

In vitro Cytotoxicity and Apoptotic Assay in HT-29 Cell Line Using *Ficus hispida* Linn: Leaves Extract

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ABSTRACT

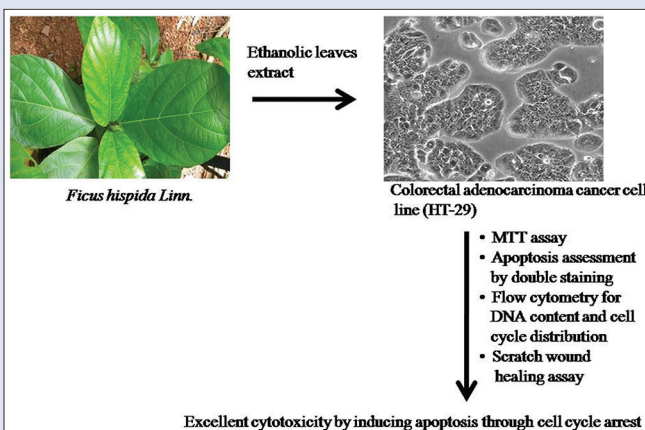
Background: *Ficus hispida* Linn. (Family *Moraceae*), well-known beneficial medicinal shrub, has been traditionally used for the treatment of various diseases such as leukoderma. **Objective:** The aim of the present study is to investigate the efficacy of *F. hispida* ethanolic leaves extract for antiproliferative, apoptotic, cell cycle blockade, and wound healing.

Materials and Methods: *F. hispida* leaves extract was treated with colorectal adenocarcinoma cancer cell line HT29 for 24 h with control. The cells were treated at varying concentration ranges of 15, 31, 62, 125, and 250 µg/ml each. The cytotoxicity effect of leaves extract was studied by 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay and their anticancer activity was further evaluated using cell cycle analysis and wound scratch assay. **Results:** The end antiproliferative result showed that HT-29 cell viability decreases in a concentration-dependent manner and the growth inhibitory effect (IC50) values are obtained at a concentration of 125 µg. The increase in number of apoptotic cell was observed after treating HT-29 cells with the sample in double-staining methods. G0/G1 phase of the cell cycle was significantly blocked by the test sample followed by the G2/M phase in a negligible manner. *In vitro* cell wound closure or contracture was not significant when compared the sample against control group. **Conclusion:** *F. hispida* Linn. ethanolic leaves extract had shown to possess excellent cytotoxic effect through inducing apoptosis, especially causing cell cycle arrest at the G0/G1 phase.

Key words: Apoptosis, cancer, cytotoxicity, *Ficus hispida*, phytochemicals, plant extract

SUMMARY

- The experiment tries to evaluate the effectiveness of *F. hispida* leaves extract as an antiproliferative, apoptotic, cell cycle inhibitor and wound healing agent. Results showed that *F. hispida* Linn extract own cytotoxic property by inducing apoptosis through cell cycle arrest.



Abbreviations used: HT 29: Human adenocarcinoma colorectal cell line; PBS: Phosphate Buffered Saline; FBS: Fetal Bovine Serum; DMEM: Dulbecco's Modified Eagles Medium; MTT: 3 [4, 5 dimethylthiazol 2 yl] 2, 5 diphenyltetrazolium bromide; NCCS: National Centre for Cell Sciences; DMSO: DiMethyl Sulfoxide; PI: Propidium Iodide; AO: Acridine Orange; EB: Ethidium Bromide; IC: Inhibitory Concentration.

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INTRODUCTION

Cancer, an important cause of mortality world wide next to cardiovascular disease, is characterized by the deregulation of signaling pathways with an initial loss of controlled cell division and growth, cell invasion, and finally, result in metastasis.^[1] Apoptosis is the important mechanism that maintains tissue homeostasis through caspases leading to cell death and its regulations are altered in many of the pathological conditions such as cancer.^[2] A recent report indicated its incidence to be 94/100,000 people with an age-standardized rate in India, and about more than half of the Indian incidence globally.^[3] Lung, intestine, breast, prostate, pancreas, ovary, liver, bladder, blood cells, and bone are the most common human location site.^[4] The advent of modern cancer synthetic treatment procedures has a major drawback of expensiveness and carries a broad list of adverse effect such as anemia, appetite loss, bleeding and bruising (thrombocytopenia), constipation, diarrhea, edema, fatigue, and hair loss (alopecia) to congestive heart failure, coronary artery disease, arrhythmia, hypertension, lung problems, endocrine problem,

