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STUDIES ON THE *IN VITRO* ANTICANCER ACTIVITY OF *TABERNAEMONTANA DIVARICATA* EXTRACT AGAINST COLON CANCER CELL LINE

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ABSTRACT

Cancer is considered to be one of the leading causes of morbidity and mortality worldwide. For the past years, plants were known to possess metastatic tumor activities against numerous neoplastic cell lines. Also, nature merchandise represents a reservoir of various templates and it is being tapped to source novel metastatic tumour agents. *Tabernaemontana divaricata* has been employed in Ayurvedic formulations in recent days. Leaves and flowers of *T. divaricata* are employed in folk medicines by the Ayurvedic practitioners. It is a native of India and also different tropical regions. It may be a common plant that has been used historically for the treatment of various diseases. Also, the plant has been reported to be helpful for the treatment of cuts, inflammation, eye and skin diseases, wounds, diarrhea, ulcer and cancers. The aim of this study is to investigate the *in vitro* metastatic antitumour activity of methanolic extract on *T.divaricata* against HT-29 human carcinoma cell line. The methanolic extracts of the leaves and flowers of *T.divaricata* were examined for its effect on HT-29 cell line. It was administered by exploitation of Tryphan blue exclusion technique. The toxicity of *T.divaricata* against HT-29 cell was evaluated by MTT assay. The cell morphology of drug treated HT-29 cell line was studied by nuclear staining technique. From the performed assays, methanolic extract of those drugs show more anticancer activity on colon cell line (HT-29).

KEYWORDS: ***Tabernaemontana divaricata, Tryphan blue exclusion technique, anticancer activity***

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INTRODUCTION

Our body cells grow, divide and die in an orderly manner. Throughout the first year of an individual's life, traditional cells divide quicker to permit a person to grow. When the person becomes an adult, most cells divide solely to exchange the worn-out, broken or dying cells. The genetic material (DNA) of a cell will be broken or modified, manufacturing mutations that have an effect on traditional cell growth and division. Once this happens, the cells do not die when they ought to die and get worn out. The additional cells might form a mass of tissue that is referred to as a tumor. Cancer could be a term used for diseases within which abnormal cells divide uncontrollably and unpredictably and square measure ready to invade different tissues.[1] The worldwide statistics of cancer incidences has been menacing within the past few decades.[2]

Colorectal cancer

Large intestine cancer, which is the third most often diagnosed cancer (only after respiratory organ cancer and skin cancer) in both men and women. But, the incidences still grow alarmingly.[3] In 2010, the calculable range of the latest cases in US with carcinoma and body part cancer is about 102,900 and 39,670, adding to a completion of 142,570 new cases of large intestine cancer. The internal organ cancer and colon cancer in urban areas are more prevalent in men and exaggerated incidences of cancers of the cervix, gall bladder and colon are more prevalent in women. The typical sites of incidences of colon cancer in humans have been illustrated in Figure 1.

Typical sites of incidences of colon cancer

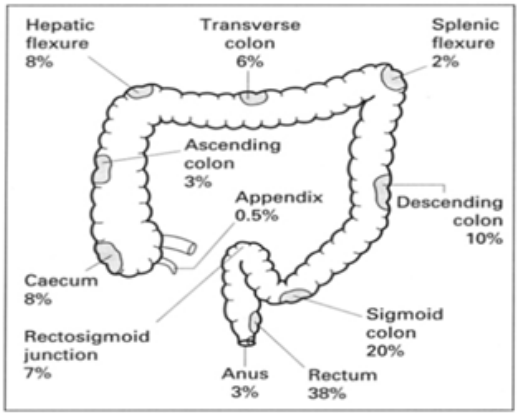

Figure 1
Schematic illustration depicting typical sites of incidences of colon cancer in humans

Causes and risk factors of colon cancer

A risk factor is anything that affects the chance of getting a disease such as cancer. Researchers have found several risk factors that might increase a person's chance of developing colorectal polyps or colorectal cancer. The risk factors include

- Being overweight or obese
- Physical inactivity
- Certain types of diet
- Smoking
- Heavy alcohol use

