

Anticancer Properties of Chrysin on Colon Cancer Cells, *In vitro* and *In vivo* with Modulation of Caspase-3, -9, Bax and Sall4

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Background: The SALL4/Sall4 is constitutively expressed in human and mice. SALL4 mRNA could be used as a marker for the diagnosis of different types of cancers. On the other hand, chrysin has diverse biological properties.

Objectives: In the present study, the effect of the chrysin was investigated on the CT26 colon cancer *in vitro* and *in vivo*. Furthermore, the expression levels of the stem cell markers; sall4 and Bax was analyzed, as well.

Materials and Methods: The cytotoxic effects and the type of cell death induced by chrysin were evaluated using a number of biological assays. The apoptotic pathway was examined by caspase-3 and caspase-9 assay. The *in vivo* antitumor efficacy of chrysin on transplanted CT26 tumor cells in BALB/c mice was investigated. In addition, mRNA expression of sall4, Bax was analyzed with RT-PCR.

Results: MTT assay and morphological characteristics showed that chrysin exerted a cytotoxic effect on CT26 cells in a dose dependent manner with $IC_{50} = 80 \mu\text{g.mL}^{-1}$. The biological assays have indicated that chrysin administrated cytotoxicity on colon cancer cells through recruitment of the apoptosis. Caspase-3 and caspase-9 colorimetric assays, in addition to Bax expression analysis, have indicated the involvement of intrinsic apoptotic pathway in the cytotoxic effect of the chrysin. The *in vivo* assay revealed a remarkable reduction of the colon tumor volume in treated mice ($8, 10 \text{ mg.kg}^{-1}$) as compared to the untreated mice. RT-PCR elucidated that chrysin attenuated tumor volume through down regulation of the sall4 and up-regulation of the Bax.

Conclusions: It was demonstrated that chrysin accomplishes anti-cancer effect on colon cancer cells via induction of the apoptosis and attenuation of the sall4 the expression. These findings introduce chrysin as an efficient apoptosis based therapeutic agent against colon cancer.

Keywords: Apoptosis; Chrysin; Colon cancer; *In vivo*; Sall4

1. Background

Colon cancer is a world-wide health problem and the second most lethal cause of cancer-related death both males and females in the world. It afflicts more than 135,000 patients per year in the world with a incidence rate of 9.4% and kills more than 55,000 patients (1). Colorectal cancer ranks the second among the most tumor incidence death (2). The incidence of the colorectal cancer is 8.5% of all cancers and may be related to the lifestyle, obesity, drinking, smoking, as well as other factors (3).

The traditional treatment of the colon cancer

includes assisting (application or administration) chemotherapeutic agents and surgery, depending on the location of the metastatic growth, however, the current chemotherapeutic agents induce serious side effects and high toxicity, which also increase the risk of death (4). Chemotherapy is one of the most important treatments for cancer patients. However, the efficacy of the chemotherapeutic drugs is still limited in most solid tumors (5).

There are more than one thousand different drugs that were found to possess significant anticancer properties (6). However, there is still continues demand for

development of the new anticancer drugs by scientific exploration among a vast variety of synthetic, biological, and natural products (7).

Natural products have been used as an important source of anti-cancer agent, which is estimated to become the major alternative for reducing the cancer death in the 21st century (6).

Pro-apoptotic potential of the natural substances has made them candidate against cancer (8). Plants represent wide variety of phytochemicals with pharmaceutical potential such as poly phenolic, terpenoids, taxol, podophyllum peltatum, and flavonoid compounds. Flavonoids are a class of compound composed of a wide range of plant pigments that are universally present in the fruits and vegetables derived foods. To date more than 4,000 types of biologically active flavonoids have been identified (9). Chrysins are composed of a large class of polyphenolic compounds present in the diet and many herbal products, which have long been associated with a variety of important biochemical and pharmacological activities in the cancer prevention and health promotion (9, 10).

A number of studies have shown that chrysins have multiple biological activities, such as anti-inflammation, anti-oxidation and anticancer effects (10). In addition, chrysins have been reported to induce apoptosis in a panel of cancer cell lines, including Hela cervical cancer cells, U937, HL-60, and L1210 leukemia cells. This plant flavonoid was also able to inhibit tumor angiogenesis *in vivo*, which is a key step in cancer cell metastasis (11). It has been reported that chrysins possess cytotoxic effect on human colon cancer *in vitro* (9).

