ORIGINAL ARTICLE

Anticancer Potential of *Tabernaemontana divaricata* Extracts against HeLa and Vero Cell Lines

J. Jovila[1], A. J. Renilda Sophy[1], Swathiga Subbiah[2], K. Murugan[3], R. Babu[4], Suresh Malakondaiah[5], Angeline Julius[5], Archana Behera[6], N. Aravindha Babu[7], Mukesh Kumar Dharmalingam Jothinathan[6]

[1]Department of Advanced Zoology and Biotechnology, Loyola College, Chennai, Tamil Nadu, India, [2]Department of Dermatology, Saveetha Medical College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India, [3]Bannari Amman Institute of Technology, Erode, Tamil Nadu, India, [4]Department of General Medicine, Vinayaka Missions Medical College, Karaikal, (Vinayaka Missions Research Foundation), Puducherry, India, [5]Centre for Materials Engineering and Regenerative Medicine, Bharath University (Deemed to be University), Chennai, Tamil Nadu, India, [6]Department of Biochemistry, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu, India, [7]Department of Oral Pathology and Microbiology, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research (BIHER), Bharath University, Chennai, Tamil Nadu, India

Abstract

Background: The history of India has recorded about its ancient medical practices, which date back to centuries when the use of green plants of various kinds was used to treat most of the diseases in such critical periods. Such one is the perennial evergreen shrub *Tabernaemontana divaricata*, which has been used as traditional medicine. It is mainly famous with its phenols and flavonoids. These compounds are associated with different pharmacological activities. **Objective:** Therefore, the leaves of *T. divaricata* were collected, extracted, and studied in this research to establish their anticancer effects. Nevertheless, its anticancer properties have received less research; hence, the objective of the research was to examine the *in vitro* anticancer effects of *T. divaricata* leaf extract isolated with the aid of a crude solvent extraction. **Materials and Methods:** Past research has demonstrated that it has possible anti-inflammatory, anti-microbial, and antioxidant properties. The anticancer activity was confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay used to evaluate the inhibition of cell growth in the *in vitro* anticancer research that was done on Vero and HeLa cell lines. **Results:** The findings revealed the anti-cancer activity of *T. divaricata* on the HeLa cell lines. In addition, docking showed that 2,2-dibromocholestanone has high binding affinity to Caspase 3, p53, and nuclear factor kappa -B and indicates potential use in anticancer. **Conclusion:** These results indicate the potential anticancer properties of *T. divaricata* leaf extract and support its possible use in anticancer research.

Key words: Anticancer, HeLa, MTT assay, plant extract, public health

INTRODUCTION

Cancer is a significant killer and still a major threat to human health and well-being over the world. Although recent breakthroughs have been made in cancer studies, it still has been very hard to manage. Several treatment methods, such as surgery, chemotherapy, radiation therapy, and targeted therapies[1] have been developed to deal with the growth of cancer and its metastasis. However, the mystery is how safe and effective drugs can be developed. One of the most common therapeutic methods applied to the treatment of cancer is the chemotherapy, which involves the use of cytotoxic agents to eliminate the cancer cells. Nevertheless, chemotherapy has its negative side as it has various side effects that affect the life of the patient. The substances that are not so natural will have less impact on the individual. Although contemporary medicine

Address for correspondence:
J. Jovila, Department of Advanced Zoology and Biotechnology, Loyola College, Chennai, Tamil Nadu, India. E-mail: jovilaj@gmail.com

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has succeeded in treating cancer, herbal medicine presents an alternative to the traditional one with a potential solution to a safer and less expensive way of treatment.[2] There is widespread study on various plant-derived metabolites in the question of their potential in the treatment of cancer, leading to the development of new therapeutic drugs. This research paper was conducted to investigate the anti-cancer effect of *Tabernaemontana divaricata* leaf extract.

As a perennial evergreen shrub, *T. divaricata* is able to produce a number of secondary metabolites to survive and develop. These are secondary metabolites that are important in plant defence, communication, and adaption to environmental stress. Plants are used as a part of the medicinal preparation in different parts, such as leaves, roots, bark, sap, and flowers. According to Das and Dubey,[2] *T. divaricata* is commonly used in traditional medicine in the treatment of various diseases, including cephalalgia, epilepsy, edema, pyrexia, abdominal neoplasms, diarrhea, ocular infections, fractures, leprosy, and inflammation.[3] In the present research paper, the evaluation of anticancer properties of the leaf extract was done through the effectiveness of *T. divaricata* in preventing cell proliferation. This analysis was on human cervical cancer (HeLa) and vero cells. These findings clearly indicate that *T. divaricata* has anticancer properties on human cervical cancer cell lines.

MATERIALS AND METHODS

Collection and extraction of sample

T. divaricate leaves were collected from the Kanyakumari district in January 2021. The specimen was authenticated and a voucher specimen (Plant Anatomy Research Center [PARC]/2021/4440) was deposited at the PARC, West Tambaram, Chennai.

