Chapter 5

Phytochemical, cytotoxicity and TLC evaluation of leaves of *Hibiscus vitifolius* L. (Malvaceae)

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Abstract: Present study investigated the phytochemical composition, cytotoxic potential, and thin-layer chromatography (TLC) profile of the leaves of *Hibiscus vitifolius* L. of Malvaceae family. Preliminary phytochemical screening revealed the presence of bioactive constituents such as tannins, saponins, flavonoids and reducing sugars using different extracts (n-hexane, ethanol and aqueous). The cytotoxic activity was assessed using the brine shrimp lethality assay, indicating significant mortality rates for n-hexane and ethanol extracts, while aqueous extract exhibited moderate cytotoxicity. TLC analysis was performed using hydroethanolic extract to establish a phytochemical fingerprint, highlighted distinct spots corresponding to various secondary metabolites. The findings suggest that *H. vitifolius* leaves possess significant phytochemical and cytotoxic properties, warranting further investigation for potential therapeutic applications.

Keywords: Bioactive constituents, cytotoxic activity, phytochemical fingerprint, therapeutic applications

Introduction

In the past few decades, the world has witnessed a significant rise in chronic and lifestyle-related diseases such as cancer, diabetes, antibiotic-resistant infections, cardiovascular and neurodegenerative disorders (Senapati et al., 2015; Balwan and Kour 2021; Jain et al., 2024). Additionally, emerging infectious diseases, environmental pollution, and drug resistance have added complexity to global health challenges (Coque et al., 2025). Many synthetic drugs used to treat these conditions come with adverse side effects, high costs, and limited accessibility, especially in developing regions (Gunjan et al., 2015). This growing burden on modern healthcare systems highlighted the urgent need for alternative approaches to disease prevention and treatment. Sustainable utilization of medicinal plants offered a promising solution to these challenges (Kumar et al., 2018). Medicinal plants are reservoirs of diverse bioactive compounds such as tannins, saponins, flavonoids, reducing sugars and alkaloids that exhibited a wide range of pharmacological activities including antimicrobial, anti-inflammatory, antioxidant, anticancer, and antidiabetic properties (Ramawat et al., 2009). By promoting ethnobotanical research, phytochemical screening, and pharmacological validation, it is possible to discover new therapeutic agents that are both effective and safer than many synthetic drugs. The Malvaceae family, comprising over 200 genera and around 2,300 species, includes several plants known for their medicinal value (Raj et al., 2022). Members of this family are widely distributed in tropical and subtropical regions (Das and Islam, 2019) and have been used traditionally to treat a variety of ailments such as inflammation, wounds, infections, and gastrointestinal disorders (Abat et al., 2017).



Figure 1: Leaves and flower of *H. vitifolius*

Among them, *H. vitifolius* L., commonly found in parts of Asia and Africa, has drawn attention for its potential pharmacological properties (Jejurkar and Chavan, 2023). Traditionally, different parts of this plant have been used in folk medicine for treating fever, ulcers, and skin diseases (Kapoor et al., 2021). However, scientific validation of these uses through phytochemical and cytotoxic evaluations remained limited. By studying the leaf extracts of *H.*

vitifolius, present study aims to identified the key bioactive compounds and assessed their cytotoxicity, providing a foundation for further pharmacological investigations.

Methodology

The leaves of *H. vitifolius* were collected from nearby Mahanadi areas of CDA-13, Cuttack, Odisha, India. The plant species was identified by the authors (Figure 1). The maceration extraction method was adopted using different solvents (n-hexane, ethanol and aqueous) for experimental analysis (Figure 2). Detection of nine secondary metabolites was conducted using standard methods (Hossain et al., 2025; Rani et al., 2025). Details of phytochemical analysis is given below.