The SALL gene family has been found to be involved in tumorigenesis. This gene family is among the most important transcriptional regulators of the stem cells. SALL4 is the most well studied SALL gene associated with the cancer. Recent studies have shown that SALL4 is an excellent target for assessment of treatment with regard to several types of cancers including, liver, ovarian primitive germ cell, and testicular germ cell tumors (12). The association between SALL4 and OCT4, NANOG, and SOX2 suggests that SALL4 might be a candidate marker for the metastatic germ cell tumors (13). SALL4 was highly expressed in several solid tumors such as breast cancer, germ cell tumor, hepato and gastric cancers, as well as colon carcinoma (14). The role of SALL4 was the focus on carcinogenesis, prevention, treatment of tumor cell lines, and tumor tissues (15).

Our aim was to investigate the anti-cancer effect of chrysins on CT26 colon cancer cells both *in vitro* and *in*

vivo. Furthermore, we were interested in achieving more insights on the role of chrysins through evaluation of the type of cell death it induces in CT26 cells; in addition to examining the expression of sall4 in colon cancer cells under treatment with chrysins, as well.

2. Objectives

In order to have a new insight into the mechanistic cytotoxicity of chrysins, the present study was designed to evaluate whether chrysins were cytotoxic against CT26 colon cancer cells via involvement of sall4 and caspase-dependent pathway.

3. Materials and Methods

3.1. Cell Culture

CT26 cells were procured from Pasteur Institute (Tehran, Iran). The cells were cultured in RPMI-1640 medium, supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco, Scotland), supplemented with the 1% antibiotic and incubated at 37°C in a humidified atmosphere of 5% CO₂.

3.2. *In vitro* Assays

3.2.1. Cytotoxicity Assay

CT26 cells were seeded in a 96-well plate overnight and treated with various concentrations of chrysins (10, 20, 40, 80, 100, and 200 µg.mL⁻¹) for 24 h and 48 h. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (5 mg.mL⁻¹ in PBS) was then added to the cells and incubated in the dark for 4 h. Finally, DMSO (dimethyl sulfoxide) was added and optical density was measured at 560 nm with a microplate spectrophotometer (epoch from Bio Tack, USA) (16).

3.3. Morphological Assessments

CT26 cells were treated with various concentrations of chrysins (10, 20, 40, 80, 100, and 200 µg.mL⁻¹) for 48 h and morphological alteration were observed under an inverted microscope (Bio Photonic, Brazil) and photographs were taken.

3.4. Apoptosis Evaluation Using PI (Propodium Iodide) Staining

CT26 cells were treated with inhibitory concentrations of chrysins (40 and 80 µg.mL⁻¹, respectively) for 48 h. Cells were harvested and washed with PBS. Afterwards, centrifugation was carried out, and the cell pellet was incubated in 500 µL of a hypotonic buffer (500 µg.mL⁻¹ PI in 0.1% sodium citrate and 0.1%

Triton X-100) overnight at 4°C and flow cytometry was performed using a BDFACS Calibur system (Becton Dickinson, USA) (17).

3.5. Apoptosis Analysis with Annexin V-FITC and Propodium Iodide Method

For quantitative evaluation of the chrysanthemum induced apoptosis, Annexin/Propodium Iodide assay (Abcam, UK) was performed according to the manufacturer instruction. Briefly, 48 h after treatment, the CT26 cells were trypsinized and resuspended in 500 μ L of the 1X binding buffer. Thereafter, 5 μ L of Annexin-V-FITC and 5 μ L of Propodium Iodide were added and incubated at room temperature for 5 min, subsequently analysis was conducted using FACSCalibur (Becton Dickinson) flow cytometry (18).

3.6. Apoptosis Assessment by DAPI Staining

The cells (1.5×10^5) were seeded in the 6-well plate and treated with different concentrations of chrysanthemum for 2 days. The cells were washed twice with PBS, and then DAPI [2,2'-diphenyl-1-picrylhydrazyl] was added and incubated for 10 min in the dark. Finally the cells were washed twice with PBS, resuspended in 1 mL methanol, and the nuclear morphology was observed under a fluorescence microscopy (18).

3.7. Caspase-3 and Caspase-9 Activity

In order to detect the induced apoptotic pathway, caspase-9 or caspase-3 colorimetric assay (Abcam-UK) was conducted as per manufacturer's instruction. CT26 cells (2×10^6) were treated with inhibitory concentrations of chrysanthemum for 48 h. Then cells were harvested and mixed with 50 μ L lysis buffer. Subsequently, 50 μ L of 2X reaction buffer was added to each cytosolic extract. Finally, 5 μ L of DEVD-p-NA (caspase-3 substrate) or LEHD-pNA (caspase-9 substrate) were added and the absorbance of samples was read at 405 nm (19).