Sequential extraction of desiccated plant material was performed using solvents of ascending polarity, including methanol, ethyl acetate, and hexane. Briefly, 180 g of dry powder was deposited in a conical flask; 800 mL (1:4 W/V) of hexane was added, and the mixture was stirred intermittently for 72 h. The extracts were filtered and the solvent was removed by vacuum distillation in a rotary evaporator. The extract is in paste form. The extracts were placed in flasks before desiccation. The remaining plant material was sequentially extracted with ethyl acetate and methanol.[3]

Phytochemical screening of *T. divaricata* extracts

Preliminary phytochemical analyses of alkaloids, coumarin, flavonoids, glycoside-sugar, saponin, steroid, tannin, phenol, and proteins were performed by the standard procedure of Duraipandiyan and Ignacimuthu.[3]

The antiproliferative effect of extracts on cancer cell lines

HeLa was acquired from the Central University Laboratory, DCAHS, TANUVAS, Madhavaram, Chennai. Stock cells were cultured in DMEM supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), 10% inactivated fetal bovine serum (FBS), and Amphotericin B (5 µg/mL) in a humidified atmosphere containing 5% CO_2 at 37°C until confluent. The cells were dissociated in TPVG solution containing trypsin (0.2%), ethylenediaminetetraacetic acid (0.02%), and glucose (0.05%) in phosphate buffered saline. The stock cultures were grown in culture flasks and were placed in 96-well plates.[4]

Formulation of analytical solutions

Each extract was dissolved in DMSO, and the volume was adjusted with DMEM containing inactivated FBS (2%) to obtain a stock solution at a concentration of 2 mg/mL, followed by sterilization by filtration. Two-fold serial dilutions were prepared from this mixture for cytotoxicity assays. The maximum DMSO concentration was <0.1%.[5]

Assessment of cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Monolayer cultures were trypsinized and the cell concentration was increased to 1.0×10^5 cells/mL in DMEM supplemented with 10% FBS. A 0.1 mL solution, approximately containing 10,000 cells, was introduced into each well of a 96-well plate. Following a 24 h period, the supernatant was discarded, the wells were rinsed, and 50 µL of different extract concentrations were administered. The plates were incubated at 37°C in a 5% CO_2 atmosphere. The supernatant was removed, 100 µL of propanol was used to dissolve formazan, and absorbance was quantified at 540 nm. Growth inhibition percentages and IC_{50} values were determined from dose-response curves. Doxorubicin functions as a positive control.[6]

$$\% \text{ of Growth inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

Analysis of extract by gas chromatography-mass spectrometry (GC-MS)

Ethyl acetate, hexane, and methanol extracts were examined via GC-MS employing helium as the carrier gas at a flow rate of 1 mL/min. Separation was achieved in a fused silica Elite-5MS column (30 m × 0.25 mm ID × 250 µmdf) using a Clarus 680 GC instrument. The injector temperature was set at 260°C, while the oven was planned to increase from 60°C (with a 2 min hold) to 300°C at a rate of 10°C/min, followed

by a 1min pause. The mass detector parameters consisted of a transfer line and ion source maintained at 230°C using electron impact ionization at 70 eV. Scans were varied from 40 to 600 Da in 0.2-s intervals. Components were identified by comparison with the GC-MS NIST (2008) database.[7]

Docking analysis

Protein preparation

The Protein Data Bank (PDB) is a reliable source for obtaining protein 2D structures at www.rcsb.org. All heteroatoms connected to the actual structure were removed to generate the protein's fundamental structure.[8] The protein coordinates of the heteroatom molecule were eliminated to optimize the quantity of binding pocket proteins (caspase3, Nuclear Factor kappa- B [NF-KB], and p53). The protein's 2D structure was observed using the Swiss-PDB viewer, and its energy was decreased in anticipation of further docking investigations.

PDB

The system functions as a unified, worldwide repository for all information related to the composition of large biological molecules. The data provided by biochemists and biologists from around the world, typically obtained through cryo-electron microscopy, nuclear magnetic resonance (NMR) spectroscopy, or X-ray crystallography, can be accessed for free on the internet via the websites of its affiliated organizations (pdbe, pdbj, and rcsb). A central database is simply known as a pdb. The primary data in this case are considered to be the vital data, such as atomic coordinates, chemistry of the macromolecule, small-molecule ligands, data collection details, structure refinement, and some structural descriptors. The maximum data items within one pdb entry are 400. PDB refers to a compilation of two-dimensional structural information for large biological macromolecules, such as nucleic acids, proteins, and their complexes. The PDB holds paramount significance for structural biologists. Protein NMR is responsible for determining approximately 12% of all structures. The PDB uses the PDB file format.[9] These files can be downloaded using web pages. The name of each PDB file begins with a numerical digit and concludes with a trio of alphabetic characters, for example, 1smt. The PDB id is commonly used for this purpose. As an illustration, the protein caspase3 was chosen with the PDB id 5jft; NF- KB was represented by 4d1m, and p53 was denoted by 1a3q.

