

DEVELOPMENT OF PHARMACOGNOSTIC AND PHYTOCHEMICAL STANDARDS FOR PSEUDOBLUB OF *BULBOPHYLLUM NEILGHERRENSE*

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ABSTRACT

Pseudobulb of *Bulbophyllum neilgherrens* Wight is used by the folklore traditions medicinally. No data is available on pharmacognostical and phytochemical standards of the pseudobulb. Pharmacognostical standards including macroscopic study, microscopic study, powder analysis and histochemical tests along with physico chemical parameter study, fluorescence analysis, qualitative and quantitative chemical assay, chromatographic fingerprinting were developed for the pseudobulb of this orchid plant in the present study. Macroscopical study showed the presence of rhizome with nodes and internodes. Solitary pseudobulb arises from the nodes of rhizome. Microscopical study showed the presence of outer thick cuticle followed by parenchymatous epidermis. Mucilage cells and raphides seen in the ground tissue are identifying features. The vascular bundles are conjoint, collateral and closed type. Powder analysis showed annular, spiral and scalariform thickened vessels, acicular crystals, fragments of pitted vessel, oil globules, starch grains, tracheids and fibers. Qualitative phytochemical assay showed the presence of alkaloids, tannins, phenols, flavonoids, steroids, saponin glycosides and reducing sugar. Tannins, sugars and alkaloids were found to be 0.828%, 8.96% and 0.3% w/w respectively. Chrysin, a flavonoid attributed with anti hyperlipidemic and anti inflammatory activity has been detected in the methanolic extract of the pseudobulb through HPTLC finger printing.

Keywords: *Bulbophyllum neilgherrens*, pharmacognosy, phytochemical, orchids

INTRODUCTION

Orchids are used in indigenous system of medicine since time immemorial. The source of prime *Ayurvedic* drugs like *Jeevaka* (*Malaxis muscifera* Lindl.), *Rishabhaka* (*Malaxis acuminata* D. Don), *Riddhi* (*Habenaria intermedia* D. Don) and *Vridhhi* (*Habenaria edgeworthii* Hook. f. ex) are orchids.¹ *Dendrobium macraei* Lindl. is considered as one of the source drug for *Jeevanti* and another orchid species *Vanda roxburghii* R.Br. is considered as the

source for *Rasna* in many of the *Dravyaguna* materia medica.²

Bulbophyllum neilgherrens Wight. is an epiphytic monocot of Orchidaceae family abundantly available in Western ghats.³ The plant is endemic to South India, which occurs in plains and in higher elevations up to 900m⁴ mainly in forests of Karnataka⁵, Kerala and Tamilnadu.⁶ It is commonly known as *Pottlekai* in Kannada,⁵ *Kalmel pullurvi* in Malayalam³ and *Purusharantha* by the tribes.⁷ The special characteristic of

the plant is the presence of green, angled pseudobulb, which is a solid bulbous enlargement of the stem to preserve water and nutrients. Pseudobulbs in this orchid are 4cm long and 2cm across, smooth, green and four angled. Leaves 10cm-15cm long, 2-3cm broad, coriaceous, elliptic to broadly oblong, obtuse at apex, base narrowed tapering into short petiole attached to the pseudobulb.^{4,5} Scape stout, from the base of the pseudobulb. Flowers in racemes, petals small, pale yellow, lip purple.⁸

Pseudobulb of *B. neilgherrense* is used by the folklore traditions for restoration of adolescence and as tonic in the form of juice,⁹ consumed to attain good health by *Valmiki*s of Visakhapatnam district.¹⁰ Paste prepared from pseudobulb and leaf is consumed along with cow's milk to treat leucoderma and heart diseases.⁹

The methanolic extract of *B. neilgherrense* leaf, stem and root showed the presence of alkaloids, tannins and phenols. Water extract of leaf showed reducing sugars and saponin glycosides.⁹ In vitro study of leaf and pseudobulb of *B. neilgherrense* showed antibacterial effect in ethanol extract, chloroform and aqueous extracts.¹¹ On a perusal of literature it was found that pharmacognostical and phytochemical standards for pseudobulb of *B. neilgherrense* is yet to be developed. Pseudobulb being useful part of the plant, pharmacognostical studies along with physico-chemical and phytochemical studies are carried out. Chrysin is a flavonoid found in orchids like *Bulbophyllum odoratissimus* Lindl. and *Cypripedium macranthos* Sw.¹² which has broad spectrum biological activities.¹³ Chrysin is taken as a marker compound to develop chromatographic fingerprinting.