3.8. In vitro Assays

3.8.1. Chrysanthemum Preparation

Chrysanthemum was purchased from Sigma (Cat no. C 80105). To prepare different concentrations of the chrysanthemum (0.5, 1, 2, 4, 8, and 10 mg.kg⁻¹), chrysanthemum was dissolved in 100 μ L DMSO (Merck, Germany) and was diluted with serum before experiments.

3.8.2. Animals

Male BALB/c mice (25 ± 5 g, 6 weeks of age) were purchased from Pasteur Institute of Iran, and were fed with chow diet (Tous, Khorasan). The mice were maintained in

standard condition at 25 ± 2 °C, 12 h light/dark cycles with a relative humidity of $55 \pm 5\%$ and a 12 h time cycle.

3.9. In vivo Antitumor Assessment

To create an allograft colon carcinoma model, CT-26 mouse colon cancer cells (1.5×10^6 cells in 0.2 mL PBS) were injected subcutaneously into the right flank of BALB/c mice (n=5). After subcutaneous tumor formation (approximately 7 days) the animals were randomly designated into 5 groups of 5 mice.

The mice were divided into group I (control, administrated PBS orally duration treatment period) and treatment groups received chrysanthemum dissolved in serum including group II (0.5 mg.kg⁻¹ chrysanthemum), group III (1 mg.kg⁻¹ chrysanthemum), group IV (2 mg.kg⁻¹ chrysanthemum extract), group V (4 mg.kg⁻¹ chrysanthemum extract), group VI (8 mg.kg⁻¹ chrysanthemum), and group VII (10 mg.kg⁻¹ chrysanthemum) via oral administration once daily for 2 weeks. The mice were sacrificed at the end of 2 week and the tumors were removed, then fresh tumors immediately were kept in an RNAlater solution (Qiagen, Hilden, Germany) at -20°C until extraction.

3.10. Total RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR

The untreated and treated tumor tissues were snap frozen in the liquid nitrogen, ground mechanically to a fine powder, and were placed in the new cryogenic tubes. Then, RNA was extracted from colon tissue samples using the RNeasy kit according to manufacturer's instruction (Denazist, IRAN), cDNA was synthesized using cDNA synthesis Kit following to the application of total extracted RNA plus oligo dT (Oligo(dT))₁₂₋₁₈ Primer is suitable for use in first-strand cDNA synthesis with reverse transcriptase. The primer hybridizes to the poly(A) tail of mRNA. It is phosphorylated on the 5' end to facilitate cloning of cDNA) as primers for the synthesis of the first-strand (Parstous, IRAN) as per manufacturer's protocols.

Briefly, cDNA synthesis was performed in the presence of random hexamer or oligo dT, then incubated at 65°C for 5 min, and followed by addition RT pre-mix, incubation at 25°C for 10 min, 50°C for 60 min and 70°C for 10 min. PCR reaction was performed in a final volume of 20 μ L containing: 10 μ L Taq pre mix, 2 μ L cDNA, 2 μ L primer (forward and reverse) and water to a final volume of 20 μ L. The sequences of primers used in this study were as follows: GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) Forward 5'-AGATGGTGAAGTCGGAGTCA-3', Reverse 5'-ATCA TTGATGGCCACCACTTG-3' was used as the house-keeping gene. The forward and reverse primer for Bax

were designed as 5'-TTTGCTTCAGGGTTCATCCA-3' and 5'-CTCCATGTTACTGTCCAGTCGT-3', forward and reverse primer for *sall4* were as follow 5'-CCAAAGGCAACTAAAGGTTCAC-3' and 5'-CCGTG AAGACCAATGAGATCTC-3'. Following to the amplification, the PCR products were electrophoresed in a 2% agarose gel and were evaluated by green viewer staining (20).

3.11. Statistical Analysis

Our data were expressed as means \pm SD. Analysis of variance and significant differences were obtained by SPSS 16 software, one-way ANOVA analysis. Values of the $P<0.05$ were considered as significant.

4. Results

4.1. Effect of Chrysin on CT26 Cell Proliferation

To determine the cytotoxic effect of chrysin, colon cancer were treated with different concentrations of the chrysin ranging from 10 to 200 $\mu\text{g.mL}^{-1}$ for 24 and 48 h. (Figure 1) shows that chrysin treatment inhibited CT26 cell proliferation as a dose dependent manner. The growth rate of CT26 cells in response to 80 $\mu\text{g.mL}^{-1}$ was 48.75% and 46.68% at 24 h and 48 h, respectively. The IC_{50} for chrysin was determined to be approximately 80 $\mu\text{g.mL}^{-1}$ which reduced CT26 cells' growth up to 50% compared to the untreated cells.

