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Original Article

ISOLATION OF PHYTOCONSTITUENT, *IN VITRO* ANTICANCER STUDY IN HELA AND MCF-7 CELL LINES AND MOLECULAR DOCKING STUDIES OF *POTHOS SCANDENS* LINN

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ABSTRACT

Objective: This study aims to isolate an active phytoconstituent from ethanolic leaf extract of *Pothos scandens* Linn., to evaluate *in vitro* anticancer activity, and to carry out molecular docking studies of the isolated phytoconstituent.

Methods: The bioactive constituent 1,1'-(4,5-dihydroxy benzene-1,2-diyl) bisoct-7-en-1-one, a phenolic compound, was isolated by using chromatographic methods and the structure was elucidated by various spectroscopic techniques. *In vitro* anticancer activity was evaluated against HeLa and MCF 7 cell lines. The viability of cells was evaluated by direct observation of cells by an Inverted phase-contrast microscope and by the MTT assay method. IC_{50} was calculated using the linear regression model.

Results: The results of anticancer studies revealed that different concentrations of the ethanolic extract of leaves of *Pothos scandens* Linn. exhibited cytotoxic activity against HeLa and MCF 7 cell lines with IC_{50} of 22.9 and 18.32 µg/ml, respectively. The anticancer potential of the plant was revalidated by *in silico* molecular docking study with Vascular Endothelial Growth Factor Receptor 2 (VEGFR2, PDB ID: 4AG8) using Discovery studio 2018. Results of the docking study showed that the ligand exhibited strong interaction with the VEGFR2 kinase with significant binding energy.

Conclusion: *Pothos scandens* linn. can be used for the isolation of potent anticancer agents.

Keywords: *Pothos scandens,* Phytoconstituent, Anticancer, MTT assay, Molecular docking

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INTRODUCTION

Cancer is a disease in which some cells of our body grow abnormally and invade other parts of the body. According to WHO, 9.6 million people worldwide are estimated to have died from cancer in 2018. Globally one out of six deaths is due to cancer (WHO, 2018). Cancer may be developed due to changes in genes that control the growth and functions of our cells (National Cancer Institute, 2007). In a cancer cell, the normal cell cycle control is disrupted by: Abnormal growth factor function, Abnormal cyclin-dependent kinase (CDK) function, Abnormal DNA synthesis, and Abnormal decreases in negative regulatory forces due to mutation in the tumour suppressor gene [1].

Anticancer agents are used for curing cancer or to prolong life or reduce symptoms associated with cancer [2]. Most anticancer agents act by affecting the process of cell division. A major limitation of commonly used anticancer agents is that they also target rapidly dividing normal cells such as hair follicle cells, bone marrow cells, digestive tract cells, etc and cause harmful side effects such as bone marrow suppression, alopecia, damage to GI mucosa, and ulceration, teratogenicity, etc [3]. Plant-derived natural products have a significant role in ameliorating many diseases of human beings. The plant-based therapeutics provide an alternative strategy for better and less toxic cancer treatment [4]. Secondary plant metabolites such as alkaloids, flavonoids, polyphenols, saponins, lignans, volatile oils, terpenes, etc exhibit significant anticancer effects either by inhibiting cancer cell-activating proteins, enzymes and modulating multiple signaling pathways such as CDK2, NF-κB, Bcl-2, cytokines, PI3K, Akt, MAPK/ERK, MMP, or by activating DNA repair mechanism [5-7].

P. scandens Linn. is a medicinal aroid that belongs to the family Araceae. The plant is widely distributed in Madagascar, the Himalayas, as well as Indo Burma region and Indochina [8, 9]. This plant is widely used throughout the world to cure various diseases [10, 11]. The stem of the plant is cut up and smoked with camphor, like tobacco, for the treatment of asthma [12]. The root of the plant cut and fried in oil is used to promote the healing of abscesses. The people of Northeast India use the whole plant for bone fracture [13]. The Kanikkar tribes of Southern Western Ghats of India use P. scandens leaves mixed with the fruits of Capsicum annum and rhizome of *Allium sativum* and the mixture is ground into a paste with coconut oil and applied topically to heal wounds created during delivery [14]. Chinese people use the plant as a blood coagulant [15].

Despite its traditional medicinal claim, extensive research on P. Scandens Linn. has not yet been conducted. Only a few reports are there regarding the pharmacological activities of the plant. The leaf extract of the plant was reported to possess anti-diabetic, bronchodilator [16], antibacterial, anthelmintic and larvicidal activity [17]. Various extracts of root, stem, and leaves of Pothos scandens Linn. were found to possess antioxidant and antipyretic activities [18].

MATERIALS AND METHODS

Authentication and collection of plant material

The plant *Pothos scandens L* was identified and authenticated by Dr. G. Valsaladevi, Curator, Department of Botany, University of Kerala, Kariavattom, and the plant specimen was deposited in the Herbarium of Department of Botany, the University of Kerala, Kariavattom with voucher No. KUBH-6029.

Fresh plants of *Pothos scandens L.* were collected from Tropical Botanic Garden and Research Institute, Palode, Thiruvanthapuram District (Kerala) during October. The collected plant material was washed thoroughly with tap water; the leaves were cut into small pieces and air-dried under shade at room temperature for two weeks. The shade-dried leaves were powdered into a coarse powder.

Preparation of extract

About 500g of the dried leaf powder of *Pothos scandens* Linn. was defatted with petroleum ether (60-80 °C) by continuous hot extraction in a soxhlet apparatus for 12 h [19]. The defatted powder material was extracted with 300 ml ethyl alcohol (95% v/v) for 48 h in soxhlet apparatus. The extract obtained was filtered and made solvent-free by using a rotary evaporator and the resulting semisolid mass was dried.

Isolation and structure elucidation of phytoconstituent

About 15g of ethanolic extract was chromatographed on a silica gel column. Gradient elution was carried out with n-Hexane: Ethyl acetate (100:0-0:100) and with Ethyl acetate: Methanol (9:1). Seven fractions were collected. Each fraction was subjected to analytical TLC and those fractions with the same Rf value were pooled together. The purity of the fractions was further assessed by HPLC analysis on a C18 analytical column using Methanol: Water (65:35) as a mobile phase with a flow rate of 0.6 ml/min. The column was maintained at 40 °C. The purest fraction obtained was then subjected to IR, NMR, and GC-MS analysis to establish the chemical structure of the isolated compound [20, 21].

