

HPLC analysis of amino acid and antioxidant composition of three medicinal plants of (pithoragarh) uttarakhand himalayas

Abstract

Antioxidant phytochemicals such as vitamin C, b-carotene, lutein, a-tocopherol, amino acid and total phenolics, were estimated in some medicinal plants using a reverse phase HPLC system. Wild plants play a vital role in the health security of Himalayan inhabitants and constitute significantly to their diet. Amino acid was analysis performed using the Waters Associates PICO-TAG method. The total amino acid content in *P. indica* was 58.80mg amino acid/g sample (dry weight), *E. thymifolia* was 123.92mg amino acid/g sample (dry weight) and *P. hirta* was 225.73mg amino acid/g sample (dry weight). The total essential amino acids in *P. indica*, *E. thymifolia* and *P. hirta* were 33.58, 57.99 and 145.82mg amino acid/g respectively. Among the three investigated plants, Carotenoids viz xanthophylls content was found 0.13 to 151.01mg/100g dry weight basis. The maximum xanthophylls content was found in *P. indica* leaves and minimum in *P. hirta* rhizomes. The b-carotene content varies from 4.62 - 374.55mg/100g on dry weight basis. DL-a-tocopherol in these medicinal plants was found 13.48mg/100g, 24.95mg/100g and 9.13mg/100g on dry weight basis in *P. hirta*, *E. thymifolia* and *P. indica* respectively. This study discovers amino acid and antioxidant content in medicinal plants. Such information will increase the understanding of the faction of these antioxidant phytochemical in lowering incidence of ageing and other chronic diseases. Results of our study suggest the great value of these species for use in pharmacy and phytotherapy. Based on this information, it could be concluded that this plant is natural sources of antioxidant substances of high importance.

Keywords: medicinal plant, amino acid, carotenoids, vitamins, phenolics, HPLC

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Introduction

The medicinal value of the plants lies in their chemical substances that produce a definite physiological action on human body. Therefore there is need to evaluate the local herbs for mineral and nutrient composition to determine the potential of indigenous source of medicine. *Pavetta indica* Linn is a shrub or small tree belongs to the family of *Rubiaceae*. The leaves very variable elliptic oblong to elliptic, lanceolate, glossy green and flowers are white.¹ The roots possess purgative, aperient, diuretic and tonic properties. The plants prescribed in visceral obstructions, jaundice, headache, urinary diseases and dropsical affections. The phytochemical investigation,² chemical composition of essential oil³ and physio phytochemical screening⁴ has been reported on this plant. The leaves of plant are used in the treatment of liver dysfunction, pile, urinary diseases and fever.⁵ The root of *Pavetta* are bitter, frequently prescribed in visceral obstructions. The roots of plant and dried ginger is given in conjunction with water in the case of dropsy of renal. Methanolic extract of leaves have been reported as antipyretic and anti-inflammatory.⁶ The plants under *Euphorbia* genus are used to treat cancer, migraine, warts, intestinal parasites, tumors etc. The *E. thymifolia* is found in tropical regions, but it is absent in North Australia.⁷ *Euphorbia thymifolia* Linn (*Euphorbiaceae*) is traditionally used as blood purifier, cough, antiviral in brachial asthma and paronychia⁸ and Water extract of this plants have antiviral activity.⁹ *Pouzolzia hirta* Linn (*Urticaceae*) is a suberact herb found in Kumaon region of India.¹⁰ The powder of the plant rhizomes has been used as binder to flour of maize and wheat by the local population of Uttarakhand. The tuberous roots of plants are eaten raw or roasted. The rhizomes of plants are eaten as a vegetable to expel worms. The rhizomes of plants have been reported to possess good antihelmintic activity.

Present investigation of arial parts of the plant (leaves, pre-mature and mature seeds and fruits) were taken to isolate amino acid and phytochemicals. The aim of the present study was to analyze the chemical composition of the medicinal plants.