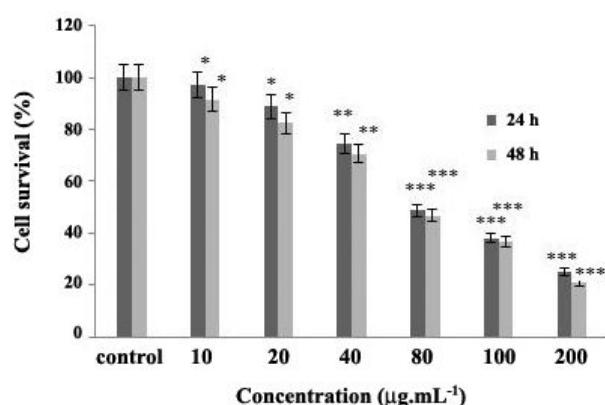


Figure 1. The inhibitory effect of chrysin on CT26 colon cancer cells viability. Cells were treated with a range of chrysin concentration (10, 20, 40, 80, 100, 200 $\mu\text{g.mL}^{-1}$) for 24 and 48 h, then MTT assay was performed. Data represents as mean \pm SD of three independent experiments. p value of * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ were considered significant

4.2. Morphological Studies by Inverted and Fluorescence Microscope

DAPI staining is a reliable apoptotic assay in chemoprevention studies and it helps to observe the apoptotic changes at DNA level. As exhibited in (Figure 2A), specific concentration of chrysin has induced apparent apoptotic morphological alterations, such as a reduction of cell volume, cell shrinkage, and apoptotic body formation in CT26 colon cancer cells

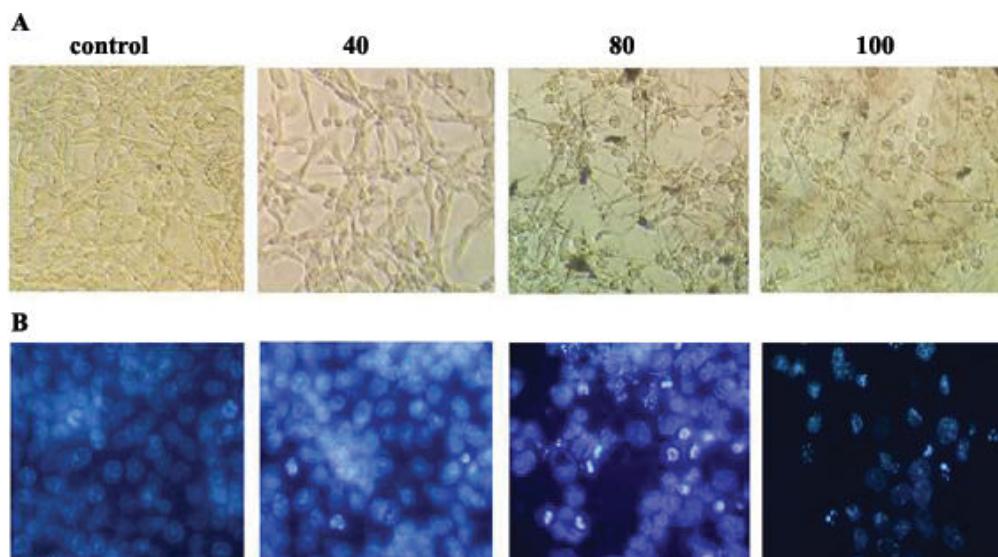


Figure 2. A: CT26 cells morphological changes following to the treatment of the cells with chrysin at different concentrations for 48 h. From the left: control cells, or cells without any treatment, and toward the right: cells that were treated with 40 $\mu\text{g.mL}^{-1}$, 80 $\mu\text{g.mL}^{-1}$, and 100 $\mu\text{g.mL}^{-1}$ of chrysin for 48 h, respectively. B: The fluorescence profiles of the corresponding CT26 cells treated with the same concentration of chrysin and stained with DAPI. Arrows indicate DNA fragmentation in the corresponding treated cells group

showing that apoptosis has been triggered under exposure with growth inhibitory concentration of the chrysin. DAPI staining showed that chrysin has induced chromatin condensation as a typical apoptotic feature in the inhibitory concentrations of chrysin (Figure 2B).

