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R E S E A R C H A R T I C L E

Evaluation of In-Vitro Cytotoxic Potential of Ficus Benghalensis Tender Prop Roots Extract

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A B S T R A C T

Introduction: Cancer can be defined as the abnormal, excessive, uncontrolled proliferation of cells under the influence of a plethora of known and unknown carcinogenic stimuli. **Aim:** To evaluate cytotoxic potential of methanolic extract of tender prop roots obtained from *Ficus benghalensis* through *in –vitro* assays like Tryphan blue method and MTT assay emploting Colo 320 cell lines. **Materials:** The tender prop roots were collected from *Ficus benghalensis* tree was collected from the same was authenticated by the Botanist at SV Govt. arts and science college, Tirupati and the specimen was preserved for reference. The Aerial prop roots of *Ficus benghalensis* were shade dried and pulverized to get coarse powder followed by sieving (Sieve Number 44) to remove the unwanted waste material and to obtain the coarse powder. **Results:** Preclinical safety testing of recent drug candidates may be a crucial step in pharmaceutical drug development and depends on anordered series of *in-vitro, in-vivo* and *in-silico* tests before administration to humans. **Conclusion:** The study concludes that tissue culture technology has virtually revolutionized cancer biology in discovering cytotoxic molecules. Our current study is a preliminary effort to evaluate cytotoxic potential of methanolic prop root extract of *Ficus benghalensis* against colorectal adenocarcinoma cell lines *Colo 320* employing two widely known *in-vitro* assays viz Tryphan blue and MTT assay.

Keywords: cytotoxic potential, *Colo 320, Ficus benghalensis, in-silico*

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Cancer can be defined as the abnormal, excessive, uncontrolled proliferation of cells under the influence of a plethora of known and unknown carcinogenic stimuli that in due course of time invades various anatomical regio

ns of body leading to fatal complications and ultimate death if not diagnosed and intervened appropriately.

Development of cancer-carcinogenesis

Normal cells are under the constant influence of several signals regulating the behaviour of the cell. Cancer is characterized by dysregulation of these intercellular signalling mechanisms. The term carcinogenesis describes the multistage process of development from a normal cell to a malignant cancer cell. This complex successive transformation involves changes at genetic and cellular levels to acquire the malignant characteristics[1-4].

Hallmarks of cancer

The hallmarks of cancer consist of six biological capabilities acquired during the multistep development of human tumors.

Cell death

It is possible to discriminate up to 12 pathways of cell death occurring in mammals, 10 of which are genetically programmed. The different types of cell death are: necrosis, apoptosis, anoikis, caspase independent apoptosis, autophagy, Wallerian degeneration, excitotoxicity, erythropoiesis, platelets, cornification, lens a nd mitotic. Catastrophe. Apoptosis, autophagy or cornication are best known among the genetically programmed forms of cell death, but necrosis or mitotic catastrophe which is not genetically programmed lead to cell death as well.

Necrosis

Necrosis occurs predominantly in pathological conditions and is the end point of very severe damage, resulting in a loss of membrane integrity, swelling and disrupture of the cells. The cellular contents are released into the cells environment leading to a strong inflammatory response. Necrosis can be secondary to apoptosis.

Apoptosis

Apoptosis is a programmed cell death which occurs in response to physiological signals during embryonic development, in maintenance of homeostasis in multicellula r organisms or to eliminate dysfunctional cells. Apoptosis is strictly regulated and can be recognized by morphological modifications such as cellular shrinking, cell deformation and loss of contact to neighbouring cells[5-9].

Carcinogenesis of colorectal cancer

Anatomy of the colon

The gastrointestinal tract consists of the upper and the lower part. The upper gastrointestinal tract consists of mouth, pharynx, esophagus and stomach. The lower gastrointestinal tract includes small intestine, consisting of duodenum, jejunum and ileum, the large intestine, consisting of caecum, colon, rectum and anal canal. In mammals, colon consists of the ascending colon, transverse colon, descending colon and sigmoid colon. The ascending colon is the part of the colon from the caecum to the hepatic flexure where the transverse colon begins and leads into the descending colon at the splenic flexure. The part from the splenic flexure to the beginning of the sigmoid colon is called descending colon, where the stool is stored[10-12].