***In vitro* evaluation of anti-cancer activity**

Ethanolic extract of leaves of *Pothos scandens* Linn. was subjected to *in vitro* anticancer evaluation against HeLa (cervical cancer) and MCF 7 (hormone-dependent breast cancer) cell lines. HeLa cell lines and MCF 7 cell lines were acquired from National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's Modified Eagle Medium (DMEM, Himedia). The cell lines were cultured in a 25 cm2 tissue culture flask containing DMEM supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine, sodium bicarbonate, and an antibiotic solution containing Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). All cell lines were cultured in a humidified incubator at 37 °C and 5% CO2 atmosphere (Galaxy® 170 Eppendorf, Germany).

The viability of cells was evaluated by direct observation of cells by an Inverted phase-contrast microscope [22] and followed by the MTT assay method [23, 24].

Different dilutions (6.25µg, 12.5µg, 25µg, 50µg and 100µg/ml) of the leaf extract were prepared by adding 95% ethanol to the extract. Positive control was prepared by using doxorubicin in 95% ethanol. Test media was prepared by adding 1% (v/v) of the various dilutions to DMEM and dissolved completely by cyclomixer. After that, the extract solutions were filtered through a 0.22 µm millipore syringe filter to ensure sterility. The test results were compared with positive control containing 1% (v/v) doxorubicin in DMEM and negative control containing 1% (v/v) 95% ethanol in DMEM.

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in a 10% growth medium. 100 µl of cell suspension (5x104 cells/ml) was seeded into each well of a 96 well tissue culture plate and incubated at 37 °C in a humidified 5% CO_2 incubator.

After 24 h the growth medium was aspirated and replaced with 100 µl of control or test medium. Each concentration was added in triplicates to the respective wells and incubated at 37 °C in a humidified 5% CO_2 incubator [25, 26].

Antiproliferative effect by direct microscopic observation

The entire plate was observed at an interval of every 24 h; up to 72 h in an inverted phase-contrast tissue culture microscope (Labomed TCM-400 with MICAPSTM HD camera) and microscopic observations were recorded as images. Any detectable change in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells, were considered as indicators of cytotoxicity.

Antiproliferative effect by MTT method

Fifteen mg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Himedia, M-5655) was reconstituted in 3 ml phosphate-buffered saline (PBS) until completely dissolved and sterilized by filter sterilization. After 24 h of the incubation period, the media in wells were removed and 20 µl of reconstituted MTT solution was added to all test and control wells; the plate was gently shaken and then incubated at 37 °C in a humidified 5% CO2 incubator for 4 h. After the incubation period, the supernatant was removed and 100 µl DMSO was added to all wells and mixed thoroughly to dissolve dark blue formazan crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on an automated microplate reader and the absorbance values were measured at a wavelength of 570 nm [27, 28].

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x100}}{\text{Mean OD of control}}$$

Determination of IC_{50} values

IC_{50} was calculated using a linear regression model by plotting the average percentage viability (triplicates per concentration) against the logarithmic value (base 10) of concentration.

Molecular docking

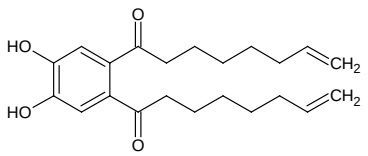
To confirm the anticancer activity exhibited by the plant extract, molecular docking study of the isolated ligand was performed with Vascular Endothelial Growth Factor Receptor 2 (VEGFR2, PDB ID: 4AG8) using Discovery studio 2018 [29]. The target and ligand were pre-processed before docking for getting the minimized structures and correct conformers. The C docking module was performed for the analysis of the molecular interactions of the targets with the ligand using charm as a force field [30, 31].

RESULTS AND DISCUSSION

Isolation and structure elucidation of phytoconstituent

After a sequence of column chromatography and HPLC analysis (fig. 1) of ethanolic leaf extract of *Pothos scandens* Linn, the purest fraction was collected and dried in a rotary evaporator. The fraction was then subjected to spectroscopic studies. The GC-MS, FTIR, and NMR spectroscopic data obtained for the isolated compound is shown in fig. (2), fig. (3), and fig. (4), respectively.

Based on the spectral characterization, the following structure is proposed for the isolated compound:



Molecular formula-$C_{22}H_{30}O_4$

IUPAC name-1,1'-(4,5-dihydroxy benzene-1,2-diyl)bisoct-7-en-1-one

Smiles notation-Oc1cc(C(=O)CCCCCC=C)c(cc1O)C(=O)CCCCCC=C

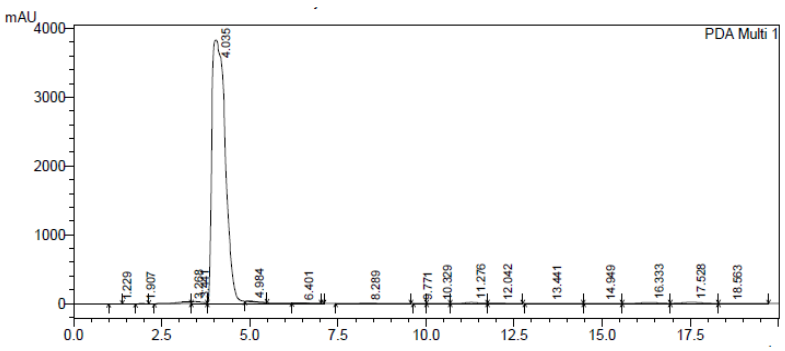


Fig. 1: HPLC of the purest fraction

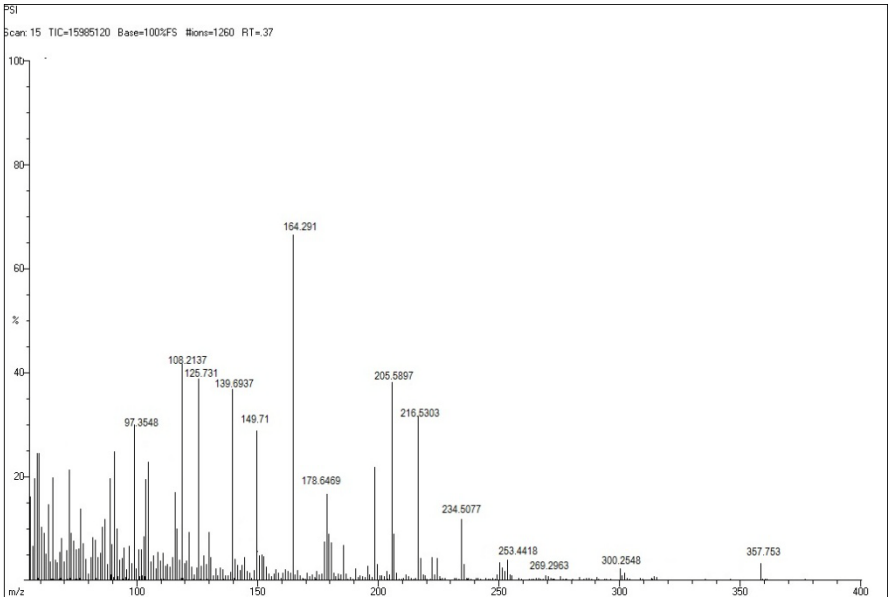
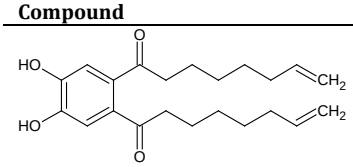
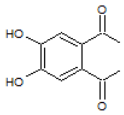
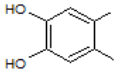
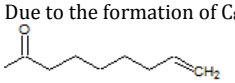


Fig. 2: Mass spectrum of an isolated compound

Table 1: Mass spectral details of the isolated compound

Compound	Peak (m/z)	Remarks
	Molecular ion peak at 357.753	Molecular weight of the isolated compound is 358
	Base peak at 164.291	Due to the formation of $C_8H_4O_4$ fragment 
	Peak at 97.3548	Due to the formation of C_7H_{13} fragment 
	Peak at 108.2137	Due to the formation of $C_6H_4O_2$ fragment 
	Peak at 125.731	Due to the formation of $C_8H_{13}O$ fragment 

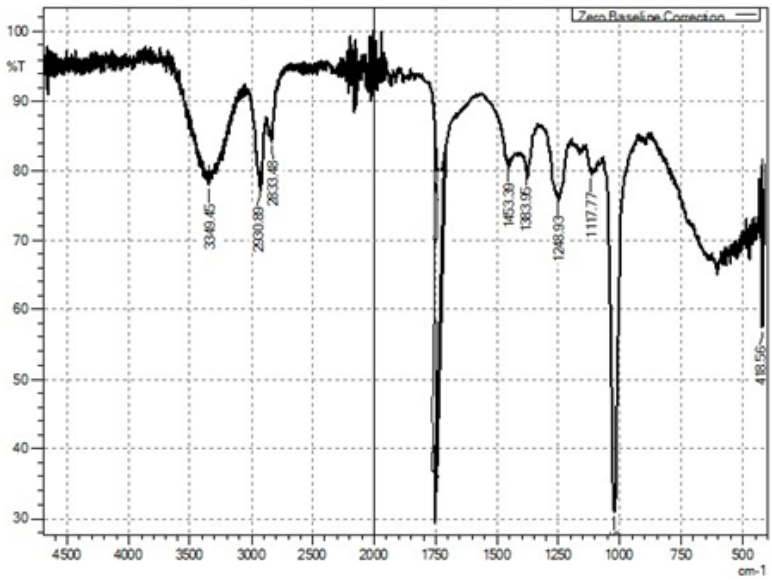
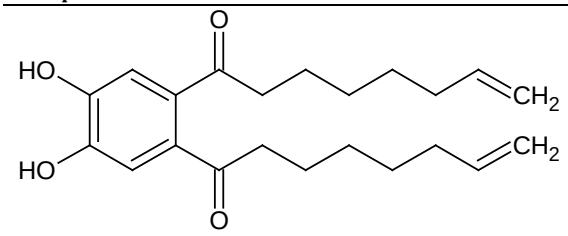


Fig. 3: IR spectrum of an isolated compound

Table 2: IR spectrum details of the isolated compound

Compound	Absorption band (cm^{-1})	Remarks
	3349.45	Due to O-H Stretch
	2930.89	Due to Aromatic C-H Stretch.
	2833.48	Due to alkenyl C-H stretch
	1750	Due to C=O Stretch
	1453.39	Due to C=C ring stretch
	1383.95	Due to In-plane O-H bend
	1248.93	Due to C-O stretch
	1000	Due to out-of-plane C-H bend

