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In-vitro cultivation and phytochemistry of *Diplazium esculentum* (Retz.) Sw.: An important Himalayan pteridophyte

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Abstract

Among the different wild edible ferns, *Diplazium esculentum* (Family: Athyriaceae), is one of the most popular species ranked as a pharmacologically diverse ethnomedicinal plant owing to its medicinal usage of circinate part and young leaves. The present article is the first comprehensive review on the *In-vitro* propagation and phytochemistry of this edible fern. The antimicrobial, antioxidant, immunomodulatory, neuro modulatory, anti-fertility, cytotoxic *etc.* are the most thoroughly studied pharmacological activities of the *D. esculentum*. This fern is consumed traditionally depending upon its indigenous nutritive and medicinal value in different areas of the world. It is a seasonal underutilized vegetable which may act as a potential functional food and also reservoir of important compounds such as alkaloids, flavonoids, saponins, Terpenoids. This fern is also rich in various micronutrients including phosphorus, calcium, iron, vitamins C, A and dietary fibres. Hence, malnutrition can be overcome by using this wild edible to some extent. The fruitful *In-vitro* multiplication of this fern was also reviewed while its genetic diversity is less inferred.

Keywords: *Diplazium esculentum*, medicinal and local uses, *In-vitro* propagation, phyto-compounds

Introduction

Beginning from the time of plant collection up to modern era, men have been exploiting the various plants and their parts from wild for their livelihood and existence. Since ages most of the food items whether it fruits, vegetables, medicines or cereals *etc.* that have been utilized by the indigenous people throughout the world have their origin in plants. An organism utilizes either wild or cultivated form of a plant. But the manner in which they are being consumed may vary or particular in a region, district, state or a country to which the particular plant is native. Additional peculiarity in preparation of certain local cuisines based on the traditions, geographical boundaries, communities, resource availability *etc.* greatly enhances this variation. As the population is growing day by day, the progressive demand of food stock on the cultivated crops increased so there is urgent need to explore more and more alternatives. Thus an attempt to fulfill this growing demand of food supply and to ensure year-round availability or balanced nutrition, every wild edible plant that is less frequently consumed need to be documented in terms of their diversified usage to human beings. Therefore, their consumption can be increased at sustainable rate and further be used as a substitute to feed this growing population. *In-vitro* propagation, various beneficial phyto-compounds and comparative genetic diversity of these wild edibles need to be explored and research involving these parameters has also increased markedly throughout the years. Among these, a plant group, under identified to its diverse role in our lives is the ferns and its allies [1].

In simple words, the ferns and its allies (not true ferns) are included in the plant group, Pteridophytes which represent the vascular cryptogams. Their mode of reproduction is through spores. The Eastern and Western Ghats Indian Himalayan regions are biodiversity hotspot area where pteridophytic variability is thought to be very high. Of the various wild edibles used in any particular area as vegetable, ferns and related ferns additionally have their distinct position in lots of cuisines, although they are not popular compared to other seeded flora.

Even description on several edible ferns from different Indian regions has not yet been documented. Therefore, a complete knowledge about naturally available vegetable ferns is much awaited from northern India [1]. But the hazardous impact on consuming these wild edibles must also be kept in mind while utilizing them.

Morphology

Diplazium esculentum is a huge perennial herb, about 1m or half, belongs to family Athyriaceae and grows along stream banks, canals, in open marshy areas, and at 2,300 m from sea level [2]. Single species of this fern forms very dense gregarious colonial vegetation under suitable environmental conditions. An erect, crawling, branched rhizome of about 15-20 cm long, 4-6 cm diameter with a narrow, lanceolate and toothed margin, apex covered with reddish brown colored scales (about 1cm long) anchors the plant to the ground (Fig. 1). Often a lean slender black colored trunk of about 1 m tall extends from rhizome part. Plant is able to re-grow if defoliated either by human or environmental interference. Leaves form a dense turf in a rosette fashion is up to 120 cm long [3]. Lamina is dark green in color, variable, bipinnate, glabrous or hairy, widely lanceolate, 60-80 X 30-60 cm with outer triangular shape, acute terminal with all the leaflets are merged. About 12-16 pairs of pinnae are arranged alternatively to form lamina,

