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RESEARCH ARTICLE

Molecular Basis of *Sida cordifolia* (L.) Induced Apoptosis in Melanoma Cell Line

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Abstract

Sida cordifolia of the family Malvaceae is widely used in traditional medicine for treating inflammation, respiratory and neurological ailments and wound healing. Its extract was found to possess effective antitumor activity in hepatocellular carcinoma and HeLa cell lines. This study was aimed at screening the anticancer activity of *S. cordifolia* and to investigate its mechanism of action. Aerial parts of the plant were subjected to hot continuous extraction by Soxhlet apparatus with ethanol as solvent. Cytotoxicity of the extract was assessed in various cancer cell lines *viz.* breast, ovarian, colon, skin, and liver cancer by MTT assay. For each cell line, the IC_{50} value was calculated. The mechanism of anticancer activity of the extract was studied in melanoma cells by exposing them to 12.5 and 25 µg/ml extract and comparing results with the control. Gel electrophoresis was used to analyse DNA laddering. Expression of TP53, Bcl and Caspase gene family proteins were determined by SDS-PAGE. Mitochondrial membrane potential was studied by the JC-1 kit. Cell cycle analysis was performed by using a flow cytometer. Statistical analysis was done by ANNOVA, and significant values were further analysed by Tucky post-hoc analysis. P value less than 0.05 was considered statistically significant. MTT assay revealed maximum cytotoxicity of the extract against melanoma with an IC_{50} value of 16.51µg/*ml*. Melanoma cells treated with the extract demonstrated dose-dependent DNA laddering. The extract also exhibited a dose-dependent increase in the level of Bax, Caspase 3, Caspase 9 and p53 proteins. Expression of Bcl2 protein was significantly reduced. Treatment of melanoma cells with the extract showed significant loss of mitochondrial membrane potential. Melanoma cell population in subG0 and G2/S was significantly elevated. From these results, we conclude that ethanol extract of *S. cordifolia* is cytotoxic to melanoma cells. It acts by inducing apoptosis via an intrinsic mechanism. The extract also arrests melanoma cells in the G2/M phase.

Keywords: Bax, Bcl2, Caspase, Cell Cycle, Mitochondrial Membrane Potential

1. Introduction

Worldwide, cancer is the second leading cause of death[1]. As stated by the International Agency for Research on Cancer, the incidence and mortality of all types of cancer in Asia are 49.34% and 58.3% respectively[2]. Chemotherapy is an important part of cancer treatment but most of the chemotherapeutic agents are cytotoxic to healthy, rapidly dividing cells[3,4]. Many times, patients develop drug resistance making chemotherapy ineffective[5]. To overcome this issue, various natural resources are extensively studied to find new sources of modern medicine. Consequently, the search for newer effective but safe chemotherapeutic agents continues.

Natural resources have been used for medicinal purposes since time immemorial. Some of the important secondary metabolites like phenols, flavonoids, steroids, and alkaloids impart medicinal properties to the plants. The efficacy and potency of these compounds for medicinal use depend not only on their concentration but also on their chemical structure. Both these factors

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are decided by their habitat, the climate in which the plant grows and the season of harvest[6]. Currently, many plant-based anticancer products and their analogues like vinca alkaloids, and paclitaxel, are available in the market[7,8].

Sida cordifolia belongs to the family Malvaceae. It is found throughout the tropical and subtropical plains of India[9,10]. In traditional medicine, it is used as a tonic, astringent, diuretic and aphrodisiac. It is also used to treat inflammation, respiratory and neurological ailments and for wound healing[10]. Pharmacological studies have proven its anti-inflammatory, antipyretic, antioxidant, anti-ulcer, and hepatoprotective activities[11]. The cytotoxic potential of *S. cordifolia* against HeLa and HepG2 cell lines has shown promising results[12]. However, its efficacy in different types of cancers has not been explored. Moreover, the data is scanty concerning its mechanism of action as an anticancer agent. Hence, the current study was designed to evaluate *in vitro* antitumor activity of *S. cordifolia* and to study the molecular mechanism involved in it.

2. Materials and Methods

The protocol was accepted by the institutional ethics committee. Chemicals used in the study were of analytical grade and purchased from Mercury's scientific chemicals industries and Hi Media laboratories.

2.1 Preparation of the Extract

Specimen material was verified by the botanist and deposited in the department. Aerial parts of *S. cordifolia* were dried and coarsely powdered. The material was extracted by a hot continuous extraction process using the Soxhlet apparatus. Serial extraction was performed by using petroleum ether, chloroform, acetone, ethanol 95% v/v and distilled water. Qualitative phytochemical analysis was performed by chemical methods[13]. Ethanol extract was selected for screening the anticancer activity due to the presence of major anticancer phytochemicals like alkaloids, steroids, phenols, and flavonoids.

