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ORIGINAL ARTICLE

Nutritional characterization and chemical composition of *Diplazium maximum* (D. Don) C. Chr.

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Abstract *Diplazium maximum* (D. Don) C. Chr. is a wild edible fern, traditionally consumed in the tribal areas of western Himalayas. The edible part of the plant (young fronds) was analyzed for its nutritional and phytochemical composition. The dried young fronds (DYF) were found to have high contents of dietary fibre (38.32 g/100 g dry basis) and crude protein (25.39 g/100 g dry basis). Branched-chain-essential-amino acids, polyunsaturated fatty acids (PUFA) (constituting more than 50% of total fatty acids), dihomo-gamma-linolenic acid (unique omega-6 PUFA) and phenolics like epicatechin, myricetin, catechin and procatechuic acid were present in nutritionally significant amounts. Hydro-alcoholic extracts of the DYF contained maximum distribution of polyphenols and flavonoids and exhibited high antioxidant capacities. Analysis of functional properties of DYF such as water and oil absorption capacity, dispersibility and swelling capacity indicated its potential application in instant convenience food products such as soup mixes. Sensory scores of soup mix prepared using DYF was similar to that of commercially available soups. In conclusion, *D. maximum* is nutritious enough to be popularized for domestication, wide consumption and inclusion in the form of instant food products in existing food basket.

Keywords Fiddlehead fern · Branched chain amino acids · Dihomo-gamma-linolenic acid · Polyphenols · Antioxidant · Instant convenience foods

Introduction

Edible ferns are a common group amongst wild food plants collected and consumed by different communities of the world (Liu et al. 2012). Although, edible ferns do not find place in mainstream agriculture for consumption, they are extensively used as supplementary diets or substitutes for staple food during famine (Maroyi 2014). Wild edible plants such as edible ferns often serve as suitable alternatives to staple crops for people inhabiting interior and agriculturally difficult terrains such as that of Himalayas (Thakur et al. 2017).

As per Chinese traditional knowledge, "Shi-Jing", edible ferns are being consumed for almost 3000 years. Even current scientific systematic records from nineteenth century indicate prolific usage of ferns like *Athyrium esculentum*, *Ceratopteris siliquosa*, *C. pteridoides*, *Dryopteris prolifera*, *Pteretis nodulosa*, *P. esculenta*, and *Helminthostachys* sp. by the inhabitants of Filipino, Malayan and Japanese regions. Extensive applications of around 144 fern species in food and ethno-medicine in China was reported by Liu et al. (2012). Fronds of *Matteuccia struthiopteris, Ophioglossum polyphyllum*, *O. nudicaule*, *Blotiella glabra, Cyclosorus gongylodes, Christella dentata* and tender leaves of *Botrychium lanuginosum* are also regarded as a delicious vegetable in sub-Saharan Africa, Nepal, Indian Himalayan terrain, New England, Alaska,

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Northern America and Southern Canada (Maroyi 2014; DeLong et al. 2011).

Among edible ferns, the fiddlehead ferns are extremely popular; and are consumed as seasonal leafy vegetable and a delicacy in different mountain tribal communities of the world such as Himalayan regions of Pakistan, India, China, Tibet and Nepal (Maroyi 2014). The edible portion of fiddlehead ferns are succulent and tender with only the young fronds being consumed by human beings. These young fronds are generally termed as 'croziers'. The genus '*Diplazium*' is a predominant group of fiddlehead ferns and includes a few popular edible species such as *D. esculentum, D. sammatii, D. proliferum* etc. In lower Himalayan regions, *D. esculentum* is a popularly consumed fern, and is locally known as 'Lungru' in western Himalaya and as 'Dhekisaag' in North Eastern Himalayan states of India (Yumkham et al. 2017). Since the edible fronds emerge only during the rainy season, the fronds are pickled, dried and stored for use throughout the year (Chakraborty and Roy 2018). The species is reported to have ethno-medicinal properties and has significant use as food, fodder and medicine. However, the local population of Mandi, Chamba and Kangra valley of Himachal Pradesh predominantly consume a lesser-known fiddlehead fern, *D. maximum*, locally called as "Khasrod or Lengadu".

It has been observed that only the young tender fronds of *D. maximum* are cooked and consumed as vegetable (Thakur et al. 2017; Rana et al. 2019). These observations interested us to evaluate the nutritional potential of this lesser known species, i.e., *D. maximum*. In order to validate *D. maximum*'s potential as a food, a systematic analysis involving proximate composition, phytochemical constituents and in vitro antioxidant activity was performed. Additionally, a shelf stable, convenient and nutritious food product was prepared using *D. maximum* to encourage its preservation, domestication and inclusion in the current food basket of the region.

Materials and methods

Plant material collection

A survey was undertaken to understand the distribution pattern of the edible fern, *Diplazium maximum* (D. Don) C. Chr., locally sold as Khasrod/Lengadu (Fig. 1a–c). Samples were collected from select western Himalayan regions such as Palampur, Kandwadi, Kulyani, Bundla, lot in district Kangra; Barot of Mandi and Churah sub-division of Chamba, considering their distribution abundance.

