

## LAWSONIA ALBA LEAVES INDUCE APOPTOSIS AND CELL CYCLE ARREST IN B16F10 MELANOMA CELLS

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### ABSTRACT

**Objective:** The present study was designed to investigate the anti-melanoma activity of the ethyl acetate fraction of *Lawsonia alba* lam leaves (ELA) against B16F10 cells.

**Methods:** Cytotoxicity of ELA on B16F10 cells was determined by MTT assay and supported with the morphology of apoptotic and necrotic cells under phase-contrast microscope, fluorescence microscopy with AO/EtBr, confocal microscopy with PI, Agarose gel electrophoresis and Annexin V-FITC/PI staining, mitochondrial membrane potential and cell cycle arrest by FACS was also performed on B16F10 cells.

**Results:** Cytotoxic effect of ELA on B16F10 melanoma cell was confirmed by MTT assay with an IC<sub>50</sub> value of 14.10µg/ml. Morphological study showed arrays of both the nuclear changes including chromatin condensation and apoptotic body formation indicating that the treatment with ELA and 5-Fluorouracil (standard) causes apoptotic changes in the melanoma cells compared to the untreated control. Agarose gel electrophoresis study showed fragmented DNA in the form of ladder. The depolarization of mitochondrial membrane potential was confirmed. Flow cytometric analysis showed appreciable number of cells in early apoptotic stage. The cells were arrested mostly in G<sub>0</sub>/G<sub>1</sub> phase of cell cycle.

**Conclusion:** Ethyl acetate fraction of *Lawsonia alba* L. leaves possesses potent apoptotic activity against B16F10 cells.

**Keywords:** *Lawsonia alba*, Cancer, Melanoma, Apoptosis

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### INTRODUCTION

Cancer is the cell proliferation independent of growth factor regulation with apoptosis resistance and angiogenesis, leading to the disruption of homeostatic condition therapy to tumorigenesis. One of the forms of cancer i.e. malignant melanoma of the skin is the most frequent cause of mortality from skin cancer and approximately 20-25% of patients with malignant melanoma die of metastatic disease. Melanoma is an aggressive and refractory cancer derived from melanocytes [1]. The incidence of melanoma has been continuously increasing worldwide and becoming a huge public health issue [2]. The standard means of treating melanoma include surgery, radiation therapy, and chemotherapy [3]. (The treatment of melanoma disease largely depends upon early diagnosis [4]. Drug resistance in melanoma mainly occurred through dysregulation of apoptosis, whereas drug transport, detoxification, and enhanced DNA repair may also exert a function [5]. Henceforth, induction of apoptosis deliberated the major target of anti-cancer chemotherapeutic strategies for melanoma [6].

*Lawsonia alba* (Lythraceae) commonly known as Henna is widely known for treating various ailments in folklore medicine. Essentially, it is a very common plant with flourishing medicinal properties including anti-microbial [7], antimalarial [8]. Our previous study on *L. alba* reported its hepatoprotective effects along with anti-oxidant property [9]. Amino derivatives of lawsone and lapachol were identified, showing the cytotoxicity against Ehrlich carcinoma and human K562 (leukemia cells). Henna when applied topically on the back skin in the UV-B initiated/TPA promoted and per-oxy-nitrite initiated/TPA promoted mouse skin carcinogenesis models antitumor activities [10]. In this study, we investigated the effects of *Lawsonia alba* leaves in melanoma cells because of its increasing incidence and high fatality rate for skin tumors. The present investigation ELA is an approach to study the apoptosis-inducing activity of the active fraction from *Lawsonia alba* leaves against B16F10 melanoma cell lines.

### MATERIALS AND METHODS

#### Chemicals and reagents

DMEM, fetal bovine serum (FBS), trypsin (Gibco, USA), 5-Fluorouracil (Sun Pharma), penicillin-streptomycin (Biowest, Germany),

gentamycin (Nicholas, India), HEPES, L-glutamine, MTT, acridine orange, ethidium bromide, agarose (Puregene), proteinase k (SRL), Annexin-V FITC (apoptosis kit), RNase, Propidium Iodide, JC-1 (Sigma), DMSO, Ethyl acetate (Merck). All other chemicals of analytical grade were procured locally.

