International Journal of Applied and Natural Sciences (IJANS)
ISSN (P): 2319-4014; ISSN (E): 2319-4022
Vol. 6, Issue 3, Apr - May 2017; 1-8

International Academy of Science, Engineering and Technology
Connecting Researchers; Nurturing Innovations

THERAPEUTIC POTENTIAL OF METHANOLIC EXTRACT OF *EVOLVULUS ALSINOIDES* PLANT FOR ANTI CANCER ACTIVITIES IN VITRO

NAZANIN SOUDBAKHSH MOGHADAM[1], ANIL KUMAR H.V[2] & MURALIDHAR.S.TALKAD[3]

[1]Research Scholar, Kalinga University, Village Kotni, Near Mantralay, Naya Raipur, Chhattisgarh, India

[2]Department of Environmental Science & Laboratory for Applied biological Sciences,
DVS College of Arts and Science, Shimoga, Karnataka, India

[3]Research Scholar, Department of Biotechnology, R&D Centre, Dayananda Sagar Institution,
Kumaraswamy Layout, Bangalore, Karnataka, India

ABSTRACT

Evidence is reviewed supporting a general hypothesis that genomic instability and essentially all hallmarks of cancer, including aerobic glycolysis (Warburg effect), can be linked to impaired mitochondrial function and energy metabolism. Most cancer cells become heavily dependent on substrate level phosphorylation to meet energy demands. Most chemotherapeutic drugs for cancer treatment are molecules identified and isolated from plants or their synthetic derivatives.

Evolvulus alsinoides Linn. (dwarf morning glory) plant is widely used as antibacterial and anthelmintic Adaptogenic (anti-stress) and anti-amnesic and a good memory tonic.

In the present investigation, determined the *in vitro* cytotoxic activity of methanol extract of *E. Alsinoides* leaves**.**

Cell death caused by the whole plant extracts is via apoptosis. methanolic extract of *E. alsinoides* plant showed particularly strong anticancer capabilities since it inhibited actual tumor progression in a breast adenocarcinoma mouse model. Our results suggest that whole plant extracts are promising anticancer reagents.

KEYWORDS: p53, MCF-7 Cells, MTT Assay, Methanolic Extract of *Evolvulus Alsinoides* Linn, Bcl2, Anti-Proliferative Activity

INTRODUCTION

Drug developers have benefited from decades of academic cancer research and from investment in genomics, genetics and automation; their success is exemplified by high-profile drugs such as Herceptin (trastuzumab), Gleevec (imatinib), Tarceva (erlotinib) and Avastin (bevacizumab). However, only 5% of cancer drugs entering clinical trials reach marketing approval. Cancer remains a high unmet medical need, and many potential cancer targets remain undrugged.

Evolvulus alsinoides Linn. (dwarf morning glory) is a perennial herb with a small woody and branched rootstock. It grows as a weed in open and grassy places throughout India. This is widely distributed in tropical and subtropical regions throughout the world

This plant contains alkaloids: betaine, shankhapushpine and evolvine. An unidentified compound has been isolated. 3 Scopoletin, scopolin, umbelliferone, 2-methyl-1, 2, 3, 4-butanetetrol, 6-methoxy-7-*O*-ß-glucopyranoside coumarin, 2-C-methyl erythritol, kaempferol-7-O-ß-glucopyranoside, kaempferol-3-O-ß-glucopyranoside and

quercetine-3-O-ß-glucopyranoside were reported from *n*-BuOH soluble fraction from the ethanol extract of *E. alsinoides*

Cancer is the major cause of death worldwide, claiming over 6 million lives every year. In the recent years alternative therapies have gained importance over conventional cancer therapies for the treatment of cancer. Chemotherapy is restricted by both intrinsic and acquired cell resistance to drugs. This has necessitated the use of natural products for the treatment of cancer. There are compelling evidences for experimental investigations on the efficacy of plant drugs against cancer. It was reported to possess anti ulcer and anti catatonic activity, and immunomodulatory activity, the cytotoxic activity was evaluated against human cervical cancer cell line (HeLa), Human laryngeal epithelial carcinoma cells (HEp-2) and mouse embryonic fibroblasts (NIH 3T3).