Development of colorectal cancer

Malignant transformations which lead to colorectal cancer occur mostly in the mucosa, where colorectal cancer usually starts as a benign polyp. Most of these hyperplastic polyps remain benign showing almost normal histopathology. However, these hyperplastic areas have an unusual high division rate leading to thicker epithelia over time and to alterations in the morphology of the cells which is called dysplastic cells or adenoma.

The higher the degree of dysplasia in an adenoma the higher is the risk of developing into a malignant carcinoma. 95% of colorectal cancers develop in this way. Polyps that grow into the colonic lumen on a stalk can be removed by colonoscopy to prevent progression to malignant adenocarcinomas. They invade surrounding colon tissue and grow into the wall of the colon. In advanced stages the cancer cells metastasize and spread to other organs, mainly the liver.

Clinical features

The clinical symptoms of advanced disease may include any of the following:

- Jaundice
- Severe weight loss
- Abdominal swelling
- Shortness of breath
- Extreme fatigue and dizziness

Side effects of colorectal cancer treatment

Surgery: The side effects from surgery for colorectal cancer may include:

• Fatigue, for an extended period of time
• Constipation or diarrhoea
• A temporary or permanent colostomy
• Sexual side effects, such as erectile dysfunction in men, after more extensive operations for rectal cancer.

Radiation therapy

Side effects of radiation therapy for colorectal cancer include mild skin irritation, nausea, diarrhoea, rectal irritation, the urge to defecate, bladder irritation, fatigue, or sexual problems. Some degree of rectal or bladder irritation may be a permanent side effect, and can lead to diarrhoea and frequent urination[13-14].

Chemotherapy

Chemotherapy drugs kill cancer cells but also damage some normal cells and the side effects depend on the type of drugs, the amount taken and the length of treatment. General side effects from chemotherapy include fatigue, nausea, vomiting, diarrhoea, loss of appetite, hair loss, swelling, rashes, mouth sores, numbness, tingling and blistering of the hands and feet. Some patients may experience low blood cell counts because chemotherapy can damage the blood producing cells of the bone marrow. This can increase the chances of infection (due to a shortage of white blood cells) and bleeding or bruising after minor cuts or injuries (due to a shortage of blood platelets).

2. Methodology

Plant Material collection and authentification: The tender prop roots were collected from *Ficus benghalensis* tree was collected from the same was authenticated by the Botanist at SV Govt.arts and science college, Tirupati and the specimen was preserved for reference

Preparation of the Aerial prop root powder for Extraction: The Aerial prop roots of *Ficus benghalensis* were shade dried and pulverized to get coarse powder followed by sieving (Sieve Number 44) to remove the unwanted waste material and to obtain the coarse powder.

Process of Extraction:

About 100 g of the *Ficus benghalensis* crude root powder was subjected to extraction by using 600ml of methanol successfully by Continuous hot percolation method by using Soxhlet apparatus at a constant temperature of about 45- 55°C. The crude powder was extracted with the solvent for 4 consecutive days. After extractions, the extract was collected and removed of solvent by using rota vapor apparatus to get well-dried extract, weighed and the percentage yield was calculated from the weighed powder of the plant. The percentage yield of the extract obtained was about 4.75%. The obtained extract was further used to evaluate for its anti cancer activities by preparing concentrations of 10,25,50,75 and 100ug/ml in double distilled water[15].

Calculation of Percentage yield:

The percentage yield of the extract after successful completion of the extraction process was calculated by using the formula and the percentage yield of the prop roots extract was found to be 4.75%.

***In-vitro* anticancer activity**

Cell lines

COLO 320 cell lines were obtained from sugen Life Sciences Pvt. Ltd., Tirupati and cultured in RPMI 1640 medium (Difco, invitrogen corp, Canada).

Characteristics

Karyo type

Marker chromosomes with homogeneously staining regions (HSR) were observed and the double minute (DM) chromosomes appeared in much lower frequency than the parental line *COLO 320*.

Clinical Data 55 years

Caucasian Female

Genes Expressed

Serotonin, norepinephrine, epinephrine, adrenocorticotropic hormone (ACTH), parathyroid hormone.