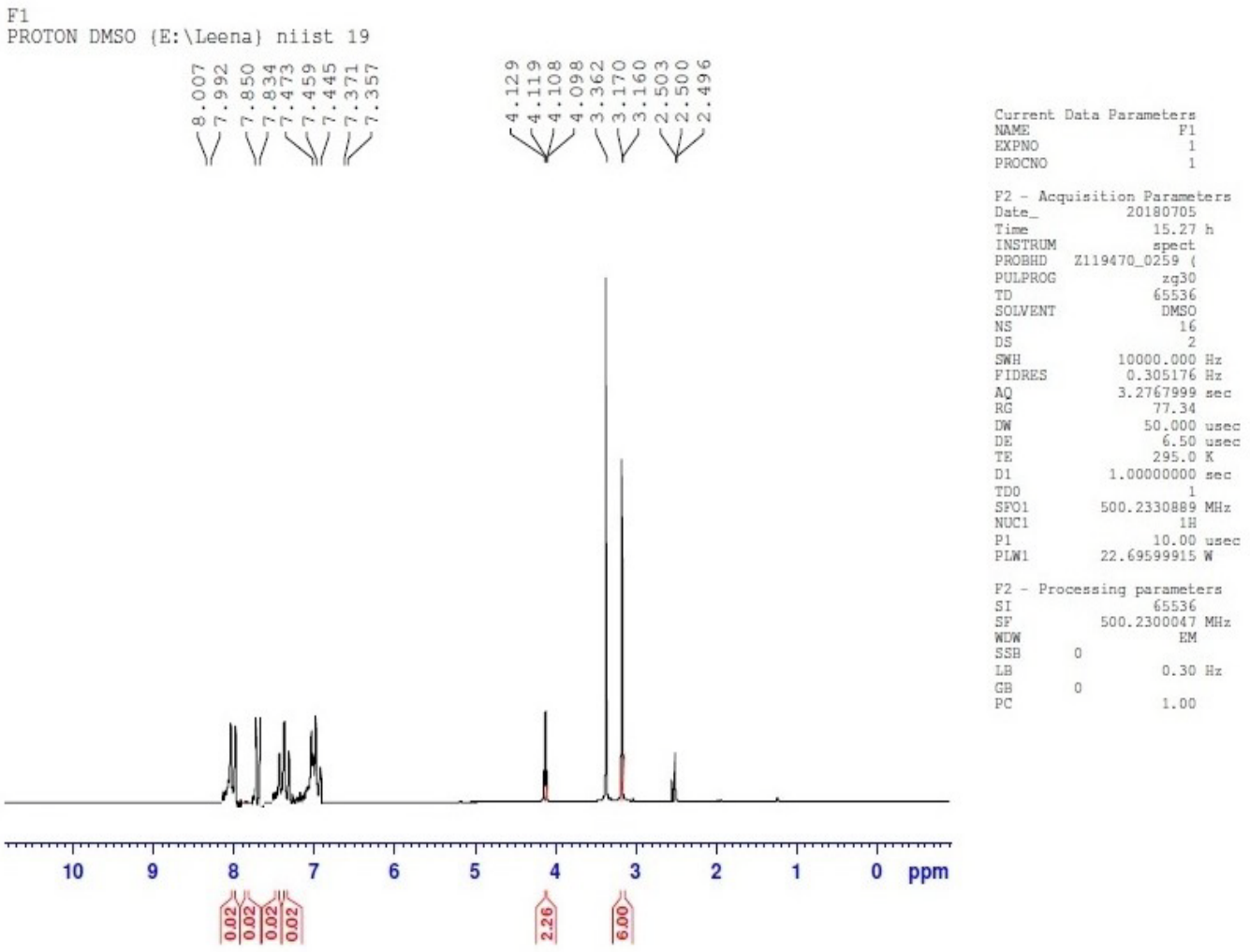
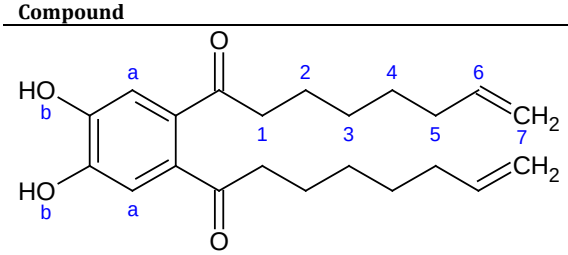


Fig. 4: NMR spectrum of the isolated compound

Table 3: NMR spectral details of the isolated compound

Compound	Chemical shift δ(ppm)	Remarks
	8.007	Due to the proton a
	7.850	Due to O-H proton b
	7.459	Due to protons 1
	7.357	Due to proton 6
	4.11	Due to protons 2
	3.36	Due to proton 7
	3.170	Due to proton 5
	2.5	Due to protons3 and 4

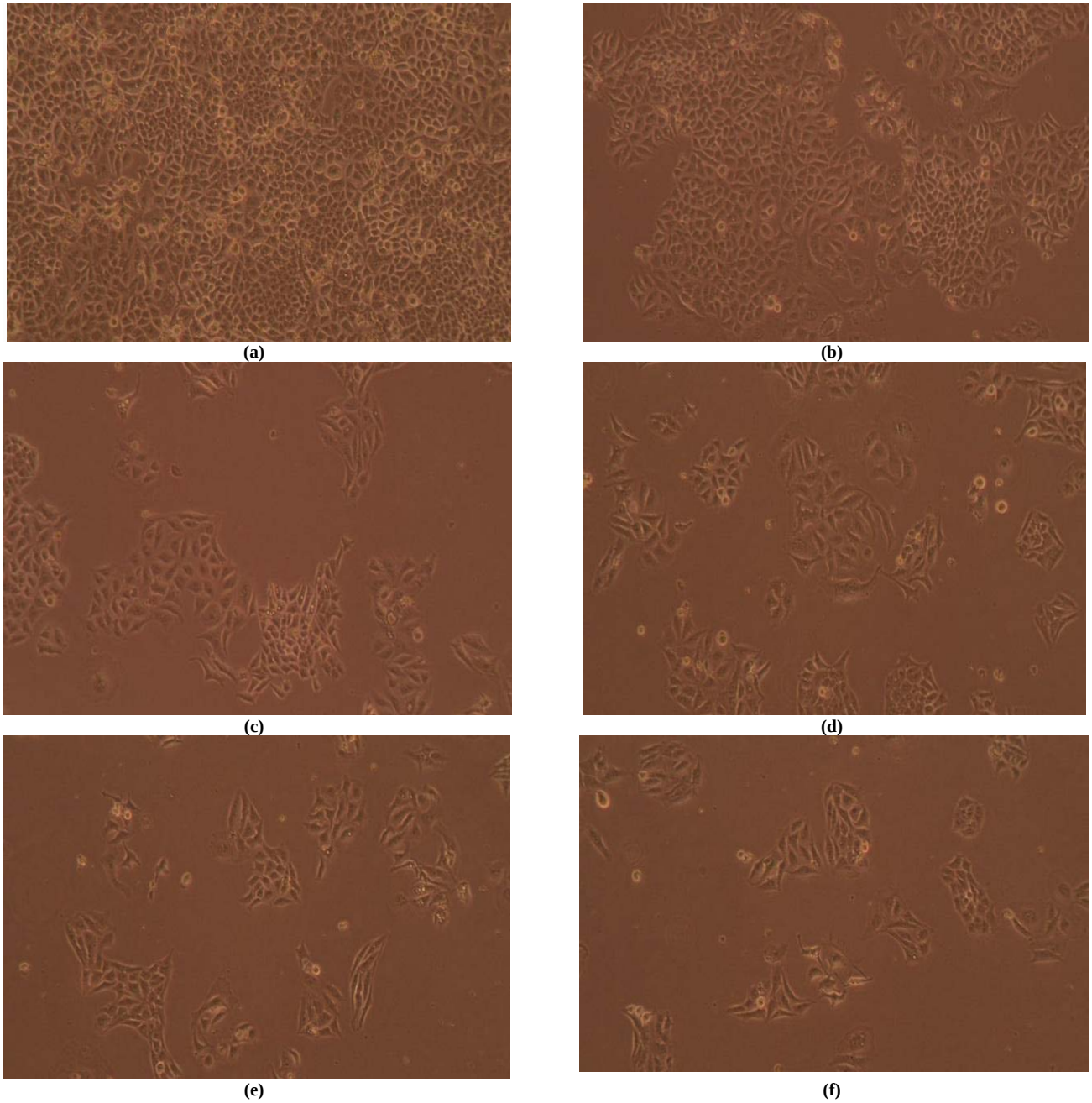


Fig. 5: Morphological changes showing inhibition of Hela cell lines by different concentrations of ethanolic extract of leaves of Pothos scandens. a: -ve control (95% ethanol), b: 6.25 µg/ml, c: 12.5 µg/ml, d: 25 µg/ml, e: 50 µg/ml f: 100 µg/ml

In vitro anti-cancer activity

In vitro anticancer actiivity of ethanolic extract of leaves of *Pothos scandens* Linn. was evaluated against HeLa and MCF 7 cell lines. The viability of cells was evaluated by direct observation of cells by an Inverted phase contrast microscope and MTT assay method. The morphological changes in HeLa and MCF 7 cells after treatment with the extract and standard doxorubicin are shown in fig. 5, 6, 7 and 8. The IC_{50} values are shown in table 4.

