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Research Article

Mussaenin A Isolated From *Mussaenda glabrata* Induces Apoptosis in the Liver Cancer Cells Via Mitochondrial Pathway

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ABSTRACT

Plants belonging to Rubiaceae family have been used in Chinese folk medicine and Ayurveda. The aim of the study was to determine the effect of Mussaenin A isolated from root of *Mussaenda glabrata* on liver cancer cell line, Hep G2. MTT assay was performed to check the ability of Mussaenin A to induce death in cancerous Hep G2 cells and normal NIH3T3 cells. Acridine Orange/Ethidium Bromide staining, Hoechst staining and DNA fragmentation assays were used to confirm the apoptosis inducing ability of MA on Hep G2 cells. RT-PCR and western blotting was performed to check the expression of pro- and anti-apoptotic factors. Calorimetric assay was done to check caspase-3 and caspase-9 activities. Mussaenin A at lower concentrations was found to induce cell death selectively in the liver cancer cells (IC_{50} = 11.38 µg/mL). The transcriptional expression studies of the pro-apoptotic Bax and anti-apoptotic Bcl-2 and Cox-2; and the western blot analysis of pro-apoptotic BAK and BAD showed that MA upregulated the expression of pro-apoptotic factors and down regulated the expression of anti-apoptotic factors in Hep G2 cells. Caspase-9 and 3 activities were found to be upregulated in the calorimetric studies. The down regulation of anti-apoptotic factors and upregulation of pro-apoptotic factors show that the Mussaenin A induced apoptosis in the liver cancer cells via the intrinsic or the mitochondrial pathway.

Keywords: Rubiaceae, Growth inhibition assay, Anti-apoptotic, intrinsic pathway.

INTRODUCTION

Natural products from plants have played a key role in lives of humans especially as source of food and medicines throughout history. Bioactive principles have gained importance recently due to the effect they are found to have on epigenetics. The search for molecules with fewer side-effects on normal cells is still on due to the harmful effects of drugs available now for treatment of cancer. Terpenes are among the most active group of plant components that has facilitated the discovery of many chemotherapeutic drugs. A large number of terpenes have been reported for their cytotoxic effect on various cancer types both *in vitro* and *in vivo*[1]. Triterpenoids, such as glycyrrhizic acid, ursolic acid, oleanolic acid, and nomilin, the diterpene andrographolide, and the monoterpenoids like limonene and perillic acid had shown immunomodulatory and antitumor activities. All of them could induce apoptosis in various cancer cells by activating various proapoptotic signaling cascades. Many of these terpenoids are found to inhibit metastatic progression and tumor-induced angiogenesis[2]. Iridoids, flavonoids and triterpenes are the common chemical ingredients distributed in *Mussaenda* species. The most recognized compounds in Mussaenda genus are the iridoids and triterpene saponins. Plants belonging to this family have been used in Chinese folk medicine and Ayurveda as well[3].

The objective of this study was to isolate Mussaenin A (MA) from the root of *Mussaenda glabrata* and to determine the effect of the Mussaenin A on the liver cancer cell line, Hep G2 and normal NIH3T3 fibroblast cell line. The apoptosis inducing ability of MA was studied using Acridine Orange/Ethidium Bromide staining, Hoechst staining and DNA fragmentation assays. The study also tried to elucidate the mechanism by which MA induced apoptosis in the liver cancer cells by studying the expression of pro- and anti-apoptotic factors.

MATERIALS AND METHODS

Isolation of Mussaenin A

The plant Mussaenda glabrata was collected from Palakkad district in Kerala and authenticated at Botanical Survey of India, Coimbatore and the specimen (BSI/SRC/5/23/2014-15/Tech-137) was deposited at the Institute. Mussaenin A was isolated using the method reported by Zhao *et al*. (1996)[4]. All chemicals and reagents used including the solvents were of analytical grade obtained from Hi-media (Mumbai, India).

Cell culture

The Human liver cancer (Hep G2) and normal (NIH3T3) fibroblast cells were obtained from NCCS, Pune. The cells were maintained in DMEM supplemented with 10 % FBS 75 and 100 mg/L streptomycin and 100 U/L

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penicillin. Cells were maintained at 37ºC in a humidified atmosphere of 5 % CO_2 and 95 % air. All cell culture reagents were purchased from Hi-media (Mumbai, India).

Cell Viability Assay

The Hep G2 and NIH3T3 cells were grown in 12-well culture plates. MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess cell viability according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). The assay was quantitated by measuring the absorbance at 570 nm[5]. NIH3T3 cells were used as control (noncancerous cells) to check the activity of Mussaenin A on normal cells. After measuring the absorbance at 570 nm at the end of the MTT assay, the % growth inhibition was determined using the formula:

% growth Inhibition = 100 – (Abs (sample) / Abs (control) X 100)

Apoptosis studies

Hoechst Staining

Exponentially growing Hep G2 cells (1 x 10^4 cells/mL) were incubated with Mussaenin A for 24 h. Apoptotic nuclear morphology was visualized using Hoechst staining[6, 7]. Cells were fixed with 4% p-formaldehyde and stained with Hoechst dye 33258 (working concentration of Hoechst was taken as 10 µg/mL, stock 1 mg/mL), incubated for 15 min and examined under fluorescent microscope (Olympus IX71) with excitation/emission at 300/380 nm, which was connected to a digital imaging system.

