Indian J. Applied & Pure Bio. Vol. 41(3), 1605-1613 (2026). **ISSN: 0970-2091**

Apoptosis induced by *Melia dubia* Cav. leaf extract through oxidative stress and mitochondrial destabilization in HeLa cervical cancer cells

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Abstract

Cervical cancer continues to pose a significant global health problems, underscoring the demand for bioactive phytochemicals capable of modulating key oncogenic pathways. *Melia dubia* Cav. - Meliaceae also known as Malabar neem is a perennial tree. The leaves contain rich source of bioactive limonoids and other major pharmacological phytoconstituents such as linoleic acid, palmitic acid, caryophyllene, probucol and aromadendrene. In this study, the anticancer potential of *Melia dubia* leaf extract was investigated in HeLa cervical cancer cells with a focus on oxidative stress-mediated apoptosis and mitochondrial dysfunction. Treatment with the extract significantly elevated intracellular reactive oxygen species (ROS) levels, disrupting the cellular redox balance. This ROS surge triggered a loss of mitochondrial membrane potential, indicating compromised mitochondrial integrity. These events collectively promoted cytochrome C release and subsequent activation of apoptotic pathways. Morphological alterations with apoptosis, including cell shrinkage, nuclear condensation and membrane blebbing were also observed. Overall, our findings demonstrate that *Melia dubia* leaf extract induces potent cytotoxic effects in HeLa cells by initiating oxidative stress and mitochondrial-dependent apoptosis, highlighting its potential as a promising candidate for cervical cancer therapeutics.

Key words : HeLa cells, *Melia dubia* Cav., Mitochondrial dysfunction, Oxidative stress, Reactive oxygen species (ROS).

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Cervical cancer is the fourth most commonly diagnosed malignancy among women worldwide, accounting for approximately 6.5% of all cancer cases. More than 90% of cervical cancer incidences are caused by persistent infection with high-risk oncogenic strains of the Human Papilloma Virus (HPV). Histologically, squamous cell carcinoma constitutes nearly 70% of cases, while adenocarcinoma contributes to about 25% [5]. The development of cervical cancer is strongly associated with oxidative stress and mitochondrial dysfunction induced by oncogenic HPV infections. The integration of HPV DNA into the host genome initiates the expression of viral oncoproteins, resulting in chronic inflammation and increased production of reactive oxygen species (ROS). These ROS cause oxidative damage to cellular macromolecules including proteins, lipids, and DNA, thereby promoting malignant transformation[21]. Persistent HPV infection disrupts key regulatory pathways that controls cell proliferation, differentiation, and apoptosis, promoting tumor initiation, progression, and metastasis. Such molecular alterations contribute to the formation of precancerous lesions, including squamous intraepithelial lesions, which may progress to invasive cervical carcinoma[17,19].

Among the viral genes, the early HPV proteins E6 and E7 are considered as the primary mediators of oncogenesis and are essential for malignant transformation. These oncoproteins exert their tumorigenic effects primarily by interfering with host cell cycle control mechanisms[21]. The tumor suppressor protein p53 plays a pivotal role in maintaining genomic stability by arresting the cell cycle in response to DNA damage thereby allow repair or trigger apoptosis if the repair fails. The HPV E6 protein binds with p53 in association with the E6- associated protein (E6AP), leading to its ubiquitin-mediated proteasomal degradation. As a result, p53 levels are significantly reduced, impairing the cell's ability to respond appropriately to genomic stress. Similarly, the retinoblastoma protein (pRb), another crucial tumor suppressor, regulates the transition from the G1 to S phase of the cell cycle. Under normal conditions, hypo-phosphorylated pRb binds to E2F transcription factors, preventing premature S-phase entry. However, the HPV E7 protein associates with hypo-phosphorylated pRb, disrupting this interaction and destabilizing its association with E2F, which in turn drives uncontrolled DNA synthesis and continuous cell proliferation[27]. The persistent expression of E6 and E7 in transformed cells induces a state of oncogene addiction, making them promising molecular targets for therapeutic intervention[13].

Conventional chemotherapeutic agents such as cisplatin and carboplatin—both platinum- based drugs—have demonstrated significant efficacy in cancer treatment. However, in advanced stages of disease, recurrence and chemo resistance remain major challenges, underscoring the urgent need for potent, cost-effective, and safer anticancer compounds[8]. Medicinal plants represent a valuable source of diverse bioactive phytochemicals with therapeutic potential. *Melia dubia* Cav., family Meliaceae, is one such promising plant. Its leaves are rich in alkaloids, carbohydrates, glycosides, phenolics, tannins, gums, and mucilages, contributing to a wide range of pharmacological activities including antibacterial, antifungal, anti-inflammatory, antioxidant, anticancer, analgesic,

antidiabetic, and antiurolithiatic effects. Additionally, the plant contains a variety of bioactive compounds such as caryophyllene, humulene, probucol, germacrene-D, linoleic acid & palmitic acid[11].