According to the American National Cancer Institute (NCI), crude extracts with an IC50<30 µg/ml are considered to be active against cancer cells [32, 33]. Different concentrations of the ethanolic extract of leaves of *Pothos scandens* Linn. exhibited potent cytotoxic activity against HeLa and MCF 7 cell lines with IC_{50} of 22.9 and 18.32 µg/ml, respectively.

Molecular docking

The crystal structure of VEGFR2 kinase domain of human with PDB ID: 4AG8 was retrieved from PDB with a resolution of 1.95A °. The protein consists of a single polypeptide chain with a sequence length of 316 amino acids. The binding site of protein interaction with its inhibitor compound Axitinib is Glu917, Cys919 and Asp1046. The preliminary docking analysis was conducted by selecting Glu917, Cys919 and Asp1046 as binding site residues. The ligand selected interacted with ALA 86, VAL 914, ASP 1046 and CYS 1045 with strong H–bond interaction. The docking score, binding energy, Hydrogen bonds, and active site residues of the target protein are shown in table 5.

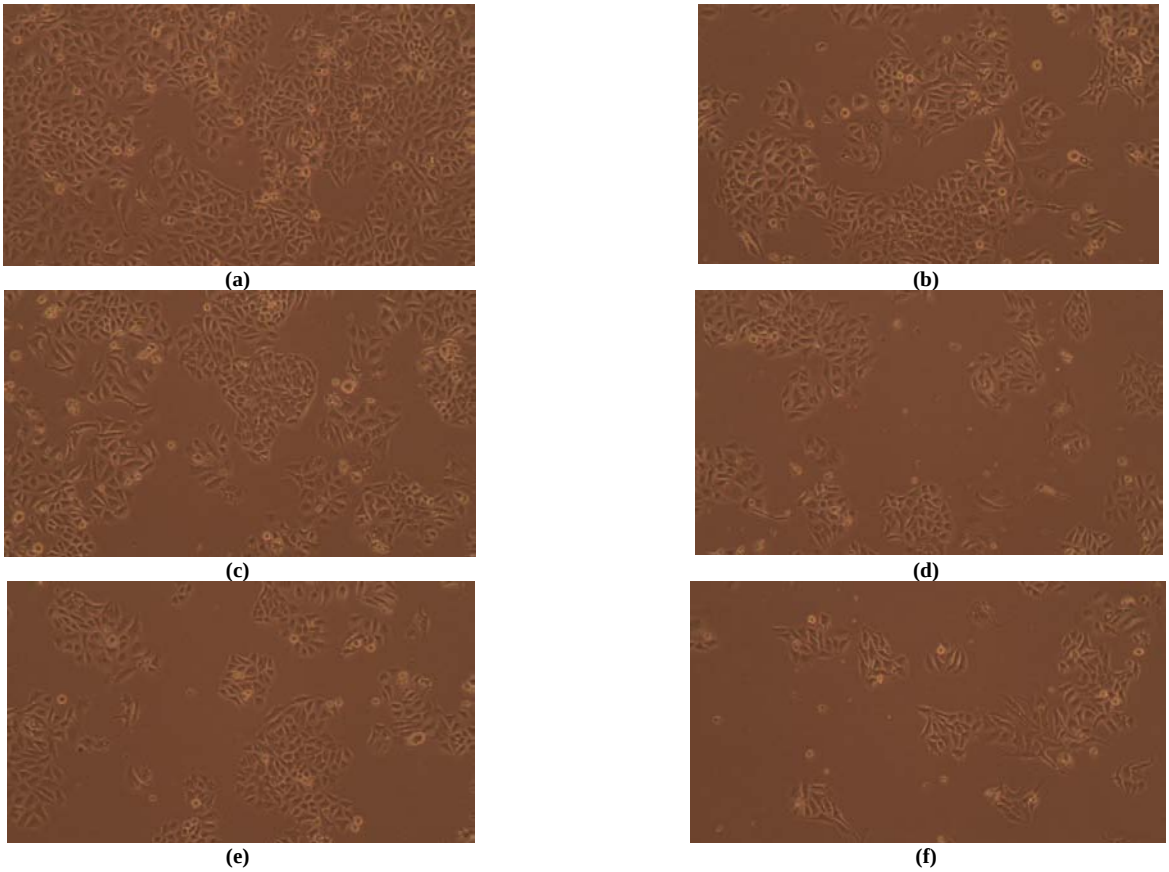


Fig. 6: Morphological changes showing inhibition of Hela cell lines by different concentrations of standard drug Doxorubicin. a: -ve control (95% ethanol), b: 0.097 µg/ml, c: 0.19µg/ml, d: 0.39 µg/ml, e: 0.78 µg/ml f: 1.56 µg/ml

