

Chapter 7

Phytochemical and cytotoxicity analysis of tubers of *Habenaria reniformis* (Orchidaceae)

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Abstract: *Habenaria reniformis*, a terrestrial orchid, underwent phytochemical analysis and cytotoxicity evaluation. The hydroethanolic extract was found to contain tannins, saponins, terpenoids, phenolic compounds, reducing sugars, and alkaloids upon phytochemical screening. Furthermore, the extract exhibited substantial cytotoxic activity against brine shrimp nauplii, with a mortality rate of 100 % at concentrations of 50 mg/ml and higher. This study highlights the potential of *H. reniformis* as a source of bioactive compounds with cytotoxic properties, meriting further research into its medicinal applications.

Keywords: Bioactive compounds, pharmacological, terrestrial

INTRODUCTION

The increasing emergence of novel health issues and the decline in therapeutic efficacy of existing drugs, largely due to natural and anthropogenic factors, necessitate the exploration of uncharted botanical sources (Kumar *et al.*, 2012; Kumar *et al.*, 2013; Kumar and Jena, 2014; Dwibedy *et al.*, 2015; Murmu *et al.*, 2016; Kumar *et al.*, 2017; Sahoo *et al.*, 2021; Singh *et al.*, 2022; Aradhana *et al.*, 2022; Jaiswal *et al.*, 2024; Palei *et al.*, 2024; Hossain *et al.*, 2025). There is a pressing need to screen unexplored plant species for bioactive compounds and pharmacological agents to address these challenges (Tripathy *et al.*, 2015; Jena *et al.*, 2025). Orchid species, with their established medicinal properties, remain largely unexplored, making them ideal candidates for the discovery of novel therapeutic agents (Marndi *et al.*, 2024). Orchids, one of the largest and most diverse families of flowering plants (Devi *et al.*, 2018; Kumar and Thorat, 2018; Kumar *et al.*, 2018; Swain *et al.*, 2019;

[Devi et al., 2019a](#); [Devi et al., 2019b](#); [Devi et al., 2020](#); [Kumar et al., 2021](#); [Kumar and Kumar, 2021](#); [Kumar, 2022](#); [Kumar et al., 2022c](#); [Sharma et al., 2024](#)), have been used as therapeutic agents ([Chakraborty et al., 2022](#); [Kumar, 2023](#)). With over 30,000 species, orchids are a rich source of bioactive compounds ([Kumar and Kumar, 2020](#); [Kumar et al., 2022a](#); [Mety et al., 2022](#)). The importance of orchids as ethnomedicinal plants is documented ([Kumar and Kumar, 2020](#)). In many traditional medicine systems, they are considered valuable for their medicinal properties ([Panda and Mandal, 2013](#); [Śliwiński et al., 2022](#); [Bazzicalupo et al., 2023](#)). The unique bioactive compounds present in orchids have been shown to exhibit pharmacological values, making them a promising source of new medicinal agents ([Marndi et al., 2024](#)). *Habenaria reniformis*, ([Figure 1](#)), a member of the Orchidaceae family, is one such terrestrial unexplored orchid ([Kumar et al., 2022b](#)).



Figure 1: Flowers of *Habenaria reniformis*

The phytochemical and pharmacological properties of *H. reniformis* remain largely unexplored. The present research focused on analyzing the phytochemical profiling of *H. reniformis* and assessing its potential toxicity using a brine shrimp lethality test. The findings of this study will contribute to the understanding of the phytochemical and pharmacological properties of tubers of *H. reniformis* and provide a basis for further research on its medicinal applications.

METHODOLOGY

The tubers of *H. reniformis* were collected from the Rourkela Forest Division, Odisha, India (Figure 2). The plant species were identified by the authors. The phytochemical analysis was carried out using different solvents by using standard methods (Devi et al., 2023; Devi et al., 2024b).



Figure 2: Collection of tubers of *Habenaria reniformis*

Test for tannin: About 1 ml of the tuber extract was taken. About 3-5 drops of 10% lead acetate solution were added. The formation of gelatinous precipitate confirmed the positive results for the presence of tannin.

Test for saponin: About 1 ml of the tuber extract was taken, and 1 ml of distilled water was added and shaken well. The persistent froth formation confirmed the presence of saponin.

Test for flavonoids: About 1 ml of the tuber extract was taken. About 2 ml of 2% NaOH solution and then diluted HCl was added. The colour initially turned to an intense yellow with the NaOH solution and later became colourless. This colour-changing transformation confirmed the presence of flavonoids.

Test for terpenoids: About 1 ml of the tuber filtrate was added with 6 drops of chloroform and placed in the water bath for a few minutes. Then, 6 drops of concentrated sulphuric acid were added. The appearance of a reddish-brown interface confirmed the presence of terpenoids.

Test for phenolic compounds: About 1 ml of the tuber extract was taken, and a few drops of 5% ferric chloride solution were added to it. The dark bluish-black appearance confirmed the presence of phenolic compounds.

Test for reducing sugars: About 1 ml of the tuber extract was taken, and about 2-3 drops of Fehling's solution A and B were respectively added and then kept in the water bath for some time. The presence of red-orange precipitate confirmed the presence of reducing sugar.

Test for steroids: About 1 ml of the tuber extract was taken. 1 ml of chloroform and 1 ml of concentrated sulphuric acid were added to it. The appearance of 2 phases, with the upper red and lower yellow with green fluorescence confirmed the presence of steroids.

Test for alkaloids: About 1 ml of the tuber extract was taken, and about 3 to 4 drops of Dragendroff's reagent was added. The formation of a reddish-brown precipitate confirmed the presence of alkaloids.

