Original Article

Evaluation of Cytotoxic and Anti-Tumor Activities of *Coscinium fenestratum* Extract

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ABSTRACT

Objective: To evaluate the anti tumor activity of *Coscinium fenestratum* extract under *in vitro* and *in vivo* conditions. **Materials and Methods:** The cytotoxic activity of *C. fenestratum* extract was tested on HeLa cells by MTT assay. The apoptosis-inducing activity of the extract was measured via mitochondtial membrane potential assay, acridine orange and ethidium bromide dual staining method, DNA fragmentation assay and caspase assay. The *in vivo* antitumor activity of the extract was tested against developed solid tumor in mammalian system. **Results:** The extract showed significant anti-proliferative activity and is dose dependent. The extract treatment clearly showed an increasing number of apoptotic events both in HeLa cells and DLA cells and collapsed the mitochondrial membrane potential and these effects were higher than that of the positive control taxol. The cellular DNA was cleaved in to multiple fragments. Membrane blebbing and nuclear fragmentation was also observed after the extract treatment. *C. fenestratum* also triggered the release of mitochondrial cytochrome -c and activation of caspases 3 and 9 in HeLa cells, indicative of its apoptotic potential and was found to be highly effective in tumor regression. **Conclusions:** These finding suggested that the potential antitumor activity of *C. fenestratum* extract.

Keywords: *Coscinium fenestratum*, Anti tumor, Apoptosis, caspases, Cytochrome-c.

INTRODUCTION

Cancer has been a leading cause of death in the developed countries. With changing standards of living and food habits and also due to availability of curative treatment for many infectious diseases, cancer is surpassing other illnesses as a

principal cause of morbidity and mortality even in developing countries[1]. Surgery, radiotherapy and chemotherapy- are the established treatment modalities for various cancers which may be employed alone or in combination with the other two methods. Chemotherapeutic agents are of synthetic or natural products. Despite enormous progress in the field of organic chemistry, currently 25% of all prescription drugs are derived from natural sources. This is more significant with regard to anti-cancer drugs in which more than 80% are plant-derived compounds[2].

Antitumor agents can exert their cytotoxic effects in many ways but all the mechanisms lead to one of the two distinguishable basic forms of cell death: apoptosis or necrosis. Necrosis is a consequence of an intensive physical or chemical insult, resulting in rapid degradation of the cell and the liberation of inflammatory mediators which deteriorate the surrounding tissues[3]. On the other hand, apoptosis is a strictly regulated and programmed self-demolition of the cell; this is a general feature seen in multi-cellular organisms which is responsible for the elimination of damaged cells and also plays an important physiological role during embryonic development[4]. This mode of death is characterized morphologically by cellular shrinkage, nuclear condensation and increased membrane permeability, and biochemically by the internucleosomal cleavage of DNA, leading to an oligonucleosomal "ladder", phosphatidylserine externalization and proteolytic cleavage of a number of intracellular substrate proteins[5].

The different apoptosis signaling pathways induced by various anticancer drugs with distinct primary sub-cellular targets and mechanisms of action may converge on mitochondria to cause mitochondrial permeability transition (MPT), release apoptogenic factors from the mitochondrial intermembrane space (IMS) into the cytosol, activate a caspase proteolytic cascade that is amplified by a positive feedback loop involving the release of mitochondrial cytochrome *c* (Cyt-c) and ultimately, triggering internucleosomal DNA fragmentation[6].

Multidisciplinary scientific investigations are making the best efforts to combat cancer, but the sure and perfect cure is yet to be brought into world of medicine. An alternative solution to western medicine associated with severe side effects, is the use of medicinal plant preparations to arrest the insidious nature of the disease. Of the 92 anticancer drugs commercially available prior to 1983 in the United States, approved worldwide between 1983 and 1994, approximately 62% can be related to natural origins[7]. Hartwell's data show that about 3000 plants having anti-cancer properties has been used as potent anti-cancer drugs[8]. Among Indian Ayurvedic herbs, some 30 herbs have shown antitumor activities, and the number may rise as more and more herbs are studied[9]. Many herbs have been evaluated in clinical studies and are currently being investigated phytochemicaly to understand their tumoricidal actions against various cancers[10]. One of the best approaches in the search for anticancer agents from plant resources is the selection of plants based on ethnomedical leads[11].

Coscinium fenestratum Colebr. (Menispermaceae), commonly known as tree turmeric, grows widely in the Western Ghats of India and Sri Lanka. The plant has been mainly used for treating diabetes mellitus in the traditional Ayurvedic and Siddha systems of medicine[12]. The stem contains berberine, ceryl alcohol, hentriacontane, sitosterol, palmitic acid, oleic acid and saponin, together with some resinous material. Isolation of tertiary alkaloids, berlambine, dihydroberlambine

and noroxyhydrastinine from the roots has also been reported[13]. Berberin is known to exhibit multiple pharmacological activities such as antimicrobial activity towards bacteria, fungi and viruses, antimalarial, anti-inflammatory, antihypertension, reduce blood glucose level, reduce blood lipid, and antiproliferative activity[14-21]. It was also shown to inhibit the *in vitro* growth of a number of human cancer cell lines[22]. Hence in present study we sought to evaluate the cytotoxic effects and anti tumor activity of *C. fenestratum* extract.

MATERIALS AND METHODS

Animals

Male Swiss albino mice of 8-10 week old weighing 25-28g, were selected from inbred group maintained under standard condition of temperature (25 ± 5) and humidity. Animals were provided with food and water *ad libitum*. All experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), [Reg:No.-1999/CPCSEA] constituted by the Animal Welfare Division of Government of India.

