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Chrysin inhibits propagation of HeLa cells by attenuating cell survival and inducing apoptotic pathways

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Abstract. – OBJECTIVE: Chrysin, one of the main active constituents of flavonoids, is known for demonstrating protective effects against various types of cancer including cervical cancer. The aim of this study was to determine apoptosis induction and antiproliferative action of chrysin on human cervical cancer cells.

MATERIALS AND METHODS: In this study, attempts have been made to establish anticancer role of chrysin on HeLa cells. MTT, mitochondrial potential, DNA fragmentation, annexin V/propidium iodide assays, qPCR and protein profiling were performed.

RESULTS: Chrysin treated HeLa cells showed time and dose dependent decrease in cell viability and demonstrated profound effects on nuclear morphology and DNA fragmentation. Chrysin treatment increased the expression of proapoptotic genes *BAD, BAX, BID, BOK and APAF1, TNF, FASL, FAS, FADD* and caspases (like *caspase 3, caspase 7, caspase 8* and *caspase 9*), whereas it decreased the expression level of antiapoptotic genes *MCL-1, NAIP, XIAP* and *Bcl-2* and cell cycle regulatory genes *CCNB1, CCNB2, CCND1*, CCND2, CCND3, *CCNE2, CDK4* and *CDK2* at transcript level. Furthermore, chrysin significantly upregulated pro-apoptotic proteins, like TRAILR2/DR5, TRAILR1/DR4, Fas/TNFRSF6/CD95, phosphoP53(S15), BAD, BAX, cleaved caspase 3, procaspase 3, HTRA2/Omi and SMAC/Diablo, while downregulated anti-apoptotic proteins like BCL-X, BCL2, XIAP and CIAPs that support chrysin mediated apoptosis in HeLa cells. Remarkably, chrysin downregulated the phosphorylated AKT pathway proteins, (p-473) AKT, (p-Ser 2448) mTOR, (p-Ser241) PDK1, (p-Ser112) BAD, and upregulated (p-Ser21) GSK3b, (p-Thr172) AMPKa, P27 (p-Thr198) and (p-Ser15) P53, which endorses chrysin mediated apoptosis.

CONCLUSIONS: Chrysin significantly inhibited proliferation and induced apoptosis by modulation of various apoptotic genes and AKT/MAPK pathway genes.

Key Words:
Chrysin, Apoptosis, AKT, MAP kinase, Anticancer, Chemoprevention.

Introduction

Aberrant molecular and biochemical behavior of cancer cells scores a relevant interest to use plant derived dietary agents, which usually act as modulators of various biological processes, such as cell proliferation, apoptosis, cell migration and inflammation that can reverse/alter the abnormal behavior of cancer[1-4]. Earlier scientific reports[1-4] indicate that polyphenols present in fruits and vegetables can inhibit the progression of cancer development. Polyphenols, such as curcumin, genistein, resveratrol, apigenin, fisetin, luteolin etc. have shown their anti-neoplastic action by targeting several hallmarks of cancer in various type of cancers[2,4-7]. Hence, their therapeutic potential is gaining more attention in the development of a better treatment regimen against cancer.

Apoptosis or programmed cell death is a vital and organized process, regulated by a series of signal transduction cascades and cellular proteins that regulate cell turnover, appropriate development, and cell homeostasis to maintain cellular development. It is characterized by distinct morphological characteristics and is induced by ener-

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gy-dependent biochemical mechanisms[8]. Cancer cells have the ability to evade apoptosis by passing the critical checkpoints in the cell cycle and can be modulated by the activation of pro-apoptotic proteins and the inhibition of anti-apoptotic genes through novel therapeutic interventions[1,7,9]. Considering the cytotoxic profile of flavonoids, and their suitability for inducing apoptosis in cancer cells, flavonoids are the potential candidates for targeting different cancers[10-14]. Flavonoids, a subclass of polyphenols have a basic 15-carbon phenyl propanoid core (C6-C3-C6 system), and are sub-grouped into several classes like flavanones, flavones, isoflavones, 3-deoxy flavonoids, flavonols and anthocyanins. They are ubiquitously present in fruits and vegetables[3,5,7,15] and are considered as potential chemo-preventive agents due to their greater bioavailability, affordability and higher specificity against cancer cells compared to the existing cancer treatments[2,3,7,15-17]. Several *in vitro* and *in vivo* studies[2,4,6,18-20] on flavones have shown their anti-proliferative potential by promoting cell cycle arrest at G1/S phase or G2/M phase and bringing about apoptosis *via* modulation of various molecular signatures including caspases, B-family proteins, nuclear factor kappa B and PI3K/AKT/mTOR, MAPK/P38 and JAK/STAT pathways.

Chrysin 5,7-dihydroxyflavone, a flavone, is a natural bioactive compound found in honey, passionflower and propolis. It has antioxidant, anti-inflammatory and anticancer properties[20]. Several reports[20-23] have shown that chrysin hampers cancer cell growth by regulation of cell cycle, induction of apoptosis and inhibition of metastasis and angiogenesis with differential effects towards cancer cells. Chrysin mediated molecular changes are demonstrated by altering abnormal multiple cell signaling pathways, which are associated with cell proliferation, cell survival, apoptosis, angiogenesis, metastasis and invasion. Chrysin inhibited cell proliferation in SW480 led to G2/M arrest and inhibited COX-2 expression *via* NF-IL-6 inhibition[5,16,18,24]. Chrysin acts as an inhibitor of HDAC2 and HDAC8, causes G1 cell cycle arrest in A375 melanoma cell line[25]. Zhang et al[24] reported anticancer effects of chrysin on three different colon cancer HT-55, HCA-7 and LoVo cell lines with IC_{50} values of 0.4, 0.4 and 0.8 mM, respectively. Chrysin is a potential anti-cancer agent but has displayed different mechanisms of action in different cancers. It induces apoptosis in leukemic U937 cells by AKT inhibition and caspase activation, whereas in C6 glioma cells, G1 arrest and apoptosis were due to upregulation of p21WAF/CIP1 expression or NFkappaB/p65 pathway in cervical cancer cells and in hepatocellular carcinoma chrysin induces apoptosis by P53/BCL2/caspase[13,24,26]. The activation of AKT *via* phosphorylation prevents apoptosis, whereas dephosphorylation initiates apoptosis. BAD and caspase 9, the vital players of intrinsic pathway get phosphorylated and inhibited by activated PI3K/AKT pathway that plays a significant role in cell survival and proliferation[26]. Chrysin has shown dephosphorylation of BAD and AKT pathway in U937 and breast cancer cells[26-27]. The precise molecular mechanism of chrysin's action is not well established in cervical cancer cells. Therefore, in this study we evaluated the efficacy of chrysin as a pro-apoptotic agent and its molecular cross talk in HeLa cells.

Materials and Methods

Cell Culture and Preparation of Chrysin Stock

Human cervical cancer cell line, HeLa, was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA; Merck KGaA Darmstadt, Germany) augmented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA; Merck KGaA Darmstadt, Germany) and 100X Penstrep (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO_2 at 37°C. Chrysin was acquired from Sigma-Aldrich (St. Louis, MO, USA; Merck KGaA Darmstadt, Germany). 1 mM chrysin was prepared in Dimethyl Sulfoxide (DMS0; Sigma-Aldrich, St. Louis, MO, USA; Merck KGaA) and aliquots were kept at -20°C. Various dilutions between 1 to 50 µM were made in complete medium for the treatment purpose.

