ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Reversal Of Promoter Hypermethylation Of *RASSF1A* Gene Caused By *Aloe Barbadensis Miller* And *Murraya Koenigii* In Cervical Cancer.

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ABSTRACT

The incidence of Cervical Cancer has been increasing since the 1940s, making it the most common malignancy among women in the developed countries. Worldwide, 10%–15% of all women canbe diagnosed with Cervical Cancer duringtheir lifetimes. The identification of novelapproaches for the prevention and treatment of cervical cancer is urgently needed. An appealing approach, in theory, for the prevention of cervical cancer is the use of natural compounds and plant extracts. Plant extracts are known to possess antiproliferative, differentiative, immunomodulatory, and apoptosis-inducing properties. *RASSF1A*is a novel tumor suppressor gene that promotes apoptosis, cell cycle arrest and reduces the tumorigenicity of cancer cell lines and inhibits tumour invasion, angiogenesis and metastasis. In the present study, we investigated the effects of plant extracts, i.e. extracts of *Aloe barbadensis miller* (also known as Aloe vera) and *Murraya koenigii* (also known as curry leaves) on the methylation status of the *RASSF1A* gene and cancer invasion in Cervical Cancer cell lines. Our results showed that treatment of Cervical cells with Aloe vera and Curry leaves reversed the hypermethylation status of the RASSF1A gene.

Keywords: *RASSF1A,* Antiproliferative, Immunomodulatory, tumorigenicity, Angiogenesis, Metastasis

https://doi.org/10.33887/rjpbcs/2019.10.4.28

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ISSN: 0975-8585

INTRODUCTION

Human body contains millions of cells; it grows, divides and dies in specific manner. Any disturbance in this system can result in uncontrolled division and multiplication of cells, which leads to cancer. Worldwide Cancer is a disease becoming a major cause of death. In India approximately four lakhs of people are affected by cancer every year and about 2 lakhs of people die every year. This uncontrolled repeated division results in formation of cluster of cells called Tumor or Neoplasm. There are two types of tumors, benign and malignant: **(a) Benign tumor** is defined as a non cancerous tumor which remained constricted to the original area of its formation. Its size ceases after attaining certain growth and it gets encapsulated in connective tissue so that it unable to affect adjacent tissues. **(b) Malignant tumor** is defined as a cancerous tumor which grows rapidly because the dividing cells continue to proliferate. Malignant tumor is not encapsulated, so these cells can reach to other parts of the body through blood and lymph. They form new malignant tumor in invaded parts. The phenomenon is called metastasis. Cervical cancer is a major health problem worldwide and is the most frequent cause of cancer in women in India. HPV is known to cause almost all cases of cervical cancer. HPV is a very common virus that can be passed on through any type of sexual contact. There are many types (strains) of HPV, many of which are harmless. However, some types can cause abnormal changes to the cells of the cervix, which can eventually lead to cervical cancer. But these infections are very common, and most women who have them do not develop cervical cancer. Most cases of cervical cancer are caused by two strains i.e. HPV 16 and HPV 18. They don't have any symptoms, so women will not realize they have it. Alteration in epigenetic mechanism is one of the significant cause in the development and progression of cancer. Epigenetics includes DNA methylation and histone modifications which are important mechanisms of gene regulation and play essential roles both independently in tumor initiation and progression.Early detection and affordable drugs with clinical efficacy is necessary to address this serious health challenge. Cytotoxic effects of most of the chemical compounds shifts the focus towards natural plant extracts for the epigenetic reversal. Phytochemicals derived from fruits and vegetables, referred to as chemo preventive agents that has potential to be used as adjuncts to current cancer therapies, include genistein, diallyl sulphide, S-allyl cysteine, allicin, lycopene, curcumin, 6-gingerol, ursolic acid, silymarin , anethol, catechins and eugenol (Dorai., 2004). Epigallocatechin3-gallate (EGCG) has been proved to cause reactivation of methylation silenced genes such as p16INK4a,*WIF-1*, retinoic acid receptor s, O6-methylguanine methyl transferase, human mutL homologue 1, and glutathione S-transferase-π in human colon, prostate, and mammary cancer cell lines (Fang et al., 2003). Genistein and lycopene which are generally found in tomato have been shown to affect gene expression in many ways that can either promote or inhibit the carcinogenic processes in breast cancer cell lines. Both genistein and lycopene, at very low, dietarily concentrations can potentially mitigate tumorigenic processes via promoter methylation modulation of gene expression (King., 2008).Plant extracts with potent anticancer effects should add to the efforts to find a cheap drug with limited clinical side effects. Keeping this very purpose in mind, an attempt has been made in this research to explore the potential of plant extracts to exhibit anti tumorigenic activity or exert cytotoxic effect in human cervical carcinoma cells.

