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Reversal of promoter hypermethylation of *CADM1* and *SOCS1* by leaf extract of *Datura metel* in cervical cancer cells

Mahek Sharan[1,2], Niraj Kumar Jha[1], Saurabh Kumar Jha[1], Abhimanyu Kumar Jha[1]*

[1] *School of Biotechnology, Sharda University, Noida, Uttar Pradesh, India.* [2]*Department of Zoology, Banaras Hindu University, Varanasi, Uttar Pradesh, India*

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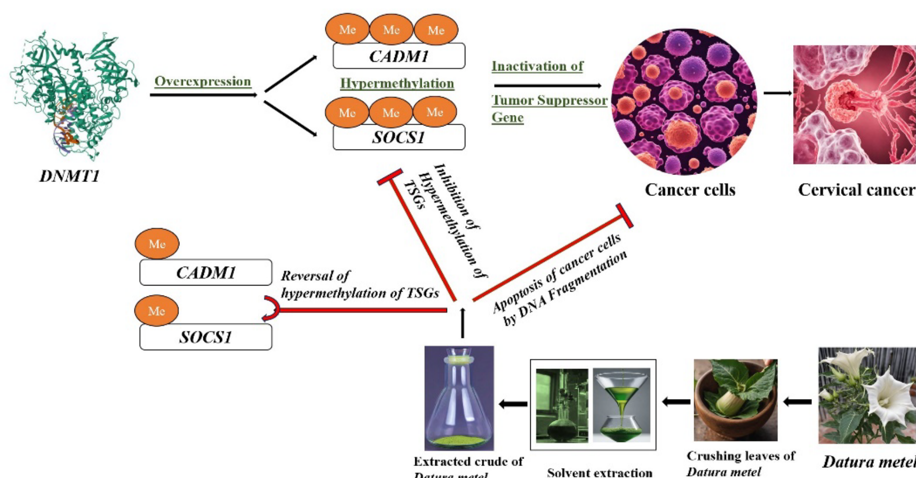
Research Article



ABSTRACT

Cervical cancer ranks as the second most fatal cancer among women in developing countries, trailing only behind breast cancer. Human Papillomavirus (*HPV*) infection, among various contributors, stands as a primary cause of cervical cancer. Overexpression of DNA methyltransferase 1 (*DNMT1*) leads to hypermethylation of Cell Adhesion Molecule 1 (*CADM1*) and Suppressor of Cytokine Signaling 1 (*SOCS1*), consequently silencing these tumor suppressor genes (TSGs) epigenetically. In this study, we explored the reversal of aberrant methylation in the squamous cervical cancer cells, SiHa using a concentration of 5 µg/ml of *Datura metel* ethanol-chloroform (E: C) leaf extract. Treating the cells with the extract for 72 hours and consecutively for six days, the DNA fragmentation study for cell apoptosis was performed. The methylation-specific PCR analyzed the DNA aberrant methylation patterns of these TSGs and their subsequent reversal and fragmentation. The results suggest that the *Datura metel* leaf extract (E: C) was able to cause DNA fragmentation and also effectively reversed the promoter hypermethylation, leading to the reactivation of *CADM1* and *SOCS1*. This was evidenced by the reduction in intensity and visual sharpness of the methylation-specific band and the unmethylation-specific band displayed an increase in width and enhanced luminosity in MS-PCR for both genes. This study marks one of the initial global reports showcasing the potential of E: C leaf extract from *Datura metel* in reversing hypermethylation and reactivating *CADM1* and *SOCS1* genes in cervical cancer cells. Further exploration into the phytochemicals of *D. metel* leaves that demethylate *CADM1* and *SOCS1* could unveil a promising candidate for reactivating suppressed genes in cervical cancer.

Keywords: Cervical cancer, Reversal of Promoter hypermethylation, Tumor suppressor genes, Datura metel, Epigenetic modifications.

