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A comprehensive approach to characterizing bioactive compounds from *Haplanthodes tentaculatus* (L.) R. B. Majumdar using HR-LCMS and MTT assay

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ABSTRACT

The bioactive compounds in *Haplanthodes tentaculatus* (L.) R. B. Majumdar has demonstrated significant potential in addressing conditions like cancer, diabetes, inflammation, and microbial infections. This study employs high-resolution liquid chromatography mass spectrometry and network analysis to investigate these chemicals. The methanolic extract revealed 92 identified compounds, many of which exhibited inhibitory effects on MDA-MB-231 breast cancer cell growth. Aclacinomycin N, Ganoderic acid Mb, Cucurbitacin E, and Hydroquinidine displayed notable inhibitory effects. The extract demonstrated an IC_{50} value of 61.41±0.692 μg, showcasing its effectiveness in impeding cancer cell development. While promising, further research is needed to unravel the specific molecular pathways involved. The identification and characterization of distinct chemicals in the extract offer potential leads for medication development. This work underscores *H. tentaculatus* as a vital source of bioactive substances with potent anticancer properties, emphasizing the need for continued exploration in advancing cancer therapies.

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INTRODUCTION

The role of plants as a source of bioactive compounds with potential therapeutic properties has been widely acknowledged. These bioactive metabolites have displayed encouraging potential in the management of various illnesses, including cancer, diabetes, inflammation, and microbial infections. Notably, cancer presents a substantial global health issue, resulting in the loss of millions of lives each year (Dziedziński *et al*., 2021). In 2008 alone, developed nations have experienced a high mortality rate due to cancer, with 7.6 million deaths recorded. However, emerging nations are also witnessing an increase in cancer cases, with 64 percent of the 7.6 million fatalities in 2008 occurring in developing countries (Ord, 2008; Seffrin *et al*., 2009) This rise in cancer cases can be attributed to cancer-causing habits such as smoking and unhealthy diets (Carocho & Ferreira, 2013).

The development, division, and death of cells in the human body are typically controlled in a regulated manner. Cell death through apoptosis, which involves caspase activation, is a programmed mechanism to maintain cellular balance. However, when cells evade apoptosis and continue to grow uncontrollably, they can undergo neoplastic transformation, leading to the formation of tumors-abnormal masses of tissue (Brown *et al*., 2023). Cancer, as a collective term, comprises over 100 different diseases, each with its epidemiology, risk factors, and originating from various cell types and organs in the body (Rindi *et al*., 2018). Cancer is characterized by uncontrolled cell proliferation that can spread to distant organs and invade neighbouring tissues. *Haplanthodes tentaculatus*, belonging to the Acanthaceae family, Lamiales order, and Magnoliopsida class, is a flowering plant found exclusively in India, primarily thriving in the biodiverse Western Ghats region. Also known as *H. tentaculatus* or occasionally identified as *H. tentaculata*, this species holds ecological significance as a distinctive component of India's rich floral diversity, particularly in the Western Ghats, showcasing the unique and varied plant life indigenous to this region (India Biodiversity Portal, n.d.; Singh *et al*., 2022) Studies have indicated that phenolics and flavonoids derived from plants

can act as potent anticancer agents by affecting the genes involved in the transformation of normal cells into malignant ones, their ability to metastasize, and their capacity for angiogenesis (Bhagya & Chandrashekar, 2020). Traditional medicine often utilizes herbal extracts with potential anticancer properties, but the mechanism of action of these extracts remains to be fully understood.

To address this, a combination synergy model centred on high-resolution liquid chromatography mass spectrometry (HR-LCMS) and network analysis has been proposed (Banerjee *et al*., 2021). This approach allows researchers to gain insight into the mechanism of action of herbal extracts used in traditional medicine (Ngoc *et al*., 2019). The study focused on *H. tentaculatus*, as there were no previous investigations using HR-LCMS data to explore its potential anti-cancer efficacy. To evaluate the anticancer activity of the chemical compounds, present in *H. tentaculatus*, the MDA-MB-231 cell line was used as a reference (Mughees *et al*., 2020). The foundation of numerous in vitro assessments concerning a cell population's reaction to external stimuli relies on the evaluation of cell viability and proliferation. The MTT Cell Proliferation Assay quantifies the rate of cell proliferation, and conversely, when metabolic processes result in apoptosis or necrosis, it gauges the decline in cell viability (Bastipati *et al*., 2021; Şenol *et al*., 2021).

