www.ijrps.pharmascope.org

ISSN: 0975-7538
Research Article

In vitro anti- cancer activity of *Solidago canadensis* L.

Ravichandiran V, Deepa N*

College of Pharmacy, Vels University, Old Pallavaram, Chennai – 600117, Tamil Nadu, India

ABSTRACT

The In-vitro anti - cancer activity of various extracts viz., Hexane, Chloroform, Ethyl acetate and 50% Aqueous – ethanol of the whole plant of *Solidogo canadensis* L was studied by Tryphan blue dye exclusion technique. And further the ethyl acetate extracts, which showed best response and activity against the tested extracts was subjected to MTT- assay and apoptosis screening with the cell lines HeLa and MCF-7. All extract showed potential cytotoxicity comparable to that of the standard drug against the tested cell lines viz., HeLa and MCF-7. The Ic50 values of the ethyl acetate extract was found to be best with 150 and 210 µg/ml with Hela and MCF-7 respectively in tryphan blue dye exclusion technique. In MTT- assay it was found to be 170 and 275 µg/ml with Hela and MCF-7 respectively. In apoptosis screening ethyl acetate (EA) extracts showed an apoptosis dose dependently in both the tested cell lines viz., HeLa and MCF-7. Ethyl acetate extract at high concentration (500µg/ml) showed apoptosis of cells representing nuclear fragmentation with 42% and 30.33% apoptotic cells against viz., HeLa and MCF-7 respectively. Thus, confirms the potent invitro anti-cancer property of the EA extract against the tested cell lines viz., HeLa and MCF-7. Hence, the present study brought to light the scientific validation of the plant *Solidogo canadensis* L. With respect to cytotoxicity. Further investigations with the ethyl acetate extract may lead a way for the identification of the entity responsible for this potential property.

Keywords: *In vitro* anticancer activity; *Solidago canadensis L.; cytotoxicity*

INTRODUCTION

Solidago canadensis L. belongs to the family Asteraceae, widely distributed across North America, occurring in almost every state of USA and throughout Canada, India, etc. Numerous interesting secondary metabolites such as flavonoids, tri-terpenoids, saponin, phenolic acids, glucosides, polysaccharides, diterpenes and essential oils (Thiem B. et al., 2001) were reported for the genus *Solidago.* Earlier investigations on the plant *Solidago canadensis* have leads to the isolation of flavonoids (Apáti P et al., 2003 and Krepinsky J et al., 1962), phenolic acids (Kalemba D et al., 1992), sesquiterpenes (Bohlmann F et al., 1980), diterpenes (Anthonsen T et al., 1969 and Reznicek G et al., 1990) and saponins (Reznicek G et al., 1990). The flowers of the plant were used in traditional American practice as an analgesic (Rousseau J et al., 1945), burns and ulcer treatment (Arnason T et al., 1981), febrifuge (Smith H. H et al., 1933), GIT (Moerman D et al., 2000 and Turner N et al., 1980) and liver (Moerman D et al., 2000) aids. In European phytotherapy for the treatment of chronic nephritis, cystitis, urolithiasis, rheumatism and as an anti-phlogistic (O'Brien J et al., 2000). In spite of the wide spread use of *S. canadensis* and phytoconstituents reported, there hardly exists any documentation on the pharmacological profile of the plant. Hence in the present study an attempt was made to illustrate the in-vitro anti-cancer potential of the plant *S. canadensis L.*

* Corresponding Author
Email: deepanatarajan@yahoo.com
Contact: +91-9841266372
Received on: 13-11-2011
Revised on: 11-01-2012
Accepted on: 27-01-2012

EXPERIMENTAL

MATERIAL AND METHODS

Plant material: The fresh plant material (whole plant) was collected fresh from the rain forest areas of Tirunelveli district and Ooty / Tamil Nadu during June 2008. And its authenticity was confirmed by Survey of Medicinal Plant Unit, Siddha. C.C.R.A.S. Govt. of India, Palayamkottai, Tirunelveli-627 002. Tamil Nadu, India. The voucher specimen of the plant *Solidago canadensis* was deposited in the herbarium (Number: D - 01062008) of the Department of Pharmacognosy, Vel's college of pharmacy, old pallavaram, Chennai - 600 117. The cell lines for the study were procured from standard laboratory maintained at Amala cancer research institute, Amala nagar, Tirussur, Kerala. And used for the study.