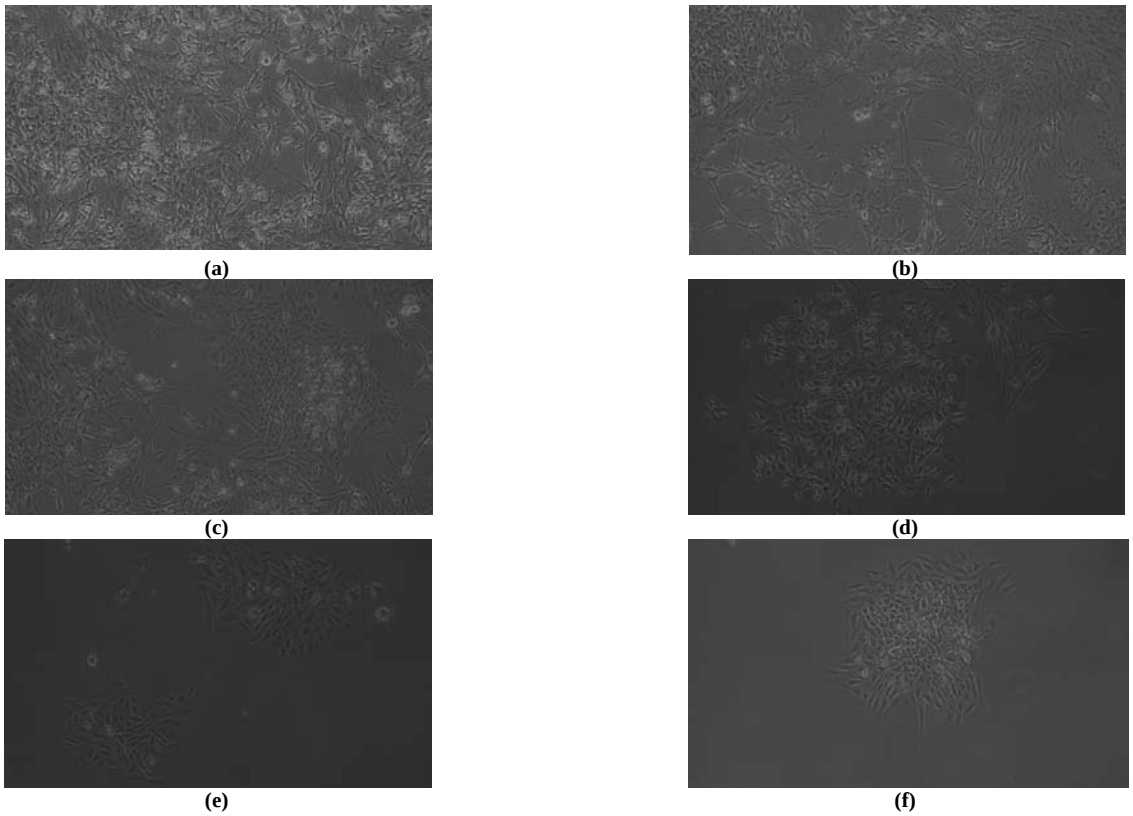


Fig. 7: Morphological changes showing inhibition of MCF 7 cell lines by different concentrations of ethanolic extract of leaves of Pothos scandens. a: -ve control (95% ethanol), b: 6.25 µg/ml, c: 12.5 µg/ml, d: 25 µg/ml, e: 50 µg/ml f: 100 µg/ml

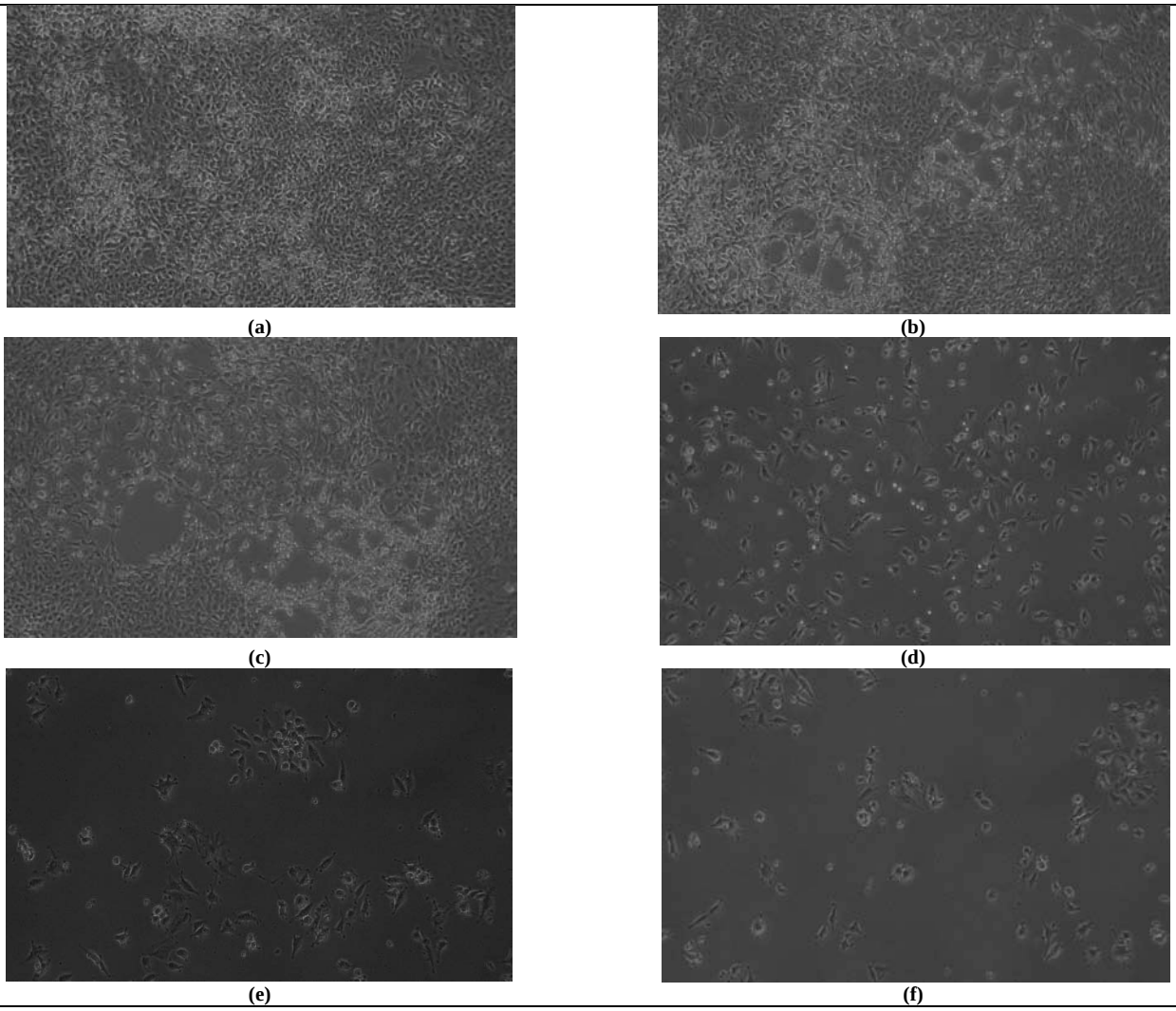
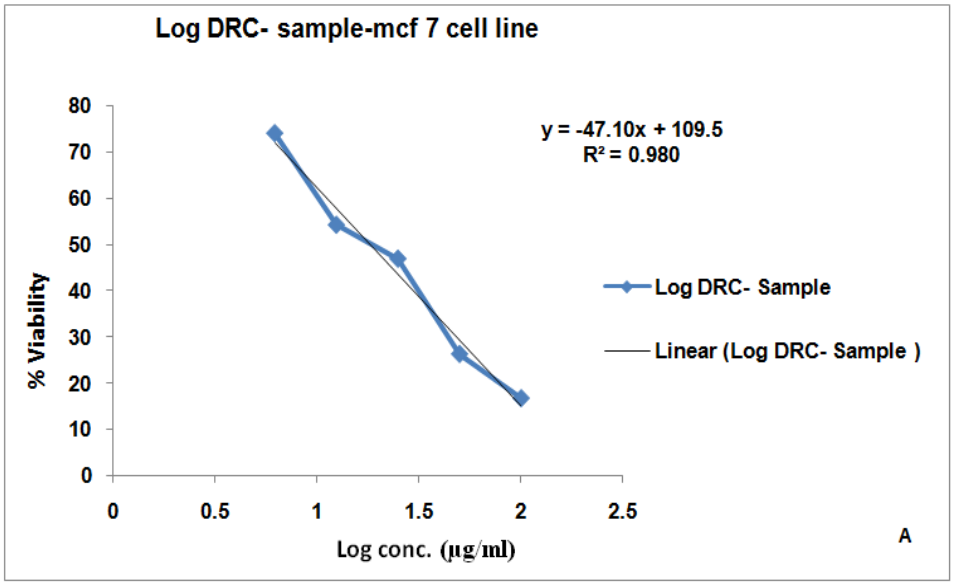
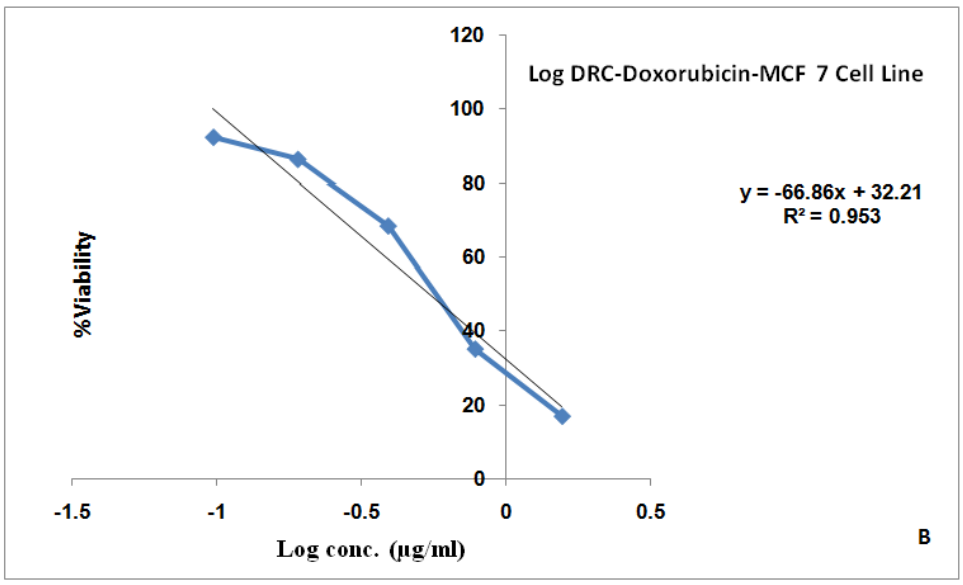


