Macro-Micromorphological, Antioxidant and Preliminary Phytochemical Studies on Two Terrestrial Orchids *Eulophia Nuda* Lindl. and *Geodorum Densiflorum* (Lam.) Schltr.

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Abstract An attempt for study of two terrestrial orchids i.e. *Eulophia nuda* (Lindl.) and *Geodorum densiflorum* (Lam.) Schltr. were carried out. Main focus was on the corms of both the orchids due to its huge medicinal properties like anticancerous, antioxidant, antidiabetic, antimicrobial, phytotoxic, etc. This is one of the prime reasons behind their threatened status along with endemic habitat. The parameters which were analysed during the study include macro- and micro-morphology, preliminary phytochemistry, and antioxidant activity (DPPH activity). In macro-morphology of both the orchids, slight difference was reported and, thus sometimes *Geodorum densiflorum* is being used as an adulterant in place of *Eulophia nuda*. During micro-morphological analysis, the oxalate crystals, raphides, mucilage ducts, starch grains, and oil granules were observed from cortical region of corm and in the mesophyll tissue of leaves in both the orchids. Stomatal Index of leaf surface was calculated along with the type of stomata, i.e. Cyclic II type. Preliminary analysis of phytochemical constituents in both the orchids was performed with three different solvent systems. A comparative antioxidant activity between leaves extract of both the orchids was carried out.

Keywords Orchid, Amarkand, Epigynous, Macro-Micromorphological, Stomata, Medicinal Properties, Preliminary Phytochemistry, Antioxidant

Activity, DPPH Free Radical Scavenging Assay

1. Introduction

The two taxa under investigation belong to the family Orchidaceae, which is one of the largest and diversified families among angiosperms [1].

- Eulophia nuda* Lindl (= *E. spectabilis* (Dennst.) Suresh) a terrestrial orchid, which usually grows in shady places along with grasses and shrubs in forests. In ayurveda, it is known as Amarkand [2]. Its corms are medicinally important and are used for curing bronchitis, tumours, scrofulous glands, and also used as vermifuge. Anticancerous, anti-oxidant, and antibiotic properties are reported [3, 4, 5]. Due to overexploitation, it is under threatened status. Due to the similar morphology of their corms, *Geodorum densiflorum* corms are used as adulterant.
- Geodorum densiflorum* (Lam.) Schltr. is mostly found in wet habitats like rainforests, moist deciduous forests, and grasslands with mostly terrestrial habit. In India, it is distributed in Kerala, Karnataka, Tamil Nadu and Maharashtra [6]. The corm extracts also shows antioxidant, cytotoxic, and

antibacterial potentials [7, 8].

In orchids, the flower is modified and diversified among the angiosperms such as zygomorphic flowers, which show variously modified labellum, epigyny, reduced stamens adhering with style and stigma to form a column (Gynandrium), pollens bound together to form a mass called as pollinia. Orchid seeds are very minute [9]. The underground fibrous corms contain important secondary metabolites like triterpenoids/steroids, saponins, proteins, anthraquinones, anthocyanins, etc [10, 1]. Out of these phenanthrene derivatives are isolated from corms of *Eulophia nuda* i.e. Nudol possess anti-cancerous, antioxidant, and antimicrobial, anti-inflammatory, antiallergic, phytotoxic properties [4, 11]. Phenanthrenes are tricyclic aromatic hydrocarbon (isomeric with anthracene) insoluble in water but soluble in most organic solvents like toluene, carbon tetrachloride, ether, chloroform, acetic acid, and benzene [11].

2. Materials and Methods

2.1. Collection of Plants

Primary step was scanning of probable regions for the availability of these two orchids- *Eulophia nuda* and *Geodorum densiflorum*. *Eulophia* was collected from Kolhapur and Melghat forest, Amravati; while *Geodorum* from Ghodazari forest (Chandrapur District) and Rawanwadi, (Bhandara District). The collected plants were identified with the help of standard literature like Flora of Maharashtra and Flora of Kolhapur [12].

2.2. Morphological Characterization

2.2.1. Macroscopic characters

Macro-morphological characters of stem, leaf, and corm were analysed and compared between the two orchids manually. Observed differentiating characters were noted.

2.2.2. Microscopic characters

For micro-morphological characters, thin transverse sections of fresh leaves and corms were taken and dipped in 70% ethanol for 2 minutes, stained with safranin for 30 minutes and then dehydrated at 95% ethanol plus 0.5% picric acid for 30 seconds. Then sections were washed with 95% ethanol and 4 drops ammonium hydroxide per 100 mL for 30 seconds. For final dehydration step dipped the sections in absolute alcohol for 10 seconds. Later counterstained with Fast green and washed with absolute alcohol to remove excessive stain. At last kept the slides in xylene solution for 20 seconds and mounted in glycerine then observed under microscope (Johansen, 1940) [13].