#### Cell line

B16F10 cells were obtained from the National Facility for Animal Tissue and Cell Culture, Pune, India. The B16F10 cells were cultured and routinely maintained in DMEM. The medium was supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100µg/ml), gentamycin (100µg/ml) and incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> inside a CO<sub>2</sub> incubator.

#### Collection and identification of plant specimen

*Lawsonia alba* Lam. leaves were collected from Ranchi, Jharkhand and identified by Dr. V. P. Prasad, Scientist-D, Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, West Bengal. The specimen authentication no. is CNH/Tech. Π/2014/75/158. The preparation of the plant extract was described previously [9]. The methanolic extract of *Lawsonia alba* was further fractionated by separating funnel using Ethyl acetate. Removal of the solvent in a rotary evaporator (BUCHI R-200) yielded 750 mg of the ELA fraction.

#### Cytotoxicity study by MTT assay

For evaluation of the cytotoxic effect of ELA against melanoma cells MTT colourimetric assay was performed [11]. B16F10 cells in the log phase were cultured in 96-well plate and treated with different concentrations of ELA (1 mg/ml) for 24, 48 and 72 h. Cytotoxicity was measured using 3-(4, 5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (MTT) assay. Standard drug 5-FU (0.087µg/ml) treated cells served as positive control [12]. The absorbance of each well was observed at a wavelength of 570 nm by microplate manager (Reader type Model 680 XR Bio-Rad laboratories Inc.) The IC<sub>50</sub> value of ELA was determined for B16F10 cells.

### Cell morphology assessment

To investigate the effect of ELA on cell morphology  $1 \times 10^6$  cells (B16F10) were seeded in DMEM media supplemented with 10% FBS. 24h post adherence, the cells were treated with  $IC_{50}$  dose of ELA and anti-cancer drug 5-Fluorouracil. The cells were then observed under a light microscope (Leica Microscope) at a magnification of 40X.

### AO-EtBr staining

The morphological changes of apoptosis were evaluated by fluorescence microscopy [13]. B16F10 melanoma cells ( $1 \times 10^6$ ) were cultured in coverslip in a small petri plate and allowed to grow for 24 h and another petri plate was kept as control. After 24 h cells were treated with  $IC_{50}$  dose of ELA, 5-Fluorouracil and control plate was left untreated. After that plates were kept in a humidified atmosphere with 5%  $CO_2$  and  $37^\circ C$  temperature for 18 h. After 18 h the cells adhered to the coverslip were washed with PBS and stained with 10  $\mu$ l of acridine orange and ethidium bromide in a ratio of 1:1. Then the coverslips were mounted on clear slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.

### Propidium iodide staining

Propidium iodide stain was used to examine the nuclear fragmentation. B16F10 cells in log phase were treated with  $IC_{50}$  dose of ELA and 5-FU for 24 h. After 24 h the control and ELA treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10 $\mu$ g/ml of propidium iodide for 5 min. The cells were mounted on slides to observe the differences in nuclear morphology between untreated and ELA treated B16F10 cells under a confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystem, Heidelberg, Germany) installed with an inverted microscope [Leica DM-7RB]. Images for propidium iodide were acquired from argon/krypton laser and UV laser line using 590 nm long pass filter for propidium iodide for images [14].

### DNA fragmentation study

To confirm the apoptotic mode of cell death, DNA fragmentation assay was performed [15]. B16F10 cells ( $1 \times 10^6$ ) were treated with  $IC_{50}$  dose with ELA and standard anti-cancer drug 5-FU for 24 h. The harvested cells were washed with PBS and re-suspended in lysis buffer, proteinase-k was added and lysis was initiated by incubation at  $50^\circ C$  for 1hr at  $37^\circ C$  overnight respectively. DNA fractionation was done following the general phenol-chloroform fractionation procedure. The isolated DNA samples were mixed with loading dye and subjected to 1% agarose gel electrophoresis for overnight at 20V using ethidium-bromide. DNA fragmentation was observed in UV transilluminator (GENEL, Bangalore Genei Pvt. Ltd.)

### Mitochondrial membrane potential by flow cytometry

B16F10 ( $1 \times 10^6$ ) cells were treated with ELA ( $1/2IC_{50}$  dose and) for 18 h to assay the mitochondrial membrane potential activity of cell

in a flow cytometry. Cells were washed with PBS, pelleted down and dissolved in prewarmed PBS. 200  $\mu$ M JC-1 stain was added and the samples were incubated at  $37^\circ C$  for 15 min. Shift in the mitochondrial membrane potential was determined by FACS (Becton Dickinson FACS Fortessa 4 laser Cytometer), fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) with the help of BD FACS Diva software (Becton Dickinson).