2.2 Anticancer Activity Screening

Cell lines used in the present study were obtained from the National Centre for Cell Sciences (NCCS), Pune. MCF7 breast cancer cell line, PA1 ovarian cancer cell line, HT29 colon cancer cell line, HepG2 liver carcinoma cell line and A375 melanoma cell lines were used in the study. Dulbecco's Modified Eagle's Medium (DMEM) was used to culture MCF7, HT29, A375 and HepG2 cell lines and Eagle's Minimum Essential Medium (EMEM) for PA1 cells. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C in the presence of 10% inactivated foetal bovine serum, penicillin (100IU/ml) and streptomycin (100µg/ml). Cell lines were used after ruling out mycoplasma contamination.

A monolayer of each cell line was treated with 100µl of the extract or standard drug (cisplatin and 5-fluorouracil) for 24 hours. Concentrations used were 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml. Negative control was also run in the experiment. Cytotoxicity of the extract against each cell line was determined by MTT assay by using standard protocol[14]. Since the extract was maximally effective against melanoma cells, further studies were conducted by treating them with 12.5µg/ml and 25µg/ml concentrations of the extract, which were close to the IC_{50} value.

2.3 Apoptosis Analysis

2.3.1 DNA Fragmentation Assay[15]

The culture medium was removed after 24 hours of treatment with the extract. Then PBS was used to wash the cells and an ice-cold buffer to rinse them. 200µl of lysis buffer was used to resuspend the cells. They were incubated at room temperature for 10 seconds to release DNA. After centrifugation of the mixture at 5000g for 5 minutes, DNA in the supernatant was extracted using 25:24:1 volume of phenol: chloroform: isoamyl alcohol. After the DNA was precipitated with ethanol; it was air-dried and dissolved in Tris EDTA buffer containing RNase A. Electrophoresis was performed to analyze the samples by using 1% agarose gel with ethidium bromide.

2.3.2 Gene Expression Study

The protein concentration of various apoptosis-related genes was analyzed by SDS PAGE by following standard protocol. All the antibodies were procured from Abcam US. The procedure in brief is as follows: Control and test groups of melanoma cells (12.5 and 25 µg/ml extract) were incubated under standard conditions for 48 hours. 200 µl triple-detergent lysis buffer was added to each plate and the mixture was scraped to a 1.5 ml tube, vortexed and kept in ice for 30-60 minutes. Tubes

were centrifuged at 14000 rpm for 10 minutes to obtain proteins in the supernatant. Gel was prepared by using 12% polyacrylamide (8% for p53). The sample was run at 200 V for 45-60 minutes. Gel was removed and proteins were transferred on the membrane (100 V, 350 mA current) and successively treated with primary and secondary antibodies and DAB. To normalize the band intensity of each protein, a loading standard, **β actin**, was used. Quantitative analysis of band intensities was done by ImageJ software.

2.3.3 Assessment of Mitochondrial Membrane Potential[16]

Mitochondrial membrane potential was determined by the JC-1 assay kit by following the manufacturer's instructions. In brief, melanoma cells were treated with the extract for 24 hours. After washing with PBS, cells were incubated for 20 minutes at 37°C in the presence of JC-1 working solution and culture medium. After washing cells twice with JC-1 staining buffer culture medium was added, and red (590nm)-green (529nm) fluorescence was measured with the help of fluorescence microscopy.

2.4 Cell Cycle Analysis

The distribution of cells in various phases of the cell cycle was determined by measuring the intensity of fluorescently stained DNA[17]. Melanoma cells treated with the extract were harvested after 48 hours and 2 x 10^6 cells were resuspended in ice-cold PBS. Cell suspension was added gently to 9 ml of cold 70% ethanol and fixed on ice for two hours. Cells were centrifuged for 10 minutes at 4°C at 200g and washed in PBS. They were resuspended in staining buffer (PBS with 100µg/ml RNase A, 50µg/ml Propidium Iodide). Plates were wrapped in foil to protect from light and incubated overnight at 4°C. Data was acquired on a flow cytometer (Becton Dickinson Aria II Flowcytometry with BD FACS™ Pre-Sort Buffer equipped with BD FACSD iva software) at 488nm.

2.5 Statistical Analysis

All the tests were run in triplicate and values were expressed as mean ± SE. IC_{50} values were derived from the sigmoid dose-response relationship by using GraphPad Prism 8 software. One-way ANOVA was used to compare means. Alpha was set at 0.05. Significant values were further tested by Tukey Post Hoc test, in SPSS16 software.

3. Results

Preliminary phytochemical analysis of *S. cordifolia* detected alkaloids, steroids, phenols, flavonoids, carbohydrates, and proteins in ethanol extract. MTT assay demonstrated a minimum IC_{50} value (16.51µg/ml) of ethanol extract against the melanoma cell line which was close to the standard anticancer drug cisplatin (2.56µg/ml). Hence, melanoma cells were selected to study the mechanism of anticancer activity of the extract.

