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ORIGINAL ARTICLE

In-vivo Toxicity Evaluation and Phytochemical, Physicochemical Analysis of *Diplazium esculentum (Retz.) Sw.* leaves a Traditionally used North-Eastern Indian Vegetable

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ABSTRACT
Diplazium esculentum (Family-Athyriaceae), commonly known as 'dhekia' in North-East India is a valuable indigenous medicinal plant and green vegetable of India.It is an excellent source of several food nutrients and phytocompounds. The World Health Organization (WHO) has emphasised the need to ensure quality and safety of herbal products using modern techniques. In context of our research endeavour, we have aimed to identify and ensure the quality, purity and safety of D. esculentum by carrying out phytochemical, physicochemical and toxicity studies of this indigenous medicinal plant. We aim to establish some standards which would help the future researchers in their upcoming research endeavours with D. esculentum. The present study intended with various phytochemical, physicochemical screening and toxicity studies were carried out on the leaves of the D. esculentum. Phytochemical evaluation of D. esculentum leaf extracts showed the presence of flavonoids, steroids, carbohydrates, glycosides, alkaloids, proteins and phenolic compounds. Physicochemical properties like total ash content, water-soluble ash content, acid-insoluble ash content and extractive value using various solvents, loss on drying of D. esculentum leaf extracts were determined. Acute oral toxicity study carried out for two weeks determined the highest dose of 5000 mg/kg body weight. The results of the various phytochemical, physicochemical and toxicity studies indicated that D. esculentum is rich in various biologically-active compounds which could serve as potential source of the crude drugs. The plant is also non-toxic to the experimental model, as determined by the toxicity studies.
***Keywords*:** *Diplazium esculentum*, Phytochemical, Physicochemical, Toxicity studies.

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INTRODUCTION

As per WHO estimates, traditional, complementary, alternative, or non-conventional medicines are used by 70–95% of global population particularly in developing countries for their health- care (WHO, 2011). Traditional medicines vastly depend on the usage of plants, compared to other natural resources. Medicinal plants have been used in traditional healing practices for treating various human ailments since time immemorial. Such traditional practices have provided the basis of scientific investigation on medicinal plants which led to the discovery of many potential drug molecules of modern medicine. Herbal medicine has therefore become the most reliable form of alternative medicine for treating human disorders around the world. In recent years, use of herbal drugs in the developed countries has increased greatly because of their easy availability and cost effectiveness, besides having desired pharmacological effectiveness with high safety level or low toxicity profile. It is estimated that world's one-fourth population is dependent on traditional herbal medicines for the treatment of various ailments s [1]. However, the lack of organized documentation and stringent quality control procedures has hindered the easy acceptance of such plant drugs to be used as herbal medicine. There is a need for the record of all the research work carried out on traditional medicines in the form of documentation. With this drawback, it

becomes extremely important to make surety about the standardization of the plant and parts of plant to be used as a medicine. For the process of standardization, we can use different techniques and methodology to achieve our goal in the stepwise manner e.g. physicochemical, phytochemical and toxicity studies. These steps and processes are helpful in identification and standardization of the plant material. Correct characterization and quality assurance of starting material is an essential step to ensure reproducible quality of herbal medicine which will help us to justify its safety and efficacy. For this purpose, we have done phytochemical and toxicity studies of *Diplazium esculentum*. In the present studies we have focus our investigations on one of the commonly available plant *Diplazium esculentum* [2].

Diplazium esculentum Retz. Sw. (Family: Athyriaceae) is the most commonly consumed fern in hill tribes of North-Eastern India along with Bangladesh and Philippines [3]. It is commonly known as *'dhekishak'* in north-east India and mostly found near river and swamp area. The genus Diplazium includes 400 known species; *D. esculentum* beingone of the most common variety [4, 5]. The young fronds, rich in iron, phosphorus, potassium and protein are stir-fried as a vegetable or used in salads [6].It is believed by the native tribes of India that the plant counteracts constipation and is used as an appetizer [7].