bone and joint problem, brain and spinal cord problems, dental, oral, and vision problems.^[5] The World Health Organization (2001) has estimated that between 80% of the populations are treated through medicines or medicinal formulations obtained from plant sources.^[6] Moreover, plant sources (phytoextracts) are commonly employed in traditional medicine are thought to be safe, better alternative to synthetic drugs without any side effects, longer usage duration, and potential in targeting diseases.^[7,8] Hence, screening for phytochemical pharmacological properties from

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different traditional plants are necessitated to identify a more efficient chemotherapeutic drug with better efficacy to target different signaling pathway in a precise, specific, and sensitive way to control cancer proliferation and induce apoptosis.

Ficus hispida Linn. (Family *Moraceae*) is one such well-known beneficial medicinal shrub or moderate-sized plant growing in damp and shady areas found in India, Sri Lanka, Myanmar, Southern region of China, New Guinea, Australia, and Andaman island.^[9] Most of this plant parts are used as a treatment remedy for various ailments such as leukoderma, skin diseases, jaundice, and as antipoisonous by Indian traditional healers. Current literature survey had shown that various parts of the plants are beneficial as antimicrobial, anti-inflammatory, analgesic, antipyretic, antitussive, antiulcer wound healing, antidiabetes, gall bladder disease, anti-diarrheal in rats (significant inhibitory activity against castor oil-induced), paracetamol-induced hepatotoxicity protective effect on rat, cancer cell lines (oral, colon and breast cancer) through phenanthroindolizidine alkaloid called O-methyltylophorinidine, cisplatin-induced nephrotoxicity, antihyperlipidemic, cardioprotective effect, and antioxidant.^[10-20] The above medical properties of the plants are due to the presence of phytochemical constituents include phenanthroindolizidine alkaloids, n-alkanes, coumarins and triterpenoids, hispidin, oleanolic acid, bergapten, β -amyryn and β -sitosterol, lupeol acetate, β -sitosterol, and β -amyryn acetate.^[21,22]

However, the leaves are of particular interest in view of anticancer property, but till date, there is limited and incomplete research has been performed on this traditional plant. Moreover, screening of crude extract or phytochemical potential to induce apoptosis has not been properly elucidated in the past as it is the best strategy for developing a novel anticancer drug. Hence, the objective of the present study is to investigate the *F. hispida* leaves potential for their antiproliferative and apoptosis inducing activities on human adenocarcinoma colorectal (HT-29) cell line.

MATERIALS AND METHODS

Chemicals

Trypsin was procured from Gibco, USA, while Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS), Dulbecco's Modified Eagles Medium (DMEM), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin-streptomycin, and ethidium bromide were purchased from Hi-Media Laboratories, Mumbai, India. Rest of the chemicals employed in the study was analytical grade.

Collection of *Ficus hispida* leaves and preparation of crude extract

The leaves of *F. hispida* Linn. were collected from Chennai. The plant specimen was deposited and authenticated by Prof. Jayaraman, Plant Anatomy Research Centre, West Tambaram, Chennai, bearing a voucher number PARC/2015/3210. The dried material was further subjected to a fine coarse powder using a blender, and then, the powder was stored in air-tight container for further use. Crude plant extract was prepared by Soxhlet extraction method. About 150 g of *F. hispida* Linn. leaf powder was weighed and macerated in 450 ml of ethanol. The powdered material was kept for occasional shaking for 72 h at room temperature. After 72 h, the supernatant was collected by filtration and the solvent was evaporated. The crude extract was stored in 4°C until further use.