3.1 Apoptosis Analysis

3.1.1 DNA Fragmentation Analysis

As shown in Figure 1, melanoma cells showed the formation of a DNA ladder in a dose-dependent manner and laddering was absent in DNA isolated from the control cells.

3.1.2 Gene Expression Study

Figure 2 shows an expression of Bcl2 family proteins after the treatment of melanoma cells with the extract. Anti-human Bax antibodies detected a band at 21 kDa and anti-human Bcl2 detected a band at 26 kDa. These were the predicted sizes for the Bax and Bcl2 proteins, respectively. The graph in Figure 2 compares normalised values of Bax, Bcl2 and their ratio in 3 groups. Expression of Bax showed a statistically significant increase in both the treatment groups, $F(2,6) = 2.994E4$, $p = 0.00$. Bcl2 expression was higher in the cells treated with 12.5µg/ml concentration of the extract. However, in cells treated with 25µg/ml extract, it reduced to less than that of the control group. These changes in Bcl2 expression were also statistically significant in all the groups, $F(2,6) = 654.58$, $p = 0.00$. Analysis of the Bax: Bcl2 ratio revealed a statistically significant increase in its value in both the treatment groups when compared with control ($F(2,6) = 3.255E3$, $p = 0.00$) indicating shifting of cellular balance in favour of apoptosis.

Figure 3 denotes the expression of caspases 3, caspase 9 and p53 proteins when melanoma cells were treated with the extract. The bar diagram in Figure 3 compares the normalised values of these proteins. Caspase 3 expression displayed a statistically significant increase

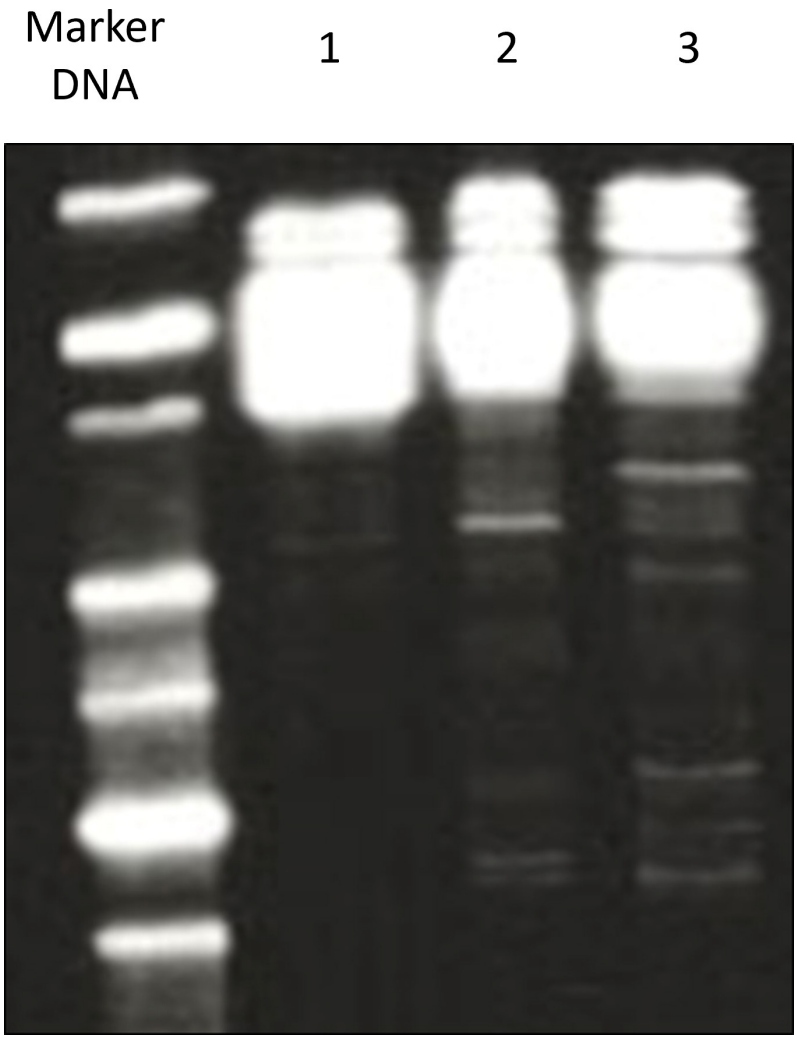

Figure 1. Apoptotic DNA ladder in A375 cell line. Lane 1: untreated cells, Lane 2 and 3: cells treated with 12.5 µg/ml and 25 µg/ml concentration of *S. cordifolia* extract. Marker DNA lane: Standard molecular size marker (2 Kb).

in a dose-dependent manner in treatment groups F (2,6) = 3.172E4, p = 0.00. Expression of caspase 9 was minimal in the control group but exhibited a significant rise in both the treatment groups F (2,6) = 1.909E3, p = 0.00. Similar pattern was observed for p53 expression F (2,6) = 1.646E4, p = 0.00.

