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Pharmacognostic and preliminary phytochemical investigation of *Eulophia herbacea* Lindl. Tubers (Orchidaceae)

Anil Tatiya[1*], Sanjay Surana[1], Snehal Bhavsar[1], Dhanshree patil[1], Yogesh Patil[1]

[1]*Department of Pharmacognosy, R.C.Patel Institute of Pharmaceutical Education and Research, shirpur-425405, Maharashtra, India*

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ABSTRACT

Objective: *Eulophia herbacea* Lindl (Orchidaceae) is an important traditional medicinal plant and widely used in treatment of variety of disease by tribes. Plant is unexplored scientifically yet for their identification and use. Therefore, the current study was carried out to perform detailed pharmacognostical and phytochemical analysis of *E.herbacea*. **Method:** Systematic pharmacognostic evaluation of tubers of *E.herbacea* has been carried out with respect to macroscopy, microscopy, WHO recommended physico-chemical parameters, florescence analysis of powder and estimation of different chemical standards. HPTLC fingerprinting of amino acid was also developed. **Result:** Tubers are fibrous, woody and perennial with numerous rootlets. Microscopic study shows the presence of cork, cortex, parenchymatus ground tissue, fibro vascular bundles and calcium oxalate crystals. Qualitative phytochemical test revealed the presence of acidic compounds, carbohydrates, amino acids, mucilage, tannins, steroids and triterpenoid. **Conclusions:** Morphological, histological and physico-chemical parameters studied in this paper may be proposed to establish the authenticity of plant *Eulophia herbacea*, which can probably, helps to differentiate the crude drug from its other species with respect to quality, purity and identification.

1. Introduction

Eulophia, a genus of perennial terrestrial orchids with fleshy tubers, rarely pseudobulbs, is distributed in the warm parts of the world, especially in Asia and Africa. About 28 species occur in India [1], several species are ornamental. *Eulophia herbacea* Lindl.(Orchidaceae) is commonly known as Kutri-kand, Kukad-kand, or umarkand [2] which is occurred in terrestrial, on hill slopes as forest undergrowth found in the area of Himalaya, Bengal, western parts of deccan peninsula[3]. Leaves linear-lanceolate or elliptic-lanceolate, glabrous, multi-nerved, plicate, 12-30cm x 2.5-8.5cm. Flowers are white, purple-nerved, in lax racemes, scape stout, 22-35 cm long. Capsules ellipsoid, obscurely ribbed. It bears flower & fruits in July-September [4]. It is traditionally used in the treatment of tumors of scrofulous glands of neck [5]. It is used as salep, dried tubers of various species of Orchids, and *Eulophia* used to make a nutritious beverage by treating the powdered preparation with hot water as tonic. Decoction of tuber is used on spermatorrhoea, urinary complaints, and menses [6]. *Eulophia herbacea* Lindl also shows multiple activities such as anti-cancer, nutritional, anti-hyperlipidemic, anti-oxidant, anti-arthritic, anti-inflammatory, antimicrobial and immunomodulator.

There is no report of systematic pharmacognostic and phytochemical studies on the tubers. In order to secure some standard for its identification; this study was carried out for pharmacognostical screening.

2. Materials and Methods

2.1. Plant material

The tubers of *Eulophia herbacea* Lindl, Orchidaceae, were collected from the subtropical hilly area of Toranmal

*Corresponding author: Dr. Anil U Tatiya, Department of Pharmacognosy and Phytochemistry, R.C.Patel Institute of Pharmaceutical Education and Research, shirpur-425405, Maharashtra, India
Tel: +91-2563251808, +91-9923070789
Fax: +91-2563-255189
E-mail: aniltatiya12171@gmail.com

region, Maharashtra, India, in the month of July-August. They were identified and authenticated by Dr. D.A.Patil, Taxonomist, Department of Botany, S.S.V.P.S College of science, Dhule, Maharashtra, India. A Herbarium specimen was deposited in Dept of Pharmacognosy, R. C Patel institute of Pharmaceutical Education and Research, Shirpur, Dhule. With the number (FIH 107154).

2.2. Macroscopic and Microscopic characters of tubers

The tubers were separated from other parts, washed, cleaned and dried for further use. The detail macroscopic characters of fresh tubers were noted including special features. Microscopic sections were cut on a microtome & by free hand sectioning. Numerous temporary and permanent mounts of the microscopical sections of the tubers were made and examined microscopically. Histochemical reactions were observed with different staining agent for the general and specific microscopic characteristic of tubers. Photomicrographs of the microscopical sections were taken with the help of MOTIC photomicroscope provided with Motic Images plus 2.0 software [7].