Acridine Orange/Ethidium Bromide Staining

The Hep G2 cells, treated with Mussaenin A for 24 h were washed with Phosphate buffered saline (PBS) and trypsinized. Twenty-five µL of cell suspension (1x10^4 cells/mL) was mixed with 1 µL of acridine orange/ethidium bromide solution (one part each of 100 µg/mL of acridine orange and 100 µg/ml of ethidium bromide in PBS) just prior to microscopy[8, 9]. The mixed suspension (10 µL) was placed on a microscope slide covered with a glass cover slip and examined under fluorescent microscope (Olympus 1X71) connected to a digital imaging system.

DNA Fragmentation Assay

Hep G2 cells grown in 60 mm culture plates were treated with different concentrations (10, 20, and 50 µg/mL) of Mussaenin A for 24 h[10]. Cells without treatment were taken as control. After 24 h, the cells were harvested and washed with PBS and pelleted by centrifugation at 300 rpm. To the cell pellet, 100 µL lysis buffer (50 mM Tris-HCl pH 8, 20 mM EDTA, 10 mM NaCl, 1% SDS) was added for 1 min and centrifuged at 2000 rpm for 5 min. The supernatant was collected and incubated with RNase A (5 µg/mL) for 1 h followed by digestion of proteins with proteinase K (100 µg/mL) for 5 h. Aliquots of lysate (20 µL) were loaded to 1.5% agarose prepared in TBE buffer (Tris-Borate 45 mM, 1 mM EDTA, pH 8) containing 3 mg/mL EtBr, and DNA was separated by electrophoresis. The separated DNA fragments in the gel were visualized using ChemiDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA).

Reverse Transcriptase-PCR

The mRNA expression levels of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (BAX) and Cyclooxygenase-2 (COX-2) were studied using reverse-transcriptase PCR[11, 12]. The Hep G2 cells were grown in 60 mm culture plates. The first set of Hep G2 cells was untreated. The second set of cells was treated with Phorbol 12-myristate 13-acetate (PMA) at a concentration of 50 ng/mL. The third set of cells was treated with Mussaenin A alone at 10 µg/mL concentration. The last, fourth set of cells, was treated with both Mussaenin A and PMA at 10 µg/mL and 50 ng/mL concentrations respectively. After incubation, total RNA was isolated from the treated and untreated cells using manufacturer's protocol (Chromous Biotech Ltd, Bangalore, India). cDNA was synthesized from 1 µg of total isolated RNA by incubation for 1 h at 37ºC with M-MLV reverse transcriptase (Promega, Madison, WI). After the 1 h incubation, the mixture was again incubated at 70^0C for 15 minutes to deactivate the reverse transcriptase. The cDNA synthesized (2 µL) was added with 5 µL reaction buffer (1X), 2 µL dNTPs (0.2 mM each), 1 µL each of Forward and Reverse primer (0.4 µM each), 3 µL $MgCl_2$, 0.2 µL Taq polymerase (Promega, Madison, WI, USA) and made up to 20 µL with nuclease free water for PCR reaction. Human specific primers were used for Bcl-2, BAX, COX-2 and beta-actin. The forward and reverse primers used for Bcl-2, BAX, COX-2 and beta-actin amplification are given in Table 1. The synthesized PCR products were separated on 1.5 % agarose gels and analyzed using ChemiDoc XRS (Bio-Rad Laboratories Inc., Hercules, CA).

Western blot analysis

The protein expression levels of Bcl-2 homologous antagonist/killer (BAK) and Bcl-2-associated death promoter (BAD) were studied using western blotting assay. The Hep G2 cells were grown in 60 mm culture plates. The first set of Hep G2 cells was untreated. The second set of cells was treated with Mussaenin A at 10 µg/mL. The last and third set of cells was treated with MA at 20 µg/mL. The protein was separated by SDS-PAGE on a 10 % separating gel and transferred to nitrocellulose membrane[13-15]. Addition of primary rabbit monoclonal anti-BAK and rabbit monoclonal anti-BAD antibodies were done after the transfer of protein on to the membrane. The nonspecific protein binding sites on the blotted nitrocellulose membrane were blocked with 5% non-fat dry milk in 0.1 % Tween-20 in PBS (pH 7.4) at 4°C overnight. The membrane was incubated with monoclonal antibodies for BAK, BAD and β-actin at the dilution of 1:1000 (in 3 % BSA), 1:2000 (in 3 % BSA) and 1:2000 (in 3 % BSA) respectively at 4^0C overnight with gentle shaking. The membrane after incubation was washed thrice with PBS-Tween-20 (5 minutes each) and incubated with goat anti-rabbit HRP antibody for BAK and goat anti-rabbit HRP antibody for BAD (in 5 % non-fat milk solution) (abcam, Cambridge, USA) at RT for 1 hour. After incubation, the membrane was washed twice with PBS-Tween-20 (5 minutes each) and for 10 minutes with the same. The protein bands were quantified by autoluminographs. The amount of protein in each well was confirmed by stripping the membrane with stripping

Table 1: Forward and reverse primers for the genes.

