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Research paper



A Comprehensive Analysis of Hedychium Spicatum for Its Phytochemical Profile, Antioxidant Activity, Antibacterial Properties, and Cancer-Fighting Potential

Kanchan Lakhera *, Swapna Kumar Srivastava

School of Biotechnology, IFTM University, Moradabad-244102, Uttar Pradesh, India
**Corresponding author E-mail: lakhera.kanchan@gmail.com*

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Abstract

Hedychium spicatum, a medicinal plant from the Zingiberaceae family, was evaluated for its phytochemical composition, antioxidant, antimicrobial, and anticancer activities using extracts prepared with solvents of varying polarity (water, ethanol, and acetone). Extraction yields were highest with water (55.8%), followed by ethanol (46.8%) and acetone (42.6%). Phytochemical screening revealed that the water tract contained the broadest range of secondary metabolites, encompassing tannins, flavonoids, glycosides, alkaloids, phenols, saponins, and carbohydrates. Quantitative analyses showed greatest total phenolic content (16.67mg/g) along with total flavonoid content(2.99mg/g) in the water extract, correlating with superior antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl (77.54%), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (89.70%), along with Ferric Reducing Antioxidant Power assay (4571.43 µM Fe (II)/g) assays. Atimicrobial study indicated that the water extract exhibited the most potent activity against Bacillus subtilis, Staphylococcus aureus, and Escherichia coli, with select extract fractions demonstrating low minimum inhibitory concentrations. Anticancer screening revealed significant cytotoxicity of water and ethanol extract fractions against HeLa and MCF-7 cancer cell lines, with water fractions 3 and 5 showing the highest activity. Water and ethanol bioactive fractions confirmed the presence of tannic acid, ferulic, and vanillic acid. The results support traditional use of H. spicatum and highlight the potential of its polar extracts and bioactive fractions for therapeutic applications in antioxidant, antimicrobial, and anticancer drug development.

Keywords: *Hedychium Spicatum; Phytochemicals; Antioxidant Activity; Antimicrobial Activity; Cytotoxicity; Solvent Extraction.*

1. Introduction

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1.1. Bioactive compounds in Hedychium spicatum

Essential oils of Hedychium spicatum are rich in monoterpenoids and sesquiterpenoids, with major components including sabinene, camphene, α-pinene, α, β-phellandrene, 1,8-cineole, and myrcene. Other notable terpenoids identified are linalool, limonene, caryophyllene, caryophyllene oxide, camphor, linalyl acetate, and borneol (Jain et al., 2022). The rhizome contains labdane diterpenes, including hedychinone, yunnacoronarin A and D, hedychilactone B and C, spicatanol methyl ether, and coronarin-E (Rawat et al., 2018; Mittal et al., 2022). Significant phenolic as well as flavonoid compounds include quercetin, chrysin, teptochrysin, and ethyl-trans-p-methoxy cinnamate (Singh & Singh, 2022; Rawat et al., 2018). The plant also contains polyphenols, contributing to its antioxidant properties (Rawat et al., 2018). Beta-sitosterol is a prominent sterol identified with antidiabetic activity (Singh & Singh, 2022). Carotenoids such as xanthophylls, α-carotene, and β-carotene, along with methylated phenols like DL-β-tocopherol and γ-tocopherol, are present and contribute to plants' capacity for antioxidants (Rawat et al., 2018; Mittal et al., 2022).

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1.2. Bioactivity of Hedychium spicatum

H. spicatum exhibits a broad spectrum of bioactivities attributed to its rich phytochemical profile. These bioactivities have been validated through various in vitro, in vivo, and silico studies, supporting its traditional medicinal uses. Extracts (essential oil, petroleum ether, chloroform, hydroalcoholic, and methanolic) from H. spicatum rhizomes show significant antifungal as well as antibacterial properties. Extracts have been efficient against various Gram-positive, along with Gram-negative bacteria, encompassing drug-resistant strains of S. aureus, which are resistant to methicillin along vancomycin, as well as harmful fungi (Bisht et al., 2006; Singh et al., 2023). Methanolic as well as hydroalcoholic extracts have demonstrated strong inhibition against pathogens like Shigella species, Bacillus cereus, Vibrio cholerae, Klebsiella pneumoniae, S. aureus, E. coli, as well as P. aeruginosa (Arora et al., 2017; Patil & Upadhye, 2024). Essential oil derived from the rhizome is particularly potent, with notable inhibition zones and low minimum inhibitory concentrations (MICs) (Tian et al., 2020). Extracts, particularly ethanolic and hydroalcoholic, exhibit excellent free radical scavenging activity, often comparable to or better than ascorbic acid in DPPH assays (Singh & Singh, 2022; Mittal et al., 2022; Gangwar et al., 2024). Compounds such as quercetin and 7-hydroxyhedychenone are identified as major contributors to this antioxidant effect (Singh & Singh, 2022; Mittal et al., 2022). In vitro as well as in silico research shows that H. spicatum extracts inhibit α-glucosidase and α-amylase enzymes, indicating potential for managing hyperglycemia. Bioactive compounds like quercetin and beta-sitosterol are linked to these antidiabetic effects (Singh & Singh, 2022; Mittal et al., 2022). Traditional and experimental studies support anti-inflammatory, antipyretic properties, along with hepatoprotective impacts (Rawat et al., 2018; Singh et al., 2023). Some extracts have shown cytotoxic effects in preliminary studies, suggesting potential anticancer properties, though more research is needed (Tian et al., 2020). Plant extract reportedly has sedative, anti-helminthic, analgesic, antispasmodic, and CNS depressant activities (Patil & Upadhye, 2024).

