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Original Article

Anti-proliferative action of chrysin in colon cancer cells and its effects on signaling pathways

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Abstract: The goal of the current study is to elucidate the anticancer potential of chrysin against a panel of colon HT-55, HCA-7 and LoVo cancer cell lines. The anti-proliferation effects were checked out using MTT and BrDu incorporation assays against all the three cell lines. The inhibition in the case of HT-55, HCA-7 and LoVo cell lines with IC_{50} values of 0.4 mM, 0.4 mM and 0.8 mM were obtained. In addition DNA synthesis were also observed with IC_{50} values of 2.5, 4.2 and 1.8 mM for HT-55, HCA-7 and LoVo cell lines, respectively. Based on the efficient activity in anti-proliferation methods, HT-55 cells were chosen to carry out further experimental studies. Various changes in signaling pathways of HT-55 cells were observed upon chrysin treatment. Changes in ERK1/2, p38, CREB and AKT were observed while testing HT-55 cells with chrysin. In vitro studies of chrysin in colon cancer cells has also been elucidated.

Keywords: Chrysin, MTT assay, colon cancer

Introduction

The initiation of cancer formation is a complicated, multiform process probably associated with many kinds of events involved at the cellular and morphological levels. In western countries, colorectal cancer is found to be second dominant cause of death due to cancer. It was found that only 40 to 50% of people those who underwent remedial surgery are getting worse again and die of this disease. Even the advanced treatments and the well understanding of mutations participated in the growth of colorectal cancer has not been favorable but the development of screening measures and efforts are continuously proceeded to find the efficient prevention of this cancer. Literature reports showed that there is a decrease in the percentage of colorectal cancer when the patients are consuming either non-steroidal anti-inflammatory drugs which are said to be NSAIDs or aspirin etc [1-3]. This indicates that these drugs can be used as an effective agent for treating colorectal cancer but the continuous usage of these drugs leads to gastrointestinal damages which may be due to the hindrance of gastric prostaglandin secretion that may be essential for regulating the integrity of gastric mucosa.

It is well known that flavonoids strongly exhibits antioxidant properties and prevent the development of cancer. Chrysin also known as 5,7-dihydroxyflavone is found to occur in many kinds of plants, propolis and in honey. Chrysin, a naturally occurring common flavonoid belongs to apigenin family. Accumulation of high level contents of chrysin flavonoid are identified in propolis and in honey [4, 5]. At present, chrysin is accessible in the form of capsules as a supplements for dietary utilized for raising lean body mass. It can also acts as a testosterone uplifting agent and has been reported to have antioxidant, anti-inflammatory, anticarcinogenic activities [6, 7]. Chrysin was recently reported as a strong inhibiting agent against enzyme-aromatase [8]. It has been found to exhibit cancer preventing ability via suppressing malignant cell multiplication through downregulation in Hela cells. Its inhibitor effect was proved against latent infection using immuno-deficiency virus activation models [9], and of altering GABAC and GABAA receptors upon binding with the benzo diazepine site present in these receptors [10].

Zhang et al showed that chrysin can suppress the cell multiplication and trigger apoptosis against HeLa cancer cells [11]. Even though the prime function of apoptosis is broadly exposed, different mechanisms seem to function in variety of cell lines. For instance, Caspase activation and AKT inactivation mediated apoptosis were studied in leukemic U937 cell lines whereas G1 arrest and apoptosis were found in C6 glioma cells and the occurred apoptosis in this case is due to the increase of p21WAF/CIP1 expression and not because of p53 expression changes [12]. Hence, the clear mechanism is not exactly proved. Also, the properties of chrysin supplement is not reported so far in an in vivo model and thus, the present study was constructed to investigate the role of chrysin against colorectal cancer.

Materials and methods

Cells and culture methods

HT-55, HCA-7, and LoVo human colon adenocarcinoma cell lines and CCD 841 CoTr human colon epithelial cell lines were ordered from American Type Culture Collection (ATCC, Menassas, VA, USA). HT-55, HCA-7 and CCD 841 CoTr cell lines were maintained in a Dulbecco's Modified Eagle's Medium (DMEM) whereas LoVo cell lines were maintained in an Eagle's Minimal Essential Medium (EMEM) in a humidified atmosphere of 95% air and 5% CO_2 at a temperature of 32°C. All these culture media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Chrysin was purchased from Sigma Aldrich (St. Louis, MO, USA) and the stock solutions of chrysin were prepared by dissolving the appropriate of substance in dimethyl sulfoxide (DMSO) for desired concentrations.