Cell lines and culture

Dalton's lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adyar, Chennai and were maintained in mice by intraperitoneal inoculation of 1x 10^6 viable cells. Human cervical adenocarcinoma (HeLa) cell lines were procured from the National Centre for Cell Science, Pune, India. The cells were grown in Dulbecco's modified Eagle's medium. All the cell culture materials were from GIBCO/BRL Life Technologies Inc., Grand Island, NY, USA. Medium was supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The cells were maintained in a humidified incubator at 37°C in 5% CO_2 atmosphere.

Chemicals

MTT (3-(4,5-dimethylthiazol- 2-yl)-2, 5- diphenyl tetrazolium bromide), dithiothreitol , protease inhibitors, PIPES-KOH, horseradish peroxidise and β-actin were purchased from Sigma Chemical Company Inc., St. Louis, MO, USA. JC-1 stain was obtained from Molecular Probes (Eugene, OR). Cleaved Caspase-9 and Cleaved Caspase-3 antibodies were purchased from Cell Signaling Technology, Inc. USA. Cytochrome-C antibody was obtained from Santa Cruz Biotechnology Inc, Santa Cruz, CA. RNAse from HiMedia Laboratories, Mumbai, India. acridine orange, ethidium bromide, EDTA, KCL, MgCl2, manitol and sucrose was obtained from Merck India Ltd., Mumbai, India. All other chemicals were of analytical grade and procured from reputed manufacturers.

Preparation of the extract

Authenticated dried stem of *Coscinium fernestratum* (Collection No. 003820, Acc.No.3418) were obtained from Amala Ayurvedic Hospital and Research Centre, Thrissur, India. The stems were powdered and 100 g was extracted with 50 % ethanol at room temperature for 24 hours. Extract was filtered through Whatman No: 1 and the supernatant was evaporated in a rotary evaporator at 50^o C under vacuum and then lyophilized. The yield of preparation was 6 %. The high performance thin layer chromatography (HPTLC) finger printing of the extract was carried out using silica gel 60 F 254 plates of E. MERK with the solvent system butanol: acetic acid: water (5:1:4) at the detection wave length of 356 nm (Figure.1).

Cytotoxic effect of *C. fenestratum* on human carcinoma cells

The cytotoxic effect of *C. fenestratum* extract against HeLa was determined by a colorimetric assay, using 3-(4,5-dimethylthiazol- 2-yl)-2, 5- diphenyl tetrazolium bromide (MTT). It is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells, into an insoluble colored formazan product, which can be measured spectrophotometrically[23]. Briefly, 100 µl of cells (1×10^4 cells) were seeded in 96 well microplates and incubated for 24 hours (37^0 C, 5% CO2 air humidified). 10 µl of prepared concentrations of extract (25 µg/mL to 100 µg/mL) were added and incubated for another 72 hours in the same condition. Taxol (12 nM) was used as a positive control. The medium was aspirated and 100 µl of fresh medium containing of MTT (1mg/ml) was added and again incubated for 4 hours at 37^0 C followed by aspiration. MTT lysing solution 100 µl (Isopropanol with 4mM HCl) was added to this and the extent of MTT reduction was measured at 570 nm (formation of formazan) and 655 nm (reference) using a Microplate Reader (Model 680, BIO-RAD). The results were presented as means ± SD from three independent experiments.

Mitochondrial apoptosis detection by JC-1 staining method

HeLa cells were used for this study. The cells were seeded in 24 well plates. The cells are treated with and without *C. fenestratum* extract (50 µg/mL and 100 µg/mL) and incubated it for 24 hours. The JC-1 stain (5 µg/mL) containing medium is added to the cells, followed by incubation for 15 min at 37°C and then washed with PBS, followed by adding fresh medium without serum. Finally the cells were observed and photographed under fluorescent (Nikon, Japan) microscope[24].

Morphological analysis of apoptotic cells by DNA double staining method

For quantification of apoptosis by morphologic criteria, DLA cells were stained with both acridine orange and ethidium bromide, as a modification of a standard assay[25]. The treated or untreated cells ($1x10^6$ cells/ml of media) were incubated for 0-8 hours at standard conditions (37^0 C and 5 % CO_2). After incubation about 500µl of the cells were taken and centrifuged at 1500 rpm for 2-3 minutes. After centrifugation discard the supernatant and 60 µl of the pellets were taken. To this added 100 µg/mL of acridine orange (AO) and ethidiuum bromide (EB) at a ratio of 1:1. The suspension was immediately (fast uptake) examined by fluorescence microscopy. Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA.

Counted minimum of 250 total cells, recording the number of each of the following four cellular states: (1) Live cells with normal nuclei (bright green chromatin with organized structure); (2) Live cells with apoptotic nuclei (bright green chromatin which is highly condensed or fragmented and membrane blubbing); (3) Dead cells with normal nuclei (bright orange chromatin with organized structure); (4) Dead cells with apoptotic nuclei (bright orange chromatin which is highly condensed or fragmented along with and membrane blubbing).

DNA fragmentation assay

HeLa cells were plated in 10cm dishes and were treated with the extract for 72 hours. The cells were harvested in the medium and centrifuged for 10 minutes at 2000 rpm. The pellets were then suspended In 1.0 mL PBS and mixed well and again subjected to centrifugation at 2000 rpm for 5 minutes. The lysing solution was then added to the pellet and incubated it for 15 minutes. After incubation it was subjected to centrifugation for 5 minutes at 1500 rpm and the supernatant was separated. To this added equal volume of phenol chloroform isoamyl alcohol, mixed well and incubated for 5 minutes. The mixture was then centrifuged for 10 minutes at 12000 rpm and the upper layer was separated and added double volume of ethanol and $1/10^{th}$ volume of potassium acetate. Mixed by inversion and kept overnight at -20^0C. Centrifuged at 12000 rpm for 15 minutes and then washed with 70 % ethanol and centrifuged at 12000 rpm. Discarded the supernatant, air dried and 50 µL of TE buffer was added (Tris HCl 10mM & EDTA 1mM). It was then treated with 4µl of RNAse (20mg/ml) for 1 hour followed 4µL of protease (20mg/ml) for an hour. DNA was finally subjected to agarose gel electrophoresis at 80V for 1 hour and the laddering was observed under UV illumination[26] (Molecular Imager Gel Doc XR Imaging System- BIO-RAD).