Preparation of plant extracts

Freshly collected plant material (whole plant) was dried in shade, then coarsely powdered. One kg of powder was extracted in an aspirated bottle with Hexane, Chloroform, Ethyl acetate and 50% aqueous –

ethanol by the cold maceration process for 3 – 7 days. All extracts were filtered through Whatmann filter paper no. 1 and evaporated on a water bath and finally dried in a vacuum to get the residue. This residue of all extracts was suitably diluted with DMSO (Dimethyl sulfoxide) to get a final concentration of 1000 micrograms / ml and used for the study.

Cell lines used: **HeLa and MCF-7**

Cyto-toxicity studies

Cytotoxicity studies involve the analysis of morphological damage or inhibition of the zone of outgrowth induced by the chemicals tested.

Trypan blue dye exclusion assay (Lillie, R.D., 1977)

Transferred 700 µl of a cell suspension to 24 well plates and incubated for 24 hours in 5% CO_2. After incubation added 300 µl of drug with varying concentration of EA (25-400 µg/ml) and incubated for 48 hours. 100 µl of cell suspension was treated with 100 µl of 0.4% Trypan Blue Solution in an Eppendorf tube and mixed thoroughly and allowed to stand for 5 to 15 minutes. Transferred a small amount of trypan blue-cell suspension mixture to both chambers of a hemocytometer. The number of stained and unstained cells are counted in one square mm area. Non-viable cells will stain blue. Keep a separate count of viable and non-viable cells. Then the percentage inhibition is calculated using the formula:

$$\%\ Inhibition = \frac{Number\ of\ non\ viable\ cells\ (Stained)}{Total\ Cells\ (Stained\ and\ Unstained)} \times 100$$

Assay for Proliferation Studies

MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) method (Scudiero DA, 1988)

MTT assay (Scudiero DA, 1988)

Materials: RPMI-1640, (Himedia, Mumbai India), TRYPSIN-0.25% (Gibcos USA), FBS (Fetal bovine serum) (Gibcos USA),MTT MTT 4mg/ml (Himedia), DMSO (Emerck India, Lysis buffer (15%SLSin 1:1DMFand water), Composition of RPMI; 9.54 g/lit, 10%FBS, 2000mg sodium bicarbonate, 250 l each of penicillin (60mg/ml), streptomycin (100mg/ml), Amphotericin (200mg/ml).

Cell lines used: HeLa -Cervical carcinoma cancer cell and MCF-7-human breast cancer cells.

Method

0.1ml of the cell suspension (containing 1 X10^5cells) and 0.1ml of the **EA** (31.25-500 µg/ml) in DMSO such that the final concentration of DMSO in media is less than 1%) were added to the 96 well plates and kept in carbon dioxide incubator with 5% CO_2, at 37^0 C for 72 hours. Blank contains only cell suspension and control wells contain 1% DMSO and cell suspension.

After 72 hours, 20 (l of MTT was added and kept in a carbon dioxide incubator for 2 hours followed by 80 (l of lysis buffer (15%SLSin 1:1 DMF and water). The plate was covered with aluminium foil to protect it from light. Then the 96 well plates are kept in rotary shaker for eight hours.

After 8 hours, the 96 well plates were processed on ELISA reader for absorption at 562nm. The readings were averaged, and viabilities of the test samples were compared with DMSO control.