2.3. Powder characteristics

Preliminary examination like behavior of powder with different chemical reagents and microscopically examination was carried out according to the method given in Khandelwal and Kokate [8, 9].

2.4. Fluorescence analysis of tuber powder

Powder material was analyzed under visible light, short and long UV light after treatment with various organic/ inorganic solvents / reagents like Petroleum ether, methanol, water, 10% aqueous NaOH, 50%HCl, 50% H_2SO_4 , acetic acid, 50% HNO_3 etc [10,11].

2.5. Physicochemical parameters

Physicochemical parameters such as percentage of total ash, extractive values and moisture content, foreign matter content, loss on drying, swelling index, foaming index were determined as per official method of the Indian pharmacopoeia and the WHO guidelines on the quality control methods of medicinal plant materials [12, 13].

2.6. Quantitative determination of heavy metal and minerals

Air dried tuber powder was kept in muffle furnace for preparing ash. Heavy metal and inorganic content of ash was determined quantitatively by atomic absorption spectrometer (AAS; Perkin Elmer 400) 19

2.7. Preliminary phytochemical screening

Powdered drug was extracted with petroleum ether, methanol and water successively. The extracts were dried and weighed. The presence or absence of different phytoconstituents viz. triterpenoid, steroids, alkaloids, vitamins, tannins, glycosides and flavonoids, etc. were detected by usual given method [8].

2.8. Phytochemical standards

Total protein content by lowery method [14], phenolic content by folin-catechu reagent method [15], total flavonoid by the aluminum chloride colorimetric method [16] and proanthocyanidin by vanillin-sulfuric acid method [17] was determined. Available carbohydrate, crude lipid, crude fiber and total mucilage were estimated by official method [18,19].

2.9. HPTLC study of amino acids

Methanolic extracts of *E.Herbacea* (MEEH) studied for amino acid finger printing pattern using HPTLC. The plate was developed in n-Butanol: Water: acetic acid (4:1:1 v/v) as mobile phase in Camag twin trough TLC chamber with lid up-to 8 cm. Derivatization of plate was done by dipping the plate in to 0.25% Ninhydrin in acetone. The plate was scanned at 366 nm using Scanner 3. In this study eleven numbers of amino acids was detected in MEEH, from that four amino acids were match with standard amino acids such as alanine, threonine, serine and aminobutyric acid.

2.10. GC-MS analysis of petroleum ether extract

An Agilent model 6890 GC interfaced to a 5973 mass selective detector was used for mass spectral identification of petroleum ether extract. HP-5MS capillary columns (30 m×0.25 mm×0.25 μm film thickness) were used for GC. The oven temperature was maintained at 60℃ for 6 min then programmed to 240℃ at 5° min-1. The carrier gas was helium, at a flow rate of 0.9 mL min-1, and the injection volume was 1 μL. In mass spectrometry electron-impact ionization was performed at electron energy of 70 eV. Components of PE were identified by comparison of their mass spectra and retention indices with those published in and contained in the NIST′98 MS computer library.

3.Results

3.1. Macroscopic characteristics

Leaves are linear-lanceolate or elliptic-lanceolate, glabrous, multi-nerved, plicate, 12-30cm x 2.5-8.5cm (Figure 1A). Fresh tubers are light brown colored, odorless with a slightly acrid taste. The tubers are found as napiform (Figure 1B) with average size of 4-6 cm in width and 5-8 cm in length. It shows prominent node like structure over the surface. The tubers are stout, shows the presence of numerous rootlets and root scars in upper parts, fractured surface is fibrous. Flowers white, purple-nerved, in lax

racemes, scape stout, 22–35 cm long (Figure 1C).

3.2. Microscopic characteristics of tubers

T.S. of *Eulophia herbacea* tubers shows the entire general characteristic with some prominent identification feature. In general it showed the presence of cork, cortex, endodermis ground tissue, scattered vascular bundle and calcium oxalate crystal. Cork represented the outermost layer of tubers, containing 2–3 layered rectangular, thin walled cork cells (Figure 2A). Cortex consists of 6–15 layers of polygonal thin walled cellulosic parenchymatus cells (Figure 2B). Cells showed the presence of starch and acicular calcium oxalate crystals. The vascular bundles were found to be scattered in ground tissue and cortex. These vascular bundles were collateral closed and partially covered with lignified fibers i.e. fibro vascular bundle (Figure 2C).Xylem represented discontinuous groups of vessels. The vessels showed largely reticulate & pitted thickenings, responsible for water conduction. Phloem of vascular bundle consists of sieve tube along with companion cells, responsible for conduction of food. Phloem occupied relatively large area than xylem, with thick walled and big parenchymatus cells. The parenchyma in the phloem region is highly lignified cells. The calcium oxalates needles were abundant throughout the section. The mucilage cells were scattered in ground tissue and also deposited in cork cells.

