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TWO EDIBLE FERNS OF WESTERN HIMALAYA: A COMPARATIVE IN VITRO NUTRITIONAL ASSESSMENT, ANTIOXIDANT CAPACITY AND QUANTIFICATION OF LUTEIN BY UPLC-DAD

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Diplazium esculentum and *Diplazium maximum* are ferns which have been consumed in tribal areas of western Himalaya. In the present study, the nutritional profile, total polyphenol content, total flavonoid content and antioxidant activity of these two species were evaluated. From the nutritional profiling results, protein content in *D. esculentum* and *D. maximum* found to be 1.73 mg/g and 1.5 mg/g respectively. The total carbohydrate content of *D. esculentum* and *D. maximum* were 0.15 mg/g and 0.16 mg/g respectively. The antioxidant activity in different extract of these ferns were assessed and highest activity was observed in methanolic extract of *D. maximum* as compared to *D. esculentum* followed by chloroform, butanol and water extracts. For standardization of the extracts, lutein was quantified in both the species by Ultra performance liquid chromatography (UPLC) and it was higher (6.1 mg/g of plant sample) in *D. maximum* as compare to *D. esculentum* (1.4 mg/g of plant sample).

Keywords: *Diplazium esculentum*, *Diplazium maximum*, Antioxidant activity, Nutrition, UPLC, Lutein

INTRODUCTION

The production of free radicals or reactive oxygen species such as superoxides ($O_2^{\cdot}$), alkyl peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and hydroxyl ($HO^{\cdot}$) in body, readily reacts with biomolecules such as lipids, proteins, enzymes etc present in body and leads to cell or tissue injury (Wiseman and Halliwell, 1996; and Chew *et al*., 2008). Excessive production of free radicals occurs due to oxidative stress or radiations, ultimately leading to the degenerative diseases such as atherosclerosis, cardiovascular disorders, cancer, diabetes mellitus, aging and neurodegenerative disorders (Pham-Huy, 2008). Occurrence of important chemical constituents in plants such as vitamins, carotenoids and polyphenols leads to a good antioxidant activity (Gil *et al*., 2002; and Chen *et al*., 2007). Due to their hydrogen donating capacity, the phenolic compounds undergo redox reaction with free radicals. The free radicals accept the proton and get stabilized (Choudhary and Swarnkar, 2011). The antioxidant properties of phenolic compounds can treat atherosclerosis and coronary heart disease, though showing selective cytotoxicity to breast cancer cells (Xiang *et al*., 2014). Intake of fruits and vegetables rich diet helps in eradication of free radicals from the body (Pham-Huy, 2008). Natural antioxidants are preferably used in food and nutraceuticals due to safety and health concerns as compare to the synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Valentao *et al*., 2002; and Wong *et al*., 2006).

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Pteridophytes, the vascular cryptograms, are the second most important group of flora in the world (Dixit, 1984). About 12000 species of pteridophytes have been identified worldwide, out of which 1000 species are distributed in India (Dixit, 2000). Ferns are being ignored in relation to flowering plants for fewer applications in medicines (Singh and Singh, 2013). Ferns contain bioactive components such as alkaloids, flavonoids, terpenoids, glycosides, sterols, phenols, sesquiterpenes and exhibit diverse phytomedicinal properties like antioxidant, anti-cancer, anti-bacterial, anti-viral, anti-fungal, rheumatism, diabetes, inflammation, anti-fertility, diuretic, hepato-protective and sedative (Kulandairaj and Britto, 2000). The pharmacological properties are already evaluated in ferns but little attention has been given to highland ferns. Higher amount of phenolic compounds and their good antioxidant activity has been previously estimated in the ferns growing at high altitudes (Spitaler *et al.*, 2008; and Rawat *et al.*, 2015). Thus, it provides a great interest to researchers to exploit *D. esculentum* and *D. maximum*, found in Kumaun Himalayas, Tripura and Orissa (Kumari *et al*., 2011). These are edible ferns, belongs to Athyriaceae family (Christenhusz *et al*., 2011). Only curled fronds of both ferns are eaten as vegetables or salads by the tribals living in western Himalayas. *D. esculentum* and *D. maximum* are known as linguda and lingura respectively, in northern India (Kumar, 2004; Upreti *et al*., 2009). Edible fronds of *D. esculentum* are rich source of proteins, carbohydrates and minerals (Seal, 2012). Preliminary analysis suggests the presence of saponins, proteins, amino acids, phenols, alkaloids, diterpenes, triterpenes, glycosides and anthraquinones in *D. esculentum* (Tongco *et al*., 2014) whereas in *D. maximum* presence of glycosides, flavonoids, triterpenoids, proteins, amino acids, saponins and resins was reported (Mir *et al*., 2013). The pharmacological activities such as anti-inflammatory (Kaushik *et al*., 2011), antioxidant (Roy *et al*., 2013), anti-cancer (Rahmat *et al*., 2004), haemolytic (Roy *et al*., 2013), antifungal (Zakiara *et al*., 2010), anti-helmentic (Semwal *et al*., 2013), and immunomodulatory activities (Roy *et al*., 2013) of *D. esculentum* have been reported.

