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IMPACT OF DIFFERENT SOLVENTS ON PHYTOCONSTITUENTS, ANTIOXIDANT AND FTIR ANALYSIS OF *DIPLAZIUM ESCULENTUM* LEAF EXTRACT FROM HIMALAYAN REGION

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Abstract

Diplazium esculentum (Family - Athyriaceae), commonly known as 'lingad' in Himachal Pradesh, India is agreen vegetable and is considered to be a valuable medicinal plant of India. This study was conducted to investigate the total phenolic, flavonoid, antioxidant potential and FTIR analysis of *Diplazium esculentum* with four different solvents *i.e.* ethanol, methanol, chloroform and aqueous. Among the solvents tested, methanol was reported as excellent extractive solvent with the highestflavonoid and total phenolic content *i.e.* 45.3 mg QE g^{-1} DE and 73.7 mg GAE g^{-1} DE respectively. Methanol also showed the best antioxidant activity in both DPPH and ABTS assay *i.e.* IC_{50} values 80.20µg/mL and 73.38µg/mL. The FTIR spectroscopic studies revealed different characteristic peak values with various functional compounds in the extracts. The current study suggested that the methanol extract has strong activity, which can be used in functional foods and pharmaceutical industries.

Key words: *Diplazium esculentum,* antioxidant, phytochemicals, total phenolic and flavonoid content.

Introduction

Medicinal plants contain certain components of the rapeutic value and have been used as remedies for human diseases and other organisms from past centuries. For medicinal purpose more than 35,000 plant species are being utilized by various human cultures around the world (Janakiraman *et al*., 2012). Long before the establishment of human habitat, people lived in forests, relying on its resources for food, clothing, shelter and medicines. Much of the medicinal use of plants seems to be developed through observation of wild animals and trial and error (Yaniv *et al.,* 2014). Recently, WHO estimated that 80% of world's population relies exclusively on traditional medicine for their primary health care needs. According to WHO, around 21,000 medicinal plants species (Maryam *et al.,* 2012). Successful treatment of various ailments using numerous medicinal plants have been reported, leading to mass screening for their therapeutic components. Now, the search for natural phyto compounds rich in antimicrobial and antioxidant properties areincreasing because of medicinal importance in treatment and control of many diseases (Halliwell *et al.,* 1996). *Diplazium esculentum* is an edible fern distributed throughout Asia and Oceania that grows wild on the bank of streams and rivers. It is known as paco in the Philippines, linguda in northern India referring to the curled fronds and dhekishak in the northern region of West Bengal, India (Archana *et al*., 2012). It possesses many medicinal activities. Phenols, triterpenoids, Steroids, flavonoids and flavones are present in this plant. The leaves of plant aremostly consumed as vegetables and the dried rhizomes used as insecticides. The decoction is used for cure of hemoptysis and cough. The vegetable shoots possess antioxidant activity. An investigation has shown anti-anaphylactic activity by reducing mast cell degranulation (Bishwadeep *et al.,* 2012). Another study shows effect of *D. esculentum* on Central Nervous System, an excellent stimulant effect due to increased loco-motor activity using aqueous leaf extract (Atul *et al*., 2012).

Therefore, this study aims to determine the effects of aqueous and other organic solvents on the extraction yield, phyto constituents and antioxidant potential of *D.*

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esculentum leaves. This will help in determining its medicinal value, which may be useful in increasing the database for the medicinal plant or could be used as an antioxidant in food, pharmaceutical and medicinal preparations.

Materials and Methods

D. esculentum was collected in February, 2020 from Totu area of Shimla district, Himachal Pradesh, India. The sample was identified and submitted in Herbarium at the School of Biological and Environmental Sciences, Shoolini University, Solan, India.

Morphological evaluation

In the morphological evaluation, various organoleptic characters such as odor, colour, shape, fracture, texture, taste and size were determined by (Gouri *et al.,* 2017).

Preparation of Extract

Fresh leaf of *D. esculentum* was washed and dried in poly house for 24 hours. The dried sample was powdered and used for extraction. Four solvents namely ethanol, methanol, distilled water and chloroform were used for extract preparation. 5gm of sample was mixed with 100mL of each solvent to make extract followed by incubation in shaker at 37°C for 48 hours. The solvents were then evaporated using water bath at 40°C to obtain plant extract (Jianguo *et al.,* 2013).