Cell viability assay of Lymphocytes and HeLa cells

The antiproliferative activity of chrysin (1-50 µM) on HeLa cells was determined by using MTT (Sigma-Aldrich, St. Louis, MO, USA; Merck KGaA Darmstadt, Germany) [3-(4,5-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA) assay as published earlier by Hussain et al[28], 2012. Briefly, in a 96 well plate ≈10,000 cells/well were plated and incubated for 24 h. Then, the cells were treated

with various concentrations (1-50 µM) of chrysin and DMSO (vehicle control). Following the treatment for 24 and 48 h, MTT assay was performed for chrysin and respective control cells. MTT 5 mg/ml was prepared and added to each well and incubated for 2 h at 37°C. The reaction was stopped by adding DMSO and the plate was read at 570 nm. The experiment was performed three times and an average of three experiments was taken for precise estimation ($p<0.05$). The cell viability was calculated by using the below formula:

$$\% \text{ Viability} = \frac{\text{Mean of three treated tests}}{\text{Mean of three controls}} \times 100$$

HiSep™ LSM 1077 (HiMedia, India; Catalogue No. 1077-LS001) was used to isolate lymphocytes. Blood was collected in heparin containing sterile tube and diluted 1:4 with PBS. 2.5 ml of HiSep™ was taken in a 15 ml centrifuge tube and overlaid with 7.5 ml of diluted blood and then centrifuged at 400 g for 30 minutes at room temperature. Hi-SEP is an iso-osmotic solution and centrifugation creates several cell layers, with the mononuclear cells (lymphocytes and monocytes) present as a band-below the plasma layer. The plasma layer was aspirated and discarded while the mononuclear interphase was transferred to a centrifuge tube. 10 ml of isotonic phosphate-buffered saline was added to the mononuclear cells layer in the centrifuge tube and mixed by gentle aspiration followed by centrifuging at 160-260 × g with brake off, at room temperature (15-25°C) for 10 minutes. This washing was done two times. This washing with isotonic phosphate-buffered saline removes HiSep™ LSM and diminishes the number of platelets. The cell pellet was resuspended in Roswell Park Memorial Institute-1640 (RPMI-1640) complete media and cells counted and plated in 96 well (~10,000 cells/well) and treated with different concentrations of chrysin ranging from 1 to 50 µM for 24 h. Cell viability assay with MTT was performed as described above and cell viability was calculated.

Microscopic Examination of Treated Cells

The changes in the cell morphology representing cell death were observed by using inverted microscope. Chrysin treated HeLa cells (1-50 µM) for 24 and 48 h were visualized using inverted light microscope.

Analyzing Changes in Nuclear Morphology of HeLa Cells

To check for the mode of cell death after chrysin treatment, morphological changes at nuclear level were analyzed using Florescent Microscope (Olympus, Tokyo, Japan). Briefly, ≈25×10^4 cells/ml were plated and incubated for 24 h for the attachment at 37°C followed by the treatment with chrysin (10 µM and 15 µM) for 48 h. Cells were then fixed in acetone: methanol (1:1) solution at -20°C for 10 min, followed by staining with propidium iodide (PI; 10 µg/ml in PBS) for 30 seconds in the dark at room temperature; afterwards, the coverslips were washed with 1× PBS and mounted with DPX and observed under the Progress Fluorescent Microscope (Olympus, Tokyo, Japan) at 515 nm. The images were captured at 100 x magnification.

DNA Laddering Assay

ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (Invitrogen, Carlsbad, CA, USA; Catalogue No: KHO1021) was used for rapid extraction of chromosomal DNA of the cells. Approximately, 1×10^6 cells were plated and treated with 10 and 15 µM of chrysin for 48 h. Untreated HeLa cells were taken as controls. The lysis of the controls and treated cells was performed by using TE lysis buffer. Then, Enzyme A was added to the lysate and vortexed, incubated at 37°C for 10 min followed by the addition of enzyme B and incubation at 50°C for 30 min. Afterwards, ammonium acetate and ethanol were added. The DNA was allowed to precipitate at -20°C for 10-15 min. Lysates were vortexed and incubated at -20°C for 15 min then centrifuged. The precipitated DNA was washed with ethanol, air dried and resuspended in DNA resuspension buffer. Thereafter, DNA samples were subjected to gel electrophoresis using 1.2% agarose gel at 80 V. The gel images were captured using G-Box, Syngene gel documentation system. The samples (15 to 30 µL) were loaded on 1.2% agarose gel containing 0.5 µg/mL ethidium bromide and running buffer (1xTBE). The gel was run at 5 V/cm for 1 to 2 h. Ethidium bromide-stained DNA was visualized by transillumination and images were saved. The experiment was performed three times.

Investigation of Cell Cycle

Chrysin treated HeLa cells were examined by using Flow cytometry to determine the DNA content in the cells and analyze the cell cycle using Propidium Iodide Flow Cytometry Kit

(Abcam: ab139418, Cambridge, UK). Approximately, 1.5×10^6 cells were plated in 25 cm^2 flasks followed by treatment with concentrations of chrysin (10 and 15 µM). The untreated cells were taken as controls. Both treated and control cells were trypsinized and washed with 1xPBS and fixed in absolute ethanol at -20°C for overnight. Prior to use, the Propidium Iodide (PI) + RNase Staining solution in PBS was prepared. The fixed cells were centrifuged at room temperature at 500xg for 5 min, the supernatant was discarded. This was followed by washing and staining of the cells with PI stain for 30 min at 37°C in the dark. Lastly, the analysis of the stained cells was performed using flow cytometry (FACS Calibur; Becton Dickinson, Brea, CA, USA) and data was analyzed using FloJo software (Version 10.1; Pasadena, CA, USA). Experiment was repeated thrice, and one-way ANOVA was used to calculate significance and *p*-value was set at $p<0.05$.

Double Staining Using Annexin V/Propidium Iodide

Cell death mediated by apoptosis in chrysin treated HeLa cells was quantitated using a FITC-conjugated Annexin V/Propidium Iodide (PI) assay kit (ab14084), Abcam, Cambridge, UK). This assay allows to quantitate the viable cells, early apoptosis (Annexin V+ PI -) and late apoptosis (Annexin + PI +). Approximately, 2.5×10^5 cells per well were plated in six well plates and treated with 10 and 15 µM of chrysin for 48 h; the untreated control and treated cells were collected and washed with 1× PBS and the staining protocol was followed as per the manufacturer's manual followed by Flow cytometry analysis (FACScan, Becton-Dickinson, Brea, CA, USA) and the data were analyzed using FlowJo software Version 10.1 (Pasadena, CA, USA). The fraction of the cells in each quadrant of the graph helped in the quantitation of early (Annexin +ve) and late apoptosis (Annexin +ve /PI +ve), and the viable cell fraction (unstained). Experiment was repeated thrice, and one-way ANOVA was used to calculate significance and *p*-value was set at $p<0.05$.

Mitochondrial Potential Assay

TMRE (Tetramethyl rhodamine, ethyl ester) Mitochondrial Membrane Potential Assay Kit (ab113852) was obtained from Abcam (Cambridge, UK). The said kit is generally used for observing the variations in the mitochondrial membrane potential in live cells using spectrophotometric analysis (Synergy H1 Bioteck Plate Reader, Winooski, Vermont, USA) and fluorescent microscopy (Olympus, Tokyo, Japan). Nearly 5×10^3 cells were plated in a 96 clear bottom plate and treated with 10 and 15 µM chrysin for 48 h. Following the treatment, TMRE was added to the control cells, treated cells and negative control cells (FCCP) and incubated for 20 min at 37°C. Afterwards, the intensity of florescence was measured using microplate spectrophotometry (Ex/Em = 549/575 nm) and microscopic pictures of TMRE stained cells were taken by using fluorescence microscope (Progress Fluorescent Microscope (Olympus, Tokyo, Japan) and images were captured at 40× magnification. The graph was plotted as % fluorescence as compared to untreated controls. Data are presented as the mean ± standard deviation of three independent repeats and significance was set at $p<0.05$.