Tumorigenic effects: Yes, in nude mice

Comments Cells are weakly positive for keratins

Culture method

The base medium for this cell line is formulated RPMI-1640 Medium. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Subculturing

Culture conditions Temperature 37^0c

Volumes used in this protocol are for 75 cm 2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes

Cryopreservation

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO

Storage temperature: Liquid nitrogen vapor phase

Culture Conditions: Temperature: 37°C

Tryphan blue dye exclusion assay Method

Experimental design

The designed study consists of three groups viz: Negitive control, Control, Test. In the Negative control group the cell lines were incubated with the medium for a period of 24 hours. This group was designed to rule out the possibility of any growth inhibitory effect of certain compounds of medium. The control group was designed to rule out the effect of any residual or traces of solvent with which the extract was prepared on the growth inhibition of cell lines. Here the solvent employed was methanol and hence it is added at the concentration of 0.1% (v/v) in distilled water. In test group different concentrations of test extract i.e, 10, 25, 50, 75 and 100µg/ml are incubated with *colo 320* cell lines for a period of 24 hours. This group was used to study the effect on cell line viability.

Seeding of cells

COLO 320 cells were cultured to reach the 80-90% confluency using RPMI 1640 medium. After reaching the desired confluency, culture was collected and centrifuged at 3000 rpm for 10 minutes to get cell pellet. The pellet was resuspended in 1ml of fresh culture media. Cell concentration was determined by Tryphan blue assay was performed by mixing 50µ litres of culture and 50 µlitres of 0.4% tryphan blue dye. Finally cells seeded in 24 well plates at the concentration 10000 cells/ml and incubated at 5% co_2 incubator at 37^0 c for 24 hours.

Drug treatment

Cells were maintained in 24 well plates in triplicate for every concentration, and treated with different concentrations (10, 25, 50, 75, 100µg/ml) of MEFB and control groups were treated with medium and methanol. The treated cells were incubated for 24 hours in 5% co_2 incubator at 37^0c.

***In-vitro* cytotoxic assay**

After 24 hours incubation the cells were collected from each well in eppendorff and centrifuged at 3000rpm for 10 min to get cell pellet, to the pellet 50µlit of each medium and tryphan blue was added and mixed well to suspend the pellet. Cytotoxicity was screened by performing tryphan blue assay. Percent of growth inhibition was calculated by using the following formula

Micro culture Tetrazolium Assay

Plant material used: Methanolic extract obtained from the prop roots *of Ficus benghalensis.*

Principle: This assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow coloured water soluble substrate 3 (4, 5 dimethyl thiazol yl) 2, 5 diphenyl tetrazolium bromi de into an insoluble purple coloured formazan product whose coloured is measured by means of ELISA reader at 540 nm. Only viable cells with active mitochondria reduce significant amounts of MTT, since reduction of MTT can only occur in metabolically active cells.

Cell lines

Human colorectal adenocarcinoma- *colo 320* were obtained by sugen Life Sciences Pvt. Ltd., Tirupati from an authenticated supplier. Stock culture of these cell lines were cultured in RPMI -1640 with 10% inactivated newborn bovine serum, Penicillin (100 IU/ml), Streptomycin (100µg/ml)) under humidified atmosphere of 5% CO_2 at 37^0C until confluent.

Table 1: Experimental design to study the effect of methanolic extract of Ficus benghalensis prop roots on *Colo 320* cell line viability by Tryphan blue assay

S.NO	Group	Treatment
1.	Negitive control	*Colo 320* cell lines with medium (RPMI 1649)
2.	Control	*Colo 320* cell lines were incubated in RPMI medium along with methanol at a concentration of 0.1% v/v.
3.	Test	*Colo 320* cell line with cultured medium and MEFB extract at different concentration

3. Results and Discussion

Table 2: Percentage yield of prop roots extract of *Ficus benghalensis*

S. No.	Name of the test substance	% Yield (mg/100 gm)
1.	Methanolic prop root extracts of *Ficus benghalensis*	4.75 ± 2.13

Table 3: Effect of methanolic extract of Ficus benghalensis on viability of *Colo 320* cell lines by Tryphan blue assay

S.NO	Groups	Concentration of MEFB (µg/ml)	% Cell viability
1	**Control**		94.43±0.3480
2	**Test**	10	85.30±0.1538[a]
		25	82.17±0.5487[a]
		50	78.83±0.4410[a]
		75	55.73±0.8192[a]
		100	54.07±0.5207[a]

All values were expressed as Mean ± SEM of (n=3) performed by ANOVA
a = p< 0.0001
All groups are compared with control.