Medicinal plants

Plants occupy a novel position on this planet since they are the inspiration for life in the world. Researchers and scientists have identified nearly 122 compounds which are used in modern medicine that were derived from plant sources. More than 80% of the medicines used today, have had an identity and related use of the active elements of the plants.[4] Some of the pharmaceutical compounds currently available to the modern day physicians are derived from medicinal plants that have a very long history of use as an active herbal remedies.[5] *Tabernaemontana divaricata* which is classified underneath the dogbane family is a decorative, flowering, evergreen bush that typically grows to an approximate height of six feet and it comes underneath the *Tabernaemontana* that consist of 100 - 110 species of flowering plants. It is a standard plant found in tropical countries, including Brazil, Egypt, India, Sri Lanka, Vietnam, Malaysia and Asian country. The flowers are white and sweetly fragrant. The leaves, flowers, roots and stem of the plant have potent medicinal compounds and are used, historically for the treatment of ulcers and rheumatism. Alternative medicinal properties of the plant embody anixolytic, antidiabetic and anticonvulsant activities.

MATERIALS AND METHODS

Preparation of extract

Leaves and flowers of *T. Divaricate* was collected from Erode, Tamilnadu in September, 2013. The leaves and flowers of *T. Divaricate* was identified and authenticated by Dr. P. Jayaraman, Director, National Institute of Herbal Science, Chennai, India. They were thoroughly washed with fresh water, shade dried and ground to a fine powder, in a mixer. Extraction of the powder (70 g) was done by Soxhlet apparatus by using solvent methanol (200 ml) for 72 hrs. The extract was evaporated to dryness by hot water bath and centrifuged at 4000 rpm at 20°C for 10 min and the superanant was collected. Then it was reconstituted in distilled water and stored at -20°C. The frozen extracts were thawed until they reached liquid state.[6,7,8,9]

Cell culture

Human colon cancer cell line (HT-29) was obtained from AIIMS, New Delhi, India. HT-29 cancer cell line was cultured in McCoy's 5A Medium. The culture medium was supplemented with 10% fetal bovine serum (FBS), 1% antibiotic and antimycotic solution (50 mg/L of streptomycin and 50,000 units/L of penicillin) and 2mM glutamine. Cultures were held in 25 cm culture flasks at 37°C, 5% CO_2 and 95% relative humidity.[10]

Trypan blue dye exclusion assay

A cell suspension in a fastened volume of cells (1ml) was first prepared initially. Although the associate antiseptic technique is not essential, throughout the various stages of this procedure, it is a good laboratory procedure to observe and keep sterility throughout the procedure. The cells were treated with various concentrations, 10-100 µg/ml of leaves and flower extract for 48 hrs. Then 50µL of cell suspension was taken and mixed with the same volume of Trypan blue.[11,12,13] The solution was mixed well using a pipette. Using a measuring device, the mixture of cell suspension and Trypan blue was placed into the haemocytometer and overlayed with a cover slip. The suspension of cells would pass beneath the cover slip by capillary action unless there is an associated bubble. The wells should not be overfilled to the extent that the cover slip is affected once it is placed on the grid. And the cell resolution is another important factor that must be taken care of. The haemocytometer was placed on the stage of an inverted magnifier. The focus and power of the microscope were adjusted till a single count square fills the sector. The number of cells per ml, and the total number of cells were calculated. The percent viability was calculated by the following formula.

% viability = (live cell count/total cell count) X 100

Cytotoxicity assay

Cytotoxicity of sample of tumor cells w as measured by microculture tetrazolium (MTT) assay.[14,15] For the assays, 96-Well microplates were seeded with 100 µl medium containing 5000 cells. After 48 hours incubation and attachment, the cells were treated with 6 fourfold dilution of crude extracts. From the stock solution (40 mg/ml), each extract sample was applied in a series of half-dozen dilutions (final concentrations starting from 15 to 500 µg/ml) with a final DMSO concentration of 0.1% and was tested in quadruplicate. Medium was aspirated and fresh medium was added to the cells with various concentrations of plant extracts (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml) and incubated for 48 hours. After 48 hours incubation, cell viability was determined by adding (Sigma) tetrazolim salt as cytotoxicity indicator; 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to every well and incubated at 37^0C for 4 hours. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO. Then the absorbance was measured at 570 nm with ELISA small plate reader.[16,17,18,19,20] Tetrazolium salts were cleaved to formazan dye by cellular enzymes (only in the viable cells). The % cell inhibition was calculated by the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

The Inhibitory concentration required for 50% cytotoxicity (IC50) value was analyzed using Sigmaplot software.

