Original Article

Chromatographic Characterization for Quantitative Measurement of Phytoconstituents in Polyherbal Suspension and Evaluation of its Nutritional Efficacy on Malnourished Rats

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ABSTRACT

Background: The present study demonstrated HPLC method development for quantitative measurement of phytoconstituents present in food-grade polyherbal suspension (PHS) and evaluated its nutritional efficacy in the rat. **Procedure:** The PHS was prepared by mixing the dried extracts of *Nelumbo nucifera* (NN) seeds, seeds of *Euryale ferox* (EF) and fruits of *Trapa natans* (TN) with other excipients. The PHS was analyzed using the HPLC method for the development and validation of the phytoconstituents present in PHS as compared to standard biomarkers. The nutritional efficacy of PHS was evaluated on malnourished rats by observing body weight, food intake, biochemical and haematological data. **Results:** Results exhibited that the developed HPLC method was simple, rapid, sensitive and cheap for the determination of phytoconstituents in the PHS. The nutritional composition of NN, EF and TN revealed that protein content was highest in TN (21.30%), NN (14.30%) and EF (13.16%) respectively. PHS restored the haematological data of malnourished rats with values of 7.37 $10^3/\mu$L, RBC; 11.75 g/dL, Hb; and 1147 $10^3/\mu$L, platelets at 400 mg/kg dose compared to the control group. The result of biochemical data exhibited that cholesterol and triglyceride levels were 36.08 and 60.28 mg/dL, respectively after treatment with PHS (400 mg/kg). PHS also enhanced the bodyweight of animals in comparison to the control group. **Conclusion:** Thus, PHS may be an alternative nutritional formulation against protein deficiency-induced malnutrition.

Keywords: Malnutrition, Polyherbal, HPLC, Validation, Biomarkers.

INTRODUCTION

Malnutrition is a problem in almost all countries despite their economic development, where people are devoid of high-quality diets.[1] The insufficient body's dietary needs may lead to decreased immunity, more susceptibility towards infection, impairment of mental and physical development and decreased productivity.[2-3] In India, malnutrition is a major concern for children below five years of age; the small villages, districts and states share a large proportion (80%) of the malnutrition burden.[4] It is also a significant issue for older patients, despite their decreased energy requirement they need the same nutrients and protein intake as equal to younger patients, however, age-related changes pose an impact to meet these requirements.[5] Public health approaches for the prevention and control of protein deficiency have been developed and expanded. The deficiency of nutrients can be assessed by executing different approaches including measurement in blood, food intake and biochemical level.[6] Nutritional supplements are an important source to regain reduced nutrition.[7] It may help to fulfil adequacy of diet, improving health parameters and quality of life.[8]

Plants and herbs as nutritional supplements have been receiving much attention in past

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decades. Medicinal plants and herbs are the best sources for nutrition, they also add aroma and flavour to the food.[9] Herbs are naturally rich in protein and preferable to animals proteins that are liable to cause obesity and other disorders.[10] *Nelumbo nucifera* (NN) is a member of the Nelumbonaceae family, in India, it is known with other names such as Indian lotus, sacred lotus and water lily.[11] The phytoconstituents present in seeds of NN are nuciferine, lotusine, dauricine, roemerine, armepavine, and neferine.[12] The seeds are rich in proteins and essential minerals to meet the daily requirement of nutrients.[13] *Euryale ferox* (EF) is an aquatic nut crop commonly known as Makhana in India. It is rich in carbohydrates and protein.[14] It has a nourishing property to the heart, ovaries, testes and uterus. It reduces chronic fatigue and gives body strength.[15] *Trapa natans* (TN) is a free-floating plant that belongs to the family Trapaceae. It is commonly known as Singhara in India and grows in ponds, shallow water and swampy lands. The fruits of *T. natans* are rich in nutrients, proteins, carbohydrates and essential minerals. It has been used in many Ayurvedic preparations due to its astringent, nutrient, diuretic, tonic, antidiarrhoeal and appetizer properties.[16]

The nutritive properties of herbs are because of active phytoconstituents that works synergistically in polyherbal formulation for producing pharmacological effects.[17] Thus, a lower dose is required for such polyherbal formulation to achieve the desired therapeutic effect with reduced toxicities. The standardization of polyherbal formulation helps in the identification of active compounds that are responsible for the therapeutic effect. The quality control method using high-performance thin-layer chromatography (HPLC) helps in the validation of these active compounds.[18] Therefore, in this study, the HPLC method was developed for the validation of phytoconstituents and to evaluate the nutritional efficacy of polyherbal formulation in malnourished rats.

Procedure

Plant details

Nelumbo nucifera, Euryale ferox and *Trapa natans* plant parts were used for the study. They were collected from Dhamatari, Chhattisgarh, India. The plant parts were identified by Dr. S. S. Chandravanshi, Krishi Vigyan Kendra, Chhattisgarh and voucher specimens were deposited in the institute (Voucher no. 0243).

Nutritional analysis of collected plant parts

Total carbohydrate

Hodge and Hofreiter, 1962, the method were used to determine total carbohydrate content in the samples.[19] Samples were hydrolyzed with HCl (2.5 N) for 3 hr and then neutralized with NaCl followed by centrifugation at 12000 × g for 10 min. The aliquot (1 ml) of supernatant was taken to add into the anthrone reagent (4 ml) and then heated the mixture for eight minutes. The developed dark green colour was measured at 630 nm. The amount of glucose present in PHS was expressed with unit mg/g.