Extraction Yield

The percentage yield of the extraction was calculated using formula: $(V_1/V_2) \times 100\%$, where V_1 is the weight of extract after evaporation of solvent and V_2 is the dry weight of plant sample.

Qualitative Phytochemical Analysis

Phytochemical tests were done to identify the presence of chemical constituents in the extracts of *D. esculentum* using standard protocol (Jovale *et al.,* 2014). These include test for alkaloids, carbohydrates, flavonoids, phenols, saponins and proteins.

i. Mayer's test: Extracts were dissolved in dilute hydrochloric acid and washed separately. 2-3 drops of Mayer (Potassium Mercuric Iodide) reagent were added to the filtrate. The presence of alkaloids was indicated by the development of a yellow color precipitate.

ii. Benedict's test: Extracts were treated and heated gently with 2-3 drops of Benedict's reagent. Development of yellow/green/red coloured precipitates showed the presence of carbohydrates.

iii. Lead acetate test: Few drops of lead acetate solution were added in the plant extract. Yellow color precipitate was formed which indicates the presence of flavonoids.

iv. Ferric chloride test: Few drops of ferric chloride solution were mixed with extract (10mg). Bluish black colour was formed which indicates the presence of phenol.

v. Froth test: 10 mL of methanol was dissolved in extracts (100mg) for making stock solutions. Added 20mL of distilled water to stock solutions to dilute stock solutions to 0.5mg/mL. The prepared solution was then shaken for 15 mins. The presence of saponin was indicated by the foam on the top of the test tubes.

vi. Millon's test: 3-4 drops of Millon's reagent were added to the extracts and heated. The presence of amino acids revealed the existence of white precipitate that changed to brick red on heating.

Total Phenolic Content

TPC was evaluated by a Folin-Ciocalteu assay as described by Roy, (2013) with slight modification. 1mg plant extract was mixed with 1mL of ethanol, methanol, distilled water and chloroform in different eppendorf. Different concentration (25µL, 50µL, 75µL and 100µL) from each sample of different solvent was diluted using distilled water to make volume of 1mL. 1.25mL folin ciocalteu reagent (1:10) and 1mL Na_2CO_3(7.5%) was added in each sample. The sample was then incubated at for 90 minutes and measured the absorbance at 765nm. All tests were performed in triplicates. The phenolic content was determined using gallic acid as a standard. The phenolic content values were expressed as mg of gallic acid equivalent per gram of dry extract (mg GAE g^{-1} DE).

Total Flavonoid Content

TFC was evaluated by the aluminum chloride method as described by (Das *et al*., 2013). 1mg plant extract was mixed with 1ml of ethanol, methanol, distilled water and chloroform in different eppendorf. Different concentration (25µL, 50µL, 75µL and 100µL) from each sample of different solvent was diluted with 95% ethanol to make volume of 1mL. 150µL of 5% $NaNO_2$ was added in each sample and incubated for 15 minutes at room temperature. After 15 minutes, the solution was treated with 150µL of 10% $AlCl_3$. Then, 1mL of 1M NaOH is added to the solution. Finally, solution was diluted by adding 200µL distilled water and incubated for 30 minutes at 37°C. Then, measured the absorbance at 510nm. All tests were performed in triplicates. The total flavonoid content was shown as mg of Quercetin equivalent per

gram of dry extract (mg QE g^{-1} DE).

Antioxidant Activity

• DPPH Assay:

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging was calculated by standard protocol (Saleha *et al*., 2014). First, DPPH solution was prepared, dissolving 2.4mg DPPH in 100ml methanol in dark condition. Sample was prepared in different solvent by dissolving 1mg extract in each solvent. Different concentration (25µL, 50µL, 75µL and 100µL) from each sample of different solvent was diluted with methanol to make volume of 1ml. 100µL of each extract sample was transferred to different test tubes and 900µL of DPPH solution was dissolved, followed by the incubation of reaction mixture for 30 minutes at room temperature in dark room. The absorbance of control and sample was measured at 517nm against blank. In relation to the control, optical density of DPPH on addition of test samples was in decreasing order which was percentage inhibition of DPPH radical, used to calculate the antioxidant activity. The capability of scavenging DPPH radical was calculated using the following equation:

$$\text{\% DPPH radical scavenging activity} = \frac{\text{A (Control) - A (Sample)}}{\text{A (Control)}} \times 100$$

Where

A (control) – Absorbance of control and

A (sample) – Absorbance of the test/standard

The free radical scavenging activity was expressed as IC_{50} value, which represented the inhibitory concentration of extract/standard required to scavenge 50% of free radicals.