However, there is no report regarding antioxidant activity as well as nutritional potential of *D. maximum* in the literature. Therefore, in current study, we focus on a comparative analysis of antioxidant activity, nutritional potential and quantification of lutein in edible ferns, *D. esculentum* and *D. maximum*.

MATERIALS AND METHODS

D. esculentum and *D. maximum* plants were acquired from Kangra district in July 2012 and August 2012, respectively. These plant materials were deposited in the herbarium of CSIR-IHBT, Palampur, Himachal Pradesh, India with voucher no. PLP-16712 and PLP-16911 respectively and were authenticated by the taxonomist, Dr. Brij Lal at CSIR-IHBT.

Extraction and Fractionation

The crushed dried plant material of *D. maximum* (840 g) was percolated with ethanol (4 × 2.5 l) for four times. The combined extract was concentrated over reduced pressure, lyophilized and the yield of extract was found to be 52.6 g. The resulted extract was dissolved in distilled water and subjected to sequential extraction with hexane (3 × 500 mL; 17.7 g), chloroform (3 × 500 mL; 3.3 g), ethyl acetate (3 × 500 mL; 0.8 g) and butanol (3 × 500 mL; 4.7 g). The water fraction was obtained as 21 g.

Isolation of Lutein

A simple isocratic method was used for the isolation of lutein. Sample with 10 mg/ml concentration using acetonitrile (ACN) solvent was prepared and filtered with 0.2 µm Millipore filter (PVDF). System equipped with dual λ absorbance detector was used for purification. Waters Prep HPLC C-8 column, (XBridge Shield; 5 µ particle size; 50 x 250 mm dimensions) was used. The mobile phase used and run time was 12% water (A) and 88% ACN (B) with 12 ml/min flow rate and 30 min respectively.

Quantification of Lutein by UPLC

Preparation of Standard and Sample Solutions

1 mg/2 ml stock solution of standard lutein was prepared with ACN. 5 mg of the both extracts (*D. esculentum* and *D. maximum*) were dissolved in 1 ml ACN and resulted solution was filtered through 0.2 µm Whatman millipore filter.

UPLC Conditions

Lutein content was quantified by an Acquity UPLC system (Waters, Milliford, MA, USA) in two species of *Diplazium*. The samples were analyzed using analytical column BEH C18 (100 mm x 2.1 mm, 1.7 µm particle size), injection volume 1 µl and operated by MassLynx v4.1 software. The isocratic solvent system with methanol and 0.1% formic acid in water (90:10, v/v) was used. Flow rate was kept for 0.28 mL/min with the run time of 7 min.

ESI-MS/MS Conditions

Mass of the compound was estimated on Q-TOF triple-quadrupole mass spectrophotometer equipped with ESI source (Micromass, Manchester, UK) operated by MassLynx v4.1 software. The parameters of mass spectrophotometer are as follows: capillary voltage (3.2 kV), sample cone voltage (30 V), extraction cone voltage (3 V), desolvation temperature (220 °C), source temperature (80 °C), auto sampler temperature (10 °C), cone gas (50 l/h), desolvation gas flow (400 l/h), scan time (1 s) and inter scan delay (0.1 s).