INTRODUCTION

Globally, cervical cancer ranks as the second most common malignant tumor among females, posing a significant threat to women's health. According to the GLOBOCAN 2020 database reports, there were 341,831 reported mortality cases among 604,127 cervical cancer cases, equating to 13.3 cases per 100,000 women per year, with a mortality rate of 7.2 deaths per 100,000 women per year. Over the past 30 years, the percentage of young women affected by cervical cancer has increased from 10% to 40%.[1] A 2018 study ranked cervical cancer as the fourth most frequently diagnosed cancer and the fourth leading cause of death due to cancer in women.[2] Reports indicate that the mortality rate in low and middle-income countries is approximately 18 times higher than in wealthier countries.[3]

The incidence and mortality rates of cervical cancer have decreased in recent years. However, the risk of developing cervical cancer remains high due to various factors. These include smoking and reproductive factors, such as engaging in multiple sexual partnerships, known to heighten a woman's vulnerability to developing cervical cancer, primarily due to an increased risk of Human Papillomavirus (HPV) acquisition.[4] Other significant contributing factors are changing sexual habits,

*Corresponding Author: Abhimanyu Kumar Jha
Tel: +91- 8285470873
Email: abhimanyu.kumar@sharda.ac.in

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high parity, and lower socioeconomic status,[5] as well as exposure to specific strains of HPV, like HPV 16/18.[6] These elements collectively contribute to the process of carcinogenesis over time, as the development of invasive cancer could extend up to 20 years.[7] HPV infections play a crucial role in this process by expressing E6 and E7 viral oncoproteins, which are known to disrupt major tumor suppressor genes, such as *p53* and retinoblastoma. Additionally, these viral oncoproteins interact with DNA methylation modifications, responsible for regulating genetic integrity, apoptosis, immune response, cell adhesion, and cellular control.[8]

Epigenetics of cervical cancer: Epigenetics, a phenomenon pivotal in various biological processes such as immune response, embryonic development, and cancer biology, encompasses significant alterations in cellular genomes during cervical carcinogenesis. These modifications include global DNA hypermethylation and hypomethylation of tumor suppressor genes, occurring in both the host cellular genomes and the human papillomavirus.[9] Malignant tumors typically exhibit regional hypermethylation and global hypomethylation patterns. The epigenetic silencing of tumor suppressor genes, initially observed with RB due to hypermethylation, later extended to other genes like *MLH1, E-cadherin, VHL,* and *p16.*[10] Among the family of tumor suppressor genes, Cell adhesion molecule 1 (*CADM1*) plays a crucial role in tumor growth suppression by demonstrating pro-apoptotic and anti-proliferative activity. However, its loss of expression, often caused by hypermethylation, has been linked to tumor formation and metastasis.[11, 12] This gene's functional loss is evident in over 90% of all high-grade cervical neoplasia and invasive cervical cancers.[13] Hypermethylation-induced *CADM1* overexpression inhibits cell proliferation and suppresses gene expression, promoting the progression of high-grade lesions and subsequent carcinogenesis.[14] Similarly, the Suppressor of Cytokine Signalling 1 (*SOCS1*), another tumor suppressor gene, displays aberrant methylation in 61% of cervical cancer cases,[15] altering the phenotype of cervical cancer.[16] Studies have indicated that human papillomavirus (*HPV*), upon attacking the host, induces promoter hypermethylation of *CADM1* through its association with *DNMT1*[17] and transcriptional inactivation of the SOCS1 gene.[18]

Reversal of Promoter Hypermethylation: In malignancies, drugs that interfere with or inhibit DNA methylation can induce re-expression and reactivation of silenced genes. Azacitidine and decitabine, both approved by the Food and Drug Administration (FDA) for myelodysplastic syndromes (MDS), have demonstrated demethylating effects. However, azacitidine treatment has been associated with certain toxicities, such as transient cytopenia (granulocytopenia and thrombocytopenia), nausea, limb pain, bleeding, neutropenia, pneumonia, dyspnea, myalgia, and weakness.[19] Similarly, decitabine usage has reported toxicities including hematologic issues (neutropenia, thrombocytopenia, and anemia), infections, constipation, diarrhea, and coughing.[20] Therefore, there's a demand for safer, non-toxic demethylating agents.