Preparation of mitochondrial and cytosolic fractions

For the determination of Cytochrome-c, HeLa cells were treated with 200 µg/mL *C. fenestratum* extract for 48 hours in 10 cm dishes followed by extracting cytosolic and mitochondrial fractions. Briefly, cells were washed twice with ice-cold PBS, pH 7.4 and scraping in PBS which followed by centrifugation at 2000 x *g* for 5 minutes. The cell pellet was then resuspended in 600 µL extraction buffer containing 200 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, and 1 mM dithiothreitol and protease inhibitors(1X). Cell suspensions were then incubated briefly with 100 µg/mL digitonin and homogenates were centrifuged at 12,000 x *g* for 10 minutes at 4°C. The supernatant (cytosolic fraction) as well as pellet (enriched for nuclei and mitochondria) were recovered. Protein concentration was measured using Bradford reagent, and samples were denatured in standard SDS sample buffer[27].

Detection of caspases

HeLa cells were seeded in 6 well plates and were treated with the extract for 48 hrs. After the incubation period the medium was removed and cells were lysed by adding SDS sample buffer followed by scraping and boiling in tubes.

Western blot analysis

Proteins resolved[28], transferred and detected by following a standard protocols[29]. Cell extracts were resolved by electrophoresis on a 10% SDS-polyacrylamide gel and transferred on to Nitrocellulose membranes (0.2 µM, Bio-Rad). For Cytochrome c 12% SDS Polyacrylamide gel was used. Membranes were blocked with 5% nonfat milk in PBS-Tween and subsequently probed with antibodies directed against cytochrome-c and cleaved caspase-3 and 9. After washing the membranes were incubated with horseradish peroxidase-conjugated antimouse or antirabbit antibodies (Jackson Immuno Research Laboratories, Inc). Proteins were detected with the enhanced chemiluminescence (ECL) reagents (Amersham, GE Healthcare Life Sciences). Further the membranes were reprobed with β-actin (Sigma) antibody to determine the equal protein loading level.

Effect of *C. fenestratum* in tumor regression

Animals were divided into two groups of six animals each. Viable DLA cell ($1x10^6$ in 0.1ml PBS) were transplanted subcutaneously into the right hind limb of mice. After 15 days, animals with tumor size around 1.1± 0.1 cm^3 were divided into two groups of six animals in each group. *C. fenestratum* extract (250, mg/kg bw, p.o) was administered for 7 consecutive days. The group received only the cell line served as the control. The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier calipers twice a week for 4 weeks.

RESULTS

Cytotoxic effect of *C. fenestratum* extract

MTT assay was used to demonstrate the cytotoxic activity of *C. fenestratum* extract *in vitro*. Taxol was used as a positive control. Data presented in figure 2 clearly showed that *C. fenestratum* extract has a marked inhibitory effect on HeLa cells and is concentration dependent. The percentage of survival was getting lower as the concentration is increased. At a concentration of 100 µg/mL, the extract had around 50% toxicity to the tumor cells.

Effect on mitochondrial apoptosis

JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazol-carbocyanine iodide) is a lipophilic fluorescent cationic dye that incorporates into the mitochondrial membrane, where it can form aggregates due to the physiological membrane potential of mitochondria. This aggregation changes the fluorescence properties of JC-1 leading to a shift from green to orange fluorescence. In healthy cells, the negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. With the increasing of the concentration of JC-1, it aggregates and become fluorescent red. When the mitochondrial potential collapses in apoptotic cells, JC-1 just exists as monomers and do not accumulate within the mitochondria. When dispersed in this manner, JC-1 remains in the cytoplasm in a green fluorescent monomeric form. It was observed from the present experiment that the cells treated with different concentrations of *C. fenestratum* extract exhibited intense green fluorescence when compared with that of the untreated cells which appeared in colour (Figure 3). From this it was clear that the cells became apoptotic after treatment with the extract. The intensity of green fluorescence was much less in the taxol treated cells when compared to that of the extract treated cells indicative of the effect of *C. fenestratum* in apoptotic induction.

Detection of apoptotic morphology by DNA double staining method

Treatment of DLA cells with *C. fenestratum* extract resulted in significant morphological changes, indicating the cytotoxic action of *C. fenestratum* extract was due to its ability to induce apoptosis (Figure 4). From the data presented in table.1 it was found that the extract induced apoptosis in time and concentration dependent manner.

DNA fragmentation assay

In order to detect DNA fragmentation, DNA samples were isolated from HeLa cells after 72 hours of incubation with the extract and separated by agarose gel electrophoresis. As shown in figure 5, after incubation of cells with the extract, oligonucleosome ladder pattern was displayed. This data indicated that the cellular DNA was cleaved into multiple

fragments upon apoptosis induction by *C. fenestratum* extract.

Induction of cytochrome-c release and caspase activation

From the data presented in figure 6, it was found that *C. fenestratum* triggered the release of mitochondrial Cyt-c in HeLa cells. Because the release of Cyt-c from the mitochondrial IMS (intermembrane space) into the cytosol, which may be a limiting factor in caspase-9 activation and represents a central coordinating step in apoptosis, the extract can activate the caspase proteolytic cascade compared to the untreated controls. The 15-kDa bands of cytosolic Cyt-c became increasingly visible 48 hours after treatments (Figure 6).