Plants were also collected from wild for identification and taxonomic authentication. The plant samples were deposited in the herbarium of CSIR-Institute of Himalayan Bioresource Technology (IHBT) Palampur, India and an authentication number, PLP-16588 was assigned to the plant. Thereafter, the plants collected from wild were established in the Experimental farm and fernery of CSIR-Institute of Himalayan Bioresource Technology, Palampur (1310 m amsl, 32.6°N and 78.19°E) (Fig. 1b).

Nutritional analysis of *D. maximum*

Freshly emerged fronds were collected from plants maintained at the Experimental farm of CSIR-IHBT. The scaly and hairy epidermal (outer) layer were removed and the young fronds were washed with running tap water and then blotted dry on a filter paper. Next, the fronds were diced to a size of 2.0 cm × 2.0 cm and dried in an industrial tray drier at 40 °C ensuring uniform moisture content of 12 g/100 g biomass. The dried biomass were pulverized to obtain free flowing powder having an average particle size of 400 µm and was denoted as dried young fronds (DYF). All experiments in the present study were conducted using the DYF (Fig. 1d). The DYF was stored under refrigerated conditions (4 ± 2 °C) throughout the study period.

The proximate composition of DYF was analyzed using standard procedures of Association of Official Analytical Chemists (AOAC 2005). The moisture content was determined by oven drying a known quantity of DYF (5.0 g) at 110 °C until constant weight. The protein content was estimated by quantifying the nitrogen content of DYF in a Kjeldahl apparatus. A standard conversion factor, (N × 6.25) was used to convert the nitrogen content into crude protein. The fat content of DYF was determined by solvent extraction with *n*-hexane in a Soxhlet apparatus. Total ash was determined by calcination at 550 °C until constant weight. The total carbohydrate content was determined by difference method. The total dietary fibre content was determined by serial enzymatic digestion method described by Pradeep et al. (2014). The starch content was determined by estimating the liberated glucose using phenol–sulphuric acid method and total sugars by digesting DYF in acidic solution. While the reducing sugars were determined by extracting DYF with 70% ethanol as described by Pradeep et al. (2014), the difference between the total sugars and reducing sugars was considered as non-reducing sugar content. The ash produced by calcination of DYF sample was used for the estimation of mineral elements such as calcium, magnesium, sodium, potassium, iron, zinc and copper by the procedure outlined by AOAC (2005). Atomic absorption spectrophotometry was used to measure the elements, and their concentrations were determined against respective standard calibration curves. All analyses were performed in triplicates.

Fig. 1 *D. maximum* **a** distribution map, **b** experimental field of CSIR-IHBT, **c** young fronds for sale in market, **d** dried powder (DYF)

Soluble proteins

The different classes of proteins from DYF were successively extracted using distilled water, 5% NaCl, 0.1 M NaOH and 70% ethanol, respectively at room temperature (25 °C). The total protein content was determined spectrophotometrically as per the method described by Wang et al. (2014).

Amino acid composition

The amino acid composition of DYF was determined by digesting DYF biomass, equivalent to 5.0 mg protein under acidic conditions. The digest was first converted to *o*-phthaldialdehyde derivatives. Next, these were subjected to reverse phase high-performance liquid chromatography (RP-HPLC) for amino acid analysis. The amino acid content was expressed per 100 g biomass and compared with common vegetables and edible mushrooms in addition to popular *Diplazium* species, the *D. esculentum*. Soya protein was used as reference protein. In another study, the aqueous, hydro-alcoholic (70%) and alcoholic (100% ethanolic) extracts of DYF were used after over-night incubation at 4 ± 2 °C. After filtering these through 0.45 µm membrane filters (Merck Millipore, United States), the samples were derivatized using *o*-phthaldialdehyde and subjected to amino acid analysis.

Fatty acid composition

The total fat obtained after Soxhlet extraction of DYF was used for the analysis of fatty acids composition. The crude fat was converted to fatty acid methyl esters (FAME) by refluxing the extracts with 5% methanolic hydrogen chloride (w/v) for 2 h (Christie 1993). The FAMEs were then extracted with *n*-hexane, and washed with 5% NaCl solution followed by 2% $KHCO_3$. These were subsequently dried over anhydrous Na_2SO_4 and concentrated under vacuum. Thereafter, the FAMEs were dissolved in HPLC grade *n*-hexane, and 0.5 µL of FAME extract was injected

into a GC/GC–MS (Agilent 7890 series gas chromatograph) equipped with a flame-ionization detector and a fused silica HP-5 column (30 m × 0.32 mm with film thickness of 0.25 µm; J&W Scientific). The temperature was programmed at 120 °C (5 min hold) to 280 °C (10 min hold) at 5 °C/min. Finally, the FAMEs were identified by comparing the retention times with standard FAME mixture and fragmentation patterns with authentic standards (C-8–C-24, FAME mix, SUPELCO).

