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Research Article

EVALUATION OF ANTICANCER ACTIVITY *TINOSPORA CORDIFOLIA* LEAVES EXTRACT AND ITS ISOLATED FRACTIONS

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ABSTRACT

The study investigated the anticancer potential of phytoconstituents isolated from *Tinospora cordifolia*, an established medicinal plant used in traditional medicine. The authenticated plant leaves were extracted with ethanol, and the crude extract along with its toluene fraction was analyzed for phytochemical constituents. Qualitative tests confirmed the presence of alkaloids, steroids, glycosides, and flavonoids. Separation through chromatographic techniques resulted in three distinct fractions. The anticancer potential of the extract and fractions was evaluated on human cervical and lung cancer cell lines using standard cell viability assays. The crude ethanolic extract exhibited considerable cytotoxic activity, while among the isolated fractions, the third fraction showed pronounced inhibitory effects on cancer cell growth comparable to the standard reference compound. LC–MS/MS profiling of the active fraction indicated the presence of magnoflorin, bergenin, stigmasterol, and 8-hydroxy tinosporide. The findings suggested that *Tinospora cordifolia* contains bioactive phytoconstituents with significant anticancer potential. The study concluded that these constituents may act synergistically to inhibit cancer cell proliferation and could serve as leads for the development of safer, plant-based anticancer agents.

Keywords: Tinospora, Alkaloids, Antineoplastic potential, Bioactive phytoconstituents, Cytotoxic evaluation.

INTRODUCTION

Tinospora cordifolia is a deciduous climbing shrub found in India, Sri Lanka, China, and Bangladesh, and is known as Amrita or Guduchi. It is commonly used in Ayurvedic medicine to enhance the immune functions (Mathew *et al*., 2016) and to treat urinary disorders, dyspepsia, and fever (Prajwala *et al*., 2019). The plant has several medicinal properties, such as stimulating bile secretion, relieving thirst and burning sensation, treating jaundice, and acting as a bitter stomachic, diuretic, and anti-emetic. It is also effective in treating scorpion sting and snake bite. *Tinospora cordifolia* contains various chemical compounds, including steroids, glycosides, alkaloids, diterpenoid lactones, and aliphatics (Joshi and Kaur, 2016; Reddy & Reddy, 2015; Sharma *et al*., 2019; Upadhyay *et al*., 2010). The plant's explored phytoconstituents and their pharmacological activities are listed in Table 1. Cancer is a disease with abnormal cell growth which has potential to spread to other body parts. The most prevalent cancer among females is breast cancer and among males is lung cancer, accounting for about 23% and 17% of total cancer cases respectively. The available chemotherapy and radiotherapy are expensive and have a number of side effects like cardiac, neurological, renal and pulmonary toxicity, as well as myelosuppression imposing serious threat to the quality of life. This addresses a need to develop better treatment options with more potency and less toxicity. The plant-based compounds are potential

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anticancer candidates due to their safety, oral bioavailability and low cost. *Tinospora cordifolia* has shown anti-cancer activity, in various animal models (Ali and Dixit, 2013; Mishra & Kaur, 2013; Verma *et al*., 2011).It is reported to be better than doxorubicin treatment(Jagetia *et al*., 1998). Crude extracts of medicinal plants are used in disease treatment, but isolation and identification of the active phytoconstituents and elucidation of the mode of action of a drug is of paramount importance. In this study, attempt has been made to isolate and identify three phytoconstituents responsible for anticancer activity from *Tinospora cordifolia*.

MATERIALS AND METHODS

Collection and Authentication of plant material

Fresh leaves of *Tinospora cordifolia* were collected from the local area of Rajgurunagar, Pune, India. The plant was identified and authenticated by the Botanical Survey of India, Western Regional Centre, Pune, Maharashtra (specimen no. BSI/WRC/cert./2015).

