RESEARCH PAPER

Phytochemical Profiling and Bioactivity Assessment of *Haplanthodes verticillatus*: A Potential Source of Antioxidant and Anticancer Agents

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Abstract:
Introduction: *Haplanthodes verticillatus,* an indigenous plant found in the Western Ghats of India, is classified under the Acanthaceae family and has been traditionally utilized for its medicinal properties. However, there is a lack of extensive research on its bioactive constituents and potential pharmacological effects. The objective of this study is to examine the chemical makeup and biological properties of *H. verticillatus*, with a specific focus on its antioxidant and anticancer characteristics. *Objective*: The primary objective of this research was to systematically extract and characterize the phytoconstituents of *H. verticillatus* and evaluate their potential antioxidant and anticancer activities using advanced analytical techniques and in-vitro assays. *Method*: The whole plant was collected, authenticated, and subjected to successive solvent extraction using hexane, chloroform, ethyl acetate, ethanol, and water. Each extract was analyzed through preliminary phytochemical screening, followed by detailed chemical profiling using Gas Chromatography-Mass Spectrometry (GC-MS). The ethyl acetate extract was further fractionated using column chromatography, leading to the isolation of specific phytoconstituents. The antioxidant activity of the extracts was assessed using the DPPH radical scavenging assay, while the cytotoxic effects on A549 lung cancer cells were evaluated using the MTT assay. *Results*: The GC-MS analysis of the ethyl acetate extract revealed a complex chemical profile with 85 compounds, including Allyltetramethoxybenzene (23.2%), Linoleyl acetate (5.89%), and Kessane (4.12%) as the major constituents. The ethyl acetate extract exhibited the highest antioxidant activity, with an IC_{50} value of 63.53 µg/mL, closely matching that of ascorbic acid (65.53 µg/mL). In the MTT assay, the ethyl acetate extract demonstrated a dose-dependent cytotoxic effect on A549 cells, with an IC_{50} value of 40.51 µM, indicating significant anticancer potential. Furthermore, a unique white compound isolated from the ethyl acetate extract was identified as stigmasterol, a phytosterol with known pharmacological properties. *Conclusion*: The findings of this study highlight the potential of *Haplanthodes verticillatus* as a valuable source of bioactive compounds with strong antioxidant and anticancer properties. The ethyl acetate extract, in particular, exhibited promising therapeutic potential, warranting further investigation into its bioactive components and possible applications in cancer treatment. The comprehensive chemical profiling and biological evaluation conducted in this study provide a solid foundation for future research aimed at developing novel therapeutic agents from *H. verticillatus*.

Keywords: *Haplanthodes verticillatus,* GCMS, *antioxidant* activity, MTT assay, A549 lung cancer cells
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Introduction

Haplanthodes is a genus of Angiosperms that is endemic to peninsular India. It is one of the 49 classified genera and is mostly found in the low altitude ranges of the Western Ghats region. (Singh et al., 2022; Surveswaran et al., 2022, 2020) Haplanthodes is a member of the Acanthaceae family, which has 242 genera and 3947 species divided into four subfamilies: Acanthoideae, Avicennioideae, Nelsonioideae, and Thunburgioideae. (Gnanasekaran et al., 2016; Surveswaran et al., 2022) The Haplanthodes species include: 1) H. neilgherryensis (Wight) R.B.Majumdar, 2) H. plumosa (T. Anderson) Panigrahi & G.C. Das, 3) H. tentaculata (L.) R.B.Majumdar, and 4) H. verticillata (Roxb.) R.B. Majumdar. The genus is found in the states of Goa, Gujarat, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Rajasthan, and Tamil Nadu. (Gnanasekaran et al., 2016; Singh et al., 2022; Surveswaran et al., 2022; Wood, 2014)

Haplanthodes verticillata (Roxb.) R.B. Majumdar, also known as H. verticillate, is a tall annual herb that reaches a height of approximately 0.5 meters.