The data presented in figure 7 confirmed that the release of Cyt-c corresponded with the activation of pro-caspase-9. Release of Cyt-c coincided with the processing and activation of pro-caspase-9 event that is known to occur in a cytosolic complex termed the apoptosome, which consists of cytochrome *c*, procaspase-9, and the adaptor protein, apoptotic protease-activating factor-1. Apoptosis induction by the extract was also determined by caspase-3 activation, which is the key downstream effector caspase proteolytically activated by the initiator caspase-9, and the result clearly indicated the activation of caspase-3 by the extract in HeLa cells.

Effect of *C. fenestratum* extract on tumor regression

The extract was also found to be highly effective against developed solid tumor. Treatment with the extract at a dose of 250 mg/kg body weight for 7 consecutive days after tumor development, showed substantial regression in tumor volume as compared to the control animals (Figure 8).

DISCUSSION

Combating cancer is of paramount importance. To this end various chemical compounds are used today. Cytotoxic compounds are one of the most important classes of drugs used for cancer treatment. There have been several attempts to get new cytotoxic agents. In this regard compounds such as colchicine, *Vinca* alkaloids and paclitaxel isolated from medicinal plants showed considerable promise. Many phytochemicals have the power to inhibit cell proliferation and also to suppress the promotion and progression cancer [30]. Our present study mainly deals with the anti cancer activity of the ethanolic extract of *C. fenestratum* both under *in vitro* and *in vivo* conditions.

MTT assay was used to evaluate cytotoxicity based on metabolic reduction of MTT. The purpose of the study was to determine whether this extract has cytotoxic effect against cancer cells. MTT based cytotoxic assay was carried out using human carcinoma (HeLa) cells. The ethanolic extract exhibited significant growth inhibition of the tumor cell lines.

Recently, it was shown that one of the mechanisms of action of several antineoplasic drugs is by induction of apoptosis[31,32]. Apoptosis is a regulated cell death used by multicellular organisms to dispose redundant cells. It is morphologically and biochemically characterized by cell shrinkage, cell membrane blebbing, nuclear chromatin condensation, and nonrandom DNA fragmentation. This type of death is altered in many pathological states and is indispensable for elimination of “sick” or not normal cells in organisms[33]. From the present experiment it was clear that the extract induced apoptosis in tumor cell lines, more over it also altered the membrane potential of mitochondria which in turn lead to the release of cytochrome-c as an

indication of caspase activation. The caspase-cascade system plays essential roles in the apoptosis. Caspases are cysteineaspartic protease in a family of cysteine proteases. Caspases exist as inactive proenzymes known as procaspases that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small subunits that dimerize to form the active enzyme. Sequential activation of caspases played a central role in the execution phase of cell apoptosis[34,35]. So we further examined the activation of caspase 9 and 3 which in turn down regulate the apoptotic pathways and it was observed that *C. fenestratum* extract induced activation of theses caspases in tumor cell lines indicative of its apoptotic activity.

An apoptosis-induction capacity rather than necrosis induction is accepted as a key feature of a potential antitumor drug. The morphological changes were detected by AO/EB double staining with fluorescence microscopy. Typical markers, including cellular shrinkage, nuclear condensation and an increased membrane blebbing, were observed after the extract treatment. In parallel with the morphological changes, the other important features of apoptosis i.e., fragmentation of nuclear DNA were also detected.

Tumor volume is one of the important criteria for direct or indirect anticancer activity. The *in vivo* experiment revealed tumor regression potential of the extract as shown by the small size of tumor volume in animals bearing solid tumors. These results suggest that the *C. fenestratum* extract contain compounds that may modulate tumorigenesis at different stages or may act at the same stage. Berberin is one of the major constituent of *C. fenestratum* extract. It was reported that, berberin has a strong anti proliferative activity[22]. Hence the presence of berberin may be one of the reason for the anti tumor activity of the extract.

It was observed that tumor cells produced more peroxides when they proliferate actively. This rise in peroxides indicated the occurrence of intensification of oxygen free radical production[36]. Cells which are equipped with enzymatic antioxidant mechanisms play an important role in the elimination of free radicals. High levels (up to 0.05 µmol/h per 10^4 cells) of H_2O_2 are constitutively released from a wide variety of human tumors[37]. Our previous study has clearly shown that *C.fenestratum* extract has a strong antioxidant and anti inflammatory activity. Hence it can be assumed that the significant antioxidant and anti-inflammatory activity of the extract may also contribute to its significant antitumor and anticancer property. Hence *C. fenestratum* extract possibly provides additive or synergistic effect in the prevention and treatment of cancer.

CONCLUSIONS

The results obtained from the present study indicate that the *C. fenestratum* extract exhibit a combination of anticancer activities both under *in vivo* and *in vitro* conditions. Hence the *C. fenestratum* extract can be regarded as a promising starting material for the development of future anticancer agents.

ACKNOWLEDGEMENT

The authors express their gratitude to KSCSTE, Thiruvananthapuram , Kerala, India for the financial support provided to them as a research grant to CKKN and and Research fellowship to DS. The authors also thank The Deanship of King Saud University, Riyad, Saudi Arabia for funding the publication of this research group No (IRG14-05).

Conflict of interest

The authors declare that there are no conflicts of interest.