The exploration of bioactive compounds from plants, such as *H. tentaculatus*, holds great promise in the search for novel and effective anticancer agents. Understanding the mechanisms by which these compounds act on cancer cells is essential for developing targeted and efficient therapeutic approaches. The utilization of advanced analytical techniques, such as HR-LCMS and network analysis, opens new avenues for research in the field of natural products and cancer treatment. By combining such techniques with cell viability and proliferation assays like the MTT Cell Proliferation Assay, researchers can gain a comprehensive understanding of the potential anticancer efficacy of plant extracts. As researchers continue to deepen their knowledge of the mechanisms behind these compounds' anticancer properties, the possibility of developing targeted and effective therapies becomes increasingly tangible, offering hope for improved cancer treatments in the future.

MATERIALS AND METHODS

Plant Material

The plant material utilized in this study was obtained from Uttan Gorai Road in Maharashtra, India, with coordinates 19.261 °N and 72.794 °E. The aerial portions of the plant were dried at room temperature in the shade to prevent direct sunlight. Blatter Herbarium at St. Xavier's College in Mumbai validated and identified the plant material, corresponding to H. Santapau's Herbarium (No. 21599).

Extraction

A total of 4 kg of pulverized plant material (*Haplanthodes tentaculatus*) underwent maceration in 20 L of 96% methanol. The maceration process involved soaking the plant material at 50 °C for 3 hours, followed by separation using a centrifuge machine. Distillation at 50 °C collected methanol and the plant extract separately.

HR-LCMS (High-Resolution Liquid Chromatography-Mass Spectrometry)

The extracted compounds were analyzed using an Agilent G6550A MS Q-TOF HR-LCMS system equipped with an electrospray ionization source (Dual AJS ESI). The analysis was performed on a C18 column maintained at 40 °C. For chromatographic separation, the mobile phase encompassed solvent A - comprising 0.1% formic acid in water and solvent B - consisting entirely of acetonitrile. The flow rate stood at 0.300 mL/min, with pressure consistently upheld at 1200 bar. For the HR-LCMS analysis, a sample injection volume of 5 µL was used. The chromatographic gradient steps were as follows: From 0 to 1 minute: The mobile phase was held at 5% - B. From 1 to 25 minutes: A linear increase to 100% - B. From 25 to 30 minutes: The mobile phase was held at 100% - B. From 30 to 31 minutes: Return to 5% - B. From 31 to 35 minutes: The mobile phase was held at 5% - B. The mass spectrometry analysis was conducted over a range of 120 to 1200 m/z (mass-to-charge ratio). The gas flow rate was set to 13 L/min, and the gas temperature was maintained at 250 °C. The HR-LCMS system was equipped with various components, including a Hip sampler, Binary pump, Column comp., and Q-TOF.

This method allows for the separation, identification, and quantification of compounds present in the methanolic extract of *H. tentaculatus* using high-resolution liquid chromatography coupled with mass spectrometry.

Anti-cancer Activity

The cancer cell lines were sourced from the National Centre for Cell Science (NCCS) in Pune and were cultivated in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% Fetal Bovine Serum and 0.5 mL/L of penicillin/streptomycin antibiotics. Incubation was performed in a humidified environment with 5% CO_2 and 95% air at 37 °C. For the MTT assay, cells were treated with a series of doses of the test compound, ranging from 5 to 100 µg/mL. Cancer cells were trypsinized and seeded at a density of 5.0×10^4 cells/well in 96-well plates, followed by overnight incubation at 37 °C. Fresh medium with varying concentrations of the test compound (5 to 100 µg/mL) was then added to respective wells in triplicates, and the cells were incubated with the compound for 48 hours in a CO_2 incubator at 37 °C. Post-incubation, the media with the test compound was discarded, and 100 µL of MTT solution (0.5 mg/mL) was added to each well, followed by a 3-hour incubation at 37 °C. During this period, viable cells with active mitochondria reduced the MTT salt to form dark purple formazan crystals. These crystals were solubilized with Dimethyl Sulfoxide (DMSO), with gentle agitation to ensure complete solubilization, and the optical density (OD) of the solubilized formazan in DMSO was measured at 570 nm using

a microplate reader. The percentage growth inhibition in cancer cells was calculated using the formula: Percentage Growth Inhibition = [(OD control - OD treated)/OD control] * 100.

The MTT assay is a valuable method for evaluating the cytotoxic potential of natural extracts on cancer cell lines. This protocol allows for accurate measurement of cell viability and cytotoxicity, making it a widely used approach in cancer research. The obtained results can provide insights into the efficacy of the tested natural extracts as potential anti-cancer agents, guiding further investigations for drug development.