The aqueous and organic extracts of fresh and boiled samples of *D. esculentum* gave higher anti-oxidative activity using the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods, compared to that of α-tocopherol (Vitamin E), which served as positive control [8].Aqueous and alcoholic extracts of the plant showed activity against human and plant pathogenic bacteria like *Escherichia coli*, *Salmonella arizonae, Salmonella typhi*and, *Staphylococcus aureus*. The reference standard antibiotic used in the study was tetracycline. All extracts mixed in equal proportion with the antibiotic were more effective against the bacteria than the antibiotic alone [9].

In our present study, phytochemical, physicochemical screening and toxicity studies, including fluorescence and thin layer chromatographic analyses, were carried out with an objective to standardize the *D. esculentum* leaves as there is no report on phytochemical and safty standardization of the leaves of this plant till date.

Figure 1. Leaves of *Diplazium esculentum.*

MATERIALS AND METHODS

Pharmacognostic Studies:

Collection and extraction of the plant material

Fresh leaves of *Diplazium esculentum (Retz.) Sw.* were collected in March 2014 from Dibrugarh forest, Dibrugarh district, Assam, India. The plant species were identified and authenticated by Botanical Survey of India, Eastern Regional Centre, Shillong, India, and a voucher specimen (BSI/ERC/2014/Plant identification/360) was deposited.

Air-dried powdered material of previously collected plant, *Diplazium esculentum* was packed in a Soxhlet extractor and extracted successively with the following solvents: petroleum ether (60- 80°C), chloroform, ethyl acetate, methanol and water. Each time before extracting with the next solvent, the powdered material was air dried first and then oven dried below 50°C. Finally, the marc was macerated with chloroform water (ratio) for 24 hours to obtain the aqueous extract. Each extract was concentrated by distilling off the solvent (in a rotary vacuum evaporator) and then evaporated to dryness on the water bath.

Reagent and Chemicals

All chemicals used in the study were of analytical grade, manufactured by Rankem Fine Chemicals Limited (RFCL), Mumbai and Himedia Laboratories, Mumbai.

Organoleptic evaluation

Various sensory parameters of the plant material (such as colour, odour and taste) were studied by organoleptic evaluation.

Physicochemical analysis

Physicochemical constants of the powdered leaves were analyzed to evaluate the quality and purity of the drug. Various physicochemical parameters like moisture content (% LOD), total ash content, and acid-insoluble ash content and water-soluble ash content were determined according to the methods specified in the Indian Pharmacopoeia. Water-soluble and alcohol-soluble extractive values were determined by cold maceration method as per WHO guidelines. The information collected from these tests was useful for standardization and obtaining the quality standards.

Phytochemical screening

The extracts obtained were subjected to qualitative tests to identify various plant constituents. Tests for alkaloids, carbohydrates, glycosides, phenolic compounds, saponins, tannins were performed according to standard procedures to identify the phytoconstituents [10].

Fluorescence analysis

Fluorescence is an important phenomenon exhibited by various chemical constituents [11]. Fluorescence analysis of leaf powder was done according to the standard protocol using various reagents, acidic and basic solvents and observed under UV chamber.

Thin layer chromatographic (TLC) analysis

Thin layer chromatography (TLC) was done using HPTLC silica gel plates 10 x 10 cm (Merck, Germany). Chromatograms were obtained in the isocratic technique of development. The best mobile phase composition was established experimentally. 0.5 ml extracts were applied using 10 ml Hamilton syringe on a plate as spots of 3-4 mm zones and the corresponding R_f values were noted [12]

Toxicity Studies:

Acute Oral Toxicity Study

Acute oral toxicity test was performed as per OECD) guidelines 423. The animals were used with the approval of the Institutional Animal Ethics Committee (Approval No.IAEC/DU/50 Dated 24.09.2013, Registration No. 1576/Go/a/11/CPCSEA dated 17.02.2012) and the study was conducted following internationally accepted principles for laboratory animal use and care. Experiments were performed using healthy young adult wistar albino rats (both male and female), nulliparous, non-pregnant and weighing 150 to 250 gm [13].