Despite its extensive traditional use and promising pharmacological profile, scientific exploration into the optimal extraction methods, solvent-dependent phytochemical profiles, and the comparative efficacy of different extracts remains limited. This work aims to address these gaps by systematically comparing the extraction yields, phytochemical profiles, and bioactivities (antioxidant, antimicrobial, and cytotoxic) of Hedychium spicatum rhizome extracts obtained using water, ethanol, and acetone, thereby providing a scientific basis for their traditional and potential therapeutic uses.

2. Materials and Methods

2.1. Material

The National Center Cell Sciences in Pune provided HeLa as well as MCF-7 breast cancer cell lines. National Center for Microbial Resources in Pune obtained bacterial cultures E. coli, P. aeruginosa, S. aureus, along with B. subtilis, with accession numbers MCC 2408, MCC 2010, MCC 2265, and MCC 3099, respectively.

2.2. Collection of plant sample and its extraction

Plants of Hedychium spicatum were collected from the Dehradun region and verified by Dr. Anamika from Vardhman College, Bijnor, from the Department of Botany. Rhizomes had been washed, shade-dried at room temperature, and air-dried. Soxhlet extraction of powdered rhizome was carried out as per Azwanida (2015). Different kinds of compounds were isolated according to their solubility by performing sequential extraction with three solvents, namely, acetone, ethanol, and water. A 1:25w/v ratio of plant powder to solvent was utilized. The extraction occurred at a temperature of 60–80°C, which mitigated some of the impediments normally associated with the process without destroying most of the compounds. The extracts were weighed for yield and then stored at 4°C after being filtered and concentrated at lower pressure in a rotary vacuum evaporator.

2.3. Qualitative estimation of phytochemicals

Phytochemical screenings were performed following the standard protocol for qualitatively estimating glycosides, saponins, phenolic compounds, carbohydrates, flavonoids, and alkaloids, among others.

2.4. Estimation of total phenolic content (TPC) and total flavonoid content (TFC)

Siddhuraju along with Becker (2003) [22] and Zhishen et al., 1999 [23] methods were used to assess the crude's phenolic and flavonoid contents, respectively. 50µl of crude extract as well as 950µl of distilled water had been combined to determine phenolic content. After 45 minutes of dark incubation, 500 µl Folin-Ciocalteu phenol reagent (1:1 with water) as well as 2500µl of 20 percent sodium carbonate solution had been thoroughly mixed with the diluted extract. Color intensity has been noted at 765nm. A gallic acid standard curve has been utilized to estimate phenolic content regarding GAE (gallic acid equivalents) per gram of dry weight.

250µl crude extract has been combined with 1.25ml distilled water for flavonoid content estimation. 75µl of 5% $NaNO_2$ solution has been added to the diluted extract following a 5-minute room temperature incubation. 150µl of 10 percent $AlCl_2$ has been added and filtered. 0.5ml 1M NaOH along with 0.275 ml of distilled water, had been mixed well with filtrate. Intensity of pink color has been noted at 510nm with a reagent blank. The quercetin linear curve was prepared to estimate flavonoid content regarding quercetin equivalents per gram dry weight. Both phenolic as well as flavonoid content were computed utilizing the following equation.

$$\text{TFC (Total flavonoid content) or TPC (Total Phenolic content)} = \frac{C*V}{m}$$

Where C=gallic acid equivalent (mg/ml)/ quercetin equivalents (mg/ml), V = plant extract volume(ml), as well as m = pure plant extract weight(g).

