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AQUEOUS EXTRACT OF AERIAL PARTS OF *CENTELLA ASIATICA* ARRESTS CELL CYCLE AT G0/G1 ON COLORECTAL CANCER CELL LINES

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ABSTRACT

Purpose: To investigate the cytotoxicity and cell cycle analysis of aqueous extract of aerial parts of *Centella asiatica* on colorectal (HCT116) cancer cell lines.

Approach: *Centella asiatica* extract was subjected to preliminary phytochemical screening and in the next stage, brine shrimp lethality assay was carried out. MTT assay was also performed on colorectal cancer cell lines to assess the in vitro cytotoxicity activity of the plant extract. Then, cell cycle arrest was also done as a part of investigation using Flow cytometry.

Findings: Phytochemical screening revealed the presence of various vital phytoconstituents like saponins, glycosides, tannins, triterpenoids, flavonoids and polyphenols. Aqueous extract of the aerial parts of *Centella asiatica* markedly increases the mortality in brine shrimp lethality assay. Further, the extract exhibited cytotoxicity on human colorectal cancer cell lines and cell cycle arrest at G0/G1 phase.

Originality: This research work throws light into the usage of the plant as an alternative to conventional chemotherapy of cancer. In vitro brine shrimp lethality assay, cytotoxicity and cell cycle analysis were all performed for the first time using aqueous extract of *Centella asiatica* aerial parts.

Conclusion: It can be concluded that the aqueous extract of aerial parts of *Centella asiatica* showed antioxidant & anticancer activities and even arrested the cell cycle at G0/G1 phase.

Key words: *Centella asiatica, brine shrimp lethality assay, MTT assay, cell cycle, HCT116, flow cytometry.*

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INTRODUCTION

Cancer affects the most of the population worldwide and the numbers of cases grew tremendously in the in the last few years. Various treatment strategies are available to treat cancer viz,. chemotherapy, radiation and surgical interventions. Several adverse drug reactions are reported with the conventional chemotherapy and this has made the researchers to look back into traditional system of medicines[1]. Various forms of herbal extracts in the form of tinctures, churnas and other kinds of crude extracts were used to treat the affected ones. Colorectal cancer is highly resistant to chemotherapy, but there is still no effective cure for patients with advanced stages of the disease. To explore the possibility of alternative treatment to cancer, researchers start using medicines from herbal origin[2]. Anticancer properties/activities have been reported in several plant species and selection of plant is based on ethno-medical knowledge and testing the selected plant efficacy as well as safety is one of the best approaches for the isolation of anticancer molecules as leads[3]. Plant derived natural products such as flavonoids, terpenoids and steroids etc. have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and antitumor activities[4]. In the herbal world, many Indigenous plants like black pepper, asafoetida, pippali, and garlic are quoted to be useful in various forms of cancer. One more such plant is the “Centella asiatica” belonging to family “Umbelliferae” is well known for its memory enhancer, wound healing and as an antidepressant in the Indian system of medicines[5]. In the