MATERIALS AND METHODS

Whole plant was collected from its natural habitat in Puttur TQ, Karnataka, India during April 2012. Botanical identity was confirmed by the botanist. A herbarium specimen is preserved in the Pharmacognosy laboratory, IPGT&RA, Gujarat Ayurved University, Jamnagar (voucher specimen number 6025\2012). Pseudobulbs were collected, cut into small pieces and dried in shade. Dry material was ground and sieved in mesh 80# (sample).

Macroscopic evaluation of fresh pseudobulb was done. Size, shape, color and texture were noted in detail. Organoleptic study of the pseudobulb powder was carried out for determining its color, odor, taste and texture.¹⁴

For microscopic evaluation, free hand sections of the fresh sample were taken and washed with chloral hydrate solution. Sections were first observed in distilled water, later stained with phloroglucinol and conc. HCl. Pseudobulb powder was viewed under microscope for the detection of various cells and their contents. Photomicrographs were taken by Carl zeiss trinocular microscope.^{15,16}

Thick sections were treated with various reagents to locate chemical constituents such as tannin, mucilage, lignin and calcium in histochemical tests.¹⁷

Physicochemical evaluation including loss on drying, ash value, extractive values and P_H of the sample was done.¹⁸

Fluorescence analysis of the sample was carried out by observing under day light and ultra violet light after treatment with various chemical and organic reagents like methanol, chloroform, hexane, 6N HCL, 4% NAOH.¹⁹

Qualitative assay for the presence of reducing and non reducing sugars, proteins, amino acids, steroids, saponin glycosides, alkaloids, tannins and phenolic compounds were done.^{20,18}

Tannins, sugars and alkaloids were quantitatively assessed in 5g, 2g and 5g of the sample respectively by the following methods^{21,22}

- Estimation of tannins- Stock solution of the sample was prepared using 5 g of sample in 100 ml of distilled water. 10 ml of this stock solution was treated with 25 ml of indigo carmine solution and diluted. Titration is done with $KMNO_4$ and percentage of tannin in was estimated.
- Estimation of sugar- Lead acetate was used to remove tannins from the water extract of sample. Sodium oxalate was used to remove excess of Lead acetate. Filtrate was collected and used for sugar estimation. Total and reducing sugar estimation was performed using Fehling solution with standard titrimetric method.
- Estimation of alkaloids- Methanolic extract of the sample was prepared. It was acidified with 6N HCL and liberated fraction was removed. Aqueous phase was basified using 10% NH_4OH . Organic phase was collected, evaporated to dryness and weighed. Percentage of alkaloids was calculated.

HPTLC¹²

HPTLC study was carried out for screening chrysin which is one of the target analyte in *Bulbophyllum neilgherrense*. For the aimed purpose, chrysin (97% purity) was procured from Sigma Aldrich. Chrysin was taken as reference material and methanol

extract of chrysin was compared with the sample i.e. methanol extract of *B. neilgherrense* pseudobulb. HPTLC analysis was performed on aluminum plates pre-coated with silica gel 60F₂₅₄ (Merk, Germany). Extract was applied on the plate of 10x10 cm as bands of 10 mm width of each with the help of CAMAG linomat IV sample applicator. The plate was developed in a CAMAG twin-trough chamber previously equilibrated with a mobile phase for 20 min. Solvent system of flavonoids i.e., chloroform: methanol: formic acid (8.8:0.7:0.5)V\V was selected. Plate was developed up to 8 cm, air dried and scanned at wavelength of 254 & 366 nm using CAMAG TLC Scanner 3. The chromatograms were recorded as densitographic profile under UV radiation. Later on derivatization was carried out for this plate and was sprayed with vanilline sulphuric acid. Color reaction was observed for sample and standard.

RESULTS AND DISCUSSION

Macroscopic characters-[Fig. 1]

Fresh pseudobulbs are cone shaped, smooth and succulent and are tetragonal on maturing, measuring about 3-3.5 cm in length and 1.5-2cm in breadth. Base of the pseudobulb is tufted with wiry fibrous roots and these bulbs are interconnected by rhizomatous stem. At the apex, it bears a single flattened leaf. Pseudobulbs appear green, shiny and smooth when young; on maturity it develops angles and wrinkles, gradually changing to yellow color on ageing. Presence of solitary cone shaped four angled pseudobulb differentiate this species from other *Bulbophyllum* members.



Fig. 1: A) *B. neilgherrense* in its natural habitat, B) Pseudobulb attached to leaf and stem.