2.3. Preliminary Phytochemistry

The leaves and corms of both the orchids were dried and crushed for preliminary phytochemical analysis. Three different solvent systems were used for comparative study as used by Maurya and Dongarwar, 2009 [14]. All the preliminary phytochemical tests were performed according to the methods given by Sadasivam and Manickam, 1996 [15]; and Harborne, 1973 [16].

2.4. Antioxidant Analysis

DPPH Free Radical Scavenging Assay

In vitro antioxidant activity of the sample extracts analysed via DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical assay [17]. The sample stocks were diluted with methanol to prepare a concentration series of 25µL, 50µL, 75µL, 100µL, and 125µL. DPPH solution prepared by adding 1.3 mg/mL. To each concentration series 75 µL DPPH solution was added, shaken vigorously, and kept under dark for 30 minutes at room temperature. The decoloration of DPPH was determined by measuring the absorbance at 517nm using Shimadzu UV 1800 spectrophotometer. The percentage of scavenging is calculated by the following equation.

$$\text{DPPH Scavenged (\%)} = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100$$

3. Results

3.1. A. Macroscopic Characters

Stem of *Eulophia* is greenish yellow and that of *Geodorum* is green in color. Leaves are membranous; leaf surface is smooth in *Geodorum* whereas it is rough in *Eulophia*. Both the orchids have fibrous underground corms. In *Eulophia*, the corm shape is ovoid while, in *Geodorum* it is sub-globose. Size of tuber in *Eulophia nuda* is 3.5-4 cm (Figure 2); in *Geodorum densiflorum* around 3-3.5 cm (Figure 1).

Figure 1. *Geodorum densiflorum* and its corm.

Figure 2. *Eulophia nuda* and its corm

3.1. B. Microscopic Characters

For studying micro characters of *Eulophia nuda* and

Geodorum densiflorum, thin sections of corm, roots, and leaf were taken.

Corm: The section of both the orchids shows epidermis with multilayered velamen tissue (Figure 3A). In ground tissue, oxalate crystals, mucilage ducts, starch grains, oil granules, raphides, and scattered conjoint closed vascular bundles are present (Figure 3 A, B, C, D, F, G, H).

Leaf: It shows thin layer epidermis surrounding mesophyll tissue containing oil granules, raphides, and closed vascular bundles (Figure 3 E, I, J). Xylem and phloem are enclosed by sclerenchymatous bundle sheath cells (Figure 3 K).

Stomata: Stomata in both the orchids is of Cyclic II type, where there are four epidermal cells, two are elongated and parallel to the long axis of the guard cells while the other two are short and rectangular cells (Figure 3 L).

VT- Vilamen tissue, OG- Oil granule, OC- Oxalate crystal, MD- Mucilage duct, R- Raphides, VB- Vascular bundle, BS- Bundle sheath, Ph- Phloem, Xy- Xylem, SG- Starch grain, EC- Epidermal cells, St- Stomata.

Figure 3. A) T.S. of *Eulophia nuda* corm; B) Enlarged view with mucilage ducts, raphides, oxalate crystals and vascular bundles; C) T.S. of *Eulophia nuda* root with scattered vascular bundles; D) An enlarged conjoint closed vascular bundles enclosed by bundle sheath cells. E) T.S. of *Eulophia nuda* leaf with detailed view; F) T.S. of *Geodorum densiflorum* corm showing oxalate crystals (dark colored), oil granules, mucilage ducts, raphides, and vascular bundles; G) Enlarged view; H) Single vascular bundle; I) T.S. of *Geodorum densiflorum* leaf; J) Detailed view of the section; K) An enlarged vascular bundle, L) Stomata type of both the orchids.

Stomatal Index

Stomatal distributions on upper and lower leaf surface were calculated through stomatal index. The stomatal index is calculated by following formula:

Stomatal Index = $\text{No. of stomata per mm}^2 \times 100 \div \text{No. of stomata per mm}^2 + \text{No. of epidermal cells per mm}^2$.

According to the data presented in table 1 the lower leaf surface of *Eulophia nuda* shows 30.95% stomatal index and that of *Geodorum densiflorum* shows 29.26%. Whereas the upper leaf surface of both the orchids show zero stomatal index which indicates absence of stomata inspite of leaf being isobilateral.

3.2. Preliminary Phytochemical Analysis

The preliminary chemical tests of the leaf powder were carried out with three different solvents. Flavonoids, proteins, tannins, carbohydrates, phenols, quinones, terpenoids and steroids were present in all the three extracts of both the orchids. While saponins and anthocyanins were confirmed from methanolic and water extract of both the orchids; volatile oils from petroleum ether extract only of both the orchids. Few chemicals were completely absent in all the extracts like cardiac glycosides, anthrene glycosides, and coumarins (Table 2).