Material and methods

Chemicals

Standard of xanthophyll, α -carotene, β -carotene and DL- α -tocopherol were procured from Sigma Chemical Co. St Louis, USA. Individual standard was accurately weighed, developed and diluted with HPLC grade ethanol. Petroleum ether, methanol, ethyl acetate and anhydrous sodium sulphate and other chemicals and reagents used in this study were purchased from Merck Chemical Co. Mumbai, India.

Plant material

The plants were first identified in the Department of Botany, Kumaun University, Nainital and then at B.S.I., Dehradun. The voucher specimen was deposited in the Herbarium section at B.S.I., Dehradun. The voucher no. 112173 for *Pouzolzia hirta* (Blume) Hassk, 112173 for *Pavetta indica* and 17195 for *Euphorbia thymifolia*. The collected plant materials were first washed with cold water to remove the soil particles and then shade dried. The dried material was finely powdered in the grinding machine and weighed in an electrical balance. Dried plant parts were cut up and stored in tight-seal dark containers until needed.

Total phenolic content

The rhizomes of each source (wild and planted) were dried in shade and powdered using electrical grinder. The amount of total phenolic content was estimated following¹¹ with modification. The reaction mixture contained 100 μ l of sample extract, 500 μ l Folin-Ciocalteu's reagent (freshly prepared), 2ml of 20% Sodium Carbonate and 5ml of distilled water. After 15min reaction at 45°C the absorbance at 650nm was measured using spectrophotometer (HITACHI, Model UV5704-SS). Results expressed as mg of Catechol equivalent per 100g of dry weight.

Ascorbic acid content

Ascorbic acid content was estimated by method¹² with modification. Dry leaves powder (2.0g) was extracted with 4% oxalic acid and made up to 100ml and centrifuged at 10,000 rpm for a 10minute. 5ml supernatant liquid was transferred in a conical flask, followed by addition of 10ml 4% oxalic acid and titrated against standard dye solution (2, 6-dichlorophenol indophenol) to a pink end point. The procedure was repeated with a blank solution omitting the sample.

Amino acid analysis

Amino acid analysis was performed using the Waters Associates PICO-TAG method with some modification¹³ with some modification an integrated technique for precolumn derivatization of amino acids using phenylisothiocyanate (PITC). The PICO-TAG technique comprises of three steps: (i) Hydrolysis of protein or peptide samples to yield free amino acids, (ii) pre-column derivatization of the samples with PITC and (iii) analysis by reverse phase HPLC. The chromatographic separation on the hydrolyzates was performed using a reverse phase Pico-Tag column (3.9 x 300mm) C18 at 40°C and a UV detector at 254nm. The solvent system consisted of two eluents, (A) an aqueous buffer and (B) 60% acetonitrile in water. Gradient elution were employed using two pumps, programmed to deliver the mobile phases eluents A and B. A gradient which was run for the separation consisted of 10% B traversing to 51% B in 10min using a convex curve (number 5). A set of amino acid standards (Merck Germany) was analyzed with each set of three experimental samples. Identification of the amino acids in the samples was carried out by comparison with the retention times of the standards.

Extraction and Isolation of carotenoids and tocopherol

Dried plant material (1.0g of each) was extracted with light petroleum ether/methanol/ethyl acetate (1:1:1, V/V/V, 4 x 30ml) until the extracts became colorless. The extract was mixed in a 250ml separating funnel, shaken vigorously and allowed to stand for phase separation. Upper layer was collected in a 100ml flask (Borosil India Co. Ltd.) and lower layer was shaken with 50ml water and 50ml petroleum ether for phase separation. Upper layer was mixed with the first extract. The organic extract was dried over anhydrous sodium sulphate (10g), filtered and evaporated to dryness in a Rotary Vacuum Evaporator under reduced pressure. The residue was dissolved in light petroleum ether (5ml) and filtered by 0.2 μ m membrane filter prior to HPLC analysis.