### Apoptosis assay by flow cytometric analysis

To verify apoptosis in B16F10 treated cells, Annexin V-FITC assay was performed [16]. Flow cytometric analysis was done by performing dot plot assay to investigate the type of cell death induced by ELA. Exponentially growing cells were seeded and exposed to  $IC_{50}$  dose of 5-FU and ELA for 18 h. The cell pellets were washed with Annexin-FITC binding buffer provided in apoptosis kit (Sigma) and stained with propidium iodide. All data were taken by Becton-Dickinson FACS LSR Fortessa 4 laser Cytometer was taken using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). Live statistics were used to align the X and Y mean values of the Annexin-V FITC or PI stained quadrant populations by compensation. Data analysis was performed by BD FACS Diva software program.

### Cell cycle arrest by flow cytometry

B16F10 ( $1 \times 10^6$ ) cells were treated with 5-FU and ELA ( $IC_{50}$  dose) for 18 h to assay the stage of cell cycle arrest in a flow cytometry. Cells were washed with PBS, fixed with cold methanol. Then, they were resuspended in cold PBS and kept at  $4^\circ C$  for 90 min. Cells were pelleted down, dissolved in cold PBS, treated with RNase for 30 min at  $37^\circ C$  and stained with propidium iodide and kept in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined by FACS (Becton Dickinson FACS Fortessa 4 laser Cytometer), fluorescence detector equipped with 488 nm argon laser light source and 623 nm bandpass filter (linear scale) with the help of BD FACS Diva software (Becton Dickinson).

### Statistical analysis

The data were analysed statistically using one-way ANOVA followed by Dunnet's t-test. The data were expressed as mean $\pm$ SEM. "P" value less than 0.05 implied significance. Values were expressed as mean \* $P < 0.05$ , when compared to control.

## RESULTS

### Cytotoxic potential

MTT assay revealed significant reduction in cell proliferation in a time and concentration-dependent manner upon treatment with ELA at the concentration of 10 $\mu$ g, 25 $\mu$ g, 50 $\mu$ g, 100 $\mu$ g and 200 $\mu$ g for 24, 48 and 72 h in B16F10 cell line. The  $IC_{50}$  value of ELA for B16F10 cell line was found to be 14.10  $\mu$ g/ml.

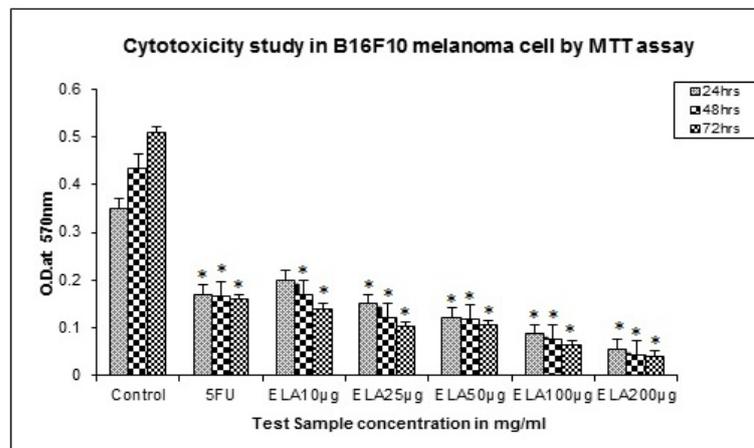


Fig. 1: MTT assay for 24, 48 and 72 h with different concentrations of ELA on B16F10 cells. The data were expressed as mean $\pm$ SEM. "P" value less than 0.05 implied significance, values were expressed as mean \* $P < 0.05$ , when compared to control

### Cell morphology monitoring by light microscopy

Light microscopic images clearly show membrane disintegration of ELA treated B16F10 cells compared to that of untreated control cells.

### Morphological characterization by AO/EtBr staining

Acridine orange and ethidium bromide stained B16F10 melanoma cells revealed nuclear chromatin condensation and apoptotic body formation thereby indicating the occurrence of apoptosis.

