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Research Article

Phytochemical Profiling of Wild and *In vitro* Derived Hardened Tubers of the Endangered Medicinal Orchid *Eulophia nuda* Lindl.

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Abstract

Eulophia nuda Lindl., a terrestrial medicinal orchid valued in traditional medicine for treating tumours, respiratory ailments, skin disorders, gastrointestinal problems, and used as an appetizer, vermifuge, and aphrodisiac, is increasingly threatened in the wild due to overharvesting and habitat degradation. Given its ethnobotanical importance and declining natural populations, understanding its phytochemical composition is critical for both conservation and sustainable medicinal use. This study aimed to evaluate and compare the phytochemical profiles of in vitro derived hardened and wild-collected tubers to assess metabolite retention under controlled propagation. Tubers from in vitro derived hardened plantlets and mature wild plants were extracted with 80% methanol, followed by qualitative analysis using Liquid Chromatography–Mass Spectrometry (LC–MS). Ten major phenolic and flavonoid compounds were detected in wild tubers, including quercetin, kaempferol, rutin, apigenin, isorhamnetin, p-coumaric acid, chlorogenic acid, ferulic acid, caffeic acid, and gallic acid. In vitro–derived hardened tubers retained eight of these compounds, with apigenin and gallic acid absent, indicating a qualitative chemical similarity of 83.3% with wild tubers. The selective loss of certain compounds was attributed to the absence of environmental stressors and ecological cues in controlled hardening conditions. The study demonstrated that in vitro propagation effectively preserved core phenolic and flavonoid pathways while highlighting stress-dependent metabolite variation, providing insights for conservation-driven utilization and sustainable medicinal exploitation of this endangered orchid species.

Keywords: Conservation; Flavonoids; In vitro Propagation; LC–MS; Phenolic Compounds

Abbreviations

ESI- Electrospray Ionization; LC-MS: Liquid Chromatography–Mass Spectrometry; m/z: Mass-To-Charge Ratio

Introduction

Orchidaceae, the second largest family of flowering plants, comprises nearly 900 genera and 28,000–32,000 species, representing the pinnacle of plant evolution and ecological specialization [1]. Orchids display remarkable variation in growth habits, floral structure, pollination mechanisms, and habitat preferences, ranging from terrestrial to epiphytic forms, with many species relying on specific mycorrhizal fungi for successful germination [19]. Their high ornamental value, long-lasting blooms, and adaptability have positioned orchids as an economically significant group in global floriculture. However, this same diversity is coupled with ecological vulnerability, as habitat degradation, overexploitation, and slow natural regeneration threaten many species in the wild [3].

In India, orchids constitute an important component of native flora, particularly enriched in the Northeastern region and the Western Ghats [13]. Traditional medicinal systems across various cultures have long utilized orchids for therapeutic purposes, including treatments for inflammation, respiratory diseases, infections, wounds, and chronic ailments [16]. Despite their wide ethnomedicinal use, the pharmacological and phytochemical potential of many orchids remains insufficiently explored, limiting the scientific understanding necessary for their responsible utilization.

Among terrestrial medicinal orchids, *Eulophia nuda* Lindl. holds notable ethnobotanical importance. Commonly known as manya, salam mishri, or goruma, the species possesses underground globose pseudobulbs traditionally used to treat tumours, scrofulous glands, bronchitis, skin rashes, rheumatoid arthritis, acidity, blood impurities, gastrointestinal disorders, piles, and snake bites [21]. Its tubers are also valued as an appetizer, vermifuge, and aphrodisiac. Recent studies have reported the presence of phenanthrene derivatives exhibiting anticancer, antioxidant, and DNA-protective properties [14]. Phytochemical investigations reveal the presence of flavonoids such as apigenin, luteolin, kaempferol, and quercetin, alongside other secondary metabolites including terpenoids and stilbenoids [6,15]. These findings support the medicinal relevance of *E. nuda*, yet comprehensive phytochemical profiling of this species remains largely incomplete, particularly when comparing wild and *in vitro* derived hardened tubers.

Wild populations of *E. nuda* are under significant threat due to unscientific harvesting practices. The extraction of underground tubers for medicinal use eliminates the entire plant, causing drastic declines in natural populations. Combined with low natural seed germination (approximately 5%) and dependence on mycorrhizal fungi, the species exhibits limited capacity for natural regeneration [16,17]. Habitat loss, forest disturbances, and overexploitation further accelerate its decline. Many orchid species, including *Eulophia* spp., are protected under CITES regulations due to increasing pressure on wild populations [19]. This urgent conservation scenario highlights the need for sustainable strategies that allow continued medicinal use while preserving biodiversity. Given the species' high traditional importance, threatened status, and limited scientific understanding of its biochemical properties, a detailed phytochemical analysis of *E. nuda* is essential. Exploring the chemical constituents of wild and *in vitro* derived hardened tubers will not only validate medicinal claims but also support conservation through sustainable resource use. Therefore, the present study focuses exclusively on the phytochemical profiling of *E. nuda*, aiming to identify bioactive compounds, highlight its medicinal potential, and strengthen the scientific basis for conservation-driven utilization.