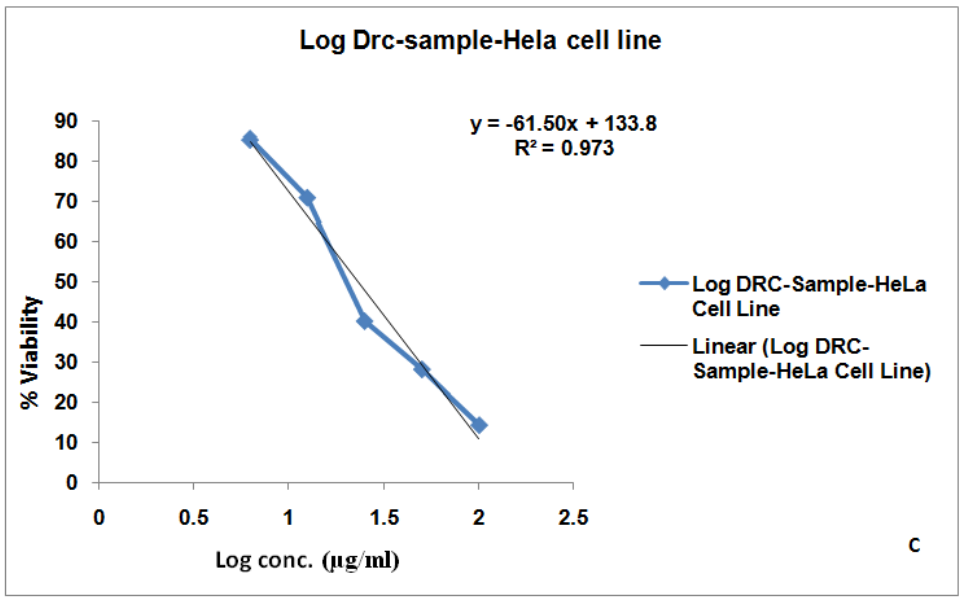
Fig. 8: Morphological changes showing inhibition of MCF 7 cell lines by different concentrations of standard drug Doxorubicin. a: -ve control (95% ethanol), b: 0.097 µg/ml, c: 0.19µg/ml, d: 0.39 µg/ml, e: 0.78 µg/ml f: 1.56 µg/ml

Table 4: IC_{50} values of ethanolic leaf extract of *Pothos Scandens* linn. and doxorubicin on MCF 7 and HeLa cell lines