SPDB viewer

The deep view application is powerful and easy-to-use molecular graphics software. It was designed to be fully compatible with the computational resources provided by an expert protein analysis system, also known as expats. The Swiss molecular biology laboratory is located in Geneva. Deepview is a highly effective analytical tool that is user-friendly for studying structures and generating visually impressive graphics. The deepview allows the model to generate simply by inputting the amino acid sequence. The deepview detects the formation of hydrogen bonds between proteins and ligands. This tool allows evaluation of the accuracy of models and maps, examination of electron-density maps obtained through crystallographic structure determination, and identification of common problems with protein models.[10]

This feature allows you to simultaneously visualize multiple models and overlay them to compare their structures and sequences. The software calculates electrostatic potentials and molecular surfaces and performs energy minimization. The Swiss PDB online server is a useful tool for submitting amino acid sequences to Expasyto identify homologous proteins when the sequence is known, but the structure is unknown. After submitting the sequence alignment, it is sent to Expasy, where the Swiss-model server constructs a homology model and provides the outcome.[11]

Computational generation of ligands

The available ligand structures were obtained from PubChem. The OpenBabel service was used to convert the SDF files of the ligands into PDB format, which contains accurate information on the ligand's coordinates.

PubChem is a chemical database

The database contains chemical molecules and their corresponding biological activities. The National Center for Biotechnology Information, a branch of the National Library of Medicine within the United States National Institutes of Health, was assigned to system maintenance. Access to PubChem is available at no cost through a web-based user interface. FTP allows for the free download of numerous compound structures and descriptive datasets, totaling millions. PubChem houses detailed descriptions of substances and small molecules that have a maximum of 1000 atoms and 1000 bonds. The PubChem database is identified by more than 80 databases.

Open babel

Open Babel is a versatile software toolkit specifically developed to interpret and process various chemical data formats. The project is a freely accessible and collaborative platform that enables individuals to analyze, search, and store data related to chemistry, molecular modeling, biochemistry, solid-state materials, and other related fields. This variant of the Babel program is used to translate chemistry files.[12] Open Babel is an open source project that is intended to add to the functionality of Babel by providing a cross-platform application and library to the conversion of various file formats of computational chemistry, molecular modeling, and other fields. Open Babel is made up of two parts: A command line utility and a C++ library. The command-line program replaces the initial Babel application, which makes it easier to convert a variety of chemical file formats. All

of the code needed to translate the file is found in the C++ library, and it has numerous utilities to help build more open-source scientific software. Babel was originally on smog.com, the computational chemistry list, and Open Babel on sourceforge.net. The original authors converted Babel into C++, which they designated as OpenBabel. The openBabel comprises two primary components.[13]

1. Pre-made software applications for converting, searching, editing, and analyzing chemical files
2. A comprehensive set of tools for programmers to facilitate the development of chemistry software.

The software can interpret, generate, and transform more than 110 chemical file formats. In addition, it can apply filters and perform searches on molecular files using smarts and other techniques.

Analysis of molecular docking

The Autodock 4.2 software was used to predict the binding of substrates or prospective drug candidates to receptors with a defined 2D structure. The software was used to create a gpf-grid and dpf-dock parameter files. This involves the incorporation of polar hydrogen, Kollman charges, and a transformation to include type atoms. The components were integrated into the receptor to enhance protein preparation for docking simulation. Autodock requires precomputed grid maps. The grid must encompass the region of interest, particularly the active site, within the macromolecule. The grid center was repositioned to include all amino acid residues inside the specified active pocket. The Autodock 4.2 program, in conjunction with Autogrid 4.2, was used to produce grid maps. The grid and docking locking logarithmic files were produced using the Cygwin program.[14] The Lamarckian genetic algorithm was selected to perform a search for the best conformers. During the docking procedure, a maximum of 10 conformers were considered for each molecule. The dlg file exhibits a docked structure throughout many runs, each characterized by a unique binding energy (b.e). The docked structure exhibiting the lowest b.e was eventually selected.

RESULTS

Here, phenols, flavonoids, and steroidsare present in all sequential extracts. Glycoside sugar is present in hexane but absent in ethyl acetate and methanol extracts. Furanoidsare present in the methanol extract but absent in the ethyl acetate and hexane extracts [Table 1].

MTT assay of *T. divaricata* leaves

The percentage of inhibition indicates a concentration gradient in all extracts. The inhibition of growth exhibited selective anticancer activity. The ethyl acetate extracts show high cytotoxicity at 1mg/ml compared with the other hexane and methanol extracts.

At a dosage of 1 mg/mL, the hexane extract of *T. divaricata* significantly inhibited HeLa cells, whereas vero cells demonstrated a comparatively lesser inhibition at the same concentration. The ethyl acetate extract of *T. divaricata* at a concentration of 1 mg/mL exhibited a significant inhibitory effect on HeLa cells, whereas vero cells showed a comparatively reduced level of inhibition at the same concentration. The methanol extract of *T. divaricata* significantly suppressed HeLa cells at a concentration of 1 mg/mL, whereas vero cells showed a comparatively modest level of inhibition at the same concentration.

The concentration gradient has been shown in the percent of inhibition in each extract. Selective anticancer efficacy in growth suppression has been found. It has been observed that ethyl acetate extract is highly cytotoxic at the level of 1 mg/mL with respect to other extracts, as shown in Figures 1-3.