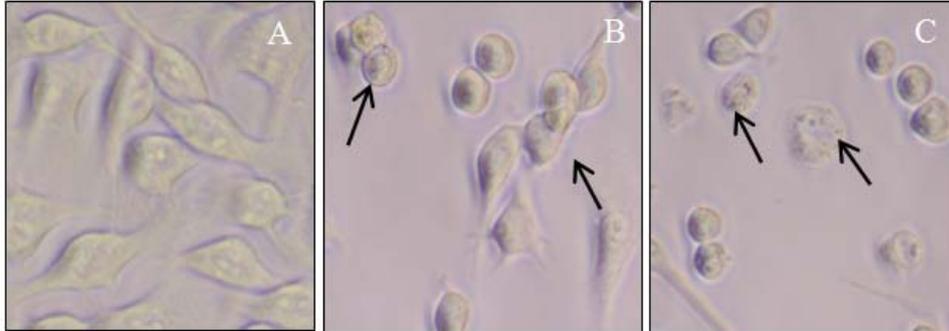


Fig. 2: (A) represents light microscopic images of control cells with intact cell membrane integrity. (B) and (C) represent cells treated with IC<sub>50</sub> dose of ELA and 5-FU which show cell shrinkage and membrane disintegration

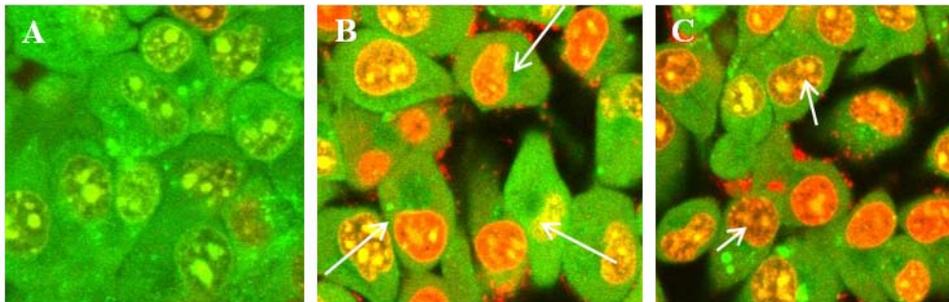


Fig. 3: Fluorescence microscopic images of B16F10 melanoma cells stained with acridine orange and ethidium bromide in 1:1 ratio. (A) represents untreated control cells with intact nuclei that exhibit green fluorescence. (B) and (C) represent cells treated with IC<sub>50</sub> dose of ELA and 5-FU. Both Fig. B and C show distinct cell shrinkage, chromatin condensation, nuclear fragmentation and membrane disintegration that are the hallmark of apoptosis

### Propidium iodide staining by confocal microscope

Propidium iodide stained B16F10 skin melanoma cells revealed the presence of apoptotic bodies in the form of nuclear fragmentation in

cells as compared to the untreated control cells. Nuclear chromatin condensation and apoptotic body formation indicate the occurrence of apoptosis.

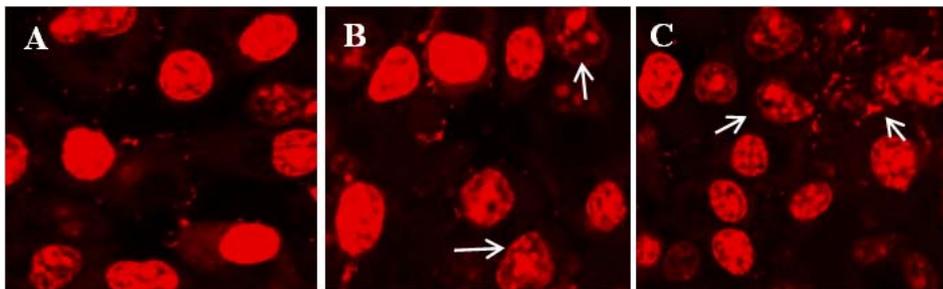


Fig. 4: Confocal microscopic images of B16F10 melanoma cells stained with Propidium iodide. A, B and C represent control, ELA and 5-FU (IC<sub>50</sub> dose) treated cells. Control cells show intact membrane integrity whereas ELA and 5-FU treated cells show condensed chromatin and fragmented nuclei that confirm the generation of apoptotic bodies

### Detection of DNA fragmentation

The gel pattern of DNA samples isolated from untreated control melanoma cells showed an intact DNA band whereas gel pattern of B16F10 cells treated with IC<sub>50</sub> dose of 5-FU and ELA showed degraded DNA bands in the form of ladder. These observations

confirmed apoptosis induction upon treatment with ELA in melanoma cells.