2.5. Antioxidative assay of extracts

For antioxidative assays, all extracts were diluted to a 1 mg/ml concentration. DPPH free radicle assay, ABTS, and FRAP assay were estimated following the protocol of Blois, 1958 [24], Re et al., 1999 [25], as well as Benzie & Strain, 1996. [26] respectively. For DPPH

assay, 100µl of extracts had been mixed well with 1.5ml of DPPH solution (0.1 mM) following a half-hour room temperature incubation in dark. Absorbances had been noted at 517nm against a reagent blank. Standard curve of ascorbic acid has also been prepared between different ascorbic acid concentrations and their % DPPH inhibition to estimate all extracts' Vitamin C Equivalent Antioxidant Capacity (CEAC).
For ABTS, 100 µL of ABTS reagent (1:1 of 7mM ABTS and 2.45mM potassium persulfate, incubated for 16 hrs in the dark) has been mixed well with 1mL of diluted extract, followed by a 1-minute incubation in the dark. Absorbances had been noted at 734nm. ABTS reagent was used as a reagent control. CEAC was estimated using an ascorbic acid standard curve. The following equation has been utilized to estimate % inhibition or scavenging of ABTS or DPPH free radicals.

$$\text{DPPH or ABTS inhibition (\%)} = \left(\frac{\text{Acontrol}-\text{Atest}}{\text{Acontrol}}\right) * 100$$

Where Acontrol is the reagent control absorbance, as well as Atest is extract absorbance.
To estimate the FRAP value of crude extracts, 70µl of distilled water as well as 900µl of FRAP reagent were combined well with 30µl of extracts. Reaction mixture has been incubated for 10min at room temperature, and absorbances have been noted at 593nm. Ferrous sulfate ($FeSO_4$) has been utilized as a standard. The mixture of acetate buffer, TPTZ solution, as well as ferric chloride was utilized as a control in a ratio 10:1:1, without adding test extracts. FRAP outcomes had been expressed in µM $FeSO_4.7H_2O$/g test extract dry weight.

2.6. Column purification of extracts

Extracts with antibacterial and anticancer activity were purified by flash column chromatography. 30g of pre-activated silica gel of mesh size 60 to 20 has been wet packed into a column (inner diameter of 18mm and length of 300mm) with chloroform. A gradient elution system used chloroform and methanol, starting from 100% chloroform to 50% chloroform and methanol solvent, at a 1ml/min flow rate to effectively separate extract components based on polarity. Column fractions were dried under ambient conditions and stored at 60 °C.

2.7. Antibacterial activity and minimum inhibitory concentration (MIC) of crude and purified

Antibacterial activity of crude extracts as well as fractions was tested on Müeller-Hinton agar following CLSI guidelines. The agar was freshly prepared, autoclaved, poured into Petri dishes, and inoculated with 0.1 mL bacterial cultures (S. aureus, B. subtilis, E. coli, P. aeruginosa) at 0.6 OD. Sterile 6 mm discs soaked in the samples had been positioned on agar as well as incubated at 37°C for 24-72 hrs. Inhibition zones had been seen, as well as active samples were subjected to MIC determination using the broth dilution method. In 96-well plates, 0.1 mL of bacterial cultures (OD 0.1) was treated with diluted extracts. Controls included wells with culture only and solvent blanks (acetone, ethanol, water). After 24-hour incubation at 37°C, absorbance at 600nm has been measured to estimate MIC, with imipenem used as a standard reference.

2.8. Screening of anticancer activity of crude and fractions and their IC50 estimations

The MTT cytotoxicity assay, as described by Mosmann (1983), was followed to screen the anticancer activity of extracts and their fractions at concentrations of 0.1mg/mL and 1mg/mL, respectively. Cancer cell lines (HeLa and MCF-7) had been seeded in 96-well plates at a density of approximately 5,000 cells per well and incubated for 24 hrs at 37°C in a 5% CO_2 atmosphere to allow cell attachment. Following incubation, cells were treated with 0.1mg/mL of crude extracts and 1mg/mL of fractions for the next 24 hours, along with appropriate controls (cell control, DMSO control, and media control). After treatment, 25µL MTT solution (5mg/mL) has been added to each well and incubated for 4 hours to assess cell viability. Formazan crystals had been solubilized in 100µL of DMSO with gentle shaking for 10 to 15 minutes, and absorbance was measured at 570nm utilizing a microplate reader. The percentage of cytotoxicity has been computed utilizing the following equation:

$$\text{\% cytotoxicity} = \left(\frac{\text{Acontrol}-\text{Atest}}{\text{Acontrol}}\right) * 100$$

Where Acontrol is the absorbance of untreated HeLa as well as MCF-7 cells, and Atest is the absorbance of treated cells.
Samples exhibiting more than 50% cytotoxicity were considered for IC_{50} estimation. For IC_{50} determination, the active fractions were diluted to 50–500µg/mL and tested using the same MTT assay procedure.

2.9. HPLC identification of fraction composition

The determination of the composition of active fractions was carried out following the protocol of Hingse et al. (2014) using an HPLC technique on an Agilent 1200 series instrument equipped with a multi-solvent delivery module and a UV-Vis detector. The separation was carried out in a reverse-phase column using a linear gradient from two mobile phases: solvent A (25% methanol in 1% acetic acid) and solvent B (75% methanol in 1% acetic acid). Increasing gradient conditions were: 0-30 min, 100% A; 30-45 min, 82% A and 18% B; 45-60 min, 72% A and 28% B. The isocratic mobile phase flow rate was further established to 0.75 mL/min. The detection took place at wavelengths of 280 and 360 nm. Characterization was carried out using tannic acid, ferulic acid, and vanillic acid as standards. Retention times for these standards were determined from chromatographic profiles and UV absorbance spectra at 9.931, 10.793, and 13.871 min, respectively.