Cytotoxicity

The potential of chrysin on cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Initially, HT-55 and HCA-7 cells at a density of 3 × 10^5 cells/ml were cultured in a 96-well plates whereas LoVo cells were cultured at a density of about 2 × 10^4 cells/ml. After 24 h incubation, the medium was changed and the desired chrysin concentrations (0.01, 0.5, 1.0, 5.0 and 10.0 mM) were added and further incubated for next 48 h. After incubation, MTT reagent of about (10 mg/ml) in PBS was added and again incubated for 2 h. The obtained formazan crystals were dissolved using sodium dodecyl sulfate buffer overnight. Absorbance were measured at 570 nm wavelength using a microplate reader (Sunrise, Tecan, USA).

5-bromo-2'-deoxyuridine incorporation assay (BrdU)

The chrysin effects on synthesis of DNA within the cells were assayed using BrdU incorporation, a colorimetric immuno assay. HT-55, HCA-7 and LoVo colon cancer cell lines at a density of 4 × 10^5 cells/ml were cultured in a 96-well plates and incubated for one day. After incubation, the medium was changed and then further treated with different chrysin concentrations (0.01, 0.5, 1.0, 5.0 and 10.0 mM). After the chrysin treatment, it was allowed to incubate for another 48 h. This is followed by the addition of BrdU using standard procedures given by the manufacturer's instructions (Cell Proliferation ELISA BrdU, Sigma-Aldrich, St. Louis, USA). The absorbance were recorded at 450 nm in a microplate reader.

LDH based cytotoxicity assay

In order to assess the cytotoxicity of chrysin against colon epithelial cells, LDH based cytotoxicity assay was carried out. Normal cells were cultured at a density of 2 × 10^4 cells/ml in a 96-well plates and incubated for one day. After incubation period the medium was removed and then various chrysin concentrations (2, 4, 6, 8, and 10.0 mM) were added and further incubated for 24 h using standard procedures given by the manufacturer's instructions according to manufacturer's procedures (Pierce™ LDH Cytotoxicity Assay Kit, Thermo Scientific, USA). The reaction was terminated using 1 M hydrochloric acid, and the absorbance at 490 nm were measured using microplate reader.

Apoptosis and necrosis

In this method, fluorescent Hoechst 33342 and propidium iodide (PI) staining were used to identify the apoptotic and necrotic cells, respectively. Cells were cultured on eight well chamber slides and then treated it with different chrysin concentrations (1, 5, 10 mM) and