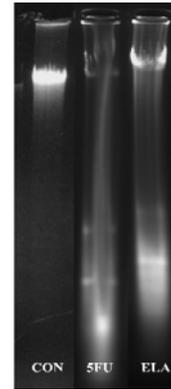
### Depolarization of mitochondrial membrane potential

Depolarization in mitochondrial membrane potential was studied by staining treated and untreated cells with JC1 dye. The depolarization

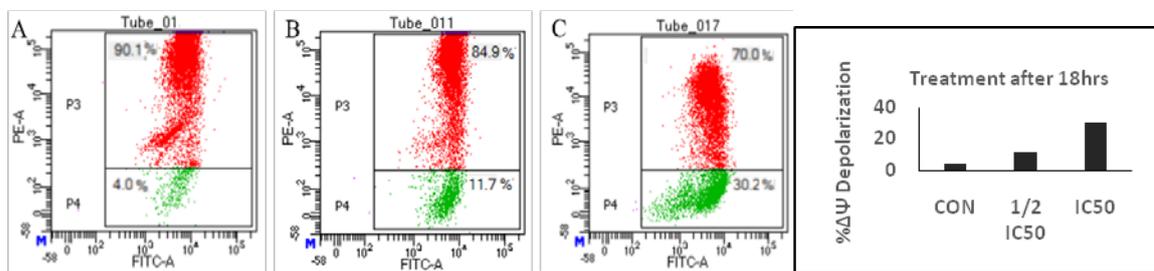
led to a shift from red to green fluorescence leading to the release of Cytochrome C. A significant shift of 11.7% and 30.2% in the mitochondrial membrane potential from red to green fluorescence was observed when B16F10 cells were treated with the 1/2 IC<sub>50</sub> dose and IC<sub>50</sub> dose of ELA for 18 h respectively.

**Detection of apoptosis by flow cytometric analysis**

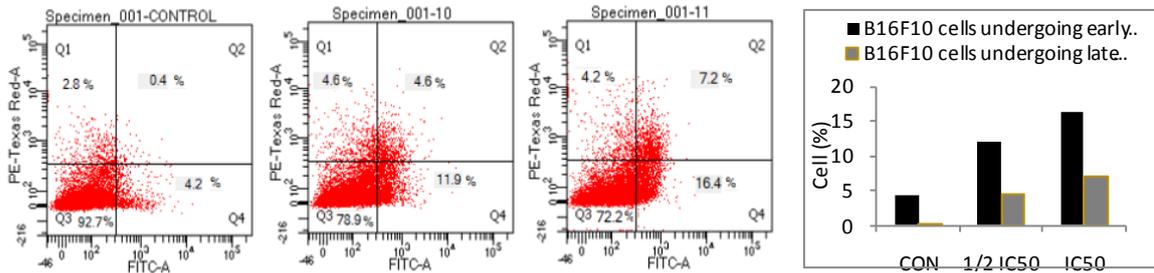
For the detection of apoptosis by flow cytometric analysis, double labelling technique, using Annexin V FITC and propidium iodide, was utilized. Lower left (LL) quadrant (Annexin V-/PI-) depicted the population of live cells, lower right quadrant (LR) (Annexin V+/PI-) was regarded as the cell population at early apoptotic stage, upper right (UR) quadrant (Annexin V+/PI+) represented the cell population at late apoptotic stage and extreme upper right (UR) and upper left (UL) quadrant (Annexin V-/PI+) was considered as necrotic cell population. Flow cytometric data analysis revealed that after treatment with 1/2 IC<sub>50</sub> dose and IC<sub>50</sub> dose of ELA for 18 h, 11.9% and 16.4% B16F10 cells were in the LR quadrant compared to 4.2% control cells indicating early apoptosis.