Method Development for UPLC

The method validation includes selectivity, inter-day, intra-day, Limit of Detection (LOD), Limit of Quantification (LOQ) and recovery experiments. The selectivity of peak in sample was identified by comparing its retention time (t_R) and UV λ_{max} with standard. The calibration curve of the standard was prepared by diluting the standard solution to different concentrations. To analyze the repeatability and reproducibility of developed method, intra-day and inter-day precision were performed. Diluted concentrations of standard were injected to the instrument for determining LOD and LOQ. Accuracy of method was evaluated by performing recovery experiment. For the evaluation of recovery, 10 mg of extract was mixed with three different (300, 500 and 800 µg) concentration of standard.

Estimation of Nutritional Potential

Determination of Protein Content

The protein content was estimated in parent extracts of *D. esculentum* (DE-PAR) and *D. maximum* (DM-PAR) using Bradford assay (Bradford, 1976). 1 mg/ml of Bovine serum albumin (BSA) was prepared and diluted to concentration 0.1 mg/ml. For the preparation of calibration curve, different concentrations (50, 100, 150, 200, 250, 300, 350 and 400 µl) of BSA were mixed with 1.6 ml of Bradford reagent. Stock solution, 10 mg/ml of both the DE-PAR and DM-PAR samples were prepared. Each sample (50 µl) was mixed with 1.6 ml of Bradford reagent and absorbance of obtained solution was measured at 595 nm with UV spectrophotometer.

Determination of Carbohydrate Content

The carbohydrate content was determined using anthrone method (Sadasivam, 1992). 5 ml of 2.5 N HCl was added to 100 mg of plant material and heated on water bath for 3 h. After cooling at room temperature, mixture was further neutralized using sodium carbonate and volume was made 100 ml with distilled water. The solution was filtered and 0.2 ml of sample was diluted with 0.8 ml of distilled water to make the volume upto 1 ml. Glucose (0.175 mg/ml) was used as a standard. Different concentrations (200, 400, 600, 800 and 1000 µl) of the standard were used. 4 ml of anthrone was also added to each sample. After heating the solution for 8 min on water bath, the absorbance was measured at 630 nm.

Total Polyphenol Content (TPC)

Folin-Ciocalteu's reagent method was used to determine TPC in DE-PAR and DM-PAR spectrophotometrically (Kalia *et al.*, 2008). For the calibration curve, different concentrations of gallic acid (20, 40, 60, 80 and 100 µg/ml) were used. 0.5 ml of 1N Folin-Ciocalteu's phenol reagent and 1.0 ml of 35% Na_2CO_3 were mixed with different concentrations of gallic acid and finally the volume made up to 25 ml with distilled water. 50 µl of DE-PAR and DM-PAR were added to same reagents as mentioned above. Absorbance was measured at 730 nm after incubation for 35 min at room temperature. Triplicate analyses were run for each extract. TPC was expressed as mg of gallic acid equivalent per gram of dry plant sample.

Total Flavonoid Content (TFC)

Different concentrations of quercetin (20, 40, 60, 80 and 100 µg/ml) for calibration curve were prepared. Each of concentrations were mixed with the 1.5 ml of 95% ethanol (v/v), 0.1 ml of 1 M potassium acetate, 0.1 ml of 10% aluminum chloride (w/v) and 2.8 ml of water in a 5.0 ml volumetric flask. The same reagents were mixed with 0.5 ml of DE-PAR and DM-PAR as described above. Absorbance was measured at 415 nm after incubation for 30 min at room temperature. Triplicate analyses were run for each extract. The total flavonoid content was expressed as mg of quercetin equivalent per gram of dry plant sample (Kalia *et al.*, 2008).