Fig 1: (A) *Diplazium esculentum* plant in its natural habitat; (B) Young fronds; (C) Abaxial surface of pinnae showing linear shaped sori.

the adaxial surface of it is of concave shape and grooved. In the lobes of pinna, around 6-10 pairs of veins, single, reaching the margin/sinus is present. The rachis is either glabrous or hairy and yellow to light brown linear indusia surrounding the linear, slightly curved minute sori, covering from half to the entire vein is present. Spores are bean-shaped, monolete, dark brown and enclosed within a polycellular, globoid, short stalked sporangia [4]. The stipe and lamina together constitute frond that is 1-2 X 0.5-1 m, erect to bow shaped. The black colored stipe is scaly and light colored at the base and above, respectively [2]. The edible part of a fern constitutes young, hairy, blackish green erect leaf stalks whose top is curled with developing leaves on them referred to as fronds (Fig.1B).

Depending upon the traditional knowledge of locals and tribes around 3-4 morphologically distinct landraces of *D. esculentum* are found in Western Ghats [5], and from Sikkim (Eastern Himalayas) 2 local landraces of this fern are 'Chiple ningro' and 'Sawane ningro' [6]. Both of them evaluated the nutraceutical and bioactive significance of ferns especially of *Diplazium*. *Diplazium esculentum* is known by several synonyms such as *Anisogonium esculentum* (Retz.) C. Presl, *Asplenium esculentum* (Retz.) C. Presl, *Athyrium esculentum* (Retz.) Copel., *Callipteris esculenta* (Retz.) J. Sm. ex T. Moore and Houlston, *Hemionitis esculenta* Retz., and *Microstegia esculenta* (Retz.) C. Presl. The common names of this fern are vegetable fern, fiddlehead fern and edible fern.

Local names

Depending upon its presence in a particular indigenous area *D. esculentum* has been known by a specific name to the natives of that area or country. For instance 'Pani nyuro or Ningro', 'Pako', 'Pucuk paku', and 'Kuwareshida' in Nepal, Philippines, Malaysia, and Japan, respectively [7]. In north and east India, young fronds are popularly known as 'lingra/lingru/lungru', 'rukja', and 'lochanch' respectively; "dekia" and "lankuth, lingade, lemkut" in Aruanchal Pradesh and Kinnaur (Himachal Pradesh), India [8, 9]. This fern is called as "kasror" and "lingdi/lingad" in Chamba and Mandi districts of Himachal Pradesh [10, 11]. In comparison to tropical region ferns, a cyclic reproductive phenology does not found in *D. esculentum.* It is hard to reach to some conclusion because studies on phenology of this species have not been conducted properly [3].

Distribution and Habitat

Diplazium esculentum is a rhizomatous fern, native to Asia. It is a pantropically distributed wild edible fern found throughout tropical, subtropical and temperate regions *viz*., India, Japan, China, Cambodia, Laos, Thailand, Taiwan, Indonesia, South Asia, Southeast Asia, Oceania, Vietnam, Polynesia and Malaysia [12]. Sometimes it become invasive and flourishes well in places having sufficient hot, humid, and shady surroundings, preferring acidic soil [4]. All over the world about 350 species are documented in genus *Diplazium.* From India around 40 of which 17 species from Sikkim alone have been reported by Kholia [13]. In India, this fern is common in states of Himachal Pradesh, Meghalaya, Uttarakhand, Arunachal Pradesh, Orissa, Tamil Nadu, Western Ghats regions of Maharastra, Karnataka, Kerala and Sikkim [14, 15]. In Himachal Pradesh, it covers districts Mandi, Shimla, Solan, Chamba, and Kangra.