ABTS Assay

ABTS was dissolvedto a concentration of 7 mmol/ Lin distilled water. By reacting ABTS stock solution with 2.45 mmol/L of potassium persulfate, ABTS radical cation ($ABTS^{+}$) was produced. Mixture was allowed to stand in dark at the room temperature for 12-16 h, before use. and results were compared with ascorbic acid as standard. Then, the reading of absorbance was measured at 734 nm after exactly 6 min. The percent scavenging activity was determined by the percent inhibition, calculated by the following formula:

$$\text{Cavenging effect (\%)} = \frac{A_o - A_s}{A_o} \times 100$$

Where, A_o is the absorbance of blank and A_s is the absorbance of sample (Dhanani *et al*., 2013).

FTIR Analysis

Table 1: Morphological characteristics of *D. esculentum* leaf.

Features	Observation
Color	Dark green
Odour	No characteristic odour
Shape	Lanceolate
Fracture	Smooth
Texture	Herbaceous
Taste	crisp, palatable
Size	20-50cm

Fourier transform infrared (FTIR) spectroscopy is an instrument used in the chemical investigation of substances. It is based on the measure of the vibration of a molecule by IR radiations at specific range of wavelengths within chemical functional groups and generates a biochemical spectrum of the sample. FTIR spectra were recorded for the sample in the middle IR region (4000-4000 cm^{-1}) using an avatar-330 FTIR instrument (Davis and Mauer, 2010; Ekpenyong, 2012).

Statistical Analysis

Total phenolic, total flavonoid content and values of IC_{50} were determined by using linear regression analysis. In triplicates, each sample was evaluated independently and the results were expressed as mean value ± standard deviation (n=3).

Results and Discussion

Morphological Features

The leaf of *D. esculentum* were 20-50 cm long, dark green in color, lanceolate in shaped, no characteristic odour, smooth fracture and crisp and palatable taste (Fig. 1 and Table 1).

Yield of Extraction

The extracts from the dried leaves of the plant were made by using different solvents. Aqueous and other

Fig. 1: *Diplazium esculentum* leaf.

Table 2: Total yield of different extracts.

Extract type	Yield (in %)
Ethanol	7.9 ± 0.86
Methanol	12.6 ± 0.70
Chloroform	3.4 ± 0.55
Aqueous	14.2 ± 0.45

organic solvents were studied for their effects on the extraction yield of *D. esculentum*. Results showed a significant difference in the extraction yield using different solvents (Fig. 2). Among solvents tested, aqueous extract resulted in the highest extraction yield (14.2%), followed by methanol (12.6%), ethanol (7.9%) and chloroform (3.4%). The weight and percentage compositions are shown in table 2. Saleha *et al.,* 2014 reported that the chloroform extract yield of plant was 12% and methanol was 18%, which is much higher than the present study.

Phytochemical screening

The preliminary phytochemical screening tests might be helpful in the identification of the pharmacologically bioactive components in the plant material and subsequently lead to discovery and development of drugs

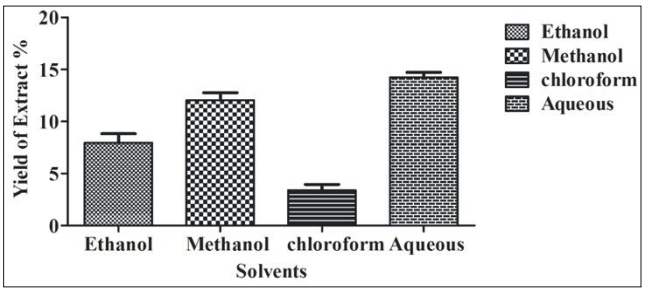

Fig. 2: Effect of different solvents on extract yield.