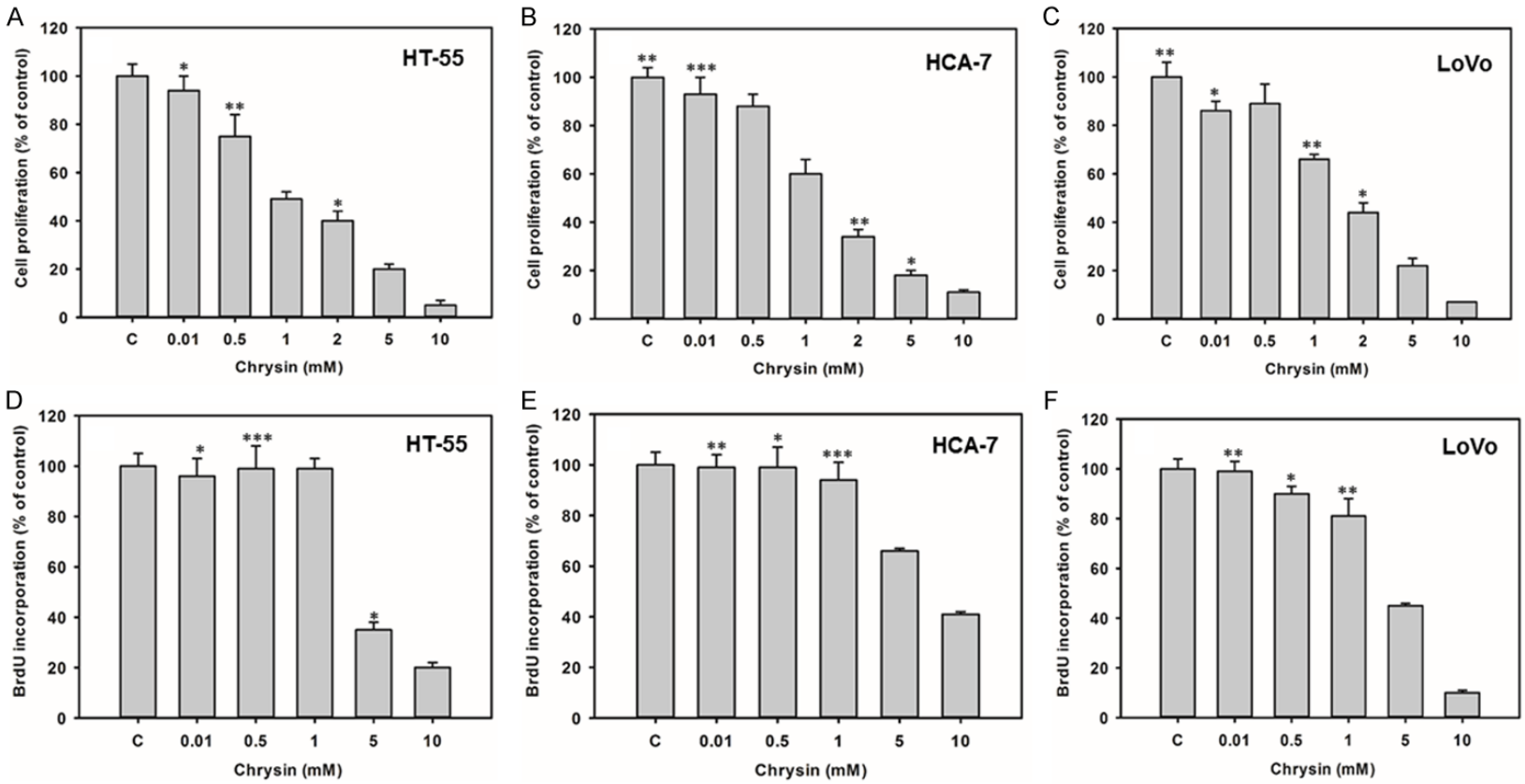

Figure 1. Inhibition of colon cancer cells proliferation by chrysin treatment. MTT assay of HT-55 (A), HCA-7 (B) and LoVo (C) colon cancer cells treated with various concentration of chrysin for 96 h. (B) BrdU test for HT-55 (D), HCA-7 (E) and LoVo (F) colon cancer cells treated with various concentration of chrysin for 48 h. Results are represented as mean (% of control) 7 S.E.M., N ¼ 6; *P<0.05, ***P<0.001.

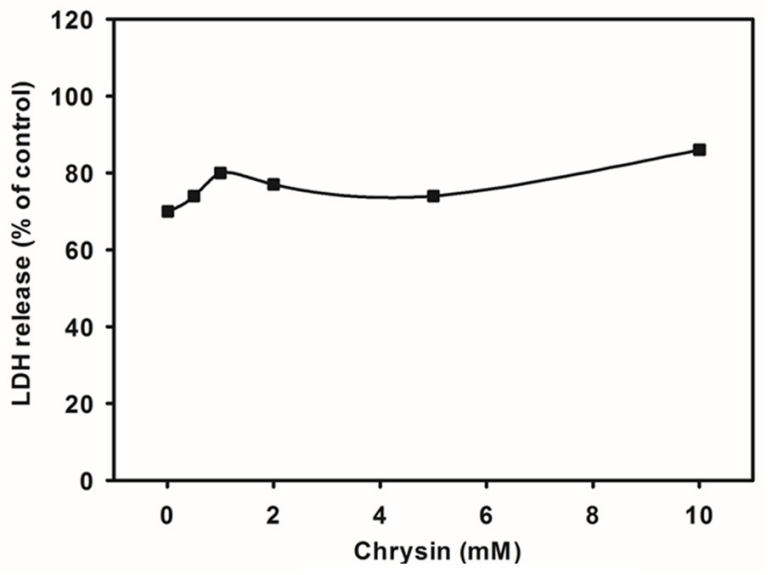

Figure 2. LDH release assay. Chrysin treated with CCD841 CoTr normal colon cells with different concentrations (2, 4, 6, 8 and 10 mM). Data are given by means of % of control considering *P<0.05 and ***P<0.001.

incubated for about 24 or 48 h. After incubation, the culture media was removed and added PBS containing Hoechst (0.1 µg/ml) and propidium iodide (0.2 µg/ml) and further incubated for another 15 min. Then the images of cells were captured using LAS X Widefield Systems (Leica Microsystems, Germany) at 400 × magnification.