GC-MS analysis

The GC-MS technique was followed for the identification of compounds present in the extract of *T. divaricata*. These compounds were separated based on their retention time (RT) values. The following Tables 2-4 and Figures 4-6 provide

Table 1: Phytochemical analysis *Tabernaemontana divaricata*

Compounds	Hexane	Ethyl acetate	Methanol
Phenol	+ve	+ve	+ve
Flavonoid	+ve	+ve	+ve
Quinone	–ve	–ve	–ve
Saponin	–ve	–ve	–ve
Tanin	–ve	–ve	–ve
Alkaloids	–ve	–ve	–ve
Steroid	+ve	+ve	+ve
Glycoside sugar	–ve	+ve	+ve
Furanoid	–ve	–ve	+ve
Protein	–ve	–ve	–ve

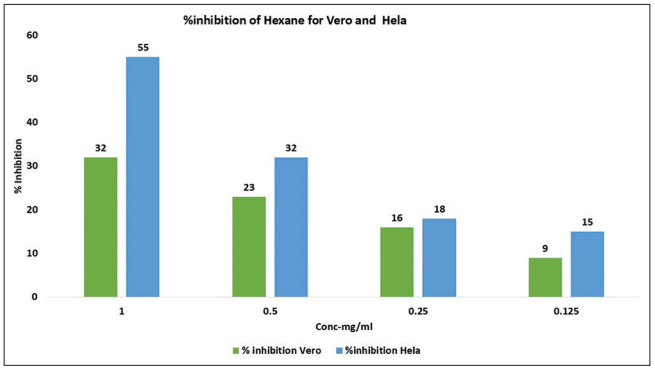


Figure 1: Percentage inhibition of hexane extracts against Vero and HeLa cell lines

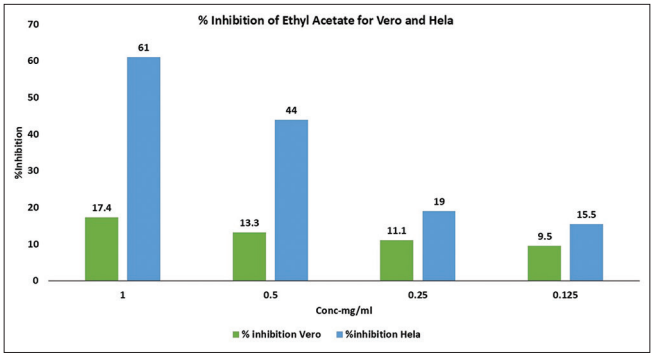


Figure 2: Percentage inhibition of ethyl acetate extracts against Vero and HeLa cell lines

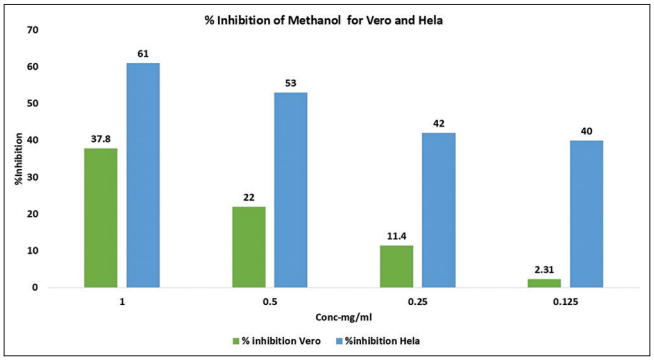


Figure 3: Percentage inhibition of methanol extracts against Vero and HeLa cell lines

information regarding the RT, molecular formula, molecular weight, compound name, and molecular structure.

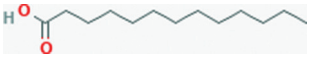
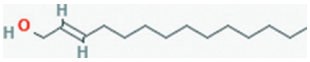
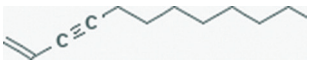
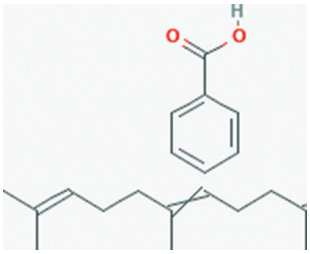
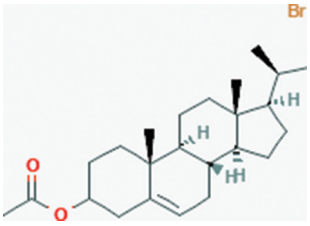
Ethyl acetate, hexane, and methanol were subjected to GC-MS analysis, which resulted in the identification of 37 different chemicals. Among these, four chromatogram peaks from the hexane extract were discovered to be unknown, followed by two peaks that were unidentified in the ethyl acetate extract, and finally, four peaks that were unidentified in the methanol extract.

Docking analysis

Considering that the extract has the highest cytotoxicity against the HeLa cell line, the components of ethyl acetate were chosen for docking.