Genes	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)
Bcl-2	ATGGACGGGTCCGGGGAG	TCAGCCCATCTTCTTCCA
BAX	CAGCTGCACCTGACG	ATGCACCTACCCAGC
Cox-2	TTCAAATGAGATTGTGGGAAAATTGCT	AGATCATCTCTGCCTGAGTATCTT
β-actin	GTTTGAGACCTTCAACACCCC	GTGGCCATCTCCTGCTCGAAGTC

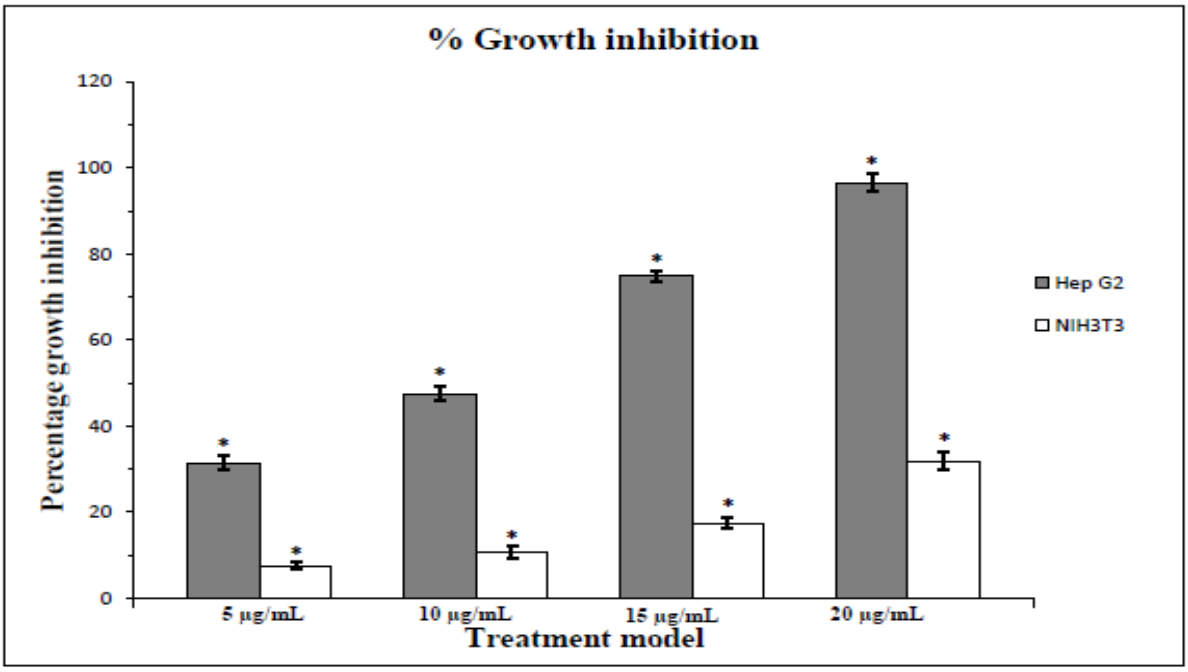

Figure 1: Dose dependent response of Mussaenin A on the cell viability of Hep G2 and normal NIH3T3 cells.

buffer (at 70°C for 1 hour) and reprobing with monoclonal antibody to β-actin by following the manufacturer's instruction (Santa Cruz Biotechnology, Inc., CA). Immunodetection of the protein expressions under study was performed using ECL prime western blotting detection reagent (Amersham Biosciences, Piscataway, NJ) and visualized in a ChemiDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA).

Caspases Activity Assay

Caspase-9 and 3 activities in the cell lysates were measured using the colorimetric assay with commercial kits according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Cells were incubated with and without Mussaenin A for 24 h. After the incubation, the cells were collected by trypsinization and lysed using the lysis buffer (250 mM HEPES, pH 7.4 containing 25 mM CHAPS and 25 mM DTT). The assay was based on the hydrolysis of the peptide substrates Leu-Glu-His-Asp p-nitroaniline (LEHD-pNA) by caspase-9 and acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac- DEVD-pNA) by caspase-3 resulting in the release of p-nitroaniline (pNA) moiety. The cell lysates were mixed with the substrate and read at 405 nm in a microplate reader[16,17]. The results were expressed as micromoles of pNA released per minute per mL.