Figure 1: Macroscopic Characters

Microscopic characters-[Fig. 2]

Transversely cut section of the pseudobulb is tetragonal to circular in outline, with number of vascular bundles distributed all over the ground tissue [Fig 2A]. Detailed study shows the outer epidermis layer is made up of single layered barrel shaped compactly arranged parenchyma cells interrupted by the presence of sunken stomata at some places [Fig 2B]. Epidermis is covered with thick waxy suberized cuticle [Fig 2C]. Hypodermis is 2-3 layered with compactly arranged sclerenchyma cells. Ground tissue is mostly made of loosely arranged parenchyma cells, interrupted by mucilage containing cells and raphides. Parenchyma cells are pitted, banded, lignified or beaded with mesh like network [Fig 2D]. Some cells contain simple starch grains, oil globules and acicular crystals of calcium oxalate [Fig 2E, 2F, 2G].

Vascular bundles are abruptly distributed all over the ground tissue. Towards the periphery vascular bundles are smaller in size and more in number where as towards the centre they are bigger size and less in number. Each vascular bundle is conjoint collateral and closed [Fig 2H]. Xylem in each bundle is arranged in Y shape with two big metaxylems, tailed by some smaller protoxylem with vacuoles. Phloem is present above the xylem consisting of

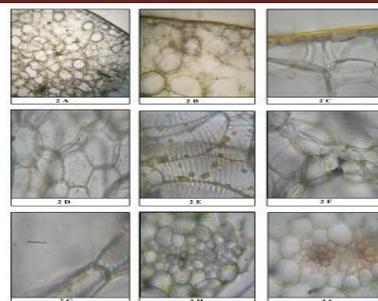


Fig. 2: Microscopic characters: A) TN of pseudobulb, B) parenchyma cells, C) thick waxy cuticle, D) band of sclerenchyma cells, E) parenchyma cells with oil globules, F) starch grains, G) acicular crystals, H) Vascular bundle, I) Companion cells.

Figure 2: Microscopic Characters

sieve tubes and companion cells. Each vascular bundle is surrounded by sclerenchymatous sheath [Fig 2I].

Epiphytic orchids are provided with specialized morphological characters like epidermis with thick waxy cuticle and sunken stomata to check the transpiration, pseudobulbs for storage of water and nutrients. Ground tissue composed of more number of mucilage cells greatly help in protecting the surrounding cellular constituents. Typical distribution of vascular bundles, presence of raphides, starch grains, oil containing cells, mucilage cells, banded parenchyma, and tannin materials are the characteristics of *B. neilgherrense* pseudobulb which will further aid in authentication and standardization of the sample.

Powder analysis [Fig. 3]

Organoleptic evaluation of the pseudobulb powder showed brownish colour, without characteristic smell. On touch, the powder was fibrous and on putting into mouth it was mucilaginous without characteristic taste.

Diagnostic characters detected under microscope were annular, spiral and scalariform thickened vessels [Fig 3A], acicular crystals [Fig 3B], fragments of pitted vessel [Fig 3C], lignified fibers with wide lumen [Fig 3D], mucilage cell, lignified parenchyma cells with oil globules

[Fig 3E], simple starch grains [Fig 3F], pitted parenchyma cells [Fig 3G], tannin

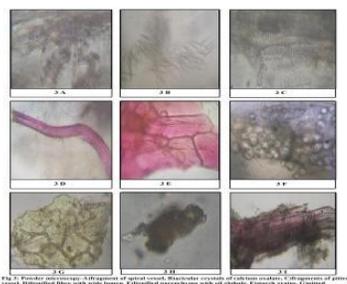


Figure 3: Powder Microscopy

Histochemical tests

The results of various histochemical tests conducted on the pseudobulb powder

[Fig 3H], tracheids and fibers [Fig 3I].

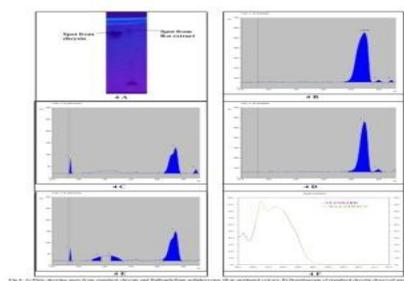


Figure 4: HPTLC

confirmed the presence of lignin, starch grains, calcium oxalate crystals, tannin and mucilage [Table1].