The percentage growth inhibition was calculated using the following formula,

$$\%\ Growth\ Inhibition = 100 - \frac{Mean\ OD\ of\ Individual\ Test\ Group}{Mean\ OD\ of\ Control\ Group} \times 100$$

Apoptosis (Bergan, R., 1996)

Hoechst staining and photo-microscopy

To analyse the morphological apoptotic changes, 1 X 10^5 cells seeded in 96-well plates (37^0 C, 5% CO_2), when *logarithmic growth phase of cells was reached, the EA* with final concentration of 500 and 750 µg/ml or 0.1% DMSO (negative control) was added, respectively. After 48 h the cells were washed in phosphate-buffered saline (PBS) and stained for 10 min at room temperature in PBS containing 40% paraformaldehyde and 10 mg/ml Hoechst. HeLa cells for Hoechst staining were grown on the sterilized cover slips and processed as described (Kerr JF., 1994.), with modifications. Briefly, after washing one time with PBS, cells were fixed with 3.7% formaldehyde in PBS for 10 min, washed one time with PBS, stained with 0.4 mg/ml Hoechst (Molecular Probes, Eugene, OR) in PBS for 15 min, washed two times with PBS, and then one time with water. Cover slips were then air-dried and mounted with Slow Fade (Molecular Probes) mounting media. Morphological evaluations of nuclear condensation and fragmentation were performed immediately after staining by the fluorescent microscope (Olympus, Japan) at 550 nm of emission.

RESULTS & DISCUSSION

Tryphan Blue Dye Exclusion Technique

Cytotoxicity studies by trypan blue exclusion method is a very simple method which can be carried out within a short time of 48 hrs. It is a precise method, which takes in order to account on the viable and also the dead cells in addition to estimation of IC_{50} concentration. The IC_{50} of EA was found to be 210 & 290 µg/ml against HeLa and MCF-7 cell lines respectively (Table 1). Among the tested cell lines, extract of EA was more selective cytotoxic against the HeLa cell line than MCF-7.

MTT Assay

Scientific strategies for the *in vitro* evaluation of natural products with biological activity have changed in the past few years. One recent development is the highly automated bioassay screening based on colorimetric methods that quantify the proliferation of cell cultures. Many biological assays require the measure-

ment of surviving and/or proliferating in mammalian cells. This can be achieved by MTT assay, a non – radioactive, fast and economical assay widely used to quantify cell viability and proliferation.

MTT is a yellow water soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water insoluble dark blue farmazan by reductive cleavage of the tetrazolium ring. MTT is cleaved by all living, metabolically active cells but not by dead cells or erythrocytes. The amount of farmazan generated is directly proportional to the cell number over a wide range, using a homogenous cell population.

In the present study, the cytotoxic activity of EA using two human cancer cell lines [i.e. cervical carcinoma (HeLa) and breast cancer (MCF-7)] were evaluated with MTT assay. When the cells were treated for 72 hours with various concentrations of EA (31.25-500 μg/ml), the relative cell survival progressively decreased in a dose dependent manner.

The IC_{50} of the extract of EA was found to be 170 & 275 μg/ml (Table 2) on HeLa and MCF-7 cell lines respectively. Among the tested cell lines, extract of EA was more selective cytotoxic against the HeLa cell line than MCF-7.

Apoptosis

Apoptosis, or programmed cell death, is a highly conserved, tightly controlled cell suicide process that is regulated by many different intracellular and extracellular events to ablate neoplastic cells in normal physiological functions. Apoptosis is controlled by two potential pathways, the mitochondrial pathway and the death receptor pathway. The mitochondrial pathway is characterized by the loss of mitochondrial trans membrane potential and release of cytochrome *c* [Hacker.H.,2007.]. The death receptor pathway is mediated by serial activation of Fas [a cell surface death receptor of the tumor necrosis factor (TNF) family of cytokines]. Apoptosis as an intrinsic suicide serves to remove excess, damaged or infected cells in metazoans. It's known that apoptosis is characterized by a variety of morphological and biochemical events, including phosphatidylserine (PS) externalization, chromatin condensation, genomic DNA fragmentation, and plasma-membrane blebbing (Jacobson MD, 1997; White E, 1996).