Hoechst 33342 staining

Chromatin condensation was studied by nuclear staining with Hoechst 33342. After treatment with varied concentration of extracts for 48 hours, cells were harvested and washed with PBS thrice. Then the cells were stained with 1 µl of Hoechst 33342 (5 mg/ml; Sigma) in 1 ml basal medium and incubated at room temperature in the dark for 15 minutes. Stained cells were imaged under a fluorescent magnifier by mistreatment 350 nm stimulation and 460 nm emissions.[21]

Dna fragmentation assay

The methanolic extract which is cultured with the HT-29 cell lines was passed to deoxyribonucleic acid fragmentation technique.[22 ,23] A distinctive feature of apoptosis at the organic chemistry level is deoxyribonucleic acid fragmentation. This methodology was used as a semi-quantitative method for measuring apoptosis. The culture medium was removed and centrifuged at 3000 x g for 5 min to gather detached cells. 2 cm^3 of cells that was centrifuged at 3000rpm was suspended in 20 µL of 1X TE Buffer and 100 µL of 100 % SDS and then it was incubated at 60ºC for 20 min; added 300 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1); mixed well, then centrifuged at 10,000 rpm for 10 min. To the supernatant added 500 µL of isopropylalcohol; added 200 µL of 70% plant extract; then centrifuged at 10,000 rpm for 10 minutes. The pellet was dried at 37^0C till there were no traces of the solution. The pellet was resuspended in 20 µL of 1xTE Buffer. Electrophoresis was performed to extract deoxyribonucleic acid on agarose gel. Agarose gel electrophoresis was carried out. For casting 1% Agarose gel, 0.8 gm of agarose was added in 80 cm^3 of diluted 1X TBE buffer. Allowed the gel to solidify; transferred the gel to 1X TBE buffer filled action tank. Added 2 µL of gel loading dye to 20 µL of sample deoxyribonucleic acid, mixed well, and then loaded the entire 22 µL of sample to gel. Connected the power cord terminals at respective positions and run the gel at 50 V until the gel loading dye migrates to more than half the length of gel. The separated sample DNA was visualized with MW marker under ultraviolet illumination Transilluminator.

STATISTICAL ANALYSIS

All the results were analyzed by One-way Analysis of Variance (ANOVA). The level of significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Tryphan blue exclusion assay

Cell viability was calculated before the experiments. The live cells excluding tryphan blue and non –viable cells

(stained cells) were counted using the haemocytometer. Cell viability results by the methanolic extract of leaves and flowers against HT- 29 for various concentrations for 48 hrs were studied. As the concentration increases, there is a decrease in the cell viability. At the concentration of 100µg/ml, in both leaves and flower extracts, 0% viability was obtained.

Mtt assay

Sensitivity of HT-29 cell line to *T.divaricata* was determined by the MTT colorimetric assay. [24,25] The inhibitory activities of these extracts were compared with standard Triton-X for HT-29 cell line. The percentage cancer cell inhibition profiles were observed to be concentration dependent which is shown in Figure 2 and 3. The maximum concentration (µg/ml) used in the study was 100µg/ml. Therefore, the *in vitro* screening of the methanolic extracts of leaves and flowers showed potent cytotoxic activity against colon cancer cell line. In this study, it was observed that the methanolic extracts of the leaves and flowers of T.*divaticata* induce a concentration dependent inhibition of HT-29 cells, with an IC 50 value of 25µg/ml after 48 hrs of incubation.