HPLC analysis

All the samples were analyzed using Shimadzu HPLC interfaced with model SPD-10 AVP Variable wavelength (190-750nm) UV- Vis detector, Column used was C18 Phenomenex® (150x4.60mm), pore size 5 μ m with solvent system 8:2:40:50 (methanol, ethyl acetate, acetonitrile and acetone), flow rate 0.7ml/min, run time 20 minutes and detector wavelength was 450nm. The HPLC condition for the estimation DL- α -tocopherol was adopted as described in¹⁴ with some modification.

Statistical analysis

The assays were run in triplicate for each sample and the results expressed as mean value \pm SD.

Results

The amino acid content of each of the three plants viz., *P. hirta*, *E. thymifolia* and *P. indica* summarized in Table 1. Quantitative determination of amino acid concentration was conducted by HPLC and the amino acid profile is shown in the chromatogram (Figure 1-4). Seventeen amino acids detected and the separation of these amino acids in the sample is reasonably resolved. All the essential amino acids i.e. methionine, leucine, lysine, cysteine, phenylalanine, tyrosine, arginine, isoleucine, threonine and valine and seven non-essential amino acids were found to be present in the three plants. The total amino acid content in *P. indica* was 58.80mg amino acid/g sample (dry weight), *E. thymifolia* was 123.92mg amino acid/g sample (dry weight) and *P. hirta* was 225.73mg amino acid/g sample (dry weight). The total essential amino acids in *P. indica*, *E. thymifolia* and *P. hirta* were 33.58, 57.99 and 145.82mg amino acid/g respectively.

The ratio of essential amino acids to total amino acid is 0.57 i.e. more than half of the amino acid in *P. indica*. The results also indicated that the ratio of essential amino acids to non-essential amino acids is 1.33. *P. indica* is rich in alanine, lysine, valine, arginine, alanine, glutamic acid, proline and aspartic acid. The ratio of essential amino acids to total amino acid is 0.47 i.e. almost half of the amino acid in *E. thymifolia* consist of essential amino acids. The results also indicated that the ratio of essential amino acids to non-essential amino acids is 0.88. *E. thymifolia* is rich in alanine, methionine, phenylalanine, valine, glycine, arginine, alanine, glutamic acid and aspartic acid.

The ratio of essential amino acids to total amino acid is 0.65 i.e. more than half of the amino acid in *P. hirta* consist of essential amino acids. The results also indicated that the ratio of essential amino acids to non-essential amino acids is 1.82. *P. hirta* is rich in alanine, glycine, phenylalanine, threonine, valine, methionine, arginine, alanine, glutamic acid, proline, and aspartic acid. In this study compared the amino acid composition of each of three specimens to that of

a World Health Organization standard protein.¹⁵ According to the WHO reference protein, the highest quality plant proteins were found in *Euphorbiathymifolia* and *Pouzolzia hirta* (Table 2) each of these scored at or above the score of the WHO standard for 5 of 7 amino acids or amino acid pairs.

Table 1 Amino acid content of three plants in mg/g dry weight basis. All values are mean of triplicate determinations expressed on dry weight basis