Evaluation of Antioxidant Activity

Sample Preparation

100 g powdered plant material of two species of *Diplazium* were extracted with methanol using soxhlet extraction method. The parent extract of *D. esculentum* (DE-PAR) was suspended in water and successively fractionated with chloroform (DE-CH), *n*-butanol (DE-BU), and water (DE-WT) separately. Different fractions of *D. maximum* were

prepared by fractionation with chloroform (DM-CH), *n*-butanol (DM-BU) and water (DM-WT). All fractions were dried under vacuum and were kept at 4 °C for further analysis. Stock solutions of all fractions DE-PAR, DM-PAR, DE-CH, DM-CH, DE-BU, DM-BU, DE-WT and DM-WT were prepared as 5 mg/ml for the evaluation of TPC, TFC and antioxidant activity.

DPPH Assay

The DPPH assay is based on the principle of hydrogen donating capacity of antioxidant to the DPPH•. After the donation of hydrogen atom to the DPPH•, deep violet colour is changed to yellow. This change in colouration showed the reduction of free radical. Ascorbic acid (0.25 mg/ml) was used as a standard. Different concentrations of ascorbic acid (20, 40, 60, 80 and 100 µg/ml) were used for the calibration curve. Different concentrations of DE-PAR, DM-PAR, DE-CH, DM-CH, DE-BU, DM-BU, DE-WT and DM-WT measuring (100, 200, 300, 400, 500 µg/ml) were prepared. 1.9 ml of 0.1mM DPPH• solution prepared in ethanol, was added to each of the sample. The mixture was allowed to stand at 25 °C in the dark for 30 min. The absorbance of resulted solution was measured at 517 nm (Kalia *et al*., 2008). All measurements were done in triplicate. Inhibition of free radical DPPH (*I%*) was determined as follows

$$I\% = \frac{(Absorbance\ of\ control - Absorbance\ of\ test\ sample)}{Absorbance\ of\ control} \times 100$$

Steady state method (Brand-Williams *et al*., 1995) was used to determine the kinetic study of DPPH assay in parent samples (DE-PAR and DM-PAR). Their stock solutions of IC_{50} values as (DE-PAR, 314 µg/ml and DM-PAR, 235 µg/ml) were prepared. 0.1 ml each of sample was mixed with 1.9 ml of DPPH solution (0.1 mM). The decrease in absorbance was monitored for 5, 10, 15, 20, 25 and 30 min at 517 nm.

ABTS Assay

Aqueous solution of 7 mM ABTS and 2.45 mM potassium persulphate (1:1, v/v) were mixed and at room temperature allowed to stand for 16 h in the dark for the generation of $ABTS^+$. The mixture was diluted with distilled water to an absorbance of 0.700 ± 0.020 at 734 nm. Different concentrations of ascorbic acid (20, 40, 60, 80 and 100 µg/ml) were used for the calibration curve. Various concentration (100, 200, 300, 400 and 500 µg/ml) of DE-PAR, DM-PAR, DE-CH, DM-CH, DE-BU, DM-BU, DE-WT and DM-WT were prepared and 0.1 ml of each sample was mixed with 1.9 ml diluted $ABTS^+$ solution. After incubation of 4 min, absorbance was measured at 734 nm. All determinations were conducted in triplicate (Kalia *et al*., 2008).

FRAP Assay

Ferric 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (Fe^{3+}-TPTZ) get reduced to ferrous, coloured form (Fe^{2+}-TPTZ) due to the presence of antioxidants. For the preparation of FRAP reagent, 0.3 M acetate buffer with pH 3.6, 10 mmol of TPTZ solution in 40 mmol of HCl and 20 mmol of iron (III) chloride were mixed in proportions of 10:1:1 (v/v/v), respectively. Ascorbic acid was used as a standard. 50 µl of DE-PAR, DM-PAR, DE-CH, DM-CH, DE-BU, DM-BU, DE-WT and DM-WT was added to 1.5 ml of the FRAP reagent. After 4 min incubation at room temperature, absorbance was measured at 593 nm. All measurements were done in triplicate (Kalia *et al*., 2008).