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literature, it was suggested that plant exhibits induction of cancer cell apoptosis and inhibition of in vivo tumor promotion and in vitro invasion of Human cancer cell lines such as cervical cancer cell lines (HeLa), breast cancer lines (MDAMB-231)[6-8]. Centella asiatica contain asiatic acid (AA) a pentacyclin triterpinoid has a wide range of biological activities. For a long period of time, AA was mainly believed to be responsible for wound healing; protection against UV induced photo aging[9]. Also Asiatic acid induce apoptosis and cell cycle arrest in several types of cancer in rats. Recently, the apoptosis inducing activity of AA in various cancer cells has drawn the attention of investigators. Presence of triterpene and steroids were also reported in the plant. Reports also suggest the possibility of AA in cell growth inhibition in breast cancer, hepatoma, glioblastoma, melanoma and gastrointestinal tumor cells[7]. The plant also appears to have antioxidant activity and ability to stimulate collagen synthesis for tissue regeneration. Some of medicinal properties of the plant are attributed to the presence of antioxidant principles such as phenolic and flavonoidal constituents[10,11]. Antioxidant principles derived from plants are reported to have antitumor activity. Hence the plant containing flavonoids are constantly being screened for antitumor activity and moreover AA found in the plant induces colon cancer cell growth inhibition and apoptosis through mitochondrial death casecade mechanism[12,13]. There are many indications that free radicals are implicated in patho physiological conditions including cancer[14]. Since dietary source is also one of the main source for medicines and they have been poorly investigated. Reports suggest the possibility of dietary source in scavenging free radicals as well as in cancer chemo preventive activities including neuroblastoma[15]. Further several lead compounds from the herbal source such as genistein (soybeans), lycopene (tomatoes), brassinin (cruciferous vegetables), sulforaphane (asparagus) are in preclinical/clinical trials. Centella asiatica contain many phytoconstituents including flavonoids and triterpenoids[16]. These constituents present in Centella asiatica have very powerful cytotoxic, antioxidant and anticancer activities[5,14]. Furthermore, the plant of the present study is extensively used in various forms of tumors. However, the details pertaining to its activity against colorectal cancer (HCT 116) is not yet clearly understood. Keeping all these in view, the study was proposed.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified eagle medium (DMEM), Trypsin-EDTA, Hank's Balanced Salt Solution (HBSS) and Foetal bovine serum (FBS) were procured from High media. Thiazolyl Blue Tetrazolium Bromide (MTT), Propidium iodide (PI), doxorubicin, DPPH, DMSO, Tris-HCl etc. were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell Lines

HCT-116 (colorectal cancer cell lines) was procured from National Centre for Cell Science (NCCS) Pune, India.

Plant Material

Plant material (Centella asiatica (CA) aerial parts) was collected from local gardens of Himachal Pradesh and was authenticated by Prof. Lakshmaya, Director of Pharmacy, GRD(PG)IMT, Dehradun. The aerial parts were shade dried at room temperature and pulverized.

Preparation of Extracts

Dried aerial parts were submitted to decoction (10% w/v, plant: water) for 15-20 min at a temperature of around 100°C to obtain the aqueous fruit extract of CA (yield: 8.40% relative to dry plant). The aqueous extract obtained was kept under refrigerated conditions until use.

Phytochemcial Screening

Preliminary phytochemical screening was performed as per the available standard protocol C[17,18] to identify the various phytoconstituents present in the extract.

Brine Shrimp Lethality Assay[19-21]

The brine shrimp lethality assay was carried out to investigate the cytotoxicity of the drug. Freshly hatched brine shrimp larvae (nauplii) were used as test organisms. The different concentrations of the plant extract 1-5000 µg/ml (wt/vol) was prepared in distilled water and used against brine shrimp larva in different sets. Artificial sea water medium was used as control. Different concentrations of drug along with 10 shrimp larva was added to each well of the micro titer plate and incubated at room temperature (30-37°C) for 24h. The numbers of dead larva in each well were counted under the light microscope. Then analyze the data by probit analysis and lethal concentration (LC_{50}) was calculated.

$$\text{Percent mortality} = \frac{\text{No. of dead nauplli}}{\text{Total no. of nauplli}} \times 100$$

$$\text{Corrected \% mortality} = \frac{(M_{obs} - M_{control})}{(100 - M_{control})} \times 100$$

In-Vitro Cytotoxicity[22,23]

Maintenance of cell lines

Cell lines were procured from NCCS Pune and grown in 25 cm^2 tissue culture flasks containing Dulbecco's modified eagles medium (DMEM) supplemented with 10% FBS, 1% (wt/vol) L-glutamate and 50µg/ml gentamycin sulphate at 37ºC in Co_2 incubator in an atmosphere of humidified 5% CO_2 and 95% air. The cells were maintained by routine sub culturing in 25cm^2 tissue culture flasks. Cell viability was checked with a small sample of the suspension by tryphan blue dye exclusion test. Then they were further trypsinzed and tumor cells from the first and second passage of transplantation were stored in liquid nitrogen in cryo vials containing

DMEM medium supplemented with 10% FBS and 10% DMSO as preservative at a concentration of 1x 10^6 cells /ml. This constituted the tumor bank. After every 10 crossings, that tumor cell line was discarded and new passage was started using the original tumor cells from the tumor bank.