Amino	P. Indica	% of Total AA	E. Thymifolia	% of Total AA	P. Hirta	% of Total AA
Aspartic acidn	1.34±0.15	2.28	5.86±0.09	4.73	12.75±0.60	5.65
Glutamic acidn	1.66±0.06	2.82	7.31±0.05	5.9	14.00±0.09	6.2
Serinen	1.19±0.08	2.02	3.47±0.58	2.8	8.03±0.06	3.56
Glycinen	0.58±0.01	0.99	1.80±0.04	1.45	8.16±0.05	3.61
Histidinen	1.23±0.01	2.09	3.65±0.04	2.95	-	-
Alaninen	17.96±0.01	30.54	43.84±0.37	35.38	36.59±0.05	16.21
Prolinen	1.26±0.07	2.14	-	-	0.38±0.03	0.17
Lysinea	3.03±1.34	5.15	-	-	1.44±0.04	0.64
Threoninea	-	-	5.99±0.04	4.83	40.74±0.08	18.05
Tyrosinea	2.20±0.06	3.74	4.51±0.13	3.64	10.06±0.10	4.46
Valinea	3.61±0.07	6.14	11.69±0.13	9.43	25.04±0.03	11.09
Methioninea	0.55±0.13	0.94	2.46±0.07	1.99	4.24±0.02	1.88
Cysteinea	0.60±0.33	1.02	0.90±0.10	0.73	1.95±0.03	0.86
Isoleucinea	1.97±0.33	3.35	8.66±0.48	6.99	17.50±0.08	7.75
Leucinea	1.93±0.09	3.28	11.71±0.14	9.45	27.90±0.01	12.36
Phenylalaninea	0.62±0.07	1.05	4.38±0.01	3.53	12.06±0.02	5.34
Argininea	19.07±0.09	32.43	7.69±0.05	6.21	4.89±0.04	2.17
TEAA	33.58	51.96	57.99	46.8	145.82	63.96
TNEAA	25.22	48.04	65.93	53.2	79.91	36.04
TAA	58.8		123.92		225.73	

±, Denotes the standard error; a-TEAA, total essential amino acid; nTNEAA, total non essential amino acid; AA, amino acid.

Table 2 Comparison of the content of selected essential amino acid of 3 plants with that of the WHO Ideal pattern

Plant Specimen	ILE	LEU	VAL	PHE+TYR	LYS	THR	MET+ CYS	Score*
WHO standard	4	7	5	6	5.5	4	3.5	---
P. indica	3.35	3.28	6.14	4.79	5.15	-	1.96	7-Jan
E. thymifolia	6.99	9.45	9.43	7.17	-	4.83	2.72	7-May
P. hirta	7.75	12.36	11.09	9.8	0.64	18.05	2.74	7-May

*This pattern is based on the essential amino acid need for the preschool child; WHO/FAO. Energy and Protein Requirements. WHO Technical Report Series, No. 522. Geneva, World Health Organization, 1973.

Table 3 Antioxidant content in medicinal plants

S.N.	Antioxidants	P. Hirta		E. Thymifolia		P. Indica	
		mg/100g	Range	mg/100g	Range	mg/100g	Range
	Total phenolics	230.59±0.33	230.15-230.95	336.73±0.55	336.25-337.50	251.52±1.00	250.23-252.68
	Xanthophyll	0.13±0.01	0.12 - 0.13	0.51±0.04	0.48-0.55	151.01±2.16	149.34-152.34
	a-Carotene	-	-	-	-	1.96±0.07	1.96-2.01
	b-Carotene	4.62±0.68	4.14-5.10	178.98±4.62	175.45-181.90	374.55±1.40	373.77-375.66
	DL-a-tocopherol	13.48±0.83	12.81-13.92	24.95±1.10	24.14-25.69	9.13±0.28	8.94-9.33
	Vitamin-C	108.40±0.32	108.05-108.83	88.48±0.95	87.37-89.68	77.49±1.83	74.90-78.90

All values are mean of triplicate determinations expressed on dry weight basis.

±: Denotes the standard error.

The nutritional analysis of the indigenous edible and fodder plants of the Uttarakhand region by chemical means gives the potential values of these foods to those populations who rely upon them as staples or supplements to their diet. The next step is to assess the bioavailability of the essential nutrients in these plants, such studies must be contemplate. These studies will focus on the composition of the biochemical, mineral, amino acid present in these plants and on the possible presence of antinutrients, such as metal chelators (e.g., phytates, oxalates) and protease inhibitors.