Statistical Analysis

Results refer to mean ± standard deviation and are average of three values per experiment; each experiment was repeated at least three times. Statistical evaluations were assessed using the Student's *t* test, and $p < 0.05$ was considered significant.

RESULTS

Effect of Mussaenin A on cell viability

Concentration-dependent study performed using MTT assay to check the effect of Mussaenin A on the cell viability of normal NIH3T3 cells and Hep G2 liver cancer cells showed that an increase in concentration of MA (0, 10 and 20 µg/mL) selectively reduced the cell viability significantly in the cancer cells than normal cells (Figure 1). The IC_{50} value of Mussaenin A was found to be 11.38 ± 0.46 µg/mL on Hep G2 cancer cells. Total (100 %) growth inhibition was observed at a concentration of 20 µg/mL of MA on the Hep G2 cancer cells whereas this concentration produced only 30 % cell death in the normal NIH3T3 cells which showed that MA has reduced cytotoxic effect on non-cancerous normal cells.

Apoptosis studies

Hoechst staining was performed for evaluation of nuclear morphology after treatment with different concentrations of Mussaenin A on Hep G2 cells. Nuclear degradation was initiated at lower concentration of Mussaenin A and significantly increased at higher concentrations (Figure 2). The results clearly indicated significant nuclear morphological changes associated with apoptosis in the Hep G2 cell line after treatment with different concentrations of Mussaenin A. The Hep G2 cells stained with acridine orange/ethidium bromide showed that the

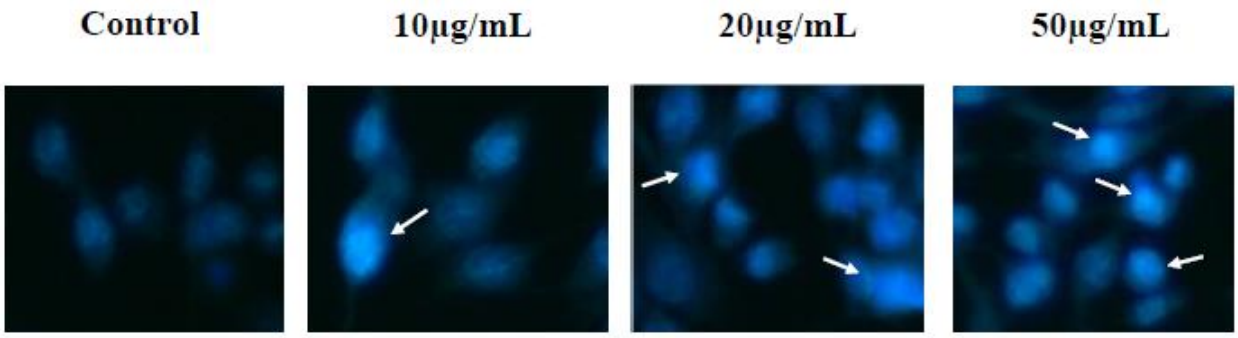

Figure 2: Hoechst staining stained Hep G2 cells after treatment with Mussaenin A.

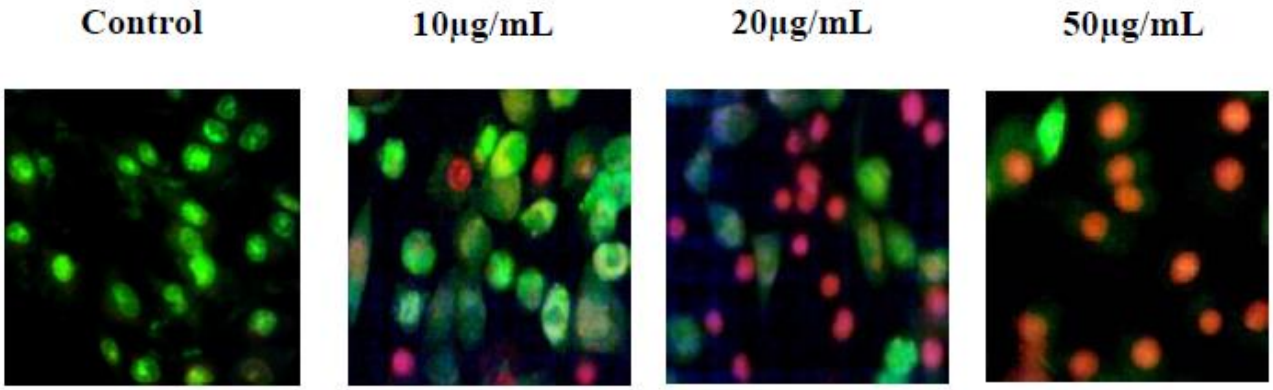

Figure 3: AO-EtBr stained Hep G2 cells after treatment with Mussaenin A.

live cells took up the acridine orange stain and fluoresced in bright green color (Figure 3). As the concentration of Mussaenin A increased, the degree of membrane damage also increased and the apoptotic cells stained as orange/red as a result of co-staining with ethidium bromide. Considerable DNA fragmentation was observed when the Hep G2 cells were treated with different concentrations of Mussaenin A (Figure 4). As the concentration of MA increased from 10 µg//mL to 50 µg//mL, the DNA fragmentation in the cells also increased confirming induction of apoptosis in the cancer cells.