Due to wide ecological variations in its habitat, *D. esculentum*, most commonly grows in humid and shady locations. This fern has also been reported in ranges from 100-1200 meters of tropical and sub-tropical forest areas [3]. In Southeast Asia, plant grows along the margin of paddy field, inside cocoa, coffee, rubber, oil, also in secondary forest and in tree plantations. As it is a common species growing around the villages and farms in South East Asia, so easily spotted at glance. Secondly, in agricultural scattered sites, the species is comparatively taller than other fern species; third easily recognizable near human settlements because of the clustered appearance of the plant, a unique feature imparted by the rosette-like arrangement of greenish leaves. Only soft earlier stages are suitable for eating. After this, the fern loses its edibility as the leaves open up, increase in size and the fleshy stems get lignified. This suggests harvesting of the fern at a particular development stage.

Local food and medicinal usage

Literature has documented more than 200 species of pteridophytes being used by different tribal population of India. They have been utilizing fern and fern allies as curative agents to counteract various ailments like fertility, anthelmintic, cancer, diabetes, inflammation, aphrodisiac, convulsant, rheumatism, diuretic, hepatoprotective, sedative [16]. Beside, tribal communities, ethnic groups and folklore are also extracting benefit from these wild edibles since ancient time throughout the world. For food and medical purpose either whole or various plant parts like rhizome, stem, fronds, pinnae and spores are being exploited in various manners. In India, newly emerged fronds of *D. esculentum* have been used as a vegetable or salad while rhizomatous part is being exploited to control insects and pests [17].

The two most important parts of this fern, rhizome and leaves are in common and wide usage throughout Asia and Oceania. Nikmatullah *et al.* [18] reported that they are being boiled and taken as a herbal drink in order to treat muscle and joint diseases in East Java, Indonesia. The concerned tribal and local communities of a region have been consuming this edible fern from years after collection of newly emerged fronds, their subsequent washing, boiling or cooking in oil or butter. The whole plant including roots is being used by the Rane Sundanese community in treatment of fever, dermatitis, and measles [19]. Another community in Philippines, Batas Island called Ivan community found effectiveness of its sporophylls and shoots against hypertension and constipation [20]. Also the vegetable and pickle prepared from the young fronds are greatly enjoyed by the residents of the central; north-western Himalayas (Himachal Pradesh and Uttarakhand) and northeastern India

(especially Sikkim). Again the cuisine, importantly pickle (FAO, 2010) is considered to be effective in counteracting both the constipation and as an appetizer by the natives of the Himalayas. In Northeast India, in Lohit district of Arunachal Himalayas, Khamptis "Chauya" traditional healers claimed *D. esculentum*, a new herbal drug effective against diabetes mellitus [8].

In Himachal Pradesh, this seasonal vegetable is consumed regularly by tribals of Kinnaur and Pangwal, Pangi valley, Chamba districts (H. P.) [9, 21]. The inhabitants of Kinnaur have been utilizing fresh leaves of this edible fern, chopped into small pieces subsequently dried and stored for winter use while tribals of Chamba still use the stem portion in the treatment of muscular pain. The decoction being prepared of whole plant is taken at night. Tender shoots of the fern were preferred for consumption based on their palatability and taste in Kinnaur than the leaves of other vegetables [9]. Thus the fern is eaten in large quantities by Himachalis and several preparations are made from it. Special curries like *DUM* and *MADHRA* with exclusive recipes are made in Himachal from it. The preparations are even served as special dishes to VIPs on special occassions like marriages. Lingad is especially popular among the members of Sood community of H.P. and they have their own recipes for lingad curries. Every year tons of "lingad" worth lakhs of rupees is sold in the vegetable market of Himachal towns. This plant entire supply comes from forests as lingad and has still not been brought under cultivation. Along with Himachal, lingad found in the forests of Kashmir and Uttaranchal is also liked by the natives [22]. In 1952, according to the wealth of India, the moisture, protein, fat, fibre, mineral and beta carotene content found in about one hundred grams of fresh fronds are 91.3 g, 1.0 g, 100 mg, 1.4 g, 600 mg, 0.98 mg, respectively. *Diplazium esculentum* has been utilized as one of supplementary food plant by the natives of upper Shimla, Himachal Pradesh. The fern is locally called as "Lingra" in Shimla and utilized in several preparations such as curry, pickle and vegetable. The fern is potentially rich in various micronutrients, mainly iron (8.40 mg/100g), manganese (5.60mg/100 g), zinc (5.30mg/100g), phosphorous, calcium and folic acid, anti-nutritional factors (tannins, phytic acids, and trypsin) [23].