Phytochemical composition analysis

Total chlorophyll and carotenoids

The powdered DYF sample (100 mg) was extracted in acetone thrice and filtered. The filtrates were pooled and concentrated under vacuum, and the extracts diluted appropriately for the estimation of both chlorophyll and carotenoids. The optical density of the extracts were measured at 661.5, 645 and 450 nm and Lichtenthaler's equation (Lichtenthaler 1987) was applied for the quantification of chlorophyll a, b, total chlorophyll and carotenoids as below:

$$\text{Chlorophyll } (a)(\mu g/mL) = 11.24 \times \text{ O.D } 661.5 - 2.04 \times \text{ O.D } 645$$
$$\text{Chlorophyll } (b)(\mu g/mL) = 20.13 \times \text{ O.D } 645 - 4.19 \times \text{ O.D } 661.5$$
$$\text{Total chlorophyll } (\mu g/mL) = [7.05 \times \text{ O.D } 661.5 + 18.9 \times \text{ O.D } 645]$$
$$\text{Total carotenoids } (\mu g/mL) = (1000 \times OD\,450 - [1.9 \times \text{Chl } a + 63.14 \times \text{chl } b.])/214$$

Total polyphenols and flavonoids

DYF was individually extracted with 70% (v/v) aqueous methanol (hydro-alcoholic), aqueous and also absolute ethanol (alcoholic) at 1:5 solid to liquid ratio assisted by homogenization at 5000 RPM. The extracts were pooled, filtered and concentrated under vacuum. The crude extract (5.0 mg/mL) was re-suspended in respective solvents for determining the contents of total polyphenol and flavonoids. The total polyphenol content was determined spectrophotometrically using Folin–Ciocalteu method of Singleton et al. (1999) and values were expressed as gallic acid equivalents (GAE). Briefly, to 500 µL of each extract, 5 mL of Folin–Ciocalteu reagent and 1 mL of 20% Na_2CO_3 solution were added. The volume of the reaction mixture was made up to 25 mL with distilled water and incubated in dark for 30 min at 37 °C in a water bath. The absorbance of the mixture was measured at 760 nm. The assay for each sample was repeated thrice in triplicates.

The total flavonoid content (TFC) was spectrophotometrically determined using the aluminum chloride method of Chang et al. (2002). Briefly, 125 µL of extract was taken and to this, 75 µL of 5% $NaNO_2$ solution was added. After incubating the reaction mixture for 6 min at room temperature, 150 µL of 10% $AlCl_3$ solution was added and re-incubated for 5 min at room temperature. Next, 750 µL of 1 M NaOH was added to the mixture and the final volume was made up to 2500 µL with distilled water. After a final incubation for 15 min at 37 °C in a water bath, the absorbance was measured at 510 nm. The total flavonoid content was expressed as quercetin equivalents, QE (mg/g) and the assay of each sample was repeated thrice in triplicates.

The crude extracts (5.0 mg/mL) of DYF were subjected to ultra-performance liquid chromatography (UPLC) for composition analysis of polyphenols and flavonoids. A UPLC system equipped with a C-18 column (21.1 × 50 mm, 1.7 µm), a suitable guard column, 600 quaternary gradient pump, PDA and a 717 auto sampler (ACQUITY UPLC, Waters) was used. The column temperature was set at 25 °C and a solvent gradient of 0.05% trifluoro acetic acid (TFA) in water and acetonitrile were used at a flow rate of 0.3 mL/min. The run time was 40 min and the separation was monitored at 280 nm. For identification of different phenolic compounds, retention time, co-injection and spectral matches with standards were used as per the method of Joshi et al. (2011).

In-vitro antioxidant activity of DYF extracts

The anti-oxidant potentials of DYF extracts (i.e., hydroalcoholic, alcoholic and aqueous) were evaluated using the slightly modified free radical scavenging assays i.e., 2,2-Diphenyl-1-picrylhydrazyl radical, (DPPH) and 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Re et al. 1999). Aqueous, hydroalcoholic and alcoholic extracts of DYF were diluted in the range of 2.5–300 µg/mL, and assayed for their free radical scavenging activity. The IC_{50} values of the extracts (i.e. 50% inhibition of free radicals) were determined using ascorbic acid as reference standards.

Functional properties of DYF

Bulk density was measured by determining the volume occupied by a known quantity (100 g) of biomass in a measuring cylinder (250 mL) as described by Pradeep et al. (2014). The 'water absorption capacity' (WAC) and the 'oil absorption capacity' (OAC) of DYF (1.0 g) were determined as per the method of Pradeep et al. (2014) and Sosulski et al. (1976), respectively. Refined groundnut oil was used for determination of OAC. Both WAC and OAC were expressed as weight of water (g) or oil (g) bound per g sample. Swelling power and solubility index were also determined by adding 10 mL water to 1.0 g of DYF

followed by 30 min incubation at 30 °C in a shaking water bath. Thereafter, the mixtures were centrifuged and the supernatant obtained was transferred to pre-weighed petriplate, and evaporated to dryness on a water bath followed by drying in a hot air oven at 105 °C for 3 h. For the calculation of swelling power and solubility index of DYF, both the residue in the centrifuge tubes and the dried Petriplates were weighed individually. The dispersibility of DYF was determined by dispersing 5.0 g DYF in 100 mL of water uniformly. Next, a 10 mL aliquot of the dispersion was filtered through a pre-weighed filter paper. The filtrate was then dried and weighed, separately. Solids remaining on filter paper accounted for the percent dispersed solids, while the dried filtrates gave percent soluble solids (Kulkarni et al. 1991).