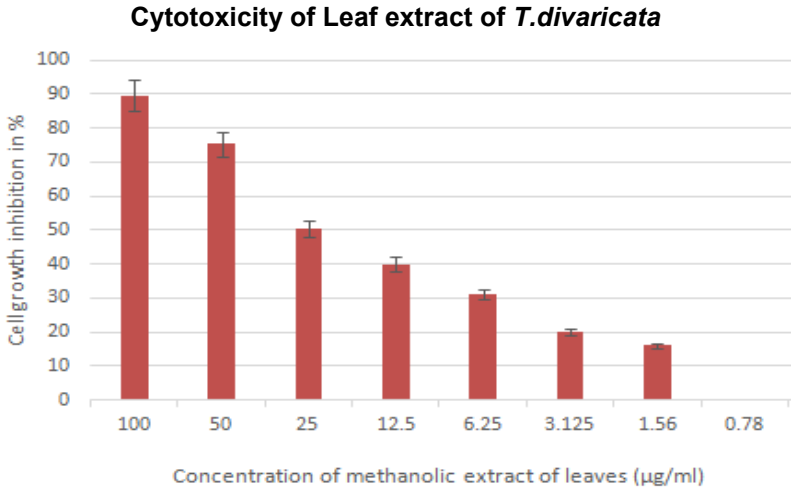

Figure 2

Cytotoxicity of Leaf extract of T.divaricata against HT29 cell line after 48 h incubation. Fresh medium was added to the cells with various concentrations of plant extracts (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml) and incubated for 48 hours. After 48 hours incubation, cell viability was determined by adding (Sigma) tetrazolim salt as cytotoxicity indicator. The error bars represents standard deviation. The level of significance was set at P<0.05.

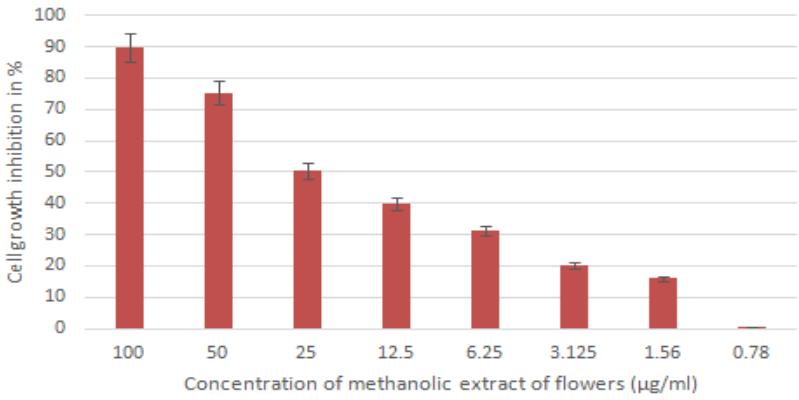

Figure 3

Cytotoxicity of Flower extract of T.divaricata against HT29 cell line after 48 h incubation. Fresh medium was added to the cells with various concentrations of plant extracts (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml) and incubated for 48 hours. After 48 hours incubation, cell viability was determined by adding (Sigma) tetrazolim salt as cytotoxicity indicator. The error bars represents standard deviation. The level of significance was set at P<0.05.

Hoechst staining

HT-29 cells were treated with the extracts of leaves and flowers of *T.divaricata* for 48 hrs. After the incubation period, the morphological changes in the cells were examined using inverted and phase fluorescent microscope. The cells displayed changes that are associated to apoptosis including membrane blubbing, chromatin condensation, cell shrinkage, apoptotic nuclei and DNA fragmentation.

Effect of Leaf extract (L) and Flower extract (F) of T.divaricata on morphology of HT-29 cells