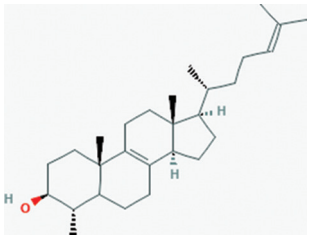
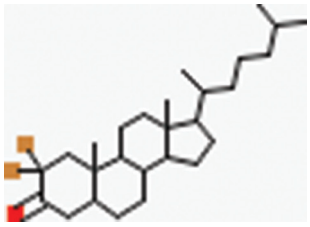
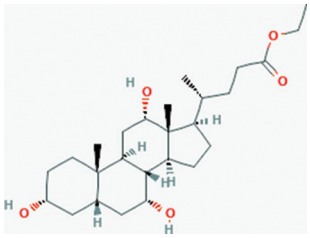
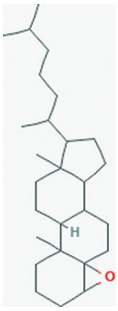
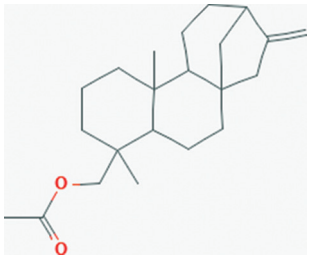
Docking analysis was performed on the three proteins selected for the study. They are Caspases 3, p53 and NF-KB, respectively. Compound 2,2-dibromocholestanone exhibits high b.e in the Caspase 3 protein; specifically, the value is −7.53 at the eighth run. In the third run, the protein p53 component2,2-dibromocholestanone exhibited a high binding affinity of −6.24. At run 9, the same di-bromocholestanone has a high binding affinity of −6.72, which is found in the NF-KB protein [Tables 5-7].

Table 2: GCMS analysis of hexane extracts of *Tabernaemontana divaricata*

RT	Name of the compound	Molecular weight (g/mol)	Molecular formula	Molecular structure
18.830	N-HexadecaonicAcid	256	$C_{16}H_{32}O_2$	
20.141	E-2- OctadecadeceN1-OL	268	$C_{18}H_{36}O$	
20.276	13-Tetradece-11- YN-1-OL	208	$C_{14}H_{24}O$	
24.252	2,6,10- Dodecatrien-1-OL,3,7,11- Trimethyl-	222	$C_{15}H_{26}O$	
25.948	Hexatricontane	506	$C_{36}H_{74}$	
27.904	22,23- Dibromo stigmas Terol Acetate	612	$C_{31}H_{50}O_2B_2$	

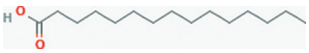
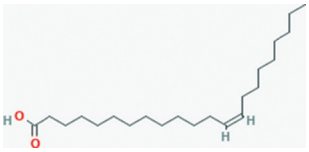
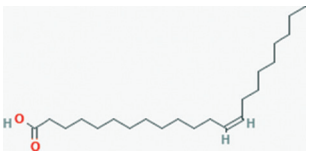
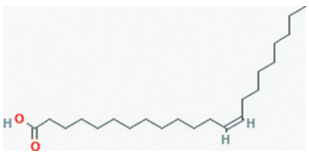
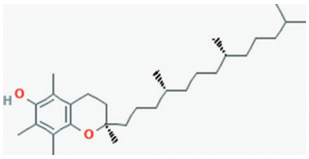
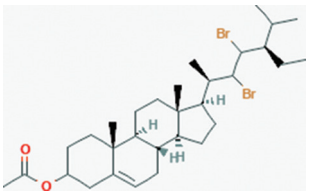
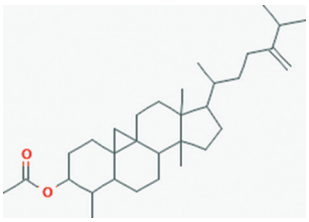
GC-MS: Gas chromatography-mass spectrometry, RT: Retention time

Table 3: GCMS analysis of ethyl acetate of *Tabernaemontana divaricata*

RT	Name of the compound	Molecular weight (g/mol)	Molecular formula	Molecular structure
9.116	Acetic Acid	60	$C_2H_4O_2$	
9.396	Acetic Acid	60	$C_2H_4O_2$	
20.631	N-Hexadecanoic Acid	256	$C_{16}H_{32}O_2$	
21.631	Hexadecanal	240	$C_{16}H_{32}O$	
26.508	Cholesta-8,24-Dien-3-ol, 4-Methyl-, (3. Beta., 4. Alpha.)-	398	$C_{28}H_{46}O$	
26.968	2,2-Dibromocholestanone	542	$C_{27}H_{44}OBr_2$	
28.824	Ethyl Iso-Allocholate	436	$C_{26}H_{44}O_5$	
29.254	Cholestane, 4,5-Epoxy-, (4. Alpha.,5. Alpha.)-	386	$C_{27}H_{46}O$	
29.809	Kauren-18-ol, Acetate, (4. Beta.)-	330	$C_{22}H_{34}O_2S$	