Soup preparation and reconstitution

Locally available vegetables such as potato, tomato, onion, coriander leaves, garlic were chosen as ingredients for soup formulations (Table S1). The vegetables were disinfected with 200 mg/kg sodium hypochlorite solution, washed and blanched for 10 min. The blanched vegetables were soaked in a 2.0 g/100 mL brine solution to prevent enzymatic browning and dehydrated as described by Ssepuuya et al. (2018). The dehydrated vegetables were pulverized to a fine flour and stored in airtight containers. The ingredient flours were mixed as per proportions outlined in formulations (Table S1). Finally, different levels of DYF (2.5, 5.0, 10 and 15%) were incorporated into the above prepared soup mix and compared with commercial soup mixes. Formulations without DYF served as control sample. The ingredient composition and nutritional facts of commercial soup mix is presented in Table S2.

Nutrition and functional analysis of instant soup product

The finished product (DYF incorporated soup mix) was then tested for nutritional composition and functional properties as per the methods described above. Thirty panelists participated in the sensory evaluation of the soup. The panelists were asked to score the acceptability of DYF incorporated soups as per 9-point hedonic scale method of Kemp et al. (2009). The flavor, thickness, taste, aftertaste mouthfeel, appearance, and overall acceptability of the product were recorded for each sample.

Statistical analysis

All the analysis were performed in triplicates and the results expressed as the mean ± standard deviation (SD). Statistical analysis of the data was performed using ANOVA followed by Duncan's multiple range test (DMRT) at significance level of $P \leq 0.05$. The statistical analysis was carried out using the software Statistica, version 7.0, Statsoft, 2004.

Results and discussion

The nutritional and phytochemical composition of the edible young fronds of *D. maximum*, a lesser-known fiddle head fern species of the genus, *Diplazium*, found to be distributed at altitudes ranging from 1200 and 2500 amsl in Himachal Pradesh, India were assessed in the present study. The findings revealed the edible fronds to be rich in several nutritional constituents with high potential for inclusion in existing food basket.

Proximate and nutrient analysis of *D. maximum*

The moisture content, an important ingredient of popular leafy vegetables, was 81.68 g/100 g in the freshly harvested, succulent young fronds of *D. maximum* (Table 1). Moisture content in the range of 80.0–89.0 g/100 g has been previously reported in the fresh fronds of other edible

Table 1 Proximate and nutritional composition of young fronds of *D. maximum*

Proximate composition	Content
Moisture[a]	11.40 ± 0.23
Total energy[b]	319.42 ± 9.22
Total nitrogen[a]	4.06 ± 0.72
Crude protein[a]	25.39 ± 0.45
Total fat[a]	1.62 ± 0.04
Soluble carbohydrate[a]	23.045 ± 0.99
Total soluble sugar[a]	1.51 ± 0.03
Reducing sugars[a]	0.7 ± 0.05
Non reducing sugars[a]	0.808 ± 0.05
Starch[a]	0.643 ± 0.09
Dietary fibres[a]	38.32 ± 1.11
Total carbohydrates[a,c]	61.36 ± 1.77
Total ash[a]	10.4 ± 0.40
Minerals	
Iron[d]	200.52 ± 5.79
Magnesium[d]	307.12 ± 8.87
Calcium[d]	277.24 ± 8.00
Zinc[d]	46.18 ± 1.33
Phosphorus[d]	1315.00 ± 37.96
Potassium[d]	1782.30 ± 51.45

Values are mean ± SD of triplicate analysis

[a]g/100 g, [b]kcal/100 g, [c]difference method, [d]mg/kg

ferns like *Diplazium, Dryoptera, Tectaria* and *Matteuccia struthiopteris* (Bushway et al. 1982; Chettri et al. 2018). Till date, edible fern species have not been characterized extensively. In the scant literature that is available on edible ferns till date, the nutritional data on ferns have been compared with commonly consumed leafy vegetables like moringa, celery and spinach. In these plants also, the moisture content ranged between 78 and 95 g/100 g (Gopalan et al. 2004).

Composition analysis of uniformly dried (12.0 g/100 g moisture content) and powdered material of *D. maximum* fronds (DYF) was also carried out. The total energy content of the DYF was found to be 319.42 kcal/100 g. This value was relatively lower than that reported in other species of *Diplazium* i.e., 416.6 kcal/100 g in *D. sammatti* (Bassey et al. 2001). The total energy content of *D. maximum* was however, significantly higher than other leafy vegetables like spinach, lettuce, moringa and fiddlehead ferns.

The total carbohydrate content of DYF was 61.36 g/100 g. While the values were similar to that of *D sammatii* (Bassey et al. 2001), the amount was significantly higher than that of fresh fiddlehead ferns i.e., 3.06 g/100 g (Bushway et al. 1982). The soluble carbohydrate content was 23.05 g/100 g and the total soluble sugar was 1.51 g/100 g. Further, 0.7 g/100 g reducing sugar, 0.81 g/100 g non-reducing sugars, and 0.64 g/100 g starch were recorded in the DYF.