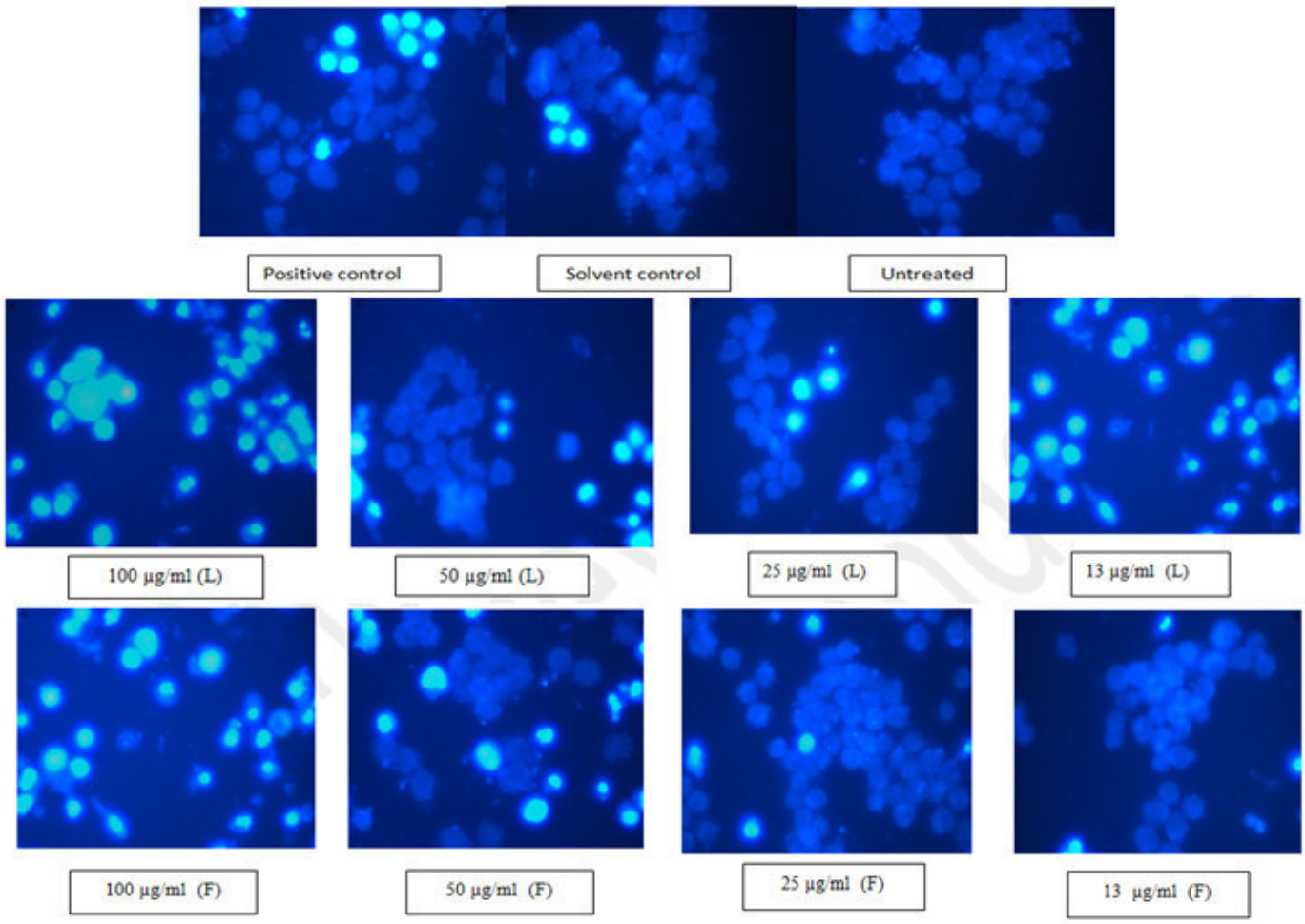

Figure 4

Effect of Leaf extract (L) and Flower extract (F) of T.divaricata on morphology of HT-29 cells. HT-29 cells were incubated with various concentrations (13, 25, 50, 100 µg/ml) of Leaf extract (L) and (13, 25, 50, 100 µg/ml) of Flower extract (F) of T.divaricata) for 48 hrs. Cells were stained with Hoechst 33342 and the stained nuclei were imaged under a fluorescent microscope.