Test for carbonyl compounds: About 1 ml of the tuber extract was taken, and about 3 to 4 drops of 2,4-dinitrophenylhydrazine (DNPH) reagent was added to it. The yellow crystal formation confirmed the presence of carbonyl compounds.

Toxicity to *Artemia salina*: For toxicity analysis, the hatching of brine cysts was the initial process. Standardization of the optimum was done at 2.0, 3.5, and 5.0 % saline concentration to check the optimum salinity for proper hatching of brine shrimp (*Artemia salina*) cysts. The brine shrimp cysts are then incubated in 3.5% saline water with proper aeration, at room temperature or between 28-35 °C, and light for 48 hours. Proper hatching could be observed within 24-48 hours depending upon the quality of the cysts, proper aeration, and light provided. Different concentrations (25 mg/ml, 50 mg/ml, 100 mg/ml, and 200 mg/ml) of the *H. reniformis* tuber extract were taken, and 3.5% saline water was used to make the volume 2 ml in each test tube. 1% DMSO was used to dissolve the crude extracts. Ten nauplii were selected and introduced to five test tubes of the extracts. Positive and negative controls were prepared using vincristine sulphate (5mg/ml) or potassium dichromate and 3.5% saline water, respectively, with a volume of 2 ml. The live larvae are highly mobile and thus differentiate from the unhatched cysts. The survivor nauplii were counted and analyzed for the death percentage (Kumar *et al.*, 2012; Nayak *et al.*, 2021; Rout *et al.*, 2024; Devi *et al.*, 2024a; Devi *et al.*, 2024b).

RESULTS AND DISCUSSION

The phytochemical analysis of *Habenaria reniformis* revealed the presence of various bioactive compounds in the aqueous-ethanolic extract. The results are presented in Table 1. The phytochemical screening indicated the presence of tannins, saponins, terpenoids, phenolic compounds, reducing sugars, and alkaloids in the hydroethanolic extract.

Table 1: Phytochemical analysis of the whole plant of *Habenaria reniformis* using hydroethanolic extract

Bioactive compounds	Hydroethanolic extract
Tannin	+ve
Saponin	+ve
Flavonoids	-ve
Terpenoids	+ve
Phenolic compounds	+ve
Reducing sugar	+ve
Steroids	-ve
Alkaloids	+ve
Carbonyl compounds	-ve

(+ve: Presence; -ve: Absence)

Table 2: Cytotoxicity analysis of the whole plant of *Habenaria reniformis* using aqueous-ethanolic extract

Extract	Concentration (in mg/ml)	Initial number of Nauplii	Number of deaths of Nauplii			Death rates (%)
			After 1 hour	After 2 hours	After 3 hours	
Aqueous-ethanolic extract	25	10	6	8	10	100
	50	10	7	10	10	100
	100	10	10	10	10	100
	200	10	10	10	10	100
	3.5% Saline	10	0	0	0	0
	Vincristine sulphate	10	10	10	10	100

The detection of tannins and phenolic compounds in the extract suggests that *H. reniformis* may possess antioxidant and antimicrobial activities (Takó *et al.*, 2020). Tannins have been reported to exhibit antimicrobial and anti-inflammatory activities, while phenolic compounds are known for their antioxidant and anticancer properties (Kaczmarek, 2020; Abotaleb *et al.*, 2020). The presence of saponins in the extract indicates that *H. reniformis* may possess antimicrobial activities (Timilsena *et al.*, 2023). Saponins have been reported to exhibit antimicrobial activities and have been used in traditional medicine to treat various ailments (Tagousop *et al.*, 2018). The cytotoxicity evaluation of the hydroethanolic extract using the brine shrimp lethality assay revealed significant cytotoxic activity. The results are presented in Table 2. The extract exhibited a death rate of 100% at concentrations of 50 mg/ml and above, indicating significant cytotoxic activity. The positive control, vincristine sulphate, also showed 100% cytotoxicity, while the negative control, 3.5% saline, showed no cytotoxicity. The cytotoxic activity of the extract may be attributed to the presence of bioactive compounds such as alkaloids, terpenoids, and phenolic compounds. These compounds have been reported to exhibit cytotoxic activity against various cancer cell lines (Matulja *et al.*, 2022). Previous studies have also explored the medicinal and pharmacological properties of *Habenaria* species. Parasher, (2021) documented the ethnomedicinal values of *Habenaria* species, and Kumar *et al.*, (2021) documented the orchid diversity of Odisha state and their medicinal and economic uses. Kumar *et al.*, (2023) reported the anti-NRLP3 inflammasome and immunomodulatory phytochemicals from the extract of *Habenaria intermedia*, while Asiri *et al.*, (2024) documented the antioxidant, anti-inflammatory, and antinociceptive effects of *H. aitchisonii*. Despite existing research on

Habenaria species, there is a notable gap in the literature regarding the phytochemical profiling and cytotoxicity evaluation of *H. reniformis*, indicating a need for further investigation.

CONCLUSION

The present study investigated the phytochemical profile and cytotoxic activity of *Habenaria reniformis*, a medicinal orchid. The phytochemical analysis revealed the presence of various bioactive compounds, including tannins, saponins, terpenoids, phenolic compounds, reducing sugars, and alkaloids. The cytotoxicity evaluation using the brine shrimp lethality assay showed significant cytotoxic activity, indicating the plant's potential as a source of anticancer agents. The study provides evidence for the medicinal properties of *H. reniformis*, supporting its traditional use in folk medicine. The bioactive compounds present in the plant may contribute to its antioxidant, antimicrobial, anti-inflammatory, and cytotoxic properties. Further studies are needed to isolate and characterize the specific compounds responsible for the plant's medicinal properties and to evaluate their potential as therapeutic agents.

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