The present study is aimed to investigate the bioactive potential of the methanolic extract of *Melia dubia* leaves in mitigating tumor growth with a particular focus on elucidating its mechanistic role in inducing programmed cell death and mitochondria mediated apoptotic pathway in HeLa cells, thereby providing valuable insight as a natural antitumor therapeutics.

Sample collection and preparation :

Fresh leaves of *Melia dubia* were collected from Forest College and Research Institute, Mettupalayam, Coimbatore, Tamil Nadu, India. The collected leaves were thoroughly washed thrice, shade dried for 7-10 days and ground into fine powder and stored in air tight amber bottle. The powdered samples were dissolved in selected solvent in 1:10 ratio and subjected to cold percolation method for 48 hours. The extracts were filtered and used for further analysis.

Cell Culture Maintenance and Treatment Protocol :

For this study, the HeLa cell line (Human cervical adenocarcinoma epithelial cells) was procured from the National Center for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% (v/v) heat- inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures were maintained at 37°C under a humidified atmosphere of 95% air and 5% CO_2 to ensure optimal growth in the logarithmic phase.

In vitro cytotoxicity activity of *Melia dubia* leaf extract :

The cytotoxicity effect of *Melia dubia* leaf extract against HeLa cell line was evaluated using MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide assay). The HeLa cells were seeded into 96-well microplates at a density of 1×10^5 cells per well and incubated at 37°C for 48 h in 5% CO_2 incubator until they reached approximately 80% confluence. The medium was then replaced, and the cells were treated with varying concentrations of the leaf extract for 24 h, while untreated cells served as controls. Morphological modifications in both treated and control cells were observed under an inverted microscope (Magnus INVI; 20× objective) after 24 h of incubation. The cells were then rinsed with phosphate-buffered saline (PBS, pH 7.4), and 20 µL of MTT solution (5 mg/mL in PBS) was added to each well. The plates were incubated at 37°C in the dark for 3 h for the formation of formazan crystals. These crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO), and the absorbance was recorded at 570 nm using a spectrophotometer. The level of formazan formed reflects mitochondrial activity, serving as an indirect indicator of cell viability in the MTT assay[14].

The percentage of cell viability was calculated using the following formula:
Cell viability (%) = Absorbance of control cells/ Absorbance of treated cells X100

The IC_{50} value depicted as the concentration that required to inhibit 50% of cell growth and was calculated using GraphPad Prism version 8 software.

Fluorescent AO/EB Staining of Apoptotic Cells :

Apoptotic morphological alterations in HeLa cells induced by *Melia dubia* leaf extract was evaluated using dual acridine orange/ethidium bromide (AO/EB) staining. Both viable and nonviable cells uptake AO stain and emit green fluorescence; however, EO is only taken up by nonviable cells that have lost membrane integrity, producing red fluorescence. HeLa cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and treated with IC_{50} concentration of leaf extract for 24 hours. Untreated HeLa cells served as controls. Post- treatment, cells were rinsed with PBS and stained with 20 µL of AO/EB staining solution (each dye at 100 µg/mL) for approximately 5 minutes. The stained cells were then visualized under a fluorescence microscope (Invitrogen EVOS FL Cell Imaging) at 40×magnification[14].

PI Staining for Cell Death Analysis :

Late-stage apoptotic and necrotic cells were detected using propidium iodide (PI) staining. PI, a fluorescent dye that intercalates with nucleic acids, facilitates visualization of nuclear alterations of apoptosis. HeLa cells were cultured in six-well plates at a density of 1×10^5 cells per well and treated with *Melia dubia* leaf extract at their IC_{50} concen-tration. After 24 hours, cells were rinsed with PBS, fixed in a 3:1 methanol: acetic acid solution for 10 minutes, and stained with 10 µL of PI (50 µg/mL) for 20 minutes. The apoptotic nucleus were then observed under a fluorescence microscope[14].