Immunoblotting

In this experiment, chrysin concentrations (1 and 5 mM) were used to analyze the western blot and it was treated with HT-55 cells. After the treatment, ice-cold PBS was used for washing followed by harvesting and subjected to lysis using lysis buffer containing tergitol (1%), SDS (0.1%), sodium deoxycholate (0.1%), NaF (20 mM), EDTA and EGTA (1 mM), Na_3VO_4 (1 mM), DTT (0.5 mM), PMSF (1 mM) and protease inhibitor for 1 h in PBS and then centrifuged at 4°C. Then the supernatants were collected and solubilized using laemmli buffer containing Tris/HCl (0.5 M), SDS (10%), glycerol (30%), beta mercaptoethanol (5%) and bromophenol blue (0.012%). About 10-12% SDS-polyacrylamide gel was used to separate 20 µg of protein and then transferred to polyvinylidene difluoride membrane membrane (PVDF). Then the blots were kept overnight with mentioned primary antibodies, antibodies against phospho-ERK, phospho-p38, phospho-CREB, phospho-AKT and β-actin, which were detected using horsereadish peroxidase conjugated secondary antibodies. The chemiluminescence detection system software (Pierce, Rockford, IL, USA). ImageJ was used to visualize the proteins.

RNA isolation and RT-PCR

HT-55 cells were treated with chrysin for 24 h and then total RNA was extracted and then reverse transcribed with RNA (2 µg) using QuantiTect Reverse Transcription Kit (Qiagen, USA) as per the manufacturer's instructions. TaqMan Gene Expression Assays were used to determine the RT PCR analyses according to the manufacturer's instructions. It was incubated on 96-well plates for 30 s at 95°C and is followed by 45 cycles for 3 s at same temperature and then at 60°C for about 30 s using MJ Mini-Opticon RT-PCR (Bio-Rad, USA). Efficiency method was used to calculate the relative expression which was normalized with β-actin (CFX Manager Software for MiniOpticon, Bio-Rad, USA).

Statistical analysis

All the data were obtained from 3 independent experimental results and the values are expressed by means of ± standard errors. The significance of the differences was analyzed by ANOVA tests and column statistics and *P value <0.05, **P value <0.01 or ***P value <0.001 were used for comparisons. Tukey's honestly significant difference (HSD) was used as a post hoc test method after ANOVA.

Results

Cytotoxicity and proliferation studies

In order to test the ability of chrysin on colon cancer cells viability, MTT assay carried out. The cell lines were incubated with chrysin for 96 h and the cell viability was observed. The IC_{50} values were found to be 0.4 mM, 0.4 mM

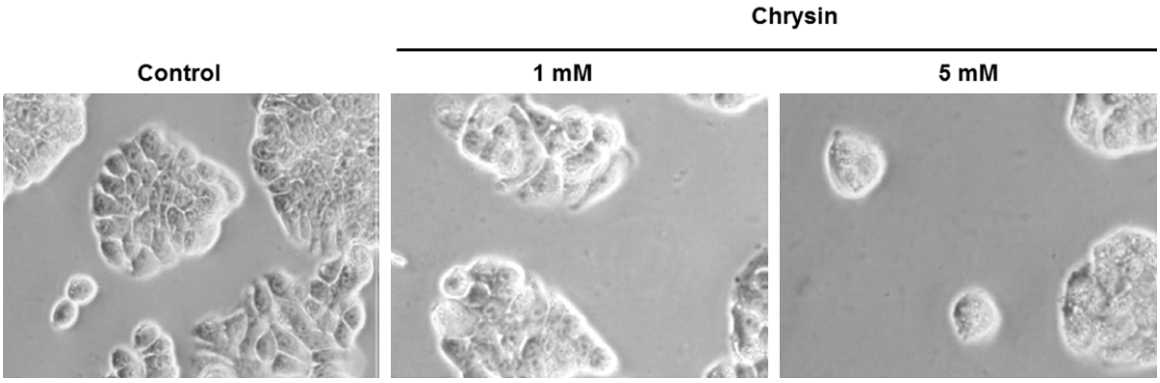

Figure 3. Observation of morphological changes. Treatment of chrysin with HT-55 cells for 48 h at a mentioned concentration. Cell images were captured at a magnification of 400 ×.

