Original Article

Evaluation of antimitotic activity of herbal extracts using plant-based model systems and their cytotoxic potential against human colon carcinoma cells

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ABSTRACT

Objective: The aim of this study was to screen plant extracts for antimitotic activity using *Vigna radiata* germination inhibition assay, followed by *Allium cepa* root tip assay and evaluation of their cytotoxic potential on colon carcinoma (HCT-116) cell lines.

Subjects and Methods: Aqueous extracts of *Aconitum heterophyllum*, *Terminalia bellirica*, *Bauhinia variegata*, *Vanda roxburghii,* and *Cassia angustifolia* were prepared by maceration method, and preliminary screening studies to check their antimitotic activity were done by *V. radiata* germination inhibition assay, followed by *A. cepa* root tip assay. Furthermore, cytotoxic actions were evaluated by cell proliferation assay. Effect of *T. bellirica* aqueous extract was analyzed to induce morphological changes, cell death, lactate dehydrogenase release, and cell survival of HCT-116 cells.

Statistical Analysis Used: The data represented were analyzed by Student's *t*-test using SigmaStat 2.0 statistical analysis software. The normality of data was tested by the Shapiro–Wilk test before the Student's *t*-test. *P* values $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ were considered as statistically significant.

Results: All the plant extracts showed promising antimitotic activity. Out of all, *T. bellirica* was highly effective on HCT-116 cells and promising effect on cell proliferation assay and Annexin-propidium iodide staining revealed that *T. bellirica* efficiently induces apoptosis.

Conclusions: *T. bellirica* inhibits cancer cell growth and induces apoptotic cell death. Collectively, it may hold potential for cancer therapeutics.

KEY WORDS: Antimitotic compounds, cancer, colon carcinoma, cytotoxicity, *Terminalia bellirica*, vincristine

INTRODUCTION

Cancer is one of the leading causes of death in both developed and developing countries and is, therefore, a serious concern worldwide. According to global cancer statistics released by the American Cancer Society, by 2050, 27 million new cancer cases and 17.5 million cancer deaths are projected to occur globally.[1] Accordingly, many efforts have been made to develop various approaches to reduce the threat caused by cancer. Conventional therapies including chemotherapy are most commonly utilized in modern cancer treatment. In addition, numerous clinically available anticancer drugs, of synthetic or natural product origin, are currently used to treat some types of leukemias, lymphomas, and solid tumors.[2,3] Despite marvelous achievements over the period of time to treat cancer, we have not been able to understand the etiology of cancer and provided poor palliation. Treatments such as chemotherapy, radiotherapy, and surgery provided limited relief for a short duration. All these instances indicate a need for alternative options for better management of cancer.[4]

Since ancient times, terrestrial plants have been used as a prominent source of medicines, and their early use in this regard in Ancient Egypt, India, China, and the Arab world has been well

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documented.[5] Previously, more than 3000 plant species have been reported for their usage to treat the various types of cancer.[6] A few years back, The Herbal Science Trust, Bengaluru, was able to resolve the problem of toxicity caused by the chemotherapeutic drugs using the formulation from *Asteracantha longifolia*. This formulation (patent no. GB 2454875 dated November 20, 2007) was successful for palliation in terminal cancer cases, and patients received a quality life before death.[7] Moreover, it must be considered that the diagnosis and prognosis of cancer are not always easy like common diseases, so claims of efficacy in treating cancer are mostly less convincing when plant-based traditional medicine is taken into consideration.[8] Due to the great variety of nontreatable cancers and their tendency to develop resistance against chemotherapeutics, there is a vital need to find new compounds and new lead structures for cancer therapeutics.[9]

Over the last decade, several novel and highly active natural products have been described that possess anticancer potential which includes the microtubule toxins such as the vinca alkaloids, Taxol, and other taxanes, all of which have proven successful in the anticancer treatment.[10,11] Taxol (paclitaxel) was originally isolated from the bark of the Pacific yew tree *Taxus brevifolia*. It stabilizes microtubules and dampens the dynamics of the polymer, thereby reducing depolymerization.[12] The vinca alkaloids were originally isolated from *Catharanthus roseus* (Madagascar periwinkle) as a class of compounds that interact with β-tubulin at a region adjacent to the GTP-binding site known as the vinca domain.[13] *Vigna radiata* (mung bean) germination assay; a primary screening method has been reported,[14] which is used for the evaluation of the cytotoxic effects of drugs or compounds. Another plant-based model system of *Allium cepa bio*assays[15] has also been proven to be a rapid, reliable, and inexpensive system by which the antimitotic effects of various cytostatic, cytotoxic, and mutagenic properties of different compounds, including anticancer drugs of plant origin, may be screened.[16-18] The results obtained by the *Allium* test could be useful in correlating the antimitotic effect of herb extracts as it is reported that the *Allium* test shows a good correlation with mammalian test systems.[19]