GC-MS: Gas chromatography-mass spectrometry, RT: Retention time

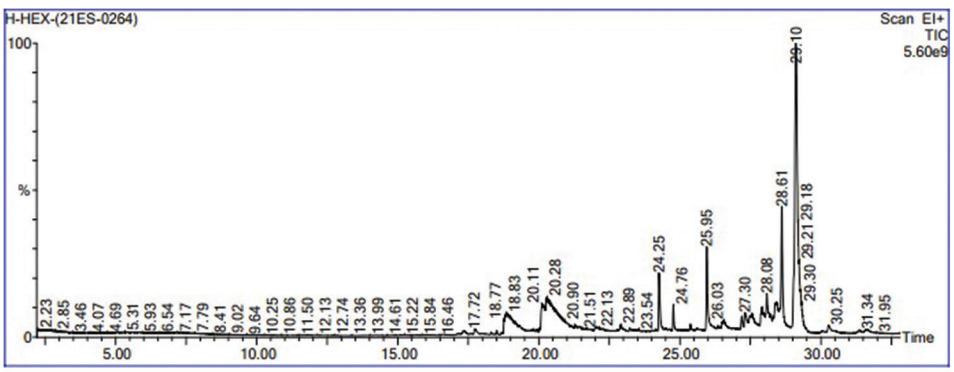
Table 4: GCMS analysis of methanol of *Tabernaemontana divaricata*

RT	Name of the compound	Molecular weight (g/mol)	Molecular formula	Molecular structure
17.249	Phytol	296	$C_{20}H_{40}O$	
19.125	N-Hexadecanoic Acid	256	$C_{16}H_{32}O_2$	
19.595	Pentadecanoic Acid	242	$C_{15}H_{30}O_2$	
19.740	Pentadecanoic Acid	242	$C_{15}H_{30}O_2$	
20.416	Erucic Acid	338	$C_{22}H_{42}O_2$	
20.596	Oleic Acid	282	$C_{18}H_{34}O_2$	
24.367	2,6,10-Dodecatrien-1-Ol, 3,7,11-Trimethyl-	222	$C_{15}H_{26}O$	
26.038	Hexatriacontane	506	$C_{36}H_{74}$	
26.643	Vitamin E	165	$C_6H_7ON_5$	
27.519	22,23-Dibromostigmasterol Acetate	612	$C_{31}H_{50}O_2Br_2$	
28.269	9,19-Cycloergost-24 (28)-En-3-Ol, 4,14-Dimethyl-, Acetate, (3. Beta.,4. Alpha., 5. Alpha.)-	468	$C_{32}H_{52}O_2$	

GC-MS: Gas chromatography-mass spectrometry, RT: Retention time

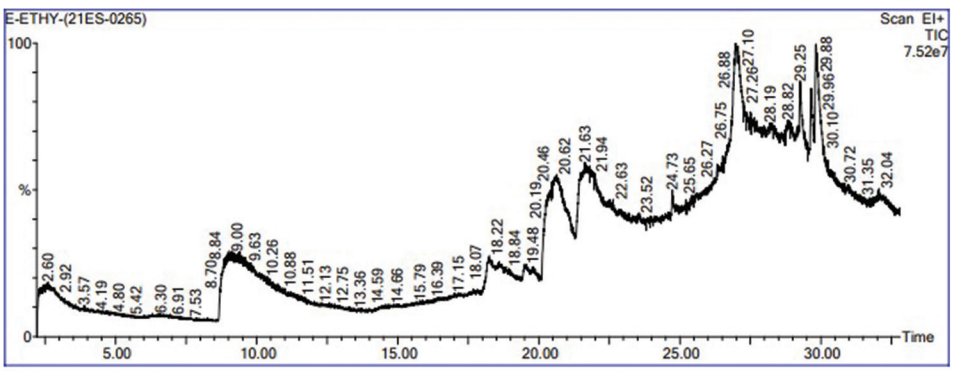
DISCUSSION

The goal of sequential plant extract analysis is to either isolate bioactive compounds for direct use as pharmaceuticals or to identify bioactive molecules that can be used as essential components in the semi-synthetic drug production process.[15] The present investigation examined hexane, ethyl acetate, and methanol leaf extracts of *T. divaricate* and demonstrated the existence of phenols, steroids, and flavonoids. Plants contain secondary metabolites, including flavonoids, steroids, and phenols,[16] which regulate cell division and proliferation. Flavonoids can counteract cancers affecting the larynx, breast, thyroid, stomach, colon, and mouth.[17]



#	RT	Scan	Height	Area	Area %	Norm %
1	18.830	3325	342,005,696	75,518,768.0	5.353	12.12
2	20.141	3587	492,319,040	71,108,136.0	5.040	11.42
3	20.276	3614	614,546,560	261,475,264.0	18.534	41.98
4	24.252	4409	1,089,261,056	56,534,752.0	4.007	9.08
5	25.948	4748	1,567,374,336	77,477,664.0	5.492	12.44
6	27.904	5139	333,721,504	33,985,100.0	2.409	5.46
7	28.079	5174	502,601,024	33,481,248.0	2.373	5.38
8	28.424	5243	341,776,480	41,376,960.0	2.933	6.64
9	28.609	5280	2,219,503,872	136,927,856.0	9.706	21.98
10	29.104	5379	5,333,296,128	622,896,704.0	44.153	100.00