Materials and Methods

Sample preparation and extraction

Tubers were collected from two sources: *in vitro* derived hardened *E. nuda* plantlets after successful secondary hardening for 60 days in the shade house, and wild plants growing naturally in their habitat. Mature wild specimens of *E. nuda* were collected from the Halasi village forest area (15°31'50"N, 74°34'24"E) in Belagavi, Karnataka, India, and their identity was authenticated by Dr. A. N. Shringeshwar, Botanist at the Mahatma Gandhi Botanical Garden, University of Agricultural Sciences, GKVK, Bengaluru. This study

was conducted in the Plant Tissue Culture laboratory, Department of Horticulture, UAS, GKVK, Bengaluru, during 2023-2025. All tubers were thoroughly cleaned to remove soil, debris, and other associated organic matter. The cleaned material was chopped into small, uniform segments to enhance drying efficiency. To ensure rapid metabolic quenching, the chopped pieces were immediately immersed in liquid nitrogen. The frozen tissues were then freeze-dried using a lyophilizer for 48 hours until they became completely dry and brittle. After lyophilisation, the tubers were ground into a fine, homogeneous powder using a sterile mortar and pestle. For extraction, 50 mg of the powdered tuber material was transferred into microcentrifuge tubes and extracted with 1 mL of 80% methanol. The samples were vortexed thoroughly and subjected to sonication for 30 minutes to facilitate efficient release of metabolites. The extracts were centrifuged, and the resulting supernatant was carefully collected and filtered through a 0.22 µm syringe filter. All filtrates were stored at –20°C until further LC–MS analysis [7]. This extraction procedure ensured high-quality recovery and preservation of delicate metabolites from *E. nuda* tubers suitable for reliable phytochemical and metabolomic profiling.

LC–MS analysis

Qualitative phytochemical profiling, with particular emphasis on phenolic and flavonoid compounds, was performed using a Waters ACQUITY UPLC system coupled to a Waters Xevo G2-XS Q-Tof Mass Spectrometer (Waters Corp., Milford, MA, USA), following a previously established method [4]. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm) with a binary mobile phase consisting of water containing 0.1% formic acid as solvent A and acetonitrile as solvent B. A 30-minute gradient elution program was employed at a flow rate of 0.2 mL/min, and the column temperature was maintained at 35°C to ensure optimal resolution of polar to semi-polar metabolites.

Mass spectrometric detection was performed using an Electrospray Ionization (ESI) source operated in both positive and negative ion modes to allow comprehensive detection of a wide range of phytochemicals. The instrument was set to scan a mass range of m/z 100–1500 in MSE continuum mode, allowing for the simultaneous acquisition of high-resolution precursor and fragment ion spectra. Data processing, including peak extraction and integration, was conducted using MassLynx V4.1 software. Compounds were putatively identified by comparing their accurate mass values (m/z) and retention time with data available in the MassBank database and previously published literature [10,12]. This approach ensured reliable annotation of metabolites in both *in vitro* derived hardened and wild tubers of *E. nuda* in the absence of authentic standards.

Results and Discussion

Detection and comparative profiling of phenolic compounds

The LC–MS based phytochemical profiling of *in vitro* derived hardened and wild tubers of *E. nuda* revealed a diverse and pharmacologically relevant array of flavonoids and phenolic acids. Phenolic secondary metabolites are critical determinants of medicinal value in orchids, contributing to their antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, neuroprotective, and anticancer activities [5,21]. These compounds also function as ecological mediators, supporting defense against pathogens, tolerance to abiotic stresses, and interactions with pollinators and soil microbiota.

Profiling of wild tubers

A total of ten major compounds were detected in the collected wild tubers of *E. nuda*: quercetin, kaempferol, rutin, apigenin, isorhamnetin, p-coumaric acid, chlorogenic acid, ferulic acid, caffeic acid, and gallic acid (Table 1). This rich phenolic composition is consistent with previous reports on medicinal orchids where orchids such as *Orchis simia*, *E. herbacea*, and *E. ochreata* were found to accumulate structurally diverse flavonoids, hydroxycinnamic acids, tannins, and bibenzyl derivatives associated with protective and therapeutic functions [7,8,20]. The predominance of hydroxycinnamic acids, including p-coumaric, chlorogenic, ferulic, and caffeic acids, indicates a well-activated phenylpropanoid pathway in

Table 1: Qualitative analysis of phenolic compounds in the *in vitro* and wild tubers of *E. nuda.*

Tentative compound name	Ret. Time	m/z values	Wild tubers	*In vitro* tubers
Quercetin	2.78	303.05	+	+
Kaempferol	2.85	287.10	+	+
Rutin	2.55	633.30	+	+
Apigenin	3.10	271.10	+	-
Isorhamnetin	3.20	317.07	+	+
P- coumaric acid	1.80	163.04	+	+
Chlorogenic acid	2.10	353.10	+	+
Ferulic acid	2.10	193.05	+	+
Caffeic acid	3.33	179.30	+	+
Gallic acid	1.25	169.01	+	-

+ Present, - Absent, m/z values ($[M-H]^-$.

naturally growing plants. Gallic acid, synthesized via the shikimate pathway, was also present and has been commonly associated with enhanced environmental stress exposure [2].