In the present study, we have screened various aqueous plant extracts for their antimitotic activity by *V. radiata* germination inhibition assay and *A. cepa* root tip assay, followed by evaluation of their anticancer potential using human colon carcinoma cells (HCT-116). Colon cancer is associated with a high mortality rate and poor prognosis worldwide. A number of therapeutic approaches were designed to combat colorectal cancer. Certainly, conventional cancer therapies lead to systemic toxicities and drug resistance. This obstacle advocates the need for the synthesis of novel compounds that restrain enormous anticancer potential. Plant extract contains a vast array of naturally occurring agents which may be the source for the novel anticancer drug. We have screened more than fifty herbal aqueous extracts using plant-based model systems including *V. radiata* germination inhibition assay and *A. cepa* root tip assay (data not shown) out of which five extracts we found more promising and we checked their anticancer potential on human colorectal carcinoma cells (HCT-116).

SUBJECTS AND METHODS

Commercially available chemicals of molecular biology grade were purchased. Roswell Park Memorial Institute (RPMI)-1640, Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin–streptomycin–neomycin (PSN) antibiotic mixture, Annexin V-FITC, and propidium iodide (PI) was purchased from Life Technologies, Invitrogen (USA). Cell culture-grade dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypan blue were purchased from Sigma-Aldrich (USA). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

Collection and selection of plant material

Lyophilized powder of parts of plant, i.e., root, stem, leaf, seed, bark, and fruit, of various herbs, plants, and trees of India was purchased from a certified dealer.

Preparation of aqueous extracts and screening of the antimitotic activity by *Vigna radiata* germination assay

100 mg/mL extracts were prepared by adding 5 g of lyophilized plant powder in water to make a final volume of 50 ml. These extracts were boiled in a microwave oven for 3 min and, after cooling, centrifuged at 10,000 rpm for 15 min. The clear supernatant was used for the assay. 0.5 ml of 100 mg/mL aqueous extracts was placed in a well of a microtiter plate and followed the modified method by Satyanarayan *et al*. We had measured the sprout length for the calculation of percentage inhibition. Vincristine, a known antimitotic drug, available as Cytocristine (brand name) by Cipla pharmaceuticals was used as a positive control (200 μg/ml) and water was used as a negative control for the assay.

Preparation of aqueous extracts and *Allium cepa* root tip assay

Lyophilized 1.6 g plant materials were used to prepare 40 ml of aqueous extract to make the final concentration of 40 mg/ml. These extracts are boiled in a microwave oven for 3 min and centrifuged at 10,000 rpm for 15 min. The clear supernatant is used for the assay.

Staining and slide preparation

The *Allium* test was performed according to the method described by Fiskesj¨o, 1988. The *A. cepa* were grown in water under laboratory conditions (light, 24°C). Onions were grown in water for 72 h, and when they develop roots of approximately 5 cm of length, they were incubated in 40 mg/mL aqueous plant extracts for 24 h. We have used four to six root tips from the analogous onions from each

