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Comparative phytochemical screening of *Rhynchostylis retusa* (L.) Blume from wild and *in vitro* origins: An investigation of secondary metabolites

Somaya Emrog Nayma, Md. Shahin Islam, Momena Khanam Mumu, Minhajur Rahman and Tapash Kumar Bhowmik*
Department of Botany, Faculty of Biological Sciences, University of Chittagong, Bangladesh

Abstract
Rhynchostylis retusa (L.) Blume is a medicinal epiphytic orchid valued in traditional medicine across South and Southeast Asia. Owing to habitat destruction, overharvesting and slow natural regeneration, *in vitro* culture techniques offer a promising alternative source for bioactive metabolites. This study comparatively evaluates the phytochemical composition of naturally grown and *in vitro* propagated *R. retusa* using qualitative screening techniques. Wild plant parts (leaf, stem, root) and *in vitro* derived tissues (plantlets, shoot buds, callus and SPSs) were analyzed for alkaloids, flavonoids, terpenoids, steroids, quinones, coumarins, tannins, saponins, phlobatannins, phenols, anthraquinones and cardiac glycosides. Marked differences were observed between wild and *in vitro* samples. Roots of wild plant parts exhibited maximal (+++) accumulation of terpenoids, quinones, steroids, flavonoids and coumarins, supporting their ethnomedicinal applicability. *In vitro* derived tissues accumulated higher levels of tannins and saponins (+++), suggesting culture induced modulation of the phenylpropanoid pathway. Glycosides were absent in all samples. The study highlights the phytochemical richness of *R. retusa* and confirms that tissue culture can generate valuable secondary metabolites, positioning *in vitro* systems as sustainable sources for pharmaceutical applications.

Keywords: *Rhynchostylis retusa*, regeneration, phytochemical screening, alkaloids, secondary metabolites

Introduction
Orchidaceae, one of the largest families of flowering plants, is renowned not only for aesthetic appeal but also for a wealth of bioactive constituents with ethnomedicinal significance [1-2]. More than 700 orchid species are used globally in traditional systems, particularly in Ayurveda, Chinese medicine and Southeast Asian folk medicine [3-4].
Rhynchostylis retusa (L.) Blume, commonly known as the "Foxtail Orchid," is an epiphytic, monopodial species widely distributed across the Indian subcontinent. Its roots, leaves, stems and flowers are traditionally used for managing fever, rheumatism, menstrual disorders, bone fractures, inflammation and respiratory ailments [5]. Pharmacological reports indicate antioxidant, antimicrobial and anti-inflammatory potential, attributed to diverse secondary metabolites including alkaloids, terpenoids, flavonoids and phenolic compounds [6-7].
Secondary metabolites in orchids are strongly influenced by ecological niche, environmental stress, developmental stage and symbiotic interactions [8-9]. *In vitro* culture offers controlled microenvironments that may enhance or suppress specific metabolic pathways; making tissue culture an important strategy for metabolite production, conservation and biotechnology [10-11]. Differences in biosynthetic profiles between *in vitro* and naturally grown orchids have been widely reported [12-13].
Despite its medicinal relevance, systematic phytochemical comparisons between wild and *in vitro R. retusa* remain rare. Understanding such differences is essential for establishing *in vitro* systems as viable alternative sources of bioactive compounds.

Materials and Methods
Plant materials
Wild specimens of *Rhynchostylis retusa* were collected from Mirsarai, Chattogram, Bangladesh. The species is characterized by a stout stem, distichous strap-shaped leaves and drooping racemose inflorescences with violet-tinged flowers [14].

In vitro culture
In vitro tissues, including callus, shoot buds, shoot primodia-like structures (SPSs) and plantlets, were developed on different media *viz.* MS [15], PM [16], VW [17] and KC [18] media supplemented with various concentrations and combinations of auxins and cytokinins. Surface sterilization of capsules for seed germination was performed using 0.2% $HgCl_2$ and 70% ethanol.

Phytochemical screening
Air-dried, powdered plant materials (wild leaf, stem, root and *in vitro* tissues) were extracted in methanol. Qualitative screening was performed using the following standard protocols:
- **Alkaloids:** Detected using Dragendroff's, Hager's, Mayer's, Wagner's and Tannic acid reagents [19-21].
- **Phlobatannins:** Detected by boiling extract with 1% aqueous HCl [22].
- **Flavonoids:** Ammonia test [22].
- **Saponins:** Frothing test [23].
- **Tannins:** Ferric chloride test [24].
- **Terpenoids and Steroids:** Salkowski test using chloroform and concentrated sulphuric acid [25].
- **Anthraquinones, Quinones and Coumarins:** Standard colorimetric tests [26].
- **Phenols:** Ferric chloride test [27].
- **Proteins:** Copper sulphate and ethanol test [28].
- **Cardiac Glycosides:** Glacial acetic acid and ferric chloride test [29].