3.1.3 Assessment of Mitochondrial Membrane Potential

The upper panel of Figure 4 shows fluorescence microscopic pictures of three different groups of melanoma cells when exposed to a JC-1 probe. There is more green fluorescence in cells treated with 12.5µg/ml and 25µg/ml *S. cordifolia* extract than that of the control group. The lower panel of Figure 4 represents a quantitative analysis of the fluorescence which indicates a significant reduction in the red/green ratio in control as well as the cells treated with the extract, F (2,6) = 663.454 p = 0.00. This is suggestive of loss of mitochondrial membrane potential in treatment groups.

3.2 Cell Cycle Analysis

DNA content of melanoma cells was measured by flow cytometry. It revealed a significant and dose-dependent increase in the cell population in the sub G0 phase than that of control, F (2,6) = 1.577E4, p = 0.00 (Figure 5). Table 1 indicates the proportion of the cells in various phases of the cell cycle, in 3 groups. It revealed a statistically significant increase in the population of cells in the G2/M phase in treatment group F (2,6) = 1.467E5, p = 0.00. This was linked

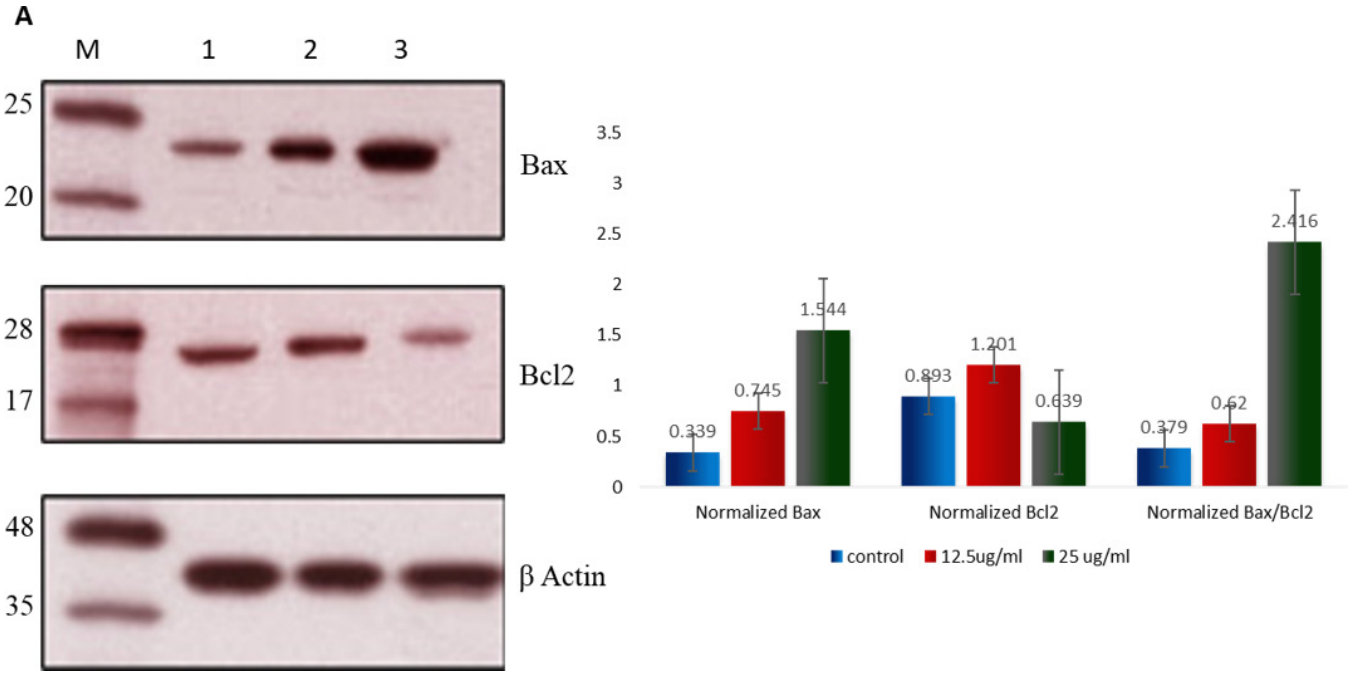

Figure 2. Western blot analysis of Bax and Bcl2 protein in melanoma cell line when treated with *S. cordifolia* extract. Panel A: Lane M: marker protein, lane 1: control, lane 2: 12.5 µg/ml and lane 3: 25 µg/ml concentration. β actin was used as a loading standard. The gel used was 12% polyacrylamide. The bar diagram indicates normalized values of Bax, Bcl2 and their ratio in control and two test groups. (*Statistically significant value by one-way ANOVA at 0.05 alpha).

with a corresponding decrease in G0/G1 and S phase population.