REFERENCES

1. Notani P. Global variation in cancer incidence and mortality. *Curr Sci* 2001; 81 (5): 465-74.
2. Aliabadi HS, Amrollah A. Cytotoxicity and antitumor properties of a marine compound, hesa-a, on cancer cells. *Daru J Pharm Sci* 2003; 11:82-87.
3. Festjens N, Berghe T V, Vandenabeele P. Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. *Biochim Biophys Acta* 2006; 1757:1371-87.
4. Weingärtner J, Lotz K, Faltermeier A Driemel O, Kleinheinz J, Gedrange T, Proff P. The role of apoptosis in early embryonic development of the adenohypophysis in rats. *Head Face Med* 2008; 4: 4-13.
5. Cruchten VS, Van Den BW. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anat Histol Embryol* 2002; 31: 214-23.
6. Nagata S. Apoptotic DNA fragmentation. *Experiment Cell Res* 2000; 256: 12-18.
7. Gordon MC, Newman DJ. Natural product drug discovery in the next millennium. *Pharm Biol*. 2001; 39:8.
8. Balachandran P, Govindarajan R. Cancer- an Ayurvedic perspective. *Pharmacol Res* 2005; 51:19-30.
9. Ramakrishnan Y, Manohor AI, Mamata P, Shreekant KG. Plants and novel antitumor agents. A review. *Ind Drugs* 1984; 21: 173-85.
10. Premalatha B, Rajgopal G. Cancer – an ayurvedic perspective. *Pharmacol Res* 2005; 51:19-30.
11. Spiridon KE. Terrestrial plant-derived anticancer agents and plants used in anticancer research. *Crit Rev Plant Sci* 2006; 25:79-113.
12. Varier PS. Coscinium fenestratum In: Indian Medicinal Plants, Compendium of 500 species. Orient Longman (Ed), Hyderabad, India.1994:191–193.
13. Datta SC, Mathur RK, Baruah JN. Minor alkaloids of Coscinium fenestratum root. *Ind Drug* 1988; 25:350.
14. Freile ML, Giannini F, Pucci G, Sturniolo A, Rodero L, Pucci O, Balzareti V, Enriz RD. Antimicrobial activity of aqueous extracts and of berberine isolated from Berberis heterophylla. *Fitoter* 2003; 74(7-8): 702-05.
15. Hayashi K, Minoda K, Nagaoka Y, Hayashi T, Uesato S. Antiviral activity of berberine and related compounds against human cytomegalovirus. *Bioorg Med Chem Lett* 2007; 17 (6): 1562-64.
16. Iwasa K, Nishiyama Y, Ichimaru M Moriyasu M, Kim HS, Wataya Y, Yamori T, Takashi T, Dong-Ung Lee DU. Structure-activity relationships of quaternary protoberberine alkaloids having an antimalarial activity. *Eur J Med Chem*1999; 34: 1077-83.
17. Lee CH, Chen JC, Hsiang CY, Wu SL Wu HC, Ho TY. Berberine suppresses inflammatory agents-induced interleukin-1β and tumor necrosis factor- α productions via the inhibition of IκB degradation in human lung cells. *Pharmacol Res* 2007; 56 (3): 193-201.
18. Liu JC, Chan P, Chen YJ Tomlinson B, Hong SH, Cheng JT. The antihypertensive effect of the berberine derivative 6-protoberberine in spontaneously hypertensive rats. *Pharmacol* 1999; 59 (6): 283-89.
19. Zhang H, Wei J, Xue R Wu JD, Zhao W, Wang ZZ, Wang SK, Zhou ZX, Song DQ, Wang YM, Pan HN, Kong WJ, Jiang JD. Berberine lowers blood glucose in type 2 diabetes mellitus patients through increasing insulin receptor expression, *Metabol* 2010; 59 (2): 285-92.
20. Zhao W, Xue R, Zhou ZX Kong WJ, Jiang JD. Reduction of blood lipid by berberine in hyperlipidemic patients with chronic hepatitis or liver cirrhosis. *Biomedicine and Pharmacother* 2008; 62 (10): 730-41.
21. Letasiov S, Jantova S, Cipak L, Múcková M. Berberine-anti proliferative activity in vitro and induction of apoptosis/necrosis of the U937 and B16 cells. *Cancer Lett* 2006; 239(3): 254-62.
22. Tungpradit R, Sinchaikul S, Phutrakul S, Wongkham W Chen ST. Antiproliferative Activity of Berberine from Coscinium

fenestratum on NCI-H838 Cell Line. *Chi Mai J Sci* 2011; 38: 85-94.
23. Mosmann T. Rapid colorimetric assay for cellular growth andsurvival: application to proliferation and cytotoxicity assays. *J Immunol Met* 1983; 65: 55-63.
24. Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not $DiOC_6$ (3) or rhodamine 123, is a reliable fluorescent probe to assess changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett* 1997; 411 (1): 77-82.
25. Duke RC, Cohen JJ. Morphological and biochemical assays of apoptosis. In Current Protocols in Immunology. vol. 1. J. Coligan, A.M. Kruisbeek, D.H. Argulies, E. M. Evach & W. Strober (Ed). John Wiley & Sons. New York. 1992; 3.17.1-3.17.16.
26. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nat* 1990; 348(6299): 334-36.
27. Jitkaew S, Trebinska A, Grzybowska E, Carlsson G, Nordström A, Lehtiö J, Fröjmark AS, Dahl N, Fadeel B. Nα-Tosyl-L-phenylalanine Chloromethyl Ketone Induces Caspase- dependent Apoptosis in Transformed Human B Cell Lines with Transcriptional Down-regulation of Anti-apoptotic HS1-associated Protein X-1. *J Biol Chemi* 2009; 284(41): 27827-37.
28. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nat* 1970; 227: 680-685.
29. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *PNAS* U. S. A 1979; 76: 4350-4354.
30. Shoba RI, Andallu B. Oxidative stress and cancer: Role of anti carcinogenic herbs and spices. *AJPCT* 2013: 3: 351-363.
31. Maldonado V, De Anda J, Melendez-Zajgla J. Paclitaxel-induced apoptosis in HeLa cells is serum-dependent. *J Biochem Mol Toxicol* 1996; 11(4): 183-88.
32. Melendez-Zajgla J, Garcia C, Maldonado V. Subcellular redistribution of Hsp 72 protein during cisplatin-induced apoptosis in HeLa cells. *Biochem Mol Biol Int* 1996; 40(2): 253-61.
33. Rathmell JC, Thompson CB. Pathways of apoptosis in lymphocyte development, homeostasis, and disease. *Cell* 2002; 109: S97-07.
34. Launay S, Hermine O, Fontenay M, Kroemer G, Solary E, Garrido C. Vital functions for lethal caspases. *Oncogene* 2005; 24: 5137-48.
35. Fan TJ, Han LH, Cong RS Liang J. (2005) Caspase family proteases and apoptosis. *Acta Biochim Biophys Sinica* 1997; 37: 719-27
36. Navarro J, Obrador E, Pellicer J. Blood glutathione as an index of radiation-induced oxidative stress in mice and humans. *Free Radic Biol Med* 1997; 22 (7): 1203-1209.
37. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 1991; 51 (3): 794-94.