RESULTS AND DISCUSSION

Organoleptic evaluation

The organoleptic study reveals that the fresh leaves of *D. esculentum* are large, palmate-shaped and green in colour, with characteristic odour and nasty taste.

Physicochemical analysis

The physicochemical parameters are given in [Table 1].

Table 1: Physicochemical parameters of *D. esculentum* leaf extract.

Sl. No.	Parameter	% w/w ± SEM*
1.	Loss on Drying (LOD)	17.36 ±0. 23
	Ash Values	
2.	Total ash	22.59 ±0.20
3.	Acid-insoluble ash	11.36 ±0.30
4.	Water-soluble ash	13.39 ±0.20
	Extractive Values	
5.	Water-soluble	23.48 ±0.26
6.	Alcohol-soluble	9.10 ±0.13

**Values are expressed as mean± SEMof three replicates*

The percentage of loss on drying, total ash, acid insoluble ash and water soluble ash are 17.36 ±0.23 (%w/w), 22.59±0.20 (%w/w), 11.36 ±0.30 and 13.39 ±0.20 (%w/w) respectively. The values of water soluble extractive and alcohol soluble extractive are 15.74% and 8.4% respectively. The determination of moisture content is important for the plant drugs because insufficient drying may lead to possible enzymatic deterioration of active principles [14]. This parameter is therefore essentially used to control the quality of crude herbal drugs and drug products. Acid-insoluble ash is a part of total ash and measures

the amount of silica present, especially as sand and siliceous earth. Water-soluble ash is the water soluble portion of the total ash [15]. The ash content gives an idea about the inorganic content of powdered leaves under investigation and thus the quality of the drug can be assessed. On the other hand, the water-soluble extractive value of the drug was found to be 23.48 ±0.26 (%w/w) which indicates the presence of water-soluble components such as sugar, acids and inorganic compounds. The alcohol-soluble extractive value was found to be 09.10 ±0.13 (%w/w) which indicates the presence of polar constituents like phenols, alkaloids, steroids, glycosides and flavonoids in the leaf extracts of *D. esculentum.*

Phytochemical screening

Phytochemical screening of the sequential leaf extract of *D. esculentum* revealed the presence of various bioactive components of which alkaloids, glycosides, Phenolic compounds, tannins, flavonoids, saponins, carbohydrates and proteins are the most prominent components and the result of phytochemical test is presented in [Table 2]. The ethyl acetate, methanol and Chloroform extract contain flavonoids compounds. Alkaloids are present in methanol extract only. The ethyl acetate and methanol extracts contain Phenolic compounds. The percentage yields of the extracts were calculated [Table 3].

Table 2: Preliminary phytochemical screening of *D. esculentum* leaf extracts using different solvents.

Sl. No.	Constituents	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Aqueous extract
1.	Alkaloids	- -	- -	--	++	--
2.	Glycosides	- -	--	+ +	--	--
3.	Phenolic compounds	- -	--	+ +	++	--
4.	Flavonoids	--	+ +	+ +	++	--
5.	Carbohydrates	- -	--	+ +	+ +	++
6.	Proteins	- -	--	--	++	++
7.	Fats and oils	++	- -	- -	- -	--
8.	Saponins	- -	--	- -	++	++
9.	Steroids	- -	--	- -	++	++
10.	Amino acids	- -	--	--	++	- -

'+ +' indicates presence;'- -' indicates absence

Table 3: Extractive valuesof *D. esculentum* leaf extracts using different solvents.

Sl. No.	Extract	Extractive values (% w/w)	Color of extract
1.	Pet. Ether extract	1.98	Black
2.	Chloroform extract	2.26	Black
3.	Ethyl acetate extract	1.87	Light Black
4.	Methanol extract	2.54	Dark brown
5.	Aqueous extract	4.17	Brown

Fluorescence analysis

The UV light produces fluorescence in many natural products (e.g. alkaloids like berberine) which do not visibly fluorescence in day light. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives by applying different reagents hence some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation. The changes in appearance and colour were observed and recorded. It is therefore an important qualitative parameter for the identification of marker components and hence considered as a useful analytical tool for the standardization of crude drugs [11, 16]. The results of fluorescence analysis were expressed in [Table 4].

