Mint Leaves Inhibits Tumour Angiogenesis via Suppression of Sonic Hedgehog Pathway by Invitro Analysis for Colorectal Cancer

Dhanasekaran. S, Sangavi. J, Ramya. R, Nithya.K.P

***Abstract*— Angiogenesis is closely related to the growth and conquest of tumours also considered as the significant mark of anticancer therapy. Angiogenesis is strongly regulated by the Sonic Hedgehog (SHH) pathway, which has been known to deed up in many types of cancer. Therefore, inhibition of angiogenesis via variation of the SHH signalling pathway has become very gorgeous for cancer chemotherapy. Mint leaves have been charity to treat innumerable cancers including colorectal cancer (CRC). Our fiction study suggested that the ethanol extract of mint leaves (EEML) is able to inhibit angiogenesis in a chick embryo chorioallantoic membrane model and tempt apoptosis of colon cancer cells. To auxiliary explain the precise mechanisms of its anti-tumour activity, in the present study we planned to use Cell line in vitro to gage the effect of (EEML) on tumour growth and angiogenesis. Our current data indicated that (EEML) reduces tumour size without affecting the Normal cells.**

***Index Terms*— Mint leaves, Colorectal cancer, Apoptosis, Angiogenesis, SHH pathway.**

I. INTRODUCTION

Cancer is a disease in which a group of cells display uncontrolled growth, invasion and sometimes metastasis. Tumour suppressor genes are then inactivated in cancer cells, resulting in the loss of normal functions in those cells, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system. HT29 cell line is a human colonic adenocarcinoma cells that are able to express differentiation features characteristic of mature intestinal cells. These cells have mainly been used for studies related to glucose metabolism and hormone receptors. The Hedgehog signalling pathway is one of the key regulators of animal development and is present in all bi laterians. Hedgehog signalling in regulating adult stem cells involved in maintenance and regeneration of adult tissues. Apoptosis is a form of programmed cell death in multicellular organisms that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin, condensation, and chromosomal DNA fragmentation. As a well-known traditional Indian folk-medicine (Mint leaves) is one of the world's most ancient herbal medicines and has been applied by Traditional Indian Medicine (TIM) practitioners for thousands of years. It has anti-inflammatory, anti-tumour, anti-mutagenic effects and this inhibited the damage to DNA in lymphocytes caused by the total particle material (TPM) extracted from cigarette tar.

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Mr. Dhanasekaran S., Department of Biotechnology, Vivekanandha college of Engineering For Women , Namakkal , India, Mobile No:9655849404.

Sangavi J., Department of Biotechnology, Vivekanandha College of Engineering For Women, Namakkal, India, Mobile No:9042925515.

Ramya R., Department of Biotechnology, Vivekanandha College Of Engineering For Women, Namakkal, India, Mobile No:9003646665.

Nithya K.P., Department of Biotechnology, Vivekanandha College Of Engineering For Women, Namakkal, India, Mobile No:9042338994.,

II. MATERIALS AND METHODS:

1. Preparation of ethanol extract from mint leaves:

Ethanol extract mint leaves was prepared by 250 g of shaded dried mint leaves & then the sample was concentrated to 1:10 ratio by heating mantle, the extract was then evaporated at the end formation of sticky substance.

2. The concentrations should range from 0.05M to 0.25M in increments of 0.05M.After that drain the column to just more than the top of the resin (do not let resin go dry).
Add 1 ml of the compound mixture and allow it to run into the capped column, and start collecting ~3 ml of effluent in each tube .Measure the absorbance of each tube at 260 nm in UV spectrophotometer.

3. Extraction of vitamin C by gel filtration:

Add 1 ml of the compound mixture and allow it to run into the capped column, and start collecting ~3 ml of effluent in each tube .Measure the absorbance of each tube at 260 nm in UV spectrophotometer.Fix the column vertically to a column stand then allow the column to room temperature. Add 4 ml of Gel Filtration Buffer to the column and allow it to drain out completely for the equilibration of the column. Store the column at 28^{o}C for next use after fixing the top and bottom caps.