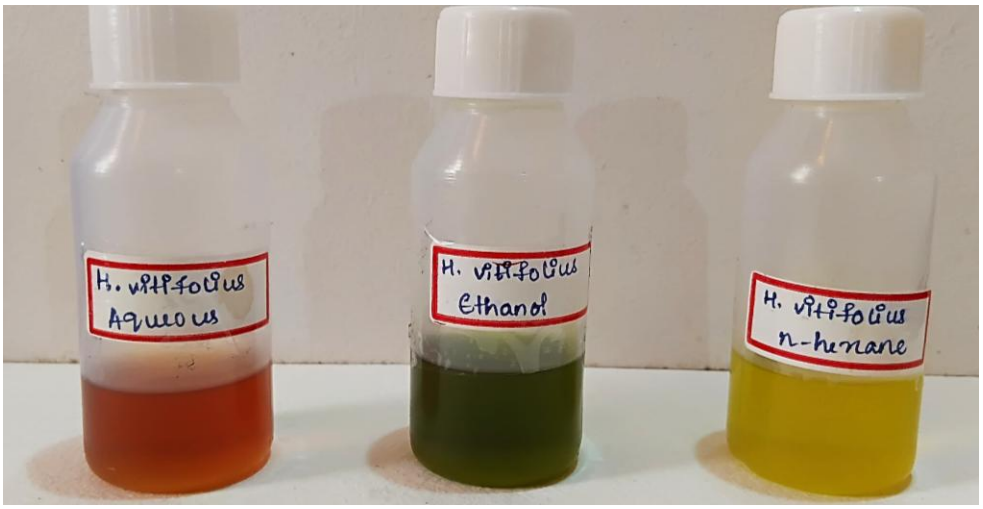

Figure 2: Filtrate extracts of *H. vitifolius* leaves for experimental analysis

Test for tannin: 1 ml of leaves extract was taken in a test tube, and 3-5 drops of 10% lead acetate solution was added to it. The gelatinous precipitate formation confirmed the presence of tannin.

Test for saponin: About 1 ml of distilled water was added to 1 ml of the leaves extract and shaken well. The formation of persistent froth was observed, confirming the presence of saponin.

Test for flavonoids: About 1 ml of the leaves extract was taken, and 2 ml of NaOH solution and 3-5 drops of dilute HCl were added to it. The color initially turned to an intense yellow color with NaOH solution and later became colorless, which confirmed the presence of flavonoids.

Test for terpenoids: To 1 ml of the leaves extract, 5-6 drops of chloroform were added. Then, 4-6 drops of concentrated sulphuric acid were added to it. The reddish-brown interface confirmed the presence of terpenoids.

Test for phenols: About 1 ml of the leaves extract was taken. A few drops of 5% ferric chloride solution were added. The dark bluish-black appearance confirmed the presence of phenolic compounds.

Test for reducing sugars: About 1 ml of the leaves extract was taken. 2 drops of Fehling's solution A followed by Fehling's solution B were added and kept in the water bath for a few minutes. The presence of red-orange precipitate confirmed the presence of reducing sugars.

Test for steroids: To 1 ml of the leaves extract, 1 ml of chloroform followed by 1 ml of concentrated sulphuric acid were added. The appearance of upper red and lower yellow with green fluorescence indicated the presence of steroids.

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

Test for carbonyl compounds: About 1 ml of leaves extract was taken, and 3-4 drops of 2,4-Dinitrophenylhydrazine (DNPH) reagent were added. The formation of yellow crystals confirmed the presence of carbonyl compounds.

Cytotoxicity analysis: For cytotoxicity test, hatching of Brine shrimp cysts was the initial process. Standardization of the optimum salinity was done at 2.0, 3.5 and 5.0 % saline concentration to check the optimum salinity for proper hatching of Brine shrimp (*A. salina*) cysts (Kumar et al., 2012). The brine shrimp cysts are then incubated in 3.5 % saline water with proper aeration, room temperature or between (28-35ºC) and light for 72 hours. Proper hatching could be observed within 24-72 hours depending upon the quality of cysts, proper aeration and light provided. Different concentrations (15.62 mg ml^{-1}, 31.25 mg ml^{-1}, 62.5 mg ml^{-1}, 125 mg ml^{-1} and 250 mg ml^{-1}) of the *H. vitifolius* leaves extracts were taken and 3.5% saline water were 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 3.5% saline water and vincristine sulphate (5 mg ml^{-1}) respectively with a volume of 2 ml. The live larvae are highly mobile and thus differentiates from the unhatched cysts. The survivor nauplii were counted and analyzed the death % (Nayak et al., 2021).

Chromatographic analysis: TLC plates were prepared on 9 cm glass slides using silica gel powder. Slides were washed with clinical laboratory detergent and dried. Clean and dried slides were wiped with ethyl acetate for removing surface adherents. 3 g of silica was taken in 20 ml of distilled water and slurry was prepared by constantly stirring and finally poured over the slides. The slides were left undisturbed till the drying of silica layer. Then the slides were activated at 50 ºC for 20 minutes before running the TLC (Mishra and Bhatnagar, 2024).