Determination of Crude Protein

The presence of crude protein in samples was determined according to the method of Sadasivam and Manickam, 1997.[20] Briefly, samples were placed in a 250 ml Kjeldahl flask for the determination of nitrogen content. The flask was containing a mixture of the catalyst including potassium sulphate with mercuric oxide and then concentrated sulphuric acid was added later. The mixture was heated in a water bath till a colourless solution was obtained. The amount of nitrogen reduced due to steam distillation was collected in boric acid solution, which was estimated by titration with HCl (0.02 N) using the mixed indicator of methylene blue and methyl red. The calculation was done using the following formula:

$$\text{Nitrogen/kg} = \frac{\text{ml (HCL)} - \text{ml(blank)} \times \text{normality} \times \text{final volume}}{\text{weight(g)} \times 14.01 \times \text{aliquot volume}}$$

$$\text{Percentage crude protein} = \text{Nitrogen \%} \times 6.25$$

Determination of Crude Fats

Fat content in the samples was determined using the soxhlet extraction method.[21] Briefly, samples were filled in a soxhlet apparatus with a flask containing anhydrous ether (75 ml). The extraction was performed for 16 hr and crude fat was collected after evaporation of solvent at 1000°C for 1 hr. It was calculated as:

$$\text{Percentage crude fat} = \frac{\text{Weight of the ether soluble material}}{\text{Weight of the sample}} \times 100$$

Estimation of Crude Fibre

The procedure was performed according to the method of Sadasivam and Manickam, 1997.[20] The pre-treated samples with ether were used and boiled with sulphuric acid (0.255 N) followed by filtration through a muslin cloth. It was boiled again with sodium hydroxide (0.313 N) then filtered through a muslin cloth and boiled again with sulphuric acid then alcohol and water successively. The residue was transferred to the china dish (C1) and dried for 2 hr at 130°C then weighted (C2) with the dish. The ash was ignited at 600°C for 30 min, cooled in a desiccator and finally weighted the ash (C3) in a dish to determine crude fibre.

$$\text{Percentage crude fiber} = \frac{[\text{Loss in weight on ignition}}{(C2 - C1) - (C3 - C1)} \times 100$$
$$\text{Weight of the sample taken}$$

Estimation of Energy

The energy value of sample drugs was calculated using following formula:

$$\text{Energy value} = [(4 \times \%\text{Protein}) + (9 \times \%\text{Fats}) + (4 \times \%\text{Carbohydrates})]$$

Estimation of Moisture Content

The moisture content in the samples was determined by recording their initial weight and dry weight (heating at 70°C). The following formulation was used to calculate moisture content in fresh weight:

$$\text{Moisture content} = \left(\frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}}\right) \times 100$$

Extraction of Collected Herbs

Extraction of Nelumbo nucifera seeds

The extraction was performed using hydroethanolic (50:50) as solvent. *N. nucifera* seeds (100 g) were filled in soxhlet apparatus and extracted for 2 hr at 50°C. It was allowed to dry using a vacuum evaporator and stored in a desiccator.[22]

Extraction of *Euryale ferox* seeds

The extraction was performed using hydroethanolic (50:50) as solvent. *E. ferox* seeds (100 g) were macerated with solvent and shaken for 16 hr over a mechanical shaker. It was allowed to dry using a vacuum evaporator and stored in a desiccator.[23]

Extraction of *Trapa natans* fruits

The extraction was performed using hydroethanolic (50:50) as solvent. *T. natans* fruits (100 g) were macerated with solvent and shaken for 15 hr over a mechanical shaker. It was allowed to dry using a vacuum evaporator and stored in a desiccator.[24]

Formulation of the polyherbal suspension (PHS)

PHS was prepared using the trituration method using dried extract and excipients. Briefly, 1g of each plant extract, 0.5% (w/v) of sodium carboxymethylcellulose, 0.2% (w/v) of methylparaben, 0.1% (w/v) Tween-80 and water up to 100 ml was used for preparing PHS. Initially, extracts were triturated individually with methylparaben and then Tween-80 was added with a small quantity of sodium carboxymethylcellulose to form a paste. The remaining part of sodium carboxymethylcellulose was added later to form a slurry and then rinsed with water to make up the volume up to 100 ml. The mixture was stirred to form a homogenous paste and PHS was stored in a refrigerator.[25]

Analysis of compounds present in PHF using HPLC technique

Preparation of stock solution of standard drug

The stock solution of the standard drug was prepared using methanol as a solvent and then stored stock solution in light-resistant or dark containers. The calibration curve was a plot for different phytoconstituents using the standard solution with a concentration containing 2-10 µg/mL of each phytoconstituents.[26]

Preparation of Sample Solution

The samples solutions of individual plants extracts (1000 µg/ml) and polyherbal suspension (1000 µg/ml) were prepared in methanol. The solution was filtered through nylon filter paper with pore size 0.45 µm. The filtrates were diluted with the mobile phase and injected directly.

Instrumentation and operating conditions

HPLC (Waters, USA) was assembled with an RP-18e column with a dimension of 250 mm length and 64.6 mm id (Merck, Germany). It was equipped with a photodiode array detector, loop manual injector (20 IL), an inline-degasser AF and software of HPLC Waters for analysis of phytoconstituents. The mobile phase was composed of methanol: formic acid (80:20 v/v) and it was used to pump at a 1 ml/min flow rate. It was monitored at 280 nm. The samples were injected in the volume of 20 lL and the column's temperature was maintained at 258°C.[27]

Validation method

Limit of quantification (LOQ), Limit of detection (LOD), and Linearity curve

Stock solutions of individual plants extracts and PHS were prepared by taking a 10 mg sample in methanol to make the concentration of 1 mg/ml solutions. The linearity curve was prepared by serial dilution of the solutions. The LOD and LOQ of the samples were determined for individual plants extracts and PHS following the standard methods.[28]

Precision

Percentage relative standard deviation was calculated by injecting six replicated injections of individual plants extracts and PHS in three different concentrations for intraday and interday precision with 11 injections.