and 0.8 mM for HT-55, HCA-7 and LoVo cells, respectively (**Figure 1**). In order to assess the chrysin influence on cell proliferation, BrdU incorporation assay was performed. The results showed that upon chrysin treatment in a dose-dependent manner, it was found that the DNA synthesis within the cells was inhibited and the IC_{50} values were found to be 2.5, 4.2 and 1.8 mM for HT-55, HCA-7 and LoVo cells, respectively. From the anti-proliferative results obtained from MTT and BrdU incorporation assays, it was revealed that HT-55 showed high antiproliferative ability and hence, HT-55 cells were selected for further experiments.

LDH measurement studies

Figure 2 clearly indicates that based on the release of LDH measurement, it was found that CCD 841 CoTr normal epithelial colon cells incubated with chrysin did not induce any cytotoxicity in a tested concentration.

Morphological studies

In this study, HT-55 colon cancer cells were treated with chrysin and incubated for 48 h. The results showed that there is a reduction in the cell count after 48 h incubation and impaired the colony formation as shown in **Figure 3**. In addition, changes in cells shape were also observed.

Hoechst and propidium-Iodide staining

In order to identify and distinguish between the apoptotic and necrotic cells, Hoechst 33342 and propidium iodide staining were done. Upon treatment of chrysin with HT-55 cells showed that no increase neither in apoptosis nor in necrosis were observed even after incubated for 24 or 48 h (**Figure 4**).

Changes in protein expression

To assess the changes in protein expression which can inhibit the cell proliferation, chrysin was treated with HT-55 cells for about 24 or 48 h and then evaluated the phosphorylation and transcription factors. The results were varied upon different time periods and concentrations (**Figure 5**). When treated with 5 mM concentration of chrysin for 24 h or 48 h, significant inhibition of ERK1/2 kinase phosphorylation was observed but when exposed to 1 mM chrysin concentration for 24 h, a slight increase in p-ERK1/2 level was observed. However, the ERK1/2 kinase strong inhibition did not relate with the decreased p-CREB protein level that was elevated upon treatment. In addition, p38 phosphorylation kinase was decreased whereas AKT kinase phosphorylation status was increased when the cells were treated with mentioned concentrations of chrysin.

Changes in CREB expression

In order to find out the CREB expression changes, RT-PCR analyses of HT-55 cells treated with chrysin (1 mM and 2.5 mM) were performed. It was observed that there is a raise in the transcript factor in chrysin treated HT-55 cells when compared to that of non-treated cells (**Figure 6**).

Discussion

In previous studies, the anticancer potential of chrysin were demonstrated. It was reported