Chemicals

Ethanol, methanol, toluene, pet ether, acetonitrile, acetone, hexane, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium hydroxide, ethyl acetate, iodine was procured from Loba Chemie Pvt. Ltd., Mumbai. HPLC grade reagents hexane, ethyl acetate, acetonitrile, methanol was purchased from Merck. HPLC grade water was obtained from Merck and was filtered through 0.2 µm filter before use. Silica gel (60-120 mesh) and Aluminum-backed TLC plates (9 cm × 2.5 cm), coated with 250 µm layer of silica gel 60 F_{254} were purchased from E. Merck, Darmstadt, Germany supplied by Anchrom Technologists, Mumbai.

Table 1. Phytoconstituents present in Tinospora cordifolia with their pharmacological role.

Phytochemical class	Phytoconstituents (molecular weight)	Plant Part	Pharmacological activity
Alkaloids	Berberine (336.36), Choline (104.14), Tembetarine (344.43), Magnoflorine (342.41), Tinosporin, Palmatine (342.40), columbin/ Isocolumbin (358.39), Aporphine (235.32), Jatrorrhizine (338.38), Tetrahydropalmatine (355.43)	Root, Stem	Anti-diabetes, Anti-viral, Anticancer, Anti-inflammatory, Immunomodulatory, Psychiatric, Neurological, conditions
Diterpenoid Lactones	Furanolactone (416.57), Clerodane derivatives, Tinosporon, 8-hydroxy tinosporide (390) Jateorine, Tinosporide-1 (374)	Whole plant	Vasorelaxant, anti-viral, anti-microbial, anti-inflammatory, antihypertensive
Glycosides	Tinocordifolioside (412.47), Tinocordiside (396.47), Cordioside, 18-norclerodane glucoside (410.77), Furanoid diterpene glucoside, Syringin (372.37), Syringinapiosyl glycoside, Pregnane glycoside (879.08), Palmatosides, Cordifolioside A-E (621.19), Bergenin (328.27), Palmatoside C/ Columbin glucoside (520.52), Palmatoside F (536.52), Amritoside A,B,C,D (626), Tinocrisposid/ Boratoside(536.57), Cordifolides B and C(575)	Stem	Neurological disorders like Parkinson's, ALS, cognitive and motor deficits, Dementia, and Immunomodulation, neuron loss in hypothalamus and spine, Anticancer
Steroids	β –sitosterol (414.70), δ-sitosterol (414.14), 20 β-hydroxyecdysone (480.63), Ecdysterone (480.63), Makisterone A (494.66), Giloinsterol, Stigmasterol (412.70)	Shoot	IgA neuropathy, early inflammatory arthritis, Anticancer
Sesquiterpenoid	Tinocordifolin	Stem	Antiseptic
Aliphatic compound	Heptacosanol (396.73), Octacosanol (410.17), Nonacosan-15-one dichloromethane	Whole plant	Anti-nociceptive, anti-inflammatory, in parkinsonism
Others	Tinosporic acid, Jatrorrhizine, Tinosporidine, Cordifelone, Giloin, Cordifol, Giloinin, N-transferuloyltyramine, Cordifolides	Whole Plant, Root	HIV (Protease inhibitors)

Preparation of ethanolic extract

The collected leaves were washed with water, kept for shade drying for 6-7 days. They were ground to fine powder and stored in an air-tight container protected from humidity and sunlight. Physicochemical analysis of the powdered drug was performed. Ethanolic extract was prepared by adding 400 ml of ethanol to 30 gm of powder in Soxhlet extractor at 45°C for 7 h (13 cycles). Ethanol was further evaporated to yield a sticky mass.

Preparation of toluene extract

About 1gm of ethanolic extract was mixed with 50 ml methanol. To 10 ml of the resulting solution, 20 ml of 2M NaOH was added. The amount of NaOH was optimized to achieve maximum precipitation. The resulting residue was separated by filtration. The residue was resuspended in 20 ml methanol; 30 ml toluene was added and mixed thoroughly. The toluene layer was separated from the methanolic layer using a separating funnel, dried and evaluated.