Recovery

The percentage recovery of the phytoconstituents present in the extracts of plants and PHS was performed

to determine the accuracy of the method. The samples of plants extracts and PHS were spiked with different amounts of phytoconstituents i.e. 1 (90, 189, 234 µg/mL), 2 (67, 119, 201 µg/mL), 3 (43, 89, 183 µg/mL) and 4 (45, 89, 200 µg/mL) before extraction. The samples were spiked three times and then analyzed.

In vitro cytotoxicity study of prepared PHS

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used for evaluation of *in vitro* cytotoxicity of PHS. RAW 264.7 cells were seeded into 96 well culture plates (2 x 10^4 cells/well). The cells were incubated with PHS in different concentrations of 12.5, 25, 50, 100, 200, 400, 800, and 1600 µg/ml for another 24 and 48 hr. The dye was added to each well in the concentration of 5 mg/ml. The cells were incubated again for 4 hr at 37°C to allow the colour change of viable cells into purple-coloured formazan crystal. The crystals were dissolved in dimethyl sulfoxide (DMSO, 150 µl) and observed the optical density (OD) at 562 nm.[29] The viability of cells was determined as follows:

$$\text{Viability (\%)} = \frac{\text{OD of sample}}{\text{OD of control}} \times 100$$

Evaluation of nutritional efficacy of PHS in malnutrition animal models

Acute oral toxicity study

OECD guideline 423 was followed to test the toxicity of PHS. The drug was administered to female Wistar rats in the doses of 50, 100, 150, 300, 1000 and 2000 mg/kg and observed the 4 hr and 24 hr for 14 days to any change in behaviour, fur colour, feeding habits, and lethargy.

Experimental Animals

Wistar rats (180-200 g) were selected for the study, obtained from the central animal house of the institute. Institutional Animals Ethical Committee approval no. was IAEC/CIP/0292. The animals were maintained with 12 hr light/dark cycle and free access to feed and water.

Experimental Protocol

The protocol of the study was designed by taking fifty male rats and divided into ten groups with an equal number of animals. Group 1 was control groups containing water. Group 2 was malnourished group received a restricted diet (200 mg/kg), Spirullina (150 mg/kg) was given to Group 3, PHS was given to Group 4 and 5 in two doses of 200 and 400 mg/kg, respectively.

Induction of malnutrition

The post-weaning animals (higher in age) that required high protein contain food for their growth and development were selected. The animals were provided with a protein-deficient diet for a month to induce malnutrition (Table 1). Haemoglobin (Hb) content and protein analysis were estimated confirmation about the induction of malnutrition. The different groups of animals will be provided with different foods as discussed in a grouping. The feeding procedure was continued for 30-45 days thereafter again haematological parameter and protein estimation was performed. It is followed by a comparison study to find out the effect of different foods. The food quantity provided to each cage was pre-weighted and non-consume food weight was determined using the weighing balance on the next day for determining the quantity of the food consumed by animals in an individual cage.[30] Figure 1 showing the handling and administration of drugs to the animals.

Bodyweight of animals

The body weight of animals of all groups was recorded daily to determine the change in weight of malnourished animals after receiving PHS.

Food intake of animals

Food intake of animals of all groups was recorded daily on a cage basis (2 rats/cage) to determine the change in food intake of malnourished animals after receiving PHS.

Analysis of blood profile

The blood profile of treated animals was observed in hematoanalyzer by collecting blood from the retro-orbital route of animals. The collected blood was used to measure the glycemic index, complete count and other cellular responses.[31]

Table 1: Nutritional composition of the experimental diets for inducing malnutrition.

Sl. No.	Nutrients	Experimental Diets (g/1000 g of chow)	
		Well-nourished	Malnourished
1	Protein (casein)	200	50
2	Gelatin	-----	150
3	Lipid (Soya oil)	70	70
4	Carbohydrate (corn starch)	532.5	532.5
5	Sucrose	100	100
6	Fiber (cellulose)	50	50
7	Mixture minerals	35	35
8	Mixture Vitamin	10	10
9	Choline	2.5	2.5

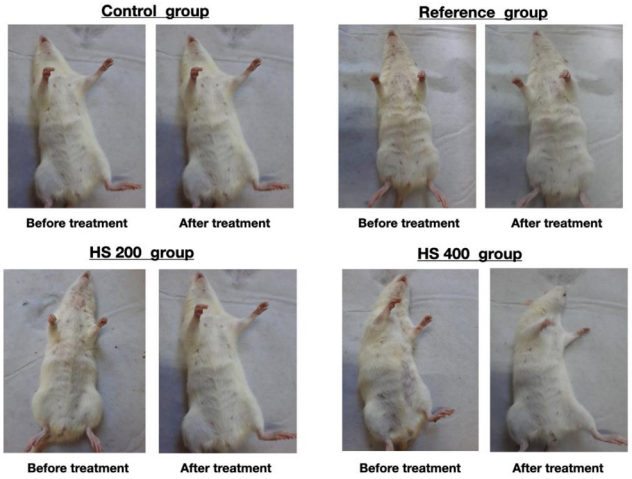

Figure 1: Handling and administration of drugs to the animals.

Biochemical estimation

The biochemical estimation was performed by isolating serum from animals blood. The biochemical parameters evaluated were total cholesterol, total protein, high-density lipoprotein, albumin and triglycerides.[31]

Euthanasia

Intravenous injection of pentobarbital was given to animals for suppressing the activity of the central nervous system followed by disrupting breathing and caused cardiac arrest.