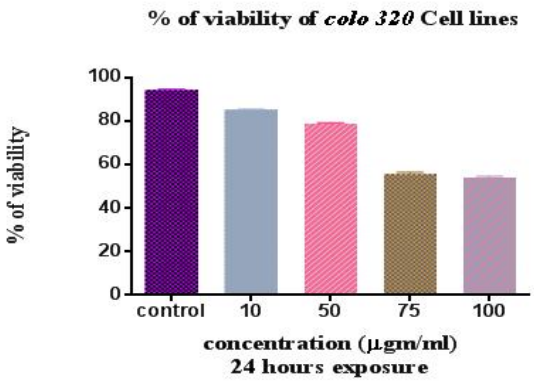

Figure 1: Effect of methanolic extract of *Ficus benghalensis* on viability of *Colo 320* cell lines by Tryphan blue assay

Single exposure of incubation of *Colo 320* cell lines to different concentrations of MEFB (10, 25, 50, 75, 100 µg/ml) for 24hours by Tryphan blue assay resulted in a significant dose dependent reduction in the viability of cells when compared with that of control (p<0.0001).

Images of *colo 320* cell lines

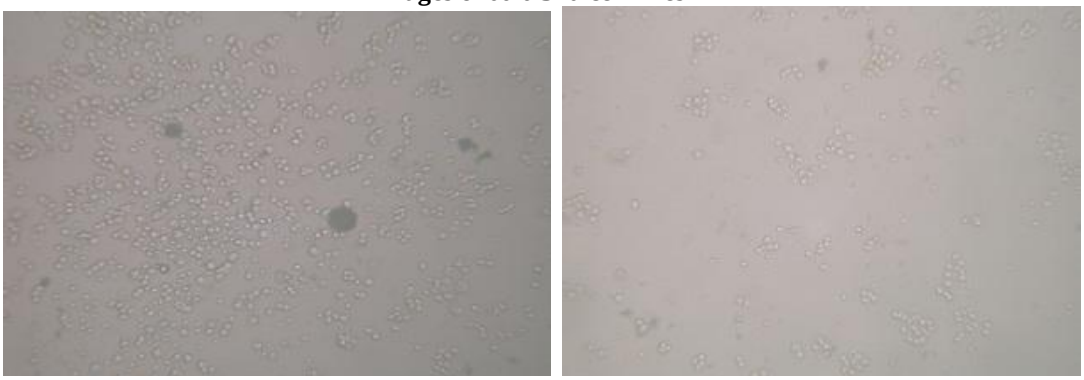

Plate I: Control **Plate II: 10µg**

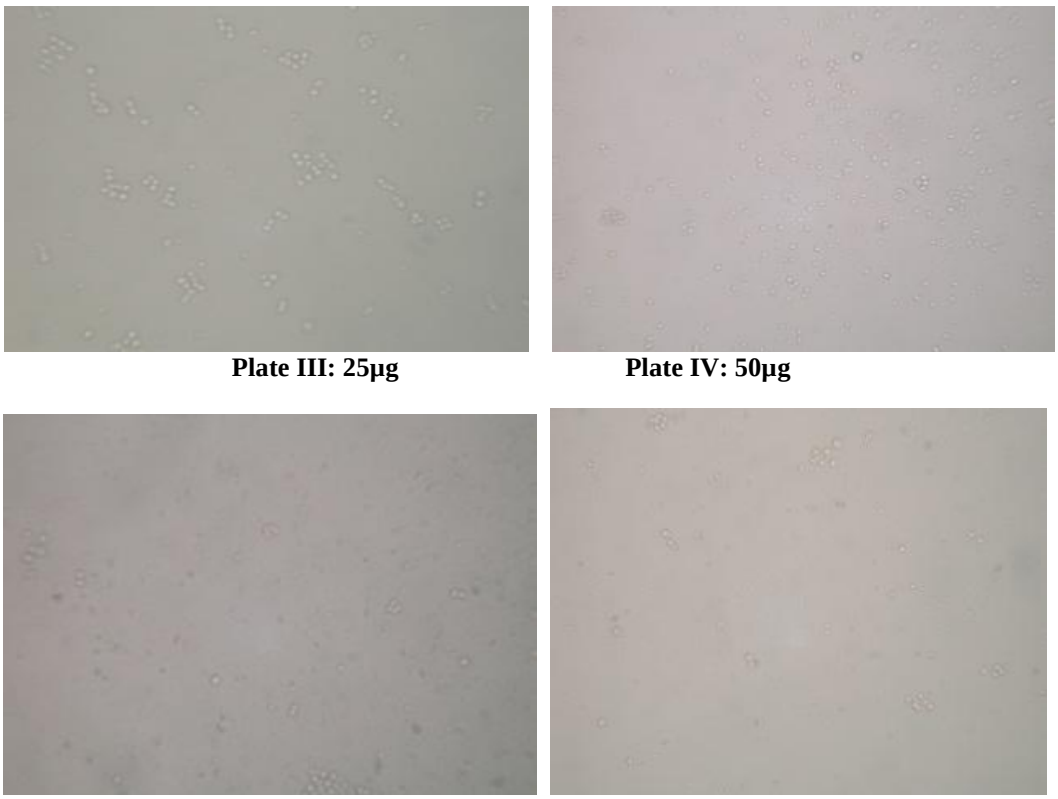

Plate III: 25µg **Plate IV: 50µg**

Plate V: 75µg **Plate VI: 100µg**

Figure 2: Images of *Colo 320* cell lines treated with MEFB

Microculture Tetrazolium Assay