2.10. Statistical analysis

All experimental measurements, including antioxidant activity assays (DPPH, ABTS, FRAP) and cytotoxicity assays, were done in triplicate (n=3) and reported as mean ± standard deviation (SD). Statistical analysis was conducted by one-way analysis of variance (ANOVA) to determine significant differences between the different types of extracts (water, ethanol, and acetone). Where ANOVA revealed statistically significant differences ($p < 0.05$), further post-hoc tests were conducted using Tukey's Honest Significant Difference (HSD) test to determine pairwise differences between groups. Execution of ANOVA and post-hoc tests was done using Microsoft Excel 2016 with Analysis ToolPak. Statistical significance was at $p < 0.05$.

3. Results and Discussion

Extraction yields of Hedychium spicatum rhizome extracts vary significantly with solvent polarity, as demonstrated in Table 1. Water (HSW) showed the highest yield (55.8%), followed by ethanol (HSEt: 46.8%) and acetone (HSAc: 42.6%). This aligns with the finding that polar solvents like water efficiently extract a broader range of polar compounds, encompassing polysaccharides as well as glycosides (Upadhyay et al., 2021; Sravani & Paarakh, 2012). The ethanolic and water extracts of Hedychium spicatum yielded only 4% and 7%, respectively, likely due to different extraction methods (Soxhlet vs. maceration) (Ghildiyal et al., 2012). Drying methods (e.g., shade vs. oven drying) also influence oil and extract composition (Gangwar et al., 2024), which could indirectly affect solvent efficiency. While water maximizes yield, acetone and ethanol may target specific bioactive compounds. For instance, acetone was optimal for phenolic content in H. spicatum (Rawat et al., 2016), and ethanolic extracts demonstrated anti-asthmatic activity (Ghildiyal et al., 2012).

Table 1: Percentage Yield of Hedychium Spicatum

Sample Abbreviation	Plant	Solvent	Sample weight (g)	Final dry weight(g)	Extraction yield (%)
HSAc	Hedychium spicatum	Acetone	5	2.13	42.6
HSEt	Hedychium spicatum	Ethanol	5	2.34	46.8
HSW	Hedychium spicatum	Water	5	2.79	55.8

Where HSAc: Acetone extract, Et: ethanol extract, and W: water extract

3.1. Phytochemical screening

Preliminary phytochemical screening of Hedychium spicatum extracts revealed solvent-dependent variation in secondary metabolite content. The acetone extract (HSAc) showed the most diverse profile, testing positive for alkaloids, flavonoids, phenols, glycosides, tannins, and saponins. The ethanol extract (HSEt) was positive for flavonoids, phenols, glycosides, and tannins, but lacked alkaloids and carbohydrates. The water extract (HSW) contained alkaloids (Dragendorff's and Wagner's), flavonoids, phenols, glycosides, tannins, saponins, as well as carbohydrates. These results are consistent with previous research employing methanol, ethanol, and water as extraction solvents (Arora et al., 2017; Singh et al., 2013; Upadhyay et al., 2021). For instance, methanolic and ethanolic extracts have been reported to contain abundant flavonoids, steroids, proteins, alkaloids, reducing sugars, phenolic compounds, oils, and saponins, supporting the findings (Singh et al., 2013; Upadhyay et al., 2021). Similarly, water as well as ethanol extracts have shown strong positivity for flavonoids, phenolics, and carbohydrates, while acetone and ethanol are particularly effective for extracting phenolics and flavonoids (Arora et al., 2017; Upadhyay et al., 2021).

Table 2: Results of Qualitative Phytochemical Screening of Different Plant Extracts

Sample	Alkaloid			Flavonoid	Phenol	Glycosides	Tannins	Carbohydrate			Saponins	steroids
	Mayer's test	Dragendorff's test	Wagner test					Molisch	Fehling's	Benedicts		
HCAc	+	+	+	++	++	+	+	-	+	-	-	-
HCEt	-	-	-	++	+	+	+	-	-	-	-	-
HCEw	-	+	+	+	+	+	+	-	+	+	-	-