Statistical calculations

Data was represented with mean±SEM. All statistical comparisons between the groups are made using One Way Analysis of (ANOVA) with Neuman-Keuls comparison tests using Graph pad Prism software. It was significantly different at $*p<0.05$, $**p<0.01$ and $***p<0.001$ as compared to the control group.

RESULTS

Nutritional Analysis of Selected Plants Parts

Table 2 shows the nutritional analysis of *N. nucifera* seeds, seeds of *E. ferox* and fruits of *T. natans*. It was revealed that moisture content was highest in the fruits of *T. natans* i.e. 61.5%; however, it was 9.13% and 11.8% in seeds of *N. nucifera* and *E. ferox*. The carbohydrate content was nearly equal in all plants parts i.e. 68.62%, 74.04% and 72.55% in *N. nucifera, E. ferox* and *T. natans*, respectively. It was found that protein content was higher in *T. natans* i.e. 21.30%, on the other hand, *N. nucifera* and *E. ferox* exhibited 14.3% and 13.16%, respectively. Interestingly, the fat content was higher in *N. nucifera* i.e. 1.78% but fat content was lowest in it i.e. 1.78% as compared to the other two herbs i.e. 2.64% and 2.13% in *E. ferox* and *T. natans*, respectively. The total calorie value in all plants parts were 364, 361 and 353 Kcal in *N. nucifera, E. ferox* and *T. natans*, respectively.

Table 2: Nutritional analysis of selected plants parts.

Contents (g/100g)	Nelumbo nucifera (%)	Euryale ferox (%)	*Trapa natans* (%)
Moisture content	9.13	11.28	61.5
Carbohydrate	68.62	74.04	72.55
Crude protein	14.3	13.16	21.30
Crude fat	3.12	0.42	0.70
Crude fiber	1.78	2.64	2.13
Calorific value (Kcal)	364	361	353

HPLC analysis PHS for determination of active compounds

The compounds identified in PHS using the HPLC technique were neferine, kaempferol, caffeic acid, gallic acid, quercetin, cyanidin-3,5-O-diglucoside, and peonidin-3,5-O-diglucoside and Buddlenol E (Table 3). The HPLC method was optimized to obtain higher separation efficiency and resolution of the peak for compounds in a short time frame. The wavelength on which PHS was tested at 330 nm using a UV spectrophotometer. The peak obtained in the HPLC chromatogram of PHS was compared with retention time and UV spectrum of standard marker solution. Figure 2 showing an HPLC chromatogram of different active compounds found in PHS.

MTT assay for PHS

In this study, cytotoxicity of PHS was evaluated in RAW 264.7. Results exhibited that PHS was non-toxic up to the highest concentration of 1600 µg/ml as compared to the control group (Table 4). RAW 264.7 cells showed 92.65% viability with IC_{50} of 13240 µg/ml at 24 hr and 88.65% with an IC_{50} of 8794 µg/ml at 48 hr after treatment with PHS.

Effect of PHS on the haematological profile of malnourished animals

Results exhibited PHS restored the normal blood profile of animals in a dose-dependent manner (Table 5). The blood parameters such as NEUT, WBC, MON, LYMP, EO, BAS, RBC and PLT were 10.92, 8.17, 7.06, 1.11, 0.48, 0.03, 6.97 and 1157 10^3/µL, respectively after treatment with PHS 400 mg/kg as compared to the control group. However, the lower dose group of PHS

Table 3: HPLC determination of active compounds present in PHS.

Sl. No.	RT [min]	Area [mV*sec]	Area %	Height [mV]	Height%	Compounds identified using standard biomarkers
1	0.5167	2.9002	0.14	0.0592	0.05	Neferine
2	2.5833	314.9299	14.79	29.4071	24.55	Kaempferol
3	2.8333	1206.008	56.62	60.3402	50.37	Caffeic acid
4	3.55	68.0983	3.2	5.541	4.63	Gallic acid
5	3.9	272.7631	12.81	11.6341	9.71	Quercetin
6	4.6667	152.8253	7.18	6.2139	5.19	Cyanidin-3,5-O-diglucoside
7	4.9167	50.0619	2.35	4.4834	3.74	Peonidin-3,5-O-diglucoside
8	9.1333	62.3639	2.93	2.1086	1.76	Buddlenol E

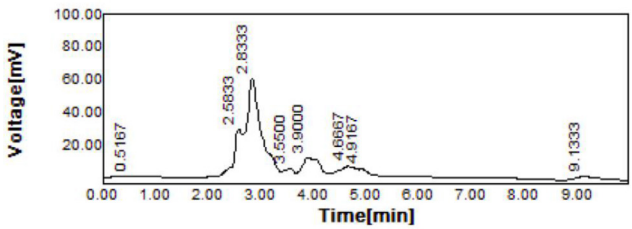

Figure 2: HPLC chromatogram for compounds identified in PHS.

Table 4: Percentage cell viability in MTT assay after treatment with PHS.

Concentration (µg/ml)	Control (%)	PHS	
		24 h	48 h
0	100.00±0.00	100.00±0.00	100.00±0.00
12.5	99.11±2.11	97.54±2.25	98.92±2.15
25	98.64±1.36	96.13±2.68	94.15±2.44
50	98.73±2.00	95.75±2.14	93.47±2.19
100	98.25±1.36	95.36±1.88	91.43±2.03
200	98.17±1.45	93.46±1.68	91.41±2.01
400	98.12±2.17	93.25±2.67	90.25±1.46
800	98.1±2.25	92.26±1.99	89.26±1.77
1600	98.06±1.89	92.65±2.73	88.65±1.36

Data are represented as mean ± SD (n=3); PHS - Polyherbal suspension

i.e. 200 mg/kg was also effective in restoring the normal blood profile of animals.