Phytochemical profile of *In vitro* derived hardened tubers

In In vitro–derived hardened tubers preserved eight of the ten compounds identified in the wild tuber of *E. nuda*. Quercetin, kaempferol, rutin, isorhamnetin, p-coumaric acid, chlorogenic acid, ferulic acid, and caffeic acid were consistently detected (Figure 1). However, apigenin and gallic acid were absent. The absence of apigenin is attributed to the downregulation of flavone synthase (FNS) activity, a key biosynthetic enzyme strongly induced by UV-B radiation and environmental stress cues—factors that are largely minimized under controlled *in vitro* conditions [18]. Similarly, the absence of gallic acid suggests a reduced metabolic flux through the shikimate pathway. In the wild, this pathway is upregulated to drive the synthesis of defense-related phenolics; however, in the nutrient-rich, pathogen-free environment of tissue culture, the plant reallocates resources away from costly defense metabolite synthesis [9].

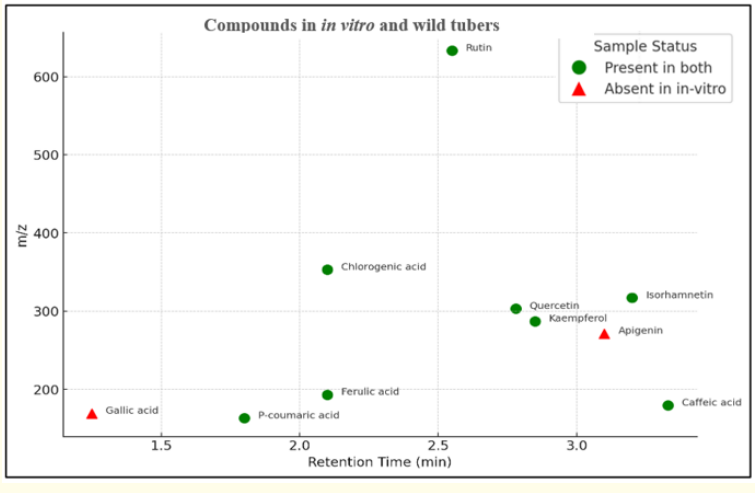

Figure 1: Detection of phenolic compounds in the *in vitro* derived hardened and wild tubers of *E. nuda.*

Comparative analysis and interpretation

Overall, the *in vitro* derived hardened tubers demonstrated a qualitative chemical similarity of 83.3% with their wild counterparts, indicating that the core phenolic and flavonoid biosynthetic pathways remain functionally active even under *ex situ* conditions. The preferential loss of apigenin and gallic acid underscores that certain metabolites are stress- or environment-dependent, requiring cues present in natural habitats but absent during *in vitro* hardening [4]. The high retention of quercetin, kaempferol, rutin, and caffeic acid demonstrates that the basal metabolic framework of *E. nuda* is conserved, ensuring maintenance of its major therapeutic properties.

The predominance of hydroxycinnamic acids in both wild and *in vitro* tubers indicates an active phenylpropanoid pathway, which serves as the precursor for flavonoid biosynthesis. The selective loss of metabolites *in vitro* reflects downregulation of stress-responsive enzymatic branches rather than a complete metabolic failure. Comparative studies in orchids, such as *Dendrobium* species, similarly reported environment-dependent variation in flavonoid and hydroxycinnamic acid profiles [11,22], highlighting the influence of ecological factors on secondary metabolite accumulation.

Conclusion

The comparative phytochemical analysis revealed that *in vitro*-derived hardened tubers exhibited a qualitative chemical similarity of 83.3% with wild *E. nuda* tubers, including quercetin, kaempferol, rutin, isorhamnetin, and key hydroxycinnamic acids. The absence of apigenin and gallic acid indicates that some metabolites require natural environmental cues such as light, stress, or microbial interactions. These results demonstrate that *in vitro* hardening effectively preserves the core phenolic and flavonoid pathways, supporting its potential as an *ex -situ* conservation strategy while maintaining medicinal value. Future work should quantify metabolites using LC–MS/MS and explore elicitation or environmental modulation to enhance retention of stress-dependent compounds.

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Conflict of Interest

Authors do not have any conflicts of interest to declare.

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