Methanol: chloroform (1:1), ethyl acetate: chloroform (1:9) and ethyl acetate: water: acetic acid (8:1:1) were used as mobile phases. Then spots were visualized under UV chamber and recorded the RF (Retention Factor) values (Kumar and Jena, 2014).

Results and discussion

The phytochemical analysis of *H. vitifolius* leaves revealed the presence of various bioactive compounds, including tannins, saponins, flavonoids and reducing sugars (Table 1: Figures 3-5). These compounds were found to be present in different extracts having various pharmacological potential. The presence of wide range of phytoconstituents suggests that *H. vitifolius* leaves may possess medicinal properties.

Table 1. Qualitative phytochemical analysis of *H. vitifolius* leaves using different extracts

Bioactive compounds	n-hexane	Ethanol	Aqueous
Tannin	Absent	Absent	Present
Saponin	Present	Present	Present
Flavonoids	Absent	Absent	Present
Terpenoids	Absent	Absent	Absent
Phenolic compounds	Absent	Absent	Present
Reducing sugars	Present	Present	Present
Steroids	Absent	Absent	Absent
Alkaloids	Absent	Absent	Absent
Carbonyl compounds	Absent	Absent	Absent

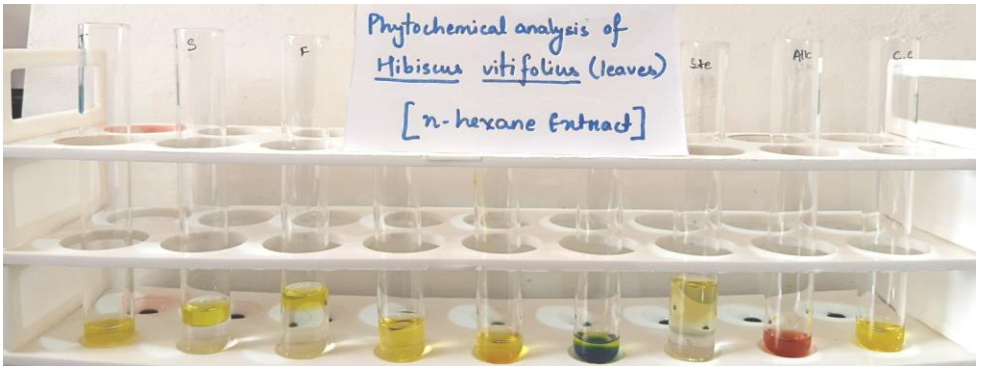

Figure 3: Detection of secondary metabolites of *H. vitifolius* leaves using n-hexane extract

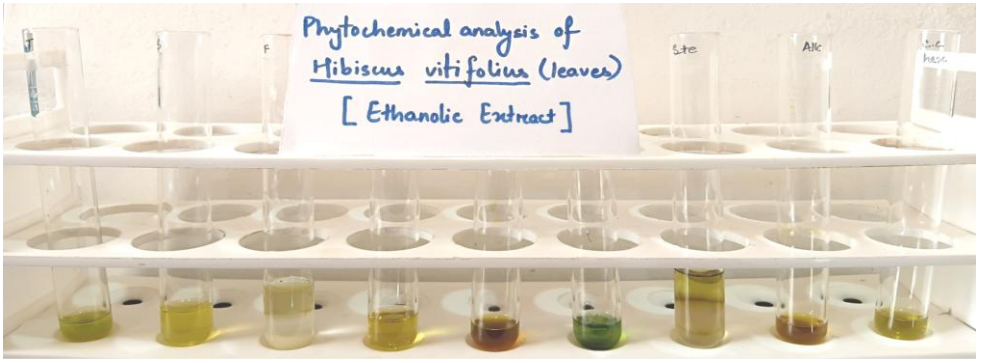

Figure 4: Detection of secondary metabolites of *H. vitifolius* leaves using ethanolic extract