Effect of PHS on the biochemistry of malnourished animals

Table 6 shows the effect of PHS on biochemical parameters of malnourished animals. Results exhibited that PHS restored cholesterol, lipid profiles and protein levels to the normal in comparison to the control group dose-dependently. The levels of cholesterol, triglycerides, VLDL, and HDL, were 34.08, 59.18, 12.06, 26.03 mg/dL, respectively after treatment with PHS 400 mg/kg to the malnourished rats. Additionally, the levels of total protein and albumin were also restored to 5.02 and 3.16 g/dL, respectively after treatment with PHS 400 mg/kg (Figure 3).

Bodyweight

Figure 4 shows the body weight of malnourished animals after treatment with PHS. It is evident from the graph that PHS 400 mg/kg significantly increased the bodyweight of malnourished animals to 176.2 g. The lower dose group of PHS 200 mg/kg was effective in increasing the weight of animals. However, the reference group was most significant to gain the weight of animals near to the control group.

DISCUSSION

In this study, we have performed the HPLC analysis for the identification of contents present in PHS which determine its nutritional efficacy on malnourished rats. Results exhibited that the developed method was optimized with good separation efficiency and resolution to identify the compounds by comparing them with the standard biomarkers. PHS was effective against nutritional deficiency in malnourished rats by restoring their haematological profile and biochemical level of enzymes. PHS was found efficient, it may be due to the presence of different active compounds present in individual extracts of plants.[32] The medicinal plants possess several bioactive compounds in their secondary metabolites responsible for therapeutic effect.[33-35] Thus, standardization and method validation of drugs derived from plants help determine the active compounds present in them.[36-37]

Results exhibited that *T. natans* showed the highest protein content in the extract followed by *E. ferox* and *N. nucifera*.

Table 5: Effect of PHS on blood of low nutrition diet induced malnutrition on rats.

Parameters	CG	MG	RG	PHS	
				200 mg/kg	400 mg/kg
WBC (10^3/μL)	11.15±1.40	7.67±1.54	11.32±0.6 [a]	9.56±0.31	10.92±0.90 [a]
NEUT (10^3/μL)	9.10±0.24	3.57±0.42	9.40±0.03 [a]	6.77±0.02	8.17±0.47 [a]
LYMP (10^3/μL)	9.21±1.49	0.35±0.03	9.54±0.19 [a]	5.86±1.05	7.06±0.44 [a]
MON (10^3/μL)	1.23±0.21	0.68±0.13	1.37±0.01	0.83±0.02	1.11±0.09
EO (10^3/μL)	0.43±0.09	0.21±0.10	0.52±0.00	0.46±0.00 [a]	0.48±0.09
BAS (10^3/μL)	0.01±0.01	0.01±0.02	0.04±0.01 [a]	0.02±0.20	0.03±0.31 [a]
RBC (10^3/μL)	6.74±0.48	2.45±0.18	7.98±0.72 [a]	5.28±0.23	6.97±0.69
HGB (g/dL)	12.38±0.72	6.26±0.19	12.22±0.19 [a]	9.65±0.31	11.75±0.96
HCT (%)	38.48±2.54	15.33±0.24	35.77±1.11 [a]	32.85±0.65 [a]	34.50±2.75
PLT (10^3/μL)	1227.0±63.39	508.40±80.53	1143±138 [a]	1027±300.0 [a]	1157±320.0 [a]

Values are presented as mean±SEM (n=6); significantly different at [a]p<0.05 in comparison to control group. CG, control group; MG, Malnourished group; RG, Reference group; PHS, Polyherbal suspension. WBC, white blood cell cunt; NEUT, neutrophils; LYM, lymphocytes; MON, monocytes; EO, eosinophil; BAS, basophils; RBC, red blood cell count; PLT, platelet count.

Table 6: Effect of PHS on biochemistry of low nutrition diet induced malnutrition on rats.

Group	Cholesterol (mg/dL)	Triglyceride (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)
CG	36.66±2.40	60.42±1.77	14.12±0.4	21.25±1.07
MG	25.02±0.79	55.08 ±1.29	11.74±1.0	36.04±0.15
RG	36.10±1.06[a]	59.05±0.89[a]	13.11±0.51[a]	25.01±0.83[a]
PHS 200	28.48±0.23	57.39±0.14[a]	11.16±0.17[a]	32.04±0.58[a]
PHS 400	34.08±0.23[a]	59.18±0.24[a]	12.06±0.18	26.03±0.18[a]

Values are presented as mean±SEM (n=6); significantly different at [a]p<0.05 in comparison to control group. CG, control group; MG, Malnourished group; RG, Reference group; PHS, Polyherbal suspension.

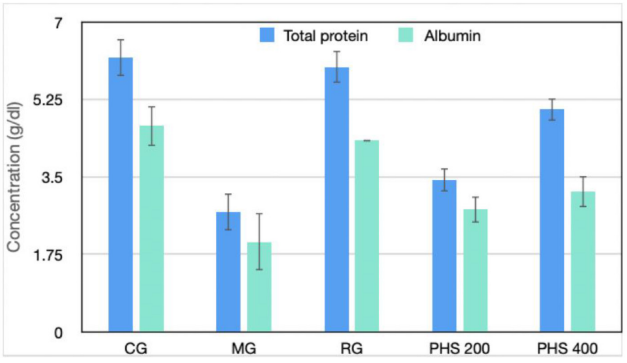

Figure 3: Effect of PHS on biochemistry of malnourished rats.
Values are presented as mean±SEM (n=6); significantly different at [a]p<0.05 in comparison to control group. CG, control group; MG, Malnourished group; RG, Reference group; PHS, Polyherbal suspension.