Table 1. Stomatal distribution on upper and lower leaf surfaces of both the orchid.

S.No.	Name of Plant	Lower Stomata			Upper Stomata		
		No. of Epidermal cells	No. of Stomata	Stomatal Index	No. of Epidermal cells	No. of Stomata	Stomatal Index
1	<i>Eulophia nuda</i>	249	105	30.95	289	0	0
		273	103		205	0	
		255	114		321	0	
		289	145		307	0	
		319	154		297	0	
		Average = 277	Average = 124.2		Average = 283.8		
2	<i>Geodorum densiflorum</i>	289	109	29.26	167	0	0
		205	116		227	0	
		321	129		169	0	
		307	120		133	0	
		297	113		149	0	
		Average = 283.8	Average = 117.4		Average = 169		

Table 2. The preliminary phytochemical observations

Phytochemicals	Chemical tests	Petroleum ether extract		Methanol extract		Water Extract	
		E. n.	G. d.	E. n.	G. d.	E. n.	G. d.
Flavonoids	Alkaline Reagent test	+	+	+	+	+	+
	Lead Acetate test	+	+	+	+	+	+
Terpenoids and Steroids		+	+	+	+	+	+
Proteins	a) Biuret Reaction	+	+	+	+	+	+
	b) Xanthoproteic Reaction	+	+	+	+	+	+
Tannin	a) Ferric Chloride test	+	+	+	+	+	+
	b) Lead Acetate test	+	+	+	+	+	+
Carbohydrates (reducing sugar)	a) Fehling's test	+	+	+	+	+	+
	b) Benedict's test	+	+	+	+	+	+
	c) Barfoed's test	+	+	+	+	+	+
Phenols		+	+	+	+	+	+
Cardiac glycosides		—	—	—	—	—	+
Anthrene glycosides		—	—	—	—	—	—
Saponins		—	—	+	+	+	+
Anthocyanins		—	—	+	+	+	+
Coumarins		—	—	—	—	—	—
Quinones		+	+	+	+	+	+
Volatile oils		+	+	—	—	—	—

3.3. Antioxidant Analysis

DPPH Free Radical Scavenging Assay

Free radical scavenging activity of petroleum ether and methanolic extracts of *Eulophia nuda* and *Geodorum densiflorum* leaves were measured by 1, 1-diphenyl-2-picryl hydrazyl (DPPH). Ascorbic acid was used as the standard reference compound. Lower absorbance of the sample extract indicated higher free radical activity (Table 3, 4). Here control absorbance was of DPPH (0.632).

Table 3. Absorbance of PE (Petroleum ether) and M (Methanol) extract of *Geodorum densiflorum* and *Eulophia nuda* at 517 nm by UV-Visible Spectrophotometer (DPPH assay).

Conc. (µg/mL)	Ascorbic acid	PE (E.n.)	PE (G.d.)	M (E.n.)	M (G.d.)
5	0.707	0.255	0.35	0.097	0.621
10	0.51	0.198	0.25	0.095	0.613
15	0.38	0.086	0.21	0.092	0.473
20	0.044	0.083	0.15	0.071	0.413
25	0.041	0.064	0.12	0.063	0.406
30	0.042	0.057	0.09	0.037	0.404

Table 4. Showing % inhibition of different PE (Petroleum ether) and M (Methanol) extract of *Eulophia nuda* and *Geodorum densiflorum* leaves with ascorbic acid as standard.

Conc. (µg/mL)	Ascorbic acid	PE (E.n.)	PE (G.d.)	M (E.n.)	M (G.d.)
5	7.94	59.65	45.98	84.65	1.74
10	33.59	68.67	60.44	84.96	3.01
15	50.52	86.39	66.77	85.44	25.11
20	94.27	86.86	76.26	88.76	34.65
25	94.66	89.87	81.01	90.03	35.75
30	94.53	90.98	85.75	94.14	36.07

4. Conclusions

In the present investigation, a detailed macro-micromorphological characteristics were studied, which revealed the difference in appearance of corm, stem, leaves in both the orchids. Micro-morphology revealed the internal arrangement of cells and presence of various substances like oil granules, oxalate crystals, starch grains, mucilage ducts. Transverse section of corm shows the presence of scattered closed vascular bundles enclosed by sclerenchymatous bundle sheath cells. The preliminary phytochemical study of leaf powder using three different solvent systems revealed the presence of flavonoids, terpenoids/steroids, proteins, tannins, carbohydrates, phenols, and quinones in all the three solvent systems. While volatile oils were observed only in petroleum ether extract; anthocyanins and saponins were observed in methanol and distilled water extracts. The antioxidant activity was analysed by DPPH free radical scavenging

assay with ascorbic acid as standard compound which showed better scavenging % in *Eulophia nuda* than *Geodorum densiflorum* in both petroleum ether and methanol extracts.

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