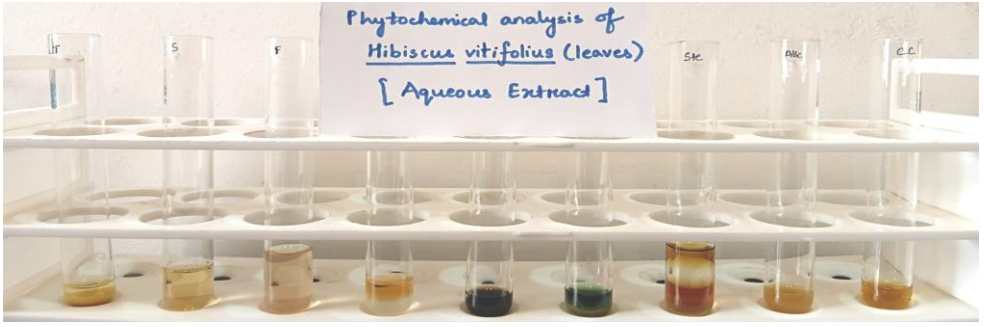

Figure 5: Detection of secondary metabolites of *H. vitifolius* leaves using aqueous extract

The cytotoxicity analysis of *H. vitifolius* leaf extracts using brine shrimp nauplii showed significant mortality rates for n-hexane and ethanol extracts, indicating potential anticancer or antimicrobial properties (Table 2; Figures 6-8). In contrast, the aqueous extract exhibited moderate to less lethality response. Present findings suggest that *H. vitifolius* leaves may possess specific bioactive compounds that contribute to toxicity.

Table 2: Cytotoxicity analysis of *H. vitifolius* leaves using Brine shrimp nauplii

Extracts	Concentration (in mg/ml)	Initial number of Nauplii	Number of Deaths of Nauplii (after hours)				Death rates (%)
			1	2	3	4	
n-hexane	15.62	10	10	10	10	10	100
	31.25	10	10	10	10	10	100
	62.5	10	10	10	10	10	100
	125	10	10	10	10	10	100
	250	10	10	10	10	10	100
	3.5% Saline	10	0	0	0	0	0
	Vincristine sulphate	10	10	10	10	10	100
Ethanol	15.62	10	10	10	10	10	100
	31.25	10	10	10	10	10	100
	62.5	10	10	10	10	10	100
	125	10	10	10	10	10	100
	250	10	10	10	10	10	100
	3.5% Saline	10	0	0	0	0	0
	Vincristine sulphate	10	10	10	10	10	100
Aqueous	15.62	10	2	2	2	2	20
	31.25	10	0	2	2	2	20
	62.5	10	0	0	0	0	0
	125	10	0	0	0	0	0
	250	10	0	0	0	0	0
	3.5% Saline	10	0	0	0	0	0
	Vincristine sulphate	10	10	10	10	10	100

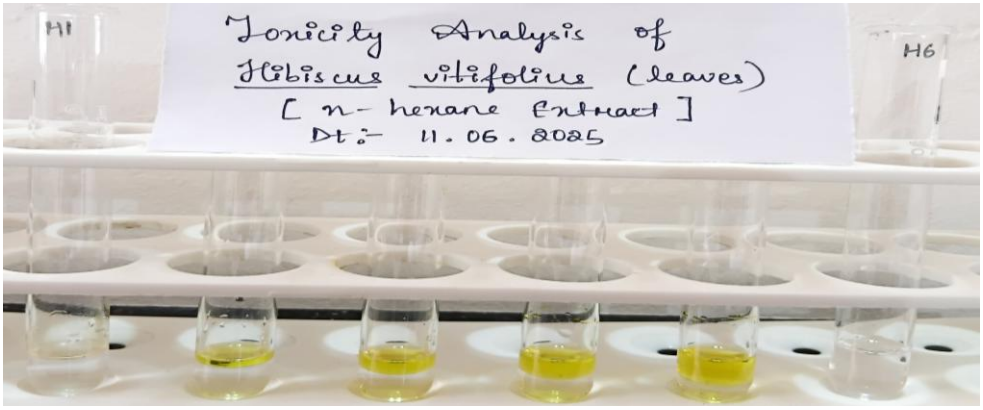

Figure 6: Toxicity analysis using Brine shrimp nauplii of n-hexane extract of *H. vitifolius* leaves



Figure 7: Toxicity analysis using Brine shrimp nauplii of ethanolic extract of *H. vitifolius* leaves



Figure 8: Toxicity analysis using Brine shrimp nauplii of aqueous extract of *H. vitifolius* leaves