Gene Expression by TaqMan Apoptosis Array

RNA extraction of chrysin treated (10 and 15 µM for 48 h) and untreated HeLa cells were performed as per the manufacturer's instructions (Gen Elute Mammalian Genomic Total RNA Kit; Sigma-Aldrich, St. Louis, MO, USA). Following the purification of RNA, gel electrophoresis was performed to examine the bands of RNA. The quantification of RNA was done by nanodrop (Nanodrop 2000c; Thermo Fisher Scientific™, Waltham, MA, USA). Afterwards, cDNA was synthesized as per manufacturer's protocol (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems™, Foster City, CA, USA). TaqMan® Gene Expression Assays (Apoptosis Array 4414072 and Oncogene array 4391524) (Thermo Fisher Scientific™, Waltham, MA, USA) were used to check the expression of various genes involved in apoptosis and signaling pathways including MAPK and AKT/MTOR/PIK3. cDNA equal to 10 µl of the treated (10 and 15 µM) and untreated samples with a concentration of 100 ng per well and 10 µl of the master-mix provided with the kit was added to each pre-plated and designed plates. PCR array analysis was performed on QuantStudio3 and analyzed by the $^{\Delta\Delta}$CT method using DataAssist™ software (Thermo Fisher Scientific™, Waltham, MA, USA). The data were normalized using a house keeping gene *GAPDH* expression for both the arrays. Relative Quantitation (RQ) indicates the fold changes in the expression of the gene

compared to the untreated control. Results were analyzed by ΔΔCT method, using global expression and RQ (fold change) was calculated. The experiment was performed two times in duplicates. The results were represented as mean ±SD of three independent experiments ($p<0.05$).

Protein Expression by Proteome Profiler Array

Proteome Profiler Array (Catalogue Number ARY009, R&D, Minneapolis, MN, USA) was used to determine the comparative levels of apoptosis-related proteins. The relative expression levels of 35 apoptosis-related proteins were investigated using this array. The cell lysate was prepared from the untreated and treated (10 and 15 μM) HeLa cells and protein was quantitated by Pierce BCA Assay (Catalogue No. 23225; Thermo Fisher Scientific™, Waltham, MA, USA). Briefly, a total of 400 μg of the protein of the diluted cell lysate was used for each membrane; the membranes were incubated with the lysates on a rocking platform overnight at 2-8°C, and then, washed to remove any unbound proteins; the captured antibodies were detected by incubation with the help of detection antibodies followed by application of Streptavidin-HRP and chemiluminescent detection reagents. The signal thus produced was quantified by chemiluminescent detector gel doc system (Bio-Rad Laboratories, Hercules, CA, USA), and analyzed by using Image Lab software program (version 6.1). The expression of the different proteins in chrysin treated samples was compared with the untreated controls and the fold change was calculated. Intensity of blot corresponds to expression of the protein and fold change was calculated after normalization with reference spots. Significance was calculated using SPSS software (SPSS Inc., Chicago, IL, USA) and was set at $p \leq 0.05$.

Caspase 3 Activity Assay

Caspases, a family of proteases enable cell death and are significant to the process of apoptosis. Caspase 3, an effector caspase, is a vital player for both intrinsic and extrinsic pathways of apoptosis. Caspase 3 Colorimetric Assay Kit (Catalogue No. CASP 3C; Sigma-Aldrich, St. Louis, MO, USA) was used for quick and efficient detection of caspase 3 activity in the cell lysate. Caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA). Briefly, 1×10^6 cells were plated and incubated at 37°C for 24 h, following which, the treatment with 10 and 15 μM of chrysin was performed for 48 h using the untreated cells as control. Both, treated and untreated cells were collected, and then lysates were prepared which were used in the assay as per the manufacturer's protocol. After overnight incubation, the plate was read at 405 nm. The OD readings corresponded to the activity of caspase 3 and fold changes against the control were calculated following the below formula: Fold change in caspase activity = (OD of chrysin treated samples/ OD of untreated controls) × 100.

The data are representative of three independent experiments and are expressed as mean ±SD, ($p<0.05$).

AKT Pathway Phosphorylation Array

AKT pathway or PI3K/AKT-MTOR pathway is one of the most vital signaling pathways that controls essential cellular processes such as survival, growth, cell migration and apoptosis. RayBio® C-Series Human and Mouse AKT Pathway Phosphorylation Array C1 (Cat# AAH-AKT-1-8; Ray Biotech, Peachtree Corners, GA, USA) was used as it is a fast, delicate, and cheap tool for simultaneous detection of the relative levels of phosphorylation of 18 AKT pathway proteins. The treated and untreated control cells were solubilized at 2×10^7 cells/ml in 1× Lysis buffer and the lysate was prepared as per the protocol. The protein quantitation was done using the Pierce BCA method and approximately 500 μg of protein per membrane was used. The membranes were blocked using the blocking buffer, followed by incubation with cell lysates overnight and the protocol was followed. The image of the blot was captured by chemiluminescent gel doc system (BioRad Laboratories, Hercules, CA, USA), and analyzed using Image Lab software (version 6.1, Hercules, CA, USA). Data are representative of three independent experiments and each protein expression was expressed as mean ± SD of three independent experiments ($p<0.05$).

Statistical Analysis

Statistical analysis was performed using SPSS software version 21 (IBM, Armonk, NY, USA). The data were analyzed using one-way ANOVA followed by Tukey's HSD post-hoc analysis. All experiments were performed in triplicate. Results are expressed as the mean ± standard

deviation of three separate experiments, $p<0.05$ was considered to indicate statistically significant differences.

Results

Chrysin Selectively Represses the Growth of HeLa Cells but Is Ineffective Against Lymphocytes

MTT assay showed that chrysin inhibits the viability of HeLa cell in a dose (1-50 µM) and time dependent manner for 24 and 48 h compared to the untreated controls. There was a significant reduction in viability at 24 and 48 h; however, there was a profound inhibitory effect even at the lower concentrations as compared to the untreated controls at 48 h. IC_{50} was found to be 15 µM at 48 h (Figure 1A). However, chrysin did not show any significant cytotoxicity on lymphocytes (Figure 1A). The cell death was observed in the chrysin treated cells, which was characterized by their rounding and detachment from the surface at 48 h in a dose dependent manner in comparison with the untreated HeLa cells (Figure 1B). The experiment was repeated three times. Results are expressed as the mean ± standard deviation of three separate experiments ($p<0.05$).

Changes of Nuclear Morphology in Chrysin Treated HeLa Cells

The induction of apoptosis by chrysin was assessed by using propidium iodide staining of untreated and treated HeLa cells (10 and 15 µM for 48 h) and observed under fluorescent microscope. It was determined that increasing doses of chrysin progressively decrease the proportion of intact cells; increased nuclear condensation and fragmentation were seen in chrysin (10 and 15 µM for 48 h) treated cells in comparison with the untreated controls, wherein uniform chromatin density was found. At 15 µM late apoptotic changes like apoptotic bodies and nuclear debris were also observed (Figure 2A).

Chrysin Mediates DNA Disintegration in HeLa Cells

Further to understand the underlying mechanism of chrysin mediated cell death, DNA fragmentation assay was performed. The untreated controls and 10 and 15 µM chrysin treated HeLa cells were subjected to the assay at 48 h and isolated DNA was analyzed on 1.2% agarose gel. A characteristic ladder pattern of inter-nucleosomal DNA fragmentation was observed in the treated cells compared to the untreated HeLa cells, wherein intact DNA was seen (Figure 2B). The experiment was performed three times.