RT-PCR

The mRNA expression level of pro-apoptotic BAX from the normal and Mussaenin A treated liver cancer cells were found to increase whereas the expression of anti-apoptotic Bcl-2 and COX-2 mRNAs were found to decrease in MA treated Hep G2 cells (Figure 5). So, it was evident that as the concentration of Mussaenin A increased, the expression of pro-apoptotic BAX increased and the expression of anti-apoptotic Bcl-2 and COX-2 decreased, which confirmed the ability of MA to disturb the integrity of mitochondrial membrane thereby leading to release of cytochrome c into the cytoplasm which would form the apoptosome complex by binding with pro-caspase-9 and Apaf-1 triggering the downstream steps of Caspase cascade in the intrinsic pathway of apoptosis.

Western blotting

The protein expression studies of BAK and BAD, showed that the Mussaenin A induced increased expression of pro-apoptotic BAK and BAD (Figure 6); which can be compared to the expression of house-keeping protein beta-actin providing a relative expression pattern (Figure 7). As these factors play a very significant role in the mitochondria mediated apoptosis pathway, the increase in the expression of BAK and BAD could be vital in the induction of intrinsic pathway of apoptosis.

Caspase-9 and 3 calorimetric assays

Finally, a significant increase in the caspase-9 and 3 activities was observed in Mussaenin A treated cells compared to control, which indicated the activation of caspase-9 and 3 in fraction induced apoptosis in the Hep G2 liver cancer cells. The activity of the Mussaenin A at 10 µg/mL was 115.85 ± 2.67 µmols of pNA released/min/mL in caspase-9 assay and 104.28 ± 5.49 µmols of pNA released/min/mL in caspase-3 assay which decreased when the concentration of the Mussaenin A was reduced (Figure 8).

DISCUSSION

In India, the earliest mention of the use of medicinal plants is to be found in Rigveda which was written between 4500-1600 BC[18]. Rubiaceae is an important family of plants with regard to its pharmacological activity. The number of described products, the structural diversity and pharmacological activities reported for various species of Rubiaceae demonstrate this family to be a promising source of new bioactive substances, which may give rise to new products as active molecules. Many of these plants have widespread use in folk medicine and some showed anti-inflammatory, analgesic, antibacterial, mutagenic, antiviral, antioxidant, effect on vascular diseases as well as activity on the central nervous

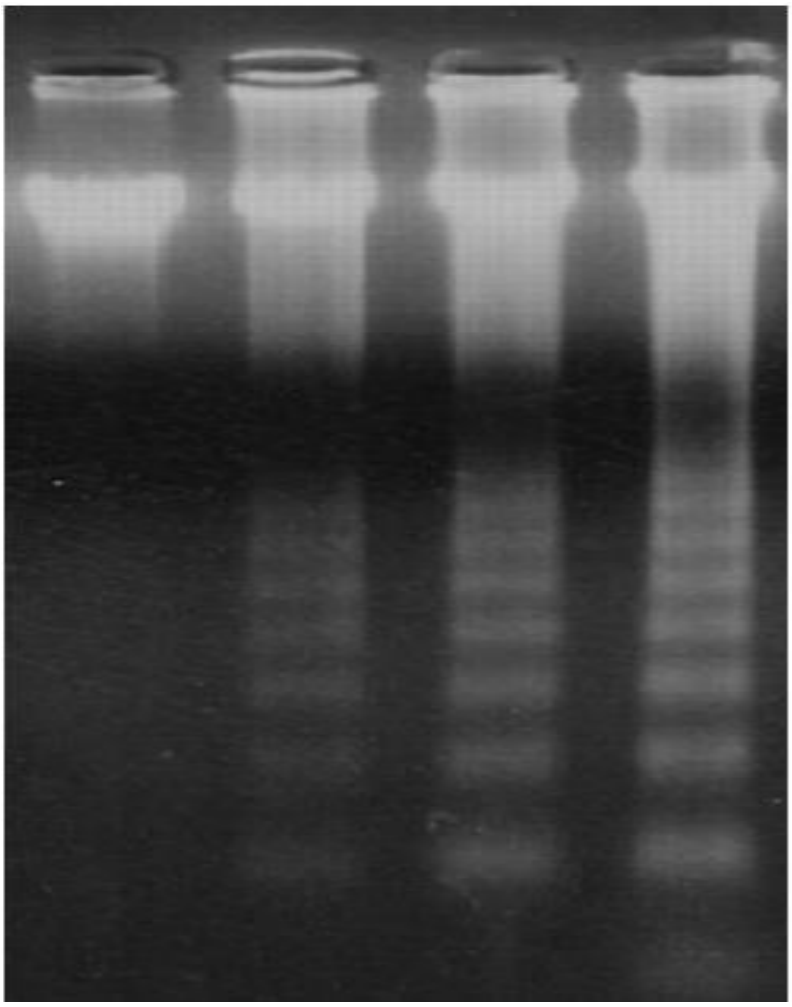

Figure 4: DNA fragmentation assay gel after treatment with Mussaenin A on Hep G2 cells. Lane 1: Control, Lane 2: 10 µg/mL, Lane 3: 20 µg/mL, Lane 4: 50 µg/mL.