Dna fragmentation assay

The agarose gel electrophoresis was performed on the cells treated with leaves and flowers extract for 24 and 72 hours. The result showed that internucleosomal DNA cleavage created no ladder pattern for the liquid extract treated cells. The deoxyribonucleic acid would possibly be intact and no DNA fragmentation was detected. The cells and untreated cells could produce a discrete band from 700 to 1000 kbp, which was not related to apoptotic DNA cleavage, but attributed to the migration of any DNA fragment larger than 700 kbp. This indicates that the DNA can be cleaved once treated, however, in a very sizable amount of nucleotide. The explanation additionally describes the presence of apoptotic bodies within the cell morphological study. An intensive deoxyribonucleic acid fragmentation could have occurred that could not be detected during this study.

DNA ladder visualized in agarose gel by ethidium bromide staining - 24 hrs

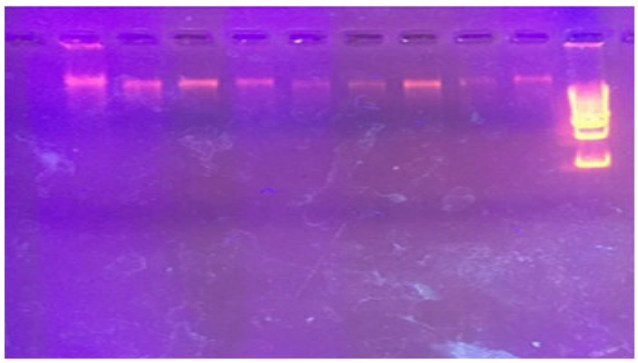

Figure 5

DNA ladder visualized in agarose gel by ethidium bromide staining - 24 hrs. Lane 1: Untreated; Lane 2: Solvent control; Lane 3: Positive control; Lane 4: 13 µg/ml Leaf extract (L); Lane 5: 25 µg/ml Leaf extract (L); Lane 6: 50 µg/ml Leaf extract (L); Lane 7: 100 µg/ml Leaf extract (L); Lane 8: 13 µg/ml Flower extract (F); Lane 9: 25 µg/ml Flower extract (F); Lane 10: 50 µg/ml Flower extract (F); Lane 11: 100 µg/ml Flower extract (F).

DNA ladder visualized in agarose gel by ethidium bromide staining - 72 hrs

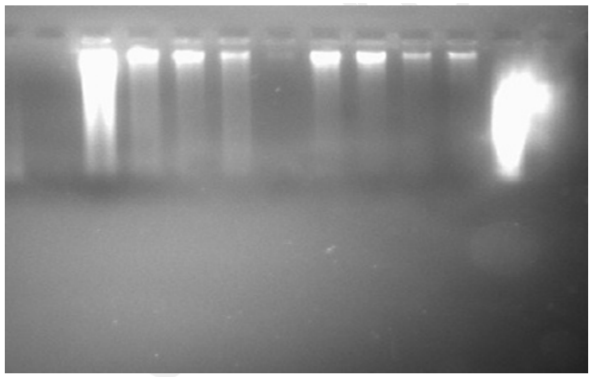

Figure 6

DNA ladder visualized in agarose gel by ethidium bromide staining - 72 hrs. Lane 1: Untreated; Lane 2: Solvent control; Lane 3: Positive control; Lane 4: 13 µg/ml Leaf extract (L); Lane 5: 25 µg/ml Leaf extract (L); Lane 6: 50 µg/ml Leaf extract (L); Lane 7: 100 µg/ml Leaf extract (L); Lane 8: 13 µg/ml Flower extract (F); Lane 9: 25 µg/ml Flower extract (F); Lane 10: 50 µg/ml Flower extract (F); Lane 11: 100 µg/ml Flower extract (F).