Its elongated, oval-shaped leaves are positioned opposite each other and measure 5-10 cm in length. Each leaf has two pointed teeth at the tip and is covered in long, spreading hairs. The flowers, which lack stalks, are densely clustered near the top of the stem in the leaf axils. Despite appearing to have five petals, the flowers actually consist of two petals that are divided into 2-3 lobes. These dark blue flowers have a light green throat and are commonly found on rocky slopes in the Western Ghats. (Sonawane et al., 2020) The *H. verticillate* locally known as kalu kariyatu (Gujarati); kala kiriyat, kastula (Hindi); kalem kiraytem (Konkani); jakara (Marathi) and ottu mudi kurinji (Tamil). (P R Kanthale and Biradar, 2012; Sonawane et al., 2020) Emeka and his colleagues conducted a study on the ethanol extract of H. verticillata leaves to identify the presence of phytoconstituents using GCMS analysis. The researchers recorded the presence of several bioactive chemicals in the ethanol-based extract obtained from the leaves. The ethanolic extract of *H. verticillata* leaves contains several compounds, with the most abundant ones being 3.beta.,17.beta.- dihydroxyestr-4-ene (10.89%), Hexadecanoic acid, ethyl ester (7.49%), 1-Methylbicyclo[3.2.1] octane (7.28%), Linoleic acid ethyl ester (6.90%), Ethyl Oleate (5.65%), and Caryophyllene oxide (5.29%). (Emeka et al., 2022) Kanthale and Biradar (2012) conducted an ethnobotanical assessment of the plant species found in the Mahur range forest in Nanded District. The researchers documented the traditional therapeutic application of *Haplanthodes* verticillata for treating asthma. The recommended dosage for treating asthma is to orally consume a tablespoon of root extract mixed with honey twice day for a duration of seven days. (Biradar, 2014; P. R. Kanthale and Biradar, 2012; LABHANE and DONGARWAR, 2014; Mubarak, 2021) Dhole et al. undertook a survey from 2007-2010 to examine the weed diversity in various maize crop fields in Nanded district. The aim was to identify the primary weed species found in kharif maize fields. The survey identified *H. verticillata* as a weed present in the maize crop areas. (Dhole et al., 2013) This research is motivated by the distinct ecological and medicinal importance of the genus Haplanthodes, specifically *Haplanthodes verticillata*. This species is native to the peninsular area of India and is mostly found in the Western Ghats. Although its traditional medicinal uses and phytoconstituents have been well-documented, there is a lack of extensive research on its bioactive components and possible pharmacological effects. The objective of this research is to address the lack of information by undertaking a thorough examination of the chemical components of *H. verticillata* in order to discover and describe its biologically active chemicals. The aims of this study are: to examine the chemical composition of *H. verticillata* using advanced techniques such as GC-MS and to analyze the plant's chemical components using In-silico and invitro analysis to assess their potential for anticancer activity.

Material and Method

Collection & Authentication of plant material

In August 2023, the whole plant of Haplanthodes verticillatus (H. verticillatus) was collected from the Northern Western Ghats (Nashik district, Maharashtra, India). The botanist at Sandip University, Nashik, India, authenticated the plant, and the herbarium was deposited under voucher specimen number SUN20230028.

Preparation of Plant material and Extraction of Phytoconstituents

The gathered plants were dehydrated/dried in a shaded area. The entire plant was dried, crushed, and powdered. Once adequately dried, the whole plants were crushed and finely powdered using a mechanical grinder. The plant material of *H. verticillatus* weighing 1000 g was extracted using a Soxhlet system with solvents of increasing polarity (Hexane > Chloroform > Ethyl Acetate> Methanol>Water). Following the initial phytochemical examination, the plant material (separately) was extracted using an all above mentioned solvents for additional study.

Concentration of Extracts

Each solvent extract was concentrated under reduced pressure using a rotary evaporator to remove the solvent, yielding crude extracts. The concentrated extracts were then collected and stored in airtight containers at low temperatures to prevent degradation and contamination.

Preliminary Phytochemical Screening

The crude extracts obtained from each solvent were subjected to preliminary phytochemical screening to identify the presence of various classes of phytoconstituents. The extracts were assessed for their physical characteristics, including color, consistency, and solubility. Standard phytochemical tests were employed to detect the presence of alkaloids, flavonoids, tannins, glycosides, saponins, and phenolic compounds.