Table 4: Effect of methanolic extract of *Ficus benghalensis* on viability of *Colo 320* cell lines by MTT assay

S.No	Groups	Concentration of MEFB (µg/ml)	% Cell viability
1	**Control**		95.07±0.233
2	**Test**	10	87.17±0.441 [a]
		25	82.73±0.3712 [a]
		50	78±0.5774[a]
		75	64.07±0.3528[a]
		100	47.87±1.139 [a]

All values were expressed as Mean ± SEM of (n=3) performed by ANOVA

a = p< 0.0001, All groups are compared with control.

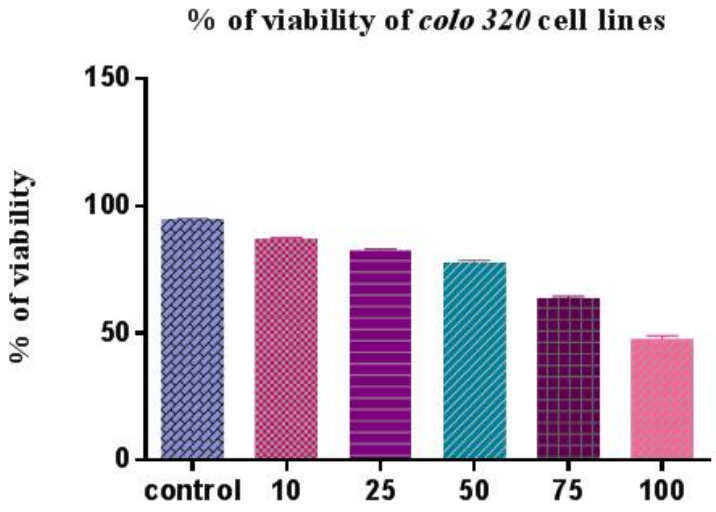

Figure 3: Effect of Methanolic extract of *Ficus benghalensis* on viability of *Colo 320* cell lines by MTT assay.

Single exposure of incubation of *Colo 320* cell lines to different concentrations of MEFB (10, 25, 50, 75, 100 µg/ml) for 24hours by MTT assay resulted in a significant dose dependent reduction in the viability of cells when compared with that of control ($p<0.0001$).