Where HCAc: Acetone extract, Et: ethanol extract, and W: water extract

3.2. Total phenolic content (TPC) and total flavonoid content (TFC)

Water was found to be most effective for extracting both TPC and TFC from the rhizome of Hedychium spicatum, with TPC as well as TFC values of 16.67mg/g and 2.99mg/g (0.1245 mg/ml), respectively (Figure 1). The result is consistent with previous research on Hedychium spicatum and related Zingiberaceae species, where aqueous and hydroalcoholic extracts have repeatedly demonstrated higher yields of phenolic as well as flavonoid compounds compared to less polar solvents (Kumar et al., 2021; Rawat et al., 2011). Total phenolic content (TPC) and total flavonoid content (TFC) differed across Hedychium spicatum extracts. Water extract (HSW) contained the maximum TPC (16.67±0.02 mg GAE/g) and maximum TFC (2.98±0.01 mg QE/g), followed by ethanol (HSEt) and acetone (HSAc) extracts. One-way ANOVA showed a difference was significant for TPC ($p=2.55*10^{-15}$) and TFC ($p=2.21*10^{-16}$). Tukey's HSD tests revealed all pairwise comparisons (HSW vs. HSEt, HSW vs. HSAc, HSEt vs. HSAc) were significant ($p < 0.05$), which indicated extraction solvent greatly influences phenolic and flavonoid yield. The findings confirm earlier findings that polar solvents, viz., water and ethanol, efficiently extract phenolic and flavonoid compounds because solvents are hydrophilic (Rawat et al., 2011; Kumar et al., 2021).

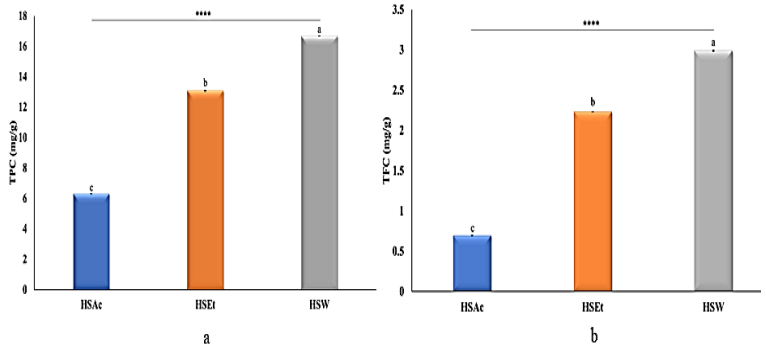


Fig. 1: Total Phenolic (TPC) (A) and Flavonoid (TFC) (B) Content of Different Plant Extracts. Data Are Represented as Mean ± Standard Deviation. Bars Represent as Mean ± SD. Different Letters (A, B, and C) Indicate Statistically Significant Differences (Tukey's HSD, P<0.05). **** Indicate P<0.0001 (One-Way ANOVA)

3.3. Antioxidant activity of Hedychium spicatum extracts

Antioxidant activity of Hedychium spicatum extracts, assessed via DPPH, ABTS, as well as FRAP assays, reveals that the water extract (HSW) exhibits the highest activity across all assays, followed by the ethanol (HSEt) and acetone (HSAc) extracts. Specifically, HSW showed the strongest DPPH scavenging (77.54%), ABTS inhibition (89.70%), and FRAP value (4571.43 µM Fe(II)/g) (Table 3), indicating a high concentration of water-soluble antioxidant compounds. These results align with earlier research by Rawat et al. (2011), who stated that aqueous and methanolic extracts of Hedychium spicatum from the western Himalaya exhibited significantly higher total phenolic content as well as antioxidant activities than less polar extracts, attributing this to the greater solubility of antioxidant phytochemicals in polar solvents. Similarly, Kumar et al. (2021) found that water and hydroalcoholic extracts of H. spicatum rhizomes showed pronounced DPPH as well as ABTS radical scavenging activities, further supporting the present observation that polar solvents maximize the extraction of antioxidant-rich compounds. One-way ANOVA and Tukey's HSD tests were applied for DPPH, ABTS, and FRAP assays. ANOVA showed differences ($p < 0.001$) in all three assays: DPPH ($p = 6.45*10^{-13}$), ABTS ($p = 4.49*10^{-14}$), and FRAP ($p = 2.02 \times 10^{-8}$). Tukey's tests validated that water extract (HSW) had superior antioxidant activity compared to ethanol (HSEt) and acetone (HSAc) extracts ($p < 0.001$). HSEt also had significantly greater antioxidant capacity compared to HSAc for all assays. These findings validate the superior bioactivity of polar extracts, particularly the water extract, and demonstrate solvent-dependence in phytochemical extraction efficiency.

Table 3: Antioxidant Activity of Different Extracts of Hedychium Spicatum Evaluated Using DPPH, ABTS, and FRAP Assays.