Procurement of cancer cell lines

The HT-29 (human colorectal adenocarcinoma) cell line was initially procured from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained in DMEM. The cell line was cultured in 25 cm² tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium

bicarbonate and antibiotic solution containing: penicillin (100 U/ml), streptomycin (100 μ g/ml). Cultured cell line was kept at 37°C in a humidified 5% CO₂ incubator (VWR, USA). Two-day-old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100 μ l cell suspension (5×10^4 cells/well) was seeded in 96-well tissue culture plate and incubated at 37°C in a humidified 5% CO₂ incubator for 24 h. The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Ethanol plant extract compound stock and test concentration

About 1 mg of plant compound was added to 1 ml of Dimethyl Sulfoxide (DMSO) and dissolved completely by cyclomixer. After that, the extract solution was filtered through 0.22 μ m millipore syringe filter to ensure the sterility. Different concentration of the test sample were prepared in serial dilution were 15, 31, 62, 125, and 250 μ g in 100 μ l of 5% DMEM. In short, after 24 h, the growth medium was removed, freshly prepared each plant extracts in 5% DMEM were five times serially diluted by two fold dilution (15, 31, 62, 125, and 250 μ g in 100 μ l of 5% DMEM) and each concentration of 100 μ l were added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO₂ incubator for 24 h.

In vitro anticancer and wound healing assay 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

The antiproliferative effect of ethanolic extract of *F. hispida* Linn. was assessed by the MTT assay against in HT-29 cell line. 15 mg of MTT was reconstituted in 3-ml PBS until completely dissolved and sterilized by filter sterilization. After 24 h of incubation period, the sample content in wells was removed. About 30 μ l of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, 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 μ l of MTT solubilization solution (DMSO) was added, and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured using microplate reader at a wavelength of 570 nm.^[23,24] Ethanolic extract efficiency for cytotoxicity was measured in terms of growth inhibition percentage for different concentration using the below formula.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of growth inhibition} = \left[\frac{\text{Mean OD samples}}{\text{Mean OD of control group}} \right] \times 100$$

Assessment of apoptosis by double staining

DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EB) were used for the morphological detection of apoptotic and necrotic cells.^[25] AO is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double-stranded nucleic acid. Only nonviable cell takes EB and by intercalation into DNA emits red fluorescence. After treatment with IC50 of plant extracts at a final concentration for 24 h, the cells were washed by cold PBS, and then stained with a composition of 100 μ g/ml each AO and EB for 10 min at room temperature. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

Determination of DNA content and cell cycle distribution using flow cytometry

Monitoring a cell ability to proliferate is critical for assessing a cell's

health during toxicity studies, and the most accurate method involves direct measurement of DNA synthesis. The MUSE cell cycle kit uses a previously mixed reagent of the nuclear DNA intercalating stains propidium iodide (PI) which discriminates cells at different stages of the cell cycle based on the differential DNA content in the presence of RNAase to increase the specificity of DNA staining in each phase (G₀/G₁, S and G₂/M).

HT-29 colorectal cancer cells were cultured as per the standard procedures and treated with IC₅₀ values of compounds for 24 h. The cell sample was transferred to a 12 mm × 75 mm polystyrene tube or 50 ml conical flask. The minimum recommended number of cells for fixation in a tube is 1 × 10⁶ cells. The samples were then centrifuged at 3000 rpm for 5 min. The supernatant was removed without disturbing the pellet. After centrifugation, the cell pellet forms either a visible pellet or a white film on the bottom of the tube.

Appropriate volume of PBS was added to each tube (i.e., 1 ml of PBS per 1 × 10⁶ cells) and the contents were mixed by pipetting several times or gently vortexing. The tubes were centrifuged at 3000 rpm for 5 min. The supernatant was discarded without disturbing the cell pellet, leaving approximately 50 µl of PBS per 1 × 10⁶ cells. The pellet was resuspended in the residual PBS by repeated pipetting several times or gently vortexing. The resuspended cells were added drop wise into the tube containing 1 ml of ice cold 70% ethanol while vortexing at medium speed. The tube was capped and freezed at -20°C.

Staining of cell cycle

After the overnight incubation, the samples were centrifuged at 3000 rpm for 5 min at room temperature. The supernatant was removed and 250 µl PBS was added to the pellet. Then, the centrifugation was done again at the same rpm. To the pellet, 250 µl of muse cell cycle reagent was added after discarding the supernatant and incubated at dark for 30 min (light sensitive) followed by flow cytometry analysis. Gating was performed with reference to untreated control cells and samples were analyzed.