Phytochemical analysis

Preliminary analysis of crude ethanolic extract and toluene extracts was carried out to identify the presence of various phytoconstituents by employing standard chemical test protocols (Evans, 2009).

Isolation of Phytoconstituents

To separate the various phytoconstituents in the toluene extract, a mobile phase was optimized using TLC. Different solvents such as hexane, ethyl acetate, acetonitrile, methanol, and water were tried in different ratios to determine the optimal mobile phase. Among all the solvent systems tested, the mixture of hexane and ethyl acetate showed the highest separation of analytes. The ratio of this solvent system was further optimized to achieve maximum resolution using TLC. The separation was performed on Silica gel 60 F_{254} plates (9 × 2.5 cm) in a TLC chamber saturated with the mobile phase for 30 minutes. The samples were run on TLC plates and observed under UV light and in an iodine chamber to identify distinct bands. The mobile phase for column chromatography was further optimized to achieve maximum resolution between different spots. Fractions were then isolated using column chromatography, with a column (30 cm × 3 cm) packed with a mixture of 300 g of silica (60-120 mesh) and 500 ml hexane. The sample was prepared by mixing the toluene extract with silica (1:1 w/w) and dried until it became free flowing. The optimized solvents for separation of fractions from TLC analysis were continuously poured on the column. The resolved fractions were collected and dried at room temperature to obtain a solid mass.

MTT assay

All cell culture experiments were carried out at the Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. Squamous cell carcinoma cell line (SiHa) and Lung carcinoma cell line (A549) were routinely cultured in RPMI 1640 medium containing 10% fetal bovine serum and 2M L-glutamine media in a 37ºC, 5% CO_2, 95% humidity incubator. Monolayers of SiHa and A549 cell were trypsinized and cells were counted. Cell suspension was diluted to 5×10^5 cells/ml in growth medium. Then 180 µl of cell suspension was added to each well of 96 well plate. This plate was incubated in CO_2 incubator for 24 hrs. until the cells reached 50-60% confluency. A stock solution of crude ethanolic extract with a concentration of 10 mg/ml was prepared. 20 µl of this solution was added to each well to obtain a final concentration of 1 mg/ml. From the stock solution of 1 mg/ml, different dilutions containing 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.0156 mg/ml were prepared in sterile PBS. 20 µl of each dilution was added to the plate in triplicates and control (without drug) was added in 6 wells. This plate was incubated for 48 hrs. 10 µl MTT dye was added in each well. Plate was wrapped in aluminum foil and again kept for incubation for 4-18 hrs in a CO_2 incubator. After incubation, the supernatant was removed without disturbing the cell layer. 100 µl DMSO and 25 µl glycine buffer were added in each well. After 10-20 min, optical density was recorded at 540 nm on ELISA plate reader. Cell viability was calculated using following formula: % Cell viability = (Average Absorbance of duplicate drug wells/ Average Absorbance of control wells) ×100

SRB assay

The SiHa and A549 cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2M L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL complete media at plating densities as describe under the MTT assay, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5 % CO2, 95 % humidity for 24 h prior to addition of experimental drugs. DMSO was used to solubilize the isolated fractions I, II and III to achieve a concentration of 100 mg/ml. Further dilution with water was carried out to 1mg/ml and stored frozen. The frozen concentrate was thawed at the time of use and diluted with complete medium to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800µg/ml. In each wells containing 90 µl of medium, 10 µl of drug was added, to give concentrations of 10- 80 µg/ml. The plates were incubated at standard conditions for 48 hrs. A 50 µl of cold 30 % w/v TCA was added to fix the cells in situ and terminate the assay followed by incubation at 4°C for 1 hr. The supernatant was removed; the plates were washed with water and dried. A Sulforhodamine B (stain, 50 µl, 0.4 % w/v in 1 % acetic acid) was added to the wells. The plates were incubated at room temperature for 20 min. Solution of 1 % acetic acid was used to remove residual dye and dried. Bound stain was eluted with trizma base (10 mM) and plate reader was used to measure the absorbance (540 nm with 690 nm as reference). Percent growth was expressed as a ratio of average absorbance of the test wells to the average absorbance of the control wells * 100. The percentage growth was calculated for each drug concentration. Percentage growth inhibition was calculated as: Percent growth inhibition = [Ti/C] x 100 where Ti and C are OD_{540} for test wells and control wells without any treatment, respectively.