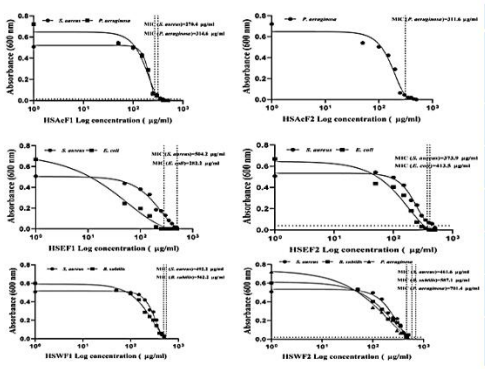
sample	% DPPH scavenging activity	CEAC (mg/ml)	% ABTS inhibition	CEAC (mg/ml)	FRAP value (µM Fe (II)/g dry wt.)
HSAc	22.46±0.24^{c}	0.08±0.001^{c}	56.74±0.12^{c}	0.0015±0.0000086^{c}	342.86^{c}
HSEt	67.01±0.13^{b}	0.17±0.001^{b}	84.97±0.12^{b}	0.0034±0.0000084^{b}	3428.57±171.43^{b}
HSW	77.54±0.39^{a}	0.23±0.002^{a}	89.70±0.07^{a}	0.0038±0.0000048^{a}	4571.43±98.97^{a}

Where HSAc: Acetone extract, Et: ethanol extract, and W: water extract of Hedychium spicatum. Values are expressed as mean ± standard deviation. CEAC: Vitamin C equivalent antioxidant capacity. FRAP: Ferric Reducing Antioxidant Power expressed in µM Fe (II)/g dry weight. Values represent mean ± standard deviation (n = 3). Within each column, values with different superscript letters (a, b, c) differ significantly ($p < 0.05$, Tukey's HSD test).

3.4. Anti-bacterial activity and MIC (minimum inhibitory concentration) of extracts

Antimicrobial activity of Hedychium spicatum extracts has been assessed against four bacterial strains, namely E. coli, B. subtilis, P. aeruginosa, and S. aureus, utilizing the zone of inhibition method. All three extracts showed antibacterial activity against the selected bacterial species. These results align with Bisht et al. (2006) and Joshi et al. (2008) findings. Bisht et al. (2006) investigated antimicrobial activity of extracts and essential oils from H. spicatum rhizomes. Their study demonstrated significant inhibitory effects against various Gram-positive and Gram-negative bacteria, including S. aureus, B. cereus, E. coli, and P. aeruginosa. Essential oils exhibited notable zones of inhibition, suggesting potent antimicrobial properties. While Joshi et al. (2008) reported that rhizome extracts of H. spicatum possess antimicrobial activity against various pathogenic bacterial and fungal strains. Their findings support the traditional use of this plant in treating infections.

Column fractions of Hedychium spicatum had been screened for their antimicrobial activity, and the potent fractions had been processed for their MIC estimation. Six fractions were collected from each extract. Only fractions 1 and 2 of each extract were effective against the selected bacterial strains. Fraction 1 of the acetone extract was effective against P. aeruginosa (1.3cm) and S. aureus (1.1 cm), while fraction 2 was effective against P. aeruginosa (1.2 cm) only. Both fractions 1 and 2 of the ethanol extract were effective against S. aureus (F1: 1.2 cm, F2: 1.3 cm) and E. coli (F1: 1.2 cm, F2: 1.6 cm). Both fractions 1 and 2 of the water extract exhibited antimicrobial activity against S. aureus (F1: 1.6 cm, F2: 1.7 cm) and B. subtilis (F1: 1.5 cm, F2: 1.6 cm). Only fraction 2 was effective against P. aeruginosa. MICs of each fraction are shown in Figure 2. Fractions HSEF1 and HSAcF2 showed the lowest MIC values, indicating higher potency.



Fractions	MIC (µg/ml)			
	S. aureus	*B. subtilis*	*P. aeruginosa*	*E. coli*
HSWF1	492.2	562.2	-	-
HSWF2	461.6	587.1	701.4	-
HSEF1	504.2	-	-	282.2
HSEF2	373.9	-	-	413.5
HSAcF1	270.4	-	314.6	-
HSAcF2	-	-	311.6	-

Fig. 2: Determination of Minimum Inhibitory Concentrations (MICs) of Extract Fractions Against Bacterial Strains. Dose Response Curves of HSWF1, HSWF2, HSEF1, HSEF2, Hsacf1, and Hsacf2 Against S. Aureus, B. Subtilis, P. Aeruginosa, and E. Coli. MIC Values Table, Representing the Lowest Concentration at Which Bacterial Growth Has Been Inhibited, Is Provided in the Table.

3.5. Anticancer activity of crude and fractions

The cytotoxicity of Hedychium spicatum fractions against HeLa (cervical cancer) as well as MCF-7 (breast cancer) cell lines reveals notable anticancer potential, particularly for water and ethanol extracts. The water fraction HSWF3 exhibited the highest cytotoxicity against HeLa cells (84.47%), while HSWF5 showed strong activity against MCF-7 cells (89.80%). Among ethanol fractions, HSEF3 demonstrated considerable cytotoxicity against HeLa cells (73.12%), and HSEF2 was active against both HeLa (61.69%) and MCF-7 (77.49%) cell lines. These results suggest that both water as well as ethanol extracts of H. spicatum contain potent bioactive compounds with selective cytotoxic effects on different cancer cell lines. Results are consistent with previous studies on Hedychium spicatum and related Zingiberaceae species. Reddy et al. (2009) reported that labdane diterpenes isolated from H. spicatum rhizomes exhibited significant

cytotoxic activity against numerous human cancer cell lines, encompassing HeLa as well as MCF-7. Similarly, Joshi et al. (2008) found that essential oils and extracts from H. spicatum demonstrated cytotoxic and antiproliferative effects in vitro. The higher cytotoxicity observed for water fractions may be attributed to the extraction of polar phytochemicals, including phenolics as well as flavonoids, which are acknowledged for their anticancer properties. Ethanol fractions also showed substantial activity, likely due to their ability to extract a broader range of secondary metabolites, encompassing terpenoids as well as alkaloids.