HeLa Cell Line

HeLa cells is an immortal cell line widely used for scientific research purposes. It is one of the oldest and most commonly used human cell line (Rahbari et al., 2009). The line was derived from cervical cancer cells taken on February 8, 1951, (Scherer et al., 1953) from Henrietta Lacks, a patient who died of cancer on October 4, 1951. HeLa cells, like most of the tumors, have defective genomes, with one or more copies of many chromosomes: a normal cell contains 46 chromosomes whereas HeLa cells contain 76 to 80 (ref) total chromosomes, some of which are highly mutated per cell. This difference is basically due to the *Human Papillomavirus* (HPV), the major cause of nearly all cervical cancers. HPV inserts its own DNA into host cells and the additional DNA results in the production of a p53 -binding protein which inhibits it and prevents native p53 from repairing mutations and suppressing tumors, causing errors in the genome to accumulate as unchecked cellular divisions occur.

ISSN: 0975-8585

MATERIALS AND METHODS

Collection of cell line

The Cervical cancer cell line HeLa, was procured from the National Centre for Cell Sciences (Pune, India).

Preparation of plant extract

Leaves of *Aloe barbadensis Miller* and *Murraya koenigii* were shade dried and made in the powder formwith the help of mortar and pestle. Powdered leaves were taken in 1.5ml centrifuge tube (1mg) and 1ml absolute alcohol added. After that extract was dried in the incubator and then dried powder was dissolved in 1ml of DMSO (Dimethyl Sulphoxide).

Cell culture

$5x10^4$ cells were seeded in 96 well culture as per the need of doses and checkpoints. The incubated cells were kept in 200µl/ml DMEM for 2hrs 30 minutes for adherence. Treat all the wells with the specific chemicals like PBS and methanol beside control and incubate for respective time periods.

Crystal violet assay

25µl of crystal violet was added to all the wells containing the treatment. 10µl of 1% SDS was added to each of the well to solubilize the stain. The wavelength was set to 590nm and the appropriate readings were taken. These readings were noted down and specific graphs were formulated. The graph made from the readings gives the IC_{50} values of the photochemical. Based on these values, efficient doses of treatment can be formulated.

DNA isolation

Cells obtained after the cell culture process were lysed in digestion buffer (10µM Tris-HCl, pH 7.6, 0.5mM EDTA, pH 8.0 and 20% SDS containing proteinase K (20µL) .DNA was then purified using standard PCI (Phenol-Chloroform-Isoamyl alcohol) extraction and then final treatment of chilled ethanol was given.

Sodium bisulphite modification

Bisulfite modification was carried out using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irwin USA). Sodium bisulphite modification was done with these specific reagents (130µl CT conversion reagent , 20µl DNA).

The reaction was then carried out in a thermal cycler following two cycles:

- 98°C for 10 min
- 64°C for 2.5 hours

ISSN: 0975-8585

Methylation specific PCR (MS-PCR)