4. Discussion

Cancer is a multifactorial disease which can influence any type of cell. Abnormal cell proliferation can be the result of inborn or acquired genetic mutations which act through changes in gene expressions that regulate cell cycle or apoptosis[18].

Ethanol extract of *S. cordifolia* demonstrated an IC_{50} value of 16.51µg/ml in the case of melanoma cell line. As per the guidelines for the plant screening program, given by the US National Cancer Institute (NCI), if the extract exhibits an IC_{50} value of < 30 µg/ml after 72 hours of treatment, it is considered a promising source for purification of the crude extract[19]. Hence, ethanol extract of *S. cordifolia* may be the potential source for purification and isolation of active principles.

DNA fragmentation is considered as the hallmark of apoptosis which is caused due to activation of endonucleases leading to cleavage at 180–200 bp[20]. It is easily visualized by agarose gel electrophoresis in the form of a DNA ladder. The presence of a DNA ladder in a dose-dependent manner confirms the initiation of apoptosis in melanoma cells.

Bax and Bcl2 belong to the Bcl2 gene family where Bax is proapoptotic and Bcl2 is an antiapoptotic protein[21]. Initiation of apoptosis is governed by the equilibrium between pro- and anti-apoptotic proteins and not by their absolute quantities[21]. Statistically significant elevation in Bax, reduction in Bcl2 and increase in the ratio of Bax: Bcl2 proteins suggest that *S. cordifolia* induces apoptosis in melanoma cells by shifting the balance in favour of apoptosis.

Muthuraman *et al* isolated 20 compounds from ethanol extract of *S. cordifolia* leaves and tested their potential to bind with Bcl2 by molecular docking mechanism. They concluded the presence of a compound which is a potential inhibitor of Bcl2. As the current study also has used ethanol extract of *S. cordifolia*, we can say that this compound may be present in our extract leading to decreased expression of Bcl2 and favouring apoptosis in melanoma cells[22].

The present study also observed a statistically significant increase in p53 expression in cells treated