Results
Alkaloid presence
Qualitative analysis confirmed the presence of alkaloids in both wild and *in vitro* tissues.

Wild plant parts: Roots exhibited the highest response (+++) to all five reagents. Stems also showed significant presence (+++), while leaves showed moderate to high reactions, particularly with Hager's and Tannic acid reagents (Table 1).

Table 1: Qualitative test for alkaloids of *Rhynchostylis retusa* (Wild plant parts)

Plant Parts	Qualitative estimation of alkaloids by different reagents				
	D	H	M	W	T
Leaf	++	+++	++	++	+++
Stem	+++	+++	+++	++	+++
Root	+++	+++	+++	+++	+++

Notes: Name of the reagents; D-Dragendroff's reagent, H-Hager's reagent, M-Mayer's reagent, W-Wagner's reagent and T- Tannic acid reagent

***In vitro* Tissues:** Shoot primodia-like Structures (SPSs) displayed the highest alkaloid intensity (+++) among cultured tissues, comparable to wild roots. Callus tissue showed high responses to Mayer's and Hager's reagents, while shoot buds exhibited moderate (++) precipitation across all reagents (Table 2).

Table 2: Qualitative test for alkaloids of *R. retusa* (*In vitro* plant parts)

In vitro Plant Parts	Qualitative estimation of alkaloids by different reagents				
	D	H	M	W	T
Callus	++	+++	+++	++	+++
Shoot bud	++	++	++	++	++
SPSS	+++	++	+++	+++	+++

Non-Alkaloid secondary metabolites

The distribution of other secondary metabolites varied significantly between wild plant parts and *in vitro* plantlets (Table 3).

Wild plant parts: Roots were the most chemically diverse, containing high amounts (+++) of terpenoids, quinine, steroids, coumarins and flavonoids. Stems also contained high levels of terpenoids, coumarins and flavonoids. Leaves contained moderate (++) amounts of terpenoids, quinine and flavonoids but lacked anthraquinones and glycosides.

***In vitro* plantlets:** Notably, *in vitro* developed plantlets synthesized the highest amounts (+++) of saponins and tannins, surpassing the levels found in wild samples. They showed moderate (++) levels of terpenoids, quinine, steroids and flavonoids. However, coumarin and anthraquinone levels were lower (+) compared to wild roots and glycosides were absent.

Table 3: Comparative qualitative phytochemical analysis

Phytochemical	Wild Plant Parts			*In vitro* Plantlets
	Leaf	Stem	Root	
Terpenoids	++	+++	+++	++
Steroids	+	++	+++	++
Coumarin	++	+++	+++	+
Flavonoids	++	+++	+++	++
Saponins	+	++	++	+++
Tannins	+	+	++	+++
Anthraquinones	-	+	+	+
Glycosides	-	-	-	-

Discussion

The study successfully established that *Rhynchostylis retusa* maintained under *in vitro* conditions retains the capacity to synthesize a broad spectrum of secondary metabolites. This aligns with findings by Molyneux *et al*. [30], who noted that orchids contain diverse phytochemicals including alkaloids, triterpenoids and flavonoids.

The ubiquitous presence of alkaloids across all tested samples, with particularly high accumulation in wild roots and *in vitro* SPSs, suggests active biosynthesis during organogenesis. This is consistent with observations in other medicinal orchids; for instance, Shrestha *et al*. [31] reported positive results for alkaloids and flavonoids in *Dendrobium amoenum*. Similarly, Banerjee *et al*. [32] conducted pharmacognostical evaluations of *Dendrobium ochreatum*, confirming the therapeutic potential of specific plant parts.

A key finding of this research is the elevated production of saponins and tannins in *in vitro* plantlets. It is hypothesized that the specific culture conditions, potentially the stress exerted by PGRs or the artificial environment, may up regulate the pathways responsible for these defense compounds. This phenomenon supports the findings of Bhowmik *et al*. [33], who observed sporadic and uneven occurrence of secondary metabolites in *Spathoglottis plicata* parts, indicating that tissue type and developmental stage significantly influence chemical profiles.

The absence of glycosides in *R. retusa* samples contrasts with their presence in other subfamilies, such as in *Vanilla planifolia*, which is famed for its glycoside vanillin [34-35]. However, the detection of coumarins and flavonoids in *R. retusa* underscores its potential antioxidant and anti-inflammatory properties, validating its traditional use in treating inflammation and skin diseases [36].

Conclusion

This comparative investigation confirms that *in vitro* propagation is a viable strategy for conserving *Rhynchostylis retusa* without compromising its phytochemical value. While wild roots remain superior sources of terpenoids and coumarins, *in vitro* plantlets show enhanced potential for saponin and tannin production. These findings concrete the way for utilizing micropropagation not only for conservation but also for the controlled production of bioactive compounds for pharmaceutical applications.

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Conflicts of interest

Authors declared that they have no conflict of interest.

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