Dietary fibres are an important proximate component of plant derived foods with numerous health benefits such as improving the intestinal flora and therapeutic effects against type 2 diabetes, dyslipidemia and other non-communicable diseases (Anderson et al. 2009). In the present study, the dietary fibre content in the DYF was found to be 38.32 g/100 g. The amount was significantly higher than that reported in the fresh and succulent croziers of *Tectaria* sp, and other species of *Diplazium* (Chettri et al. 2018).

The total nitrogen content of DYF was 4.06 g/100 g, and the crude protein was 25.39 g/100 g. Interestingly, 2.5 times higher content of proteins were recorded in the DYF of *D. maximum* as compared to young pinnae and croziers of *D. sammatii* and *D. esculentum* (10–10.5 g/100 g) on dry weight basis (Bassey et al. 2001; Tongco et al. 2014); and 5.5–6.0 g/100 g on fresh weight basis (Wali et al. 2016; Chettri et al. 2018). Based on the above, *D. maximum* appears to be a potential source of protein for wide consumption.

Detailed studies on the extractability of proteins is of commercial importance, considering the higher contents of crude protein in ferns as compared to fleshy and leafy vegetables. Maximum protein yield was obtained with alkali extraction (pH 9.00) $>$ 45% recovery (2.91 g/100 g DYF) as compared to water, salt and alcohol (Fig. S1). Requirement of alkali conditions indicate presence of strong linkages of disulfide bonds and protein–sugar interactions in this fern. Higher protein yield under alkaline conditions were also reported in other edible ferns such as *Nephrolepis* sp., *Arthropteris* sp. (Greeshma and Sridhar 2019). The observation of poor protein extractability in milder condition is common among plant proteins. The soluble protein yield obtained in the present study was 10 times higher as compared to yields reported by Wali et al. (2016). The higher yield in the present study could be attributed to finer particle size of biomass, optimum solid to solvent ratio and method of extraction.

The amino acids composition of DYF and its extracts are presented in Table 2; Table S3. The total amino acids (TAA) content was 23.75 g/100 g of biomass; and 40.29% of this constituted the essential amino acids (EAA). The TAA content with respect to total proteins was almost 34.7% higher in the DYF of *D. maximum* than that in *D. esculentum* (15.49 g/100 g TAA biomass). However, the EAA content in *D. esculentum* fronds was higher than that in *D. maximum*. On the other hand, nutritionally important branched chain amino acids (BCAA) viz, leucine, isoleucine and valine in DYF were higher (4.91 g/100 g biomass) than that in *D. esculentum* (3.16 g).

The amino acid profile of DYF biomass was also compared with *Spinach oleracea*, edible button mushrooms and soybean as reference protein sources. The TAA and EAA contents of DYF proteins were almost 50% lower than that of soybean proteins. The soybean proteins fared better with respect to certain EAAs such as lysine, methionine and aromatic amino acids. Yet, the EAA profile of *D. maximum* was either comparable and/or superior to spinach, *D. esculentum* and button mushrooms. Hence, *D. maximum* appears to be a potential amino acid rich food for regular consumption. Comparative analysis of the different extracts of DYF revealed significant variations in free amino acid composition. The presence of non-polar amino acids like alanine, valine, glycine and phenylalanine in alcoholic extract as compared to aqueous extract and also appreciable amounts of EAAs like lysine and methionine, phenylalanine in the aqueous extract of DYF were recorded (Table S3).

Isolation of high quality green leaf protein is favorable for commercial applications owing to their low lipid and low fiber content as compared to proteins obtained from other plant parts (Greeshma and Sridhar 2019). The present study clearly indicates the potential of *Diplazium maximum* as source of leaf protein concentrate owing to significantly higher crude protein content, better protein extractability under alkaline conditions and amino acid profile similar to traditionally consumed vegetables.

The total fat (ether extractives) content of DYF biomass was 1.62 $\pm$ 0.04 g/100 g DYF. The total fatty acids constituted 30% palmitic acid, and 57% of polyunsaturated

Table 2 Composition of amino acids in *Diplazium maximum* as compared to *D. esculentum* (edible fiddlehead fern), *Spinach oleracea* and *Agaricus* sp. (button mushroom) (g/100 g biomass)

Amino acids	*Diplazium maximum*[a]	*D. esculentum* (fiddlehead fern)	Button mushroom	*Spinach oleracea*	Soya white
Alanine	1.35 ± 0.04	1.25	0.21	0.14	1.64
Arginine[#]	1.23 ± 0.03	0.83	0.24	0.12	2.77
Aspartic acid	2.26 ± 0.06	1.02	0.38	0.20	4.31
Cysteine and cystine	0.07 ± 0.00	1.29	0.04	0.02	0.54
Glutamic acid	3.43 ± 0.10	0.09	0.53	0.32	6.34
Glycine	2.72 ± 0.08	1.69	0.20	0.12	1.47
Histidine*	0.56 ± 0.02	0.37	0.10	0.05	0.96
Isoleucine*	1.63 ± 0.05	0.84	0.16	0.09	1.73
Leucine*	1.95 ± 0.05	1.30	0.25	0.19	3.08
Lysine*	1.4 ± 0.04	1.35	0.25	0.10	2.71
Methionine*	0.17 ± 0.01	0.23	0.06	0.03	0.58
Phenylalanine*	1.24 ± 0.03	0.99	0.17	0.11	1.89
Proline	0.99 ± 0.03	1.10	0.14	0.10	1.22
Serine	1.49 ± 0.04	0.89	0.16	0.09	1.75
Threonine*	1.04 ± 0.03	0.68	0.16	0.10	1.47
Tyrosine	0.64 ± 0.02	0.53	0.12	0.08	1.25
Valine*	1.33 ± 0.04	1.01	0.20	0.12	2.10
Tryptophan*	0.25 ± 0.01	–	0.05	0.03	0.63