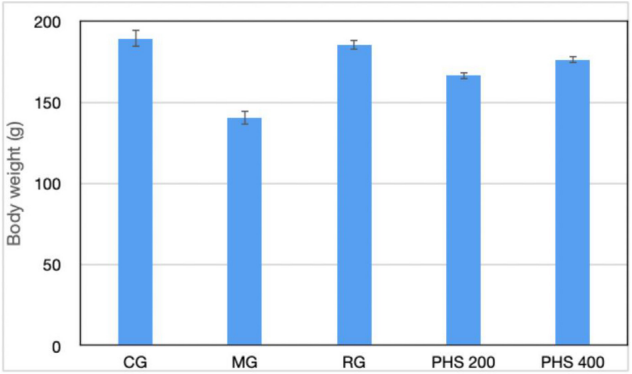

Figure 4: Effect of PHS on body weight of malnourished rats.
Values are presented as mean±SEM (n=6); significantly different at [a]p<0.05 in comparison to control group. CG, control group; MG, Malnourished group; RG, Reference group; PHS, Polyherbal suspension.

The protein content of plant extracts may be responsible for restoring the deficiencies of malnourished rats. A diet rich in proteins, calories and other nutrients helps relieve protein-energy malnutrition.[38] The prepared PHS was a mixture of all three extracts and thus, restored the haematological profile of rats significantly. Malnutrition affects the immunity of an individual by impairing lymphoid-haematopoietic organs; this haematological modification may result in hypoplasia and leucopenia.[39] It has reported that RBC count, Hb level and reticulocyte index decrease significantly due to protein-energy malnutrition.[40-41] Our results were in agreement with the study of findings Díaz Galván *et al.*, 2021 showing that polyherbal mixture increases growth performance.[42]

The analysis of the biochemical profile of malnourished rats revealed that PHS significantly restored the level of cholesterol, triglyceride, VLDL, HDL, total protein and albumin. Similar results were reported by Marimuthu *et al.*, 2019, evaluating the efficacy of polyherbal

formulation on protein-energy malnourished Cobb 430 broilers.[43] The decrease in body weight due to the reduction of body fat and muscle mass is a sign of malnutrition. It also leads to declines in muscle function.[44] Results exhibited that PHS administration to malnourished rats increased their body weight. The findings of our study were similar to the work reported by Amin and Nagy, 2007.[45] Thus, PHS may be used as an effective formulation to regain nutrients lost due to protein-energy malnutrition.

CONCLUSION

It can be concluded from the study that PHS was effective in regaining the normal nutritional status of malnourished rats. The HPLC analysis displayed the presence of several phytoconstituents in PHS in variable concentrations. PHS was found to be non-toxic for oral administration to animals up to 2000 mg/kg. The haematological and biochemical parameters were restored to normal after treatment with PHS. Thus, PHS may be an alternative nutritional formulation for protein-energy malnutrition conditions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PHS: Polyherbal formulation; **NN:** *Nelumbo nucifera*; **EF:** *Euryale ferox*; **TN:** *Trapa natans*.