The aim of this work was to characterize the antioxidant value of the medicinal plants with particular attention to carotenoids, phenolics and vitamins. In this study, we observed that xanthophyll, a-carotene,

b-carotene, vitamin C, and DL-a-tocopherol contents are present in these medicinal plants (Table 3). The retention time of xanthophyll, a-carotene, b-carotene and DL-a-tocopherol were found to be 2.045, 10.947, 11.495 and 11.780 minutes respectively (Figure 5-13).

Among the three investigated plants, Carotenoids viz . xanthophyll content was found 0.13 to 151.01mg/100g dry weight basis (Table 3). The maximum xanthophyll content was found in *P. indica* leaves and minimum in *P. hirta* rhizomes. The a-carotene content in *P. hirta* and *E. thymifolia* was below detection limit (BDL), but in *P. indica* it was found 1.96mg/100g dry weight basis. The b-carotene content varies from 4.62-374.55mg/100g on dry weight basis. *P. indica* contains more b-carotene content than *P. hirta* rhizomes. a-Carotene and β-carotene

were found more in the leaves of *P. indica* as compared to other two plants, but DL- α -tocopherol was found more in *E. thymifolia* and the range was 3.48 to 24.14mg/100g on the dry weight basis. This is the first study for quantitative variation of antioxidant in these three medicinal plants, so we could not correlate above data with earlier workers.

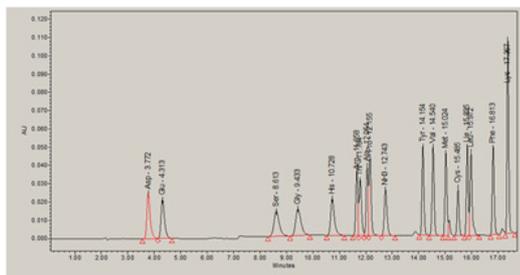


Figure 1 Amino acid profile of standard.

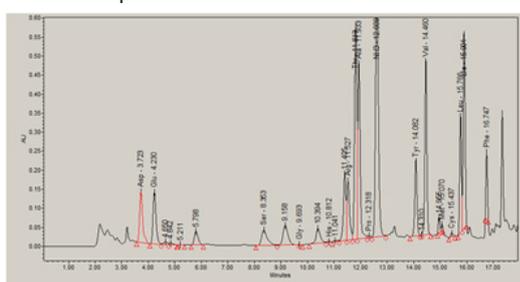


Figure 2 Amino acid profile of *Pavetta indica*.

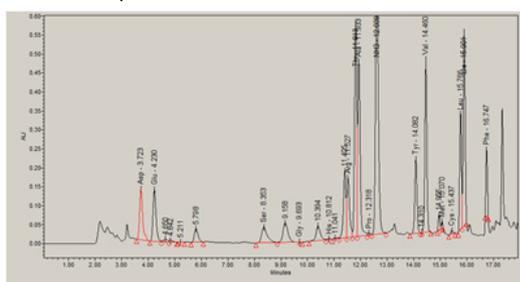


Figure 3 Amino acid profile of *Euphorbia thymifolia*.

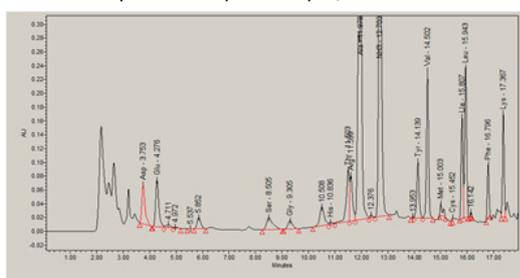


Figure 4 Amino acid profile of *Pouzolzia hirta*.

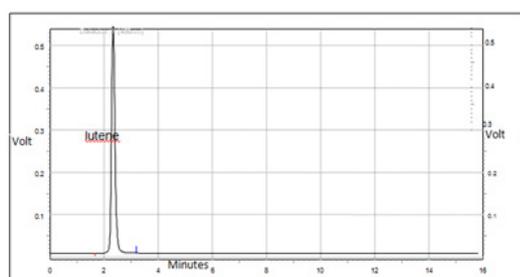


Figure 5 Chromatogram of standard peak of xanthophyll.