Chrysin Triggers Cell Cycle Arrest at G2/M Phase

To identify the regulatory role of chrysin in the cell cycle progression, cell cycle analysis was performed in chrysin treated (10, and 15 µM at 48 h) and untreated HeLa cells. The untreated HeLa cells showed a proper distribution of cells, whereas chrysin treatment induced significant build-up of cells in G2/M phase with increase in cell num-

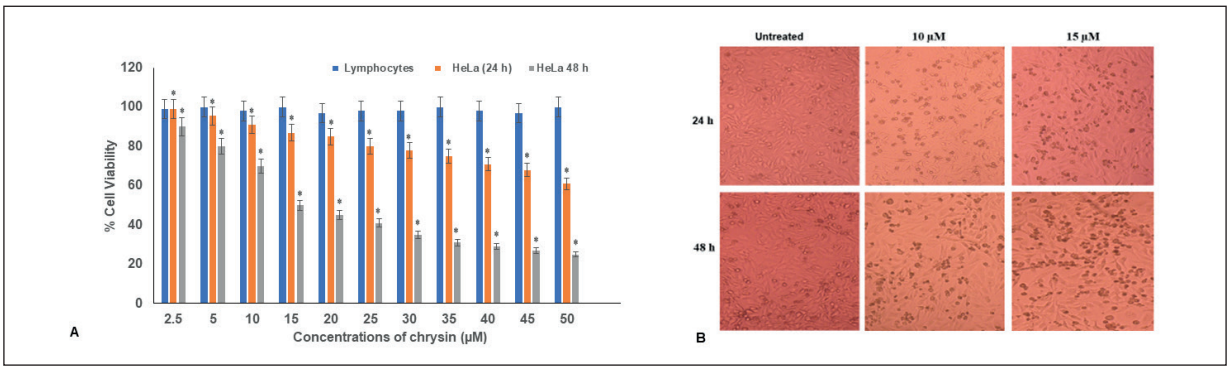

Figure 1. A, Differential induction of cytotoxicity by chrysin at various concentrations and time points: Graph is representing dose and time dependent decrease of cell viability of HeLa cells after the treatment with chrysin (1–50 µM) for 24 and 48 h, respectively as compared to the untreated controls. The IC_{50} of chrysin was found to be 15 µM at 48 h whereas chrysin is showing no significant toxicity on lymphocytes. Data are averages with SD (error bars) from three independent experiments (*$p\leq0.05$). **B,** Microscopic examination of the treated cells: Chrysin treated HeLa cells at various concentrations (5, 10 and 15 µM)) and time points (24 and 48 h) show the characteristic feature of rounding off of the cells, indicating apoptosis (Magnification 10×).

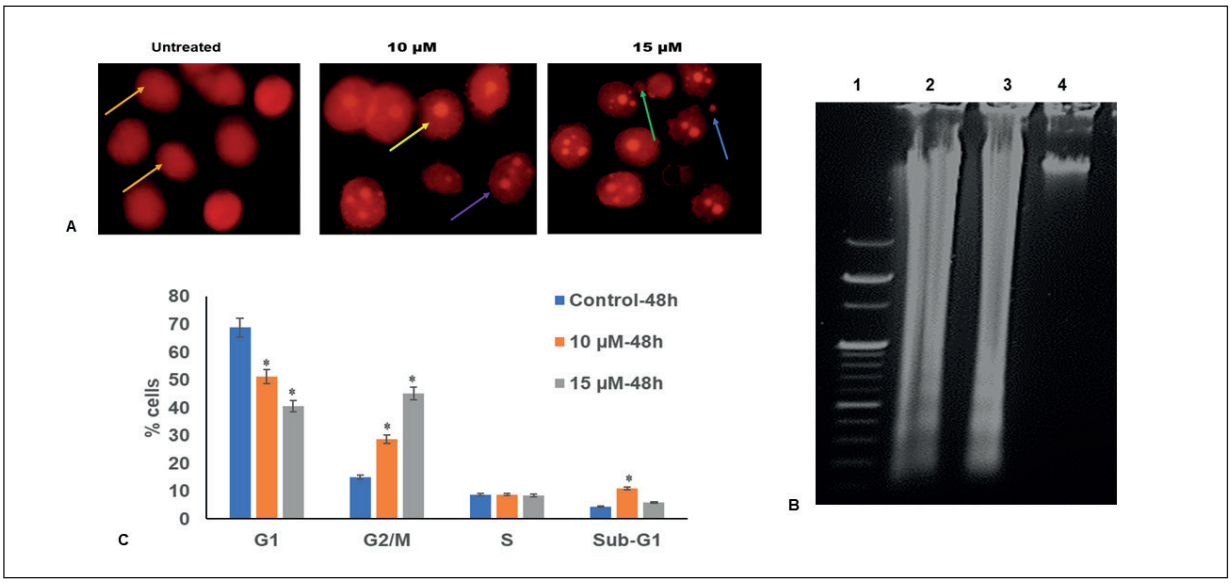

Figure 2. A, The changes in nuclear morphology of treated HeLa cells (10 and 15µM) as compared to the untreated controls. The figure depicts dose dependent increase in the apoptotic index, which is characterized by the features such as nuclear condensation, nuclear blebbing, nuclear fragmentation and apoptotic bodies). Yellow = large and prominent nuclei, purple = nuclear fragmentation, green = blebbing, white = Apoptotic bodies. **B,** DNA fragmentation of HeLa cells exposed to chrysin. The fragmentation of genomic DNA in HeLa cells treated with 10 and 15 µM of chrysin for 48 h. DNA laddering pattern was observed on ethidium bromide-stained gel (2 in treated samples as compared to untreated controls. Lane 1 shows DNA Ladder, Lane 2, 3 Depict 15µM and 10µM chrysin treatment, respectively, Lane 4 shows the control. **C,** Assessment of cell cycle was performed using flow cytometry analysis, after PI staining of the HeLa cells. The cells were treated with 10 and 15 µM chrysin for 48 h or media and cell cycle was examined with flow-cytometry after PI staining in treated and untreated samples. Chrysin induced G2/M phage arrest on HeLa cells. Cell cycle proportions were determined by flow cytometry after staining with propidium iodide. Statistical analysis of cell cycle phase distribution. Each value represents the mean of three experiments ±SD *p<0.05.

ber. The proportion of cells in control compared to 10 and 15 µM chrysin treated HeLa cells was 28 and 45%, respectively, at G2/M phase, which meant 13 and 30% increase after 10 and 15 µM chrysin treatment, respectively compared to the control (Figure 2C). Data are represented as mean ± SD of three experiments ($p < 0.05$).

Chrysin Promotes Apoptosis in HeLa Cells

Annexin V-PI double staining procedure was used to assess the initiation of apoptosis by chrysin (10 and 15 µM chrysin for 48 h) in HeLa cells. The percentage of the live cells decreased by 21.6 and 31% on 10 and 15 µM of chrysin treatment for 48 h as compared to the untreated control samples, respectively. In contrast, the early apoptotic cells (Annexin V+/PI–) increased expressively from 2.0 (control) to 7.57 and 11.3% at 10 and 15 µM after 48 h treatment, respectively. The proportion of late-stage apoptotic cells (Annexin V+/PI+) increased expressively from 1.8 (control) to 6.99 and 11.5% at 10 and 15 µM, respectively after 48 h treatment (Figure 3). Hence, chrysin significantly induced apoptosis and the percentage of early and late apoptotic cells is increased with increasing concentration of chrysin. The experiment was performed three times and data represented as mean ± SD, $p < 0.05$.