RESULTS AND DISCUSSION

Separation and Identification of Compound (1)

For the isolation, 200 mg of chloroform extract was subjected to semi-preparative HPLC using isocratic solvent system; 12% water (A) and 88% ACN (B). A peak was eluted at t_R 21.03 min and its purification was confirmed by thin layer chromatography. The collected fraction was dried under reduced pressure and yielded 3.2 mg of pure compound (1). By comparing its spectral data with literature, compound (1) was characterized as lutein (Kiplimo *et al*., 2011).

Optimization of UPLC Method

The chromatographic conditions were optimized for achieving better resolution and peak shape using standard and sample solution (Figure 1). Several trials with ACN, methanol and water in different proportions were carried out for optimizing the mobile phase. A mobile phase consists of ACN (A): 0.1% formic acid in water (B), (90:10, v/v) was selected for the better selectivity of peak and the column temperature was set at 25 °C. Lutein (t_R=4.06 min) in the sample was identified by comparing UV λ_{max} and t_R with standard compound.

Method Validation and Quantification of Lutein

Table 1 showed the result of UPLC validation method. The linearity of method was evaluated by injecting nine different concentrations. The calibration curve of standard was plotted, regression coefficient was observed to be R^2 = 0.999 which showed good linearity. LOD and LOQ were found to

Figure 1: UPLC- DAD Chromatograms of (a) Standard, (b) *D. maximum*, and (c) *D. esculentum*

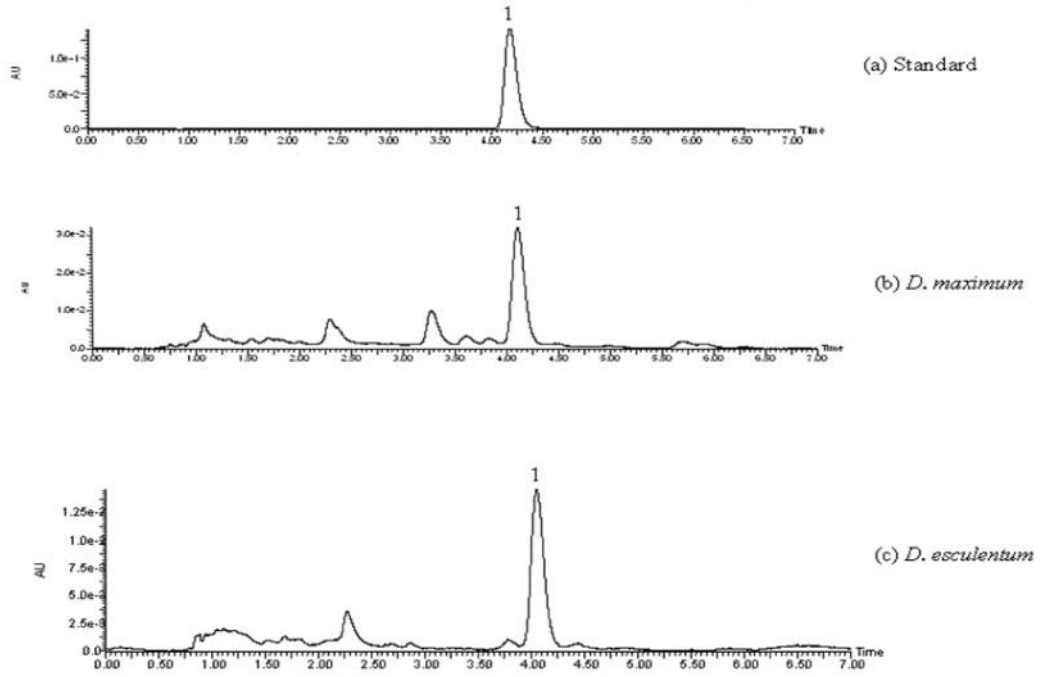


Table 1: Method Validation Data for Lutein

Compound	Regression Equation	R^2	Linearity (µg/mL)	LOQ (µg/mL)	LOD (µg/mL)	Inter-day (n=3)	Intra-day (n=6)	Average Recovery (%)±RSD (%)
Lutein	y=39.761-154.62	0.9993	3.91-500	2.93	0.98	0.175	0.2	97.99±1.86