**Fig. 5: DNA fragmentation by Agarose gel electrophoresis assay. Lane 1 indicates control cells, lane 2 and 3 indicate 5-FU and ELA treated B16F10 cells**



**Fig. 6: Flow cytometric analysis of Mitochondrial membrane potential on B16F10 cells in both control (A) and ELA 1/2 IC<sub>50</sub> (B) and IC<sub>50</sub> treated cells (C) respectively after 18 h treatment. A significant shift of 11.7% and 30.2% from red to green fluorescence was observed**

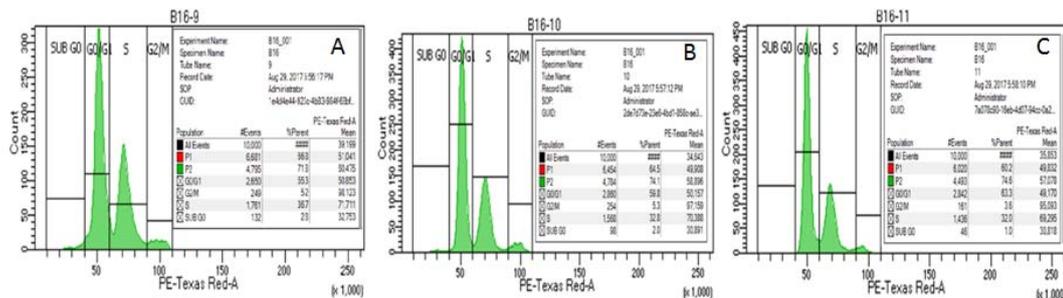


**Fig. 7: Detection of apoptosis by Flow cytometric analysis. Control, ELA 1/2 IC<sub>50</sub> and IC<sub>50</sub> dose treated cells after 18 h treatment. Staining was done with Annexin V FITC and Propidium iodide. Dual parameter dot plot of FITC-fluorescence (x-axis) vs. PI fluorescence (y-axis) shows logarithmic intensity**

**Cell cycle arrest by flow cytometric analysis**

Flow cytometric analysis of B16F10 cells when treated with 1/2IC<sub>50</sub> and IC<sub>50</sub> dose of ELA for 18 h revealed a peak change in the G0/G1

phase. DNA content of 59.8% and 63.3% were obtained for 18 h respectively in G0/G1 phase. These results confirmed ELA treatment arrested cell cycle mostly at G0/G1 phase.



**Fig. 8: Flow cytometric analysis of cell cycle phase distribution in control and ELA 1/2 IC<sub>50</sub> (B) and IC<sub>50</sub> treated cells (C) after 18 h treatment. Histograms represent various contents of DNA with the actual number of cells (x-axis denotes the fluorescence intensity of PE-Texas red and y-axis denotes count)**

## DISCUSSION

Several natural products and their analogues have been identified as potent anti-cancer agents and the identification of anti-cancer property of various plants is being unravelled in a significant manner. Our results clearly indicated that Ethyl acetate fraction of *Lawsonia alba* leaves (ELA) exert cytotoxicity on B16F10 melanoma cells through apoptosis. Its anti-melanoma effect was also compared with 5-Fluorouracil, a standard anti-cancer drug. ELA renders anti-cancer property against B16F10 cells which were supported by the morphological studies and depolarization of the mitochondrial membrane potential thereby leading to apoptosis. These outcomes represent reliable evidence supporting the administration of ELA in melanoma treatment *in vitro*.

MTT assay revealed a significant reduction in cell proliferation in a time and concentration-dependent manner upon treatment with ELA in B16F10 cell line. The IC<sub>50</sub> value of ELA for B16F10 cell line was found to be 14.10 µg/ml. (fig. 1) under phase contrast microscope, the cells in the control group were observed to be spindle-shaped, in close contact with neighbouring cells, and adhered to the surface (fig. 2). Post-treatment with ELA, there was a change in the morphology marked by cell shrinkage, with rounded or irregular cell outlines and many vacuole-like structures in the cytoplasm (fig. 1B, C). Morphological changes of B16F10 cells treated with ELA and 5-FU for 24 h at IC<sub>50</sub> were also analyzed by dual acridine orange/ethidium bromide (AO/EB) fluorescent staining. Acridine orange and Ethidium bromide are two cationic fluorescent DNA intercalating dyes. AO stains the viable cells green whereas EtBr stains the apoptotic cells orange, fragmented green nucleus represent early apoptotic cells, and orange/green dots of condensed nuclei indicate late apoptosis [17]. Cells treated with ELA upon AO/EtBr staining exhibited characteristic changes of apoptosis e. g. cell shrinkage, nuclear condensation, fragmentation and formation of apoptotic bodies (fig. 3). In order to aid the nuclear visualization, cells were stained with PI and nuclear morphology changes were observed under the confocal microscope. Cells treated with ELA showed nuclear margination and chromatin condensation, membrane blebbing, nuclear fragmentation and membrane loss (fig. 4). Fragmentation of chromatin into units of single or multiple nucleosomes that form the nucleosomal DNA ladder in agarose gel is an established hallmark of programmed cell death or apoptosis [18]. DNA fragmentation as detected by agarose gel assay suggests induction of apoptosis following exposure to ELA (fig. 5).