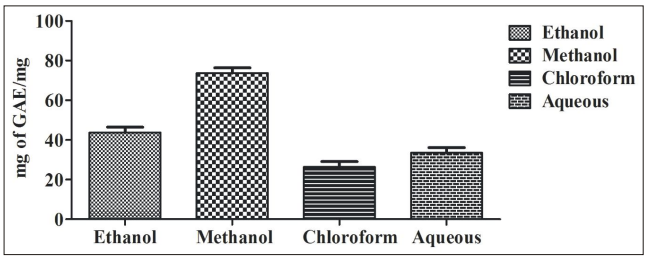

Fig. 3: TPC in different extracts of *Diplazium esculentum* leaves.

Table 3: Phytochemicals analysis in different extracts of *Diplazium esculentum.*

Phytochemical Tests	Test Name	Eth-anol	Meth-anol	Chlor-oform	Aqu-eous
Alkaloids	Mayer's test	-ve	+ve	-ve	-ve
Carbohydates	Benedict's test	+ve	+ve	-ve	+ve
Flavanoids	Lead acetate test	+ve	+ve	+ve	-ve
Phenol	Ferric chloride test	+ve	+ve	+ve	+ve
Saponins	Froth test	+ve	-ve	+ve	+ve
Proteins	Millon's test	+ve	+ve	+ve	+ve
+ve = present; -ve = not detected					

Table 4: TPC of different extracts of *Diplazium esculentum* leave.

Extract	Phenolic content (mg GAE g^{-1} DE)
Ethanol	43.7 ± 2.6
Methanol	73.7 ± 2.6
Chloroform	26.4 ± 2.7
Aqueous	33.5 ± 2.5

(Bhandary *et al*., 2012). The phytochemical screening test results showed the presence of alkaloids, flavonoids, phenols, saponins, carbohydrates and proteins (Table 3). The presence of these secondary metabolites in *D. esculentum* extractsare in agreement with the previous reports (Jovale *et al.,* 2014).

Large amounts of both primary and secondary metabolites were observed in most of the extracts. However, alkaloids were present only in methanol extract, carbohydrates were absent in chloroform extract, flavonoids were absent in aqueous extract and saponins were absent in methanol extract. Similar findings were also reported by (Julfikar *et al.,* 2015).

Total Phenolic and Flavonoid Content

Total phenolic content was calculated from the standard curve of gallic acid using the equation: y= 0.741x+0.1326, while total flavonoid content was calculated using standard curve of quercetin using the equation: y=0.965x+0.0362 contents.

Total phenolic content obtained was in the range of 26.4 to 73.7 mg GAE g^{-1}. The highest phenolic content was achieved by methanol extract (73.7 mg GAE g^{-1} DE), followed by ethanol (43.7 mg GAE g^{-1} DE) and aqueous extract (33.5 mg GAE g^{-1} DE). The lowest phenolic content was obtained from chloroform extract (26.4 mg GAE g^{-1} DE) (Fig. 3 and Table 4). These findings are in good agreement with the previous study, which reported methanol as an effective solvent for extraction of antioxidant and phenolic compounds (Das *et al.,* 2013).

Total flavonoid content was in the range of 11.7 to 45.3 mg QE g^{-1}. The highest flavonoid content was found in methanol extract (45.3 mg QE g^{-1} DE), followed by ethanol (23.4 mg QE g^{-1} DE), chloroform (12.7 mg QE g^{-1} DE) and aqueous extract (11.7 mg QE g^{-1} DE) (Fig. 4 and Table 5). Das *et al.,* 2013 reported that ethanolic extract of *D. esculentum* contained 151.90±5.01 mg/gm and aqueous extract contained 131.42±3.7 mg/gm phenolic content. While ethanolic and aqueous extract contained 67±0.28 mg/gm and

Table 5: TFC of different extract of *Diplazium esculentum* leaves.

Extract	Flavonoid content(mg QE g^{-1} DE)
Ethanol	23.4 ± 1.5
Methanol	45.3 ± 2.7
Chloroform	12.7 ± 1.7
Aqueous	11.7 ± 1.9

Values represent mean ± SD of triplicates; TPC = total phenolic content; TFC = total flavonoid content; mg GAE g^{-1} DE: mg gallic acid equivalents per gram of dry extract of the sample; mg QE g^{-1} DE: mg quercetin equivalents per gram of dry extract of the sample.

Table 6: IC_{50} values of different extracts of *Diplazium esculentum* leaves (DPPH).