Chrysin Mediates Depolarization of Mitochondria

The induction of apoptosis is linked with mitochondrial dysfunction, mitochondrial perforation, release of cytochrome c and decrease of $\Delta\psi m$ that are possibly early events in the self-programmed death. The effect of chrysin on mitochondrial potential was determined; treated and untreated HeLa cells were stained with TMRE dye for 30 min, and then, washed with the assay buffer and observed under fluorescent microscope. In the normal cells, the dye concentrated in the mitochondrial matrix forming red fluorescent aggregates, whereas in chrysin treated cells there was a sharp decrease in fluorescent intensity with increase in chrysin concentration. The fluorescent intensity

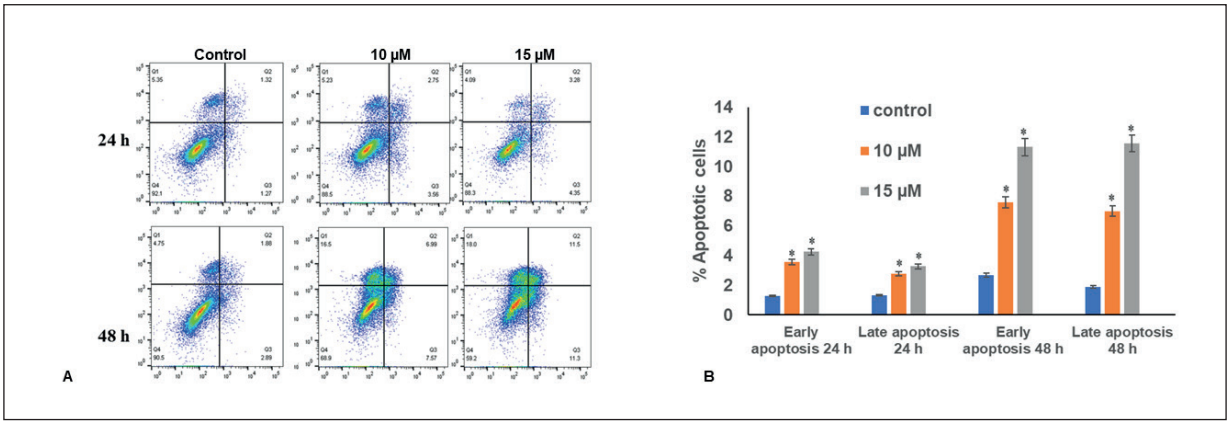

Figure 3. Assessment of apoptosis by Annexin V/PI on human cervical cancer cells (HeLa). **A,** The cells were treated with 10 and 15 µM chrysin for 48 h and apoptosis was examined by flow cytometry after Annexin V-PI double staining. **B,** Statistical representation of percentage of cell death based on the assessment of apoptosis by Annexin V/PI (*$p \leq 0.05$). The proportion of early and late apoptotic cells increased in a dose-dependent manner. Data are presented as the mean ± standard deviation of three independent experiments *$p < 0.05$.

of TMRE in the treated cells decreased to 70 and 50% of the control at 10 and 15 µM treatment of chrysin for 48 h, respectively. Results are expressed as the mean ± standard deviation of three separate experiments. The images were captured by using inverted fluorescent microscope at 40 X magnification (Figure 4).

Chrysin Modulates Cell Cycle Regulatory Genes Expression

Chrysin treatment at 10 and 15 µM for 48 h remarkably downregulated genes involved in G2-M stage of the cell cycle namely *CCNB1, CCNB2* and *CDK2*, which is consistent with the observed G2-M arrest, whereas *CCND1, CCND2,*

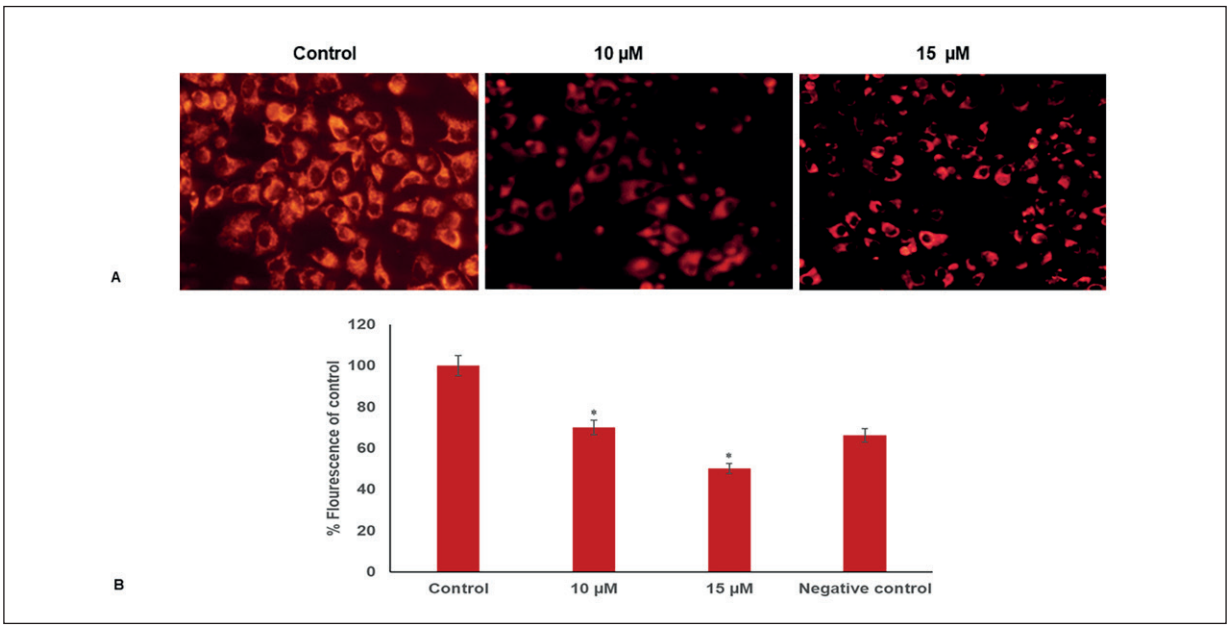

Figure 4. Mitochondrial membrane potential was determined using TMRE membrane potential assay kit. High intensity red fluorescence represented high mitochondrial membrane potential and decrease in intensity with increasing concentrations of chrysin treatment was observed. **A**, Control Cells were treated with vehicle only. Cells were preincubated with 10 µM and 15 µM chrysin for 48 h followed by TMRE staining. **B,** Plot showing decrease in fluorescence with increase in concentration of chrysin in comparison to vehicle control (*$p \leq 0.05$).

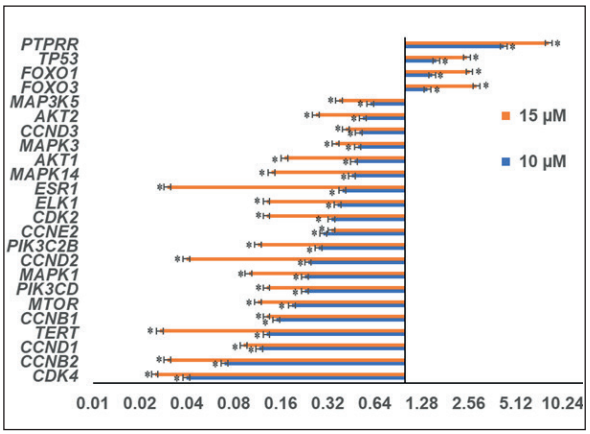

Figure 5. Expression analysis of cell cycle regulatory genes and AKT and MAPK pathways genes after treatment with 10 and 15 µM of chrysin for 48 h as compared to the untreated controls. The data are represented as mean ± SD from three independent experiments. (*p<0.05).

CCND3, CDK4 and *TERT* expressions were also downregulated. The genes, *CCND2* and *CDK4* are substrates for *SMAD3* and *SMAD4*, together they are the regulators of WNT pathway. *hTERT* gene is the most important regulator of telomerase activity and highly expressed in cancer cells, its downregulation inhibits replicative property of HeLa cells as inhibition triggers the shortening of the telomere, hence leads to cell death by apoptosis (Figure 5). [RQ ≥ 1.5 for upregulation and RQ ≤ 0.5 for downregulation].