Table 1. Effect of *C. fenestratum* extract on apoptotic morphology

Treatments	% dead cells	% apoptotic cells
0 hour		
Control	1.4	1.6
C. fenestratum (50µg/ml)	3.4	3.0
C. fenestratum (100µg/ml)	6.2	9.4
2nd hour		
Control	3	2.4
C. fenestratum (50µg/ml)	8	13.2
C. fenestratum (100µg/ml)	11	28.6
4th hour		
Control	5.4	5.4
C. fenestratum (50µg/ml)	12.2	25.6
C. fenestratum (100µg/ml)	17	36
8th hour		
Control	9.8	10
C. fenestratum (50µg/ml)	20	30.6
C. fenestratum (100µg/ml)	32.2	48.8

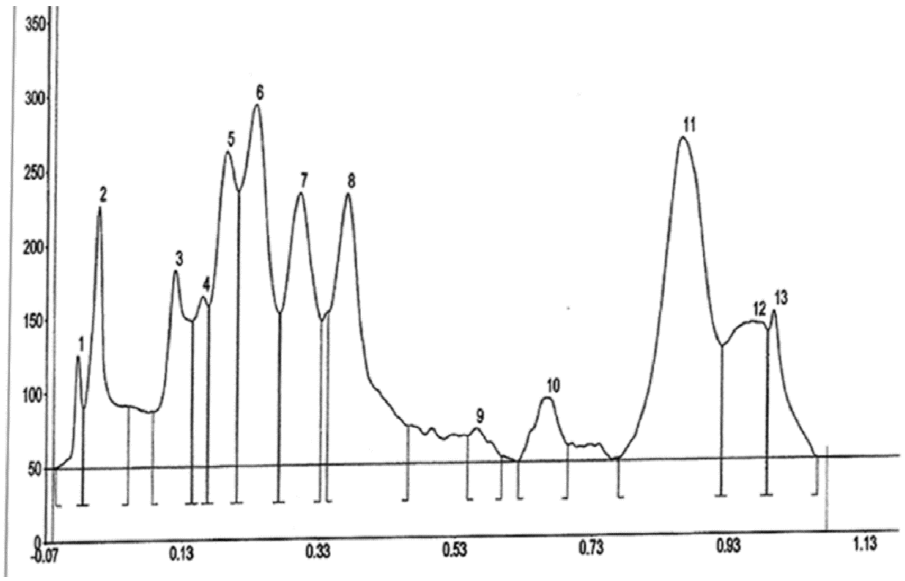

Figure 1. HPTLC profile of *C. fenestratum* extract

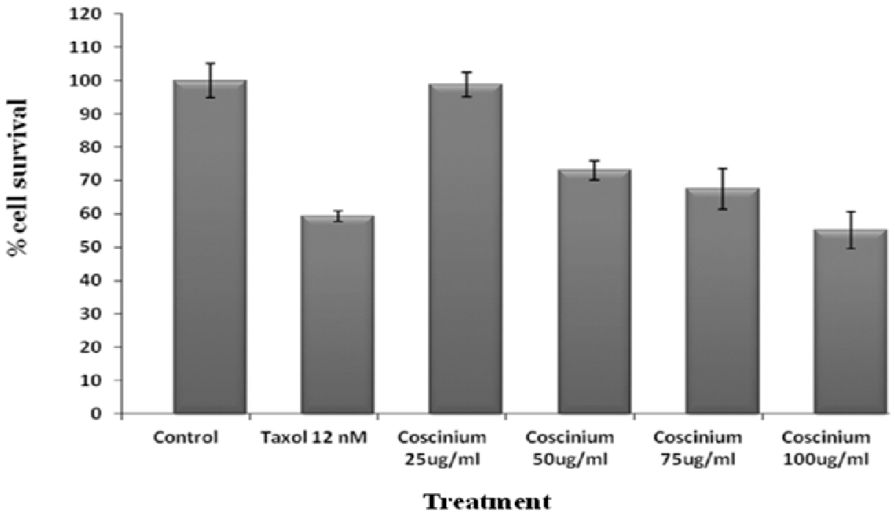

Figure 2. *C. fenestratum* extract induces growth inhibition in HeLa cells. The cells were incubated with different concentrations of extract for 72 hrs. A dose-dependent growth inhibition was observed at concentrations ranging from 25 to 100 µg/ml. Data are expressed as mean ± SD for three independent experiments