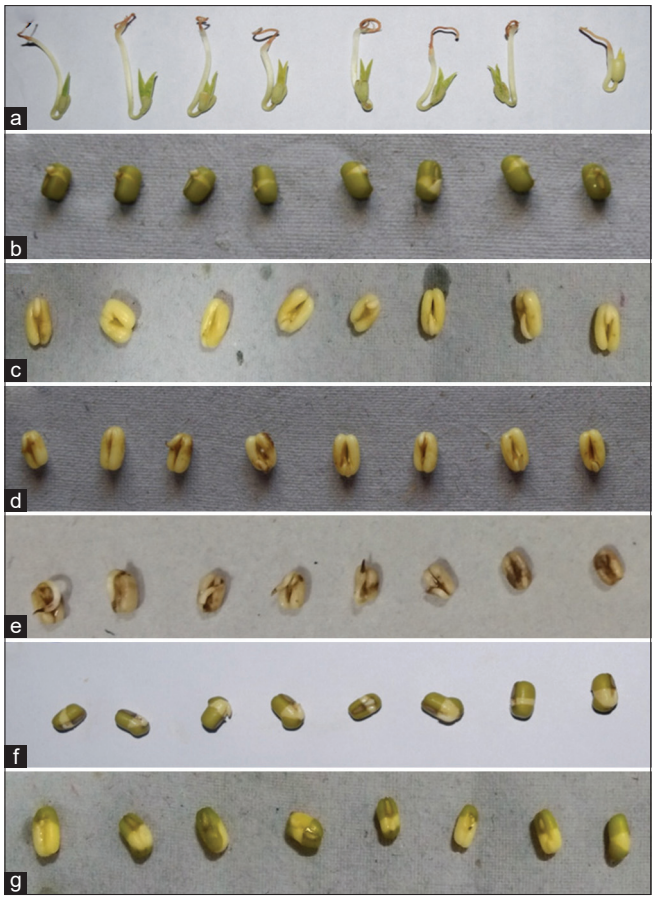

Figure 1: *Vigna radiata* germination inhibition assay: Images of seeds (n = 8) incubated for 72 h. Healthy sprouts of *Vigna radiata* were observed in negative control. (a) Seed germination is inhibited in positive control by vincristine 200μg/ml. (b) Inhibition of germination by the extracts of *Aconitum heterophyllum*, *Terminalia bellirica*, *Bauhinia variegata*, *Vanda roxburghii,* and *Cassia angustifolia* (c-g)

concentration and were fixed using Carnoy's fixative, macerated using HCl, and stained with acetocarmine stain; then, root tips were squashed in drop of 45% acetic acid on a glass slide and observed under the EVOS microscope (×65 magnification) having camera attachment, and photographs of several fields were captured. Chromosome morphology and their changes were observed, and the mitotic index was calculated using the following formula.

$$\text{Mitotic index}\,(\%) = \frac{\text{No. of dividing cells} \times 100}{\text{Total no. of cells}}$$

Cell lines and cell culture

Human colorectal carcinoma (HCT-116) cell line was obtained from National Center for Cell Science, Pune, Maharashtra, India. HCT-116 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% PSN, an antibiotic mixture (Life Technologies, USA). The cells were cultured in a humidified condition of 5% CO_2 at 37°C. The entire study was carried out on exponentially growing cells.

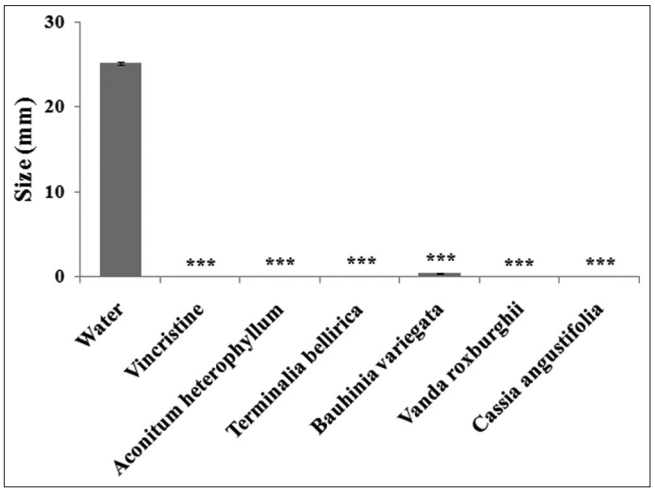

Figure 2: Comparison of the potential of germination inhibition: Compared to positive control, all the aqueous extracts had shown the significant (***$P \leq 0.001$) reduction in seed germination indicative of cytotoxic/antimitotic potential of plant extracts

Treatment of extracts

All the extracts were freshly prepared in cell culture-grade DMSO. Exponentially growing HCT-116 cells were exposed to *Terminalia bellirica* (1:250 and 1:100) or vehicle control (DMSO) for 24 h.

Morphological analysis

The change in cellular morphology of HCT-116 cells was observed by bright-field microscopy. Briefly, HCT-116 cells were treated with *T. bellirica* (1:500, 1:250, and 1:100) or vehicle control (DMSO) for 24 h. The cells were washed with DPBS, and more than 150 cells from three different fields were observed under a bright-field microscope (DP-71, IX81, Olympus, Japan).

Cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay)

The inhibitory effect of isolated extracts on cell proliferation was determined by MTT assay. Briefly, HCT-116 cells were treated with extracts (1:500, 1:250, and 1:100) or DMSO (vehicle control) for 24 h, and inhibition in cell proliferation was determined.[20]

Cell viability assay

Cell death was evaluated by dye exclusion assay using trypan blue. HCT-116 cells were treated with *T. bellirica,* as described earlier. The treated cells were harvested and washed with DPBS. 10 μl of cell suspension was mixed with 10 μl of trypan blue. Subsequently, the dead cells and total cells were counted. The percentage of cell death was determined by the following formula (percentage of cell death = number of dead cells/total number of cells ×100).