Table 3: TLC analysis of *Hibiscus vitifolius* leaves using different mobile phases

	Mobile Phases	Rf value of Spots					
		S1	S2	S3	S4	S5	S6
Ethanolic aqueous	Methanol: Chloroform (1:1)	0.64	0.68	0.72	0.75	0.82	0.91
	Ethyl acetate: Chloroform (1:9)	0.81	0.82	0.84	-	-	-
	Ethyl acetate: Water: Acetic acid (8:1:1)	0.57	0.71	0.82	0.91	-	-

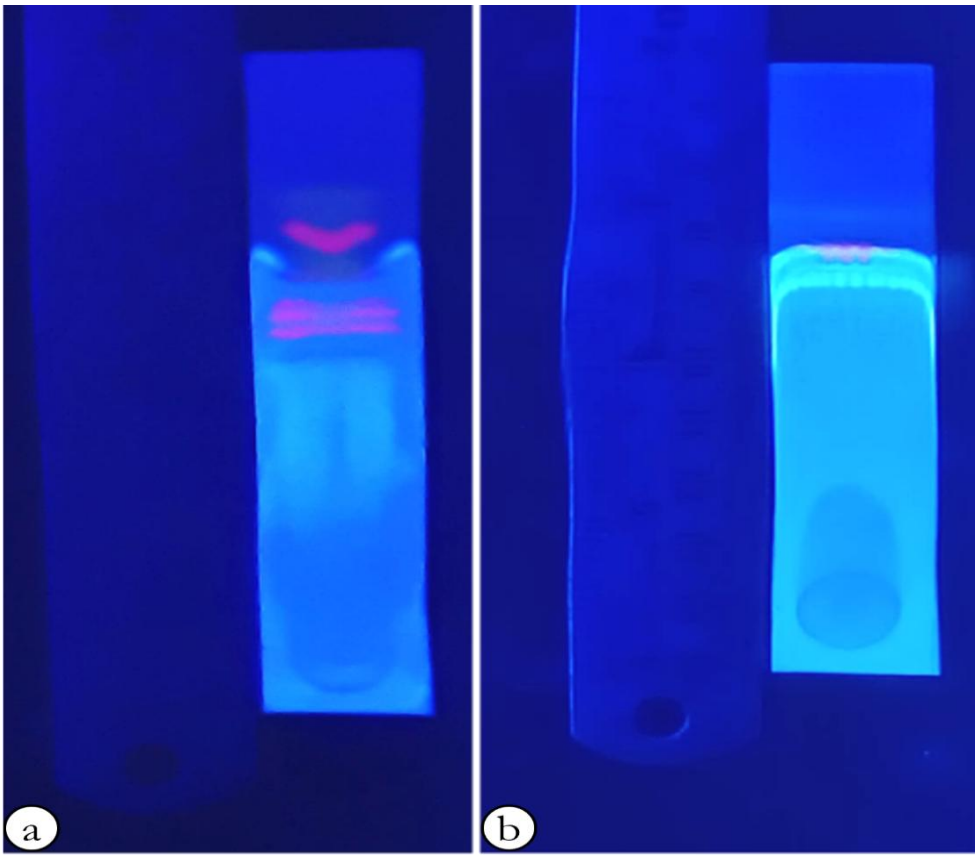

Figure 9: TLC analysis of hydroethanolic extract of *H. vitifolius* leaves (mobile phase: a) Ethyl acetate: Chloroform and b) Chloroform: Methanol

The chromatographic analysis of hydroethanolic extract of *H. vitifolius* leaves revealed distinct bioactive components, with different mobile phases separating various phytochemicals based on their Rf values. The presence of different spots in the TLC plates confirmed the presence of bioactive compounds in *H. vitifolius* leaves, which may be responsible for its medicinal properties (Table 3; Figure 9).

Conclusion

Present study on the leaves of *H. vitifolius* highlighted its potential as a valuable source of bioactive compounds with medicinal significance. Phytochemical analysis confirmed the presence of key secondary metabolites, while the cytotoxicity assay indicated significant biological activity, supporting its traditional uses in herbal medicine. Additionally, the TLC profiling provided a preliminary chemical fingerprint that can aid in further isolation and characterization of active constituents. These findings bring out the importance of *H. vitifolius* as a candidate for future pharmacological research and support the sustainable exploration of medicinal plants for drug discovery and therapeutic development.

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