3.3. Powder characteristics

Macroscopic and Microscopic

The tuber powder is light brown in color, slightly rough in touch with slight aromatic odour. Addition of small quantity of water, a mucilaginous mass was formed which indicates presence of considerable amount of mucilage. Pressing a little amount of powder between filter paper, no greasy stain was found, indicating absence of fatty oils. Behavior of powder with different chemical reagents is shown in Table 1. Microscopical examination the powder showed lignified fibers, Fibro vascular bundles, xylem, and phloem, as shown in Figure 3A, B, and C. The fluorescence analysis observed in visible, short and long ultra violet was depicted in Table 2.

Figure 1.a) *E.herbacea* plant; b) tuber; c) flowers and leaves

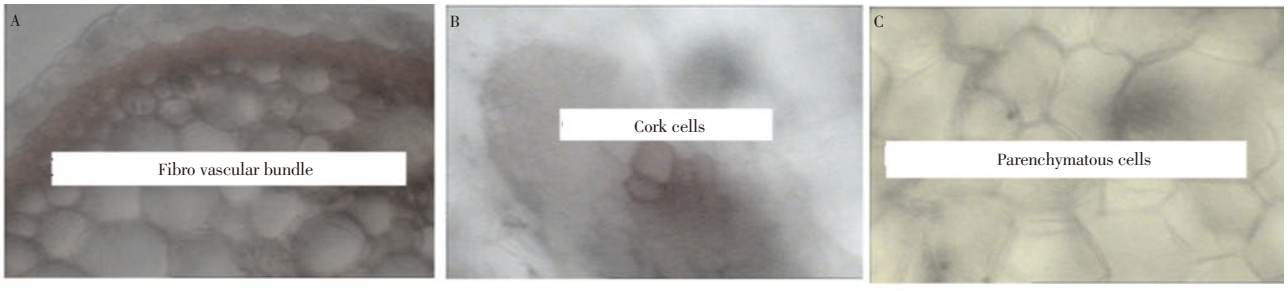

Figure 2. Microscopic characteristics of *E.herbacea* tubers

a) Fibro vascular bundles; b) cork cells; c) parenchymatus cells in cortex region

Table 1

Behavior of E. herbacea tuber powder with different chemical reagents

Reagent	Colour/	Precipitate	Constituent
Conc. sulphuric acid	Reddish		Steroid present
Aq. ferric chloride (5%)	Black colour		Tannin present
Iodine solution	Blue		Starch present
Picric acid solution	No Yellow ppt		Alkaloid absent
Aq.mercuric chloride solution	No Brown colour		Alkaloid absent
Magnesium– HCl	No change		Flavonoid absent
Aq. silver nitrate solution	No ppt		Proteins absent
Ammonical solution	No change		Anthraquinone glycoside absent
Ruthenium red	Red colour		Mucilage present

Table 2

Fluorescence analysis of powdered tubers of *E.herbacea*

Treatment	Visible Light	Short UV (254nm)	UV Light (365nm)
Powder as such	Light Brown	Brown	Dark Brown
Powder + 10%NaOH (Aqueous)	Green	Green	Light Green
Powder+NaOH(Alc.)	Green	Light Green	Turbid
Powder+ 50%HCl	Pale Green	Slight Turbid	Turbid
Powder+Ammonia	Light Green	Green	Dark Green
Powder+Iodine	Light Yellow	Light Green	Light Green
Powder+FeCl3	Dark Green	Dark Green	Dark Green
Powder+50%H2SO4	Green	Dark Green	Dark Green
Powder+Acetic acid	Yellowish Green	Turbid Green	Slightly Turbid
Powder+50%HNO3	Lemon Yellow	Yellowish Green	Yellowish Green