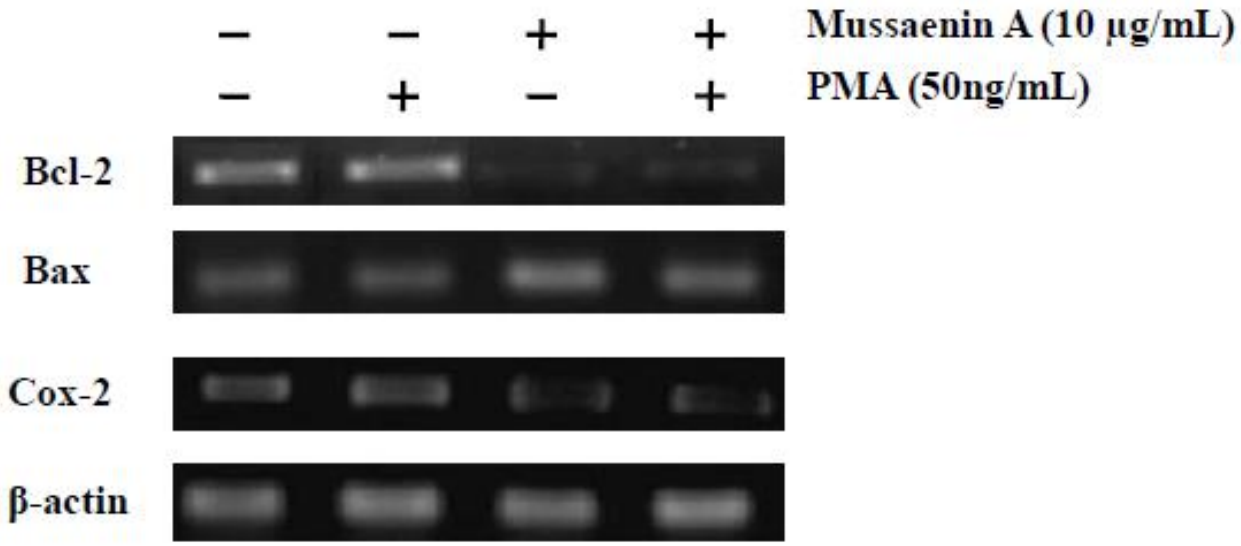

Figure 5: RT-PCR gel showing mRNA expression of pro-apoptotic BAX and anti-apoptotic Bcl-2 and COX-2.

system[19,20]. Terpenes are among the most cited phytochemical constituent to possess anti-proliferative activity. Mussaenin A isolated from the root of *Mussaenda glabrata* was found to be rich in triterpenes mainly[21].The cell viability assay using MTT showed that Mussaenin A induced growth inhibition in Hep G2 cells with an IC_{50} value of 11.38 ± 0.46 µg/mL. MTT assay is generally used as a preliminary assay to measure the growth inhibitory activity of a chemical on cells. The result showed that Mussaenin A almost completely

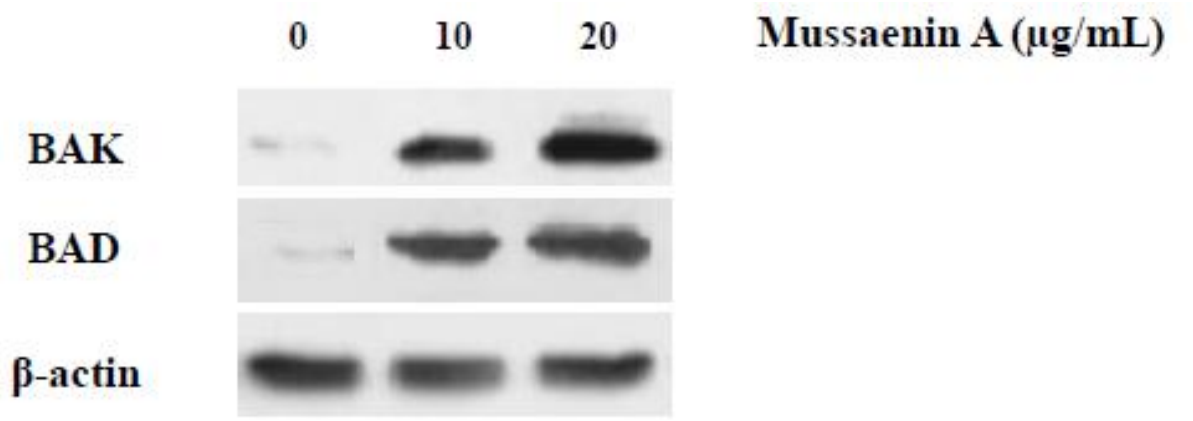

Figure 6: Western blot of BAK and BAD.