However, to the best our knowledge, the in vitro anticancer activity of this plant has not been investigated. In the present investigation, we therefore focused on *in vitro* cytotoxic activity of methanolic extract of *E. alsinoides* plant**.**

MATERIALS AND METHODS

Plant Materials and Extraction

The plant material was collected from the local rural area of Tumkur district in Karnataka state, India. The plant materials were identified and authenticated. The Plant material were air-dried and pulverized in course powder and was loaded in the Soxhlet apparatus for defating with petroleum ether and followed by extraction with methanol. The methanolic extract of *Evolvulus alsinoides* was subjected to fractionation and dry concentrated extract were used for the study

`MCF-7 cells, 96 well tissue culture plates (Corning), DMEM medium, Fetal bovine serum (FBS), MTT (Sigma), DMSO

PROCEDURE

Trypsinized the cells from T-25 flask; counted the cells using haemocytometer, and Seed $5x10^3$ cells (MCF-7 or HeLa) in 100µl media per well in a 96 well plate then incubated overnight at $37^oC/5\%$ CO_2. Leave 3 wells without cells for blank. Prepared 4mg/ml stock of the test samples in medium, then serially diluted the samples to 2mg/ml, 1mg/ml, 0.5 mg/ml, 0.2 mg/ml, and 0.1 mg/ml in medium. Then Added 100µl of these diluted samples to cells in triplicate, 100 µl of medium was used as negative control and 1µg/ ml Doxorubicin was added as internal positive control for the assay. Mixed and incubated at $37^oC/5\%$ CO_2 for 48 hours. Added 20 µl of 5mg/ml MTT in PBS to each well and incubated at $37^oC/5\%$ CO_2 for 4 hours. Aspirated the medium and then added 200 µl of Dimethyl sulfoxide (DMSO) to each wells, followed by measuring the optical density of each well using Microplate reader at 570 nm. Percentage inhibition is calculated as follows:

% Inhibition = 100 - [(Mean OD for test sample / mean OD for the control) x 100]

Negative values were considered as no inhibition or lysis. The concentration at which the sample exhibits 50% of its maximum activity (ED_{50}) was calculated using the ED_{50} plus v1.0 software.

RT-PCR FOR P53 AND BCL-2 GENE EXPRESSION

Effective Dosage for 50% inhibition (ED50) of cell proliferation of test compound was obtained by MTT assay. Approximately 0.4 X 10^6 MCF7 breast cancer cells were plated per well of 6 well tissue culture plate and incubated at 37

^{o}C/ 5% CO_2 overnight. These cells were treated with 844.833 µg / ml of the test sample dissolved in DMEM containing 10% FBS. As a negative control DMEM containing 10% FBS was used and as a positive control cells were treated with 50 µg/ml Doxorubicin hydrochloride. Plates were incubated for further 48 hours. Total RNA was isolated from these cells and mRNA expression levels of p53 and BCl-2 were carried out using semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as follows. The results were normalized with expression of *β*-actin.

RNA Isolation

- Add 0.5 ml of GITC solution directly to the wells of 6 well plate containing cells.
- Add 50 µl of 2 M Na-acetate pH 4.0 and mix gently.
- Add 500 µl of water-saturated phenol and mix gently.
- Add 150 µl of 49:1 Chloroform: Isoamyl alcohol and mix gently 25 times by inversion.
- Keep on ice for 10 min for the phases to separate.
- Spin at 12,000 rpm/2 °C/20 min.
- Remove the supernatant carefully and add 1 µl of glycogen (20 mg/ml) and mix.
- Add 550 µl of isopropanol and mix gently.
- Flash freeze in liquid nitrogen to precipitate RNA.
- Thaw at RT and spin again at 12,000rpm, 2 degrees C/,20 min.
- Discard the supernatant and add 0.8 ml of 70% ethanol.
- Spin at 12,000 rpm, 2 degrees C, 10 min.
- Discard the supernatant and air dry.
- Dissolve the RNA pellet in 20µl of DEPC treated water.

Reverse Transcription (RT)

Reverse transcription of total RNA from MCF7 cells control, MCF7 cells treated with 844.833 µg / ml samples or MCF7 cells treated with 50 µg/ml Doxorubicin were performed separately using 200 units of RevertAid™ M-MuLV Reverse Transcriptase (Fermentas Life Sciences) in a total volume of 20 µl.

PCR for β-Actin

The Sequences of the Primers Used for β-Actin are as Follows

Forward: 5'-GTGGGGCGCCCCAGGCACCA -3'

Reverse: 5'- CTCCTTAATGTCACGCACGATTTC-3'

PCR was performed as follows in a total volume of 50 µl in a 0.2 ml thin walled PCR tube.

Distribute 48 µl in 3 PCR tubes labeled as negative, positive, Tests A, B, and C.

Add 2 µl of cDNA samples into correspondingly labeled tubes.