Assessment of Nuclear Morphology Using DAPI Fluorescence :

Nuclear morphological alterations, such as chromatin condensation and nuclear fragmentation, in *Melia dubia* leaf extract treated HeLa cells were assessed using DAPI (42 , 6- diamidino-2-phenylindole) staining[22]. DAPI exhibits strong affinity for adenine–thymine–rich regions of DNA and can stain both live and fixed cells. For the assay, HeLa cells ($1 \times 10u$ cells/well) were seeded in a 6-well plate and treated with the IC_{50} concentration of leaf extract for 24 h. After incubation, the cells were rinsed with PBS, fixed in 3% paraformaldehyde (50 µl) for 10 min, and permeabilized with 0.2% Triton X-100 (50 µl) for 10 min at room temperature. Subsequently, the cells were stained with 10 µl of DAPI solution (0.5 µg/ml) for 5 min and observed under a fluorescence microscope[14].

Fluorescent Detection of Reactive Oxygen Species :

The intracellular production of reactive oxygen species (ROS) *Melia dubia* leaf extract treated HeLa cells was evaluated through the oxidative conversion of the non-fluorescent, cell-permeable probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) into the highly fluorescent compound 22 72 - dichlorofluorescein (DCF)[26]. For this experiment, HeLa cells (1×10^5 cells/well) were seeded in a 6-well plate and were

exposed to the IC_{50} concentration of leaf extract for 24 h. After treatment, the cells were incubated with 1 µl of DCFH-DA (1 mg/ml) for 20 min in the dark. The intracellular formation of fluorescent DCF was then visualized under a fluorescence microscope[14].

Evaluation of Mitochondrial Membrane Potential (Δψm) :

Alterations in mitochondrial membrane potential (MMP; Δψm) serve as a key indicator of apoptosis, mitochondrial dysfunction, and overall cell health. The cationic fluorescent dye Rhodamine 123 was employed to assess Δψm levels[23]. After exposure to the IC_{50} concentration of *Melia dubia* leaf extract, HeLa cells were rinsed with PBS and subsequently stained with 50 µl of Rhodamine 123 (10 µg/ml) for 30 min. Changes in cell morphology and mitochondrial membrane integrity were then observed under a fluorescence microscope at 40×magnification[14].

Fluorescent AO/EB Staining of Apoptotic Cells :

Apoptosis is the primary form of programmed cell death that occurs in response to cytotoxic stress. It represents a tightly regulated mechanism of cellular self-destruction initiated by cytotoxic stimuli. This process is characterized by distinct morphological and molecular features, including plasma membrane blebbing, chromatin condensation, DNA fragmentation, and the cleavage of vital cellular proteins[10,12]. To evaluate both early and late apoptotic stages, as well as necrotic events, HeLa cells treated with the methanolic leaf extract of *Melia dubia* were subjected to acridine orange/ethidium bromide (AO/EB) dual staining and analysed under a fluorescence microscope. Untreated control cells exhibited uniform green fluorescence with intact nuclei, indicating healthy cells with no signs of apoptosis and *Melia dubia* treated HeLa cells exhibited marked apoptotic changes. Cells in the early stage of apoptosis displayed light green nuclei, whereas late apoptotic cells exhibited bright orange chromatin condensation, clearly distinguishing them from necrotic cells. Necrotic cells, in turn revealed intense red fluorescence, indicative of disrupted membrane integrity caused by the cytotoxic action of the *Melia dubia* methanolic leaf extract against HeLa cells (Fig. 1). Similarly nimbolide, a compound derived from the leaves and flowers of *Azadirachta indica* A. Juss exhibited cytotoxic effects on human choriocarcinoma (BeWo) cells, a placental cancer cell line[20]. Similarly, linalool proved the antitumor potential in prostate cancer cells by inducing apoptosis and causing cell cycle arrest in the sub-G1 phase. This treatment led to extensive DNA fragmentation, as well as morphological changes such as cell shrinkage and membrane blebbing hallmarks of apoptosis[25].

PI Staining for Cell Death Analysis :

The development of cancers and its resistance to conventional therapies are largely linked to defective apoptotic signaling pathways, highlighting the critical role of apoptosis in maintaining tissue homeostasis[6,15]. To evaluate membrane integrity and apoptotic cell death, propidium iodide (PI) staining was employed on HeLa cells treated with the methanolic leaf extract of *Melia dubia* The analysis revealed significant increase in PI-positive cells following treatment with the extract, while only a few apoptotic cells were detected in the

untreated control group. The intense red fluorescence observed in treated samples indicated substantial membrane damage and cell death (Fig. 1). Similarly, linalool has been reported to induce apoptosis and promote differentiation in human leukemia HL-60 cells. Moreover, recent investigations have depicted that linalool can counteract doxorubicin resistance in human breast adenocarcinoma cells and inhibit the growth of various solid tumours, including melanoma, renal cell adenocarcinoma, and HepG2 hepatocellular carcinoma. Notably, linalool selectively triggers apoptosis in human leukemia cells without affecting the proliferation of normal hematopoietic cells[25].

