measurements

The results of the present study are safe to consume even after 3 months of storage[42]. There was no significant difference in the pattern of microbial load in the blank samples for the period of three months whereas there was growth recorded in the pasta which was supplemented with LPC (Table 20). The growth pattern among the same percentage of supplementation did not show a significant difference but on contrary with that of the blank sample there was a

significant growth. The results are in line with the study of Babak et al.[43] where they observed growth for aerobic organisms, mould and yeast growth between pasta enriched with heated and unheated wheat germ. In the present study during the first month of pasta production there was no observable growth of yeast and mould. There was increase in the growth observed in the first month and hence fourth but there was no statistical difference found in the pasta that were supplemented with different percentage of LPC and remained microbiologically stable and acceptable.

Nine point hedonic scale sensory evaluation proclaimed the pasta supplemented with 20% to be the best among all the pasta supplemented with different percentage of LPC based on the overall acceptability.

Conclusions

Many efforts have been done for the pasta enrichment by different ingredients, LPC of *Diplazium esculentum* is a good alternative source of protein. SEM study revealed the compactness of the structure of protein with the gradual increase of LPC. Although there was a slight differences between the appearances and taste of enriched pasta with blank. The overall scores were not significantly different.20% pasta scored the highest in sensory evaluation Storage upto three months did not significantly affect the cooking quality and proximate analysis. Pasta samples stored for three months were safe to consume even from microbiological point of view. The degree of hardness as measured by texture analyzer showed that on gradual increase of protein percentage the degree of harness increased. From the biochemical, cooking and microbiological point of view it can be recommended that three month of shelf life study can be performed instead of one year which is common for extruded products.

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Annexure-XI

Final Consolidated Statement of Expenditure

1. Sanction Letter/ Order No and date of sanctioning the project: **SERB/MoFPI/0030/2014, Dated: 12/12/2014**
2. Total Project Cost: **Rs. 21,55,978.00 (Rupees twenty one lacs/ fifty five thousand/ nine hundred / seventy eight) only**

   (Sanctioned/ Revised Project Cost, if applicable)
3. Date of Commencement of Project: **01.04.2015**
4. Date of Completion of Project: **31.03.2017**
5. Grant received in each year (financial year):
   a. 1st Year: Rs. 15, 77,789.00
   b. 2nd Year: Rs. 5, 00,000.00
   c. Interest, if any: Rs. 4183.00
   d. Total (a+b+c): Rs. 20,81, 972.00

Finance Officer
Tezpur University

Final Consolidated Statement of Expenditure

(Submitted financial year wise i.e. DOS* 01.4.2015 31.3.2016 and 1.4.2016 to 31.3.2017)

Sr No	Sanctioned Heads	Funds Allocated (sanctioned)	Total Released amount	Expenditure Incurred		Total Expenditure	Balance as on (31.3.2017)	Requirement of Funds up to 31st March next year	Remarks (if any)
				1st Year (1.4.2015 - 31.3.2016)	2nd Year (1.4.2016 – 31.3.2017)				
(I)	(II)	(III)	(IV)	(V)	(VI)	V + VI = (VII)	IV –VII = (VIII)		
		(Rs.)	(Rs.)	(Rs.)	(Rs.)	(Rs.)	(Rs.)	(Rs.)	
1.	Manpower costs	3,84,000.00	3,05,811.00	1,76,000.00	2,08,000.00	3,84,000.00	(-)78,189.00	**44,181.00**	
2.	Consumables	5,16,380.00	5,16,380.00	2,60,016.00	2,58,046.00	5,18,062.00	(-)1682.00		
3.	Travel	60,000.00	60,000.00	28,515.00		28,515.00	31,485.00		
4.	Equipment	9,99,600.00	9,99,600.00	9,99,576.00		9,99,576.00	24.00		
5.	Interest, if any	-	4183.00				4183.00		
6.	Overhead expenses	1,95,998.00	1,95,998.00	98,000.00	98,000.00	1,96,000.00	(-)2.00		
	Total	**21,55,978.00**	**20,81,972.00**	**15,62,107.00**	**5,64,046.00**	**21,26,153.00**	**(-)44,181.00**		