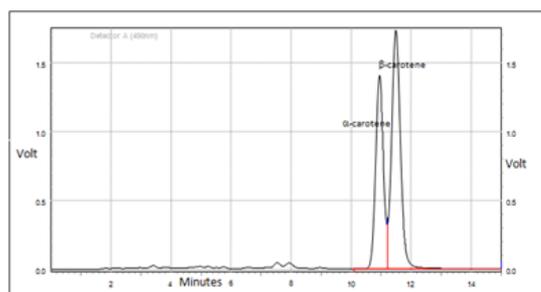


Figure 6 Chromatogram of standard peak of α -carotene and β -carotene.

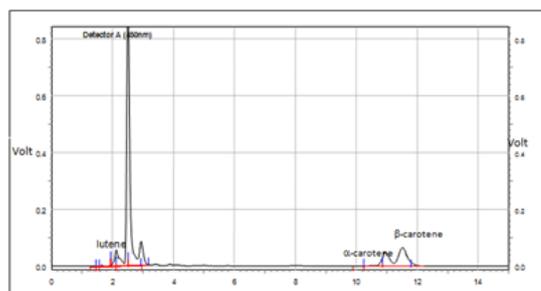


Figure 7 Chromatogram of *Pavetta indica* leaves.

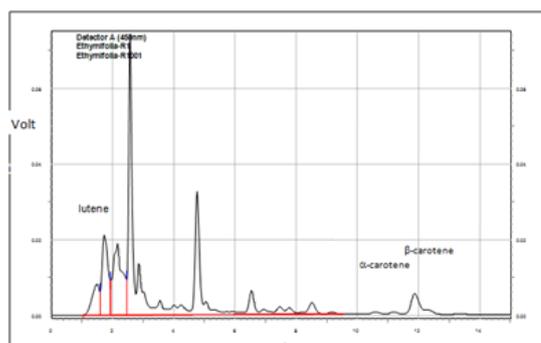


Figure 8 Chromatogram of *Euphorbia thymifolia* aerial parts.

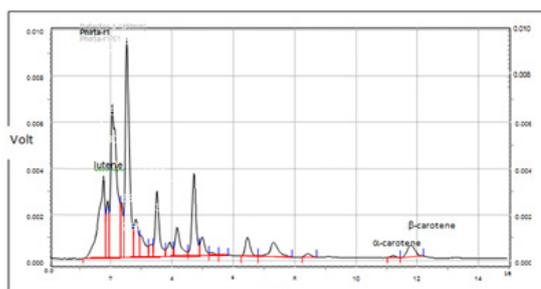


Figure 9 Chromatogram of *Pouzolzia hirta* rhizomes.

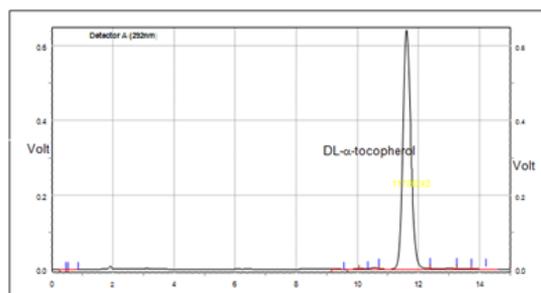


Figure 10 Chromatogram of standard peak of DL- α -tocopherol.

