HIMALAYAN FERNS (DIPLAZIUM ESCULENTUM (RETZ.) SW): EVALUATION OF ANTIOXIDANTS, ANTIMICROBIAL, ANTI-INFLAMMATORY, AND CYTOTOXIC POTENTIAL, AND ITS PHYTOCHEMICAL CORRELATION.

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Abstract

Diplazium esculentum is an edible fern found in Himalayas. Its fronds are traditionally used in headache, pain, fever, wounds, dysentery, glandular swellings, diarrhoea and various skin infections. This study investigated the antioxidant, anti-inflammatory, antimicrobial and cytotoxic properties of the D. *esculentum* young fronds using different in vitro experimental models and detection of phytochemicals present. The results of the present study suggest that the leaf of *D. esculentum* possess significant cytotoxic, antimicrobial and antioxidant properties and recommend its use as a chemoprotective dietary supplement, however further in-depth studies needed to elucidate its mode of action as well as its toxicity profile.

Keywords: Antioxidant, Antimicrobial, Cytotoxic, *Diplazium esculentum*, Fern, Phytochemical.

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1. INTRODUCTION

Ferns are one of the planet's most extensively utilized wild edible plant categories. Ferns may be used to make food, medicine, fiber, crafts and construction materials, abrasives, and decorations. Ferns are a primitive vascular plant category that may be found all over the planet. Many studies in ethnobotany have been conducted since the therapeutic properties of various ferns in India were fully characterized (Sen & Ghosh, 2011). *Diplazium esculentum* (vegetable fern or Himalayan fern) (Family-*Athyriaceae*) is an edible fern, pan-tropical in distribution and occurs widely and commonly throughout India, China, Cambodia, Laos, Thailand, Vietnam, and Malaysia. [1]

In India, people use the boiled young fronds of *D. esculentum* as vegetables with boiled rice for laxative purposes to treat colitis and constipation. It is used for wound healing and as an ailment of headache in Indonesia. One of the most important properties of *D. esculentum* investigated so far is the acetylcholinesterase inhibitory activity, which can explain the neuroimmune function of this plant. [2, 3]

Bioactivities of some traditional medicinal ferns were analyzed, and they were reported to have various properties, such as antioxidant, antimicrobial, antiviral, anti-inflammatory, antitumor, and anti- HIV. [4]

Currently, intensive research is being conducted to discover the existence of phytochemicals and nutrients in foods and to comprehend the advantages of these compounds to human health via diet. Many recent papers have focused on the dietary consumption of phenolic compounds, which are advantageous to humans because they serve as antioxidants, anti-inflammatory, anticancer, and antibacterial agents. Thus, information on food composition is required to evaluate diet quality and to design and implement food-based dietary recommendations, making public health nutrition a helpful tool. [5] The current research aims to analyze the nutritional aspects and phytochemicals of *Diplazium esculentum*.

2. MATERIAL AND METHODS

Collection And Identification of the Plant
young frond portion of the plant (*D. esculentum)* were collected from different areas of the North Bengal University campus and the adjoining regions of Darjeeling, West Bengal, India. These were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal, and three voucher specimens (Accession No. 10401, 10402, and 10403) were submitted to his herbarium center.

Preparation of Crude Extract:
Raw aqueous preparations of the plant material (Crude *D. esculentum*; CDE) was prepared using young fronds of *D. esculentum*. Fronds were washed carefully with tap water, cut into small pieces, and finely mixed with a mixer. The slurry was mixed in a round-bottom flask and concentrated under reduced pressure in a rotary evaporator. The concentrated extract was then lyophilized. The residue was transferred to a pre-weighed sample container for storage kept at –20°C for future use. [6]

Preparation of the methanolic plant extract:
Samples were prepared according to a previously described method (Hazra et al., 2008). Briefly, CDE was mixed with 500 ml methanol: water in a ratio 7:3 using a shaker for 15 h; then, the mixture was centrifuged at 2850× g, and the supernatant was decanted. The pellet was mixed again with 500 ml methanol-water, and the entire process was repeated, i.e., the extraction procedure was done twice. Double-distilled water (Milli-Q grade) was used as the solvent for the lyophilized extract in all the experiments. This preparation (MDE) has been used in most in vitro experiments. [7]

Phytochemical analysis of *D. esculentum* (MDE):
Qualitative analysis of phytochemicals was carried out for *D. esculentum* to identify the presence of different phytoconstituents. The MDE was subjected to qualitative tests as per the standard procedure to identify various phytochemical constituents. [8]

Assessment of the Antioxidant potential of *D. esculentum* MDE was used to assess the antioxidant and free radical scavenging activities by DDPH and FRAP assay. [9]