Assessment of Nuclear Morphology Using DAPI Fluorescence :

Nuclear alterations and chromatin condensation key hallmarks of apoptosis, were examined in *Melia dubia* leaf extract–treated HeLa cells using DAPI staining that specifically binds to A-T-rich sequences in double-stranded DNA [3]. Treatment with the *Melia dubia* methanolic leaf extract resulted in prominent nuclear changes, including DNA condensation, chromatin reorganization, and loss of cellular architecture, which were visualized as bright blue fluorescence with condensed nuclear regions. In contrast, untreated control cells exhibited normal intact nuclei. DAPI staining confirmed that, the cytotoxic effects of the terpenoid and other major constituents present in *Melia dubia* extract would have likely contributed to DNA damage and apoptosis[2]. These observations indicate that *Melia dubia* leaf extract effectively induces apoptosis in HeLa cells by promoting DNA and chromatin disruption (Fig. 1). Similarly, DAPI staining proved apoptosis in breast cancer cells treated with nimbolide compound from ethanolic neem *Azadirachta indica* A. Juss leaf extract. The treated cells exhibited mitochondrial membrane potential loss and highly condensed nuclei, indicative of apoptotic cell death as the ethanolic extract triggered apoptosis in human breast cancer cells as evidenced by DNA fragmentation and associated morphological changes[7].

Fluorescent Detection of Reactive Oxygen Species :

The production of intracellular reactive oxygen species (ROS) in HeLa cells was assessed by DCFH-DA staining using *Melia dubia* leaf extract. The results depicted a significant increase in fluorescence intensity, indicating enhanced oxidative stress. ROS such as superoxide anions, hydrogen peroxide, and hydroxyl radicals are known to cause damage to lipids, proteins, and DNA, ultimately triggering apoptosis[28]. Treatment with methanolic leaf extract of *Melia dubia* (IC_{50} = 54.39 ± 0.00 µg/ml) resulted in a notable increase in ROS generation compared to untreated control cells. The leaf extract modulates the redox balance in HeLa cells by elevating intracellular ROS levels, leading to ROS-mediated cell death (Fig. 1). The presence of terpenoids and other bioactive phytoconstituents in the extract likely contributed to these effects, as such compounds can act as either antioxidants or pro- oxidants depending on their concentration and the cellular environment[14,18]. Excessive ROS accumulation can induce oxidative stress, causing DNA strand breaks, lipid peroxidation, and apoptotic cell death[9]. Similarly, the ethanolic leaf extract of *Azadirachta indica* A. Juss was found to induce mitochondrial dysfunction and apoptosis in human colorectal adenocarcinoma (HT-29) cancer cells. The

oncogenic cells were treated with the ethanolic leaf extract along with the oxidative stress marker H_2 DCFDA that exhibited elevated ROS levels after 48 hours as evidenced by increased green fluorescence[24].

Evaluation of Mitochondrial Membrane Potential (Δψm) :

HeLa cells mitochondrial membrane potential loss was analyzed using the lipophilic fluorescent dye Rhodamine-123 using *Melia dubia* leaf extract. Elevated ROS levels are known to compromise mitochondrial membrane integrity, resulting in mitochondrial dysfunction and the release of cytochrome c which subsequently activates the apoptotic pathway[16]. Experimental observations revealed that *Melia dubia* leaf extract treated cells exhibited a marked reduction in dye uptake compared to untreated controls. The decreased green fluorescence intensity in treated cells indicates mitochondrial membrane depolarization, signifying severe mitochondrial impairment—an early and irreversible event in the process