It occurs under physiological conditions (e.g., tissue and organ development, tissue maintenance), and dysregulated apoptosis has been associated with autoimmune disease and cancer (Kerr JF, 1972, Wyllie AH., 1992). Toxic stimuli capable of inducing apoptosis in susceptible cells include irradiation, DNA-damaging drugs, and activation of the Fas antigen (Kerr JF, 1994). The importance of understanding apoptosis is underscored further by the fact that the "therapeutic index" of different anti-neoplastic therapies may correlate with the differential capacity of tumor and normal cells to undergo apoptosis (Itoh M., 1991). "Necrosis" is a form of cell death that differs from apoptosis (Fisher DE., 1994). Such death usually results from overwhelming damage to cells, leading to their death without the involvement of a genetically encoded suicide program (Majno G., 1995).

The nature of accelerated Ho342 staining of apoptotic cells has been controversial. Most likely, alterations in the plasma membrane permeability (passive inward diffusion) account for these finding (Lillie R.D., 1977), but diminished rates of active dye extrusion by the P-glycoprotein pump (2, 3) and structural changes of the chromatin (Lillie R.D, 1977) may also contribute to it.

Among the morphological features differentiating this form of cell death from apoptosis are Hoechst fluorescences compared with untreated cells. Extensive apoptotic alterations were observed *in* EA (500 μg/ml) that increased over time following the induction of apoptosis.

We assessed the nuclear morphological changes by Hoechst staining and Plate 3, more than 40% of HeLa cells underwent apoptosis upon a single dose (500μg/ml) of the drug after 48 h and fragmented nuclei were detected by fluorescence. Cells treated with high (500 μg/ml) doses of the drug were prone to apoptosis after 48 h. In Plate 1 & 2, the viable cells are uniform, whereas the apoptotic cells are red and contain bright red dots in their nuclei, representing the nuclear fragmentation (Plate 3).

Morphological effects observed in MCF-7 cells treated in the same experimental conditions. Hoechst fluorescence assay was employed to detect typical DNA condensation. Morphological changes induced in MCF-7 cells by extract treatment were evidenced by using PI staining and fluorescent microscopy. At 48 h of treatment with 500 μg/ml, we observed a marked increase in the number of MCF-7 cells with the typical features of apoptosis, consisting in cells with condensed and fragmented nuclei (Plate 4). Moreover, many cells were starting to detach from the substrate (not shown).

Graphs shown the percentage of apoptic cells after staining with Hoechst from these data, EA at 500 μg/ml concentration shows 42 % and 30.33% apoptic cells against HeLa & MCF-7 respectively (Table 3). EA was produced dose dependent apoptosis in HeLa & MCF-7 cells.

We utilized several methods to measure the effect of the EA on HeLa cell apoptosis. After treatment with the EA for 48 h, morphologic study by PI staining detected the typical morphology of apoptosis from cultured HeLa & MCF-7 cells, chromatin condensation, and nuclear fragmentation (Plate 3 and 4). Condensed chromatin and fragmented nuclei could also be found in many treated cells, which are classic characteristics of apoptotic cells.