be 0.98 µg/ml and 2.93 µg/ml respectively which showed good sensitivity of method for its quantification. The precision of method was determined by intra-day and inter-day variability test; expressed as % RSD, obtained as 0.2% and 0.175%, respectively. The recovery was found in the range of 96.07% - 98.2% with RSD 1.86%. The mass spectra showed *m/z* at 568 $[M]^+$ and fragmentation at *m/z* 551 $[M+H-H_2O]^+$ indicated the loss of a water molecule (Sowmya *et al*., 2014). UV λ_{max} for lutein were observed at 445 nm and 473 nm. The UPLC quantification results showed that lutein was present in higher amount in *D. maximum* as compared to *D. esculentum* (Table 2).

Determination of Nutritional Potential

The nutritional value of both ferns was determined by amount of protein content and carbohydrate content present in them. Protein content and total carbohydrate content were estimated by Bradford assay and anthrone assay respectively showed that both the ferns contain comparable amount of protein and carbohydrate contents. *D. esculentum* was found to have 1.73 mg/g of protein content whereas *D. maximum* have slightly lower content 1.50 mg/g. Total carbohydrate content was expressed as mg equivalent of glucose per gram of plant sample. Slightly higher amount of total carbohydrate was observed in *D. esculentum* (0.16 mg/g) as compare to *D. maximum* (0.15 mg/g). However the

Table 2: Comparison of Amount of Identified Compound in Two Species of Diplazium

Compound	Amount (mg/g)	
	D. esculentum	*D. maximum*
Lutein	1.4	6.1

previous report on *D. esculentum* from Meghalaya region showed higher amount of protein and carbohydrate content (Seal, 2012).

Total Phenolic Content (TPC)

Phenolic compounds are the important constituents since they can act as reducing agents to free radicals and inhibit the oxidative reactions that lead to the chronic diseases (Stagos *et al*., 2012). TPC in the *D. maximum* and *D. esculentum* was determined using Folin-Ciocalteu reagent. The results of TPC are shown in Table 3. The higher TPC was found in *D. maximum* 2.5 mg of GAE/g of dry plant sample and lower in *D. esculentum* 2.3 mg of GAE/g of dry plant sample. The low polyphenolic content in the present investigation was found might be due to extraction with less polar solvent as compared to previous reports regarding phenolic content in *D. esculentum* (Seal, 2011). However perviuos reports showed higher phenolic content (15.01 ± 0.32 GAE/g) in aqueous methanol extract of *D. esculentum* (Seal, 2011). Different reports on *D. esculentum* showed phenolic content to be 125.6 ± 13.4 mg GAE/100 g of sample for ethanol extract (Tongco *et al*., 2014), 126.67±8.16 mg GAE/g of dry plant (Roy *et al*., 2013) and 151.90 ± 5.01 mg GAE/g of dry ethanol extract (Das *et al*., 2013).

Total Flavonoid Content (TFC)

The TFC in *D. maximum* was found to be higher 7.5 mg QE/ g of dry plant sample and lower in *D. esculentum* 7.0 mg QE/ g of dry plant sample (Table 3). The TFC was earlier reported in *D. esculentum* as 34.81± 0.003 mg QE/g. Flavonoid content estimated in *D. esculentum* in other reports was found to be as 94.33 ± 6.12 mg QE/g of dry plant, 67.0 ± 0.28 mg QE/g of dry ethanol extract (Das *et al*., 2013) and 110.8 ± 11.2 mg QE/ 100 g of sample for ethanol extract (Tongco *et al*., 2014).

Evaluation of Antioxidant Activity

The antioxidant activity of leaves of *D. esculentum* and *D. maximum* were determined by using three assays: ABTS, DPPH and FRAP assays. Radical scavenging activity of different extracts of *D. esculentum* and *D. maximum* has been presented (Table 4). The antioxidant activity of each extract was expressed in terms of IC_{50} (the concentration required to inhibit free radical up to 50%). Standard (ascorbic acid) showed IC_{50} (17.45 µg/ml) and (8.44 µg/ml) for DPPH assay and ABTS assay, respectively. Previously, antioxidant activity of *D. esculentum* was determined (Gupta *et al*., 2014) and there is no report regarding the *D. maximum* in literature.