Phytochemicals have emerged as potential candidates, showcasing demethylating properties with evidence of reversing promoter hypermethylation in Tumor Suppress Genes (TSGs). Unlike synthetic drugs, phytochemicals don't induce cytotoxicity or neutropenia,[19] Curcumin, for instance, has been documented to inhibit HPV infection by targeting the *DNMT1* gene, suppressing methylation, and reversing hypermethylation of the *p15* promoter.[21] Genistein has shown potential in demethylating RARβ2 in cervical cancer cells,[22] while capsaicin has been reported to reverse promoter hypermethylation of CADM1 and SOCS1 genes in the HeLa cells.[23]

Datura, a perennial herbaceous plant in the Solanaceae family, known for its pharmacological properties, includes species like Datura metel, also called Hindu datura, Devil's trumpet, and Indian thorn-apple (Figure 1). Different extracts from *Datura metel* leaves have exhibited strong anti-cancer activity in breast cancer cells (MCF-7) and kidney cancer cells (Vero).[24] Other species like Datura inoxia and Datura stramonium, rich in phenolic and flavonoid content, have shown anti-cancer properties in leukemia[25] and caused THP-1 cytotoxicity.[26,27]

Figure 1. Datura metel

EXPERIMENTAL PROCEDURES

Plant material

Datura metel leaves were obtained from around 10-month-old plants in October at Ghaziabad, Uttar Pradesh, India.

Preparation of Crude extract

The leaves of *Datura metel* were dried in the shade for 10 days. After the leaves were dried, they were grounded using mortar and pestle and converted into powder. This powdered dried leaf weighed 3.011gm.

Preparation of ethanol: chloroform extract

The measured leaf powder of *Datura metel* was dissolved in 3ml of Ethanol: Chloroform (EC). The mixture was put in a shaker for 24 hours. The mixture was filtered twice using Whatman filter paper No. 1 after 24 hours of shaking. Using a rotary evaporator, the crude extract was obtained after 30 minutes and used for treating the cervical cancer cells at 5µg/ml which is less than IC_{50}= 8.9µg/ml.[26]

Cell Culture

SiHa cells were purchased from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured based on the protocols standardized by Freshney in 1994 in DMEM supplemented with 10% FBS at 37℃ in 5% CO_2.

Isolation of DNA

The cells that were obtained from SiHa cells were lysed using a digestion buffer (Tris-HCl, NaCl, EDTA) with Proteinase K (0.2mg/ml). The DNA was purified using standard phenol-chloroform extraction and precipitation method.

DNA fragmentation

DNA fragmentation is known as the hallmark of cell apoptosis. The DNA was isolated from normal SiHa cells and Datura-treated cells for 48 hours and was run along with a 100bp ladder in gel electrophoresis.

Sodium Bisulphite Treatment and Methylation Specific PCR (MSP)

DNA (20µL) dissolved in TE buffer underwent sodium bisulfite treatment from the kit (Zymo Research), according to the instructions provided by the manufacturer. The eluted DNA with 0.1µM unmethylated and methylated specific primers (Table 1) undergoes Methylation-specific PCR. The initial denaturation step at 95˚C for 5 minutes was followed by 35 cycles of denaturation at 95˚C for 10 seconds, annealing at 54˚C for 45 seconds, extension at 72˚C for 30 seconds, and final extension at 72 ˚C for 10minutes with a 109bp product size of both methylated and unmethylated DNA. The PCR products were separated in 1.5% agarose gel.

Similarly, the eluted DNA with 0.1µM unmethylated and methylated specific primers (Table 1) undergoes Methylation-specific PCR. The initial denaturation step at 95˚C for 10 minutes was followed by 40 cycles of denaturation at 95˚C for 30 seconds, annealing at 57˚C for 40 seconds, extension at 72˚C for 45seconds, and final extension at 72 ˚C for 10 minutes with a product size of 93bp for methylated and 105bp for unmethylated DNA. The PCR products were separated in 1.5% agarose gel.

RESULTS

DNA Fragmentation analysis

Inter-nucleosomal DNA in SiHa cells treated with 5µg/ml *Datura metel* leaf extract was observed to be fragmented in agarose gel electrophoresis against 100bp ladder and control DNA (Figure 2).