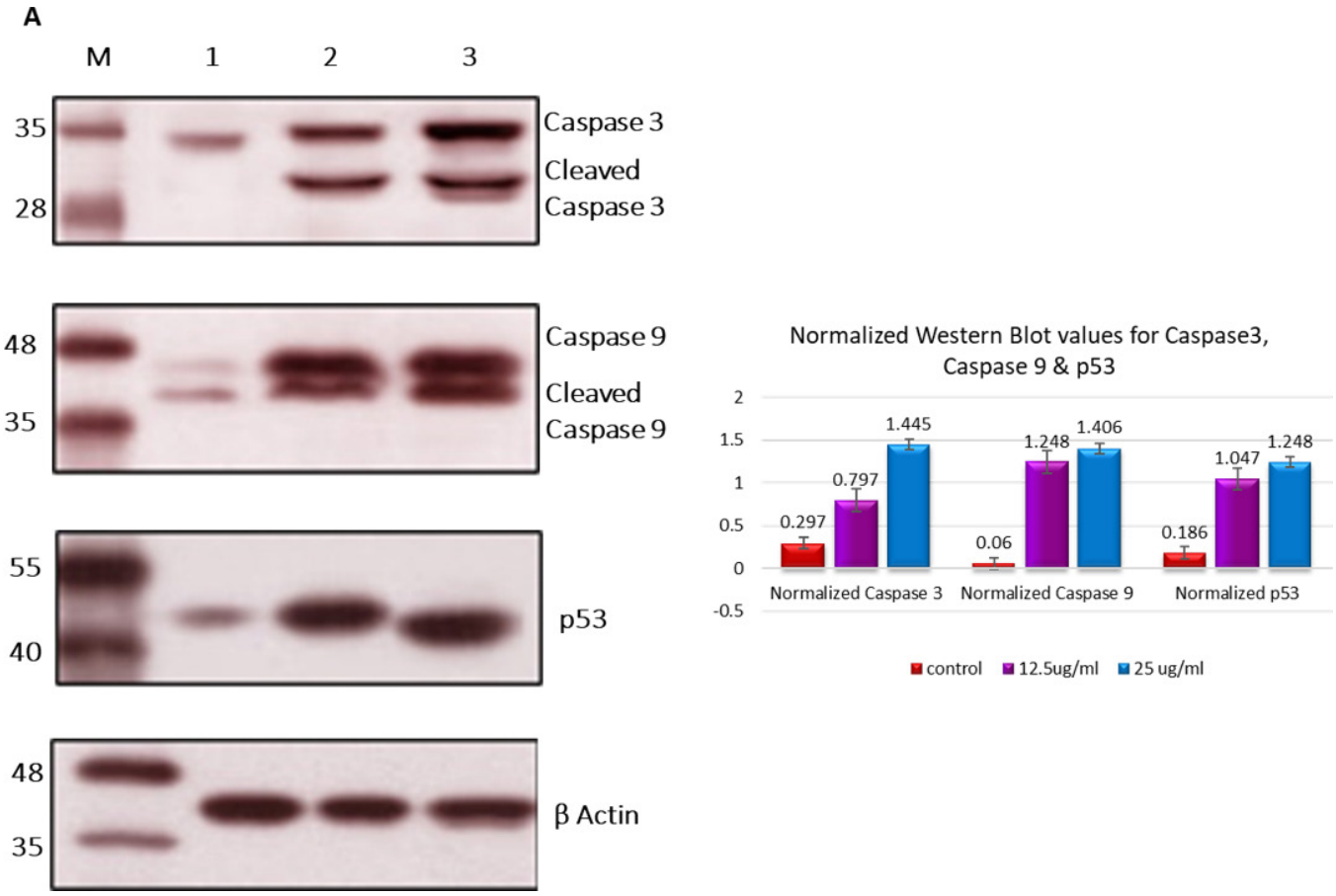

Figure 3. Western blot analysis of Caspase 3, Caspase 9 and p53 protein in melanoma cell line when treated with *S. cordifolia* extract. Panel A: Lane M: marker protein, lane 1: control, lane 2: 12.5 µg/ml and lane 3: 25 µg/ml concentration. β actin was used as a loading standard. The gel used was 12% polyacrylamide for caspase and 8% for p53. The bar diagram indicates normalized values of Caspase 3, Caspase 9 and p53. (*Statistically significant value by one-way ANOVA at 0.05 alpha).

with the extract. p53 is involved in the regulation of the cell cycle and DNA repair process. The elevated p53 level in the present study suggests that ethanol extract of *S. cordifolia* may activate the TP53 gene or CDKN2A gene and hence p53 expression[23,24]. Increased p53 protein expression is said to upregulate the expression of Bax and induce apoptosis in case of severe DNA damage[25]. This finding is in line with elevated Bax in melanoma cells when treated with the extract.

Caspase 3 is the key executioner of apoptosis because of its ability to activate a vast array of proteins. A statistically significant increase in the expression of caspase 3 supports the finding that the extract initiates apoptosis in melanoma cells. The study also revealed a statistically significant increase in the expression of caspase 9. It is one of the initiator caspases. When cytochrome c is released from mitochondria, it binds with Apaf-1 and caspase 9 to form apoptosome and activates caspase 9. Activation of caspase 9 further activates caspase 3[25]. As caspase 9 is associated with an intrinsic pathway of apoptosis, we can say that *S. cordifolia* extract induces apoptosis in melanoma cells via an intrinsic pathway.

Loss of mitochondrial membrane potential causes the release of cytochrome C and SMAC and initiates the intrinsic pathway of apoptosis. Hence, measurement of mitochondrial membrane potential is crucial in confirming the apoptosis initiation by intrinsic mechanism and is so widely used in cytotoxicity research[26]. The present study showed a statistically significant reduction in the red: green fluorescence ratio in a dose-dependent fashion, indicating a significant loss of mitochondrial membrane potential when melanoma cells were treated with the extract. Bcl2 proteins are attached to the outer