Extract	DPPH radical IC_{50} (µg mL^{-1})
Ethanol	135.2
Methanol	80.20
Chloroform	138.2
Aqueous	184.6
Ascorbic acid	22.53

Table 7: IC_{50} values of different extracts of *Diplazium esculentum* leaves (ABTS).

Extract	ABTS radical IC_{50} (µg mL^{-1})
Ethanol	94.8
Methanol	73.38
Chloroform	118.8
Aqueous	141.4
Ascorbic acid	28.3

Each value represents the mean ± SD of triplicates.
IC_{50} = half-maximal inhibitory concentration.

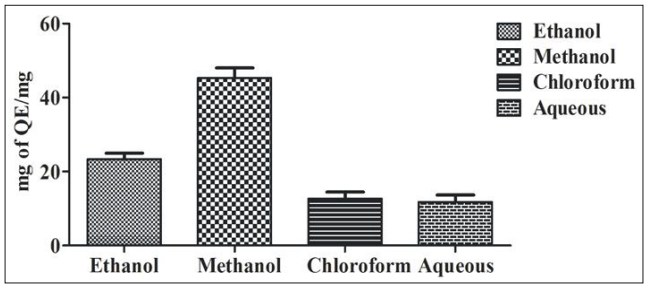

Fig. 4: Total flavonoid content in different extracts of *Diplazium esculentum* leaves.

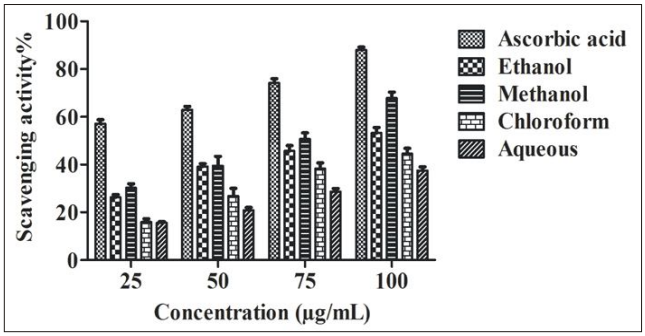

Fig. 5: DPPH assay of different extracts of *Diplazium esculentum* leaves.

64.02±0.56 mg/gm flavonoid content respectively.

Tapan *et al.,* 2012 reported that the phenolic content varied from 3.31±0.10 to 27.67±0.16 mg/g in the aqueous methanol extract of *D. esculentum* and flavonoid content varied from 8.11±0.071 to 52.14±0.004 mg/g in the same solvent system.

Antioxidant Activity

All the extracts of *D. esculentum* exhibited good antioxidant properties, which varied with the type of solvents. The best activity for DPPH assay was observed in methanol extract (IC_{50} = 80.20 µg mL^{-1}), followed by ethanol (IC_{50} = 135.2 µg mL^{-1}) and chloroform extract (IC_{50} = 138.2 µg mL^{-1}) (Fig. 5 and Table 6).

In case of ABTS assay the best activity was observed in methanol extract (IC_{50} = 73.38 µg mL^{-1}), followed by ethanol (IC_{50} = 94.81 µg mL^{-1}) and chloroform extract (IC_{50} = 118.8 µg mL^{-1}). The lowest activity was observed in aqueous extract with IC_{50}value of 184.6 and 141.4 mg mL^{-1} for DPPH and ABTS assay. Ascorbic acid was used as a standard, which exhibited an IC_{50}value of 22.53 and 28.30 µg mL^{-1} for DPPH and ABTS assay, respectively (Fig. 6 and Table 7).

Our results showed that there is a significant correlation between phytochemical and antioxidant assays could be attributed to the different mechanism of the radical antioxidant reaction. (Das *et al.,* 2013) have also reported statistical correlation between the TPC and IC_{50} values, which confirmed that phenolic content contributes to the free radical scavenging activity of the plant metabolites. Das *et al.,* 2013 reported that the ABTS scavenging activity of ethanolic and aqueous extract of *D. esculentum* was 57.84% and 52.29% respectively. Juneho *et al.,* 2018 reported that the DPPH scavenging activity of *D. esculentum* was 32.81 ± 0.42% at 50 mg/mL and 89.73 ± 0.21% at 250 mg/mL.