DPPH Radical Scavenging Activity: The DPPH scavenging effect was assayed in the methanol extract of *D. esculentum* (MDE) by UV-Vis spectroscopy. To a methanolic solution of DPPH (20 µm), 0.05 ml of the test compound dissolved in ethanol was added at different concentrations (100 - 500µg). An equal amount of ethanol was added to the control. After twenty minutes, IC_{50} values of the MDE and standard ascorbic acid were calculated at 517 nm, and IC_{50} was determined. [9]

Ferric Reducing Antioxidant Power (FRAP) Assay: The FRAP procedure described by Benzie and Strain (1996) was followed in the methanol extract of fern. The FRAP reagent contained 5 ml of a (10 $mmolL^{-1}$) TPTZ (2, 4,6-tripyridyl- S- triazine) solution in 40 $mmolL^{-1}$ H, HCL, and 5 ml of $FeCl_3$ (20 $mmolL^{-1}$) and 50 ml of acetate buffer, (0.3 $molL^{-1}$, pH 3.6) and was prepared freshly and warmed at 37°C. The sample extracts (0.5-2.5 µ/ml) were mixed with 3 ml of FRAP reagent, and the absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for ten minutes. The IC_{50} values of the methanol extract of D. esculentum and standard ascorbic acid were calculated. [10]

Antimicrobial Analysis [11]

Test Microorganisms: Selected bacterial strains were *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeuriginosa*. *B. subtilis* and *S aureus* are Gram +ive bacteria, while *E coli* and *P. aeuriginosa* are Gram -ive bacteria.

Media preparation for antimicrobial assay: Nutrient agar medium was used for the antibacterial study. It was prepared by adding 9.35 g nutrient agar and 3g agar in 300 ml distilled water taken in a 500 ml beaker. Then it was boiled and cooled. Media was autoclaved at 121 °C for 15 minutes, poured into sterile petri dishes under aseptic condition, and stored.

Antimicrobial study: The disc diffusion method was used for the antimicrobial study. The different concentrations (0.5, 1, and 2 ml/10 disc) of methanol extract of *D. esculentum* (MDE) were loaded on 5 mm sterile individual discs. The loaded discs were placed on the medium's surface, the extract was allowed to diffuse for five minutes, and the plates were kept for incubation at 37°C for twenty-four hours. Negative control was prepared using the respective solvent. For evaluation of antibacterial activity, Gentamycin was used as standard.

Cytotoxic studies [12]

Cell lines: HCT116 were obtained from MCOPS, Mangalore, India.

Culture Media: The culture media for HCT116 (colorectal carcinoma cells; ATCC CCL247) cell lines were prepared by supplementing high glucose containing 'Dulbecco's modified eagle's medium (DMEM-high glucose)' (Hi-Media, Mumbai, India) with 10% (v/v) fetal bovine serum (FBS; Hi-Media, Mumbai, India) and 100 IU/mL Antibiotic antimycotic solution (100X liquid): (A002, Hi-Media, Mumbai, India). Cells were maintained and cultured in a 5% CO_2 in a humidified atmosphere at 37 °C. [12]

In-vitro Cell Viability Study: Cell viability was determined by the Trypan Blue Exclusion Test. Briefly, cells were treated for 48 hrs and collected in the exponential phase. 50µL of the sample was mixed with 50µL of 0.4% trypan blue (TC193, Hi-Media, Mumbai, India) by gently pipetting, and then 20µL of the mix was loaded into each chamber of the hemacytometer. Counts were performed in triplicate. [13]

MTT Assay: HCT116 cell suspensions were dispensed (100 µL) in triplicate into 96-well culture plates at optimized 1.5 X 10^5 cells/ml concentrations. After a 24-hr recovery period, the cisplatin standard or HIME was diluted with distilled water were added. Seven dilutions of HIME were tested (100, 50, 25, 12.5, 6.25, 3.125, and 1.5 µg/ml), and to control wells, only culture medium (100 µl) was added, followed by an incubation period of 48 h. Later, the medium in each well was aspirated and replaced with 20 µl of MTT working solution (MTT) stock solution mixed with medium to attain a final concentration of 0.5 mg/ml. MTT powder was dissolved in Dulbecco's PBS to form a stock solution of MTT (5 mg/ml). The cells were incubated at 37 °C for four h, and then the medium was aspirated and replaced with 100 µl DMSO to dissolve the formazan crystals formed. The culture plates were shaken

for 5 min, and the absorbance of each well was read at 490 nm with 655 nm as the reference wavelength. [14]

Evaluation of Albumin Denaturation Inhibitory Activity[15]