LCMS/MS analysis of Fraction 3

LC analysis of fraction 3 was performed on Agilent binary LC 1260 equipped with C_{18} column (Zorbax Eclipse plus, 1.8µm, 2.1×50mm) maintained at 40℃. The mobile phase

consisted of aqueous formic acid (0.1%) and acetonitrile and was used at a flow rate of 0.3 ml/min for 30 minutes. The injection volume was 1µL. MS-MS was performed using Agilent 6540 Q-TOF equipped with ESI source with collision energy of 20 V. The data integration was performed using Masshunter workstation software v. B. 05.01

RESULTS AND DISCUSSION

The powder of dried leaves contained 3.8% w/w foreign matter. The total ash, acid insoluble ash, and water-soluble ash were 6.12%, 1.08%, and 1.22% w/w, respectively. The loss on drying was 7.3% w/w, which falls within the standard range of 6.5-11.5%. Upon ethanolic extraction, 30 g of powder yielded 4 g of extract (13.33% w/w). The toluene extract yield was 40% of the ethanolic extract. Phytochemical analysis revealed the presence of alkaloids, glycosides, flavonoids, and steroids in the ethanolic extract, while the toluene fraction contained alkaloids and steroids (Table 2).

Table 2. Phytochemical analysis of extract

S. No.	Phytoconstituent	Test	Inference	
			C.E.E.	T.E.
1	Alkaloid	Dragendorff's test, Mayer's test, Hager's test	+	+
2	Glycoside	Keller – killiani Test, H_2SO_4 test	+	-
3	Flavonoids	Ferric chloride test	+	-
4	Steroid	Salkowski reaction	+	+

C.E.E.: Crude ethanolic extract, T.F.: Toluene fraction

During the TLC study, a hexane and ethyl acetate mobile phase was used, and three separate spots were observed. To improve the resolution between different bands, various ratios of hexane and ethyl acetate were utilized. The ratios were altered by decreasing the amount of hexane while simultaneously increasing the amount of ethyl acetate. A hexane:ethyl acetate ratio of 90:10 resulted in the resolution of first spot. When the ratio was changed to 80:20, the distance between the first and second spots increased. A ratio of 70:30 was able to separate the second and third spots. Finally, using a hexane:ethyl acetate ratio of 60:40 increased the distance between the second and third spots. Column chromatography was then utilized to separate the phytoconstituents after optimizing the mobile phase using TLC. A hexane:ethyl acetate gradient was employed to resolve the three fractions. The first fraction was eluted using a hexane:ethyl acetate ratio of 90:10 while a ratio of 80:20 was used to resolve the second band. Finally, with a hexane:ethyl acetate ratio of 60:40, the second and third bands were optimally separated and three fractions were isolated. Crude ethanolic extract was tested for anticancer activity with MTT assay on cervical cancer SiHa cell lines and A549 lung cancer cell lines. Figure 1 shows results of MTT assay. Results showed that crude ethanolic extract has shown promising anticancer activity on both cell lines. Still, the activity on lung cancer cell line was more promising. Anticancer potential of isolated fractions was further evaluated using SRB assay.