The amplification was performed in a QUANTA BIOTECH Thermocycler using the following program. Initial denaturation 94 degree 30 sec; cycles 35 times, final denaturation at 94 degree 30 sec,annealing 64degree 1min,extension 72 degree 30 secs:final extension 72 degree 2min, with expected size of product will be 540 bp.

PCR for p53 Gene Expression

The Sequences of the Primers used for p53 are as Follows

Forward: 5'-CTGAGGTTGGCTCTGACTGTACCACCATCC -3'

Reverse: 5'-CTCATTCAGCTCGGAACATCTCGAAGCG -3'

PCR was performed as follows in a total volume of 50 µl in a 0.2 ml thin walled PCR tube.

Distribute 48 µl in 3 PCR tubes labeled as negative, positive, Tests A, B, and C.

Add 2 µl of cDNA samples into correspondingly labeled tubes.

The amplification was performed in a QUANTA BIOTECH Thermocycler using the following program

Initial denaturation of 94 ^{o}C for 2 minutes followed by 40 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 69 ^{o}C for 1 min and extension at 72 ^{o}C for 1.2 min and terminated after final extension of 72 ^{o}C for 8 min.

Expected Size of Product: 371 bp

PCR for Bcl-2 Gene Expression

The Sequences of the Primers Used for Bcl-2 are as follows

Forward: 5'- GTTCGGTGGGGTCATGTGTGTGGAGA-3'

Reverse: 5'- GCTGATTCGACGTTTTGCCTGAAGAC-3'

PCR was performed as follows in a total volume of 50 µl in a 0.2 ml thin walled PCR tube

Distribute 48 µl in 3 PCR tubes labeled as negative, positive, Tests A, B, and C.

Add 2 µl of cDNA samples into correspondingly labeled tubes.

The amplification was performed in a QUANTA BIOTECH Thermocycler using the following program

Initial denaturation of 94 ^{o}C for 2 minutes followed by 40 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 64 ^{o}C for 1 min and extension at 72 ^{o}C for 1.2 min and terminated after final extension of 72 ^{o}C for 8 min

Expected Size of Product: 462 bp

All PCR products were run on 1.5% agarose gel along with 100bp DNA ladder.

RESULTS AND DISCUSSION

The ED_{50} value as calculated by MTT assay was found to be 844.833µg/ml

Expression of p53 and Bcl-2 Genes

Quantitation of each band was performed by densitometry analysis software ImageJ and the results expressed as

the ratio p53/β-actin or Bcl-2/β-actin in percent to the medium (control). As shown in Figure 1 and Table 1 in comparison to the medium control, the p53 gene expression is not significantly changed in the presence of samples and the Bcl-2 gene expression is significantly decreased in the presence of sample.

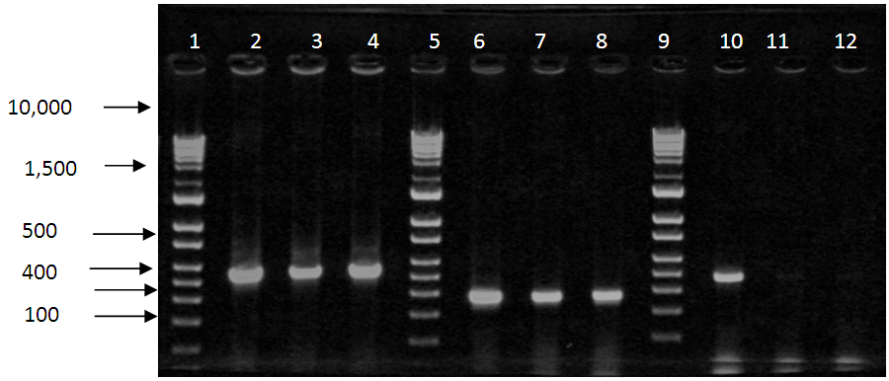

Figure 1: Agarose Gel Electrophoresis of RT-PCR Products. M- 1000 bp DNA Ladder

1. Size marker
2. β-Actin no treatment
3. β-Actin positive control
4. β-Actin sample treated(0.844mg/ml)
5. Size marker
6. p53 no treatment
7. p53 positive control
8. p53 sample treated(0.844mg/ml)
9. Size marker
10. BCl2 no treatment
11. BCl2 positive control
12. BCl2 sample treated(0.844mg/ml)

Table 1: Densitometric Analysis of PCR Amplicons

Sl. No.	Gene (Treatment)	Ratio to β-Actin	% to Medium
1	p53 (medium)	0.997597811	0
2	p53 (control)	0.94025207	20.41101251
3	p53 (sample)	0.817453681	21.41474611
4	Bcl-2 (medium)	0.685038899	0
5	Bcl-2 (control)	0.057740366	92.88248371
6	Bcl-2 (sample)	0.038580586	94.5988407

Comparative analysis of gene expression by semi-quantitative RTPCR indicates that test compound has induced expression of tumor suppressor p53 gene in MCF7 cancer cells and has suppressed the expression of BCl_2 gene. This clearly indicates that those constituents of test sample are having anti-proliferative activity upon cancer cells.