Table 4: Fluorescence analysis of powdered leaves of *D. esculentum.*

Sl. No.	Powdered drug/Treatment	Visible/Day light	Short UV (254 nm)	Long UV (365 nm)
1.	Powder drug	Greenish brown	Light brown	Dark green
2.	Powder + Methanol	Blackish brown	Yellowish brown	Brown
3.	Powder + 1% Glacial acetic acid	Brown	Dark violet	Dark
4.	Powder +10% NaOH	Dark brown	Yellowish brown	Brown
5.	Powder + Dil. NH_3	Brown	Green	Black
6.	Powder + Conc. HNO_3	Dark Green	Yellowish Green	Green
7.	Powder + Dil. NH_3 + Conc. HNO_3	Brown	Dark violet	Light Brown
8.	Powder +1M H_2SO_4	Black	Dark green	Black
9.	Powder +1M HCl	Brown	Violet	Dark Brown
10.	Powder + 10% $FeCl_3$	Yellowish Green	Yellowish brown	Brown

TLC analysis

The TLC fingerprint profile of different solvent extracts of *D. esculentum*, the number of spots obtained and their relative R_f values are shown in [Table 5]. TLC chromatograms depicted in [Figure 1]. Show certain distinct spots with their relative intensities. The colors of the spots were recorded as black, green, red and violet.

Table 5: Thin layer chromatographic analysis of *D. esculentum* leafextracts using different solvents.

Sl. No.	Extract	No of spots		R_f value	
		Plate A	Plate X	Plate A	Plate X
1.	Petroleum ether	-	1	-	0.60
2.	Chloroform	-	2	-	0.22, 0.91
3.	Ethyl acetate	3	-	0.50, 0.84, 0.44	-
4.	Methanol	3	4	0.50, 0.83, 0.45	0.22, 0.54, 0.78, 0.93

'-' indicates absence

TLC analysis also suggests the presence of different kinds of phytochemicals in leaves extract. Methanolic extract gives 3 spots in plate A and 4 spots in plate B, which indicates the presence of various phytoconstituents like alkaloids, flavonoids, saponins, and phenolic compounds in *D. esculentum* leaves. Therefore with the help of TLC fingerprinting, this traditional indigenous medicinal plant can be identified as well as standardized [17].

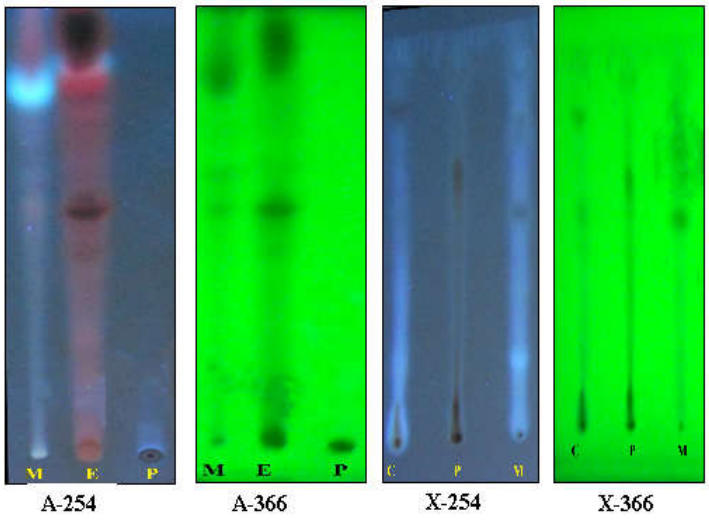

Figure 2: TLC fingerprint profile of *D. esculentum* leaf extract at 254 nm (A-254& X-254) and 366 nm (A-366 & X-366).