Evaluation of cytotoxicity by MTT Assay[24,25]

This is a colorimetric assay to measure the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase. The MTT enter the cells and then passes into the mitochondria where it is reduced to an insoluble, dark purple colored formazan product. The cells were mixed with isopropyl alcohol and the released solubilized formazan was measured spectro-photometrically. The reduction of MTT can only occur in metabolically active cells. One T-25 flask was trypsinized and added 5 ml of complete media to trypsinized cells. Cells were counted and recorded as cells per ml. Cells were diluted to give 75,000 cells per ml. Then added 100 µl of cells (7500 total cells) into each well of 96 well plate and the plates were incubated overnight. The cells were treated after 24h with standard or drug, media has been removed carefully. Final volume should be 100 µl per well and incubation was continued for further 24h. After 24h, MTT reagent 20 µl of 5 mg/ml was added to each well. in such a way that one set of wells with MTT but control with untreated cells, MTT and solubilizing buffer. The plates were incubated for 3.5 hours at 37 °C in culture hood. Remove the media carefully. Do not disturb cells and do not rinse with PBS. 80 µl DMSO was added. Covered with a foil and the cells were agitated on orbital shaker for 15 m. Finally the absorbance was read at 540 nm using ELISA reader.

Calculation

Percentage cytotoxicity calculated from the formula: [(AC - AB) - (AT - AB)]/ (AC –AB) *100

Where AC, AT and AB are absorbance of control, test and blank respectively. IC_{50} values can be determined from the dose response curves, by linear regression method.

Cell Cycle Analysis using Flow cytometry[26]

1 x 10^6 cells were seeded in 25cm^2 flasks and after overnight adherence, incubated with indicated test compounds for 24h. The adherent cells were detached by Trypsinization and wash once in 5-10ml 1X PBS to remove residual serum and trypsin. Cell number per sample should be 1-2x10^6 cells. Cells were spin around 1400 rpm for 5min at temperature 4ºC. The cells were then washed once in 5-10ml 1X PBS. Each cell pellet was resuspended in 0.5ml 1X PBS. The tubes were vortexed and 4.5ml ice cold 70% ethanol was added drop wise gently over 30 second to a mute. The cells were incubated at -20ºC overnight. Again cells were spin at 1400 rpm for 5min at 4ºC. Supernatant was removed. Cells were washed twice with 5-10ml 1X PBS. Cells were pellet down at 1400 rpm for 5 m at 4ºC. Remove the supernatant and resuspended cells in 0.5ml PI staining solution (or 1X PBS solution for –PI negative controls). Then the cells were incubated for 20 min at 37ºC. Filtered and stained cells were analyzed using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using excitation at 488nm and emission at 575/40nm and data analysis was done by using BD Accuri™ C6 software.

RESULTS

Preliminary Phytochemical Screening

The preliminary phytochemical screening showed the presence of various phytoconstituents in the extract. The extract had given positive tests for saponins glycosides,

Brine Shrimp Activity

The cytotoxic activity of plant extract was determined using brine shrimp lethality assay. The cytotoxicity levels (LC_{50}) of the drug was found to be 67.3µg/ml. The results were shown in the Table 1 and graphically depicted in Fig.1.

Table 1 : *Brine Shrimp Lethality Activity of aqueous extract of CA*

S.No.	Conc.	Log conc.	Dead Napulli	Alive Napulli	Death %	Probit Value	Corrected %
1.	4	0.602	2	8	20	4.16	11.1
2.	9	0.954	3	7	30	4.48	22.2
3.	19	1.278	4	6	40	4.75	33.3
4.	78	1.89	5	5	50	5.00	44.4
5.	156	2.19	6	4	60	5.25	55.5
6.	312	2.44	7	3	70	5.52	66.6
7.	625	3.09	7	3	70	5.52	66.6
8.	1250	3.39	8	2	80	5.84	77.7
9.	2500	3.69	9	1	90	6.18	88.8

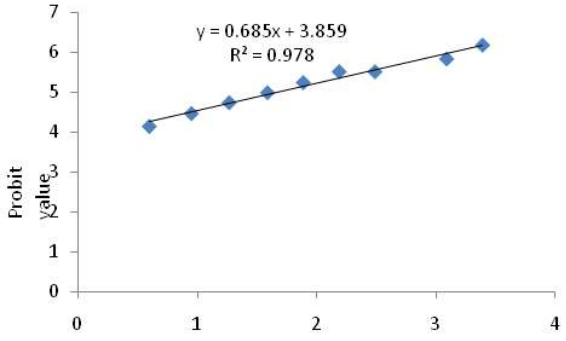

Fig. 1 : *Determination of LC_{50} of aqueous extract of CA*

In –Vitro Cytotoxicity

In this study, we examined the cytotoxicity of Centella asiatica aqueous extract on colorectal (HCT-116) cancer cell lines using MTT assay. Doxorubicin, a commonly

used drug in the treatment of wide range of cancers was used as a positive control in our cytotoxic study.