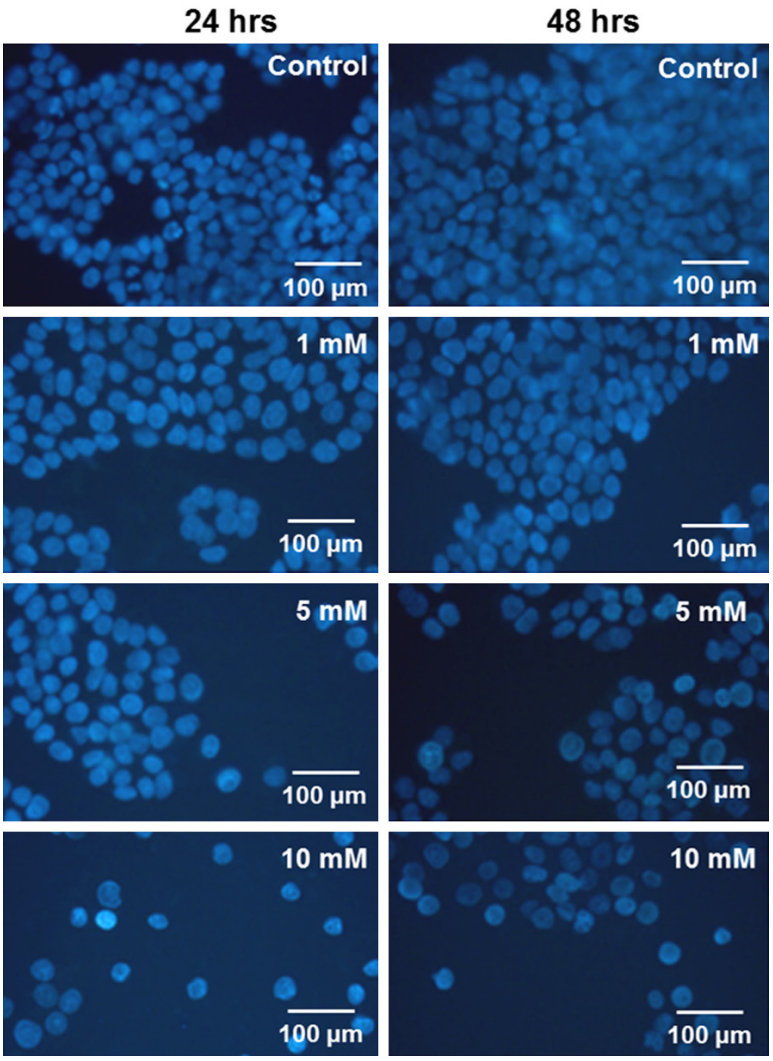

Figure 4. Hoechst and propidium iodide staining. Treatment of chrysin with HT-55 cells at a mentioned concentration; Left side panel (24 h) and right side panel (48 h). Images were captured at 400 × magnification.

that the two essential roles for apoptosis regulation and the cell cycle such as trypsin-like and chymotrypsin-like proteasomes were inhibited by chrysin [13]. The panel of HL-60, L1210 leukemia cancer cells [14-16], KYSE-510 oesophageal squamous cancer cells [17], Hela cervical cancer cells [18], OE33 oesophageal adenocarcinoma cells [19] were found to induce apoptosis and cell cycle arrest upon chrysin treatment. In the current study, an attempt was made to further explore the anticancer properties of chrysin against colon cancer cell lines.

Firstly, the dose dependent reduction behavior in the metabolic rate was found out in all the three selected colon cancer cells upon treatment with chrysin. The results of MTT assay showed that chrysin inhibited HT-55, HCA-7 and LoVo cancer cells with the IC_{50} values 0.4 mM, 0.4 mM and 0.8 mM respectively. In addition, BrdU incorporation assay was also studied to reveal the growth inhibition property was also associated with the impaired DNA synthesis within the cells upon drug treatment. Also, the toxicity of the drug was tested by treating it with normal epithelial colon cells and the results indicated that the chrysin treatment in inhibitory concentrations was non-toxic.

From the results of anti-proliferation studies, it was revealed that HT-55 cells possess great anti-proliferative potential and hence, HT-55 cells were selected to carry out the further experiments in order to explore the mechanisms involved. HT-55 cells were stained with Hoechst and propidium iodide to assess whether the cell multiplication inhibition observed after chrysin treatment causes cell death. However, the obtained results clearly revealed that neither apoptotic nor necrotic cells were increased upon chrysin treatment and this suggests that the mechanism involved is not the cell death in the anti-proliferation activities.

To detect the exact molecular mechanism involved, signaling pathways regulating the growth and proliferation processes were checked in HT-55 cells. It is known that there are several signaling pathways exists for the transmission of proliferative signals which includes mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase/AKT (PI3K/AKT), etc. One of the key factor maintaining the cell proliferation, differentiation, and gene expressions involved in various systems is the ERK signaling path-