*Essential amino acids, [#]conditionally essential amino acid

[a]Values are mean ± SD of triplicate analysis

fatty acids (PUFAs). Among PUFAs, linoleic, alpha linolenic acids and a unique omega 6 fatty acid i.e., dihomo-gamma-linolenic acid (DGLA, C20:3, 8,11,14-eicosatrienoic acid) were predominant in the DYF (Table 3), followed by linoleic acid and arachidonic acid. DGLA is an important intermediary metabolite that can be converted into prostaglandin E1. The latter is known to inhibit platelet aggregation and is also reported to have vasodilatory effect (Koga et al. 2002). DGLA inhibits the production of eicosanoids of arachadonic acid by competing with arachadonic acid for cyclooxygenase and lipoxygenase (Wang et al. 2012). This unique fatty acid has been earlier reported in the fiddlehead fern, *Matteuccia struthiopteris* (DeLong et al. (2011) but it was not observed in the commonly consumed *D. esculentum*.

Interestingly, the unsaturated fatty acids, especially, the alpha linolenic acid in *D. maximum* was similar to the leafy vegetable, purslane. Purslane is considered to be the ''gold standard'' for omega-3 content in vegetative tissue (Simopoulos et al. 1992). However, the ratio of omega 3/omega 6 in purslane is higher than *D. maximum*. These findings revealed a high percentage of unsaturated fatty acids in DYF thereby, indicating the immense therapeutic benefits of *D. maximum*. Additionally, the unique fatty

Table 3 Fatty acid composition of young fronds of *D. maximum*

Fatty acids composition				Content (g/100 g)	% composition
Common name	IUPAC	Nomenclature	Type		
Palmitic acid	Hexadecanoic acid	C16:0	Saturated	0.47	30.51
Stearic acid	Octadecanoic acid	C18:0	Saturated	0.1	6.49
Oleic acid	octadec-9-enoic acid	C18:1	Omega-9	0.1	6.49
Linoleic acid	9,12-octadecadienoic acid	C18:2	PUFA (omega-6)	0.34	22.07
α-Linolenic acid	cis-9,12,15-octadecatrienoic acid	C18:3 n3	PUFA (omega-3)	0.20	12.98
Dihomo-γ-linolenic acid	8,11,14, -Eicosatrienoic acid	C20:3	PUFA	0.23	14.93
Arachidonic acid	5,8,11,14-Eicosatetraenoic acid	C20:4 n6	Omega-6	0.1	6.49

acids in the fern can be used as chemotaxonomic markers for identifying and validating fern species that have not been studied till date.

The total ash content of DYF was 10.4 ± 0.40 g/100 g. The amounts of elemental phosphorus and potassium were also significant. The major minerals comprised of magnesium (307.12 ± 8.87 mg/kg) and calcium (277.24 ± 8.00 mg/kg), while the amounts of trace elements viz, iron and zinc were 200.52 ± 5.79 and 46.18 ± 1.33 mg/kg (Table 1). The values recorded in the DYF of *D. maximum* were similar to those in other species of *Diplazium* and fiddlehead ferns (Chettri et al. 2018). The potassium levels were also similar to that of *D. sammatii* (Bassey et al. 2001).

Phytochemical analysis

Ferns are a unique category, bridging the gap between lower and higher plants. However compared to angiosperms, ferns are under-explored as well as under-utilized for their potency of phytochemicals. Interesting observations from literature indicate that most of the edible ferns do not contain toxic chemical moieties such as alkaloids and anti-nutrition factors such as cyanogenic glycosides, lectins and enzyme inhibitors (Greeshma and Sridhar 2019). One of the major classes of phyctochemicals attributed to ferns are polyphenols and flavonoids with anti-oxidant potential. Therefore, it was imperative to characterize and screen for bioactive phytochemicals in this lesser known species.

The total chlorophyll content of DYF was 0.43 mg/g, while the total carotenoid content was 0.065 mg/g. As reported earlier by Wali et al. (2016), lutein was found to be the major carotenoid in DYF in the present study.

Phenolic compounds are effective scavengers of free radicals and are known to inhibit the oxidative reactions that lead to chronic diseases (Stagos et al. 2012). Therefore, in present study, the total polyphenols (TPC) and flavonoids (FC) of DYF were analyzed. Among the solvents used for extraction, the hydroalcoholic extract yielded 22.70 mg/g GAE and 7.43 mg/g QE. These values were 2–3 times higher than the yield obtained from aqueous or alcoholic extracts (Table 4). Although these values are similar to those reported by Seal (2012), yet they are higher than those reported by Wali et al. (2016). As per the reports of Roy et al. (2013); Tongco et al. (2014), the total phenolic content in different species of *Diplazium* range between 12 and 16 mg/g.