Cell survival assay

The survival ability of cells was monitored by cell survival assay using crystal violet stain.[20] HCT-116 cells were treated

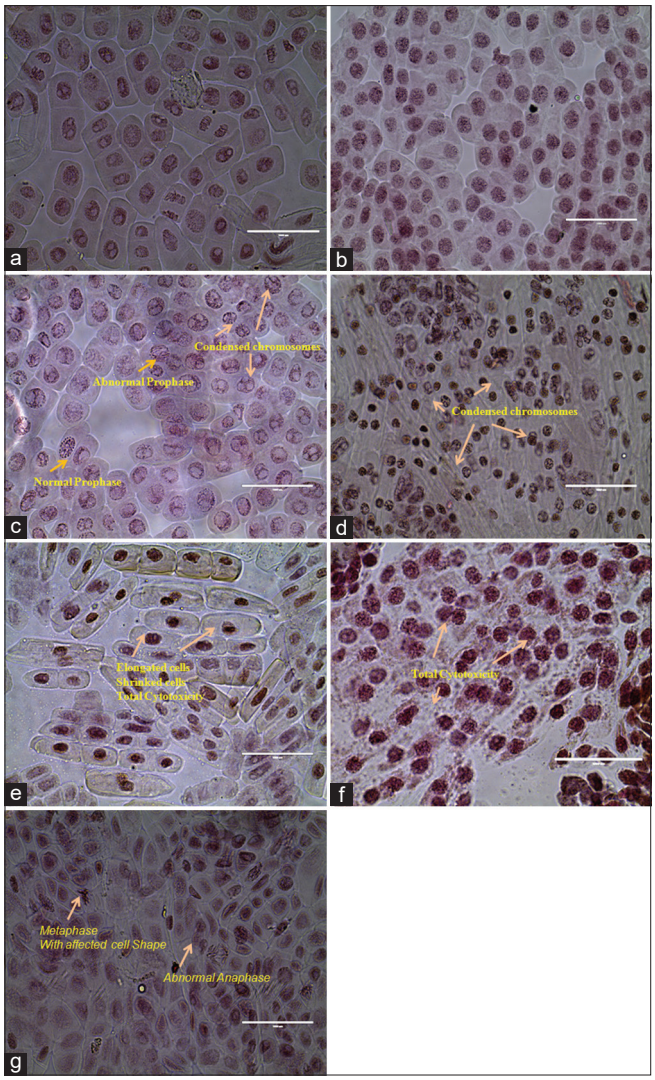

Figure 3: Microscopic images of *Allium cepa* root tip assay: Normal well-defined cell division in *Allium* root tip meristematic cells were observed. (a) Antimitotic effect in vincristine-treated tips indicated by arrested prophase. (b) *Aconitum heterophyllum*-treated tips shown cytotoxicity. (c) *Terminalia bellirica*-treated cells shown condensed chromosomes lead to failure in the cell division. (d) *Bauhinia variegata*-treated meristematic cells. (e) *Vanda roxburghii*-treated cells (f) and *Cassia angustifolia*-treated cells (g)

with *T. bellirica* (1:250 and 1:100) for 24 h. Thereafter, the cells were harvested, washed, and resuspended into the culture medium. One thousand cells were seeded in 6-well plates and incubated for 10 days until the visible colonies were found. The colonies were fixed with 100% methanol and subsequently stained with 0.4% crystal violet. Excessive stain was washed. The plates were scanned, and the colonies were counted. The percentage of plating efficiency (PE) was counted using the formula: Percentage PE = (number of colonies formed/number of cells seeded) ×100.

Apoptosis assay

The apoptotic cell death was determined by Annexin V/PI staining.[21] In brief, 1 × 10^5 HCT-116 cells were seeded on

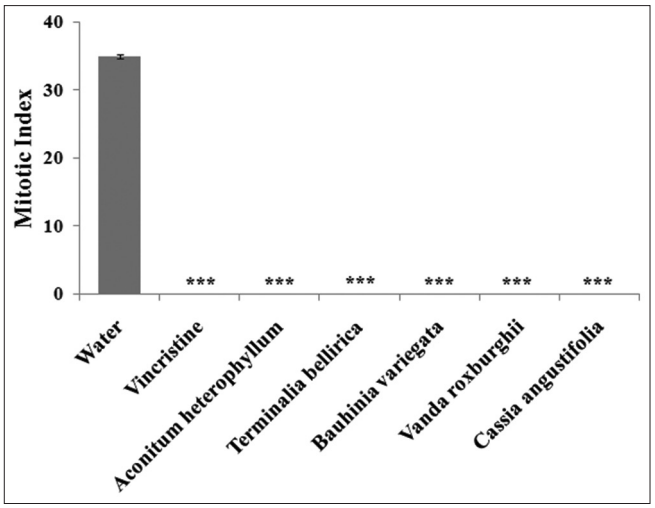

Figure 4: Evaluation of antimitotic potential by mitotic index: Comparison of the mitotic index with positive and negative control to various plant extracts shown a significant decrease in the values of mitotic index (***$P \leq 0.001$)