The determination of the IC_{50} value was conducted employing a linear regression equation:

y=mx+c. In this context, the values of y=50, as well as the parameters m and c, were extrapolated from the viability graph.

RESULTS

The HR-LCMS analysis of the *H. tentaculatus* extract in methanol revealed the identification of 92 distinct chemical compounds. Notable among these compounds, including Aclacinomycin N, Ganoderic acid Mb, Cucurbitacine E, Hydroquinidine, and others, demonstrated potential cytotoxic effects against MDA-MB-231 cells. Aclacinomycin N was detected at a retention time (Rt) of 6.279 minutes with a mass-to-charge ratio (m/z) of 811.3334 and a molecular weight (MW) of 813.9 (C42H55NO15). Ganoderic acid Mb exhibited a retention time of 10.46 minutes, m/z of 629.3682, and MW of 630.375 (C36H54O9). Cucurbitacin E was identified at 19.41 minutes, with an m/z of 555.2961 and MW of 556.303 (C32H44O8). Hydroquinidine was detected at 19.98 minutes, with an m/z of 324.1916 and MW of 326.198 (C20H26N2O2).

Furthermore, our study identified compounds like Quinic acid, Chlorogenic acid, and Bestatin, known for their potential cytotoxic effects in different cell lines. These compounds also possess various beneficial attributes, such as antioxidant, anti-inflammatory, anti-bacterial, and anti-fungal activities (Qian *et al*., 2016; Miao & Xiang, 2020; Benali *et al*., 2022). Additional compounds, including Esmeraldic acid, Nitrendipine, and Ohioensin-A, showcase diverse activities, such as anti-bacterial, anticonvulsant, neuroprotective, antioxidative, and anti-inflammatory properties (Conley, 1996; Gómez-Aldapa *et al*., 2018; Kim *et al*., 2020). It's important to note that while our study observed the cytotoxic effects of these compounds against cancer cells, further validation is required to establish their efficacy as anti-cancer agents. The MTT assay used in our study provides valuable insights into potential cytotoxicity but does not definitively prove anti-cancer activity. Thus, these findings serve as preliminary indicators warranting further investigation through in vivo studies or clinical trials to confirm their anti-cancer properties.

A comprehensive account of these compounds, along with their detailed information, is presented in Table 1. The diversity of functions and applications demonstrated by these compounds highlights the complexity and potential of the *H. tentaculatus* extract. This study not only underscores its potential as a source of compounds with cytotoxic effects but also uncovers promising avenues for its utilization in various fields. The chromatogram presents the negative electrospray ionization mass spectrometry (ESI MS) analysis of the methanol extract obtained from *H. tentaculatus*, as illustrated in Figure 1.

The *H. tentaculatus* extract exhibited notable cytotoxic effects against MDA-MB-231 cells, with an IC50 value of 61.41±0.692. In comparison, the standard chemotherapeutic agent, Cisplatin, exhibited an IC50 value of 23.47±0.423. While these results suggest potential cytotoxicity, further research is essential to confirm their anti-cancer efficacy. The study focuses on evaluating the cytotoxic potential of a crude plant extract using absorbance measurements at 570 nm. The inhibitory effects of the extract on cancer cell viability, notably at a concentration of 25 μg, underscore its efficacy at this dosage. The presented findings underscore the potential of the *H. tentaculatus* extract as a valuable source of compounds with cytotoxic effects.

However, to fully understand their therapeutic potential, further research is required to elucidate the precise molecular mechanisms responsible for the observed cytotoxic effects. Additionally, the isolation and characterization of individual compounds warrant exploration to assess their potential as drug candidates for cancer treatment.