Moreover the laxative use of the boiled young fronds with rice was witnessed by Kagyung *et al.* [24] from Debang Biosphere Reserve of Arunachal Pradesh, India. Rhizome is considered as a crucial plant part used as an anti-cough, anti-fever, anti-dysentric module also beneficial in the treatment of stomachache, diarrhoea, asthma, cough, phthisis, and dyspepsia. This fern anti-oxidative property is much greater than alpha-tocopherol form of Vitamin E [25]. The cultivated young fronds were used in preparation of cusines such as *Palai Sak* by the ethnic communities in North East India [1]. Traditionally leaf of *D. esculentum* is used against pain, wounds, glandular swellings, diarrhoea, headache, fever, dysentery, and several dermal infections [26].

In-vitro Propagation

Usually the fronds you have seen in local and supermarkets on sale are being collected from the wild species of *D. esculentum* throughout Southeast Asia. However, vegetative reproduction of the plant is easy and results into production of large cluster of individuals with several leaves. The successful *In-vitro* culturing of this rare medicinal fern using circinate part-"crosiers" as explant from Western Ghats (Kerala) has been performed by Nair *et al* [27]. This fern is consumed there as one of leafy vegetable by the Paniya and Chetti tribes. The culturing was performed on half-strength Murashige and Skoog (MS) medium alone or supplemented with different concentrations, and combinations of various phytohormones. The economical and rapid method of *In-vitro* multiplication of *Diplazium* sp. using spore explant could make conservation of this species possible. Another investigation on successful gametophytic development (observed up to prothallus stage) from *in vitro* spore cultured *D. esculentum* was observed in a very short period in Philippines [4]. For consumption, commercial and horticultural purpose, small scale cultivation of *D. esculentum* was reported in 7 Indian north eastern states, excluding Sikkim [1].

Phytochemistry

The chemical compounds which may be intermediate molecules formed during plant(s) normal metabolic pathways or processes are referred to as phytochemical compounds often termed as "secondary metabolites". These molecules are present in their natural form. Therefore, they are considered as best and safe alternative for synthetic drugs and used as a preventive molecule against different types of diseases. They are effective against viruses, microbes, fungi, parasites, allergy, inflammation, hyperglycemia and therefore have different types of medicinal properties making them very useful in pharmacy and medical fields.

Overall phytochemicals investigation in a concerned plant species has been done by collection and subsequent extract preparation of the different plant parts which then analyzed for total plant compounds presence. Globally various eminent researchers or scientists have performed the phytochemicals evaluation studies on *D. esculentum*. And these analysis revealed presence of several types of potential and important phyto-compounds namely nutritional molecules, phenolics, alkaloids, glycosides, cardiac glycosides, glucosides, saponins, tannins, proteins, terpenes, anthraquinones, cyanidins, flavonoids, leucoanthocyanins, and many more in this fern species[15, 7, 26]. Out of which flavonoids, alkaloids, saponins, terpenoids, and polyphenols are major compounds functioning as anti-inflammatory and anti-aging agents. Among these also alkaloids form the major group, acting as a nervous system stimulators, memory boosters in animals, possess acetylcholinesterase inhibition activity. Therefore, it has been confirmed that *D. esculentum* consists of considerable concentration of several important phytochemical compounds. Many of these have varying usefulness in human life.