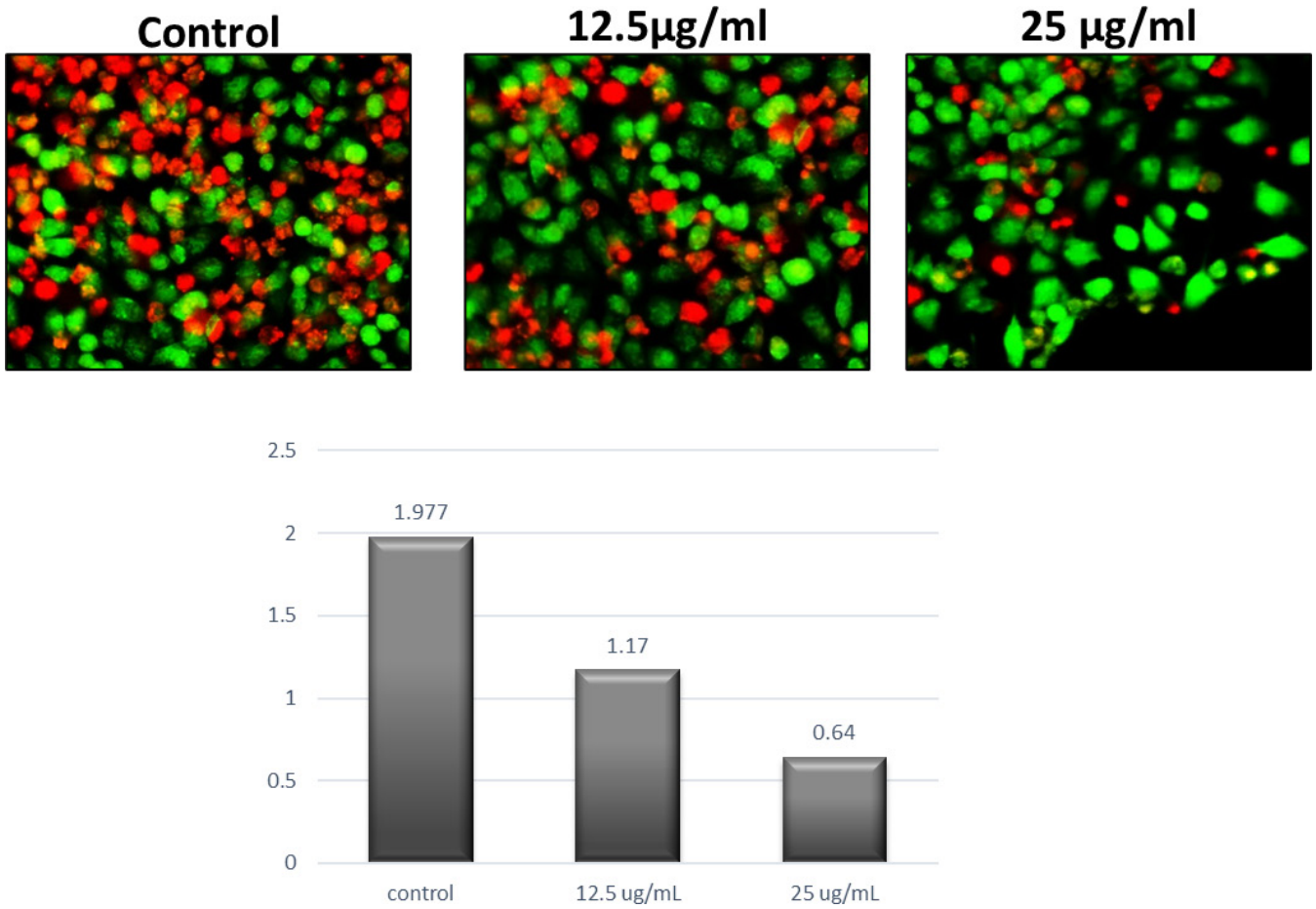

Figure 4. Fluorescence microscopic photographs and their quantitative analysis in melanoma cells when treated with 12.5 µg/ml and 25 µg/ml *S. cordifolia* extract. The control sample is melanoma cells without treatment with the extract. The bar diagram represents a quantitative analysis of red/green fluorescence in three samples. (*Statistically significant value by one-way ANOVA at 0.05 alpha).

mitochondrial membrane and prevent the formation of pores in it. Cellular stress causes inhibition of Bcl2 proteins and triggers outer mitochondrial membrane permeabilization and leakage of ions and fluid within the mitochondria. If this loss of mitochondrial membrane potential lasts for sufficient duration, it initiates the release of cytochrome c from mitochondria and starts the intrinsic pathway of apoptosis by activating caspase 9[27]. Loss of mitochondrial membrane potential in our study further supports the finding that ethanol *S. cordifolia* initiates apoptosis via an intrinsic pathway.

Flow cytometric analysis of the DNA content of melanoma cells treated with the extract revealed a statistically significant increase in cell fractions in the sub-G0 as well as G2/M phase. The sub-G0 fraction of the cells represents the apoptotic and debris population[28]. An upsurge in this fraction suggests a higher degree of apoptosis in the extract-treated cells. A statistically significant rise in G2/M cell fraction indicates that ethanol extract of *S. cordifolia* halts the cell cycle in the G2/M phase. The transition of cells from G2 to the M phase is regulated by the cyclin B1-CDK1 complex[29]. Increased G2/M fraction of cells suggests that *S. cordifolia* may suppress the expression of cyclin B1 or inhibit CDK1. It may also imply that; the extract interferes with the microtubule assembly necessary for the spindle formation, chromosomal attachment to the spindles or arrest of the cell cycle due to DNA damage. It has been suggested that phytochemicals like flavonoids, phenolic acids, tannins and alkaloids exhibit pro-apoptotic and anti-angiogenic properties[30]. Like vinca alkaloids, alkaloids in *S. cordifolia* extract may be responsible for alterations in microtubule functions leading to G2-M phase arrest. Flavonoids