4. Quantification of vitamin C by HPLC

The mobile phase consisting of Acetonitrile : Double distil water (70:30) were filtered before use through 0.55 micro membrane filter and was pumped by the dual plunger reciprocating pump (L-7100 Lunchroom, Hitachi) at a flow rate of 1 ml/min in the ratio of 90:10. The separation was carried out on a C18 column (5µm, 250 (L) X 4.6 mm i.e., Kromasil) attached with a C18 guard column temperature was maintained at 28^{0}C then the sample was injected through a Redone injector at 20 microliter and was analysed by variable wavelength detector set at 290 nm. The data was acquired, stored and analysed using winchrom software.

5. In vitro cytotoxic activity of extract

Substance was subjected for in vitro anti-cancer screening using HT-29 cell line then the cell culture was trypsin zed &

suspended to 0.1ml in each well of micro titre plates & kept for 24 hours then the aqueous layer was flicked off after that 100µl of different test compounds were added in plates & kept incubation at 37°C for 72 hours and cells were cyclically checked for granularity, contraction & swelling.

6. Cell line cytotoxicity screening

After 72 hours the sample solution in wells was flicked off and 50µl MTT dye was added to each well. The plates were gently shaken & incubated for 4 hours at 37°C then the supernatant was removed 50µl of propanol was added and the plates were gently shaken to solubilise the formed Formosan. The absorbance was measured at 490 nm by using micro plate reader the percentage growth inhibition was calculated by using the formula:

% cell inhibition=100-{(absorbance value of test sample-absorbance value of blank)/(absorbance value of blank-absorbance value of control)}*100

Data interpretation:

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

%cell survival= {(At-Abs)/ (Ac-Abs)} x100

% cell inhibition= 100-cell survivals

III. RESULTS AND DISCUSSION

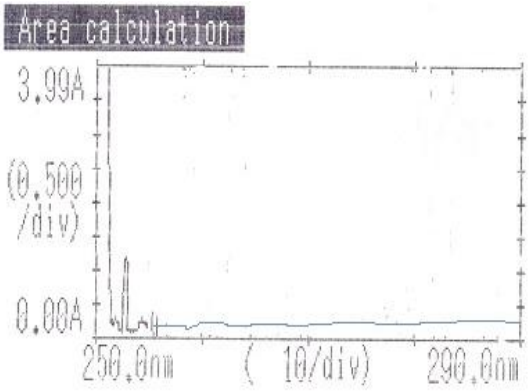
1. Preparation of ethanol extract from mint leaves

Crude extract of mint leaves was prepared by using ethanol extraction method and crude extract was stored at 20°C

2. Purification of crude vitamin by DEAE cellulose:

The crude vitamin was extracted from fractional precipitation are purified using Column Chromatography using DEAE Cellulose column and eluted using five different concentrations of solution such as 0.05M , 0.1M , 0.5M,0.1M and 0.5M of Tris HCL buffer. The purified extracts are scanned using UV Spectrophotometer and the peaks obtained were found.

Graph 1: UV spectrophotometer – purified crude vitamin of *Mint leaves*



3. Extraction of Vitamin C by gel filtration:

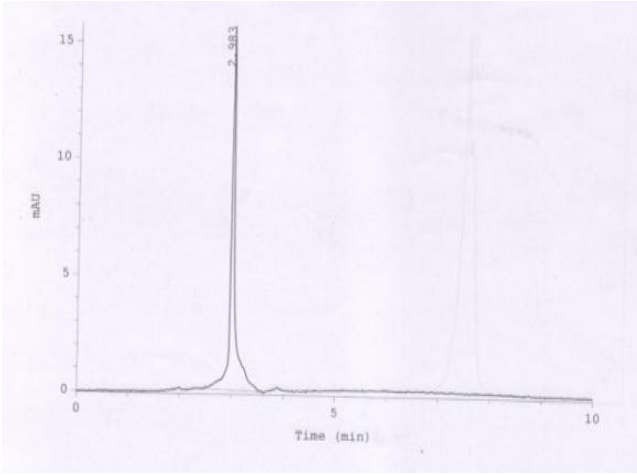
From the 0.2 ml of purified vitamin C was extracted through gel filtration chromatography. Sephacryl S-200 HR gel was used in the gel filtration column and 0.025 M phosphate buffer was used as an elution buffer to eliminate the absorbent part. Non absorbent part was eluted during the process. All non absorbent and absorbent part was collected in the different tubes for HPLC analysis.