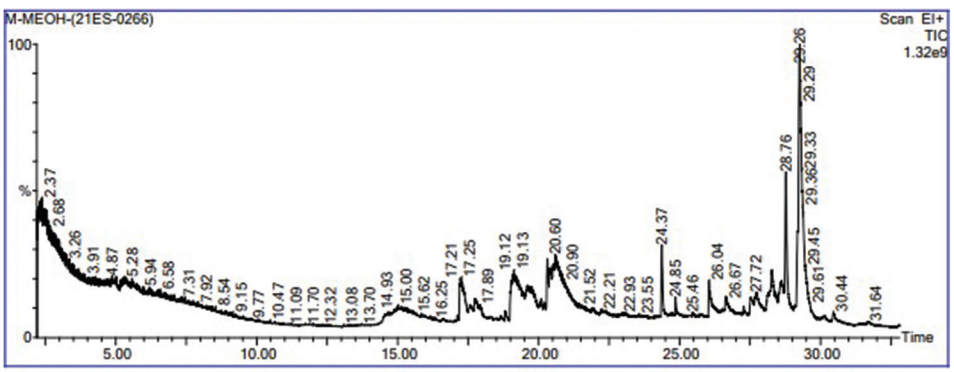
Figure 4: Gas chromatography-mass spectrometry analysis of hexane extracts of *Tabernaemontana divaricata*



#	RT	Scan	Height	Area	Area %	Norm %
1	9.116	1383	14,721,306	8,327,951.5	6.260	25.83
2	9.396	1439	13,203,563	6,174,819.0	4.641	19.15
3	20.631	3685	24,676,802	19,690,460.0	14.801	61.08
4	21.631	3885	23,570,138	23,293,858.0	17.509	72.26
5	26.508	4860	11,367,040	4,309,864.5	3.240	13.37
6	26.968	4952	40,064,332	32,237,838.0	24.232	100.00
7	28.189	5196	19,033,522	8,453,782.0	6.355	26.22
8	28.824	5323	19,370,908	8,191,309.5	6.157	25.41
9	29.254	5409	29,130,818	7,909,995.5	5.946	24.54
10	29.649	5488	27,025,108	3,852,022.2	2.895	11.95
11	29.809	5520	38,244,888	10,594,042.0	7.963	32.86

Figure 5: Gas chromatography-mass spectrometry analysis of ethyl acetate extracts of *Tabernaemontana divaricata*

The results of Kalaimagal[17] proved that the *T. divaricata* ethanolic extract of the flower possesses anticancer activity against the HeLa, HepG2, and A549 cancer cells. In the experiment, Naidoo *et al*.,[18] concluded that the hexane extract of Tabernaemontana *ventricosa* was an important inhibitor of HeLa cells with a concentration of 1 mg/mL. However, at the lower concentration of 0.125 mg/mL, the inhibiting activity on vero cells decreased. The ethyl acetate extract of *T. divaricata* had the highest level of HeLa inhibition than the other extracts in this paper.



#	RT	Scan	Height	Area	Area %	Norm %
1	17.249	3009	182,201,136	32,685,764.0	5.707	18.70
2	19.125	3384	216,561,616	77,651,688.0	13.559	44.43
3	19.595	3478	122,465,752	20,542,230.0	3.587	11.75
4	19.740	3507	110,273,328	17,599,656.0	3.073	10.07
5	20.311	3621	230,819,168	14,113,861.0	2.464	8.08
6	20.416	3642	188,583,792	16,500,566.0	2.881	9.44
7	20.596	3678	244,109,728	100,852,016.0	17.610	57.71
8	24.367	4432	318,172,640	16,470,788.0	2.876	9.43
9	26.038	4766	157,176,784	13,119,414.0	2.291	7.51
10	26.643	4887	67,552,176	7,315,472.5	1.277	4.19
11	27.519	5062	77,482,328	8,802,031.0	1.537	5.04
12	27.734	5105	83,019,832	11,869,073.0	2.073	6.79
13	28.269	5212	118,599,776	9,981,798.0	1.743	5.71
14	28.614	5281	98,844,136	12,794,273.0	2.234	7.32
15	28.764	5311	584,379,520	37,632,992.0	6.571	21.53
16	29.259	5410	1,166,966,016	174,755,888.0	30.515	100.00

Figure 6: Gas chromatography-mass spectrometry analysis of methanol extracts of *Tabernaemontana divaricata*

Table 5: Docking results for Caspase 3 5JFT protein

Ligand	Protein	Run	Binding energy
Acetic acid	Caspase 3	10	−2.82
N-hexadecanoic acid	Caspase 3	8	−5
Hexadecanal	Caspase 3	4	−4.06
Cholesta-8,24-dien-3-ol, 4-methyl-, (3. Beta.,4. Alpha.)-	Caspase 3	7	−5.61
2,2-dibromocholestanone	Caspase 3	8	−7.53
Ethyl iso-allocholate	Caspase 3	6	−7.84
Cholestane, 4,5-epoxy-, (4. Alpha.,5. Alpha.)-	Caspase 3	9	−6.4
Kauren-18-ol, acetate, (4. Beta.)-	Caspase 3	7	−7.34