FTIR Analysis

The FTIR spectrum can be used to identify the

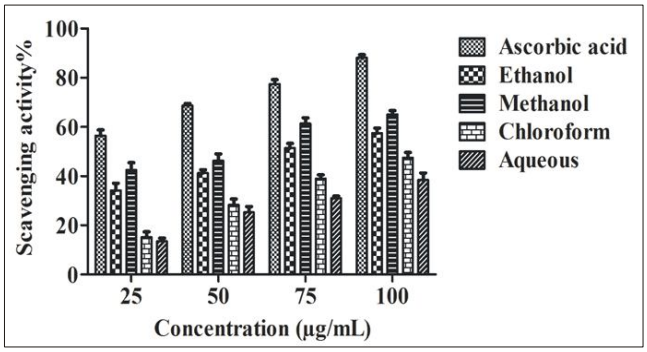

Fig. 6: ABTS assay of different extracts of *Diplazium esculentum* leaves.

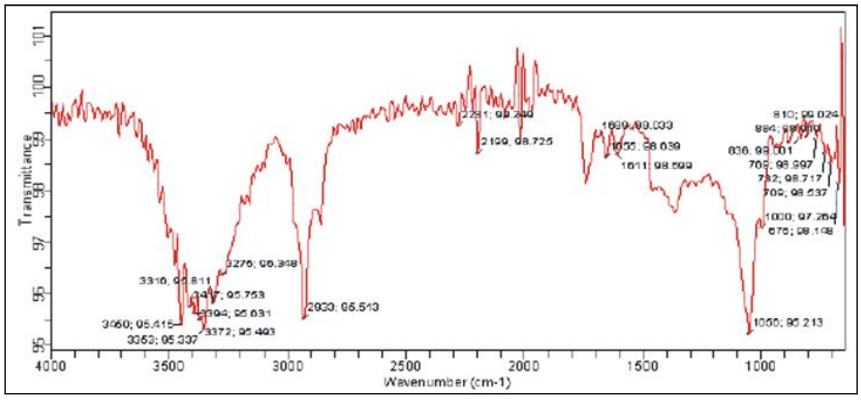

Fig. 7. FTIR peak of *Diplazium esculentum* (ethanol) leaf extract.

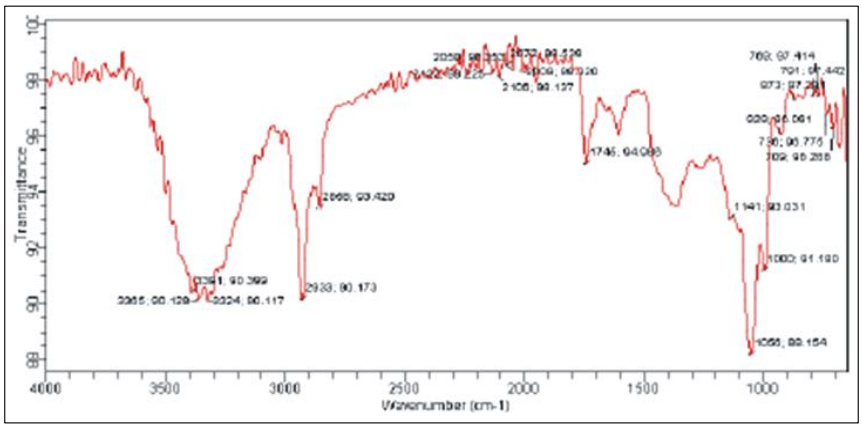

Fig. 8. FTIR peak of *Diplazium esculentum* (methanol) leaf extract.

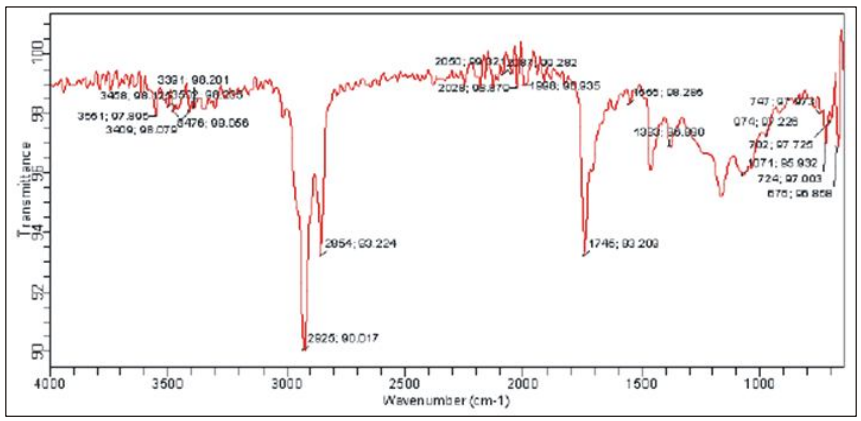

Fig. 9: FTIR peak of *Diplazium esculentum* (chloroform) leaf extract.