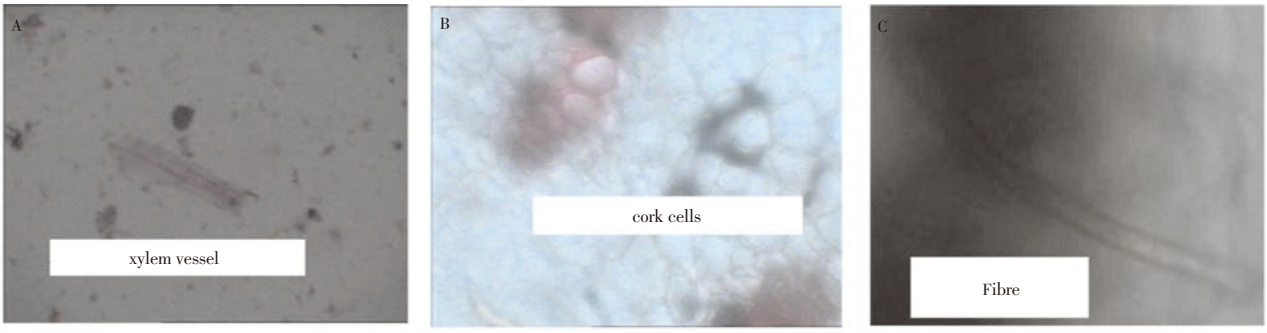

Figure 3. Powder characteristics of *E.Herbacea*; a) Xylem vessel; b) cork cell; c) fiber

3.4. Physiochemical parameters

The physiochemical parameters were shown in Table 2, such as total ash value was found to be 7.6%, water soluble ash 9.07%, acid insoluble ash 0.98%, swelling index 4 mL, Moisture content 84 % and foreign matter was 0.20 % w/w. The extractive values are mainly useful for the determination of the exhausted or adulterated drug. Petroleum ether soluble drug was 2%, water soluble 18% and methanolic soluble 14 % w/w (Table 3).

3.5. Heavy metal and mineral analysis

AAS analysis of tuber powder showed the presence of heavy metal namely cadmium and lead; and minerals such as calcium, potassium, magnesium, iron and sodium.

Table 3

Physicochemical parameters of E. herbacea tubers

Parameters	Mean± SEM (%)
Total ash	7.6± 0.208
Acid-insoluble ash	0.98±0.023
Water- soluble ash	9.07±0.231
Sulphated ash	1.02± .067
Moisture content (Fresh tubers)	83.67± 0.86
Foreign organic matters	0.20±.002
Crude fiber content	31.5±1.322
Swelling index	4ml
Pet. ether soluble extractive value	2± 0.001
Methanol soluble extractive value	14± 0.021
Water soluble extractive value	18± 0.002

3.6. Phytochemical screening and standards

Preliminary phytochemical screening mainly revealed the presence of amino acid, carbohydrates, flavonoids, Fixed oils, proteins, Saponins, steroids, tannins and vitamins. Different chemical standard such as Total protein content, phenolic content, flavonoid, Proanthocyanidin, available carbohydrate, crude lipid, crude fiber and total mucilage (Table 4 and 5).

Table 4

Heavy metal and mineral content analysis of *E.herbacea* tubers

Heavy metal &mineral content	Values
Arsenic (Ar)	N.D
Cadmium (Cd)	0.85mg/kg
Lead (Pb)	2.5mg/kg
Mercury (Hg)	N.D
Calcium (Ca)	1.71%
Potassium (K)	2.48%
Magnesium(Mg)	2.48%
Iron (Fe)	164 mg/kg
Sodium (Na)	3.42%

3.7. HPTLC study of amino acid

HPTLC study of various amino acids in methanol extracts of *E.herbacea* are presented in Figure 4 ;Ninhydrin spraying reagent was used as a developer. HPTLC studies of methanol extract revealed thirteen peaks at various Rf, Out of these, the most prominent peak of maximum area was at Rf 0.18,0.20,0.23 and 0.90 corresponding to that of marker

Table 6

GC- MS analysis of methanolic extract of *Eulophia herbacea* tubers

Compound	Mol .Formula	Rt (min.)	Conc.	M+	Base
Dehydroabietane	$C_{20}H_{30}$	24.76	9.11	270	255
Dehydroabietic acid	$C_{20}H_{28}O_2$	25.90	8.23	300	285
1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	26.22	4.02	256	57
3′,8,8′-Trimethoxy-3-piperidyl-2,2′-binaphthalene-1,1′,4,4′-tetrone	$C_{28}H_{25}NO_7$	26.75	7.48	487	149
β -Sitosterol	$C_{29}H_{50}O$	29.36	8.19	414	43
tert-Hexadecanethiol	$C_{16}H_{34}S$	30.78	7.86	257	57
Stigmasterol	$C_{29}H_{48}O$	32.17	6.38	412	55
3-Eicosene	$C_{20}H_{40}$	35.48	2.10	280	57

compound alanine, Threonine, serine and aminobutyric acid . The other peaks at Rf 0.36, 0.45, 0.60, 0.70, 0.72, 0.74, and 0.80 were also significantly prominent.