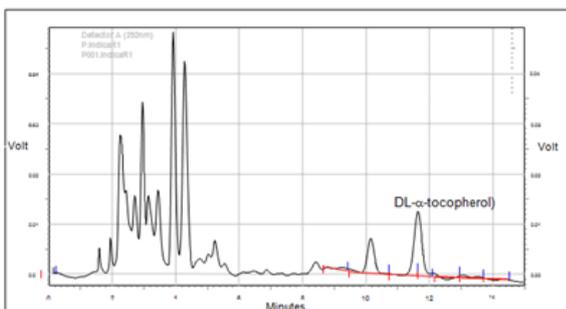


Figure 11 Chromatogram of *Pavetta indica* leaves.

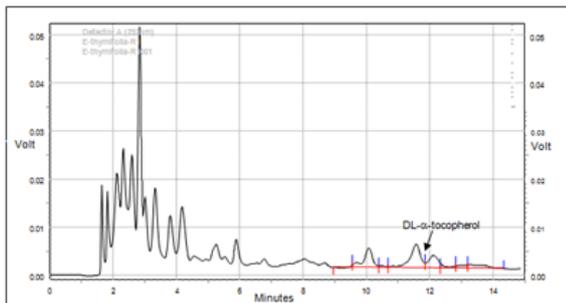


Figure 12 Chromatogram of *Euphorbia thymifolia* aerial parts.

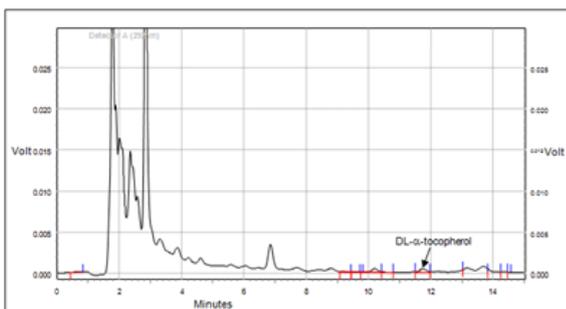


Figure 13 Chromatogram of *Pouzolzia hirta* rhizomes.

The amount of total phenolics content varies between three plants rhizomes/leaves (Table 3). The phenolics content (336.73mg/100gm) was found higher in *E. thymifolia* leaves as compared to *P. hirta* rhizomes (230.59mg/100g), while (251.52mg/100g) was found in *P. indica* leaves. As such phenolics are known for their antioxidant activity. The phenolic acids have repeatedly been implicated as natural antioxidants in fruits, vegetables and other plants. For example, caffeic acid, ferulic acid, and vanillic acid are widely distributed in the plant kingdom, resmarinic acid, an important phytochemical has been found to be potent active substances against human immunodeficiency virus type1 (HIV-1). The amount of vitamin C content varied between three plants rhizomes/leaves (Table 3). The vitamin C contents (108.40mg/100gm) was found higher in *P. hirta* rhizomes as compared to (77.49mg/100g) *P. indica* leaves, while (88.48mg/100g) was found in *E. thymifolia* leaves.

The analytical data on crude protein, crude fat, gross energy and amino acid profiles of *Pavetta*, *Euphorbia* and *Pouzolzia* clearly suggested their high potentials as cheap source of alternative proteins for humans and animals. Because of the simplicity of technology involved in leaf protein concentrates production, its incorporation into local food production systems is recommended as a practicable, sustainable and ameliorative intervention strategy for the endemic protein under-nutrition in this region.

The leaves/ rhizomes of the plant from the data, reveals that it contains an appreciable amount of proteins, minerals, fats, fibres, amino acids, antioxidants, carbohydrates, caloric value and low levels of toxicants whose value can be reduced by cooking. Since it contains substantial amount of nutrients, it can therefore be concluded that these plantleaves/ rhizomes can contribute significantly to the nutrient requirements of man and should be recommended.

Conclusion

The results shows these medicinal are good source of antioxidant. Antioxidant plays an important and maintaining body balance. The study will also help to generate a database of species, which can be exploited scientifically and judiciously in the future by local people, and so that ecological balance is maintained. The results data obtained in the present study suggest that some antioxidants and amino acid possess strong medicinal activities, which can be utilized for treatment of certain diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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