Scratch wound healing assay

Exponentially, growing cells were trypsinized and seeded at a density of 200,000 cells per well into 12-well plate for 24 h incubation (~90% confluence). The scratch wounds were made by a sterile 1 mL pipette tip through a premarked line. After removal of the resulting debris from five lineal scratches, the HT-29 cell monolayer was subsequently rinsed three times with PBS followed by incubation with sample for 24 h. The wound areas were displayed by taking images just above the interchanges between scratched wound areas and premarked lines and the effect of sample on wound closure was determined microscopically (×4 magnification, Olympus CKX41) after 24 h of incubation. The effect of sample on wound closure was captured using MRI-Image software.

Statistical analysis

All the experimental samples were done in triplicate to validate the test results. Statistical analyses were performed using latest SPSS version. The mean values of reading were obtained from the test replicate. The efficiency of the test sample was compared between treated and untreated HT-29 colorectal cancer cells by employing independent sample *t*-test and paired sample *t*-test. The *P* < 0.05 was considered as statistically significant.

RESULT

Measurement of cell viability

The experimental cells which showed the viability above 95% was carried out for further experiments.

3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay

Antiproliferative potential in terms of anticancer assessment was done on *F. hispida* for the cell viability against HT-29 cell line at varying concentration ranges of 0 µg, 15 µg, 31 µg, 62 µg, 125 µg, and 250 µg. The end result showed that HT-29 cell viability decreases in a concentration-dependent manner, and the growth inhibitory effect (IC₅₀) values are obtained at a concentration of 125 µg [Figure 1].

Apoptosis assessment

DNA-binding dyes acridine orange (AO) and ethidium bromide (EB) were used to test the ethanolic sample extract for the apoptosis induction ability by treating with HT-29 cells. The cells were divided into four categories as follows: live cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation), and necrotic cells (uniformly orange-stained cell nuclei). The test result showed the control cells and apoptotic cells as in Figure 2. The presence of necrotic cells was not observed in the experiment.

Flow cytometry analysis

The efficiency of the ethanolic leaf extract on the cell cycle progression through apoptotic induction was measured by flow cytometry after staining with PI. The result shows that 52.4%, 24.9% and 18% of the cells were in G₀/G₁, S and G₂/M phase of the cell cycle in the control cells. Flow cytometry DNA histogram had showed a peak at G₀/G₁ phase of the cell cycle. When we compared between control and IC₅₀ test sample group, we observed a statistical significance at G₀/G₁ and G₂/M phase of the cell cycle [Figure 3]. Thus, the result clearly indicates that the ethanolic leaf extract possess the inhibitory effect on cell cycle progression at G₀/G₁ phase and suggesting its interaction.

Wound scratch healing assay

HT-29 cell line was tested through the scratch assay to determine the capacity of these cells to migrate under phytoextract stimulus. The test drug at IC₅₀ value showed negligible difference in closure of wound area when compared between the control after 24 h of treatment time as shown in Figure 4.

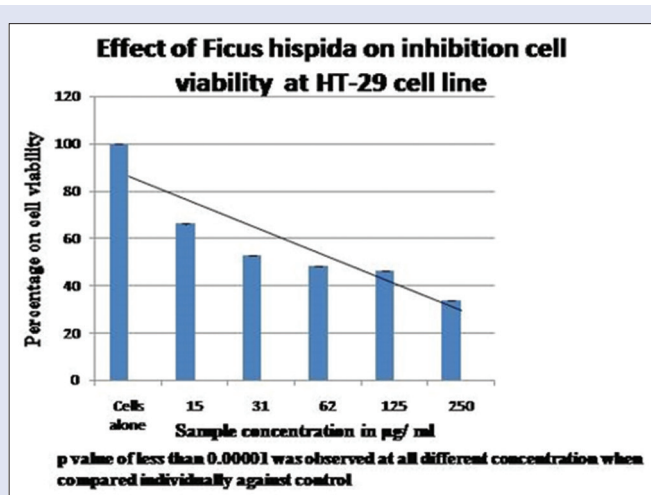


Figure 1: Cell viability percentage measured by 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay

DISCUSSION

Apoptosis, programmed cell death, and a well-structured physiological process to eliminate unregulated/abnormal or damaged cells remain as golden screening endpoint for discovering anticancer drugs.^[26] They are the common targets for currently clinically used and promising anticancer agents. It is more rational for the scientist to employ the screening method through apoptosis so that the agents that induce unspecific cytotoxicity can be excluded without wasting time and resources.^[27] Broad range of natural substance which has the potential to induce apoptosis for different tumor cell of origin has been documented.^[28,29] These natural substances of clinical values are often present in plant sources and consumed by humans in lesser quantities. Hence, it becomes essential to probe for the inducers of apoptosis from such plant resources.^[30]

Antioxidant available from nature possesses a broad range of biochemical functions including inhibiting reactive oxygen species, scavenging free radicals, and intracellular redox potential modifications.^[31]

Recently, Ali and Chaudhary^[32] have reviewed *F. hispida* based on its pharmacognostic and ethnomedicinal properties and concluded that further studies (preclinical and clinical) need to be performed to elucidate the maximum potential of this plant.

Pratumvinit *et al.*^[16] had extracted the methanol fractions from the dried stems of *F. hispida* L. and screened for anticancer activity (MTT assay with more dilution, colony forming assay, and cell cycle analysis) in four human breast cancers cell lines (SKBR3, MDA-MB435, MCF7, and T47D). The study had demonstrated the presence of cell line growth inhibition in dose-dependent manner, an emergence of apoptotic populations (G1 subset phase of the cell cycle) and suggested that *F. hispida* is beneficial in the treatment of breast cancer, especially in T47D. In similar, our study also confirms that the ethanolic leaf extract also provides anticancer activity against human colorectal cancer (HT-29 cell line) during the G0/G1 phase. In our study, we had used Muse Millipore Cell analyzer for assessing the cell cycle phase only using forward scattering measurement and their results are exported in that format. Hence, sub-G1 phase is not shown in standard format. The above similar study had also determined the IC50 value of the extract to be 110.3 µg/ml and in our study, we had observed to be at 125 µg/ml. The ideology behind the choice of using this *F. hispida* Linn. extracts against HT-29 cells would be the presence of hormone (estrogen or progesterone)-based target present in this cell line, but the exact mechanism of apoptosis still remains unclear.

Bhagat *et al.*, in the year 2010, investigated the leaf extraction process and assessed the *in vitro* cytotoxicity against oral (KB) and colon (COLO 205) human cancer cell lines. They had also proved that the growth inhibition demonstrated by all extracts and fractions were in dose-dependent manner and concluded that the *F. hispida* have ideal cytotoxicity against oral and human colon cancer cells; moreover, the active molecule are present in nonpolar nature.^[33] The above findings correlate with our findings regarding the growth inhibition.

Singh R *et al.* reported that the methanolic leaves extract of *F. hispida* in an artificially created wound in animals had caused excellent wound healing process activities and proposed that leaves are responsible for the

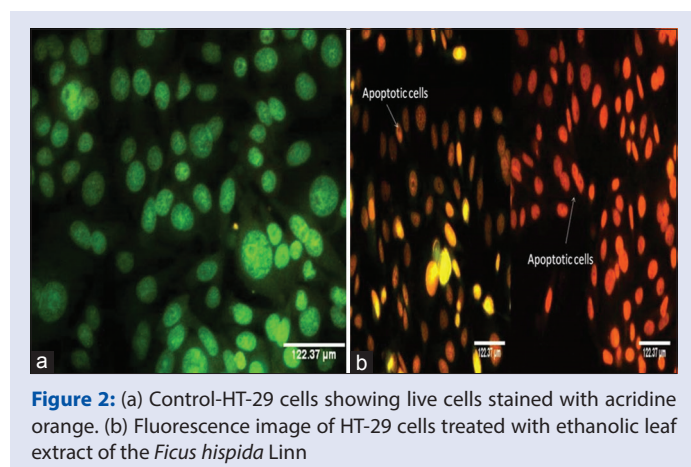


Figure 2: (a) Control-HT-29 cells showing live cells stained with acridine orange. (b) Fluorescence image of HT-29 cells treated with ethanolic leaf extract of the *Ficus hispida* Linn