Table 3: Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in Two Species of Diplazium

Sample	TPC (mg of GAE/g)	TFC (mg of QE/g)
D. esculentum	2.3	7
D. maximum	2.5	7.5

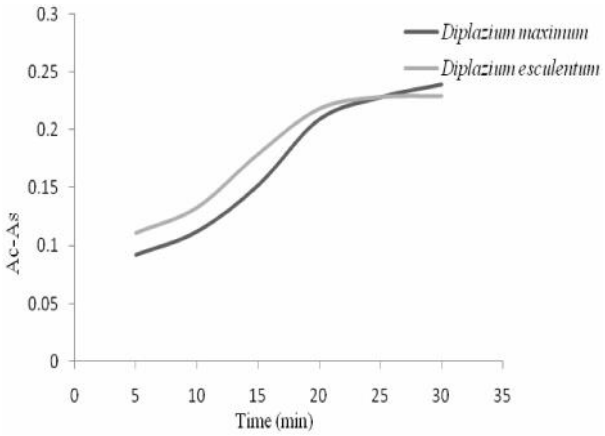
Table 4: Radical Scavenging Activity of Different Extracts of *D. esculentum* and *D. maximum*

Sample	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP mg of Ascorbic Acid/ g
Ascorbic acid	17.45	8.44	-
DE-PAR	314.4	273.44	0.51
DE-CH	287	191.62	0.55
DE-BU	289	234	0.53
DE-WT	355.4	251	0.44
DM-PAR	235	164.94	0.48
DM-CH	311.1	225.62	0.47
DM-BU	393.5	251.07	0.57
DM-WT	404.6	394.52	0.5

DPPH Assay

It is widely used assay for the antioxidant activity assessment of fruits, vegetables, plants and food samples. The main advantage of using DPPH is that it remains unaffected by side chain reaction like metal chelation and enzyme inhibition. DPPH has the hydrogen donating capacity to the antioxidants and get stabilized. The DPPH have deep purple colour, after reacting with the reducing agents in plant DPPH gets stabilized. The reduction was monitored at 517 nm. The higher antioxidant showed by parent extract of *D. maximum* (235 µg/ ml) followed by DM-CH (311.1 µg/ml), DM- BU (393.5 µg/ml) and DM- WT (404.62 µg/ml) and lower in parent extract of *D. esculentum* (314.4 µg/ml) followed by DM-CH (287 µg/ml), DM-BU (289 µg/ ml) and DM- WT (355.4 µg/ml). The free radical scavenging is previously reported in *D. esculentum* was 1.73±0.38 mg/ ml (Gupta *et al*., 2014). The kinetics of parent solutions (DE-PAR and DM- PAR) has been compared and presented (Figure 2). Basically, DPPH reaction is kinetic driven process. The graph clearly shows that the scavenging activity of both the solutions of *D. esculentum* and *D. maximum*

Figure 2: Kinetic Behaviour of DPPH Assay



increases with increase in concentration and time. The activity of *D. maximum* was started at a lower concentration (< 0.1 µg/ml) as compared to *D. esculentum* (> 0.1 µg/ml). But parent solutions of both the ferns appeared to reach steady state saturation between 20 and 25 min.

ABTS Assay

$ABTS^+$ was generated by the reaction of potassium persulfate with ABTS. $ABTS^+$ have greenish blue colour but when reacted with reducing agents which were present in plant extract lead to discoloration of free radical. This particular reaction was monitored at 593 nm. The higher antioxidant activity was observed in parent extract of *D. maximum* (164.94 µg/ml) followed by DM-CH (225.62 µg/ml), DM-BU (251.07 µg/ml) and DM- WT (394.52 µg/ml) and lower in parent extract of *D. esculentum* (273.44 µg/ml) followed by DE-CH (191.6 µg/ml), DE-BU (234 µg/ml) and DE-WT (251 µg/ml). The antioxidant activity by ABTS method in *D. esculentum* reported as 0.03±0.03 mg/ml (Gupta *et al*., 2014).