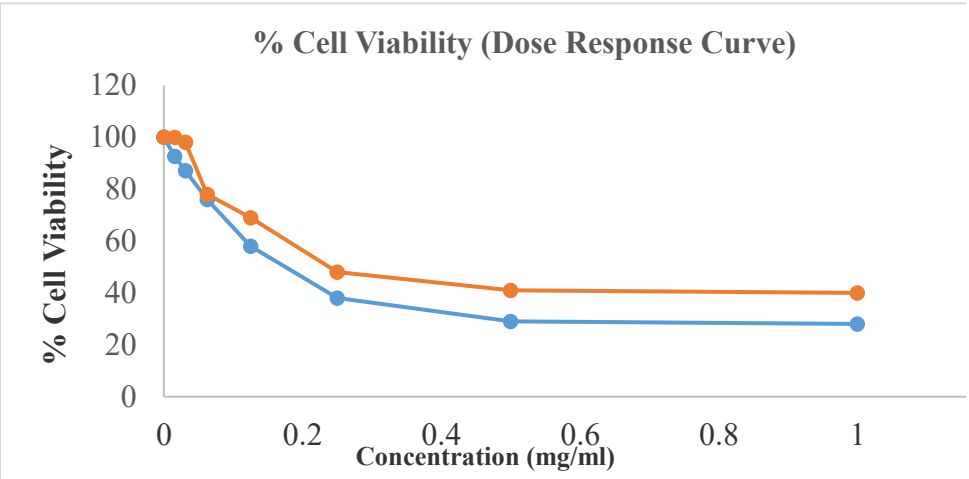


Figure 1. Dose Response Curve of TC leaves extract against SiHa and A549 cell lines.

Crude extract, Fractions 1, Fraction 2, and Fraction 3 were evaluated for anticancer activity with curcumin as a standard. The cell viability was measured using SRB assay. All the environmental conditions were maintained throughout the experiment for all the groups. The assay was performed in triplicate for each of the extracts. The growth curve was plotted against molar drug concentration of isolated constituents and % control growth. Figure 2 shows growth curve of human cervical (SiHa) and lung cancer cell line (A549). Table 3 shows % control growth of samples with different concentrations. Morphology of SiHa cells under inverted phase contrast microscopy is shown in Figure 3. The image of fraction 3 showed significant growth inhibition, cell rounding and disappearance of normal spindle shape as that of curcumin which was used as reference standard. Fraction-1 do not exhibit anticancer activity, while small amount of growth inhibition was found to be associated with fraction 2. The result of above assay shows that fraction 3 has comparable anticancer activity against SiHa and A549 cell lines as that of curcumin. Hence it was decided to carry out qualitative analysis of fraction 3.

Table 3. Result of cytotoxicity testing of various fractions and crude extract against SiHa and A549 cell lines.

	% Control growth (SiHa cell lines)				% Control growth (A549 cell lines)			
Conc (µg/ml)	**10**	**20**	**40**	**80**	**10**	**20**	**40**	**80**
Curcumin	108.0	104.1	87.2	69.4	107.2	105.6	90.3	81.7
F-I	112.4	115.7	114.6	113.6	105.4	101.6	89.2	90.3
F-II	97.6	107.2	95.9	91.2	81.7	90.0	81.5	76.7
F-III	101.1	99.6	101.6	77.4	83.5	88.0	73.8	61.1
Extract	101.3	114.6	114.6	116.6	102.4	101.2	95.8	93.5