P: Petroleum Ether extract, **C**: Chloroform extract, **E**: Ethyl acetate extract, **M**: Methanol Extract**,** Mobile phase for Plate **A**- Toluene: Ethyl Acetate: Formic Acid (4:5:1)**,** Mobile phase for Plate **X**- Methanol: Chloroform (5:1).

Acute Toxicity Study

There were no signs of toxicity or mortality was observed in the acute toxicity studies. All the rats were in healthy condition, maintained normal behaviors and usual movement patterns until the end of the experimental period. The major signs of toxicity noticed within 24 Hours included difficulty in breathing, loss of appetite and general weakness. These signs were not seen in 2500 mg/kg b.w. dose group but progressed and became increasingly pronounced as the dose increased towards 5000 mg/kg b.wt. *D. esculentum* hydro-alcoholic leaf extract is safe to be used as a medicinal plant with no toxic effects and the LD50 of this plant was therefore estimated to be above 5000 mg/kg b.wt [18].

General behavioral

The General behavioral of rats were observed first 6 h and followed by 14 h after the administration and the rats in both vehicle-treated (saline) and extract-treated groups were normal. There were no differences in General behavioral between the control and treated rats [Table 6].

Table 6: General behavioral observations for control and treated groups of albino rats

Sl. No.	Observation	Control group		Test Group	
		6 h	14 h	6 h	14 h
1.	Tremors	Normal	Normal	Normal	Normal
2.	Eyes and Skin	Normal	Normal	Normal	Normal
3.	Mucous membrane	Normal	Normal	Normal	Normal
4.	Behavioral patterns	Normal	Normal	Normal	Normal
5.	Salivation	Normal	Normal	Normal	Normal
6.	Lethargy and Sleep	Normal	Normal	Normal	Normal
7.	Allergic reaction	Normal	Normal	Normal	Normal
8.	Diarrhea	Normal	Normal	Normal	Normal
9.	Mortality and Coma	Normal	Normal	Normal	Normal

Body weight measurement

The body weight of rats were measured weekly until the end of acute toxicity studies. There were no significant differences in the body weight changes between the control and treated rats [Table 7].

Table 7. Body weights of the animals during the 14-day treatment with *Diplazium esculentum* hydro-alcoholic leaf extract.

Sl. No.	Group	0 day	7 day	14 day
1.	Control group	232.66 ± 0.23	231.12 ± 0.45	234.78 ± 0.64
2.	**Test Gr I (**2500 mg/kg body weight)	234.56 ± 0.54	232.73 ± 0.32	235.23 ± 38
	Test Gr II (5000 mg/kg body weight)	238.98± 0.67	23.56± 0.35	241.92±0.11

Biochemical analysis

[Tables8].show the results of various biochemical tests performed on the sera of control and treated rats from the acute toxicity studies. There were no significant differences in the outcome of the biochemical tests analyzed between the control and treated rats in acute toxicity evaluation [19].

Table 8 Results of biochemical tests performed on the serum of control and treated group in the acute toxicity study for 14 days.

Groups	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Triglyceride (mg/dl)	Cholesterol (mg/dl)
Control group	63.78 ± 1.45	71.11 ± 7.23	132.33 ± 3.22	167.38 ± 3.57	226.96 ± 1.64
Test Gr-I	66.87± 4.55	69.77 ± 3.56	138.76 ± 1.21	165.79 ± 4.33	229.61±1.44
Test Gr-II	68. 54± 2.87	71.91 ± 5.76	137.84 ± 2.37	169.37 ± 5.22	230.81 ± 2.11

CONCLUSION

The findings of this study indicate the presence of various phytochemicals in the plant extracts, which may be responsible for the pharmacological activity. In view of the popularizing the use of medicinal plants in complementary and alternative medicine, it is necessary to carry out scientific research for

standardization of medicinal plants with respect to their safety and toxicity assessment in laboratory animals to ascertain their safety for human use.

We sincerely believe that our study will be important to herbal drug researchers, ethnopharmacologists, medicinal chemists, pharmacologists, toxicologists, pharmacognosists and ethnobiologists the way for upcoming research on the indigenous medicinal plant of North-East India *Diplazium esculentum.*

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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