FRAP Assay

Ability of extracts of *D. maximum* and *D. esculentum* to reduce the ferric ions was determined using FRAP assay. The reduction of ferric ions to ferrous was observed at 593 nm. The presence of reducing agents in the extracts helps leads to reduction of ferric ions to ferrous. The results demonstrate that the parent extract of *D. maximum* showed higher antioxidant activity 0.48 mg of ascorbic acid/g of dry plant sample followed by DM-CH, 0.47; DM-BU, 0.57 and DM-WT, 0.50 mg of ascorbic acid/g of dry plant sample, respectively. The parent extract of *D. esculentum* showed 0.51 mg of ascorbic acid/g of dry plant sample followed by DE-CH, 0.55; DE-BU, 0.53 and DE-WT, 0.44 mg of ascorbic acid/g of dry plant sample, respectively. Although in previous report, FRAP method showed 2.12 ± 0.008 µg ascorbic acid equivalents/mg in *D. Esculentum* (Gupta *et al*., 2014).

DISCUSSION

D. esculentum and *D. maximum* are the edible ferns of western Himalaya. We have isolated lutein from *D. esculentum* by using semi-preprative HPLC. For the first time lutein is isolated from the ferns and characterised by using LC-ESI-MS and NMR spectroscopy. In addition, for quantification of lutein in both *Diplazium* species, a new UPLC-DAD quantification method was developed. The developed UPLC method is simple and sensitive for the lutein quantification in two ferns.

Method validation and quantification of lutein results showed that developed analytical method was reproducible and have recovery value well within the acceptable limits. Overall quatification and validation of lutein showed the good quality, reliability and consistency of method. The importance of lutein has been well reported for food and pharmaceutical industries (Johnson, 2004). There is very limited scientific data related both these edible species. Therefore, we have studied the nutritional profile and antioxidant activity of both ferns. Firstly we have studied the nutritional profile by determining the protein and carbohydrate contents. Their results showed that *D. esculentum* having higher carbohydrate contents than *D. maximum*. For the first time nutritional value of *D. maximum* has been evaluated. Phenolic and flavonoid content were evaluated for both ferns using spectrophotometeric methods. The results showed that the higher TPC is found in *D. maximum*. However, there is no report regarding the TPC and TFC of *D. maximum* species present in literature. Earlier reports on TPC and TFC of *D. esculentum* showed higher content (Roy *et al*., 2013; and Tongco *et al*., 2014). Results from present study showed lower content in *D. esculentum*. This wide variation of phenolic content as compared to earlier reports might arise due to place of cultivation, climatic condition, fertilization, sample collection, sample preparation and method of analysis (Rop *et al*., 2011). Higher amount of TFC is found in *D. maximum* then *D. esculentum*. The higher content of flavonoid as compared to phenolic was estimated might be due to the extraction with methanol not with aqueous methanol.

D. maximum showed higher antioxidant actvity as compare to *D. esculentum*. The results of this study demonstrates that *D. maximum* showed slightly higher phenolic and flavonoid contents as well as antioxidant potential as compared to *D. esculentum*. The behaviour of kinetics of DPPH assay suggests that antioxidant activity of *D. maximum* started at a lower concentration as compared to *D. esculentum*. However the present comparetive study showed that *D. esculentum* and *D. maximum* have slight difference in their nutritional potential, phenolic content and flavonoid contents.

CONCLUSION

A simple and sensitive UPLC-DAD method has been developed for the lutein quantification in two ferns. The quantification of lutein showed *D. maximum* has higher concentration as compared to *D. esculentum* which has been well known for its uses. The present research showed that there is no much difference in nutritional potential, phenolic content and flavonoid content of both pteridophytes. The results of this study demonstrates that *D. maximum* showed slightly higher phenolic and flavonoid contents as well as antioxidant potential as compared to *D. esculentum*. The behaviour of kinetics of DPPH assay suggests that antioxidant activity of *D. maximum* started at a lower concentration as compared to *D. esculentum*. Therefore, all results of this study suggest that *D. maximum* could be nutritious food and may be used as natural antioxidant by the food industries to reduce oxidative induced diseases.

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