Protein denaturation assay was performed: Various concentrations of test samples were prepared at 100, 200, and 300 µg/mL, and each reaction mixture was prepared by mixing with 0.5 mL of 1.5 mg/mL bovine serum albumin (BSA) and incubating at 37 °C for 20 min. The reaction mixtures were then heated for 3 min at 57 °C. Phosphate buffer (0.5 M, pH 6.3) with a volume of 250 µL was added to each mixture, which was thoroughly mixed. Then, after equal distribution of extracts in each reaction mixture, 100 µL of each mixture was transferred into separate test tubes, and *Folin–Ciocalteu's* reagent was added in the same proportion by volume. After 10 min of incubation at 55 °C, the tubes were allowed to cool, and absorbance was determined at the wavelength of 650 nm using a Multimode Microplate Reader (Tecan Sunrise, USA). Diclofenac sodium (100 µg/mL) was used to evaluate the recorded measurements as a reference drug.

Statistical Analysis[16]: Percentage data were transformed to arcsine values, and the analysis of variance was carried out (ANOVA). Means were compared for significance using Duncan's Multiple Range test (DMRT; P= 0.05).

3. RESULTS AND DISCUSSION

Preliminary Phytochemical Analysis: The study results showed several secondary metabolites (**Table 1**). It was observed that the methanol extract of *D. esculentum* (MDE) contained a higher concentration of secondary metabolites[3] like Terpenoids, Saponins Flavonoids, Glycosides, Phytosterol, Tannins, which have already been reported to possess antioxidants as well as cytotoxic properties. [17]

Table 1 Qualitative phytochemical analysis of methanol extract of *D. esculentum* (MDE)

Phytochemical	Alkaloids	Flavonoids	Glycosides	Phytosterol	Saponins	Tannins	Terpenoids
MDE	++	+++	+	++	+	++	+++

Antioxidant potential methanol extract of *D. esculentum* (MDE)The reductive capabilities of the *D. esculentum* were compared to ascorbic acid as standard. The reducing power of the plant extract was 85.43 % at a concentration of 12.5ng/ml, whereas the reducing power of the standard ascorbic acid was only 58.3 %. IC_{50} value in methanol extract in *D. esculentum* {23.32 µg/ml) was more potent than ascorbic acid (39.41 µg/ml).

The methanolic extract of *D. esculentum* exhibited a higher FRAP radical scavenging activity of 98 % at a concentration of 200) µg /ml than ascorbic acid; hence, its scavenging potency is higher than standard. IC_{50} value of DME was 36.11 µg /ml, showing high antioxidant potentiality.

Antibacterial Activities: The antibacterial activities of the methanol extract of Diplazium esculentum in terms of the diameter of the inhibition zone are presented in Table 2. Five bacteria were used to evaluate the antibacterial activity of the methanol extract of D. esculentum. There was significant variation in the antibacterial activities with the diameter of the inhibition zone (DIZ values) in different concentrations of methanol extract.

Table 2: Antibacterial Activity of Methanol Extract of Diplazium esculentum (DME)

Test Organism	Diameter of Inhibition Zone in mm (DIZ)				
	DME			Control	Standard
	M_1 (2ml)	M_2(1ml)	M_3 (0.5ml)		Gentamicin
B. subtilis	2	1	-	-	30
E. coli	1	-	-	-	28
P. aeuriginosa	3	1	1	-	32
S aureus	2	1	-	-	48

Albumin denaturation inhibitory activity of DME: *Diplazium esculentum* extracts had given more than 50% inhibition. It was 70.58 ± 0.004% while, the non-steroidal anti-inflammatory drugs (NSAIDs) had 94.90 ± 0.004%. Results indicate that the fern could have a good anti-inflammatory potential due to presence of phenolics.

Cytotoxicity of DME on Colon Cancer Cell Lines: The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations, the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in-vitro cytotoxic effects of drugs on cell lines or primary patient cells.[12] In the present study, MTT assay showed that the incubation of cancer cells lines with methanol extract of *Diplazium esculentum* (DME) reduced the viability of cancer cells and the dead cells were significantly increased with extract concentration ($P<0.05$).Also, the extract of H. indicus exhibited high cytotoxicity of 60.4%.

4. CONCLUSIONS

In the present study, an attempt was made to elucidate the immunopathological, anti-oxidant, anti-microbial, anti-inflammatory and cytotoxic potential of the fern *Diplazium esculentum* young fronts and the extract showed a promising result. Furthermore, these effects are attributed to the presence of secondary metabolites most notably flavones and terpenes. More studies are need to elucidate the exact mechanism of action and also presence any anti-metabolites or poisons.

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