Colorectal cancer cell lines (HCT116)

In human colorectal cancer cell lines the maximum cell death was found to be at 200µg/ml. The IC_{50} was found to be > 200µg/ml. This was depicted in Table 2a & 2b and graphically presented in Fig. 2a & 2b.

Table 2a : *IC_{50} value of aqueous extract of CA on HCT116 after 48 h*

Conc. µg/ml	Absorbance at 540 nm			% Cell Death			% Cell viability			Mean Cell Viability ± SEM	IC50 µg/ml
25	0.192	0.212	0.228	15.4	6.6	-0.4	84.6	93.4	100.4	92.8±4.6	
50	0.169	0.220	0.227	25.6	3.1	0.0	74.4	96.9	100.0	90.5±8.1	> 200
100	0.164	0.174	0.177	27.8	23.3	22.0	72.2	76.7	78.0	75.6±1.7	
200	0.147	0.148	0.144	35.2	34.8	36.6	64.8	65.2	63.4	64.5±0.5	

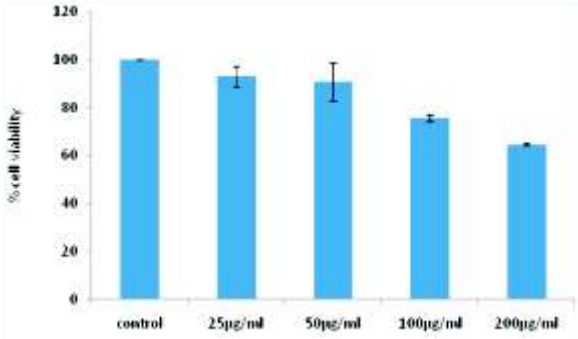

Fig. 2a : *Bar graph showing the effect of aqueous extract of CA on cell viability of colorectal cancer cells after 48 h*

Table 2b : *IC_{50} value of Doxorubicin on HCT-116 after 48 h*

Conc. µg/ml	Absorbance at 540 nm			% Cell Death			% Cell viability			Mean Cell Viability ± SEM	IC50 µg/ml
0.05	0.205	0.214	0.210	9.7	5.7	7.5	90.3	94.3	92.5	92.4 ± 1.1	
0.5	0.180	0.145	0.130	20.7	36.1	42.7	79.3	63.9	57.3	66.8 ± 6.5	4.5
5	0.094	0.088	0.099	58.6	61.2	56.4	41.4	38.8	43.6	41.3 ± 1.4	
50	0.079	0.099	0.067	65.2	56.4	70.5	34.8	43.6	29.5	36.0 ± 4.1	

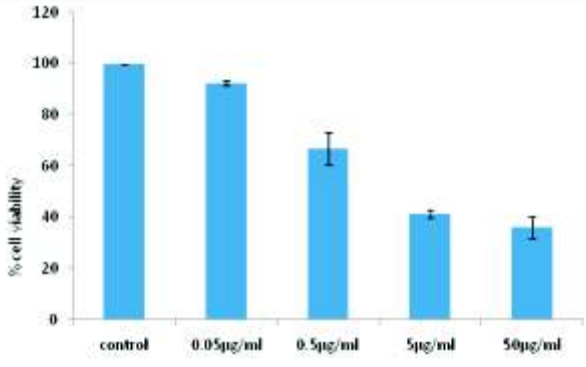

Fig. 2b : *Bar graph showing the effect of Doxorubicin on cell viability of colorectal cell lines (HCT-116) after 48 h*