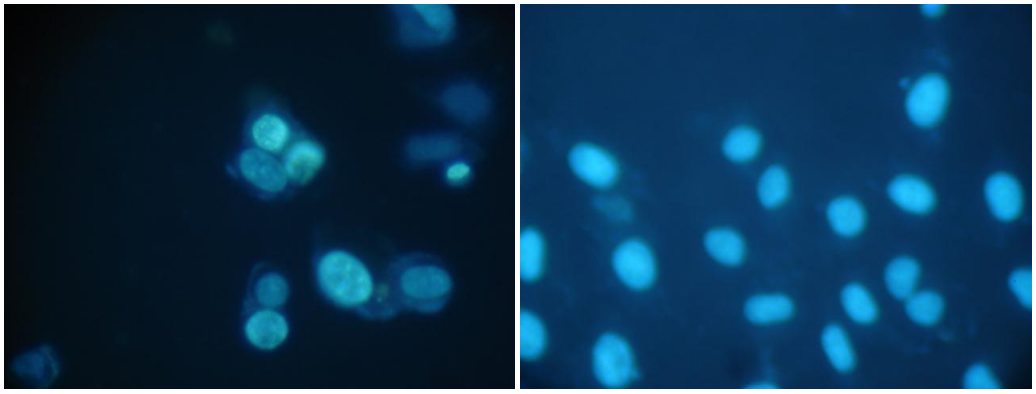

Plate 1 Plate 2

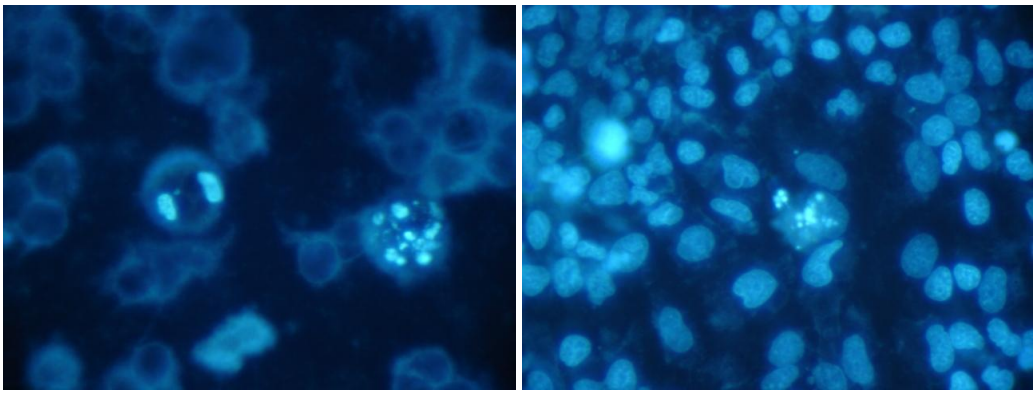

Plate 3 Plate 4

Figure 1: Fluorescence Photomicrograph of MCF 7 & HeLa Cells Stained with Hoechst

Table 1: Cytotoxic effect of extracts of *S. Canadensis* L. on HeLa & MCF-7 by Tryphan Blue Dye Exclusion Technique

Test Drug	IC_{50} (µg/ml)			
	n-Hexane	$CHCl_3$	EtOAC	Alcohol
HeLa	190	280	150	205
MCF-7	370	285	210	240

Average of 3 determinations, 3 replicates

$IC_{50,}$ Drug concentration inhibiting 50% cellular growth following 72 h of drug exposure.

This population appeared late after treatment and consisted of apoptotic bodies. Taken together, these data suggest that distinct stages of apoptosis *can* be identified by staining of cells with Hoechst. This assay should be useful for the detection and further characterization of cells at different stages in the apoptotic process.

Figure 1, Plate 1 & 2 are Photomicrographs of Hoechst stained cells that had not been exposed to EA (magnification X 10).

Figure 1, Plate 3 & 4 are Photomicrographs of Hoechst-stained cells that were exposed to EA for 48 h (magnification X 100).

Table 2: Cytotoxic effect of EA S. Canadensis L. on Human cancer cell lines by MTT Assay

Test Compound	HeLa IC_{50} (µg/ml)	MCF7 IC_{50} (µg/ml)
EA	170	275

Table 3: Percentage of apoptosis on HeLa & MCF 7 cells by treatment with EA of S. Canadensis L.

	control	HeLa	MCF-7
percentage death	07	38	32
	09	45	29
	08	43	30
Avg	8.00	42.0	30.33
SD	1.00	3.60	1.85

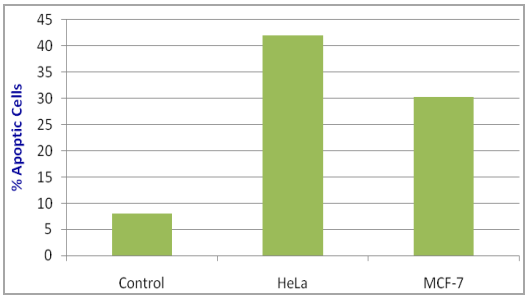

Figure 2: % Apoptic effect of EA on HeLa & MCF-7 by Hoechst staining method

CONCLUSION

These results suggest that extract of EA may exert its antitumor effects associated with two fundamental processes: suppression of cell proliferation and induction of apoptosis on human HeLa & MCF-7 cell *in vitro*. Therefore, it is concluded that the *EA* possesses potent antitumor on human HeLa & MCF-7 cancer cells.