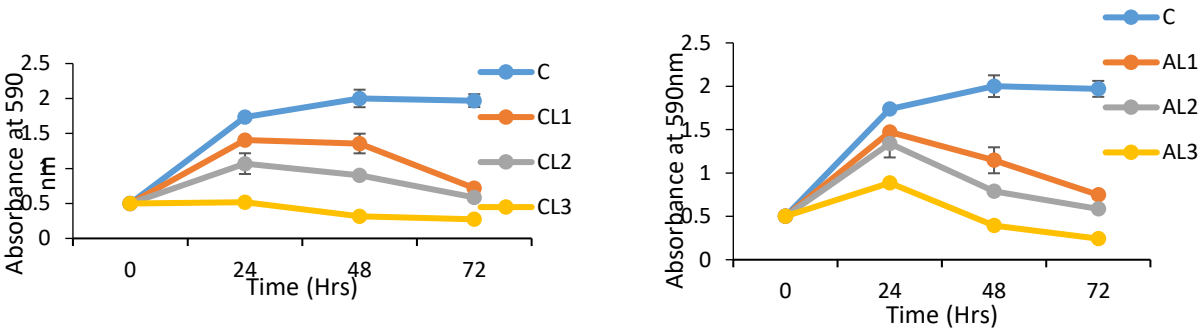
Table 1: Sequence of primers

Primers	Sequence	%GC	Volume (100µM)	T_M (°C)
RASSF1A methylated forward	GGGTTTTGCGAGGCGCG	66.7	288.6	55
RASSF1A methylated reverse	GCTAACAAAGCGGGAACCG	55.6	219.6	50
RASSF1A unmethylated forward	GGTTTTGTGAGAGTGTGTTTAG	40.9	280.0	52
RASSF1A unmethylated reverse	CACTAACAAACACAAACCAAAC	36.4	308.3	49

DNA isolated from the cell lines was modified with sodium bisulphite and MS-PCR was carried out using specific primers for methylation and unmethylation for the RASSF1Agene (Table1.) The amplified products were further run on a 2% agarose gel.

RESULTS

The IC_{50} values of *Murraya koenigii* and *Aloe barbadensis miller* in HeLa cells were found to be 25 and 11.5 µg/ml, respectively with the help of crystal violet assay.The concentration of *Aloe barbadensis miller* and *Murraya koenigii* was increased with the time period and intensity of bands were found to be decreased.



a. ***Murraya koenigii*** **b.** ***Aloe barbadensis Miller***

IC_{50} = 11.5µg/ml ***IC_{50} = 25µg/ml***

Fig.1 Treatment of plant extracts under different time periods (24, 48 and 72hours)
(% viability of HeLa cell line on treatment with plant extract)

ISSN: 0975-8585

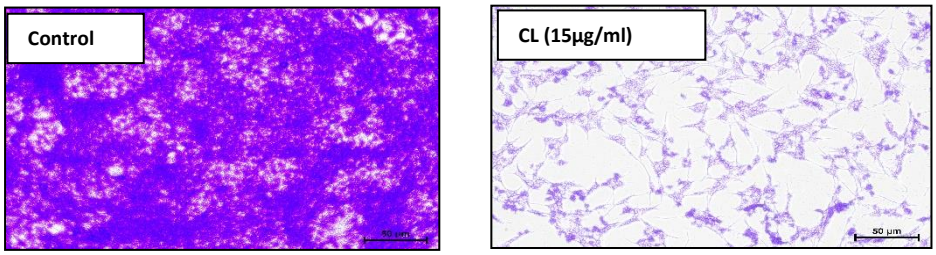


Fig.2 Morphological changes observed in HeLa cell line after treatment with *Murraya koenigii* DMSO extract (6 days treatment)

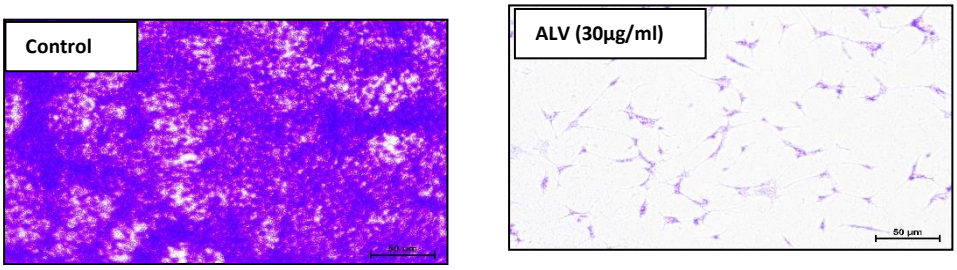


Fig.3 Morphological changes observed in HeLa cell line after treatment with *Aloe barbadensis* DMSO extract (6days treatment)

Crystal violet assay reveals the apoptosis which took place in HeLa cell line in different doses of treatment of drugs. The intensity of methylation specific band (MSB) was observed to be high in HeLa cells which were not treated with any of the plant extracts (control) , but the intensity of MSB showed a prominent decrease after 6 days of treatment with the selected plant extracts.