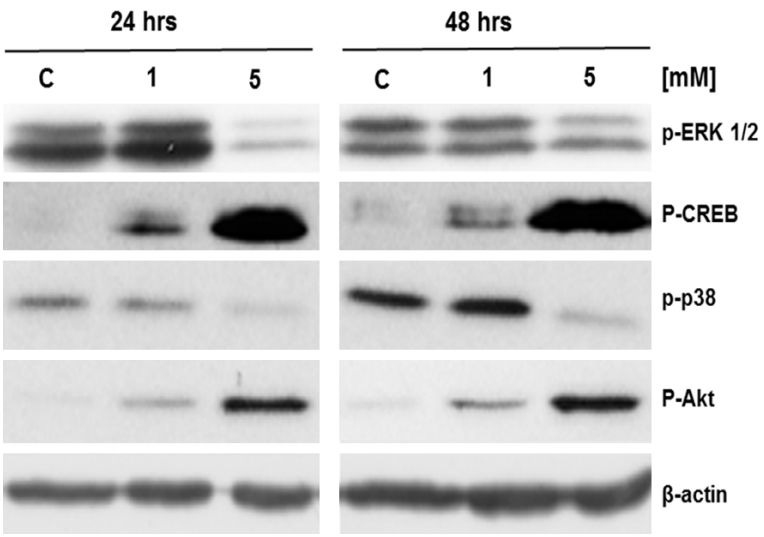

Figure 5. Western blot analysis. Treatment of HT-55 cells with chrysin at a mentioned concentrations for 24 and 48 h. β-actin acts as a control.

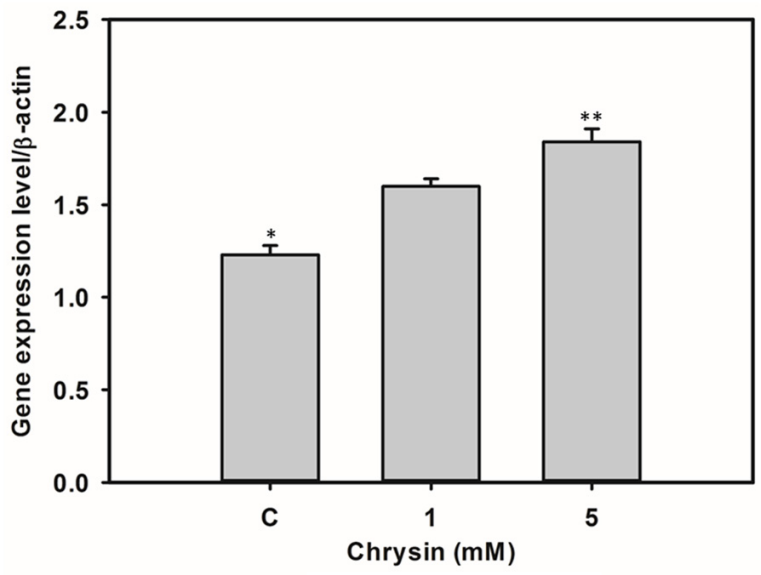

Figure 6. RT-PCR analyses. Treatment of HT-55 cells with chrysin at a mentioned concentrations for 24 h.

way operated by several mitogens maintaining many enzymes and the transcription factors. In our case, in HT-55 cells, it was found that ERK1/2 and p38 MAP kinases were markedly inhibited by chrysin but the inhibition effect was varied with different time periods and doses used. Being chrysin treatment did not show any stimulatory effect against colon cancer cells, the ability of chrysin to assist cancer cell growth cannot be eliminated. The well-accepted concept is the proliferation, apoptosis retardation and metastasis factors of colon cancer cells were affected by the AKT pathway activation and hence, involved in the colon cancer pathogenesis and its chemoresistance [20] it After 24 or 48 h chrysin treatment, AKT phosphorylation kinase was found to be increased in HT-55 colon cancer cells. Reports on clinical samples showed that enhancement of AKT activation of about 46% was found with colorectal carcinomas [21]. The transformation from polyps to carcinoma also enhance the AKT activation which is inversely related tumor suppressor protein (PTEN) known to hinder the PI3K/Akt pathway in colon cancer [22]. However, we did not find out the marked differences between HT-55 and HCA-7 cells obtained from other grade cancers (data not shown). It was proved that not only the AKT activation but also their cellular positions are responsible for determining the biological response to transduction of a particular signal by using these kinase [23, 24]. Based on these facts, except pro-apoptotic potential of chrysin in mentioned concentration, this mechanism is not related to HT-55 cells with chrysin treatment. An enhancement in ERK1/2 phosphorylation was observed after chrysin treatment and CREB overexpression was also observed along with it.

Conclusions

In conclusion, chrysin inhibited the proliferation of HT-55, HCA-7 and LoVo colon cancer cells. It was found that HT-55 colon cells influenced important signaling pathways: ERK, p38-MAPK, PI3K/AKT participated in various cellular processes involving cell proliferation

and cell death. Even though the molecular mechanisms were not completely elucidated, it can be suggested that the ERK1/2 and p38-MAPK phosphorylation inhibition may act an essential role in this process.

Disclosure of conflict of interest

None.

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