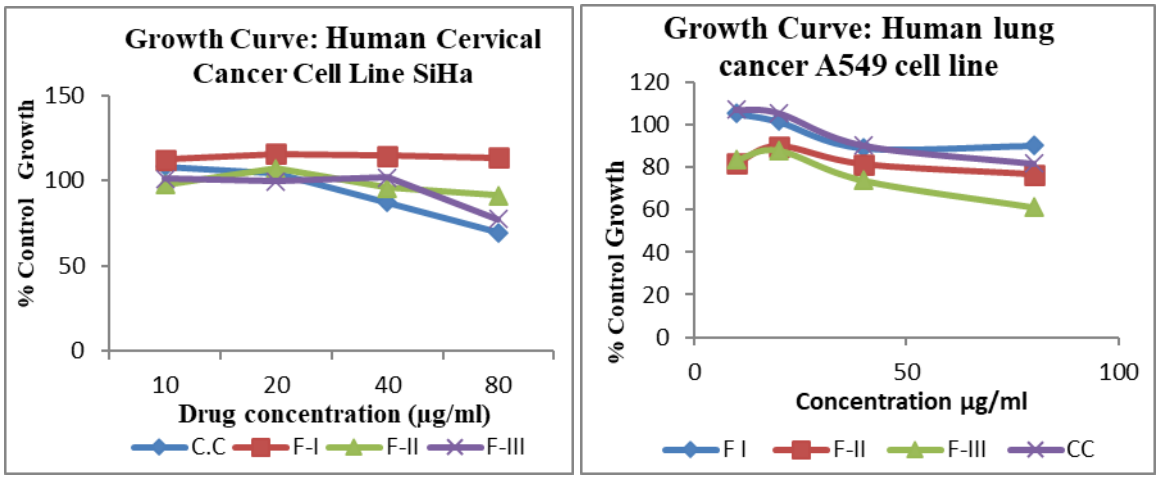


Figure 2. Growth curve of test samples on SiHa and A459 cell lines

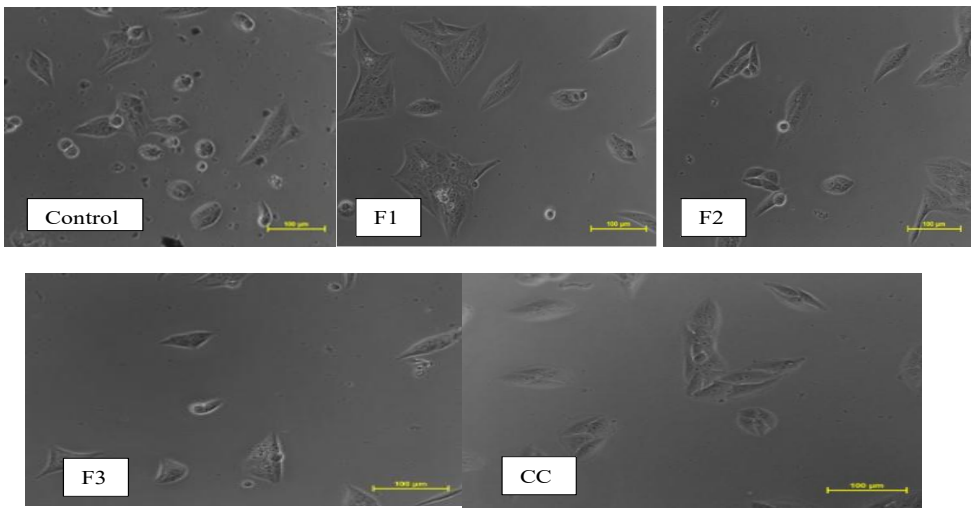


Figure 3. Assessment of cell morphology after treatment of human lung adenocarcinoma cell line A549.

LCMS/MS analysis of fraction 3 was performed, the total ion chromatogram showed 2 major peaks. Figure 4 shows LCMS/MS spectra of Fraction 3 which shows various daughter ions.