Table 1: Compounds identified by HR-LCMS from methanol *H. tentaculatus* extract

S. No.	Compound Name	Rt	MW	Formula	[m/z]	DB diff (ppm)	Hits (DB)
1	Esmeraldic acid	5.883	518.153	$C_{30}H_{22}N_4O_5$	577.167	10.09	10
2	Aclacinomycin N	6.279	812.340	$C_{42}H_{55}NO_{15}$	811.333	1251.35	2
3	Nitrendipine	6.633	360.129	$C_{18}H_{20}N_2O_6$	419.143	6.58	8
4	Ohioensin-A	7.277	372.093	$C_{23}H_{16}O_5$	431.107	16.99	3
5	Fructoselysine 6-phosphate	7.559	388.125	$C_{12}H_{25}N_2O_{10}P$	447.139	-2.3	3
6	Prunitrin	7.906	446.130	$C_{22}H_{22}O_{10}$	445.122	-19.66	9
7	Diacetylfusarochromanone	8.504	376.161	$C_{19}H_{24}N_2O_6$	435.175	5.79	9
8	Ganoderic acid Mb	10.46	630.375	$C_{36}H_{54}O_9$	629.368	2.24	7
9	Chaksine	11.111	450.293	$C_{22}H_{38}N_6O_4$	449.286	4.65	10
10	Bestatin	12.304	308.174	$C_{16}H_{24}N_2O_4$	367.188	-1.31	3
11	Quinic acid	12.295	192.063	$C_7H_{12}O_6$	237.061	2.28	1
12	Cucurbitacin E	19.410	556.303	$C_{32}H_{44}O_8$	555.296	1.08	6
13	Hydroquinidine	19.981	326.198	$C_{20}H_{26}N_2O_2$	325.191	2.59	9

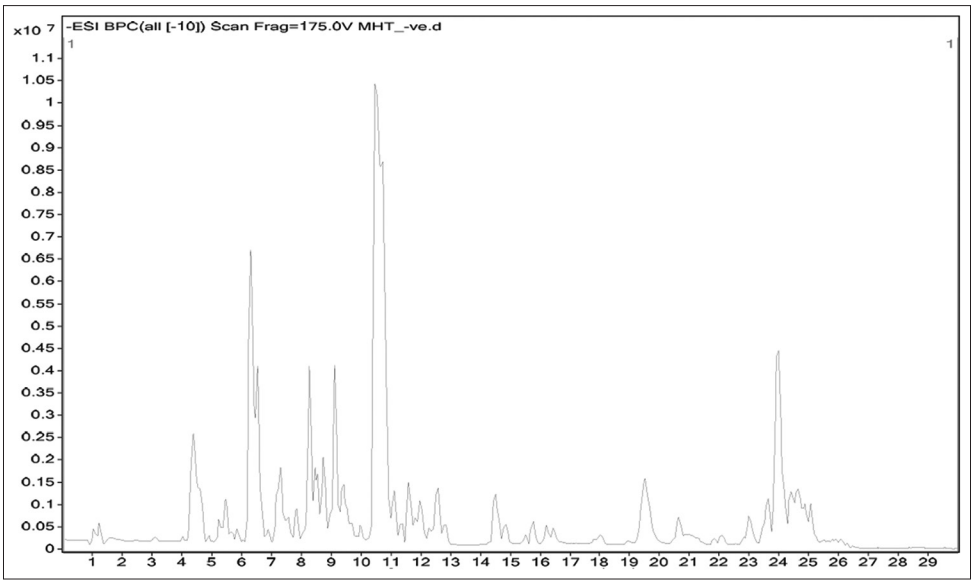

Figure 1: Chromatogram of *H. tentaculatus* methanol extract HR-LCMS

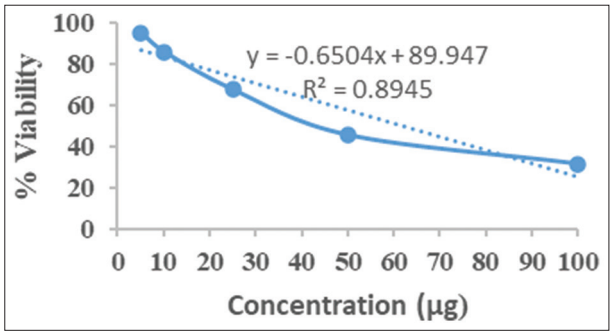

Figure 2: Graph Concentration (µg) against % Viability

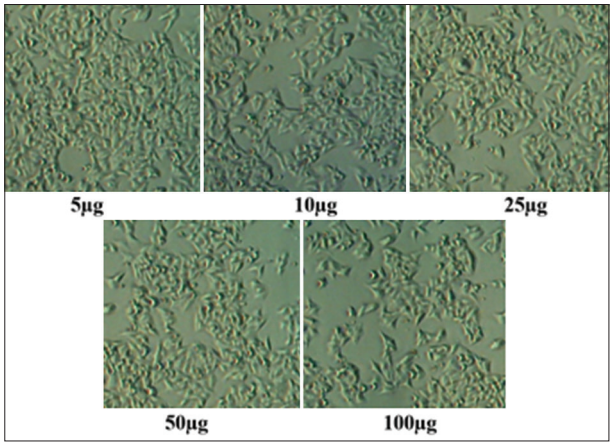

Figure 3: Cell growth of *H. tentaculatus* extract MDA-MB-231 breast cancer cell line