The wide array of bioactive molecules elucidated from earlier studies include esculentic acid, lutein, phosphoric acid, phytol, pterosin B, 2,6,10-trimethyl,14-ethylene-14-pentadecene, hexadecanoic acid methyl ester, ptaquiloside, pentadecanoic acid, acetate, Stigmasta-5,22-dien3-ol, beta-ocimene, 1,2- Benzenedicarboxylic acid, BIS(2-Methylpropyl)ester, 1-Heneicosanol, 5,8,11,14-eicosatetraenoic acid, ergost-5-en-3-ol, (3beta) and stigmast-5-en-3ol, (3beta) [28, 17]. *D. esculentum* fronds were examined for total flavonoids, phenolics, antioxidant potential and Fourier Transform Infrared Spectroscopy (FTIR) analysis of the samples collected from (Totu area) Shimla (H.P.), India by using four different solvents *i.e.*

ethanol, methanol, chloroform and aqueous. They concluded presence of highest concentrations of phenolics and flavonoids in methanolic extract than other solvents [29]. The biologically active components were assessed relatively in cooked and uncooked fronds of *D. esculentum* sampled from Western Ghats (Karnataka), India. And the results showed that on cooking, the bioactive constituents and effectiveness against oxidative species got affected differentially. Moreover, the geographical differences have also affected their (flavonoids, phenols, ascorbic acid) amount, hence, antioxidant potential of the fern [5]. The quantification of flavonols (kaempferol, myricetin, quercetin and morin) was done by Chao *et al* [30]. from methanolic acidic hydrolysate of *D. esculentum* from Taiwan. The previous study of Das *et al* [31]. revealed its effectiveness in preventing the anaphylactic shock and as a stabilizer of mast cell in sensitized Wistar rats. This fern was also probed to act as an immunosuppressant and boiled form of it has hemolytic properties in Swiss albino mice [32].

Roy [2] investigated immuno pharmacological properties of *D. esculentum* from West Bengal, India. Further, this fern anti-inflammatory [33]; anti-diabetic, hepato protective [34]; and analgesic [35] activities were reported earlier. Hence, *D. esculentum* and its active constituents have found their medical role against several type of diseases.

Neuromodulatory activity

The effect of *D. esculentum* on mouse central nervous system (CNS) was studied and found that the locomotor activity get enhanced by the aqueous leaf extract of *D. esculentum* in a dose-dependent manner. The fern was found to be a potent analgesic here. And when compared with control this extract has stimulated the CNS[33]. Besides effect on CNS, Chawla *et al* [35]. noticed the marked effect of *D. esculentum* against peripheral and inflammatory pain models. Roy *et al* [36]. observed that the neurodegenerative disorders caused due to the oxidative stress can be managed by the use of *D. esculentum*.

Larvicidal activity

Halimatussakdiah *et al* [37]. first observed the potential larvicidal activity of *D. esculentum* (methanolic extract) against third and four instar *Culex quinquefasciatus* and *Anopheles gambiae* larvae. They concluded that extract was most efficacious to *A. gambiae* third instar larvae.