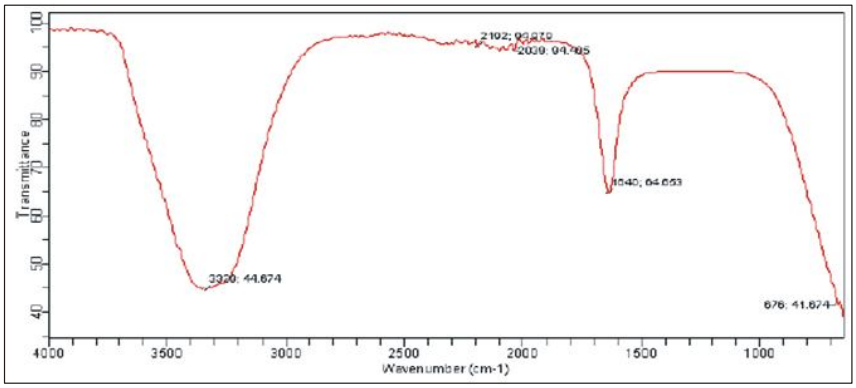

Fig. 10: FTIR peak of *Diplazium esculentum* (aqueous) leaf extract.

functional groups of the active components based on the absorption band values in the region of infrared radiations. IR radiation is passed through a sample. Some infrared radiations are absorbed by the sample and remaining are transmitted through it. The resulting spectrum shows the transmission andmolecular absorption by generating a molecular fingerprint of the sample. In the present, FTIR analysis was done to identify different functional groups present in *D. esculentum* extract made in different solvent.

Linda *et al.,* 2020 reported a broad peak at 3399 cm^{-1}, indicating the presence of hydroxyl group (OH). The vibrational band of alkyl (CH) and C = C group appeared at 2918 and 1658 cm^{-1}, respectively. Upon the adsorption, several new peaks at 1587 cm^{-1} (C = C stretch of benzene ring), 1366 cm^{-1} (C–N stretch of aromatic tertiary amine) and 1170 cm^{-1} (C–N vibration) were also observed.

In fig. 7 ethanol extract showed the broad intensity band at 3310 and 2281 cm^{-1} which is due to N-H and N=C=O stretching vibration of secondary amine and isocyanate respectively. C=O stretching at 1690cm^{-1} and CO-O-CO stretching at 1050 cm^{-1} determined the presence of primary amide and anhydride respectively.

In fig. 8 methanol extract, the highest peak was shown at 2224 and 2100 cm^{-1} which is due to the presence of nitrile (CΞN) and alkyne (CΞC) functional group. Lower spectra were shown at 1745 and 1141 cm^{-1} due to cyclopentanone (C=O) and aliphatic ether (C-O) respectively.

In fig. 9 Chloroformextract showed the maximum spectra at 3501 and 2954 due to N-H stretching of amine salts. N=C=S stretching at 2050 cm^{-1} and C=O stretching at 1745cm^{-1} due to isothiocyanate and esters respectively.

In fig. 10 aqueous extract, maximum spectrum was at 3320 cm^{-1} due to N-H stretching that showed the presence of

secondary amine. Strong N=C=S stretching appeared at 2102 and 2050 cm^{-1} that showed the presence of isothiocyanade functional group. Nitro compound (N-O) and halo compound (C-Br) at 1540 and 676 cm^{-1} frequency were present.

Conclusion

This study was carried out to test the medicinal profile of the *D. esculentum* byextracting secondary metabolites with aqueous and organic solvents. On the basis of theresultsobtained in the present study, it can be concluded that methanolic extract of *D.esculentum* contained a highest amount of phenolic and flavonoid compounds ascompared to other solvents and also exhibited best free radicalscavenging activity. From the results, it was concluded that this plant could be useful in the future for drug development for treating or preventing diseases.

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