GC-MS analysis

The GC-MS study of *H. verticillatus* extracts (obtained all extracts) was carried out using Gas Chromatography coupled with Mass Spectroscopy detector (Shimadzu GCMS-QP Series, Model GCMS-QP2020) and equipped with an AOC–20i autosampler. A Sh-Rxi-5Sil MS capillary column (30 m × 0.25 mm × 0.25 μm) (Shimadzu) was used for chromatography. For the study, a detector was operated at 70eV (Ionization energy) with electronic impact ionization (EI) mode over a scanning range of 35-600 atomic mass units (2000 Scan Speed). The carrier gas was helium with a steady 1.0 mL/min flow rate. Injector temperature was 260 °C, and the ion source temperature was 280 °C. 5 µL of the sample was injected, using

spitless mode. At a heating rate of 8 °C/min, the GC oven temperature was ramped from 50 °C (held for 3 min) to 150 °C (held for 10 min); from 150 °C to 250 °C at a heating rate of 8 °C/min (held for 10 min); from 250 °C to 280 °C at a heating rate of 10 °C/min (held for 10 min). The total run time was 56 minutes. The relative percentage of the phytoconstituents has been expressed as a percentage with peak area normalization.

Identification of Compounds

The constituents of the extract were identified by comparing their mass spectral fragmentation patterns with those documented in the literature and stored in the MS Library (NIST/EPA/NIH Mass Spectra Library 2017) using a similarity search. The retention indices of the compounds were cross-referenced with those reported in the literature to verify their identification.

Isolation of Phytoconstituent by Column Chromatography

The ethyl acetate extract, known for its diverse array of phytoconstituents, was subjected to further fractionation via column chromatography. A glass column measuring 60 cm in height and 3 cm in diameter was filled with silica gel ranging from 60 to 120 mesh, reaching a height of 45 cm. The silica gel was activated by subjecting it to a temperature of 110°C for a duration of 2 hours before using it. The ethyl acetate extract, weighing 10 grams, was diluted in a small quantity of ethyl acetate and combined with silica gel to create a powder that flows easily. This powder was subsequently placed on the upper part of the silica gel column. Elution was performed using a gradient of solvents with increasing polarity, starting with hexane and progressing through various hexane: ethyl acetate mixtures, ultimately reaching pure ethyl acetate. The fractions were obtained in 75 ml increments, concentrated using a rotary evaporator under decreased pressure, and then kept in sealed containers at low temperatures for future analysis.

In-Vitro Study

Antioxidant activity :DPPH radical scavenging activity

The different extract of *H. verticillatus* were analyzed using a 1,1-diphenyl-2-picryl hydrazyl (DPPH) method. The stock solution was produced by dissolving 24 milligrams of DPPH in 100 mL of methanol. The *H. verticillatus* extracts (100 µL) were combined with 3 mL of DPPH working solutions in a test tube. A standard/blank is typically prepared by combining 3 mL of a solution containing DPPH with 100 µL of methanol. Subsequently, the tubes were subjected to a period of 30 minutes in which they were maintained in a state of total absence of light. The measurement of absorbance was consequently conducted at a wavelength of 517 nm. The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated using the following equation: (Akgül et al., 2022; Baliyan et al., 2022; Chaves et al., 2020)

$$\text{DPPH radical scavenging activity } \% = [(Ac - As)/Ac] \times 100$$

Where, Ac is the absorbance of the control reaction, As is the absorbance of *H. verticillatus* extracts.

Anticancer Activity

***In-vitro* cytotoxicity activity**

Cell cultures

The A549 (Lung Cancer) cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, India, and cultured in Dulbecco's modified Eagles medium (DMEM).

***In vitro* cytotoxicity (MTT Assay)**

The cell viability was assessed through direct observation using an inverted phase contrast microscope, followed by the MTT assay technique. The process of seeding cells in a 96 well plate involved utilizing a two-day-old confluent monolayer of cells. The cells were trypsinized and suspended in a growth medium with a concentration of 10%. A 100 µl cell suspension containing $5x10^4$ cells per well was then added to the 96 well tissue culture plate. The plate was kept at a temperature of 37ºC in a humidified 5% CO2 incubator.

The cell viability was assessed by the standard MTT assay after 24 hours of incubation. The ethyl acetate extract of *H. verticillatus* extract (EA-HVE) was added to the cells at a final concentration of 25 µg, 50 µg, 100 µg, 200 µg, and 400 µg in 500 µl of 5% DMEM (Dulbecco's modified Eagle's Medium) from a stock of 1 mg/ml (dissolved in DMEM using a cyclomixer). Additionally, control cells that were not treated were preserved. The inverted phase contrast tissue culture microscope was used to observe the entire plate after 24 hours of treatment, and the microscopic observations were recorded as images. The 15 mg of MTT was reconstituted in 3 ml of Phosphate Buffer Saline (PBS) until it was fully dissolved, and the mixture was sterilized using a filter. 30µl of reconstituted MTT solution was added to all test and cell control wells after the 24-hour incubation period. The plate was gently agitated, and it was then incubated at 37ºC in a humidified 5% CO2 incubator for 4 hours. The sample content in the wells was removed. The formazan crystals were solubilized by gently mixing the wells by pipetting up and down after the incubation period, followed by the removal of the supernatant and the addition of 100 µl of MTT Solubilization Solution (Dimethyl sulphoxide). The microplate reader was employed to measure the absorbance values at a wavelength of 540 nm. (Fithrotunnisa et al., 2020; Kumar et al., 2021; Padmini et al., 2022)