REFERENCES

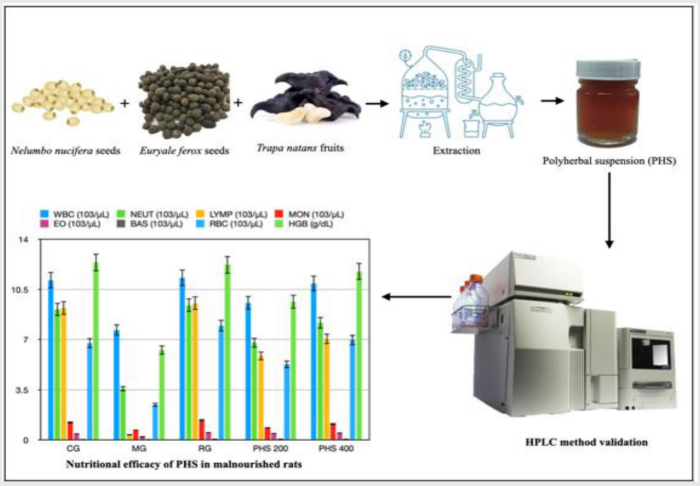
1. Webb P, Stordalen GA, Singh S, Wijesinha-Bettoni R, Shetty P, Lartey A. Hunger and malnutrition in the 21st century. BMJ. 2018;361:k2238. doi: 10.1136/bmj.k2238, PMID 29898884.
2. Arthur SS, Nyide B, Soura AB, Kahn K, Weston M, Sankoh O. Tackling malnutrition: A systematic review of 15-year research evidence from INDEPTH health and demographic surveillance systems. Glob Health Action. 2015;8:28298. doi: 10.3402/gha.v8.28298, PMID 26519130.
3. Dipasquale V, Cucinotta U, Romano C. Acute malnutrition in children: Pathophysiology, clinicaleffects and treatment. Nutrients. 2020;12(8):2413. doi: 10.3390/nu12082413, PMID 32806622.
4. Sahu SK, Kumar SG, Bhat BV, Premarajan KC, Sarkar S, Roy G, *et al*. Malnutrition among under-five children in India and strategies for control. J Nat Sci Biol Med. 2015;6(1):18-23. doi: 10.4103/0976-9668.149072, PMID 25810629.
5. Cheah K, Illsley A. What you need to know about malnutrition in older adults. Br J Hosp Med (Lond). 2020;81(9):1-8. doi: 10.12968/hmed.2020.0393, PMID 32990078.
6. Kreutz JM, Adriaanse MPM, van der Ploeg EMC, Vreugdenhil ACE. Narrative review: Nutrientdeficiencies in adults and children with treated and untreated celiac disease. Nutrients. 2020;12(2):500. doi: 10.3390/nu12020500, PMID 32075276.
7. Taylor N. Overview of oral nutrition supplements and their use. Br J Community Nurs. 2020;25(Sup8);Suppl 8:S12-s15. doi: 10.12968/bjcn.2020.25.Sup8.S12, PMID 32936702.
8. Visser J, McLachlan MH, Maayan N, Garner P. Community-based supplementary feeding for food insecure, vulnerable and malnourished populations - an overview of systematic reviews. Cochrane Database Syst Rev. 2018;11(11):CD010578. doi: 10.1002/14651858.CD010578.pub2, PMID 30480324.
9. Majumdar A, Shukla SS, Pandey RK. Culinary and herbal resources as nutritional supplements against malnutrition-associated immunity deficiency: The vegetarian review. Futur J Pharm Sci. 2020;6(1):47. doi: 10.1186/s43094-020-00067-5.
10. 10. Lonnie M, Hooker E, Brunstrom JM, Corfe BM, Green MA, Watson AW, *et al*. Protein for life: Review of optimalproteinintake, sustainabledietarysources and the effect on appetite in ageingadults. Nutrients. 2018;10(3):360. doi: 10.3390/nu10030360, PMID 29547523.
11. Paudel KR, Panth N. Phytochemical profile and biologicalactivity of Nelumbo nucifera. Evid Based Complement Alternat Med. 2015;2015:789124. doi: 10.1155/2015/789124, PMID 27057194.
12. Chen G, Zhu M, Guo M. Research advances in traditional and modern use of Nelumbo nucifera: Phytochemicals, health promoting activities and beyond. Crit Rev Food Sci Nutr. 2019;59(sup1);Suppl 1:S189-209. doi: 10.1080/10408398.2018.1553846, PMID 30633540.
13. Sridhar KR, Lotus BR. A potential nutraceutical source. Agric Technol. 2007:143-55.
14. Jana BR, Srivastava A, Idris Md. New makhana (Euryale feroxSalisb.) processed products for health benefit. J Food Process Technol. 2019;10(4):1-4.
15. Sidh V, Sharma O. Medicinal use of makhanna (Euryale feroxSalisb.): A review article. WJPMR. 2019;5(9):103-4.
16. Chaudhary J, Jain A, TrapaNatansBS. (Water chestnut): Anoverview. Int Res J Pharm. 2012;3.
17. Parasuraman S, Thing GS, Dhanaraj SA. Polyherbal formulation: Concept of Ayurveda. Pharmacogn Rev. 2014;8(16):73-80. doi: 10.4103/0973-7847.134229, PMID 25125878.
18. Seo JH, Kim JE, Shim JH, Yoon G, Bang MA, Bae CS, *et al*. HPLC analysis, optimization of extractionconditions and biologicalevaluation of CorylopsiscoreanaUyekiFlos. Molecules. 2016;21(1):94. doi: 10.3390/molecules21010094, PMID 26784157.
19. Hodge JE, Hofreiter BT. In: Whistler, Be Miller JN, RL, editors. Methods in carbohydrate chemistry. New York: Academic Press; 1962.
20. Sadasivam S, Manickam A. Vitamin S. In: Sadasivam S, Manickam A, editors, New Age International (P) Limited, New DelhiBiochemical methods.2nd ed; 1997. p. 185-6.
21. Yamini R, Kannan M, Thamaraisevi SP, Uma D, Santhi R. Phytochemical screening and nutritional analysis of *Nelumbo nucifera* (Pink lotus) flower petals and seeds. Int J ChemStud. 2019;7(3):3540-5.
22. You JS, Lee YJ, Kim KS, Kim SH, Chang KJ. Anti-obesity and hypolipidaemic effects of Nelumbo nucifera seed ethanol extract in human pre-adipocytes and rats fed a high-fat diet. J Sci Food Agric. 2014;94(3):568-75. doi: 10.1002/jsfa.6297, PMID 23824592.
23. Haleema K, Srigiripura CV, Urooj A. Antioxidant content and *in vitro* antioxidant activities of extracts from popped Euryale ferox seeds. Agric Sci Research [journal:2016];6:86-92.
24. Aidew L, Buragohain AK. Antimycobacterial and antioxidant activities of the fruit of TrapanatansL. Var. Bispinosa reveal its therapeutic potential. Am J Phytomed Clin Ther. 2014;2(10):1234-45.
25. Dandagi PM, Patil MB, Mastiholimath VS, Gadad AP, Dhumansure RH. Development and evaluation of hepatoprotectivepolyherbal formulation containing some indigenous medicinal plants. Indian J Pharm Sci. 2008;70(2):265-8. doi: 10.4103/0250-474X.41474, PMID 20046731.
26. Bishnoi RS, Kumar M, Shukla AK, Jain CP. Development and validation of novel HPLC method for the estimation of Rutin in crude hydromethanolic leaf extract of Prosopis cineraria. J Drug Deliv Ther. 2018;8(6):68-73. doi: 10.22270/jddt.v8i6.2016.