coverslips and treated with *T. bellirica* for 24 h. Thereafter, the cells were stained with 5 µl of Annexin V-FITC for 20 min in dark and subsequently 1 µl of PI for 5 min in dark. The stained cells were observed under a fluorescent microscope. More than 100 cells from three random fields were examined for the apoptotic cell death. The images were acquired and analyzed by standard method.[21]

Statistical analysis

The data represented were analyzed by Student's *t*-test using SigmaStat 2.0 statistical analysis software. The normality of data was tested by the Shapiro–Wilk test before the Student's *t*-test. *P* values *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ were considered as statistically significant.

RESULTS

Antimitotic activity of selected plant extracts using plant-based model systems

We had screened aqueous extracts from more than fifty plants (unpublished data) using *V. radiata* seed germination inhibition assay, out of which *Aconitum heterophyllum*, *T. bellirica*, *Bauhinia variegata*, *Vanda roxburghii* and *Cassia angustifolia* had shown maximum inhibition of germination. These findings clearly indicated the antimitotic potential of the selected plant extracts as they were showing significant inhibition of germination comparable to the positive control [Figures 1 and 2].

The plants which had shown maximum inhibition in the *V. radiata* germination inhibition assay were also tested for their antimitotic activity by A. *cepa* root tip assay. Actively dividing cells of roots of *A. cepa* (showing different stages of cell divisions, i.e., prophase, metaphase, anaphase, and telophase) and nondividing healthy cells without chromosomal aberrations