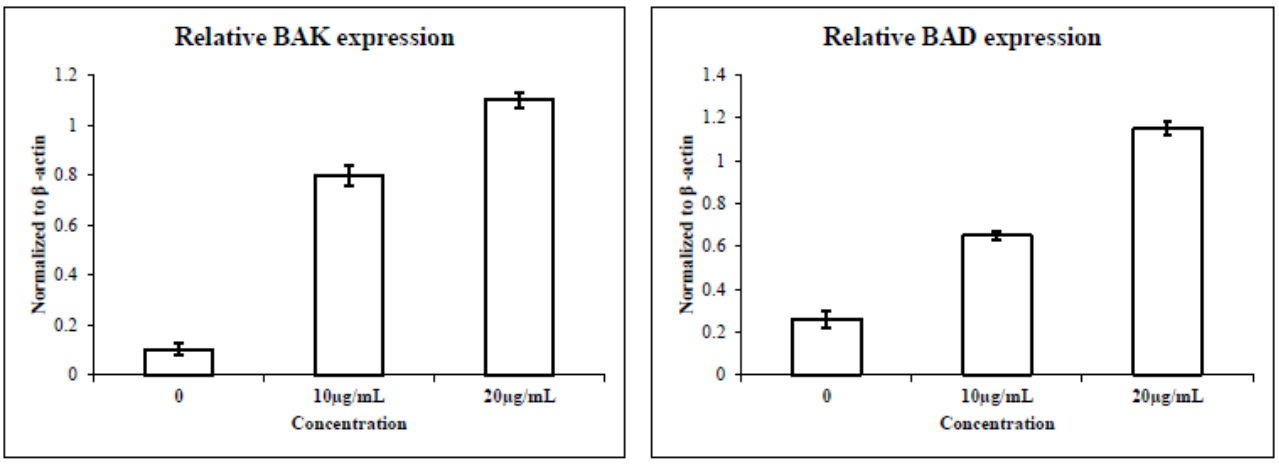

Figure 7: Relative protein expression of BAK and BAD compared to β-actin.

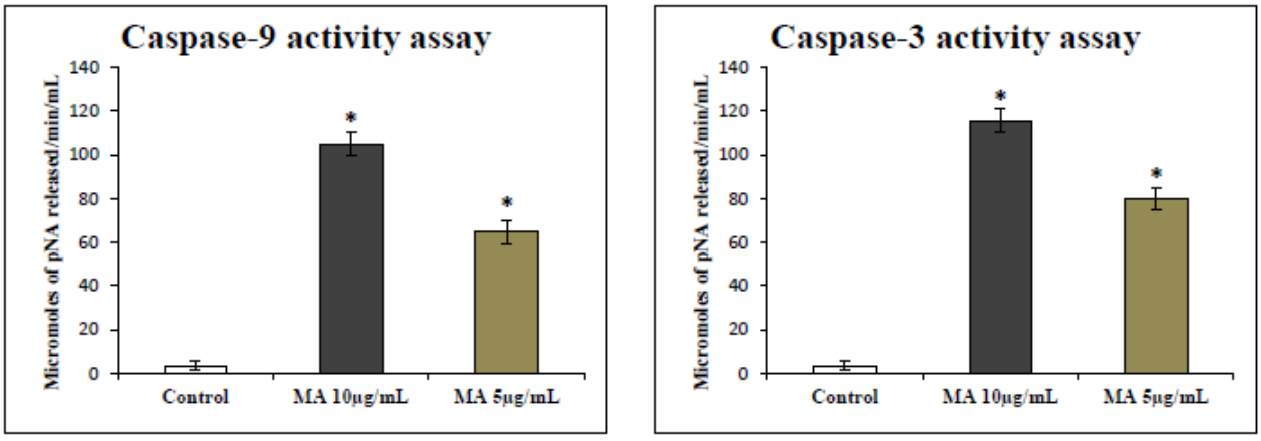

Figure 8: Effect of Mussaenin A on the Caspase-9 and 3 release in Hep G2 cells when treated with different concentrations.

inhibited the growth of Hep G2 cells at 20 µg /mL. Further, MA was found to induce lower growth inhibition in normal NIH3T3 cells. At 20 µg /mL, Mussaenin A was found to induce only 30% growth inhibition in the NIH3T3 cells which shows that MA acts on the cancer and normal cells differentially.