Cell Cycle analysis by flow cytometry

The aqueous extract of Centella asiatica significantly inhibits the growth of colorectal cells in a dose dependent manner. It was observed that normal control shows maximum percentage of cells in G0/G1 phase and it was found to be 70.3 % and aqueous extract of Centella asiatica (100µM) shows accumulation of cells about 79.6%. The results were depicted in Table 3a & 3b and graphically represented in Fig. 3a & 3b

Table 3a : *Effect of Normal control on Human colorectal cancer cell cycle (Plot 3)*

Phases of cell cycle	Count	Volume (µL)	% of this plot	% of All	Mean FL2-A	CV FL2-A
Plot	3,630	13	100.00	8.09	490,839.04	30.14%
G0/G1	2,551	13	70.28	5.69	405,336.66	6.53%
S	425	13	11.71	0.95	569,119.29	9.11%
G2/M	674	13	18.57	1.50	767,162.68	7.94%

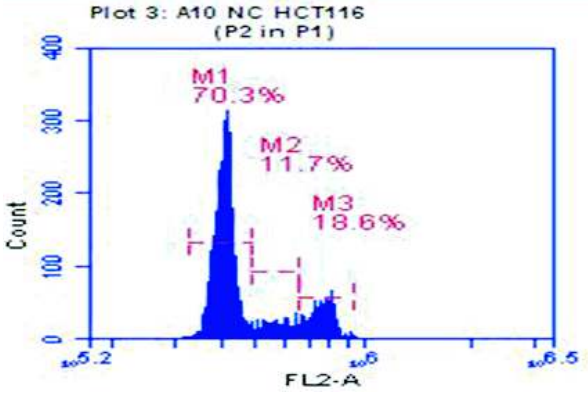

Fig. 3a : *Histogram of propidium iodide v/s cell count of normal control*
(M1=G0/G1 phase, M2=S phase, M3=G2 phase, NC=Normal Control)

Table 3b : *Effect of Aqueous extract of CA on colorectal cancer cell cycle*

Phases of cell cycle	Count	Volume (µL)	% of this plot	% of All	Mean FL2-A	CV FL2-A
Plot	4,134	4	100.00%	29.52 %	474,811.05	28.27 %
G0/G1 phase	3,291	4	79.16 %	23.50 %	415,663.00	6.76 %
S phase	304	4	7.35 %	2.17 %	585,250.96	9.33 %
G2/M phase	530	4	12.82 %	3.78 %	787,519.42	7.62 %

(Plot 3)

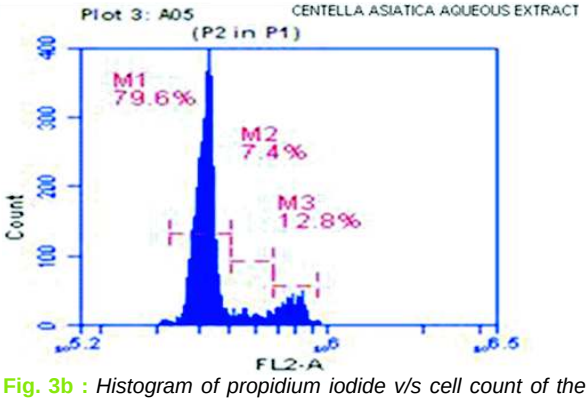

Fig. 3b : *Histogram of propidium iodide v/s cell count of the aqueous extract of aerial parts of CA*

DISCUSSION

C. asiatica has been used since ancient times to treat wide range of disease. It is extensively used to treat neurodegenerative disorders and even possesses cardioprotective, radioprotective, hepatoprotective, and antitumor activities etc[27]. The plant possesses different phytochemicals with different biological activity. Different phytochemicals like saponins, tannins, flavonoid and polyphenols present in the plant may help in protecting various chronic diseases[28]. Since the plant possesses various useful active principles, it was proposed to study the anticancer potential of this plant.