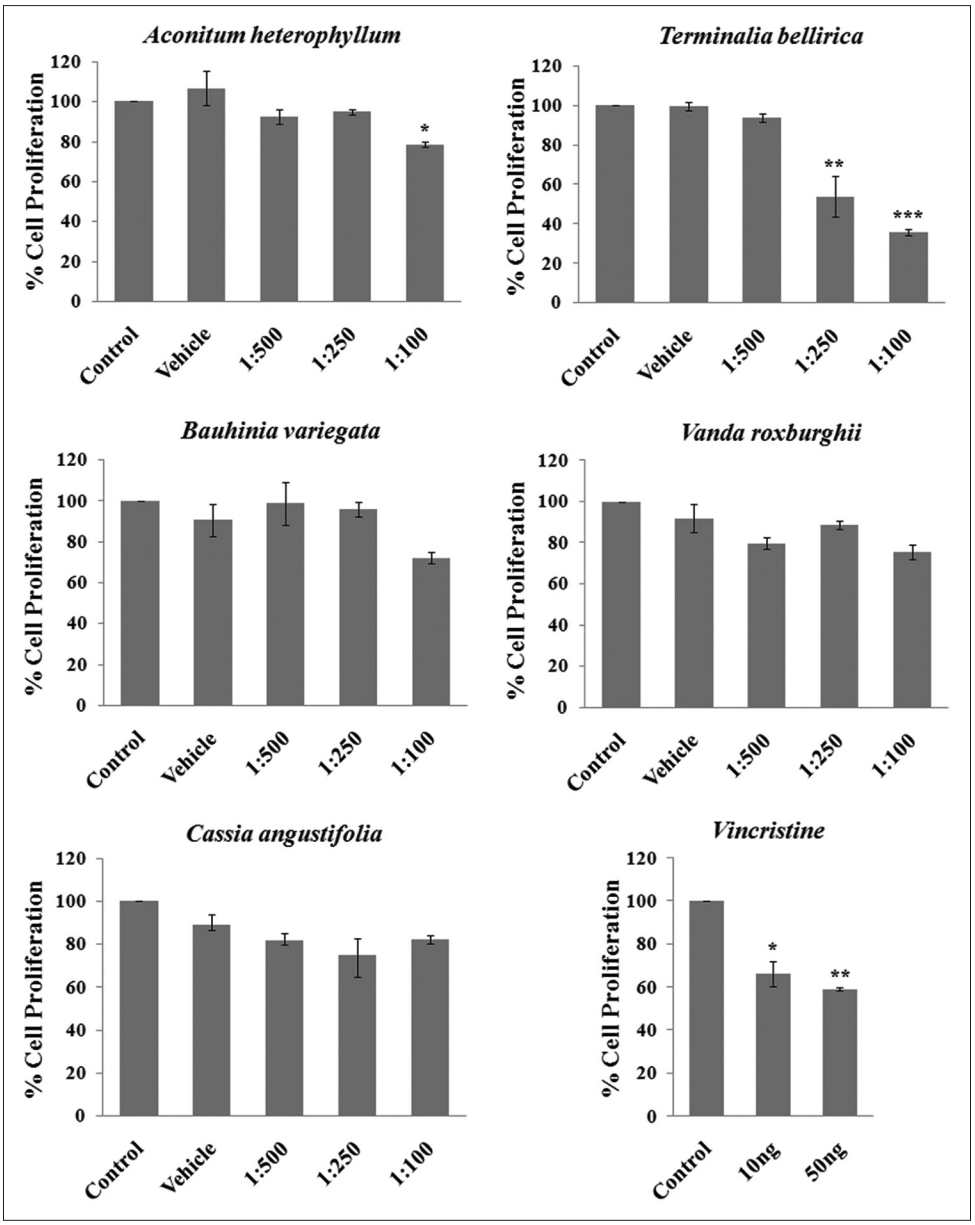

Figure 5: Antiproliferative effect of various extracts in HCT-116 cells. The antiproliferative effect of various extracts was determined by the MTT assay. HCT-116 cells were treated with various dilutions of extracts 1–5 (1:500, 1:250, and 1:100) for 24 h. One percent dimethyl sulfoxide was used as vehicle control. The percentage of cell proliferation was determined by MTT assay. Error bars correspond to the mean ± standard error of the mean of three independent experiments. Significant difference indicated as $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ between untreated and treated cells (Student's *t*-test)

were observed with mitotic index 34.9 ± 0.96 in the negative control (water) group. We observed poor mitotic index, which was close to zero in vincristine-treated root tips as it is known to affect the actively dividing cells and as expected because of its antimitotic activity. Majority of the cells were unable to complete cell division and shown abnormalities, namely disrupted cells, aggregated chromosomes, and binucleated cells with no dividing cells [Figure 3]. Aqueous extracts of *A. heterophyllum*, *T. bellirica*, *B. variegata*, *V. roxburghii,* and *C. angustifolia* had shown significant reduction ($P \leq 0.001$) in mitotic index [Figure 4].

Microscopic images of the *Allium* test [Figure 3b-g] have shown the cells that were arrested in the prophase and found some common abnormalities such as irregular cells, binucleated cells, aggregated chromosomes, and disrupted cells. These findings indicated the cytotoxic effect of these plant extracts on the actively dividing root cells.

Primary screening methods have clearly shown antimitotic activity in these extracts which made us extend our studies to check the potential of these extracts on human colon carcinoma HCT-116.

Terminalia bellirica inhibits cell proliferation and cell survival

The cell proliferation was evaluated by MTT assay after 24 h of treatment on HCT-116 cells. MTT assay showed that the *T. bellirica* efficiently inhibits cell proliferation of HCT-116 cells as compared to the other extracts [Figure 5]. Based on these results, we selected *T. bellirica* for further experiments. First, we analyzed the morphological changes by microscopic analysis and found that the cells treated with *T. bellirica* (1:500, 1:250, and 1:100) exhibit considerable morphological changes [Figure 6a]. Next, cell death was confirmed by trypan blue dye exclusion assay. The results revealed that *T. bellirica* (1:500, 1:250, and 1:100) exhibits a higher percentage of cell death [Figure 6b]. The lactate dehydrogenase (LDH) release is a common phenomenon of necrotic cell death. Further, we quantified the release of LDH after the treatment with *T. bellirica*. We found that LDH was not released upon the treatment of *T. bellirica* in HCT-116 cells [Figure 6c]. Therefore, the LDH assay confirms that *T. bellirica* does not execute necrotic cell death. To confirm the mode of cell death, we performed Annexin V/PI staining. We observed prominent Annexin V and PI-positive cells after the treatment of *T. bellirica*, which corroborated cell death from apoptosis [Figure 7]. Moreover, we evaluated the survival potential of HCT-116 cells upon the treatment of *T. bellirica* by cell survival assay. Our results revealed that *T. bellirica* efficiently reduces the ability of survival and colony formation ability of HCT-116 cells [Figure 8a and b]. Collectively, these results suggest that the *T. bellirica* significantly restricts cell proliferation and induces apoptosis in HCT-116 cells. Thus, the *T. bellirica* may hold the potential to target cancer cells and could be an efficient candidate for further studies.