Ten different phenolic compounds were recorded in the UPLC analysis of DYF extracts (Table 4; Fig. S2.). Irrespective of the extracts, four compounds namely, epicatechin, catechins, procatechuic acid and myrecitin were predominant. The maximum number of compounds were however, obtained in hydroalcoholic extracts. Myricetin, epicatechin and catechin followed by procatechuic acid and naringin were predominant in the aqueous extract while epicatechin and catechin dominated the ethanolic extract. While naringin was absent in hydroalcoholic extracts; rutin and chlorogenic acids were absent in aqueous extract. In contrast, both caffeic acid and rutin were absent in the alcoholic extract. These compositional differences of the three extracts is probably due to differences in solvent characteristics like extractability and polarity. The high phenolic content found in hydro-alcoholic extracts of *D. maximum* is worthy of investigations from nutraceutical perspective. Further, the suitability of utilizing DYF biomass in regular foods was indicated by enhanced extractability of polyphenols in aqueous medium over alcohols.

In-vitro anti-oxidant activity of *D. maximum* extracts

The radical scavenging activity of the various extracts of DYF revealed highest radical scavenging activity in the hydro-alcoholic extracts. This was evident from the lower IC_{50} values in DPPH (48.49 ± 0.94 µg/mL) and ABTS (22.16 ± 0.25 µg/mL) assays (Fig. S3). High antioxidant activity of hydro-alcoholic extracts is probably due to the presence of a range of individual phenolic acids and high contents of both TPC and FC. Partial extraction of carotenoids and other phytochemicals in hydro-alcoholic extracts probably contributed towards higher free radicals scavenging activity.

Functional properties of *D. maximum*

The ultimate aim of nutritional characterization of any plant material is in its use as food ingredient. Although several research papers are available on nutritional quality of edible ferns, only handful reports are published on functional properties of edible ferns. Assessment of functional, mainly hydration, properties like water and oil absorption capacities (WAC & OAC), swelling index, solubility, dispersibility are important as these can affect the food product quality (Wu et al. 2009). The bulk density of DYF was 0.62 g/mL and total soluble solids was 20.05 g/100 g, thereby indicating DYF as a highly insoluble fibrous material in simple aqueous medium. Dispersibility indicates a food material's ability to reconstitute water thereby, resulting into a fine consistency in a mixture. The dispersibility of DYF was 79.85 g/100 g indicating its ability to disperse into a fine consistency in aqueous medium. Water and oil absorption capacities are crucial parameters affecting the flavor and texture of foods as the intrinsic proteins or fibers interact with water and oil

Table 4 UPLC profiling of phenolic acids of young fronds of *Diplazium maximum*

Phenolic acids (mg/g)	Hydroalcholic extract	Aqueous extract	Alcoholic extract
Procatechuic acid	2.16 ± 0.062^{d}	0.81 ± 0.265^{f}	0.578 ± 0.024^{hi}
Kaempherol	0.253 ± 0.007^{mn}	0.124 ± 0.010^{op}	0.174 ± 0.042^{no}
Rutin	0.541 ± 0.010^{ij}	nd	nd
Chlorogenic acid	0.663 ± 0.019^{gh}	nd	0.192 ± 0.005^{no}
Caffeic acid	0.464 ± 0.014^{ijk}	0.315 ± 0.009^{lm}	nd
Epicatechin	3.847 ± 0.237^{a}	2.438 ± 0.070^{c}	3.269 ± 0.061^{b}
Myrecetin	0.730 ± 0.021^{fg}	3.319 ± 0.096^{b}	0.837 ± 0.024^{f}
Catechin	0.379 ± 0.011^{kl}	2.227 ± 0.064^{d}	1.031 ± 0.030^{e}
Cinnamic acid	0.013 ± 0.000^{p}	0.003 ± 0.000^{p}	0.009 ± 0.000^{p}
Naringin	nd	0.436 ± 0.014^{jk}	0.142 ± 0.008^{no}
Total phenolics	22.70 ± 0.91^{a}	11.36 ± 0.41^{b}	6.25 ± 0.24^{c}
Total flavonoids	7.43 ± 0.33^{a}	3.13 ± 0.58^{b}	2.89 ± 0.34^{b}

The values are mean ± SD. Values having different superscript are statistically significant at $p \leq 0.05$

nd not determined

significantly. The WAC of DYF is 6.28 g/g biomass. This value is unusually high amongst leafy vegetable powders and can be directly attributed to higher protein and fiber concentrations. The WAC of flours represent their ability to reconstitute under limited water conditions. Higher WAC indicates better reconstitution abilities of a material and its suitability in the preparation of 'ready to reconstitute products' (Ssepuuya et al. 2018). The ability to absorb oil is another important functionality that influences the taste of product. The OAC of DYF was found to be 2.96 g/g. This value was two times lower than that of WAC. The swelling capacity of DYF was 4.02 g/g, thereby indicating the hydration ability of powdered DYF. The functional properties of DYF is presented in Table S4.