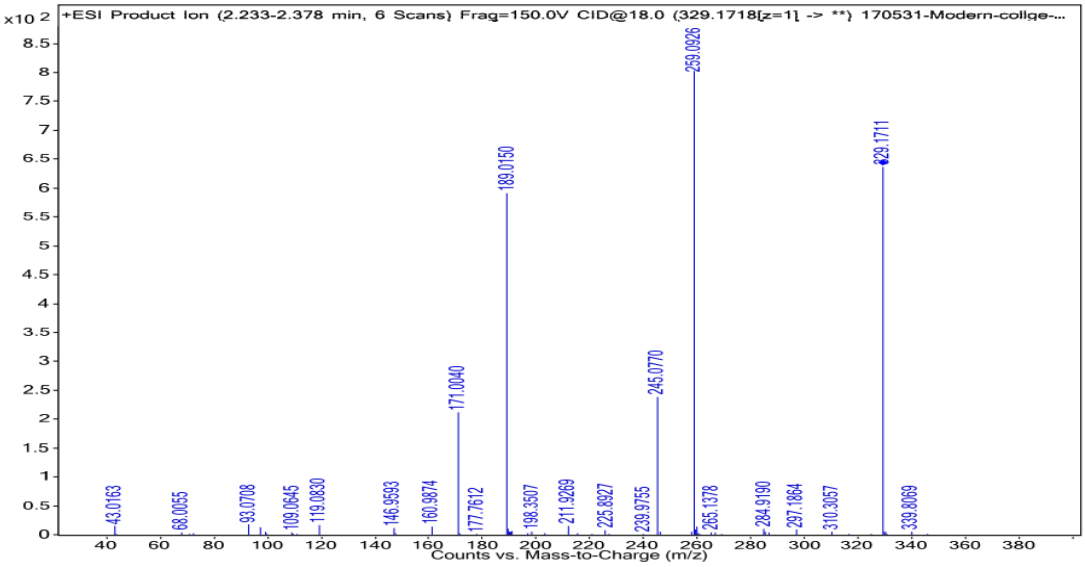
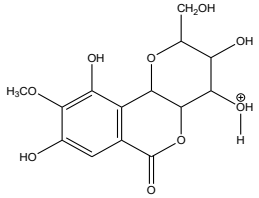
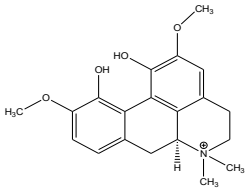
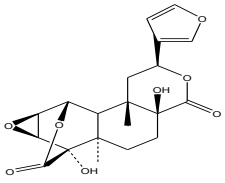
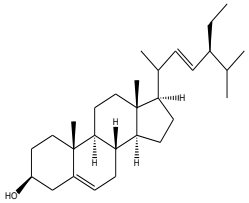


Figure 4. The LCMS/MS spectra of molecular ion with m/z of 329.171 at Rt 2. 31 min.

Table 4 lists possible phytoconstituents present in fraction 3 with their structures. LCMS/MS analysis shows some possible Phytoconstituents like Magnoflorin, Bergenin alkaloids, 8-hydroxy Tinosporide and Stigmasterol.

Table 4. List of possible Phytoconstituents present in Fraction 3 with their structures.

Rt (min)	Experimental mass	Theoretical mass	Chemical formula	Name of possible phytoconstituent	Structure
1.78 (Major)	223.096, 315.156	-	-	-	Unidentified
2.31 (Major)	329.171	329.086	$C_{14}H_{16}O_9$	Bergenin	
1.1 (Minor)	342.41	342.170	$C_{20}H_{24}NO_4$	Magnoflorin	
2.12 (Minor)	390.0	390.131	$C_{20}H_{22}O$	8-hydroxy Tinosporide	
2.43	412.70	412.370	$C_{29}H_{48}O$	Stigmasterol	

CONCLUSION

Based on the results of the study, it can be concluded that *Tinospora cordifolia* ethanolic extract has demonstrated potential anticancer activity on SiHa and A549 cell lines. The phytochemical analysis of the crude ethanolic extract revealed the presence of various phytoconstituents such as steroids, alkaloids, glycosides, and flavonoids. The study also compared three fractions of the extract with curcumin for anticancer activity and found that fraction 3 had the highest activity. The LCMS/MS analysis of fraction 3 suggested that it may contain magnoflorin, bergenin, 8-hydroxy tinosporide and stigmasterol, which may be responsible for the observed anticancer activity. These findings indicate that *Tinospora cordifolia* may be a potential source of natural compounds with anticancer properties and further studies are needed to explore the mechanisms behind its activity and potential use in cancer treatment.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

FUNDING

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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