JC-1 is a novel cationic carbocyanine dye that accumulates in mitochondria. The loss of mitochondrial membrane potential is a hallmark of apoptosis. In healthy cells, the dye stains the mitochondria bright red. The negative charge established by the intact mitochondria membrane potential allows the lipophilic dye bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria. In these cells, JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells showing primarily green fluorescence are easily differentiated from healthy cells which show red fluorescence. JC-1 staining of B16F10 cells treated with ELA shows a significant shift in the transmembrane potential from red to green fluorescence. Hence, our data suggest a change in the transmembrane potential, thereby triggering apoptosis (fig. 6). Quantitative estimation of cells undergoing early and late apoptosis was performed by Annexin V-FITC staining. The translocation of phosphatidylserine molecules to the outer surface of the cell allows determination of apoptosis when Annexin-V labeled with FITC is applied to the cell environment [19]. Annexin V-PI-considered as viable cells, while Annexin V+/PI-staining patterns showed early apoptotic cells; whereas Annexin V+/PI+ exhibited late apoptotic cells due to a loss of plasma membrane integrity and Annexin V-FITC-/PI+ was considered as necrotic cells [20, 21]. B16F10 cells when treated with 5-FU and IC<sub>50</sub> dose of ELA demonstrated cells undergoing early apoptosis (fig. 7). Cell cycle and apoptosis together play an important role in the sensitivity of cancer cells. Cell cycle checkpoints are important regulatory mechanisms that ensure the proper passage of cells through the cell cycle. The G<sub>2</sub>/M and G<sub>1</sub>/S checkpoints are critical in

maintaining DNA integrity and essential in executing the proper cell cycle events. It was known that loss of regulation of these checkpoints resulted into transformation and progression of cancer cells. The mode of cell death of cancer cells by ELA predominately followed apoptosis. A possible arrest of the G<sub>0</sub>/G<sub>1</sub> phase might have triggered apoptosis (fig. 8).

In conclusion, the present study demonstrated that ELA could inhibit the proliferation of B16F10 cell line *in vitro* and induce apoptosis by causing a shift in the mitochondrial membrane potential. Therefore, the results from this study provide critically important experimental facts to suggest that ELA may be a potential therapeutic agent for treating melanoma.

## CONCLUSION

Ethyl acetate fraction of *Lawsonia alba* demonstrated dose-dependent anti-proliferative properties in B16F10 melanoma cells. We also observed membrane blebbing chromatin condensation, membrane loss and DNA fragmentation in the form of the ladder in ELA treated B16F10 cells. The morphological characteristics indicated the induction of apoptosis. Post ELA treatment, the transmembrane potential shift from red to green also triggered apoptosis. Annexin V-FITC staining demonstrated cells undergoing early apoptosis. Cell cycle study observed by flow cytometry revealed a possible arrest of the G<sub>0</sub>/G<sub>1</sub> phase, indicating this phase arrest might have triggered apoptosis. These results have important clinical implications as they suggest commendable anti-proliferative activities against B16F10 melanoma cells. Ethyl acetate fraction of *Lawsonia alba* can be considered as an effective adjuvant therapeutic agent. Further study of proteins involved in apoptosis will improve our findings and give useful results to establish *Lawsonia alba* as a novel drug against melanoma.

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## AUTHORS CONTRIBUTIONS

The corresponding author Dr. Shila Elizabeth Besra, has generated the idea for the experiment and has critically examined and corrected the manuscript. Nilanjana Deb, the first author has conducted the experimental work and written the manuscript. Other authors Anita Hansda, Soumyasree Dutta and Dr. Ashok Pattanaik have helped during the experimental work and in the interpretation of the results. All authors read and approved the final manuscript.

## CONFLICT OF INTERESTS

The authors declare they have no conflict of interest regarding the publication of this paper.

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