27. Sinha AK, Verma SC, Sharma UK. Development and validation of an RP-HPLC method for quantitative determination of vanillin and related phenolic compounds in Vanilla planifolia. J Sep Sci. 2007;30(1):15-20. doi: 10.1002/jssc.200600193, PMID 17313136.
28. ICH. Guideline on the validation of Analysis Procedures.methodology. International Conference on Harmonization. Vol. Q2B; 1996.
29. Trinh TA, Park J, Oh JH, Park JS, Lee D, Kim CE, *et al*. Effect of Herbal Formulation on Immune Response Enhancement in RAW 264.7 Macrophages. Biomolecules. 2020;10(3). doi: 10.3390/biom10030424, PMID 32182890.
30. Alaverdashvili M, Li X, Paterson PG. Protein-energy malnutrition causes deficits in motorfunction in adultmalerats. J Nutr. 2015;145(11):2503-11. doi: 10.3945/jn.115.216382, PMID 26423735.
31. Pacanaro CP, Dias SR, Serafim LR, Costa MP, Aguilar E, Paes PR *et al*. Evaluation of biochemical, hematological and parasitological parameters of protein-deficient hamsters infected with Ancylostoma ceylanicum. PLOS Negl Trop Dis. 2014;8(9):e3184. doi: 10.1371/journal.pntd.0003184, PMID 25254370.
32. Karole S, Shrivastava S, Thomas S, Soni B, Khan S, Dubey J, *et al*. Polyherbalformulationconcept for synergicaction: Areview. J Drug Deliv Ther. 2019;9(1-s):453-66. doi: 10.22270/jddt.v9i1-s.2339.
33. Jain P. Secondary metabolites for antiulcer activity. Nat Prod Res. 2016;30(6):640-56. doi: 10.1080/14786419.2015.1036269, PMID 25920371.
34. Kumar V, Rathore K, Jain P, Ahmed Z. Biological activity of *Bauhinia racemosa* against Diabetes and Interlinked Disorders like Obesity and hyperlipidemia. Clin Phytosci. 2017;3(1):7. doi: 10.1186/s40816-017-0044-9.
35. Rao SP, Amrit I, Jain P, Singh V. Antiulcer activity of AgnimukhaChurna. Int J Ayur Pharmres. 2014;2(2):40-6.
36. Folashade O, Omoregie H, Ochogu P. Standardization of herbal medicines – a review. Int J BiodversConserv. 2012;4:101-12.
37. Rao SP, Jain P, Rathore P, Singh VK. Larvicidal and knockdown activity of Citrus limettaRisso oil against dengue virus vector.Indian. J NatprodResour.2016;7(3):256-60.
38. Anang JK. Treatment of protein energy malnutrition (PEM)-the Ghanaian traditional medical practitioners' (TMPs) Approach. Med Anthropol Newsl. 1980;11(2):12-4. doi: 10.1525/maq.1980.11.2.02a00110.
39. Borelli P, Blatt SL, Rogero MM, Fock RA. Haematological alterations in protein malnutrition. Rev Bras Hematol Hemoter. 2004;26(1):49-56. doi: 10.1590/S1516-84842004000100010- 84842004000100010.
40. El-Nawawy A, Barakat S, Elwalily T, Abdel-Moneim Deghady A, Hussein M. Evaluation of erythropoiesis in protein energy malnutrition. East Mediterr Health J. 2002;8(2-3):281-9. PMID 15339115.
41. Grover Z, Ee LC. Protein energymalnutrition. Pediatr Clin North Am. 2009;56(5):1055-68. doi: 10.1016/j.pcl.2009.07.001, PMID 19931063.
42. Díaz Galván C, Méndez Olvera ET, Martínez Gómez D, Gloria Trujillo A, Hernández García PA, Espinosa Ayala E, *et al*. Influence of a polyherbal mixture in dairy calves: growth performance and gene expression. Front Vet Sci. 2020;7:623710. doi: 10.3389/fvets.2020.623710, PMID 33575280.
43. Marimuthu S, Selvam R, Souza PD, Bran DR. Evaluation of polyherbal formulation on high energy and low protein diet induced fatty liver syndrome: Implications on performance, carcass characteristics, biochemistry and liver histopathology in Cobb 430 broilers. Int J Vet SciAnim Husbandry. 2019;4(5):40-5.
44. Saunders J, Smith T. Malnutrition: Causes and consequences. Clin Med (Lond). 2010;10(6):624-7. doi: 10.7861/clinmedicine.10-6-624, PMID 21413492.
45. Amin KA, Nagy MA. Effect of carnitine and herbal mixture extract on obesity induced by high fat diet in rats. Diabetol Metab Syndr. 2009;1(1):17. doi: 10.1186/1758-5996-1-17, PMID 19835614.

SUMMARY

In India, malnutrition is a major concern for children below five years of age; the small villages, districts and states share a large proportion (80%) of the malnutrition burden. It is also a significant issue for older patients, despite their decreased energy requirement they need the same nutrients and protein intake as equal to younger patients, and however, age-related changes pose an impact to meet these requirements. Nutritional supplements are an important source to regain reduced nutrition. It may help to fulfill adequacy of diet, improving health parameters and quality of life. Medicinal plants and herbs are the best sources for nutrition, they also add aroma and flavour to the food. The nutritive properties of herbs are because of active phytoconstituents that works synergistically in polyherbal formulation for producing pharmacological effects. The present study demonstrated HPLC method development for quantitative measurement of phytoconstituents present in food-grade polyherbal suspension (PHS) and evaluated its nutritional efficacy in the rat. The developed HPLC method was simple, rapid, sensitive and cheap for the determination of phytoconstituents in the PHS. The HPLC analysis displayed the presence of several phytoconstituents in PHS in variable concentrations. The nutritional composition of NN, EF and TN revealed that protein content was highest in TN (21.30%), NN (14.30%) and EF (13.16%) respectively. Results exhibited that *T. natans* showed the highest protein content in the extract followed by *E. ferox* and *N. nucifera*. PHS was effective against nutritional deficiency in malnourished rats by restoring their haematological profile and biochemical level of enzymes. The analysis of the biochemical profile of malnourished rats revealed that PHS significantly restored the level of cholesterol, triglyceride, VLDL, HDL, total protein and albumin. PHS also enhanced the bodyweight of animals in comparison to the control group. Thus, PHS may be an alternative nutritional formulation against protein deficiency-induced malnutrition. Thus, PHS may be used as an effective formulation to regain nutrients lost due to protein-energy malnutrition.

PICTORIAL ABSTRACT



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