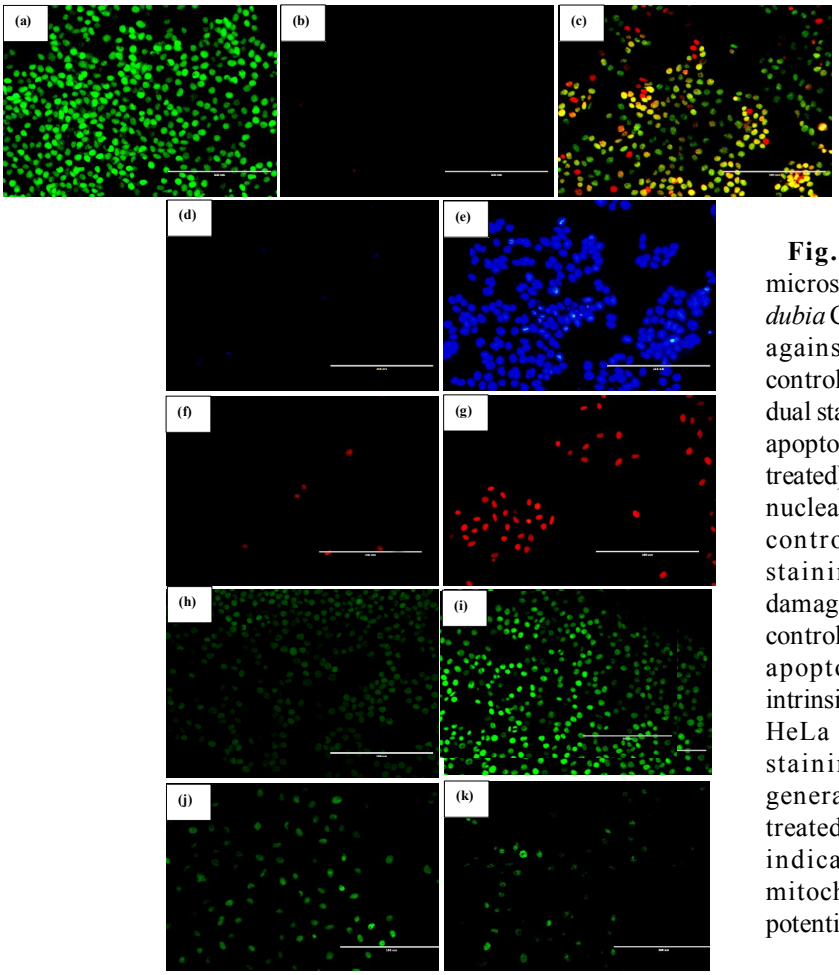

Fig. 1. Fluorescence microscopy images of *Melia dubia* Cav. leaf extract treated against HeLa cells (a, b-control, c -treated) -AO/EB dual staining for induction of apoptosis, (d - control, e - treated)- DAPI exhibiting the nuclear fragmentation, (f-control, g-treated)-PI staining revealing cell damage and cell death, (h - control, i - treated)- Induced apoptosis associated intrinsic modification against HeLa cells DCFH-DA staining revealing ROS generation, (j-control, k-treated) - Rh-123 staining indicating the loss of mitochondrial membrane potential.

of programmed cell death[1] (Fig. 1). Similar findings have been reported with ethanolic neem leaf extract of *Azadirachta indica* which induced activation of caspases and migration of apoptosis-inducing factors into the nucleus, indicating disruption of mitochondrial transmembrane potential in chronic lymphocytic leukemia cells. Flow cytometric analysis has revealed strong fluorescence from tetramethylrhodamine methyl ester (TMRM) in untreated cells, while cells treated with ethanolic leaf extract of *Azadirachta indica* exhibited diminished fluorescence, confirming mitochondrial membrane potential loss[4].

In the conclusion, the rising incidence of diseases and the emergence of multidrug-resistance have become a serious global health concern within the human population. In this research, the phytocompounds present in the *Melia dubia* Cav. leaf extract exhibited remarkable antioxidant activity, excellent stability and notable biocompatibility, demonstrating strong anticancer efficacy against HeLa cervical cancer cells with exhibited negligible toxic effects on normal cells. The predominant presence of terpenoids and other major phytoconstituents in the leaf extract has contributed to the induction of apoptosis and necrosis by causing DNA damage within cervical cancer cells. Their pro-apoptotic potential was further substantiated through fluorescence imaging, measurement of intracellular ROS production, and assessment of mitochondrial membrane potential loss. These findings reveal that methanolic leaf extract of *Melia dubia* possesses anticancer effect. The extensive *in vivo* investigations are imperative to elucidate the underlying molecular mechanisms of action and to substantiate the clinical translational potential of these phytocompounds in cancer therapy in the future research.

The authors thank the Principal, Ethiraj Centre for Research, Innovation and Creativity (ECRIC), Head, Faculty members, Supporting Staff of the Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women (Autonomous), Chennai for their valuable support and encouragement. We would like to thank Forest College and Research Institute (FC&RI), Mettupalayam, Coimbatore for providing the samples. We would like to extend our thanks to Avigen laboratory for the cell line studies.

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