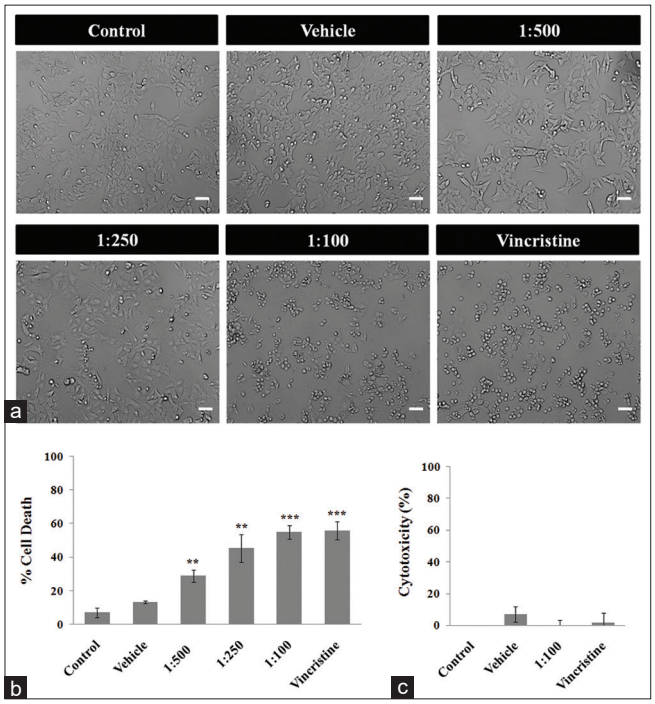

Figure 6: Effects of *Terminalia bellirica* on cell death of HCT-116 cells. Cells were treated with extract of *Terminalia bellirica* as described earlier. (a) Morphological changes upon treatment were analyzed by bright-field microscopy. (b) Percentage of cell death was determined by trypan blue dye exclusion assay. (c) Percentage of cytotoxicity as determined by lactate dehydrogenase assay. Error bars correspond to the mean ± standard error of the mean of three independent experiments. Significant difference indicated as $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ between untreated and treated cells; (Student's *t*-test). Scale bar represents 20 μm

DISCUSSION

Aqueous extracts of *A. heterophyllum* (Ativisa), fruit of *T. bellirica* (Baheda Chhal), bark of *B. variegata* (Kachnar), aerial roots of *V. roxburghii (Syn.-Vanda tessellate,* Rasna Pan), and leaves of *C. angustifolia* (Sonamukhi) plants had shown total inhibition of seed germination in *V. radiata* assay. These findings were further supported by a drastic reduction of mitotic index in *A. cepa* root tip assay. These results had clearly shown that the abovementioned plant extracts had an excellent antimitotic activity which led us to evaluate their anticancer activity using human cell lines.

In order to evaluate the anticancer efficiency of extracts prepared from *A. heterophyllum*, *T. bellirica*, *B. variegata*, *V. roxburghii (Syn.-Vanda tessellate*), and *C. angustifolia*, we first determined the antiproliferative efficacy of extract on HCT-116 cells. The antiproliferative activity was evaluated by MTT assay, and our result showed that the *T. bellirica* was highly effective on the HCT-116 cell line. Based on the results of the MTT assay, we selected the *T. bellirica* for further experiments. Morphological analysis indicates that the *T. bellirica* induces significant changes in the morphology of HCT-116 cells.

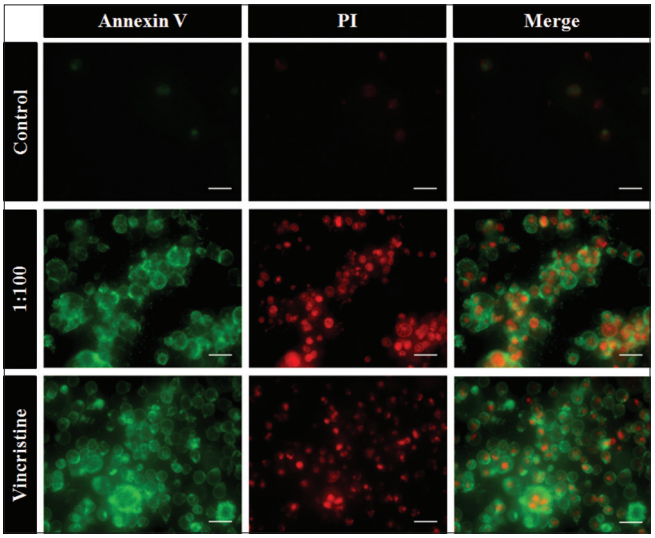

Figure 7: Apoptotic potential of *Terminalia bellirica* on HCT-116 cells. Determination of apoptosis by fluorescent microscopy. HCT-116 cells were treated with extract 2 for 24 h. Similarly, 1% dimethyl sulfoxide was used as vehicle control. The cells were stained with Annexin V-FITC for 20 min and subsequently propidium iodide for 5 min. Annexin V and propidium iodide-stained cells were observed under a fluorescent microscope. Scale bar represents 20 μm