In conclusion, this study provides valuable insights into the chemical composition and potential cytotoxic effects of *H. tentaculatus* extract in methanol. The identified compounds hold promise as leads for future drug development, but further validation is needed to confirm their anti-cancer properties. The full Table 2, 3 and Figure 2 containing detailed compound

Table 2: *In vitro* cytotoxicity testing of *H. tentaculatus* extract by MTT assay on MDA-MB-231cell line

Concentration (µg)	Absorbance (570 nm)	% Inhibition	% Viability	IC_{50} (µg)
5	0.503	4.73	95.27	61.41±0.692
10	0.454	14.01	85.99	
25	0.357	32.38	67.62	
50	0.241	54.35	45.65	
100	0.167	68.37	31.63	
Untreated	0.528	0	100	
Blank	0	0	0	

Table 3: Cytotoxicity and cell growth inhibition parameter

S. No.	Sample Name	IC_{50} (µg/mL)
		MDA-MB-231
1	Sample	61.41±0.692
2	Cisplatin (µg)	23.47±0.423

data and IC50 values are available in the original study. A visual depiction, as shown in Figure 3, portrays the relationship between cell growth inhibition of the MDA-MB-231 breast cancer cell line and varying concentrations of *H. tentaculatus* extract (5 μg, 10 μg, 25 μg, 50 μg, and 100 μg). Notably, the concentration of 25 μg exhibited the most promising outcome at the IC50 concentration, signifying a significant inhibitory effect against the cancer cells.

DISCUSSION

The results of this study provide significant insights into the anti-cancer potential of the methanolic extract of *H. tentaculatus*. The HR-LCMS analysis elucidated the chemical composition of the extract, revealing the presence of 92 distinct chemical compounds. Notably, Aclacinomycin N, Ganoderic acid Mb ($C_{36}H_{54}O_9$), Cucurbitacin E ($C_{32}H_{44}O_8$), and Hydroquinidine ($C_{20}H_{26}N_2O_2$) demonstrated remarkable anti-

cancer potential against MDA-MB-231 cells. These compounds exhibited distinctive characteristics, including retention time, mass-to-charge ratio (m/z), and molecular weight (MW).

A compelling IC_{50} value of 61.41±0.692 µg for the *H. tentaculatus* methanolic extract was observed in the assessment of anti-cancer activity against MDA-MB-231 cells. This value signifies the concentration at which a 50% reduction in cancer cell viability was achieved, highlighting the extract's notable inhibitory effect. In comparison, the established chemotherapeutic agent Cisplatin exhibited an IC_{50} value of 23.47±0.423, indicating the promising potential of the extract in reducing cancer cell proliferation.

The diverse activities observed among the identified compounds, including Aclacinomycin N ($C_{42}H_{55}NO_{15}$), Ganoderic acid Mb, Cucurbitacin E, and Hydroquinidine, underline their potential as potent anti-cancer agents (Bourhia *et al.*, 2021; Kerru *et al.*, 2017; Yang *et al.*, 2018; Yavuz *et al.*, 2023). The graphical representation in Figure 2 illustrates the concentration-to-viability relationship of the *H. tentaculatus* methanolic extract against MDA-MB-231 cells, providing a clear visualization of the dose-dependent nature of the extract's anti-cancer effects. In summary, the study's results underscore the anti-cancer potential of the methanolic extract of *H. tentaculatus*. The identification of compounds like Aclacinomycin N, Ganoderic acid Mb, Cucurbitacin E, and Hydroquinidine with notable anti-cancer effects highlights their potential in cancer therapy. These outcomes provide valuable data for further exploration and the potential development of novel anti-cancer agents from natural sources. However, additional research is essential to unravel the mechanistic basis of these compounds' anti-cancer effects and to validate their suitability as candidates for therapeutic applications in cancer treatment.

CONCLUSION

This research showcases the potential of plant-derived compounds, specifically from *H. tentaculatus*, as effective inhibitors of cancer cell growth. Through advanced analytical techniques and cell viability assays, the study identifies key compounds like Aclacinomycin N, Ganoderic acid Mb, Cucurbitacin E, and Hydroquinidine with significant inhibitory properties. The findings emphasize the promise of natural sources in developing targeted therapies for cancer treatment. Further research is needed to understand the mechanisms underlying these compounds' effects and to harness their potential for future drug development.

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