3.8. GC-MS analysis of Petroleum ether extract

GC-MS analysis of extract gives the idea about the presence of nature of chemical compound in the extracts. The results showed the presence of Dehydroabietane, Dehydroabietic acid,1-Hexadecanol, 2-methyl-,3′,8,8′-Trimethoxy-3-piperidyl-2,2′-binaphthalene-1,1′,4,4′-tetrone, β -sitosterol, tert-hexadecanethiol, stigmasterol,3-eicosene (Table 6).

Table 4

Preliminary Phytochemical Investigation of tubers extracts

Test	Pet. Ether Extract	Methanolic Extract	Aqueous Extract
Test for Carbohydrates	-	+	+
Test for Proteins	-	+	+
Test for Alkaloids	-	-	-
Test for Glycosides	-	-	-
Test for Saponins	-	-	+
Test for Flavonoids	-	+	+
Test for Tannins &phenolic	-	+	+
Test for Amino acids	-	+	+
Test for Steroids	+	+	-
Test for Fat & oil	+	-	-
Test for Mucilage	-	-	+
Test for Vitamins			
B1 (Thiamine)	-	+	+
C (Ascorbic acid)	-	+	+
E (Tocopherol)	-	+	+

(+) present, (-) absent

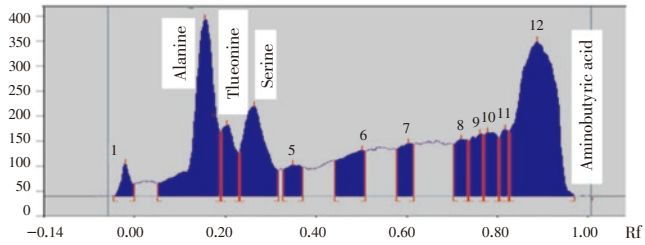

Figure 4. HPTLC chromatogram of amino acid; alanine, threonine, serine and aminobutyric acid present in *E.herbacea* extract recorded at 366 nm after treatment with Ninhydrin reagent, n-butanol: acetic acid: water (BAW) as solvent system.

Table 5

Phytochemical Standards of *Eulophia herbacea* tubers

Parameter	%(Mean± SEM)
Total protein	5.238±0.023
Total mucilage	22.000±1.45
Total carbohydrate	43.420±0.004
Total phenolic	12.600±0.028
Total flavonoids	7.746±0.023
Total proanthocyanidin	2.100±0.020
Total saponin	3.500±0.011
Crude lipid content	1.531±0.025

4. Discussion

In tubers some diagnostic characters are present in order to identifying the plant material. Some reliable characters like fibro vascular bundle, xylem and phloem were found in plant. The other commonly applied parameter for the identification is estimation of ash value, which establishes the quality and the purity of the drug. Ash value can also detect the nature of the material added to the drug for the purpose of adulteration [19] .The extractive values in different organic solvents is based on the quantity, which are soluble in them. It finds variation in the chemical constituents may cause a change in the extractive values. The variation in the extractive values may be possible due to the presence of specific compound, according to the solubility. The percentage weight of loss on drying, which is an indication of the moisture content of the material. The crude fiber is composed of many different compounds like cellulose, hemicelluloses and wood wool. Most of them are

polysaccharides. Generally, the herbal drugs are used in powder form and adulteration in the powdered drug can be detected by observing the powder under the ultraviolet light, because the fluorescence characteristic of any powder drug is very distinctive and helpful in distinguishing features for the determination of a drug. Microscopic and physicochemical studies are carried out on herbal crude drugs sample in order to establish appropriate data that may be utilized not only for identification but also to establish the purity and standard of plant sample, those supplied in powder form [20]. They are standard pharmacognostic parameters that can be used to differentiate closely related plant species or varieties with similar constituents or pharmacological activities. The phytochemical screening of the drug is a very responsive feature in the process of standardization and quality control because the constituent vary qualitatively and quantitatively not only from plant to plant but also in different samples of the same species depending upon various atmospheric factors and storage conditions. GC-MS detection, has found a variety of analytical uses, which are performing quality control analysis in the Pharmaceutical industries. In the last two decades HPTLC method has emerged as an important tool for the qualitative and quantitative phytochemical analysis of herbal drugs and formulations. This analysis is the first step towards understanding the nature of active principles and their detailed phytochemistry [21].

In the conclusion, present study on pharmacognostic evaluation of *E.herbacea* will provide useful data for identification. macroscopy, microscopy and physiochemical standards and phytochemical investigation discussed here which may help in authenticating the genuine plant along with nature of phytoconstituents present in the single drug and in polyherbal formulation.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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