In the first phase, the plant extract was subjected to preliminary phytochemical screening and it was observed that the extract revealed the presence of tannins, polyphenols, flavonoids, triterpenoids, and saponins. In the next phase of the study, it was planned to check the lethality level of the extract at different concentrations by brine shrimp lethality assay. It was clear from the Fig.1 that the cytotoxicity level of the plant extract (LC_{50}) was found to be 67.3µg/mL. This indicated the toxicity level of the drug and lethality was increased in a concentration dependent manner. Diet rich in antioxidant reduces oxidative damage to DNA. Chemopreventive was a good approach for controlling the growth of cancer, which involves the use of certain natural or synthetic product to inhibit the cancer growth. Tocopherol, vitamin-C, quarcetine, vitamin-E, β-carotene etc. are the various natural sources from which antioxidants are being obtained to control the growth of cancer. Furthermore, our plant showed rich concentrations of antioxidant principles like asiaticoside A, asiaticoside B, madecassic acid and asiatic acid. The asiaticoside present in the plant is responsible for anticancer properties[29,30]. Keeping all these active principles of the plant in view, it was further planned to investigate its effects on cytotoxicity and cell cycle blocking effects (anticancer activity).

In the final phase of the study, we have investigated the anti-cancer activity of aqueous extract of *Centella asiatica,* the popular indigenous medicinal plant against human colorectal cancer cell line (HCT116). In MTT assay for the evaluation of cytotoxicity of the extract, it was observed that the aqueous extract showed dose-dependent cytotoxicity activity against HCT116 cells. The IC_{50} value of aqueous extract was found to be greater than 200 µg/mL indicating cytotoxic effects on colorectal cancer cells. Anti-cancer drugs from many sources are designed to eliminate rapidly growing cancerous cells, and therefore, they tend to exhibit cytotoxicity and induce apoptosis in cancer cells[31]. Apoptosis is well organized cell death characterized by loss of cell membrane phospholipid asymmetry, enzymatic cleavage of the DNA into oligonucleosomal fragments, and ultimately the segmentation of the cells into membrane-bound apoptotic bodies[32]. Majority of anti-cancer drugs are known to cause their effects by preventing/arresting cell cycle[33]. Cancer cell cycle specific drugs have shown considerable value as they act on specific cancer cell cycle checkpoints (G0/G1 phase, S phase, G2/M phase) and inhibit cancer cell proliferation (G0/G1 phase arrest or DNA replication (diminished S phase) or mitosis (G2/M phase arrest). In this, we investigated the cell cycle specific pharmacological effects of aqueous extract against human colorectal cancer cells (HCT116) by propidium iodide (PI) based cell cycle analysis using flow cytometry. The data revealed that more number of cell population is accumulated at G0/G1 phase and hence extract caused a significant arrest of cells at G0/G1 phase (M1). However further studies are required to confirm apoptosis.

CONCLUSION

From the results and discussion, it can be concluded that the aqueous extract of *Centella asiatica* arrests cell cycle at G0/G1 phase.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Tiwari Sushma, Gehlot Sangeeta. Centella Asiatica: A Concise Drug Review with Probable Clinical Uses. Journal of stress Physiology & Biochemistry. 2011; 7: 38-44.
2. Jamil S Shakir, Nizami Qudsia. Centella asiatica (Linn.) Urban A Review. Natural Product Radiance. 2007;6 (2):158-170.
3. Renehan AG, Bach SP, Potten CS. The relevance of apoptosis for cellular homeostasis and tumorigenesis in the intestine. Can J Gastro Enterol. 2001; 15:166-76.
4. Gupta Y.K., Verendra Kumar M.H., Srivastava A.K. Effect of *Centella asiatica* on pentylene tetrazole-induced kindling, cognition and oxidative stress in rats. Pharmacology Biochem and Behav. 2003; 3: 579-585.
5. Johnson IT. Anticarcinogenic effects of diet related apoptosis in the colorectal mucosa. Food Chem Toxicol. 2002; 40:1171-8.
6. King RW, Deshaies RJ, Peters J-M, Kirschner MW. How proteolysis drives the cell cycle. Science. 1996; 274:1652-9.
7. Junli Zhang, Lisha Ai1 et al. Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022.
8. Maquart FX. et al. Triterpenes from *Centella asiatica* stimulate extracellular matrix accumulation in rat experimental wounds. Eur J Dermatol. 1999; 9: 289-296.