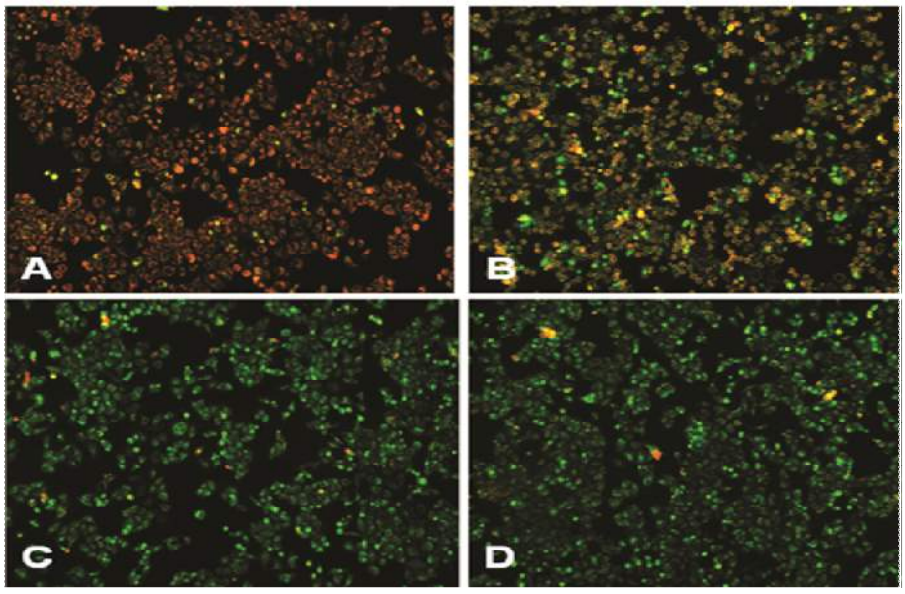

Figure 3. Fluorescence microscopy (mitochondrial membrane integrity by JC-1 staining) images showing appearance of HeLa cells for 24 hrs treatment. A) Control (DMSO), B) Taxol (12 nM), C) *C. fenestratum* Extract (25µg/ml) and D) (100 µg/ml). The illustrated fluorescence images are representative of three separate experiments

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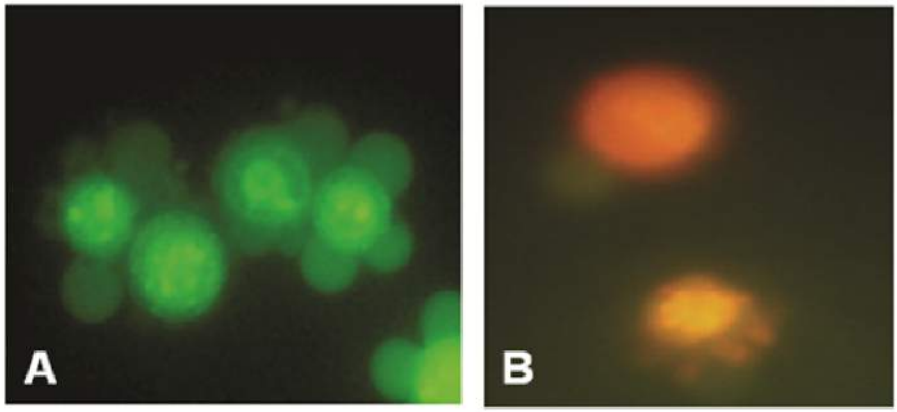

Figure 4. *C. fenestratum* extract induced apoptosis in tumor cell lines (DLA) detected by double staining method. (A) Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence (Live apoptotic cells), (B) Ethidium bromide is taken up only by nonviable cells and emits red fluorescence (Dead apoptotic cells)

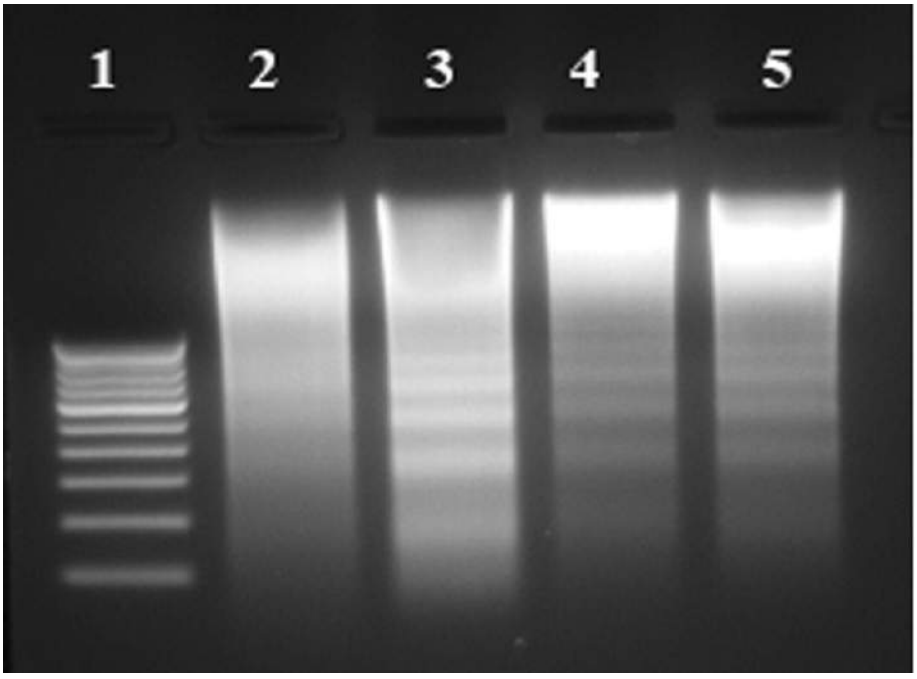

Figure 5. DNA fragmentation induced by *C. fenestratum* extract in HeLa cells (72hr).1). Marker, 2). Control, 3). Taxol (12 nM), 4). *C. fenestratum* extract (50µg/ml), 5). *C. fenestratum* extract (100µg/ml). DNA was stained with ethidium bromide after electrophoresis on a 2% agarose and then visualized under UV light. DMSO was used as the control