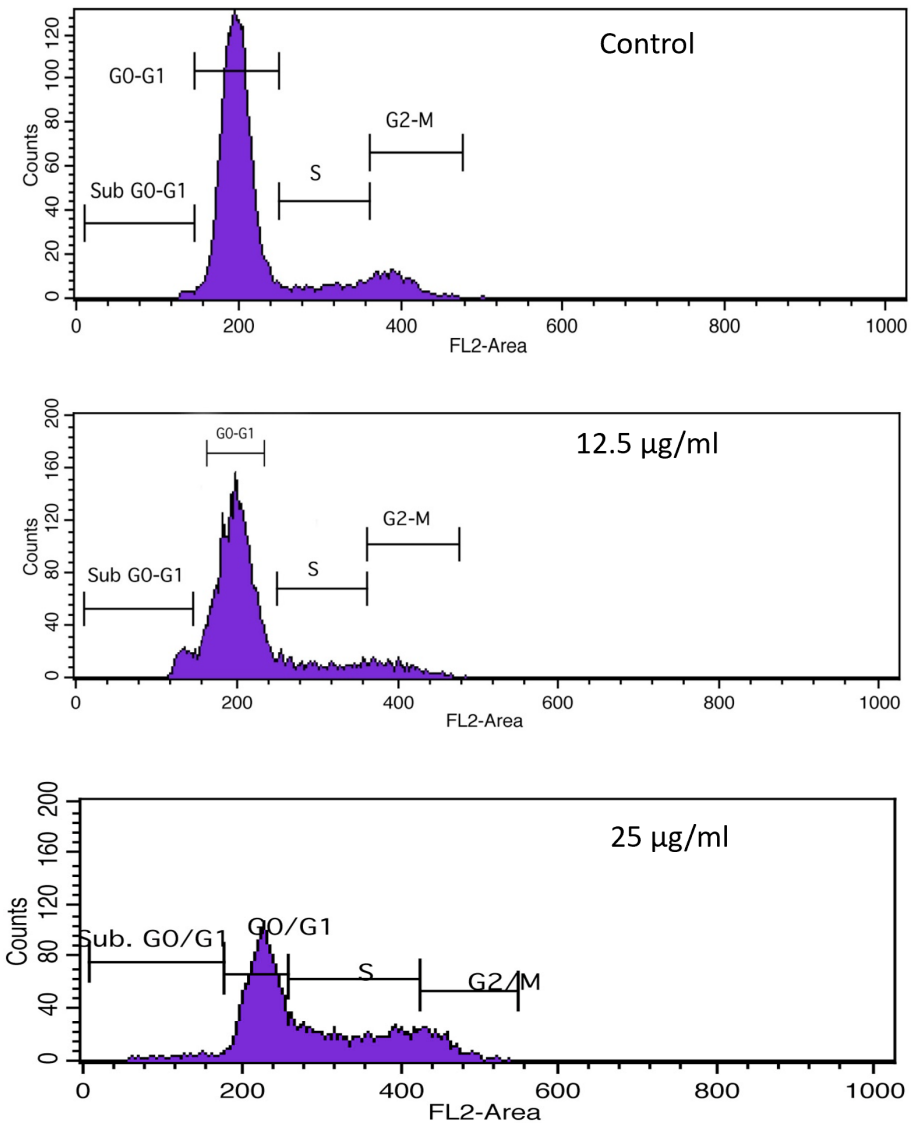

Figure 5. Effect of Ethanol extract of *S. cordifolia* on melanoma cell cycle progression. The top panel represents a histogram of cells treated with vehicle (DMSO) (control); the middle panel indicates cells treated with 12.5 µg/ml concentration of the extract and the lower panel represents cells treated with 25 µg/ml concentration of the extract when stained with Propidium iodide.

Table 1. Distribution of melanoma cells in different phases of the cell cycle when treated with ethanol extract of *S. cordifolia* for 48 hours

	Percentage of gated cells	Percentage of cells in different phases of the cell cycle (Mean ± SE)			
		Sub G0	G0/G1	S	G2/M
Control	89.630±0.286	4.85±0.046	68.52±0.029	7.85±0.023	8.41±0.017
12.5 µg/ml	91.250±0.289*	14.52±0.063*	37.92±0.040*	10.25±0.046*	28.56±0.040*
25 µg/ml	89.740±0.116	16.47±0.035*	32.78±0.035*	5.02±0.035*	35.58±0.046*
F value	13.813	1.577E4	3.055E5	5.317E3	1.467E5
p-value	0.006	0.00	0.00	0.00	0.00

*Statistically significant value (<0.05). Post hoc analysis done by Tukey HSD test.

may have inhibited the expression of cyclin B and or CDK1 causing G2-M arrest.

5. Conclusion

Ethanol extracts of *S. cordifolia* have potent anticancer activity against melanoma (A375) cells. As indicated by a statistically significant increase in p53, Bax, caspase 3 and caspase 9 and a decrease in Bcl2 proteins and loss of mitochondrial membrane potential, we conclude that ethanol extract of *S. cordifolia* inhibits melanoma cell growth by inducing apoptosis via intrinsic pathway. It also arrests the cell cycle in the G2/M phase.

6. Limitations and Future Scope

The present study has evaluated the ethanol extract of *S. cordifolia* for its anticancer mechanism. Further studies can be undertaken to isolate the active principles in the extract and their role in the anticancer mechanism. Also, *in vivo* studies can enlighten the efficiency of the extract in the presence of various bodily reactions.

7. Acknowledgements

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