DISCUSSIONS

The next pair of articles focuses on another major anticancer signalling pathway: that of EGFR. Lo and Hung describe EGFR's lesser studied and more contentious role in the nucleus, where it upregulates gene expression by interacting with transcription factors. The nuclear EGFR pathway might be associated with more aggressive tumours, as it is involved in increasing proliferation and accelerating cell cycle progression the crosstalk between the EGFR and Notch pathways, and how these converge on the transcriptional co-repressor Groucho (Hasson. P et al 2006).

The epigenome is emerging as a major factor contributing to tumour formation and progression, its role in affecting oncogene and tumour suppressor gene expression and the potential for cancer therapy in targeting these epigenetic genes (Esteller. M 2006). the different covalent modifications that underlie epigenetic changes, and focus on the contribution of histone modifications to cancer and their role as cancer targets (Allis CD, et al 2007).

Finally, the recently discovered microRNAs (miRNAs), which negatively regulate mRNAs and are involved in processes that are important in tumorigenesis, such as proliferation, differentiation and apoptosis. Indeed, miRNAs have been found to act directly as oncogenes and tumour suppressors (Hwang HW, et al 2006).

The major constituent of green tea, (–)-epigallocatechin-3-*O*-gallate (EGCG), has been shown to have cancer-preventive and therapeutic activities. Furthermore, a phosphodiesterase 5 inhibitor, vardenafil, synergizes with EGCG to induce cancer cell death.

This is a provocative observation with important implications for cancer therapy. It also raises several issues for further investigation, such as the mechanism by which EGCG specifically activates 67LR (Chung S. Yang et al 2013).

More than 30% of cancers are caused by modifiable behavioral and environmental risk factors, including tobacco and alcohol use, dietary factors, insufficient regular consumption of fruit and vegetable, overweight and obesity, physical inactivity, chronic infections from *Helicobacter pylori*, hepatitis B virus (HBV), hepatitis C virus (HCV) and some types of human papilloma virus (HPV), environmental and occupational risks including exposure to ionizing and nonionizing radiation (Cancers, NMH Facts sheet 2010).

However, due to the increasing rate of mortality associated with cancer and adverse or toxic side effects of cancer chemotherapy and radiation therapy, discovery of new anticancer agents derived from nature, especially plants, is currently under investigation. Screening of medicinal plants as a source of anticancer agents was started in the 1950s, with the discovery and development of vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins (Cragg, G. M.et al 2005).

Methanolic extracts of *Hypericum perforatum* (L.) (St. Johns wort) possessed strong antiproliferative activity in the human prostate cell line (PC-3) and the major constituents, hyperforin and hypericin, synergistically contributed to the reduction of the PC-3 cells proliferation (Martarelli, D et al 2004).

Maslinic acid, a triterpene from *Olea europaea* (L.) (Olive) has shown to be significantly inhibitory in cell proliferation of the human colorectal adenocarcinoma cell line (HT29) in a dose dependent manner (Fernando J et al 2009).

The major components in the extract were identified to be oleuropein, hydroxytyrosol, hydroxytyrosol acetate, luteolin-7-*O*-glucoside, luteolin-4'-*O*-glucoside and luteolin. All these phytochemicals inhibited the proliferation of cancer and endothelial cells with IC50, at the low micromolar range (Goulas, V et al 2009).

Methanolic leaf extract of *Plantago lanceolata* (L.) (Ribwort plantain) inhibited the growth of three different cell lines; human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines and the MCF-7 was totally inhibited (Gálvez, M et al 2003).

Further, the ethanolic extract of *P. lanceolata* (L.), produced by maceration with ethanol: water, showed significant antiproliferative activity on cervix epithelioid carcinoma (HeLa), breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29) and human fetal lung carcinoma (MRC-5) (Beara, I. N et al 2012). Several chemical constituents from *Silybum marianum* (Milkthistle) have been isolated and their cytotoxic and anticancer potential has been investigated, *in vitro*, using both cancer and normal healthy cell lines.