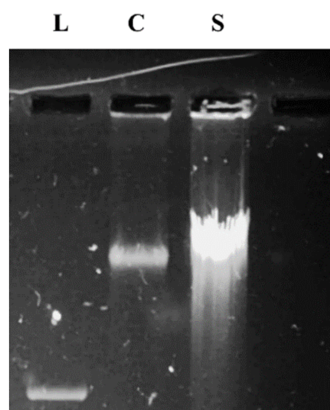

Figure 2. Inter-nucleosomal fragmented DNA of EC leaf extract of Datura metel-treated SiHa cells for 48 h (L: 100bp Ladder, C: control, S: extract treated sample DNA).

Table 1. Primer sequences, amplicon sizes, annealing temperature, and conditions for PCR analysis

Gene	Primer sequence	Annealing temperature	Cycles	Product size
CADM1	Methylated Forward 5'-TAGTATTTTATTAGTTGTTCGTTC-3'			
	Methylated Reverse 5'-GCACACTAAAATCCGCTCG-3'	54°C	35	109
	Unmethylated Forward 5'-TTAGTATTTTATTAGTTGTTTGTTT-3'			
	Unmethylated Reverse 5'-CCACACACTAAAATCCACTCA-3'			
SOCS1	Methylated Forward 5'-TAGTATTTTATTAGTTGTTCGTTC-3'			93
	Methylated Reverse 5'-CGACACAACTCCTACAACGACCG-3'	57°C	40	
	Unmethylated Forward 5'-TTATGAGTATTTGTGTATTTTTAGGTTGGTT-3'			115
	Unmethylated Reverse 5'-CACTAACAACACAACTCCTACAACAACCA-3'			

Methylation Reversal Analysis

Treatment with 5µg/ml *Datura metel* leaf extract resulted in the reversal of *CADM1* methylation at 6 days of treatment. This was analyzed by the reduction in the intensity of the methylation-specific band (MSB) and brighter unmethylation-specific band (UMSB) after the treatment (Figure 3).

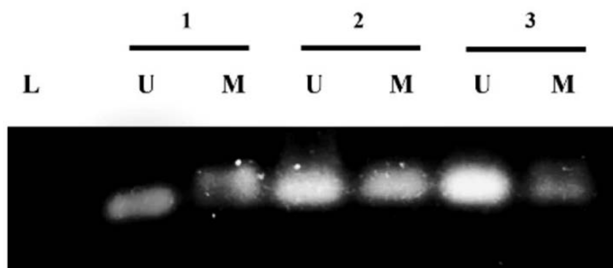

Figure 3. MS-PCR product of CADM1 treated by D. metel EC leaf extract [L- Ladder 100bp, U-Unmethylation-Specific band (UMSB), M-Methylation-Specific Band (MSB), 1-U- Unmethylated DNA at 48 hours, M- Methylation-Specific Band at 48 hours, 2- U- Unmethylated DNA at 72 hours, M-Methylation-Specific Band at 72 hours, 3-U- Unmethylated DNA at 6 days, M- Methylation-Specific Band at 6 days.

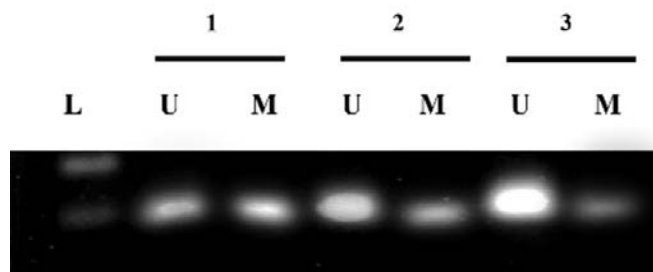

Figure 4. MS-PCR product of SOCS1 treated by D. metel EC leaf extract [L- Ladder 100bp, U-Unmethylation-Specific band (UMSB), M-Methylation-Specific Band (MSB), 1-U- Unmethylated DNA at 48 hours, M- Methylation-Specific Band at 48 hours, 2- U- Unmethylated DNA at 72 hours, M-Methylation-Specific Band at 72 hours, 3-U- Unmethylated DNA at 6 days, M- Methylation-Specific Band at 6 days.