Table 6: Docking results for protein p53 1A3Q

Ligand	Protein	Run	Binding energy
Acetic acid	p53	10	−3.22
N-hexadecanoic acid	p53	1	−3.32
Hexadecanal	p53	8	−2.64
Cholesta-8,24-dien-3-ol, 4-methyl-, (3. Beta.,4. Alpha.)-	p53	3	−3.08
2,2-dibromocholestanone	p53	3	−6.24
Ethyl iso-allocholate	p53	8	−5.89
Cholestane, 4,5-epoxy-, (4. Alpha.,5. Alpha.)-	p53	8	−3.72
Kauren-18-ol, acetate, (4. Beta.)-	p53	1	−5.54

The most effective method of analyzing unknown bioactive compounds in plants is the GC-MS analysis. The binding affinity of hexadecanoic acid is very high and ethyl acetate was selected as the extraction reagent in GC-MS analyses due to the fact that it has a high inhibition rate.[20] Eight active compounds were in all obtained with the ethyl acetate extract. Konappa *et al*.,[20] have found six compounds in the leaf extract of *Anaphalis nilgiricium*. Identifying the active components under assessment is done by the mass detector. The located range of the substances will be stored in the NIST library and used to determine the nomenclature and molecular mass of the compound, its belonging to the classes of compounds, including flavonoids, alkaloids, triterpenoids, and gemaric acid.[21]

The present research indicated that N-Hexadeconic acid can be employed as an anticancer agent in the future. The same was reported in the flora of *Kigelia pinnata* and the results indicate that the cytotoxic effects of

Table 7: Docking results for protein NF-KB 4D1M

Ligand	Protein	Run	Binding energy
Acetic acid	NF-KB	9	−2.83
N-hexadecanoic acid	NF-KB	8	−4.44
Hexadecanal	NF-KB	5	−4.51
Cholesta-8,24-dien-3-ol, 4-methyl-, (3. Beta.,4.alpha.)-	NF-KB	10	−6.28
2,2-dibromocholestanone	NF-KB	9	−6.72
Ethyl iso-allocholate	NF-KB	3	−6.12
Cholestane, 4,5-epoxy-, (4. Alpha.,5. Alpha.)-	NF-KB	5	−4.14
Kauren-18-ol, acetate, (4. Beta.)-	NF-KB	6	−3.92

NB- KB: Nuclear Factor kappa- B

N-hexadeconic acid are due to its interaction with DNA topoisomerase-I.[22-24] This interaction might also be explored by further research to determine its possibilities as an anticancer agent by acting on other proteins that are processes in cancer drug therapy.[25] The cytotoxic effect in the present study could be explained by the fact that N-Hexadecanoic acid, which is a famous anticancer agent, was contained in the ethyl acetate extract. *In vitro* studies also confirmed this hypothesis.[26-28]

The approaches to be used in this research are GC-MS analysis, molecular docking, *in vitro* cytotoxicity assay, and phytochemical analysis. The *in vitro* experiment performed on HeLa and vero cell lines indicated that the extract of *T. divaricata* had a higher inhibitory impact on HeLa cell growth to the vero cells at all concentrations, indicating that the extracts have anticancer effects.[29] The ethyl acetate extract was found to have the best cytotoxicity toward HeLa cells. *T. divaricata* extracts are rich in secondary metabolites, which are probably the source of anticancer activity.

It explored the binding mechanism of ligands identified by the GC-MS analysis with the receptor proteins of the cancer cell using the molecular docking analysis. Anantram *et al*.,[30] showed that such proteins, such as caspase 3, p53, and NF-KB, can be successfully analyzed by molecular docking. Proteins that were investigated in the study included caspase 3, p53, and NF-KB. The anticancer properties of acetic acid, hexadecanal, cholesta-8, 24 -Dien-3-Ol, 4-Methyl-, (3Beta., 4Alpha)-, kaaren-18-Ol acetate, and other compounds that bind strongly to receptor proteins have been known. Ethyl iso-allocholate and N- hexadeconic acid are anticancer agents. These substances were probably involved in the fact that the extract was able to prevent the development of cancer cells.[31] The results of the present research would be useful in future studies on naturally obtained compounds that may be used in cancer treatment.

CONCLUSION

This paper investigated the anticancer effects of the leaf extracts of *T. divaricata* through GC-MS analysis, molecular docking, *in vitro* cytotoxicity analysis, and phytochemical analysis. Ethyl acetate extract showed maximum cytotoxicity against the HeLa cells due to the availability of biochemicals, such as N-hexadecanoic acid, ethyl iso-allocholate, and the rest of the metabolites. Ethyl acetate extract showed eight active chemicals with the use of N-hexadecanoic acid that exhibits a high potential in operating as an anticancer drug through its interaction with the DNA topoisomerase-I. This agrees with the previous studies on the cytotoxic effects. The study of phytochemicals suggested the presence of phenols, flavonoids, and steroids that have been known to control cell growth and have anticancer effects. The effectiveness of the discovered ligands was validated by the molecular docking analysis with references to their targeting of cancer-related proteins. The findings of this research work explain the therapeutic potential of *T. divaricata* and provide an emphasis on its relevance in developing natural, plant-based anticancer drugs. Further research of these substances can help to improve cancer treatment procedures.

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