9. Babu TD and Paddikkala J, Cytotoxic and anti-tumor properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban, J Ethnopharmacol. 1995; 48(1): 53-57.
10. Park BC, Paek SH, et al: Inhibitory effects of Asiatic acid on 7, 12-dimethylbenz [a] anthracene and 12-O-tetradecanoylphorbol 13-acetate-induced tumor promotion in mice. Biol Pharm Bull. 2007; 30: 176-179.
11. Smith M and Boon HS: Counseling cancer patients about herbal medicine. Patient Educ Couns. 1999; 38: 109-120.
12. Lee YS, Jin DQ, Kwon EJ, et al: Asiatic acid, a triterpene, induces apoptosis through intracellular Ca2+ release and enhanced expression of p53 in HepG2 human hepatoma cells. Cancer Lett. 2002; 186: 83-91.
13. Tang XL, Yang XY, Jung HJ, et al: Asiatic acid induces colon cancer cell growth inhibition and apoptosis through mitochondrial death cascade. Biol Pharm Bull. 2009; 32: 1399-405.
14. Singh RP, Dhanalakshmi S, Agarwal R. Phytochemicals as cell cycle modulators-ales toxic approach in halting human cancers. Cell Cycle. 2002; 1:156-61.
15. Biedler, J. L., Helson, L.et al. Morphology and Growth, Tumorigenicity, and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture, Cancer Research. 1973; 33: 2643-2652.
16. Mori H, Sugie S, Yoshimi N, Hara A, Tanaka T. Control of cell proliferation in cancer prevention. Mutat Res. 1999; 428:291-8.
17. Kokate CK, Practical Pharmacognosy, Vallabh Prakashan, 15th Ed, 2005.
18. Trease GE, Evans WC. Pharmacognosy, Saunders, 15th Ed, 2002.
19. Thiele CJ. Neuroblastoma: In (Ed.) Masters, J. Human Cell Culture. Lancaster, UK: Kluwer Academic Publishers. 1998; 1: 21-53.
20. Voute, P. Neuroblastoma. In: W. Sutow, D. Fernbach, and T. Vietti (eds.), Clincal Pediatric Oncology, St. Louis: Mosby, CV, 1984: 559.
21. Young, J., Ries, L., and Silverberg, E. Cancer incidence, survival and mortality for children younger than 15 years, Cancer 1986; 58: 598.
22. Biedler, J. L., Helson, L.et al. Morphology and Growth, Tumorigenicity, and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture, Cancer Research. 1973; 33: 2643-2652.
23. Gupta Y.K., Verendra Kumar M.H., Srivastava A.K. Effect of *Centella asiatica* on pentylene tetrazole-induced kindling, cognition and oxidative stress in rats. Pharmacology Biochemistry and Behaviour 2003; 3: 579-585.
24. Babu TD and Paddikkala J, Cytotoxic and anti-tumor properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban, J Ethnopharmacol. 1995; 48(1): 53-57.
25. Rai Neha, *Centella asiatica* Extract Exhibit Anticancer Activity against Different Types of Tumors: International Journal of Pure & Applied Bioscience. 2014;2 (1):122-127.
26. Schoene NW, Kelly MA, Polansky MM, Anderson RA. Water-soluble polymeric polyphenols from cinnamon inhibit proliferation and alter cell cycle distribution patterns of hematologic tumor cell lines. Cancer Lett. 2005;230:134-40.
27. Argal, A. and Pathak, A. KCNS activity of *Calotropis gigantean* roots. J. Ethnopharmacoogyl. 2006; 106: 142-145.
28. Augusti, K.T. and Cherian, S. Insulin sparing action of leuco pelargonidin derivative isolated from *Ficus bengalesis* Linn. Indian J. Exp. Biol. 2008; 33: 608-611.
29. Mandal, S.C., Maity, T.K., Das, j., Saba, B.P. and Pal, M. Anti-inflammatory evaluation of *Ficus racemosa* Linn. Leaves extract, J. Ethnopharmacol. 2009; 72:87-92.
30. Mondal SK, Chakaraborty G, Gupta M. In-vitro antioxidant activity of Diospyrous malabrica Kostel bark. Indian J ExpBiol. 2006; 44:39-44.
31. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Experimental Cell Research. 2000;256(1): 42-49.
32. Cotter TG. Apoptosis and cancer: the genesis of a research field. Nature Reviews Cancer. 2009;9: 501-507.
33. Stewart ZA, Westfall MD, Pietenpol JA. 2003. Cell-cycle dysregulation and anticancer therapy. Trends in Pharmacological Sciences 24(3):139-145.