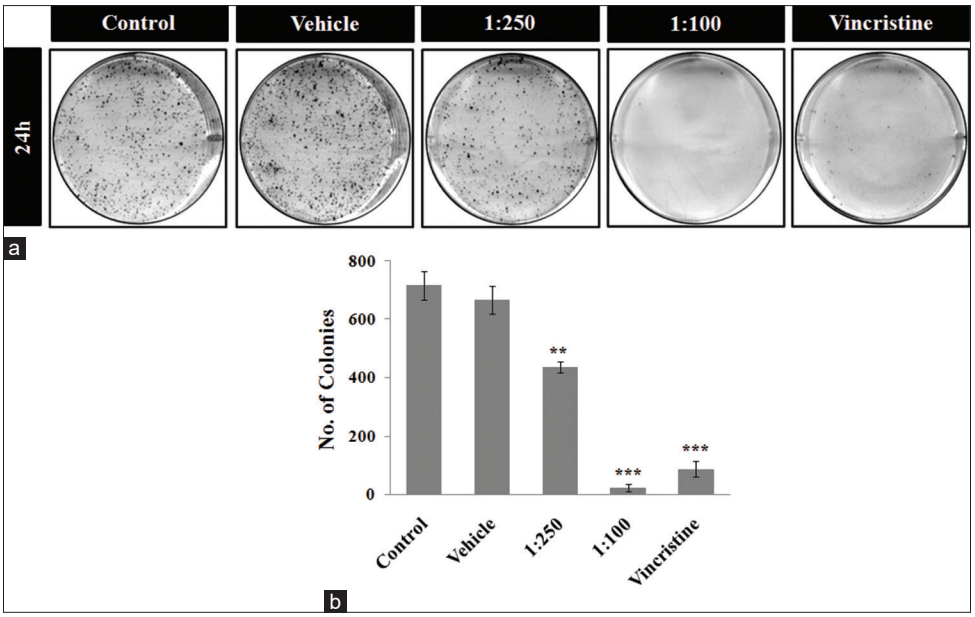

Figure 8: Effects of *Terminalia bellirica* on clonogenic ability of HCT-116 cells. (a) The clonogenic ability of HCT-116 cells upon treatment was determined by crystal violet staining. Qualitatively analysis, images represent the stained colonies of the survived cells. (b) Quantitative analysis, relative plating efficiency was determined by counting the colonies and subsequent calculation using the standard formula, percentage of plating efficiency = (number of colonies formed/number of cells seeded) ×100. Significant difference indicated as $^{**}P \leq 0.01$, $^{***}P \leq 0.01$ between untreated and treated cells (Student's *t*-test)

Furthermore, the cell death determination by trypan blue assay indicates that *T. bellirica* potentially exhibits higher cell death in a dose-dependent manner. As the *T. bellirica* was able to induce massive cell death, we further wanted to explore the mode of cell death. For the same, we performed LDH assay, which indicates the level of LDH released during cell death and ultimately its cytotoxic effects. Our results of the LDH assay showed a very minute amount of LDH released upon the treatment of *T. bellirica*. These indicate that *T. bellirica* may not induce necrotic cell death. In order to confirm the mode of cell death, we performed the annexin V-FITC/PI staining. Our result shows prominent annexin V-FITC/PI staining in treated cells which confirms apoptotic cell death. Moreover, we also analyzed the ability of cells to survive after the treatment of *T. bellirica* by cell survival assay. We found that the clonogenic ability of cells treated with *T. bellirica* reduces after the treatment.

In India, *T. bellirica* is also known as Vibhitaki, Karshaphala, and Kalidruma in Sanskrit. *T. bellirica* has diverse applications such as a laxative, astringent, antihelminthic, and antipyretic useful in hepatitis, bronchitis, asthma, dyspepsia, piles, diarrhea, and coughs.[22] *T. bellirica* is also one of the three constituents of Triphala Choorna, which is claimed to be highly beneficial for the treatment of various digestive tract problems in traditional ayurvedic medicines. Triphala Choorna is administered orally simply along with drinking water, and our study also indicated the potential application of an aqueous extract of *T. bellirica* in the prevention of human colon carcinoma which supports the traditional practice of ayurvedic medicine and deserves detailed studies for the development of efficient therapeutics.

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Conflicts of interest

There are no conflicts of interest.

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