The results of this study support the efficacy of *T.divaricata* as an antineoplastic agent for HT-29 cell line. From the present study, it has been concluded that the methanolic extract of leaves at the concentration of 13 µg/ml and the methanolic extract of flowers at the concentration of 13 µg/ml of *T.divaricata* shows 50% anti neoplastic activity in HT-29 cell line. It acts as a potential treatment to the current chemotherapeutic agents and might be employed in the treatment of HT-29 cell line. Further analysis and studies have to be done. It also justifies the medicinal uses and claims regarding the therapeutic values of this plant as a curative agent. The purification and characterization of the phytochemicals along with clinical investigations in animal models are required to produce some further insight into the *in vivo* cytotoxic activity of the plant. It serves as a chemotherapeutic agent. The results of this study support the efficacy of *T.divaricata* as an antineoplastic agent for HT-29 colon cancer cell line. It acts as a potential adjuvant treatment with current chemotherapeutic agents and might be employed in the treatment of HT-29 colon and clinical studies have to be done. From this, it is said that attributable to the presence of some anti neoplastic parts, it shows 50% activity. In future the components present in *T.divaricata* may act as a drug. Further, *in vivo* studies ought to be carried out. Considerable amount of work [26,27] has been done on the medicinal plants to treat cancer. The leaf extract of the Malayan *Tabernaemontana corymbosa*, a related species, was studied for in vitro cytotoxicity against human KB cells.[28] Study was also done to test the effect of Taberna extract on breast and colon cancer cell lines.[29] Tabernamontana is very rich in Tabernaemontanine, coronaridine fractions which showed good inhibition against Sarcoma cells. In addition, the chloroform sub-fraction and coronaridine also showed selective cytotoxic effect against Chinese hamster cells.[30] The extract of *Cymbopogon citrates*, a related species, was found to induce apoptosis in several hematopoietic cancer cell lines.[31] It has been reported, that the methanolic extract of *Vigna unguiculata* has a high cytotoxic activity against HepG-2 cells.[32] The leaf extract of *Ocimum basillicum* is known for it's chemopreventive and anticarcinogenic properties.[33] The aqueous leaf extract of *Ocimum basillicum* has been reported to show chemopreventive and antiproliferative activity on Hela cells.[34] The ethanolic extract of *Ocimum* leaf also has been shown to have significant modulatory influence on carcinogen metabolizing enzymes including cytochrome P450, cytochrome b5, and aryl hydrocarbon hydroxylase, glutathione-S-transferase.[35] Therefore, some of these medicinal plant products are also being marketed as antineoplastic medication.[36] Medicinal plants have been used in the treatment of cancer and as the basis for drug discovery. In recent days, more than 70% of anticancer drugs have been derived from medicinal plants. Scientists have studied various herbal remedies employed in colorectal cancer treatment. These plants may promote host resistance against infection by re-stabilizing body equilibrium and learning the body tissues. Medicinal plants promise a source of natural antimicrobial agents and It has been reported that the antimicrobial activity of plants is related with the defense mechanism of the phytochemical compounds against microorganisms.[37] The utilization of natural antioxidants are intensively performed to find potent compounds in combating many of the age related chronic diseases like cancer. Several reports describe that the antineoplastic activity of these plants can be attributed to the presence of antioxidants.[38,39] Almost 66 alkaloids have been isolated from this species and the yield of alkaloid fraction obtained from even the dry stems of *T. divaricata* was found to be 0.98 per cent.[40] It is these alkaloidal part of phytochemical activities that justify its use in traditional medicines. The alkaloidal compounds of *T. divaricata* could indeed play important roles in many of the pharmacological activities of the leaf and flower extracts of *T. divaricata*. The antioxidant effects of T. divaricata have been studied in depth by various medicinal plant investigators using the carbon tetrachloride (CCl_4)-induced hepatotoxicity model.[41,42] The hepatotoxic effect of T.divaricata is due to the metabolite of CCl_4 which is a free radical that causes the peroxidative effect of lipids in the endoplasmic reticulum that leads to the cell death.[43,44] Phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer properties are extensively extracted from many of the medicinal plant species. The potent use of these novel bioactive plant extracts for

cancer prevention is still dominating over the use of synthetic chemicals.[45,46]

CONCLUSION

There are still numerous alkaloids and their derivatives from *T.divaricata* whose pharmacological activities have not yet been studied. It is possible that these compounds may contain useful pharmacological properties. Therefore, in vivo studies pertaining to their medicinal properties could provide more useful insights into the benefits of *T.divaricata* for future clinical management of many of the dreadful human diseases.

CONFLICT OF INTEREST

Conflict of Interest declared none.

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