Silymarin demonstrated to have marked inhibition of cell proliferation with almost 50% inhibition in a time dependent manner on the human breast cancer cell line (MDA-MB 468), at 25 µg/mI concentration, after five days of treatment. Its potential anticancer activity was dose dependent and showed a complete inhibition of cancer cells at 50 and 75 µg/mI concentrations at the beginning of Day 2 of exposure (Zi, X., et al 1998). Induction of apoptotic cell death of human prostate cancer (DU145) treated with silibinin is shown to be due to activation of caspase 9 and caspase 3 enzymes (Agarwal, C., et al 2007).

HeLa cells treated with 75 µg of a crude leaf extract of (Graviola) *A. muricata* showing 80% of cell inhibition (Jeno Paul et al 2013).

CONCLUSIONS

Methanolic extract of E. alsinoides plant has induced expression of tumor suppressor p53 gene in MCF7 cancer cells and has suppressed the expression of BCl_2 gene, hence the therapeutic potential of methanolic extract of E. alsinoides plant for Anti cancer activities in vitro, proven to be effective to treat cancer progression

REFERENCES

1. Agarwal, C., Tyagi, A., Kaur, M. and Agarwal, R., Silibinin inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of human prostate carcinoma DU145 cells. Carcinogenesis, 2007; 28(7) 1463–1470.
2. Allis CD, Berger SL, Cote J, et al. New nomenclature for chromatin-modifying enzymes. Cell. 2007; 131:633–6
3. Beara, I. N., Lesjak, M. M., Orcic, D. Z., Simin, N. Đ., Cetojevic-Simin, D. D., Bozin, B.N. and Mimica-Duki, N. M., Comparative analysis of phenolic profile, antioxidant,anti-inflammatory and cytotoxic activity of two closely-related Plantain species: Plantago altissima L. and Plantago lanceolata L. LWT - Food Science and Technology,2012; 47: 64-70.
4. Cancers, NMH Facts sheet. World Health Organization; 2010.
5. Chung S. Yang and Hong Wang. Cancer therapy combination: green tea and a phosphodiesterase 5 inhibitor? *J Clin Invest.* 2013; 123(2):556–558.
6. Cragg, G. M. and Newman, D. J., Plants as a source of anti-cancer agents, Journal of Ethnopharmacology, 2005; 100: 72-79.

7. Fernando J., Eva, R. Z., Rufino-Palomares, E., Lupiáñez, J. A. and Cascante, M., Maslinic acid, a natural triterpene from Olea europaea L., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway. Cancer Letters, 2009; 273: 44–54

8. Gálvez, M., Mart´ın-Cordero, C., López-Lázaro, M., Cortés, F. and Ayuso. M. J., Cytotoxic effect of Plantago spp. on cancer cell lines. Journal of Ethnopharmacology, 2003; 88: 125–130.

9. Goulas, V., Exarchou, V., Troganis, A. N., Psomiadou, E., Fotsis, T., Briasoulis E. and Gerothanassis. I. P., Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells, Mol. Nutr. Food Res. 2009; 53: 600 – 608.

10. Esteller M. Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. Br J Cancer. 2006 Jan 30; 94(2): 179–183.

11. Hasson P, Paroush Z. Crosstalk between the EGFR and other signalling pathways at the level of the global transcriptional corepressor Groucho/TLE. Br J Cancer (2006) 94(6):771–510.

12. Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer. 2006 Mar 27; 94(6):776-80.

13. Jeno Paul, R. Gnanam, R.M. Jayadeepa, and L. Arul. Anti Cancer Activity on Graviola, an Exciting Medicinal Plant Extract *vs* Various Cancer Cell Lines and a Detailed Computational Study on its Potent Anti-Cancerous Leads. Current Topics in Medicinal Chemistry, 2013, 13, 1666-1673

14. Martarelli, D., Martarelli, B., Pediconi, D., Nabissi, M. I., Perfumi, M. and Pompei. P., Hypericum perforatum methanolic extract inhibits growth of human prostatic carcinoma cell line orthotopically implanted in nude mice. Cancer Letters, 2004; 210: 27– 33

15. Zi, X., Feyes, D. K. and R., Agarwal, Human breast cancer cells MDA-MB 468: induction of G1 arrest through an increase in Cip1/p21 concomitant with a decrease in kinase activity of cyclin-dependent kinases and associated cyclins. Clin Cancer Res, 1998; 4: 1055-1064.