The percentage of growth inhibition was calculated using the formula:

% of viability = (Mean OD Samples/ Mean OD of control group) x 100

Results

Collection & Authentication of plant material

The whole plant of *Haplanthodes verticillatus* (*H. verticillatus*) was collected from Northern Western Ghats (Nashik district, Maharashtra, India) in August 2023. The plant was authenticated by the botanist at Sandip University, Nashik, India, and herbarium was deposited under voucher specimen no SUN20230028.

Extraction of Phytoconstituents

The collected specimens of *H. verticillatus* were first subjected to a meticulous drying process conducted under shaded conditions to preserve the integrity of the phytoconstituents. Post-drying, the whole plants were meticulously crushed and finely powdered to facilitate efficient extraction. A total of 1000 grams of the powdered plant material was subjected to successive solvent extraction using a Soxhlet apparatus, employing solvents of increasing polarity, starting from non-polar to polar solvents.

Initially, the plant material was extracted with hexane, a non-polar solvent, to remove lipophilic compounds such as fats, oils, and waxes. Following the hexane extraction, the marc was subjected to chloroform extraction, targeting moderately polar compounds including alkaloids and some glycosides. Subsequently, the remaining plant material was extracted with ethyl acetate, aiming to isolate flavonoids, tannins, and other semi-polar compounds. Finally, methanol and water, polar solvents, were used sequentially to extract polar constituents such as phenolics, glycosides, saponins, and other hydrophilic compounds.

Each solvent extraction cycle was conducted for a sufficient duration, typically 24-48 hours, ensuring exhaustive extraction of respective phytoconstituents. The extracts obtained from each solvent were then concentrated under reduced pressure using a rotary evaporator to remove the solvents, yielding crude extracts. These crude extracts were then stored at low temperatures in airtight containers to prevent degradation and contamination.

Each extract was subjected to preliminary phytochemical screening to identify the presence of various classes of phytoconstituents. This methodical approach allowed for the systematic extraction and preliminary characterization of a broad spectrum of phytochemicals present in *H. verticillatus*, laying a foundational basis for further bioactivity-guided fractionation and detailed phytochemical analysis. This comprehensive extraction process ensures a thorough investigation into the plant's medicinal potential, aiding in the discovery of novel bioactive compounds with therapeutic relevance (Table 1).

Table 1 : Percentage yeild of Soxhlet Extraction

Sr Number	Extract	% Yeild
1	Hexane	1.7
2	Chloroform	0.8
3	Ethyl Acetate	1.9
4	Ethanol	2.7
5	Water	3.4

Preliminary Phytochemical Screening

Preliminary phytochemical screening was conducted on the crude extracts obtained from each solvent to detect the presence of a variety of phytoconstituent classes. The extracts were assessed for their physical characteristics, including color, consistency, and solubility. Standard phytochemical tests were employed to detect the presence of alkaloids, flavonoids, tannins, glycosides, saponins, and phenolic compounds (Table 2).

Table 2: Preliminary Phytochemical Screening of all extracts

Chemical Tests	Hexane Extract	Chloroform Extract	Ethyl Acetate	Ethanol	Water
Alkaloids	-	+	-	-	-
Carbohydrates	-	-	+	+	+
Flavonoid	-	-	+	+	+
Glycosides	-	-	+	-	-
Proteins	-	-	-	-	-
Steroids	+	-	+	+	-
Triterpenoid	+	-	+	+	+

Note: (+) Indicates present; (-) indicates absent.