Chrysin Triggers Apoptosis by Extrinsic and Intrinsic Pathway

The apoptotic effect of chrysin was further verified by checking the expression analysis of various genes involved in apoptosis process by using TaqMan® Gene expression assays. The results indicated upregulation of various pro-apoptotic genes by many folds, whereas downregulation of anti-apoptotic genes was observed at the given concentrations of chrysin. The expression of the genes of *BCL2* family genes like *BCL10, BCL2L14, BAD, BAX, BOK* and *BID* increased significantly and the expression of *BCL2, MCL1, XIAP* and *NAIP* was also decreased implicitly (Figure 6A). Also, most prominently chrysin treatment upregulated *APAF1, DAPK1, NOD1, NOD2, Diablo and REL* (Figure 6B) expression

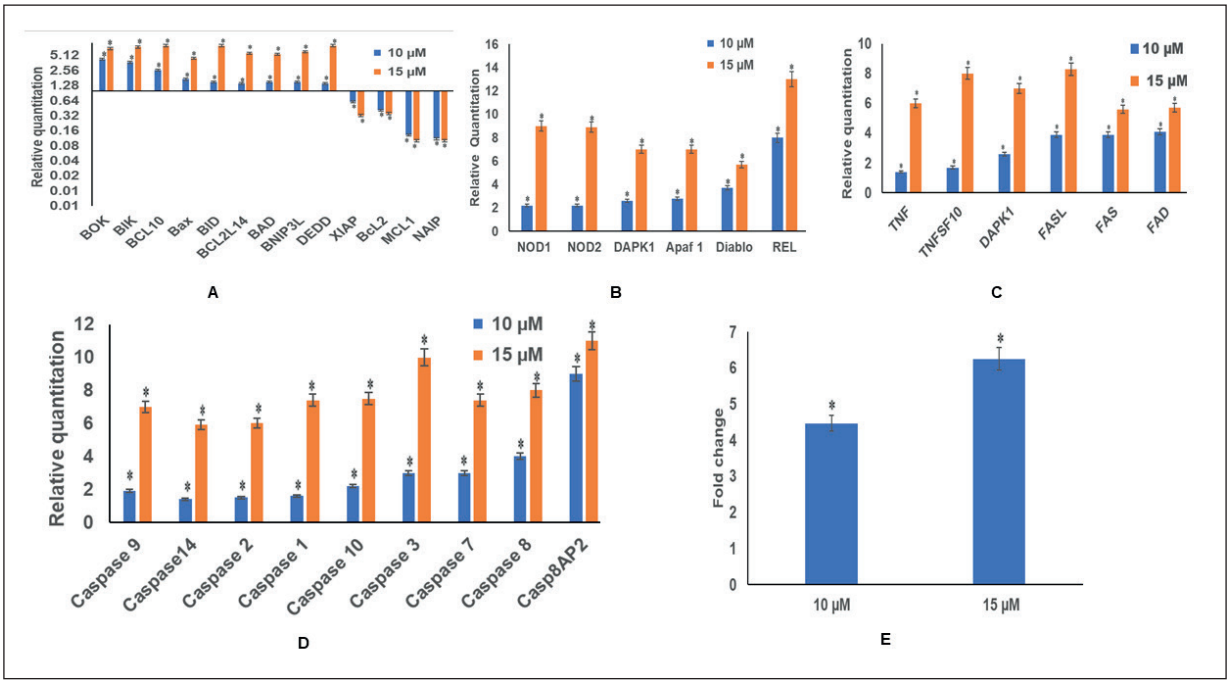

Figure 6. A, Expression analysis of the genes involved in apoptosis. Relative Quantitation values of BCL family genes after treatment of HeLa cells with 10 and 15 µM chrysin. **B,** Relative quantitation of intrinsic apoptosis related genes. **C,** Relative quantitation of receptors and ligands related to extrinsic pathway of apoptosis. **D,** Relative quantitation of the caspases involved in extrinsic and intrinsic pathway of apoptosis **E,** Caspase 3 Activity assay of chrysin treated HeLa cells showed many folds increase in caspase activity as compared to untreated controls. Caspase activity increased 4 and 6 folds after the treatment of HeLa cells with 10 and 15 µM of chrysin for 48 h. The data are represented as mean ± SD (error bars) from at least three independent experiments (*p<0.05).

at transcript level, as well as induced expression of caspase 1, caspase 2, caspase 3, caspase 7, caspase 9, caspase 8, caspase 8AP2, caspase 10 and caspase 14 (Figure 6D). The expression of FASL, TNF and their receptors FAS, FADD, TNF, TNFS10 (Figure 6C) increased significantly after chrysin treatment. The transcript level of BNIP3L increased after chrysin treatment. Likewise, P53, FOXO1 and FOXO3 were also upregulated (Figure 5). [RQ ≥ 1.5 for upregulation and RQ ≤ 0.5 for downregulation].

Chrysin Modifies Protein Expression of Pro- and Anti-Apoptotic Proteins

Proteome profiler array was used to check the expression of apoptosis and cell cycle-based proteins. A dose-dependent modulation of various proteins involved in the cell cycle and apoptosis, which is consistent with the mRNA expression was observed. An upregulation of TRAILR1/DR4, TRAIL/DR5, BAX, BAD, cleaved caspase 3, cytochrome, FAS, HTRA2/omi, SMAC/Diablo, phosphor-P53 (s46), phosphoP-53 (s-15), phospho-RAD17 (s635) was observed, on the contrary, a notable decrease of XIAP, CIAP1, CIAP2, BCLX, Clusterin, Pon2, HSP70, Claspin, HIF-1α, HO-2/HMOX2, HER 1 and HER3 (Figure 7) was found after chrysin treatment in comparison to untreated controls.

Chrysin Enhances Activity of Caspase 3

The stimulation of *caspase-3* after chrysin treatment was examined by colorimetric assay using exact chromophores, DEVDpNA (definite substrate of caspase-3). Chrysin treatment induced the activation of caspase-3 in HeLa cells in a dose-dependent manner. It was found that chrysin treated cells (10 and 15 µm) for 48 h displayed increase in caspase-3 activity by 4 and 6 folds compared to the control, respectively (Figure 6E). The experiment was performed three times and the data represented as mean ± SD (p<0.05).

Chrysin Inhibits AKT Pathway

The activated Akt phosphorylates BAD and pro-caspase-9 thereby inhibits heterodimerization with Bcl-2 at mitochondrial membrane, whereas dephosphorylation of BAD activates the interaction between them and leads to apoptosis. Chrysin at 10 and 15 µM inhibited PRAS 40 (P-Ther246), GSK3a (P-ser21), PTEN (P-ser380), BAD (p-ser112), Mtor (P-ser2448), ERK1 (p-T202/Y204), ERK2 (P-Y185/Y187), AKT (P-ser473), whereas P27 (P-Thr198), AMP-Ka (P-thr172) and P53 (P-ser241) were found to be upregulated in a dose dependent manner and GSK3b (P-ser9) showed just a marginal increase. Some others like P70S6k (P-Thr421/ser424, 4E-BP1 (P-Thr36), RISK2 (P-ser386) and PDK1 (P-ser241) showed only a marginal decline