Table 1: Histochemical test results

Sl. No.	Reagent	Observation	Characteristics	Result
1	Phloroglucinol+Conc. HCl	Red	Lignified cells	++
2	Iodine	Blue	Starch grains	++
3	Phloroglucinol+Conc. HCl	Dissolved	Calcium oxalate crystals	++
4	Fecl3 solution	Dark blue to black	Tannin cells	++
5	Ruthenium red	Red	Mucilage	++

Physicochemical evaluation

Results of physicochemical parameters are given in Table 2. Presence of low moisture content and absence of foreign matter is observed. Acid insoluble ash is very less compared to total ash content. Low total ash and acid insoluble ash signifies low level of inorganic matter and silica content. Water soluble extractive value is higher

indicating the presence of high water soluble constituents. The drug is acidic with p^H being 5. Fluorescence analysis in visible light and UV light at 254 nm and 366 nm when treated with different chemical reagents is depicted in Table 3. These parameters help in detecting adulteration and identification of the purity of the sample.

Table 2: Physico chemical parameters

Sl. No.	Parameters	Pseudobulb
1	Foreign Matter	Nil
2	Loss on Drying % w/w	3.045
3	Total Ash Content% w/w	4.2
4	Acid Insoluble Ash % w/w	0.1
5	Water Soluble Extractive Value % w/w	30
6	Alcohol Soluble Extractive Value % w/w	6.2
7	pH	5

Table 3: Fluorescence analysis

Treatment	Observation under		
	Ordinary light	UV light	
		254 nm	366 nm
1 g powder + Methanol	Yellow	Purple	Purple
1 g powder + Water	Yellow	Colorless	Fluorescent green
1 g powder + Chloroform	Orange	Purple	Pinkish
1 g powder + Hexane	Light orange	Colorless	Purple
1 g powder + 6 N HCL	Yellow	Brown	Green
1 g powder + 4% NaOH	Orange	Colorless	Fluorescent green

Alkaloids, tannins and phenols were found in water and methanolic extract of the pseudobulb. Water extract also showed the presence of saponin glycosides and reducing sugar. Flavonoids and steroids were also detected in the methanol extract [Table 4]. These findings will aid in establishing standards for the drug. Quantity of alkaloids,

tannins and sugars are shown in Table 5. Percentage of non reducing sugar is higher than the reducing sugar. Quantification of compounds ascertains purity, identity and standardizes the drug. Alkaloids and flavonoids are most important orchid chemicals for their biological properties.

Table 4: Qualitative analysis

Sl no	Parameters	Result
1	Alkaloids (M.E.)	+
2	Alkaloids (W.E.)	+
3	Saponin Glycosides (W.E.)	+
4	Tannins & Phenols (M.E.)	+
5	Tannins & Phenols (W.E.)	+
6	Flavonoids (M.E.)	+
7	Steroids (M.E.)	+
8	Reducing sugar(W.E.)	+

M.E-Methanol extract; W.E- Water extract

Table 5: Quantitative estimation

Sl. No.	Parameter	% w/w
1	Tannin	0.828
2	Reducing Sugar	2.12
	Non Reducing Sugar	6.84
3	Alkaloids	0.3

HPTLC fingerprinting

HPTLC profiling was done for screening of chrysin in sample. Chrysin was detected in sample using silica gel F₂₅₄

HPTLC pre coated plates with mobile phase chloroform:methanol:formic acid (8.8:0.7:0.5)V/V [Fig 4A]. In 254nm short wave UV, 3 spots were detected in standard

chrysin at R_f 0.77, 0.87 and 0.97, and in plant extract also 3 spots were detected at R_f 0.01, 0.79 and 0.97 [Fig 4B, 4C]. 2 spots were detected with R_f 0.77 and 0.88 in chrysin where as 4 spots were seen in plant extract with R_f 0.01, 0.24, 0.33, 0.79 at long wave 366 nm [Fig 4D, 4E]. Spectral comparison of standard chrysin and plant extract showed overlay at R_f 0.07 [Fig 4F] which indicate the presence of chrysin in the pseudobulb.

Polyphenols like chrysin, pinobanksin seen in orchid species of genus *Bulbophyllum* may have antihyperlipidemic effect.²³ This supports the use of drug in heart diseases by the folklore traditions. Chrysin is also reported to have anti inflammatory effect by inhibiting COX-2 expression and via IL-6 signalling²⁴ and also considered to have broad spectrum biological activities¹². Developed pharmacognostical and phytochemical standards may help in authenticating the drug thereby introducing new herb useful in the management of certain lifestyle diseases.

CONCLUSION

Bulbophyllum neilgherrense Wt., a less known orchid, endemic to South India is used medicinally by certain tribes. The pharmacognostical and phytochemical study reports may help in establishing standards in identity, purity and quality of the drug. Quantification of chrysin, detection, isolation and quantification of other bio-active principles are the scope for further research on this plant. In the light of reported phytoconstituents of *B. neilgherrense*, there is a need for experimental and clinical studies to provide scientific validation of the recorded folklore claims.

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