GC-MS analysis

The GC-MS analysis of the ethyl acetate extract of *H. verticillatus* revealed a complex chemical profile with 85 identified peaks (Figure 1 & Table 3). The most abundant compound was Allyltetramethoxybenzene, constituting 23.2% of the extract, followed by Kessane at 4.12% and Linoleyl acetate at 5.89%. Other notable compounds included Muurola-4,10(14)-dien-1β-ol (2.8%), Myristicin (2.31%), and Tetrapentacontane (2.33%). Several minor constituents were also detected, such as Ammonium acetate (1.46%), δ-Cadinene (2.05%), and Squalene (0.71%), among others. The diverse array of compounds, including hydrocarbons, esters, alcohols, and ketones, suggests the extract's potential bioactive properties, such as anti-inflammatory, antimicrobial, and antioxidant activities. This chemical profile provides valuable insights into the pharmacological potential of *H. verticillatus* and underscores the

need for further research into its therapeutic applications.

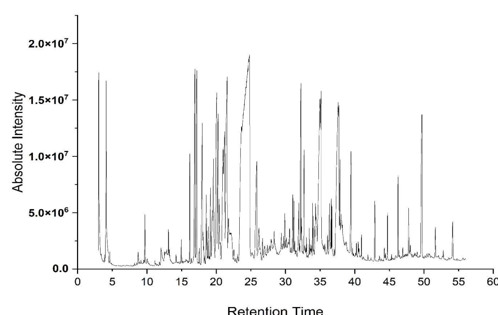

Figure 1: GCMS Chromatogram of ethyl acetate extract of *H. verticillatus*

Table 3: GCMS Table of ethyl acetate extract of *H. verticillatus*

Peak	R. Time (min)	Area %	Name of Compound
1	3.3	1.46	Ammonium acetate
2	3.96	0.04	Toluene
3	4.525	1.85	Toluene
4	4.745	0.05	2-Methyl-2-heptene
5	8.88	0.09	β-Myrcene
6	9.87	0.29	β-Phellandrene
7	11.2	0.01	Undecane
8	12.44	0.33	Cyclononanone
9	12.55	0.02	4-Isopropylcyclohexanone
10	12.94	0.02	(1,2,3-Trimethyl-cyclopent-2-enyl)-methanol
11	13.18	0.13	2-Cyclohexen-1-one, 4-(1-methylethyl)-
12	13.26	0.02	Dodecane
13	14.295	0.04	Linalyl acetate
14	14.91	0.02	4-Hexen-1-ol, 5-methyl-2-(1-methylethenyl)-, acetate
15	15.03	0.1	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)
16	15.115	0.02	Cyclohexasiloxane, dodecamethyl-
17	16.275	0.6	α-Cubebene
18	16.475	0.02	Aciphyllene
19	16.77	0.03	1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl-5-(1-methylethyl)-, [1S-(1α,2α,3aβ,4α,5α,7aβ)]
20	17.025	2.21	Copaene
21	17.355	2.12	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane-rel
22	17.635	0.09	(1R,4S,5S)-1,8-Dimethyl-4-(prop-1-en-2-yl)spiro[4.5]dec-7-ene
23	18.035	1.63	Caryophyllene
24	18.265	0.45	Caryophyllene
25	18.425	0.07	cis-Thujopsene
26	18.625	0.55	(E)-β-Famesene
27	18.745	0.23	Lavandulyl isobutyrate
28	18.975	0.34	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
29	19.075	0.06	2H-2,4a-Ethanonaphthalene, 1,3,4,5,6,7-hexahydro-2,5,5-trimethyl-
30	19.265	0.66	(1R,4R,5S)-1,8-Dimethyl-4-(prop-1-en-2-yl)spiro[4.5]dec-7-ene
31	19.505	0.75	Ylangene
32	19.825	1.31	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
33	20.135	3.06	Eudesma-4(14),11-diene
34	20.335	1.86	gamma.-Selinene
35	20.535	0.76	(3S,3aS,8aR)-6,8a-Dimethyl-3-(prop-1-en-2-yl)-1,2,3,3a,4,5,8,8a-octahydroazulene
36	20.715	0.19	Benzene, 1-methyl-4-(1,2,2-trimethylcyclopentyl)-, (R)-
37	21.045	2.05	delta.-Cadinene
38	21.275	2.31	Myristicin
39	21.66	4.12	Kessane
40	21.96	0.96	4-Isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahydronaphthalene
41	22.46	1.1	Elemicin
42	22.76	0.15	4-Isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahydronaphthalene
43	22.9	0.05	β-Oplopenone