Images of *colo 320* cell lines

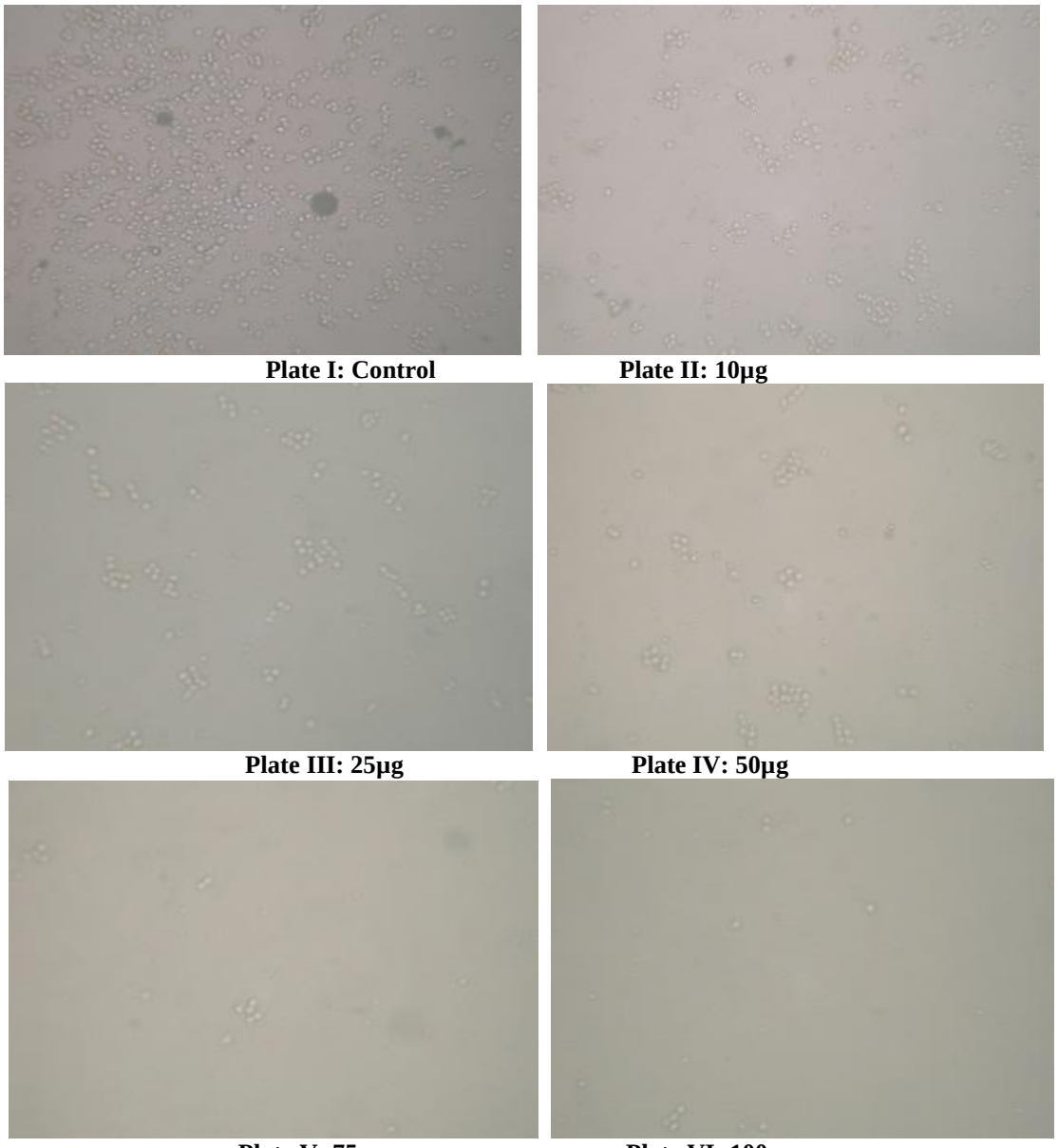

Plate I: Control **Plate II: 10µg**

Plate III: 25µg **Plate IV: 50µg**

Plate V: 75µg **Plate VI: 100µg**

Figure 4: Images of *Colo 320* cell lines treated with MEFB

IC_{50} value of Methanolic extract of *Ficus benghalensis* was calculated by linear interpolation method using the formula

$$IC_{50} = \frac{50-A}{B-A} \times (D-C)+C$$

where A = The first point on the curve, expressed in percent inhibition, that is less than 50%
B = The first point on the curve, expressed in percent inhibition, that is greater than or equal to 50%
C = The concentration of inhibitor that gives A% inhibition; and
D = the concentration of inhibitor that gives B % inhibition.

$$IC_{50} = \frac{50-35.9}{52.1-35.9} \times (100-75)+75 = 87.03 \mu g/ml$$

Historically the complexity of cancer biology has necessitated an incredible amount of research that has resulted in significant advances in our understanding of the disease pathology. This complexity of the disease is unable to match with the prevention and therapeutic approaches to shut down a wide range of immortalized cells by acting on many different mechanisms and pathways. Colorectal cancer is one of the most devasting malignancies in the

world which originates from the epithelial cell lining of the gastrointestinal tract and undergoes sequential mutations in specific DNA sequences, thereby disrupting the normal mechanisms of proliferation and self renewal. Throughout different civilizations humans have relied on nature to accomodate their basic needs, of which some are used for treatment of wide spectrum of diseases and cancer is one among those with which has taken large share from the nature.