Treatment with 5µg/ml leaf extract of *D. metel* resulted in the reversal of *SOCS1* methylation after 72 hours with increasing demethylation after further 6 days of treatment. This was

analyzed by the unmethylation-specific band (UMSB) with enhanced luminosity and lighter methylation-specific band (MSB) after the *D. metel* extract treatment (Figure 4).

Discussion

Epigenetic alterations, particularly promoter methylation of tumor suppressor genes, significantly impact tumor development. *DNMT1,* upregulated by *HPV* E6 protein, crucially initiates *de novo* methylation and degrades *p53* in cervical cancer.[28] Yanantatsaneejit et al. (2020) discovered that *HPV*'s E7 protein forms a complex with *DNMT1* at the *CADM1* promoter, inducing *CADM1* methylation.[17] Additionally, *SOCS1*'s involvement in cervical cancer phenotype has been documented.[16]

To reduce the uncontrollable cell proliferation caused by *CADM1* hypermethylation as well as the tumorigenesis caused by *SOCS1* overexpression, with no side effects and normal cell apoptosis, these epigenetic modifications must be reversed by initiating the demethylation of the *CADM1* and *SOCS1* genes. The two characteristics of apoptosis are DNA breakage and chromatin condensation.[29] Through *p53* and other proteins including *Fas, TNF,* and *TRAIL* receptors, which are the mediators of apoptosis in an extrinsic signaling route, the cell death machinery is triggered. Contrary to synthetic drugs, natural compounds lack cytotoxic effects on normal cells. Phytochemicals such as alkaloids, polyphenols, and flavonoids exhibit diverse chemo preventive activities against various cancers. Many phenolic compounds have shown various effects on antiangiogenic factors present in tumors as well as healthy normal cells.[30] There are many dietary chemo preventative compounds like resveratrol, quercetin, curcumin, and ellagic acid that have shown inhibition of cancer progression by inducing apoptosis in the cells.[31] Various studies on natural compounds like capsaicin,[23] genistein,[32] and curcumin[33] have shown reversal potential, while investigations into different families of plants and their phytochemicals are been conducted to observe their potential in cancer treatment. For example, members of Fabaceae have certain phytochemicals that have shown effects like apoptosis, cell cycle arrest induction, immune system regulation, and anti-oxidant stress.[34,35] Similar studies are being conducted on different plants and herbs to reveal their anti-cancer properties.

DNA fragmentation is one of the primary techniques to investigate cellular apoptosis due to exposure of the cell to any extrinsic chemical. In the present study, it was observed that *Datura metel* leaf extract induced apoptosis in the squamous cervical cancer cells. The targeted apoptosis of the carcinoma cells could be marked as one of the crucial steps to inhibit carcinogenesis. Following the confirmation of apoptosis, the focus of the study shifted to assessing the ability of the extract to reverse aberrant methylation in tumor suppressor genes. Treatment in SiHa cells by E: C leaf extract of *Datura metel* for 48 hours, 72 hours, and 6 days at 5µg/ml showed promising reversal effects as the bands obtained after performing Methylation-Specific PCR showed a reduction in intensity and visual sharpness of the methylation-specific bands and the unmethylation-specific band displayed an increase in width and enhanced luminosity for both *CADM1* and *SOCS1*. As the methylation-specific band became lighter indicated the level of methylation in the particular gene was getting reduced and the increase in brightness and intensity of the unmethylated bands indicated that the methylation level after the treatment with leaf extract in the SiHa cells had increased significantly as the time of treatment progressed. Thus, signifying the potential reactivation of the downregulated Tumor Suppressor Genes (TSGs).

Identifying the specific molecules present in the E: C leaf extract of *Datura metel* that is responsible for this reversal could offer an exciting treatment avenue. Therefore, the highlight of the study is that the *Datura metel* leaf extract could be considered a potent candidate for reversing hypermethylation in *CADM1* and *SOCS1* genes within cervical cancer cells. We also propose its potential synergistic use with other natural compounds for enhanced effectiveness against cancer cells.

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Conflict of Interest statement

The authors declare that there is no conflict of interest for current research work.

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