	85		
44	23.235	0.11	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-
45	25.035	23.2	Allyltetramethoxybenzene
46	25.535	0.34	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene
47	26.585	2.8	Muurola-4,10(14)-dien-1β-ol
48	26.885	0.28	Lavandulyl caproate
49	27.185	0.32	τ-Cadinol
50	27.535	0.34	(1S,4S)-4-Isopropyl-1,6-dimethyl-1,2,3,4-tetrahydronaphthalen-1-ol
51	28.185	0.93	Agarospirol
52	28.985	1.02	Ergost-25-ene-3,5,6,12-tetrol, (3.beta.,5.alpha.,6.beta.,12.beta.)-
53	29.535	0.64	1-Oxacyclopentadecan-2-one, 15-isopropenyl
55	30.285	0.96	1-((1S,3aR,4R,7S,7aS)-4-Hydroxy-7-isopropyl-4-methyloctahydro-1H-inden-1-yl)ethanone
56	30.935	0.93	3-cyclohexene-1,1-dimethanol, 4,5-dimethyl-2-(2-methyl-1-propen-1-yl)-
57	31.735	1.33	beta.-Bisabolol
58	32.585	2.84	Neophytadiene
59	32.985	1.12	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
60	33.135	0.13	Neophytadiene
61	33.535	0.3	Curcumenol
62	34.185	0.91	Cyclodecasiloxane, eicosamethyl-
63	34.635	1.39	Linolenic acid
65	35.885	0.3	Propanoic acid, 3-phenyl-, 2-oxo-2-phenylethyl ester
66	36.985	1.7	Bergapten
67	37.785	5.89	Linoleyl acetate
68	38.635	2.52	Octadecanoic acid
71	40.835	0.32	l-(+)-Ascorbic acid 2,6-dihexadecanoate
72	43.285	0.45	Dotriacontane
73	44.585	0.29	l-(+)-Ascorbic acid 2,6-dihexadecanoate
74	44.885	0.31	Dotriacontane
75	45.385	0.08	Linalyl methyl-ethyl-acetate
76	46.635	0.89	Dotriacontane
77	47.685	0.53	Tetracosane
78	48.285	0.71	Squalene
79	49.335	0.51	2-Pentanone, 5-phenyl-
80	49.935	2.33	Tetrapentacontane
81	50.985	0.46	Tetracosamethyl-cyclododecasiloxane
82	51.785	0.49	Tetrapentacontane
83	53.285	0.39	Arachidic acid, 3-methylbutyl ester
85	54.485	0.44	Tetrapentacontane

Isolation of Phytoconstituent by Column Chromatography

The ethyl acetate extract of *H. verticillatus*, which typically contains a broad spectrum of phytoconstituents, was selected for further fractionation using column chromatography. The chromatography process was meticulously carried out, resulting in the collection of 55 fractions, each of 75 ml volume (Table 4).

Table 4: Gradient Scheme of Hexane: Acetate for Fraction Collection During Column Chromatography

Fraction Number	Gradient Scheme Hexane: Ethyl Acetate
1-5	100:0 (100 % Hexane)
6-10	95:05
11-15	90:10
16-20	85:15
21-25	80:20
26-30	75:25
31-35	70:30
36-40	60:40
41-45	50:50
46-50	40:60
51-53	25:75
54-55	0:100 (100% Ethyl Acetate)

After performing column chromatography on the ethyl acetate extract, a total of 55 fractions (each containing 75 mL) were successfully obtained by using a gradient elution of hexane and ethyl

acetate. The gradient gradually intensified from 100% hexane to 100% ethyl acetate, enabling the segregation of the phytoconstituents according to their polarity. The initial fractions (1-5), which were separated using 100% hexane, mainly consisted of non-polar chemicals. However, the following fractions showed a progressive rise in polarity, which correlated with the higher concentration of ethyl acetate in the solvent used for separation. The fractions were subjected to concentration at reduced pressure using a rotary evaporator, resulting in dried residues that were then stored in airtight containers at low temperatures.

Analysis of obtained fractions

To find and validate the existence of phytoconstituents, GC-MS analysis was performed on each of the 55 fractions. The analysis revealed a diverse array of compounds distributed across the fractions. Notably, fractions numbered 26 to 32 yielded a distinctive white-colored compound. This specific compound was isolated for further analysis due to its unique appearance and potential significance.

Identification of the White Compound

The white compound obtained from fractions 26 to 32 was analyzed using GC-MS. The GC-MS chromatogram of this compound displayed a single, well-defined peak, indicating a high degree of purity. To confirm the identity of this compound, Direct Insertion (DI) mass spectrometry was performed using the same instrument. The DI method provided accurate mass measurements, which, in conjunction with a library search, facilitated the identification of the compound (Figure 2). The mass spectral data and library matching identified the compound as Stigmasterol. Stigmasterol is a well-known phytosterol with significant pharmacological properties.