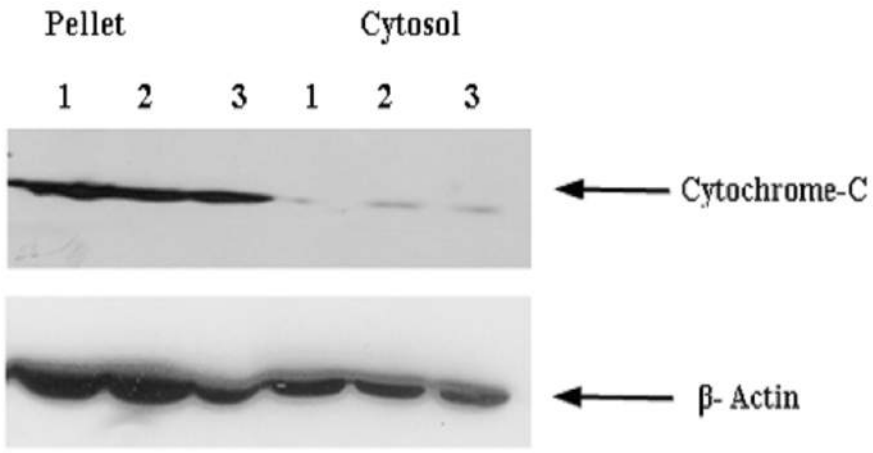

Figure 6. Effect of *C. fenestratum* extract on the release of cytochrome-c. The HeLa cells were treated with *C. fenestratum* extract for 48 hours and cytosolic fractions were used to detect cytochrome-c by western blot analysis. 1, Control (DMSO). 2. Taxol (25nM) 3.*C.fenestratum* extract (100µg/ml). The blots were also probed for β -actin as indicated to ensure that equal loading of protein

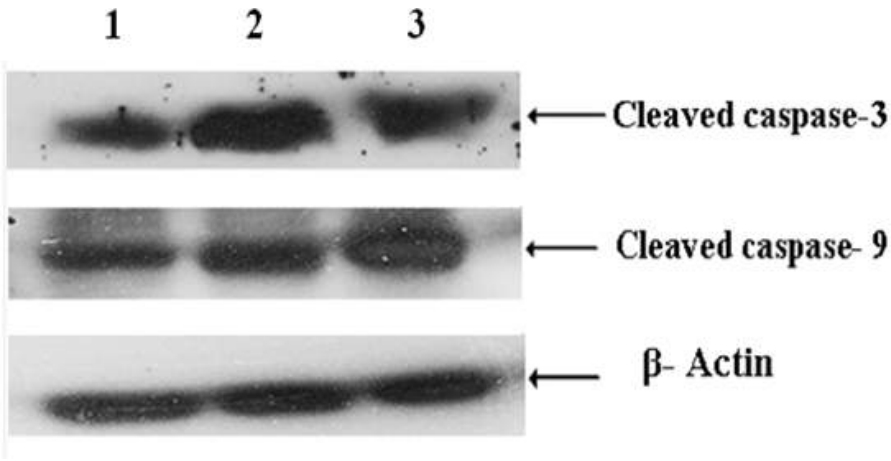

Figure 7. *C. fenestratum* extract induced activation of caspase 3 and 9 in HeLa cells. The cells were treated with the extract for 48 hrs and western blot analysis were carried out. The same blots were used to probe for β-actin was to detect equal amount of protein loading

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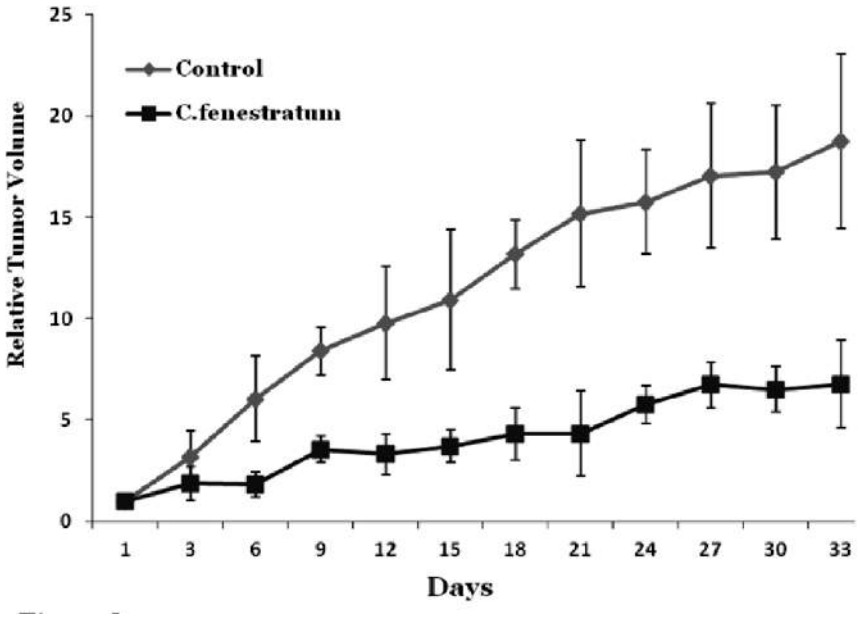

Figure 8. Effect of *C. fenestratum* extract (250 mg/kg) on tumor regression in mice. Each point represents mean ± SD