Table 4: Cytotoxic Activity of Hedychium Spicatum Extract Fractions Against HeLa and MCF-7 Cell Lines

Sample	Solvent	Fractions	% Cytotoxicity Hela	MCF-7
Hedychium spicatum	Water	HSWF3	84.4741	-
		HSWF5	-	89.8007
	Ethanol	HSEF2	61.6861	77.4912
		HSEF3	73.1219	-

Cytotoxicity percentages of water and ethanol-derived fractions of Hedychium spicatum rhizome extracts against HeLa (cervical carcinoma) as well as MCF-7 (breast adenocarcinoma) cell lines. Results represent mean cytotoxicity values expressed as percentages.

Table 5: Half Maximal Inhibitory Concentration (IC50) Of Different Fractions of Hedychium Spicatum Against HeLa and MCF-7 Cell Lines

Fractions	IC50(µg/ml) Hela	MCF-7
HSWF3	82.4306	-
HSWF5	-	320.156
HSEF2	279.693	315.819
HSEF3	310.274	-

Only fractions 3 and 5 of the water extract and fractions 2 and 3 of the ethanol extract exhibited anticancer activity against either the HeLa or MCF-7 cell lines, and they had been subjected to IC50 estimation. Cytotoxicity of all fractions increases dose-dependently, with varying potency across fractions and cell lines. HSWF3 is most potent against HeLa cells, while HSWF5 is more effective against MCF-7 cells (Figure 3 B). These values are greater than those reported for some pure compounds or essential oil fractions, which have shown IC50 values as low as 48.77 ppm for HeLa cells (Lamichhane et al., 2012). The variation may be because of the extraction method and solvent (water, ethanol, essential oil, etc.), fraction composition and phytochemical profile, cancer cell line sensitivity, and geographical and genetic variation in plant material. Several crucial oils from H. spicatum rhizomes, rich in compounds like spathulenol, 1,8-cineol, cubenol, eu-desmol, as well as α-cadinol, have shown significant in vitro cytotoxic activities against a range of human cancer cell lines, including HeLa as well as MCF-7 (Mishra et al., 2016). Several studies demonstrated that H. spicatum crucial oil induces apoptosis in cancer cells (notably prostate cancer PC-3), involving mitochondrial depolarization, activation of caspases, ROS accumulation, along with modulation of pro- as well as anti-apoptotic proteins (Ray et al., 2023).

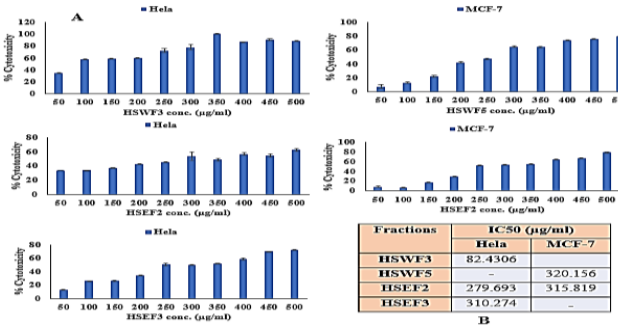


Fig. 3: Cytotoxic Activity of H. Spicatum Fractions Against HeLa and MCF-7 Cell Lines. A: Dose-Dependent Cytotoxicity of Fractions (HSWF3, HSWF5, HSEF2, And HSEF3) on HeLa as Well as MCF-7 Cell Lines. The percentage of Cytotoxicity Was Measured at Increasing Concentrations (50–500 µg/mL). B: IC50 Values (µg/Ml) Calculated for Each Extract.

3.6. HPLC detection of the major compound in the active fraction

The chromatograms of Hedychium extracts by HPLC confirm the presence of major phenolic acids, such as tannic acid, ferulic acid, and vanillic acid, by comparison with the standard retention times (6.943, 9.931, and 13.871 min, respectively). Among the samples, HSWF5 presented all three compounds clearly, reflecting a dense polyphenolic profile of this water fraction. HSEF3 and HSWF3 exhibited vanillic and tannic acids, and HSEF2 exhibited potential ferulic acid-like peaks, emphasizing the effect of solvent and fraction on compound recovery. These results are consistent with the established antioxidant and medicinal potential of Hedychium, providing experimental data for the occurrence of certain phenolic acids that are infrequently observed in the literature.