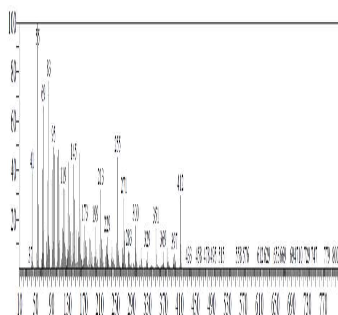

Figure 2: Mass spectra of Stigmasterol

In-Vitro Study

Antioxidant activity- DPPH radical scavenging activity

The antioxidant activity of several *H. verticillatus* extracts was tested using the DPPH radical scavenging assay, with ascorbic acid acting as the standard reference. The results showed clear variations between the extracts, as indicated by the IC_{50} values and percentage inhibition (the concentration needed to block 50% of DPPH radicals) (Figure 3). Ascorbic acid showed a dose-dependent increase in DPPH radical scavenging activity, with inhibition ranging from 21.73% at 20 µg/mL to 84.68% at 120 µg/mL, and an IC_{50} value of 65.53 µg/mL. The ethyl acetate extract demonstrated the highest antioxidant activity among the extracts, with percentage inhibition values ranging from 30.54% to 72.49% across the same concentration range, and an IC_{50} value of 63.53 µg/mL, indicating a strong radical scavenging potential comparable to that of ascorbic acid. The aqueous extract also exhibited significant antioxidant activity, with inhibition values increasing from 21.28% to 80.06% and an IC_{50} value of 67.92 µg/mL, closely resembling the standard. In contrast, the ethanol extract showed moderate antioxidant activity, with inhibition ranging from 14.61% to 66.22% and an IC_{50} value of 85.66 µg/mL. The hexane extract displayed lower antioxidant activity, with inhibition values from 2.80% to 61.52% and an IC_{50} value of 97.47 µg/mL. Antioxidant activity was most weakly demonstrated by the chloroform extract, with inhibition values ranging from 7.81% to 63.55% and an IC_{50} value of 93.05 µg/mL.

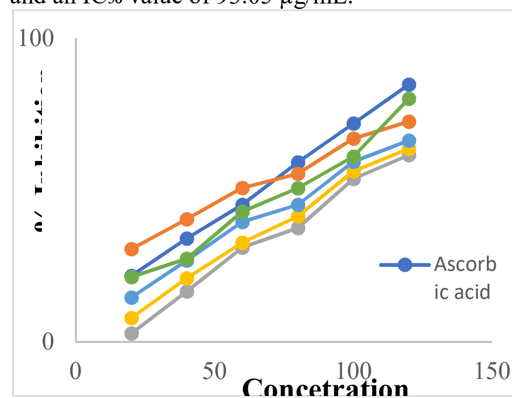

Figure 3: Percentage inhobition of all extract for DPPH method

Anticancer Activity- *In-vitro* cytotoxicity activity

The MTT assay was employed to assess the cytotoxic effects of the extract on the A549 cancer cell line. Cell viability is evaluated through the MTT assay, which is widely recognized for its ability to correlate with cell density and absorbance through the reduction of MTT by mitochondrial dehydrogenase in viable cells. Ideally, absorbance readings between 0.9 and 1.0 indicate a suitable range for evaluating both the stimulation and inhibition of cell proliferation. The optimal densities of A549 cells to be seeded for cytotoxicity assay were 16,000 cells/100 µL (Table 5).

Table 5: Cytotoxic effect of ethyl acetate extract of *H. verticillatus* against A549 cancer cell line

	B	Un	Cispl	Concentration of

	lank	treated	atin 25 µM	Extract				
				25 µM	50 µM	100 µM	200 µM	400 µM
Reading 1	0.03	0.879	0.411	0.543	0.408	0.36	0.285	0.192
Reading 2	0.05	0.865	0.409	0.531	0.413	0.364	0.279	0.183
Mean	0.04	0.872	0.41	0.537	0.4105	0.362	0.282	0.1875
Mean OD - Mean B		0.832	0.37	0.497	0.3705	0.322	0.242	0.1475
Standard deviation		0.009899	0.001414	0.008485	0.003536	0.002828	0.004243	0.006364
Standard error		0.007001	0.001	0.006001	0.0025	0.002	0.003	0.0045
Viability %		100	44.47115	59.73558	44.53125	38.70192	29.08654	17.72837