Name and Signature of Principal Investigator:
Date: 1.11.17

Professor
Deptt. of Food Engineering & Technology
Tezpur University, Napaam-784028
Dist-Sonitpur (Assam)

Signature of Competent financial/ audit authority:
(with seal) Finance Officer Tezpur University
Date: 23.11.17

* DOS : 01.4.2015

UTILISATION CERTIFICATE
FOR THE FINANCIAL YEAR 2016-17

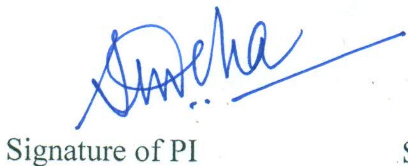
1.	Title of the Project/ Scheme	**Development of value added food products from leaf protein concentrate of green leafy vegetables of Assam**
2.	Name of the Institution	**Tezpur University, Assam**
3.	Principal Investigator	**Prof. Sankar Chandra Deka**
4.	Department of Science & Technology sanction order No & date sanctioning the project	**SERB/MoFPI/0030/2014 Dated12/12/2014**
5.	Head of account as given in the original sanction order	**Nonrecurring** (Equipment) **and Recurring** (Salaries, Consumables, TA/DA, Institutional Charges)
6.	Amount brought forward from the previous Financial year quoting DST letter no and date in which the authority to carry forward the said amount was given	i. Amount **Rs. 15,682.00** ii. Letter No **SERB/MoFPI/0030/2014** iii. Date **12.12.2014**
7.	Amount received during the financial year (Please give DST letter/order no and date)	i. Amount **Rs 5,00,000.00** ii. Letter/Order No **RTGS/NEFT UTR/Transaction No UBINH 16250132578/SAA216475810** ii. Date **06.09.2016**
8.	Interest earned (14.3.15 to 31.3.15)	**Rs. 4183.00**
9.	Total amount that was available for expenditure (excluding commitments) during the financial year (Sr. No. 6+7)	**Rs. 5,19,865.00**
10.	Actual Expenditure (excluding commitments)Incurred during the financial year (up to 31st March)	**Rs. 5,64,046.00**
11.	Balance amount available at the end of the financial year:	**(-) Rs. 44,181.00**
12.	Unspent balance refunded, if any (please give details of cheque no etc.):	**NIL**
13.	Amount to be carried forward to the next financial year (if applicable):	**NIL**

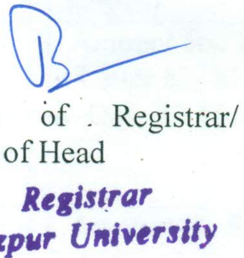
Contd.

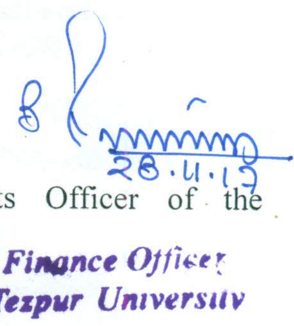
Finance Officer
Tezpur University

UTILISATION CERTIFICATE (2016-17)

Certified that out of **Rs. 5, 00,000.00** of grants-in-aid sanctioned during the year **2016-17** in favour of **Tezpur University, Assam** under this Ministry/ Department letter/ order No **RTGS/NEFT UTR/Transaction No UBINH 16250132578/SAA216475810 Date 06.09.2016** and **Rs 15,682.00** on account of unspent balance of the previous year, and an interest earned **Rs. 4183.00**, a sum of **Rs. 5,64,046.00** has been utilized for the purpose for which it was sanctioned and that the excess expenditure amount of **Rs. 44,181.00 is to be reimbursed to the Tezpur University, Assam by SERB, New Delhi.**







Signature of PI	Signature of Registrar/ Signature of Head	Accounts Officer of the Institute
Date 1.11.17	Date	Date
Professor Deptt. of Food Engineering & Technology Tezpur University, Napaam-784028 Dist-Sonitpur (Assam)	Registrar Tezpur University	Finance Officer Tezpur University

(To be filled in by DST)

Certified that I have satisfied that the conditions on which the grants-in-aid was sanctioned have been fulfilled/ are being fulfilled and that I have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned:-

Kinds of checks exercised.

1.
2.
3.
4.
5.

Signature:___________

Designation:___________

Date:___________