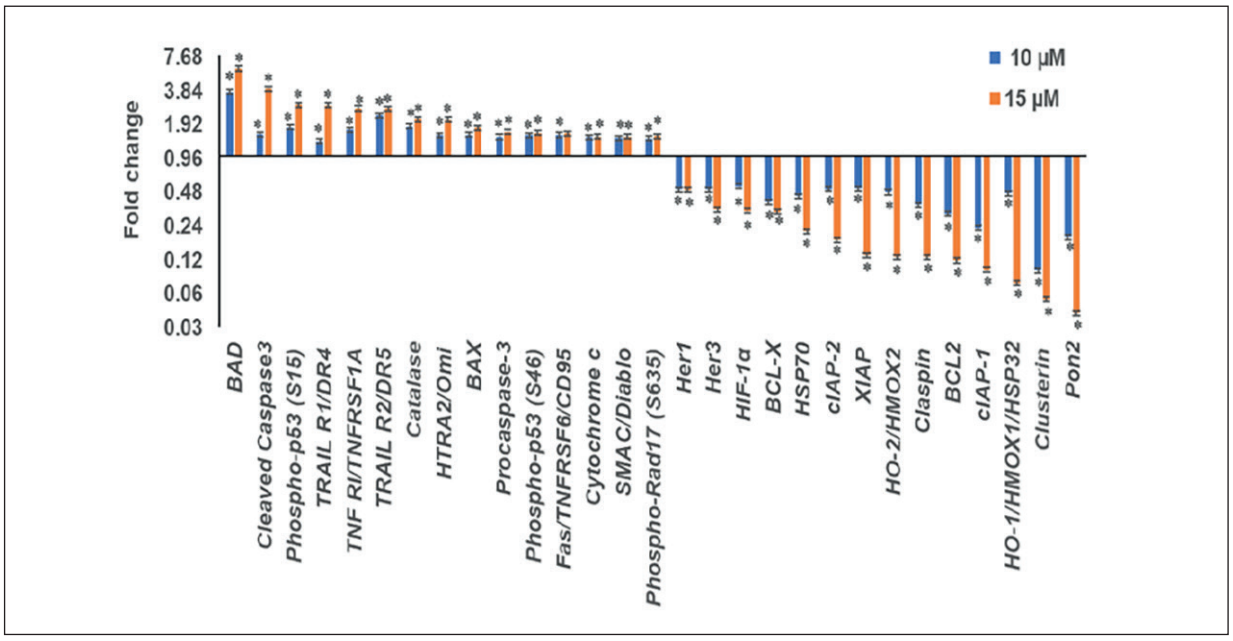

Figure 7. Protein expression analysis of various apoptotic genes. Figure shows the relative expression of these genes as compared to untreated control. The data are represented as mean ± SD of three independent experiments (*p<0.05).

after chrysin treatment (Figure 8). The expression of the proteins was normalized with the reference spots of each blot and the fold change was calculated by the formula: Fold change = Normalized blot intensity treated/Normalized blot intensity untreated control.

Chrysin Suppresses MAPK and PI3K Pathway

Chrysin (10 and 15 μM) inhibits different signaling pathways in HeLa cells at 48 h. After chrysin treatment, there was a decrease in *AKT1, AKT2, PIK3C2B, PIK3C2D, MTOR, MAPK1, MAPK14, MAP3K5, ESR1* and *ELK1* gene expression along with an upsurge in PTPRR, which is an antagonist of MAPK pathway, which advocates a direct inhibition of MAPK and PI3K/MTOR pathways (Figure 5).

Discussion

Cervical cancer is the most prevalent cancer type especially in middle age women and its incidence has been reported to increase even in young females[29]. The conventional modes of its treatment are surgery followed by chemotherapy or radiotherapy, however, these methods of treatment are associated with various side effects[30,31]. Hence, the focus of the current research is shifted towards cancer treatment strategies that are more specific, less toxic and sustainable, one such approach is phytochemicals mediated chemoprevention plan, which includes flavonoids that have been found to be anti-cancerous, anti-proliferative, anti-inflammatory, antioxidant and cell cycle modulators and can therefore be used to develop anti-cancer regimen[16,20,24-26]. In this study, it was found that chrysin inhibits cell proliferation in both, dose and time dependent manner in HeLa cells and IC_{50} was found to be 15 μM at 48 h (Figure 1A). A similar range of IC_{50} value for chrysin was reported in earlier studies, which documented that chrysin inhibited proliferation with IC_{50} of 14.2 μM at 48 h in HeLa cells, IC_{50} of 16 μM at 48 h in U937 cells, and IC_{50} of 19.5 and 9.2 μM at 48 and 72 h, respectively for MCF-7 cells[16,27]. However, in the present study chrysin did not show significant cytotoxicity on lymphocytes, similar result has been reported by Xue et al[20] on fibroblast cells. Microscopic examination of chrysin treated cells depicted rounding off and detachment of the treated cells from the surface compared to the control cells in dose and time-dependent manner (Figure 1B). Further, fluorescent microscopic analysis showed that chrysin treatment of HeLa cells induced apoptosis as the untreated HeLa cells displayed large and distinct nucleus, whereas the treated cells depicted nuclear condensation, nuclear fragmentation along with the formation of apoptotic bodies (Figure 2A) at 10 μM and 15 μM concentration of chrysin and indicated that chrysin mediated apoptosis induction, which is consistent with the previous findings[13,14,32]. Further DNA fragmentation as detected by DNA laddering assay suggests the activation of apoptosis following the exposure of HeLa cells to 10 and 15 μM chrysin for 48 h (Figure 2B). Earlier studies have reported that flavonoids triggered DNA fragmentation in different cell lines, such as leukemia (U937 cells), human cervical cancer (HeLa), prostate esophageal squamous carcinoma (KYSE-510), malignant glioma and breast carcinoma[13,14,16,20,21,26,32,33]. Additionally, chrysin treatment at 10 and 15 μM for 48 h followed by Annexin/PI staining showed that chrysin induced early and late apoptotic effects (Figure 3) and also instigated the depolarization of mitochondrial membrane potential in HeLa cells at the above concentrations for 48 h (Figure 4); similar results have been reported in the past by other research group[22].

The failure of cell cycle control is a feature of cancer cells thus cell cycle regulation is a major event to halt cell proliferation. In order to repair the damaged DNA, G2/M checkpoint halts the cell cycle and signifies a probable target for can-

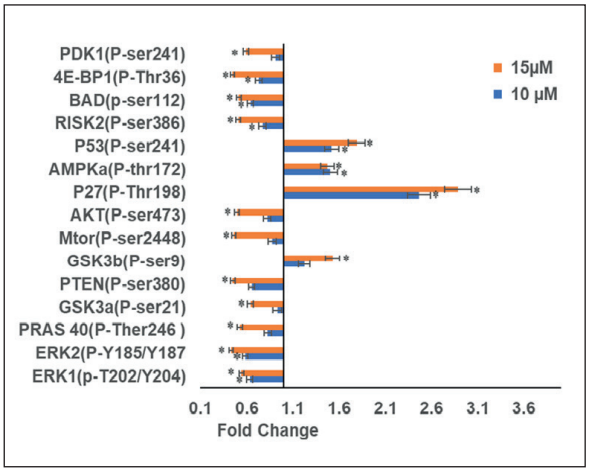

Figure 8. Protein expression analysis of phosphorylated proteins of AKT pathway proteins; figure shows the fold changes after 10 and 15 μM chrysin treatment in comparison to vehicle treated cells. Data are presented as the mean ± SD of three independent experiments (*p<0.05).

cer therapy. It is well established that flavonoids initiate cell death or cell cycle arrest at G2/M phase or G1/S[15,26]. This study showed chrysin induced G2/M arrest at different concentrations with the proportion of cells increasing in a concentration dependent manner, which falls in line with various studies[18,26,30,34]. In the present study we found 28 and 45% of cell proportion at G2/M at 10 and 15 µM chrysin treatment, respectively after 48 h (Figure 2C). To further evaluate the anti-proliferative and cell cycle arrest mediated by chrysin, it was determined that verified concentrations of chrysin downregulated the expression of different cell cycle related genes like *CCNB1*, *CCNB2, CCND1, CCND2, CCND3, CDK4, CDK2*, *TERT*. Interestingly *CCNB1, CCNB2* and *CDK2* (Figure 5) are involved in transition from G2 to M phase of the cell cycle. The upregulation of Phospho-Rad17 (S635) is also linked to G2/M arrest discussed later in this paper. Further *TERT,* which is overexpressed in various cancers is accountable for increasing the length of telomere[35]. Interestingly, in this study, chrysin at 10 and 15 µM dose promoted significant downregulation in *TERT* transcript expression, thus explaining the anti-replicative property of chrysin (Figure 5).