4. Quantification of vitamin C by HPLC:

The samples and standard of Vitamin C were analysed by HPLC separation with column C18 (250×4.6) mm internal diameter (id). The mobile phase was acetonitrile (ACN) 100% with a flow rate of 0.5 ml/min. Injection volume for sample and standard solution was 10 mL. The pH was adjusted to 3.5. The detection occurred at UV light at 290 nm wave length.

The using of HPLC analysis to measure the degree of Vitamin C purity, the results showed the purification method efficiency, especially for the *Mint leaves*, the retention time were appeared, single peaks was 3.100 (graph 4) and when compared with the retention time of the standard Vitamin C (graph 3), so the peak is represented the Vitamin C because of closely related with that of standard curve.

Graph 2: HPLC standard curve for Vitamin C



Graph 3: HPLC analysis of *Mint leaves* samples

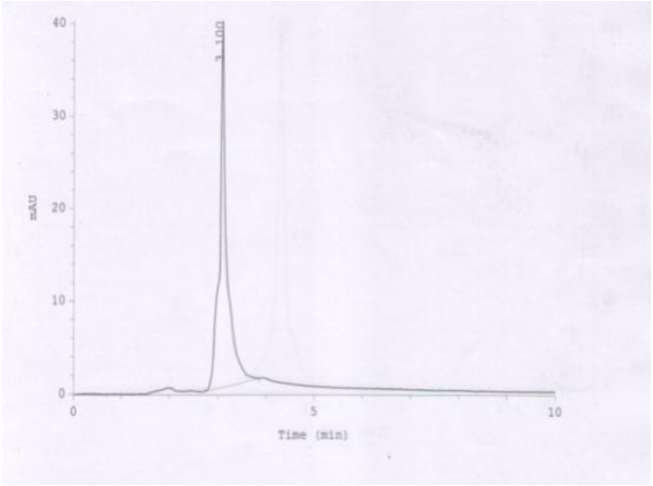


Table 1: Standard Vitamin C analysis by HPLC

Ret.time [min]	Start [min]	End [min]	Compound	Amount	Units	Area [mAU*min]	Height [mAU]	Width [min]
2.983	2.5	3.62		0		1.50567	15.631	0.058

Table 2: Sample of mint leaves analysis by HPLC

	Ret.time [min]	Start [min]	End [min]	Compound	Amount	Units	Area [mAU*min]	Height [mAU]	Width [min]
1.	3.100	2.73	3.87		0		6.67605	39.5656	0.083

5. Report of in vitro anti-cancer activity for the given extract

The given extract does not show any cytotoxicity against the normal (Rat skeletal muscle cell line) L6 cell lines. It showed 59.65±0.1452.23±0.17 percentage of cytotoxicity and 45.35±0.17 percentage of cell viability at the very high dose i.e. 3500 µg/ml. the extract showed IC 50 Value of 3500 µg/ml. In conclusion the given extract does not show any cytotoxicity against L6 cell line.

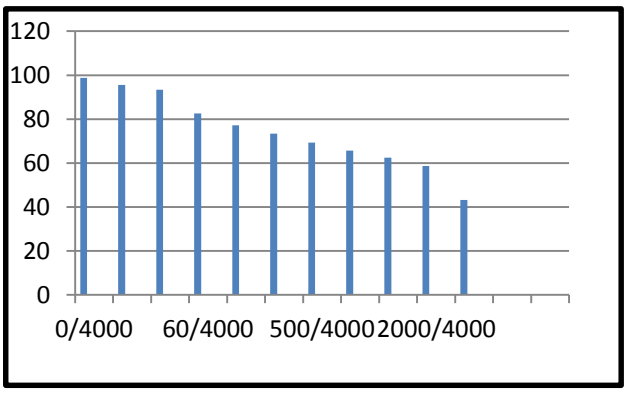
Table 3: Valuation of Cytotoxicity and Cell Viability for EXTRACT on L6 cell lines:

S.no	Conk (µg/ml)	% Cell Viability	% Cytotoxicity	IC 50 Value (µg/ml)
1.	Control	93.15±0.18	1.22±0.19	
2.	15	92.35±0.24	4.56±0.52	
3.	30	90.24±0.30	8.90±0.28	
4.	60	88.42±0.17	15.25±0.22	
5.	120	80.32±0.15	19.65±0.22	
6.	240	76.33±0.24	27.33±0.26	3500
7.	500	72.42±0.26	29.32±0.30	
8.	1000	66.42±0.28	35.38±0.26	
9.	1500	61.32±0.28	39.42±0.20	
10.	2000	55.38±0.36	46.38±0.30	
11.	4000	45.35±0.17	59.65±0.17	

Graph 2 : Concentration Vs. %cell viability

X-axis: Concentration

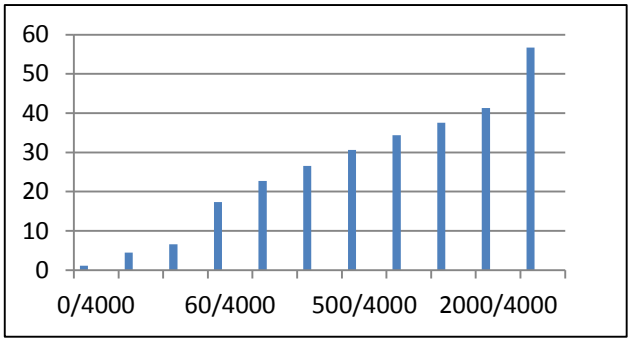
Y-axis:%cell viability



Graph 2(a)- Concentration Vs. % cytotoxicity

X-axis: Concentration

Y-axis: % cytotoxicity



The Extract showed significant anti-cancer activity against the HT-29 cell line. The control group showed only 3.82±0.04 percentage of cytotoxicity and it showed 96.32±0.01 percentage of cell viability. The extract at the dose of 15 µg/ml showed 19.56±0.21 percentage cytotoxicity on gradual increase in the dose, the extract showed gradual increase in the percentage of cytotoxicity. At the dose of 4000 µg/ml the extract killed almost 99.48±0.19 percentage of HT-29 cell lines. It showed only about 0.50±0.42 percentage of cell viability at the dose of 3500 µg/ml. the IC50 value was found to be 120 µg/ml.

Table 4: Evaluation of Cytotoxicity and Cell Viability for EXTRACT on HT-29 cell lines using MTT assay:

S.no	Conk (µg/ml)	% Cell Viability	% Cytotoxicity	IC 50 Value (µg/ml)
1.	Control	96.32±0.01	3.82±0.04	
2.	15	85.34±0.28	21.34±0.19	
3.	30	77.65±0.32	26.21±0.38	
4.	60	57.42±0.25	38.29±0.27	120
5.	120	47.32±0.19	58.43±0.20	
6.	240	35.28±0.32	69.52±0.29	
7.	500	27.42±0.34	75.58±0.35	

8.	1000	18.65±0.39	86.38±0.27	
9.	1500	10.18±0.33	90.54±0.57	
10.	2000	5.20±0.18	96.86±0.22	
11.	3500	0.50±0.42	99.48±0.19	

IV. CONCLUSION

The Chinese Mint leaves flower of Scutellaria barbata D .Don was found to have anti-oxidant and anti-cancer activity . Initially the crude vitamin was extracted and purified. Then Vitamin C was separated using gel filtration followed by HPLC quantification was done. The vitamin C showed potent cytotoxicity against the HT-29 cell line when compared to the control group. We concluded that the extract showed, it does not affect the normal cell lines. The IC50 value was found to be 120 µg/ml. In conclusion the extract has dose dependent cytotoxicity against the HT-29 cell line and not showed Rat skeletal muscle L6 cell lines.

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