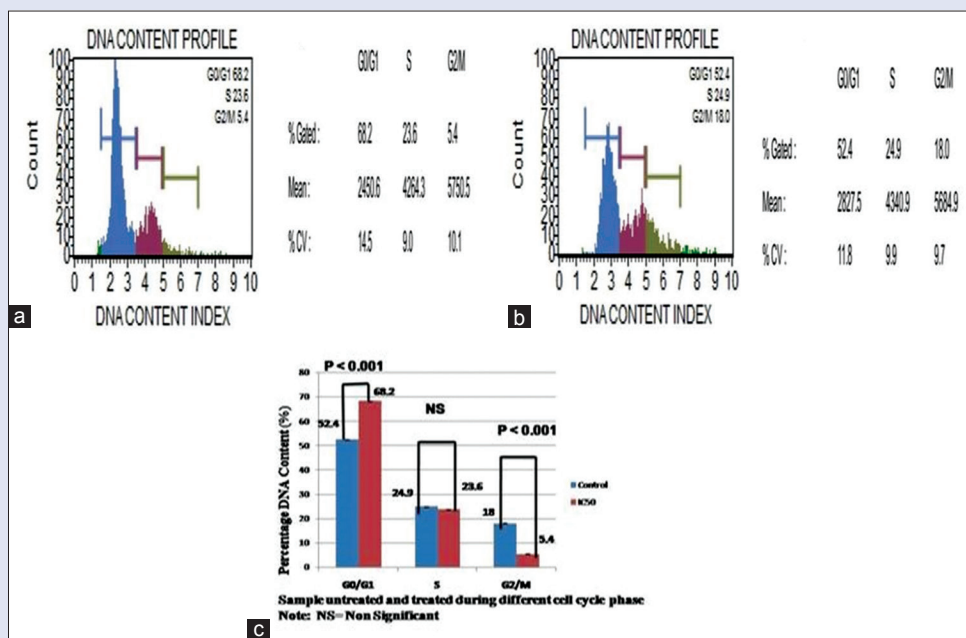


Figure 3: (a) Cell cycle analysis of untreated cells. (b) DNA Content profile during various stages of cell cycle in ethanolic leaf sample treatment against HT-29 cell lines. (c) Comparison of DNA content between control and treated HT29 cells



Figure 4: Wound closure after 24 h of treatment (control cells vs. treated cells)

upregulation of collagen in the wound site causing healing apart from other studies^[32,34] as in contrast to our study.

Abubakar *et al.*^[35] analysed the synergistic efficacy of antiproliferative effects caused by individual alkaloid extracts of *Ficus fistulosa*, *F. hispida*, and *Ficus schwarzii* combined with d- and g-tocotrienols against colorectal adenocarcinoma (HT-29) cells, lung adenocarcinoma (A549), and human brain glioblastoma (U87MG). This paper confirms that leaves of *F. hispida* provides a higher concentration of alkaloids compared to other species but both the bark and the leaf extract had less potency and cytotoxicity against HT-29 cells as contrast to our study.

CONCLUSION

This study demonstrates that *F. hispida* Linn. ethanolic extract alone induce growth inhibition effect on the HT-29 cell lines in a dose-dependent manner. Earlier reports had shown the cytotoxic efficiency of individual *F. hispida* Linn. to be low or excellent with the synergistic effect of another compound. Our study concludes that apoptosis plays role in *F. hispida* Linn. induced antineoplastic activities which might be due to the presence of effective phytoconstituents such as alkaloids, flavonoids, polyphenols, tannins, and saponins in the test drug which are normally extracted in higher quantities compared to other parts of the plant. In our study, we did not observe any wound healing assay positive but the past literature had shown positive results in animals. The limitation of the current study is that annexin V-FITC/PI flow cytometry assay was not performed to confirm the cell apoptosis as a additional confounding information due to economic constraint. Hence, further studies are required at preclinical and clinical level following a molecular investigation to explain the bioactive components and its associated mechanism that could trigger growth inhibition through this herbal extract.

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Conflicts of interest

There are no conflicts of interest.

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