Ficus benghalensis is a tropical tree belonging to the family of Moraceae. A number of preclinical studies have demonstrated various pharmacological activities of the extracts derived from Ficus species. Many novel agents have revolutionized the therapy of colorectal cancer, unfortunately most of them met with severe adverse effects. In this regard our present effort is a preliminary one to detect anti-cancer activity of a widely distributed tree in the Indian subcontinent viz. *Ficus benghalensis* employing *in vitro* cytotoxic assays like Tryphan blue and MTT assay. The specimen was taxonomically authenticated and the crude powdered form of shade dried tender prop roots of Ficus tree was extracted with methanol. Phytochemical screening was reported from earlier studies hence not gone for the screening. The methanol based extraction procedure used in the present study has been routinely followed for exploring therapeutically valuable phytochemicals. Further the solvent used for extraction i.e methanol was likely possess anti -bacterial activity and thus prevent the culture from contamination and development of fungal spores. However more refined extraction methods are needed to resolve the anticancer activity in future studies.

Preclinical safety testing of recent drug candidates may be a crucial step in pharmaceutical drug development and depends on anordered series of *in-vitro*, *in-vivo* and *in-silico* tests before administration to humans. Currently, *in-vivo* testing may be an important part of safety assessment, and maybe a regulatory requirement before a drug will progress into clinical trials. However, in recent years, many *in- vitro* assays have been developed and validated for early-stage screening aimed toward filtering out molecules with the next potential for toxicity and in some cases replacing or reducing the utilization of certain *in vivo* tests as an adherence to the 3Rs, a set of principles by ICH that outlines the replacement, reduction and refinement of the utilization of animals in research. Cell culture techniques play a key role in the development of new anticancer drugs .The majority of cytotoxic anti-cancer drugs in use today were selected on the basis of animal screening system usually employing mice with transplantable tumours. It is not always possible to extrapolate the results of *in vivo* animal studies to humans due to mysterious biology of cancer cells in different system. The ICH guidelines stress the need to reduce the number of animals, refinement of existing procedures and replacement of animals have paved the way for alternative screening procedures like employing cell lines in disease research. Further the high throughput nature of *in vitro* cytotoxic and anti-proliferative assay form an indispensible link in different components of drug discovery.

In these *in-vitro* assays, we have employed *colo 320* cell lines which were economically effective and practical in identifying growth inhibitors as potential therapeutic agents. The *colo 320* cell line grows in suspension and is easy to monitor its growth in micro culture plate. It is possible to screen compounds at only one single concentration and obtain statistically meaningful results with *colo 320*cell lines. Further the growth kinetic data show that they are having a mean doubling time of 12 hours. In the present study we have chosen twomost widely used *in -vitro* methods for assessing cytotoxicpotential of root extract viz: Tryphan blue and MTT assay. Tryphan blue is a vital stain which when added to the culture leaves non viable cells with a distinctive blue colour while visible cells appear unstained. This is based on the fact that viable cells have intact cell membrane and hence do not take in the dye from the surrounding medium. The dose dependent reduction in the number of viable cells in this assay indicates that the plant extract may be responsible for disturbing the cell membrane permeability. Hence the cells with altered membrane allow the dye to enter inside. The perturbation might be due to redistribution of phosphotidyl serine to the external side of the cell membrane which is the main biochemical feature of apoptosis.The very little reduction of viability in the control group (i.e, *colo 320* cell lines with ethanol) might be attributed to the effects of ethanol slightly damaging the cell membrane.