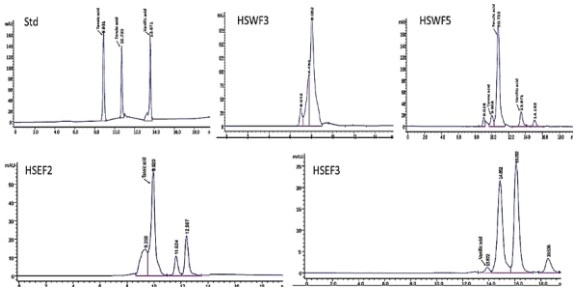


Fig. 4: HPLC Chromatogram of Bioactive Fractions of Water and Ethanol Extracts of H. Spicatum.

3.7. Geographic diversity and comparative phytochemical analysis

The current investigation utilizes Hedychium spicatum rhizomes sourced from the Uttaranchal region (Uttarakhand, India) but does not directly compare phytochemical profiles or bioactivities with those from different regions. It has been established through previous research that environmental factors such as altitude, climate, soil type, and season of harvest can influence not only the quantity but also the composition of essential oil and secondary metabolites (Rawat et al., 2018; Mishra et al., 2016). For example, H. spicatum from the Western Himalaya has been reported to yield essential oils with markedly different terpenoid composition and cytotoxicity (Mishra et al., 2016), and regional differences in the antiproliferative activity of Himalayan accessions of this taxon were reported by Lamichhane et al. (2012). Although the current investigation reports considerable antioxidant and cytotoxic activity in Uttarakhand samples, it does not include a comparative assessment. Incorporating a comparison of regions into future research would increase the understanding of the impact of variation of phytochemicals on pharmacological effect and may help to isolate the most bioactive ecotypes for medicinally useful applications.

3.8. Mechanistic consideration and future consideration

Reports indicate Hedychium spicatum extracts have antioxidant, antimicrobial, and anticancer activity, but the mode of action is unknown. Phenolic compounds function as effective antioxidants as electron donors to neutralize harmful free radicals, thereby preventing oxidative damage. They also inhibit the generation of ROS by chelating metal ions that catalyze radical formation, and by breaking oxidative chain reactions (Rawat et al., 2011). Recent studies have indicated that Polyphenols can disrupt microbial cell membranes, increase cell permeability, and inhibit essential microbial enzymes and nucleic acid synthesis. These interactions lead to leakage of intracellular components and inhibition of microbial growth (Rawat et al., 2018). Phytochemicals such as quercetin and hedychinone (Singh & Singh, 2022; Rawat et al., 2018) have action on target molecules. Quercetin causes apoptosis in cancer cells through PI3K/Akt, MAPK, and caspase pathways (Russo et al., 2012) and may inhibit microbial growth by membrane disruption or inhibition of DNA gyrase (Dabbagh-Bazarbachi et al., 2014). Labdane diterpenes such as hedychinone cause cytotoxicity through mitochondrial membrane depolarization and oxidative stress-mediated cell death (Reddy et al., 2009; Ray et al., 2023). These modes were not explored in this study. Future research should incorporate assays such as mitochondrial membrane potential, flow cytometric apoptosis or cell cycle analysis, gene expression profiling, and molecular docking to investigate interactions with microbial and cancer targets. These measurements would reveal H. spicatum's pharmacological significance and could allow the development of target-based therapies.

4. Conclusion

The current research highlights the effect of solvent polarity on extraction efficacy, phytochemical profile, as well as biological activities of Hedychium spicatum rhizome extracts. Water proved to be the most effective solvent, yielding the highest percentage of extraction (55.8%) and having the most superior antioxidant, antibacterial, and anticancer activities. Phytochemical screening showed the presence of secondary metabolites in all these extracts, with the highest range being seen in aqueous and acetone extracts. The water extract displayed the highest total phenolic (16.67 mg/g) and flavonoid (2.99 mg/g) content, coinciding with the strong antioxidant activity. It was also confirmed by antimicrobial testing, as it proved to be broad-spectrum, emphasizing its effects against B. subtilis, S. aureus, and E. coli, with MIC analysis of specific fractions further emphasizing the effectiveness of some of the fractions. The water fractions HSWF3 and HSWF5 exhibited significant anticancer activity against the HeLa as well as MCF-7 cell lines, respectively, and ethanol fractions (HSEF2, HSEF3) also showed strong effects in cytotoxicity assays. The findings, thus, support the ethno-therapeutic application of H. spicatum and underscore its promise of being a potential natural source of antioxidant, antimicrobial, and anticancer agents. The extracts of H. spicatum have the potential to be formulated into oral dosage forms or creams, gels etc., for topical application as an antimicrobial and are a good candidate for combination therapy. Future studies should entail the isolation, structural elucidation of the active components, research on the mechanism of action, and validation.

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