In this study, A549 cells were treated with varying concentrations of the extract (25 µM, 50 µM, 100 µM, 200 µM, and 400 µM) alongside a positive control (Cisplatin at 25 µM) for 48 hours (Figure 4). The untreated cells served as a negative control to establish baseline viability. The results demonstrated a dose-dependent cytotoxic effect of the extract on A549 cells. The absorbance values decreased as the concentration of the extract increased, indicating a reduction in cell viability. Specifically, the mean optical density (OD) for untreated cells was 0.872, while the mean OD for cells treated with 400 µM of the extract dropped significantly to 0.1875. The "Mean OD - Mean B" values, which account for background absorbance, followed a similar trend, with untreated cells showing the highest corrected OD of 0.832 and the 400 µM treated cells showing the lowest at 0.1475.

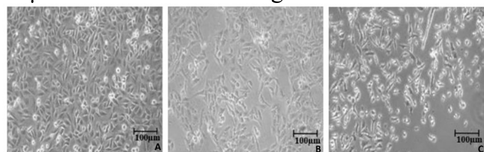

Figure 4: Morphological characteristics of A549 cancer cell line treated with ethyl acetate extract of *H. verticillatus* and (Std) cisplatin (25 µM) (A- Untreated B- Std, C- EA-HVE)

Statistical analysis revealed that the standard deviation and standard error were generally low across all concentrations, indicating consistent and reliable data. The calculated viability percentages further confirmed the cytotoxic potential of the extract, with cell viability decreasing from 100% in untreated cells to as low as 17.73% in cells treated with 400 µM of the extract. The IC_{50} value was found to be 40.51 µM.

Summary

The study focused on the detailed phytochemical investigation and biological evaluation of *Haplanthodes verticillatus*, a plant collected from the Northern Western Ghats in Maharashtra, India. A Soxhlet apparatus was employed to conduct a systematic extraction process of the plant material, which involved the use of solvents of increasing polarity—hexane, chloroform, ethyl acetate, ethanol, and water. The plant material was authenticated. Each solvent targeted specific phytoconstituents, leading to the isolation of a broad spectrum of compounds, including lipophilic compounds from hexane and polar compounds like phenolics and glycosides from ethanol and water. The extraction yielded crude extracts that were further concentrated and stored for analysis.

Preliminary phytochemical screening of these extracts revealed a rich diversity of bioactive compounds, with the ethyl acetate extract showing a particularly broad range of constituents, including flavonoids, tannins, and triterpenoids. The GC-MS analysis of the ethyl acetate extract identified 85 distinct compounds, with Allyltetramethoxybenzene (23.2%), Linoleyl acetate (5.89%), and Kessane (4.12%) being the most prominent. These compounds are known for their potential therapeutic properties, including anti-inflammatory and antimicrobial effects.

The extracts were tested for antioxidant activity utilizing DPPH radical scavenging and ascorbic acid as a reference. The ethyl acetate extract had the strongest antioxidant activity, with an IC_{50} value of 63.53 µg/mL, comparable to ascorbic acid (65.53 µg/mL). Other extracts, such as the aqueous and ethanol extracts, also showed significant antioxidant potential, though slightly lower than the ethyl acetate extract.

In addition to the antioxidant studies, the cytotoxic effects of the extracts were evaluated using the MTT assay on A549 lung cancer cells. The ethyl acetate extract exhibited a dose-dependent cytotoxic effect, with a marked reduction in cell viability observed at higher concentrations. The IC_{50} value was calculated to be 40.51 µM, indicating a potent anticancer effect. These findings were supported by low standard deviation and error values, ensuring the reliability of the data.

Moreover, the study included the isolation of a unique white compound from the ethyl acetate extract using column chromatography. This

compound was identified as stigmasterol through GC-MS and Direct Insertion mass spectrometry, a phytosterol known for its cholesterol-lowering and anticancer properties, adding further value to the therapeutic potential of *H. verticillatus*.

Conclusion

The study highlights the potential of *Haplanthodes verticillatus* as a source of bioactive compounds with significant antioxidant and cytotoxic properties. The ethyl acetate extract, in particular, exhibited strong radical scavenging and anticancer activities, indicating its potential for further exploration as a therapeutic agent. The identification of numerous phytoconstituents through GC-MS provides a foundation for future research aimed at isolating and characterizing the active compounds responsible for these biological effects.

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