Formulation of instant mixes using DYF of *D. maximum*

The DYF biomass was utilized for the formulation of ready to use convenience food premixes. Traditionally, fiddlehead ferns are consumed in the form of pickles and curries in Himalayan regions involving deep frying of fresh or dried croziers with spices (Thakur et al. 2017). In the present study, dehydrated fronds (DYF) were mixed in different proportions with various ingredients listed in (Table S2) towards formulation of instant soup mixes. The formulation was standardized linearly and it was found that DYF could be incorporated up to 10 g DYF per 100 g formulation with an optimal functional and sensorial properties. Uniform dispersibility, optimal water absorption capacity, color and after taste were mainly considered for product formulation. The present work is one of a first attempt towards developing a convenience food product utilizing edible fern *Diplazium maximum* and its nutritional and functional characterization.

Nutritional and functional properties of instant soup mix

The nutritional composition and functional properties of the above developed formulation containing 10% DYF was characterized (Table 5; Table S5) and compared with commercial soup samples. The average moisture content of soup mix was 6.8 g/100 g, whereas, the product and bulk density was 0.44 g/cm. The water and oil absorption capacity were 2.63 g/g product and 2.55 g/g product, respectively. The dispersibility of the product was 44.22 g/100 g product. A reconstitution ratio of 1:5 with hot water was found to be optimal. The protein content of the instant soup was 14.71 g/100 g product, with one serving (30 g) of soup providing 4.45 g protein. It was found to meet 7.5% of the recommended dietary allowances (RDA) of protein in an adult consuming standard 2000 kcal diet. The protein content of soup mix incorporated with *D. maximum* was higher than commercially available soup samples (Table 5). The total carbohydrate content of formulation was 53.40 g/100 g product. This was lower than commercial samples, while the total sugars content (25.68 g/100 g product) was higher than commercial samples. The reducing sugars was 1.65 g/100 g product and the non-reducing sugars was 24.03 g/100 g product. The dietary fibre content at 4.65 g/100 g product was also higher as compared to commercial products (Table 5). The total fat content (2.4 g/100 g product) was at par with commercial samples claiming low fat content. The ascorbic acid content of instant soup mix was 13.29 mg/100 g product while total polyphenol content was 122.96 mg/100 g product.

Table 5 Nutritional composition of instant soup mix

Parameter	Instant soup mix*	Commercial sample 1[#]	Commercial sample 2[$]
Moisture content[a]	6.80 ± 0.17	–	–
Total proteins (N × 6.25)[a]	14.71 ± 0.36	6.7	10.20
Total fat[a]	2.40 ± 0.06	2.50	6.50
Total carbohydrates[a]	53.40 ± 1.33	69.30	62.90
Total sugars[a]	25.67 ± 0.64	22.70	19.00
Reducing sugars[a]	1.65 ± 0.04	na	na
Non-reducing sugars[a]	24.07 ± 0.59	na	na
Dietary fibre[a]	4.65 ± 0.11	3.20	4.4
Ash[a]	2.79 ± 0.06	na	na
Ascorbic acid[b]	13.29 ± 0.33	na	na
Total polyphenols (GAE)[b]	122.96 ± 3.06	na	na

Values are mean ± SD of triplicate analysis

*Instant soup mix formulated at 10% incorporation of DYF

[#]Knorr classic sweet corn vegetable soup (low in fat claim)

[$]Knorr classic mixed vegetable soup (low in fat claim)

[a]g/100 g

[b]mg/100 g

na not available

From the experimental study, it is clearly evident that addition of *Diplazium maximum* DYF biomass has enhanced the protein content of the soup premixes. Further, presence of nutraceuticals such as polyphenols, carotenoids and polyunsaturated fatty acids in *Diplazium maximum* renders opportunity to develop functional foods in convenient formats. The total functional properties of instant soup mixes are presented in Table S5.

Sensory acceptability of formulated soups

All the formulations were uniformly reconstituted at 1:5 ratio (30 g soup mix and 150 mL hot water). The sensory attributes such as thickness, flavor, taste, aftertaste, mouthfeel, appearance and overall acceptability of the formulated soup mix were determined using 9-point Hedonics scale (Fig. S4). The dehydrated vegetables and DYF powder were brought to similar particle sizes to have uniform mouthfeel. The results revealed that the formulations containing DYF beyond 10% had settling of particles, lower scores for sensory attributes, a mild bitter aftertaste and very fibrous mouthfeel with the commercial soup scoring highest in terms of sensory attributes. Yet, the sensory scores of the formulated soups having 2.5, 5.0 and 10% DYF were similar to that of control formulation (commercial soups). Overall, the formulated soup had scores similar to that of commercially available soups.

Conclusion

Diplazium maximum is an edible fern, rich in several nutritional characteristics, that are either significantly higher or similar to many commonly consumed vegetables and mushrooms. The unique characteristics of the species are higher crude protein, high dietary fibre and PUFA content in the edible portions. Further presence of unique omega 6 fatty acid di-homo-gamma linolenic acid and polyphenols indicate its potential nutraceutical application. Acceptable sensory scores of the soup prepared with *D. maximum* biomass also indicate its suitability for instant foods production and inclusion in existing food basket. Thus, in conclusion, the study demonstrates the prospective benefits of the fern species and promote its preservation, domestication and utilization as a popular food.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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