Sample	Conc: (µg/ml)	Average asorbance	Percentage viability	IC_{50} (µg/ml)
Ethanolic Leaf Extract (MCF 7 Cell Line)	Control	0.937	100	**18.32**
	6.25	0.692	73.888	
	12.5	0.508	54.18	
	25	0.439	46.887	
	50	0.247	26.396	
	100	14.941	16.88	
Doxorubicin (MCF 7 Cell Line)	Control	0.931	100	0.541
	0.097	0.859	92.231	
	0.19	0.803	86.251	
	0.39	0.637	68.457	
	0.78	0.326	35.016	
	1.56	0.156	16.756	
Ethanolic Leaf Extract (HeLa Cell Line)	Control	0.745	100	**22.9**
	6.25	0.637	85.51	
	12.5	0.53	71.109	
	25	0.3	40.295	
	50	0.211	28.265	
	100	0.107	14.356	
Doxorubicin (HeLa Cell Line)	Control	1.056	100	0.93
	0.097	0.9	85.288	
	0.19	0.784	74.293	
	0.39	0.69	65.359	
	0.78	0.562	53.192	
	1.56	0.432	40.887	


Log DRC- sample-mcf 7 cell line
y = -47.10x + 109.5
R² = 0.980
% Viability
Log DRC- Sample
Linear (Log DRC- Sample)
Log conc. (µg/ml)
A


Log DRC-Doxorubicin-MCF 7 Cell Line
y = -66.86x + 32.21
R² = 0.953
%Viability
Log conc. (µg/ml)
B


Log Drc-sample-Hela cell line
y = -61.50x + 133.8
R² = 0.973
% Viability
Log DRC-Sample-HeLa Cell Line
Linear (Log DRC-Sample-HeLa Cell Line)
Log conc. (µg/ml)
C

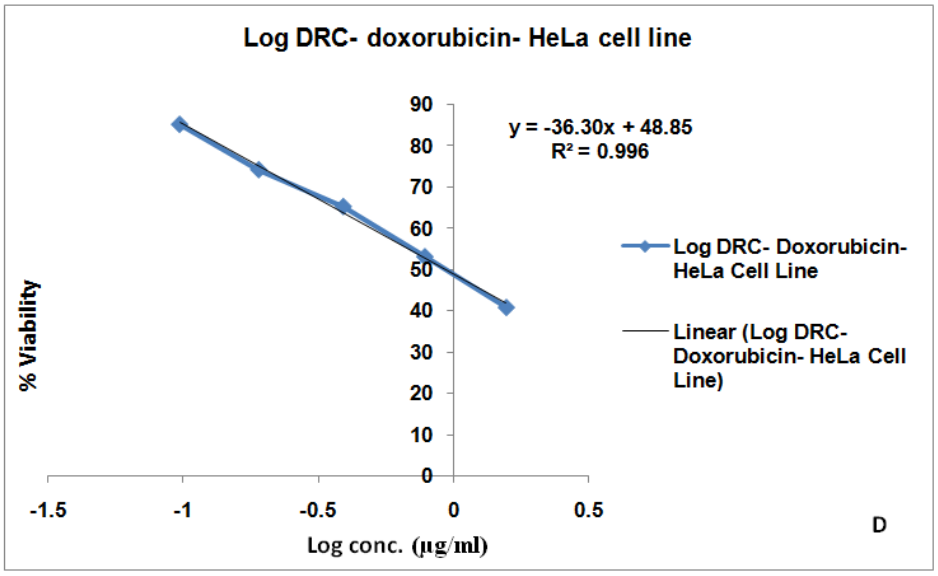
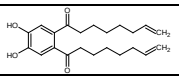


Fig. 9: Log dose-response curve of ethanolic leaf extract of Pothos scandens linn. and doxorubicin, A and B: log DRC of the extract and doxorubicin in MCF 7 cell lines, C and D: log DRC of the extract and doxorubicin in HeLa cell lines

Table 5: Docking result

Target protein PDB ID	Ligand selected	C dock score	C docker interaction energy	H-bond interaction	Active site residues
4AG8		39.9993	61.1657	Cys1045,Asp1046,Val914,Ala866	Glu917,Cys919 Asp1046

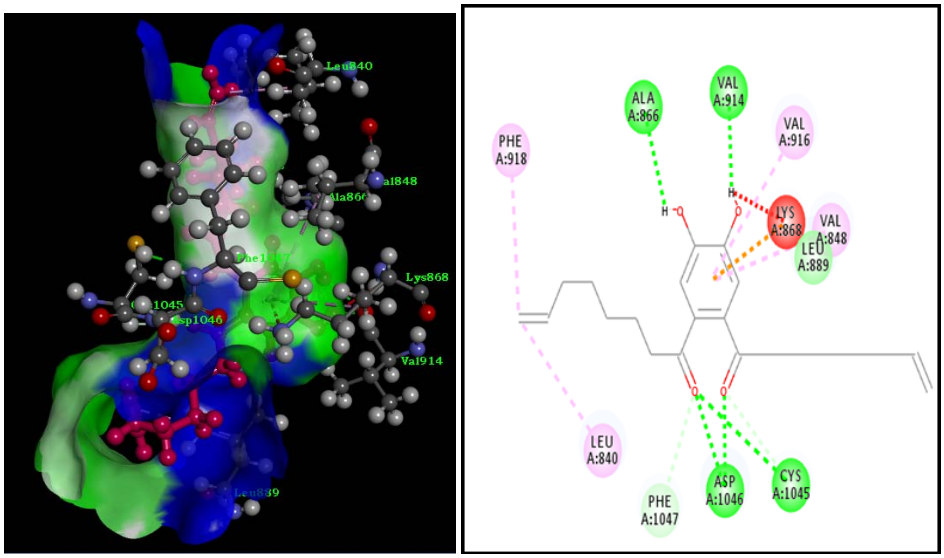


Fig. 10: Docked pose of the selected ligand with the target protein

CONCLUSION

A phytoconstituent 1,1'-(4,5-dihydroxy benzene-1,2-diyl)bisoct-7-en-1-one was isolated from ethanolic leaf extract of *Pothos scandens* Linn and its structure was elucidated with the help of spectral data. The *in vitro* anticancer activity of the extract was evaluated using HeLa and MCF 7 cell lines. The findings of the study revealed that the extract is cytotoxic against the selected cell lines. The anticancer potential of the plant was further confirmed by molecular docking studies. The docking score showed that the isolated compound interacted strongly with 4AG8. The study suggests that *Pothos scandens* Linn. can be used for the isolation of potent anticancer agents. Detailed *in vivo* studies are required for further validation.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors do not have any conflict of interest.

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