Hoechst staining assay used to study apoptosis induction, revealed that the nuclear condensation, which is a feature of apoptosis, was visible prominently and increased in a dose dependent manner in the Hep G2 cells[22]. The results showed that the nuclei of untreated control cells emitted lower fluorescence intensity compared to MA treated cells which showed typical features of apoptosis like chromatin condensation which leads to the increase in emission of intense fluorescence from the nucleus. The Acridine orange-Ethidium bromide staining showed that

Mussaenin A altered the membrane stability of MA treated cells, whereas the membranes of untreated cells were unaffected. The untreated cells fluoresced green due to the binding of Acridine orange to the intact cell membrane. But as the concentration of MA increased from 10 µg/mL to 50 µg/mL, the cells were found to take up more Ethidium bromide and fluoresce red due to the disturbance in the membrane integrity caused by the action of Mussaenin A. Finally when the DNA fragmentation assay was performed, apoptosis induction in Mussaenin A treated cells was confirmed when the DNA isolated from the treated cells formed a ladder like pattern in the gel. The DNA which gets cut into approximately 180-200 base pairs or its multiples forms a ladder like pattern when electrophoresed. The DNA gets cut at the internucleosomal linker sites by the action of Caspase activated DNase (CAD) and forms the ladder pattern which is a classic feature of apoptosis[23,24].

Molecular studies of pro-apoptotic BAX and anti-apoptotic BCL-2 and COX-2 mRNA showed that Mussaenin A regulated their expressions. The expression of BAX was found to be upregulated in Mussaenin A treated cells whereas the expression of BCL-2 and COX-2 was found to be down-regulated in the treated cells. Mitochondrial membrane permeability changes when the ratio of BCL-2 family members changes[25]. When the pro-apoptotic members are up-regulated and anti-apoptotic members are down-regulated, this leads to the release of cytochrome-c from mitochondria, which activates the caspase cascade[26]. COX-2, is an anti-apoptotic factor, which is found to be up-regulated in various carcinomas and is reported to have a central part in tumorigenesis[27]. Overexpression of COX-2 in rat intestinal epithelial cells was found to reduce the rate of apoptosis and increased the expression of the antiapoptotic protooncogene BCL-2[28]. COX-2 is reported to be a determinant of the differentiation grade of Hepatocellular carcinoma and inhibition of COX-2 was found to induce growth suppression in hepatomas[29]. Here, Mussaenin A was found to decrease the expression of anti-apoptotic BCL-2 and COX-2 mRNA and also increase the expression of pro-apoptotic BAX mRNA thus shifting the ratio towards the pro-apoptotic events which induces apoptosis in the Hep G2 cells.

The mitochondria-anchored BAK and BAD proteins are found to be pro-apoptotic in nature and induce apoptosis in the cancer cells[30,31]. The BAK and BAD proteins induce apoptosis by destabilizing the mitochondrial membrane and creating pores in the mitochondrial outer membrane thereby initiating release of cytochrome-c into the cytosol which triggers caspase cascade[32,33]. The BAK and BAD protein expression was found to increase in Mussaenin A treated Hep G2 cells. This increase in expression in BAK and BAD proteins initiates apoptosis by forming pores in the mitochondrial outer membrane.

Finally, the activities of both caspase-9 and 3 were found to increase in the Hep G2 cells when treated with Mussaenin A. Caspase-9 and 3 are the major enzymes involved in the mitochondrial mediated or intrinsic pathway of apoptosis[26, 34]. The activity of both these enzymes which was studied calorimetrically proved categorically that Mussaenin A has the ability to activate these enzymes and thus induce apoptosis in the Hep G2 liver cancer cells.

Members of *Rubiaceae* family and Genus Mussaenda is reported to possess anti-inflammatory and anti-proliferative activity but no study has been conducted till date on Mussaenin A isolated from the root of *Mussaenda glabrata*. Mussaenin A induced considerable lower percentage of cell death in normal NIH3T3 cells compared to the cancerous Hep G2 cells, thus showing its ability to differentiate between normal cells and rapidly dividing cancerous cells. Our study shows that Mussaenin A induces apoptosis in the liver cancer cells via the intrinsic or the mitochondria mediated pathway by down regulating the anti-apoptotic factors and up regulating the pro-apoptotic factors.

CONCLUSION

The growing interest in biological activities of phytochemicals is a consequence of, among others, an increasingly high incidence of various cancers and a need to find safe and effective method for prophylaxis and therapy. In this study, Mussaenin A isolated from the root of *Mussaenda glabrata* was found to induce apoptosis in the Hep G2 liver cancer cells via the mitochondria-dependent pathway. However, Mussaenin A induced lower apoptotic activity on the normal NIH3T3 cells showing that MA acted on different cells differentially. This is the first report on the mechanism of apoptosis induction by Mussaenin A isolated from the root of *Mussaenda glabrata* and these findings would provide yet another method of targeting cancer cells using phytochemicals.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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