Apoptosis is an intentional cell death that can occur by either external stimuli (extrinsic pathway/death receptor mediated) or internal signals (intrinsic/mitochondrial pathway)[13]. Several reports[27,29,33] have indicated that chrysin sensitizes apoptosis induced cell death by tumor necrosis factor (TNF) or TNF-related apoptosis-inducing ligand (TRAIL) in HepG2, A549, HeLa, HCT cancer cells. In this study, it was observed that ligands and receptors of extrinsic pathways like *FAS, FASL* and *TNFSF10, TNF* showed upregulation (Figure 6C) at the transcript level, which was supported by the protein level expressions, wherein TRAILR2/DR5, TRAILR1/DR4, TNFR1/TNFRSF1A, Fas/TNFRSF6/CD95 were upregulated after chrysin treatment (Figure 7). Further, chrysin enhanced the expression of p53 at both transcript and protein level. Likewise, *caspase 8* was also induced at transcript level. DR5 activation recruits Fas associated death domain and activates pro-caspase-8 to form DISC (Death Inducing Signaling Complex) leading to extrinsic pathway of apoptosis.

Various scholars[36] have shown that the positive correlation between flavone treatment and initiation of self-programmed death through stimulation of cysteine-aspartic proteases and release of cytochrome c through a caspase 3 dependent mechanism. Caspases are a family of protease enzymes playing important protagonists in programmed cell death. Zhang et al[13] reported that chrysin brought about apoptosis in hepatocellular carcinoma (HCC) cells by modulating p53 dependent Bcl-2/caspase-9 pathway, similar results were obtained in this study and it was found that chrysin treatment caused upregulation of caspases, i.e., caspase 1, caspase 2, caspase 3, caspase 7, caspase 8 and caspase 9 at transcript level (Figure 6 D). Concurrently, BAX, BIK, BOK, BID BAD, BCL10, Diablo, Apaf1, NOD1 and NOD2 also showed significant upregulation, whereas transcriptional decrease of Bcl2, Mcl1, NAIP and XIAP was detected after the treatment of HeLa cells with 10 and 15 µM of chrysin (Figure 6A and B). These results resemble with reports from other studies of chrysin using different cell lines[13,32,34].

Caspase 3 is the most important executioner caspase and is activated by both intrinsic and extrinsic pathways and mediates DNA fragmentation and PARP cleavage[37]. Overall, chrysin mediated apoptosis, which is normally executed by caspase 3, was evident in this study. Chrysin depicted a significant increase of caspase 3 activity in a concentration dependent manner with 4 and 6 folds increase after 10 and 15 µM chrysin treatment of HeLa cells, respectively, for 48 h (Figure 6E). Earlier studies have also reported the initiation of apoptosis by chrysin through stimulation of caspases and modulation of other apoptotic proteins in different cell lines[13-16,27,32,37].

These results were further endorsed by checking the expression of the other key proteins involved in apoptosis process and in this study it was found that chrysin treatment leads to upregulation of pro-apoptotic proteins such as BAD, BAX, cytochrome c, cleaved caspase 3, procaspase 3, SMAC/Diablo, Phospho-p53 (S46), Phospho-Rad17 (S635) and HTRA2/Omi and downregulation of anti-apoptotic proteins like HO-1/HMOX1/HSP32, HIF-1α, cIAP-2, HO-2/HMOX2, BCL-X, Claspin, HSP70, cIAP-1, Pon2, BCL2 and Clusterin (Figure 7). Seydi et al[38] have demonstrated that flavonoids induced cytotoxicity in HepG2 cells by depolarizing the mitochondria and increasing the protein expression of BAX, caspase 3, caspase 9 and decreasing the expression of Bcl2. In the present study, it was also found that HeLa cells treated with 10 and 15 µm chrysin for 48 h exhibited decrease in mitochondrial potential and it was well correlated with the increased expression of the pro-apoptotic genes

and inhibition of anti-apoptotic genes at transcript and protein levels. Hence, depolarization of mitochondria, increased expression of Caspases, BAX, BAD, Smac/Diablo, release of cytochrome c and reduced expression of Bcl2, MCL1, XIAP, cIAPs are indicative of apoptosis induction *via* intrinsic mode.

In this study, it is well recognized that chrysin induces apoptosis by both extrinsic and intrinsic pathways, which is in accordance with the various reports on gastrointestinal cancer, breast cancer, A549 and cervical cancer cells (HeLa) and hepatocellular and antiproliferative potential were supported by its ability to modulate AKT/MTOR, WNT and MAPK pathways[5,16,22,24,26,39]. Chrysin treatment transcriptionally decreased *AKT1, AKT2, mTOR* and *PIK3CD* and further was verified by validating the effect of chrysin on phosphorylated proteins of AKT pathway, whereas chrysin inhibited pro-survival phosphorylated proteins like (p-473) AKT, (p-Ser 2448) mTOR, (p-ser241) PDK1, (p-Ser112) BAD and (p-Ser 21) GSK3a, upregulated the phosphorylation of anti-survival proteins such as (p-Thr172) AMPKa, and (p-ser15) P53 (Figure 8). AKT is stimulated by the receptor tyrosine kinases and deactivates pro-apoptotic proteins and promotes cell survival by inhibiting pro-apoptotic proteins and forkhead (FoxO1/3a) transcription factors[40]. Notably, chrysin in the present study increased transcription levels of the *FOXO1/3*, tumor suppressor genes that restore the function of pro-apoptotic and cell cycle regulatory genes[41]. MAPK pathway is primarily responsible for cell proliferation; In this study chrysin treatment inhibited several members of this pathway; substantial reduction was noticed in *MAPK1, MAPK3*, *MAPK14* and *ELK 1* (activator of oncogene c-fos), with increase in *PTPRR*, PTPRR is an inhibitor of MAP pathway and leads to inhibition of cell proliferation[42].

Overall, it can be concluded that chrysin inhibits AKT/mTOR and MAPK pathways and thus activates apoptosis, as both are important players for phosphorylation of *BAD* (Figure 10). The activated AKT phosphorylates BAD at Ser-136, whereas upstream regulators of MAPK -that is Ras and RAF lead to phosphorylation of BAD at Ser-112[43]. P-BAD (Ser-136 and Ser-112) isoforms are downstream molecules of AKT/MTOR and MAPK pathways and are found to be overexpressed in many cancers, out of which (ser 112) P-BAD was downregulated by chrysin treatment showing the inhibition of above said pathways (Figure 5). This is the first study which is showing modulation of many AKT pathway proteins. Earlier studies have reported decreased levels of phosphorylated AKT in leukemia cells, A549, HEPG2 after chrysin treatment[26,44]. Wang et al[45] reported that Caov-3 cells treated with chrysin showed decreased expression of phosphorylated AKT, MTOR and 4EBP1 expression. Woo et al[46] stated that chrysin induced apoptosis by inhibiting AKT/MtOR and activating caspase in leukemia cells.

Conclusions

Based on the current findings it can be concluded that chrysin efficiently reduces the survival rate of cancer cells by the inactivation of AKT/MTOR/PI3K and MAPK pathway genes and promotes apoptosis by significantly modulating all the important cell cycle and apoptosis related genes (Figure 9). This is the first study that comprehensively documents the modulation of major pro-apoptotic genes and AKT/mTOR, MAPK pathways genes. Validation of AKT/mTOR pathway was further achieved by protein quantitation studies. This study supports the use of chrysin as a chemo preventive and treatment agent and we recommend pharmacokinetic investigations to be performed before chrysin can be used for therapeutic purposes.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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