This group served the purpose to rule out the effects of ethanol with which the extract was prepared. The MTT assay was based on the reduction of MTT (3-[4, 5- dimethyl thiazol-2yl]-2,5- diphenyl tetrazolium bromide) by mitochondrial dehydrogenase system to water insoluble purple coloured formazan in actively respiring cells. Hence this *in vitro* assay was widely recommended for examining the cytotoxic effect of xenobiotics, assessing proliferation rates and analyzing cell activity in 96 well plate format. This method truly distinguishes between healthy cells and cells that are alive but loosing function i.e, MTT reduction can be attributed to the mitochondrial activity, although non-mitochondrial, cytosolic and microsomal enzymes were also responsible for reduction of MTT but to a lesser extent. The results showed that at 10, 25, 50, 75, 100µg/ml of test extract there was 4.9%, 12.8%, 17.2%, 22%, 35.8%, 52.1% of inhibition of growth. This indicates a significant and dose dependent reduction in the growth of cell lines. This reduction of cell viability may be due to loss of mitochondrial inner transmembrane potential which is generally observed in the earlier stage of apoptosis. Collapse of this potential is believed to coincide with the opening of mitochondrial permeability transition pores, leading to the release of cytochrome C into the cytosol. In cytoplasm, cytochrome C combines with Caspase-9, Apaf-1 and dATP to form the apoptosome complex which in turn activates Caspase-9, Caspase-3, Caspase-7 and leads to apoptotic cell death. Thus the human colorectal adenocarcinoma cell lines may provide the framework for

studies leading to a deeper understanding of the control of differentiation and in a broader perspective, the control of normal cellular differentiation. Future studies with these cell lines and selected variants may shed new light on the cell biology of *Colo 320* and perhaps lead to new therapeutic modalities.

IC_{50} is one important and most commonly determined parameter in *in vitro*cytotoxic assays. IC_{50} value can be defined as the concentration of the test substance at which it reduces the number of surviving cells by 50% and is estimated from the dose dependent curves. The IC_{50}value of the methanolic extract of *Ficus benghalensis*on *colo 320* cell lines was found to be 87.03µg/ml by MTT assay[16-19].

The reduction in the survivability of *colo 320* cell lines byMEFB for a single exposure can be due to various bioactive secondary metabolite that either simply or in synergy with other constituents may have exerted the cytotoxic effect such as flavonoids, glycosides, triterpenoids Etc. Flavonoids have been demonstrated to inhibit proliferation in many kinds of cultured human cancer cell lines, where as less or no cytotoxic to human normal cells. The molecular mechanism of anti proliferation may involve the inhibition of the prooxidant process that causes tumor promotion and studies established that the formation of growth promoting oxidants acts as a major catalyst of tumor promotion and progression stages. In addition inhibition of polyamine biosynthesis could be a contributing mechanism to the antiproliferative activities of flavonoids. Ornithine decarboxylase is a rate limiting enzyme in polyamine biosynthesis, which has been correlated with the rate of DNA synthesis and cell proliferation in several tissues. Several experiments have shown that flavonoids can inhibit ornithine decarboxylase induced by tumor promoter and then cause a subsequent decrease in polyamine and inhibition of DNA/ protein synthesis. Furthermore, it is established that flavonoids are also effective in inhibiting signal transduction enzymes like protein tyrosine kinases, protein kinase C etc which are involved in cell proliferation. The other probable mechanisms for inhibition of cancer cell proliferation by extract can be mitotic block, cell cycle arrest, tubilin structure disruption. The present investigation indicated that *colo 320* cells when treated with MEFB may undergo phytochemical specific programmed cell death and did not induce non specific necrotic death as control group without extract has not significantly affected cell viability. Therefore this study form an important preliminary base to carry further investigation to isolate, characterize bioactive natural agents for the treatment of human colorectal adenocarcinoma with lower toxicity and higher effectiveness and could serve as a new source for lead molecule[20].

4. Conclusion

The study concludes that tissue culture technology has virtually revolutionized cancer biology in discovering cytotoxic molecules. Our current study is a preliminary effort to evaluate cytotoxic potential of methanolic prop root extract of *Ficus benghalensis*against colorectal adenocarcinoma cell lines *Colo 320* employing two widely known *invitro* assays viz Tryphan blue and MTT assay. The study revealed a dose dependent reduction in the number of viable *Colo 320* cells upon single exposure for 24hours indicates that the extract might be able to alter membrane permeability and functioning of mitochondrial dehydrogenase synthase. Further studies were implicated to identify individual phytoconstituents of methanolic extract of *Ficus benghalens is* responsible for cytotoxicity and elucidation of probable mechanism of action may lead to the development of promising agent of natural origin for the treatment of colorectal adenocarcinoma

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