REFERENCES

Anthonsen T., McCabe P. H., McCrindle R., Murray R. D. H., (1969). *Tetrahedron*, **25**, 2233—2239.

Apáti P., Szentmihályi K., Kristo Sz. T., Papp I., Vinkler P., Szoke É., Kéry Á., (2003). *J. Pharmaceut. Biomed.*, **32**, 1045—1053.

Arnason T., Hebda R. J., Johns T., (1981). *Can. J. Bot.*, **59**, 2189—2325.

Bohlmann F., Fritz U., King R. M., Robinson H., (1980) *Phytochemistry*, **19**, 2655—2661.

Fisher DE: (1994). Apoptosis in cancer therapy: Crosing the threshold. Cell 78:539-542.

Hacker, G., Paschen, S.A., (2007). Therapeutic targets in the mitochondrial apoptotic pathway. EEApert. Opin. Ther. Targets 11, 515–526.

Itoh N, Yonehara S, lshii A, Yonehara M, Mizushima S, Sameshima M,Hase A, Seto Y, Nagata S, (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66233-243.

Jacobson MD, Weil M, Raff MC. (1997). Programmed Cell Death in Animal Development. Cell, 88:347-54.

Kalemba D., (1992). *Pharmazie*, **47**, 471—472.

Kerr JF, Winterford CM, Harmon BV, (1994). Apoptosis. Its significance in cancer and cancer therapy. Cancer 73:2013-2026.

Kerr JF, Wyllie AH, Currie AR, (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26239-257.

Krepinsky J., Herout V., (1962). *Chem. Commun.*, **27**, 2459—2462.

Lillie, R.D. (ed.), (1977). *H.J. Conn's Biological Stains*, **9th Ed.,** The Williams and Wilkins Company, p. 158.

Majno G, Joris I, (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 146:3-15.

Moerman D. E., (2000). "Native American Ethnobotany," 3rd ed., Timber Press Inc., Portland, United States, p. 536.

O'Brien J., Wilson I., Orton T., Pognan F., (2000). *Eur. J. Biochem.*, **267**, 5421—5426.

Reznicek G., Jurenitsch J., Kubelka W., Korhammer S., (1990). Haslinger E., Hiller K., *Planta Med.*, **56**, 554.

Rousseau J., (1945). "Études ethnobotaniques québécoises: Le folklore botanique de Caughnawaga," Contributions de l'Institut botanique de l'Université de Montréal, **55**, Les Presses de l'Université de Montréal, Montréal, Canada, pp. 7—72.

Scudiero DA, Shoemaker RH, Paul KD. (1988). Evaluation of soluble Tetrazolium/formazan assay for cell growth and drug sensitivity in cultures using human and other tumor cell lines. *Cancer Res.*, 48:4827-4833.

Smith H. H., (1933). "Ethnobotany of the Forest Potawatomi Indians," Bulletin of the Public Museum of the City of Milwaukee **7**, Milwaukee, United States, p. 53.

Thiem B., Wesolowska M., Skrzypczak L., Budzianowski (2001). J., *Acta Pol. Pharm.*, **58**, 277—281.

Turner N. J., Bouchard R., Kennedy D. I. D., (1980) "Ethnobotany of the Okanagan-Colville Indians of British Columbia and Washington," British Columbia Provincial Museum, Occasional Paper Series, **21**, p. 84.

White E. (1996). Life, death and pursuit of apoptosis. Genes Dev, 10:1-15.

Wyllie AH, (1992). Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: An overview. Cancer Metast Rev 11:95-103.