Antibacterial activity

The method employed for antimicrobial activity determination was disc diffusion and broth dilution assay, performed in order to evaluate the minimum inhibitory concentration (MIC) against 3 gram positive and 5 gram negative bacteria [26]. Almost all the alcoholic and aqueous extracts of *D. esculentum* parts restricted the growth of certain human and phyto-pathogens *e.g., Salmonella arizonae*, *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus* [16]. The accumulative and strong effect of fern extract along with tetracycline was seen on the bacterial growth than using antibiotic alone. Rhizome extract possessed maximum inhibitory effect against *Staphylococcus aureus* and *Salmonella arizonae* and found to be more effective than leaves [16]. Further the bacterial growth *e.g., Salmonella typhimuriun* followed by *Klebsiella pneumonia* was observed to be inhibited by the rhizome aqueous extracts of *D. esculentum* whereas, no inhibition in case of leaf extract [26]. The highest inhibition of cholroform extract against *Sarcina lutea* followed by *Salmonella typhimurium* showed the strong antimicrobial activity of *D. esculentum*. Also it is effective in control of other bacteria *e.g., Bacillus subtilis*, *Klebsiella pneumonia*, *Shigella boydii*, and *Vibrio cholera*.

Anti-oxidant activity

Several types of assays such as ABTS (Antioxidant activity assay by 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), H_2O_2 radical scavenging, total antioxidant capacity, cuprac reducing antioxidant activity, and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay were performed to determine the antioxidant activities of plant extracts [15, 26]. The potential anti-oxidant activity and total polyphenols quantification with emphasis on HPTLC concluded the presence of considerable amount of polyphenols and saponins which might together be responsible for various pharmacological use of *D. esculentum* [15]. First report on the isolation and identification of four types of phenolics namely (2R)-3-(4′-hydroxyphenyl) lactic acid, protocatechuic acid, trans-cinnamic acid [38] and rutin; three ecdysteroids *i.e.*, amarasterone A1, makisterone C and ponastroneA were known from fiddle head portion of this fern.

Anthelmintic activity

The investigation of three extracts *i.e.* aqueous, ethanolic and petroleum ether of *D. esculentum* for possible anti-helmintic activity on adult Indian earthworm unmasked the fact that with increase in polarity of extract gradual enhancement in this activity was noticed[39]. The standard used was piperazine citrate.

Cytotoxic potential

By screening the enormous amount of bioactive constituents in *D. esculentum* from Tangail district, Bangladesh, Akter *et al.* [26] confronted the presence of significant amount of antimicrobial, cytotoxic, and antioxidant molecules. They used brine shrimp lethality bioassay and pointed out the concentration dependent enhancement in their death rate. Therefore, proved the cytotoxic effect of both chloroform and methanol extracts of its leaves.

Total phenolic compounds

Wali *et al.*[28] assessed the nutritional profile, total polyphenols and flavonoid content along with antioxidant activity of two species of *Diplazium* namely *D. maximum* and *D. esculentum* from Western Himalayas. They found that former species possessed highest antioxidant activity and lutein content than latter.

In some animals, certain pathological effects have been reported to be associated with *D. esculentum* intake. For instance, use of boiled, the human consumptional form of *D. esculentum*, possessed some toxic properties that can hamper the male reproductive function in Swiss albino mouse. Therefore, the fern should be evaluated further as a potential anti-fertility agent. Furthermore, the amount of various liver enzymes like LDH (lactate dehydrogenase), ALP (alkaline phosphatase) *etc.* and urea or creatinine (kidney) in the blood of these mice was observed to be enhanced as revealed by serum biochemistry [40].

Conclusion

When the comparison of *D. esculentum* is done with other primitive vascular plants, it is observed that the fern has adapted itself with the course of evolution and has survived from the Paleozoic times. Therefore, the phyto-chemicals associated with this medicinal herb are supposed to have much importance than other plants. Not all the ferns are edible, but it is interesting to note that all the *D. esculentum* parts either leaf, stem, young fronds, rhizome, occasionally the whole plant is used for food throughout the world. But local mass extraction of this species directly from wild and habitat degradation due to some natural calamities and human interventions intensified the research towards species conservation and sustainable use. From earlier studies done so far *D. esculentum* requires more precise expertise to explore its mechanism of action, *In-vitro* propagation and genetic diversity assessment. And eventually could result into Himalayan socioeconomic development therefore, income generation to poor tribal populations can be enhanced by considering these approaches.

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