DISCUSSION

Epigenetic silencing of TSGs is emerging as a well established oncogenic process (González et al., 2005). Epigenetic alteration is a reversible process, and this phenomenon establishes the potential use of RASSF1A as a smart concept of cancer therapy (Lyko F, Brown, R., 2005) Much of contemporary research focused on the study of epigenetic changes such as promoter hypermethylation of GSTs and RARβ (cervical cancer type1 susceptibility protein), resulting in carcinogenesis and their possible reversal using natural compounds such as curcumin and tea polyphenol EGCG. This study was designed to analyze reversal of RASSF1A promoter hypermethylation by *Aloe barbadensis miller* and *Murraya* leaves in cervical cancer cell line HeLa in dose-dependent manner. MSP was carried out to study the ability of these two extracts to cause reversal of hypermethylation and led to reactivation of RASSF1A protein expression in HeLa cells. The IC_{50} of *Aloe barbadensis* was found to be 25µg/ml and *Murraya* to be 11.5µg/ml. Therefore, it indicates that at these concentrations, reversal of hypermethylation occurred because these concentrations could not be enough to inhibit growth of the enzymes. The intensity of methylation-specific band as well as protein expression of RASSF1A was detected to maximum at 20 and 10 µg/ml concentration of *Aloe barbadensis* and *Murraya* after the 6 days of treatment. Although a large number of synthetic drugs are being added to the world of modernpharmacopoeia, but still no system of medicine in the world which can solve all the healthproblems. Therefore the search for new therapeutic constituents from plants is genuine andurgent. There are large numbers of indigenous plants left which have not been investigatedthoroughly from modern scientific view or their curative values have not been recognized. And therefore *Aloe barbadensis Miller* and *Murraya koenigii* can be considered as a potent plant for the prevention of cervical cancer without showing any side effects.

ISSN: 0975-8585

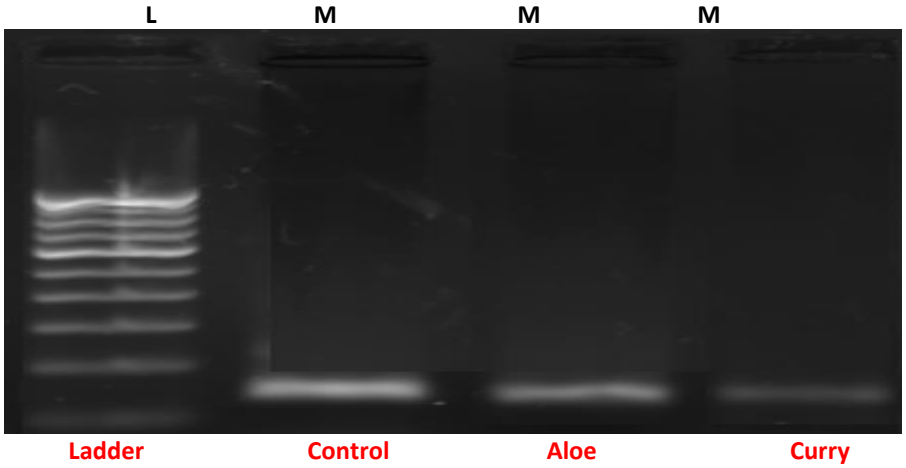


Fig. 4: Methylation Specific Bands after the 6 days treatment with 20µg/ml of *Aloe barbadensis miller* and 10µg/ml of *Murraya koenigii*

Lane 1. Ladder (L)
Lane 2. Methylation specific band (M) – control after 6 days of treatment
Lane 3. Methylation specific band (M) – *Aloe barbadensis Miller* after 6 days of treatment
Lane 4. Methylation specific band (M) – *Murraya koenigii* after 6 days of treatment

In the present study, we tried to calculate the effect of *Aloe barbadensis miller*and *Murraya koenigii* extracts on promoter hypermethylation of RASSF1A gene. The methylation specific band(MSB) was observed even after the treatment with these extracts. However, the intensity of MSBs showed a prominent decrease after the treatment with these plant extracts dissolved in DMSO (Dimethyl Sulphoxide).

Hence it can be concluded that HeLa cells do show hypermethylation for promoter region of RASSF1A gene. The reversal of hypermethylation was observed after the treatment with the *Aloe barbadensis miller* and *Murraya koenigii* as compared to control. It needs to be further checked whether it leads to the reactivation of tumor suppressor gene, RASSF1A that had been silenced in the selected plant extracts due to promoter hypermethylation. These findings can also act as the useful source for future development as cancer preventive agents.

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