4.3. Detection of Apoptosis by PI Flow Cytometry

The apoptosis inducing effect of the chrysin was analyzed using flow cytometry according to the exclusion of PI by viable cells. As shown in the (Figure 3A), chrysin treatment has significantly increased the sub-G1 peak, which is an indicator of apoptosis induction resulted from DNA fragmentation, and endonuclease activation that is considered as a crucial anti-proliferative mechanism of chrysin treatment.

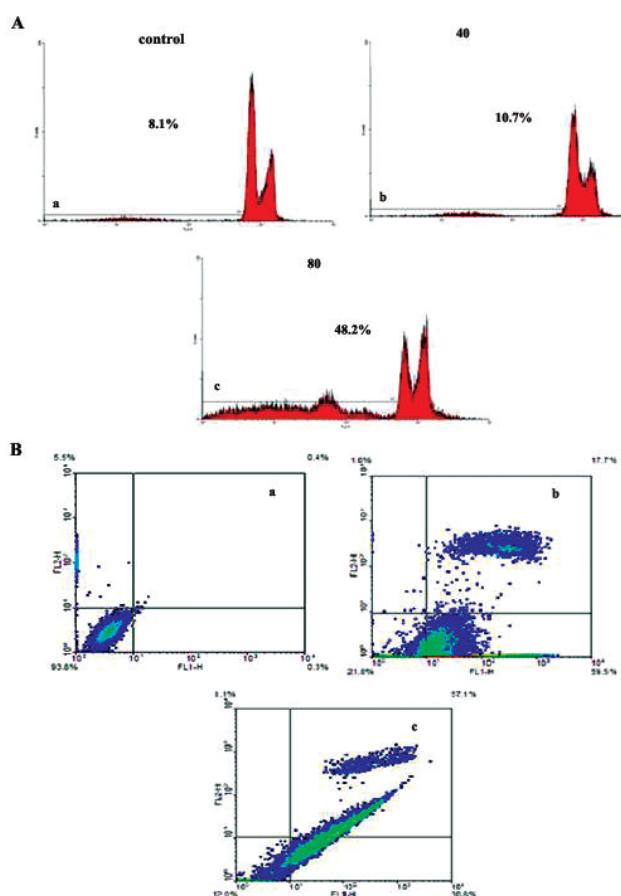


Figure 3. A: Flow cytometry histograms of apoptosis induce by PI method in CT26 cells, a: untreated cells, b: cells treated with 40 $\mu\text{g.mL}^{-1}$ chrysin, and c: treated with 80 $\mu\text{g.mL}^{-1}$ chrysin for 48 h. Apoptotic cell death can be quantified by DNA content frequency histograms (sub-G1 peak). B: Quantification of apoptosis cell death by Annexin-V/PI double-staining assay. Flow cytometry charts of CT26 cells, a: untreated or control, b: treated with 40 $\mu\text{g.mL}^{-1}$ chrysin, and c: 80 $\mu\text{g.mL}^{-1}$ chrysin, respectively

4.4. AnnexinV FITC Assay

The apoptotic alterations of the plasma membrane can be perceived through Annexin V-FITC (Fluorescein isothiocyanate) staining (18). To verify the apoptotic morphological changes as a result of treatment with chrysin (80 $\mu\text{g.mL}^{-1}$), annexin V-FITC/PI staining was performed. Flow cytometry diagrams determined that the percentage of annexin positive cells were gradually increased as the concentration of the chrysin was increased; from the live cells, to an early apoptotic, the late apoptotic, as well as necrotic cell death where it enhanced remarkably. Hence, the percentage of apoptotic cells was 0.7% in the control group, 77.2% in 80 $\mu\text{g.mL}^{-1}$ and 85.7% in 100 $\mu\text{g.mL}^{-1}$ treated groups, which is the hallmark of apoptosis induction under treatment with IC_{50} concentration of the chrysin on CT26 colon tumor cells (Figure 3B).

4.5. Determination of the Type of Apoptotic Pathway Induced by Caspase -3 and -9 Assay

To determine the type of apoptotic pathway involved, the level of caspase -3 and -9 were calculated colorimetrically. Our results elucidated that cytotoxic effect of chrysin administrated through an increase in the caspase-3 and caspase-9 activity in the treated cells in a concentration-dependent manner as compared with the control cells. Therefore, it could be concluded that chrysin exerted its cytotoxicity effects on the CT26 colon cancer cells via intrinsic apoptotic pathway (Figure 4).

4.6. Effect of Chrysin on the Tumor Volume

As was shown, chrysin suppresses tumor cell growth. We have further investigated its antitumor

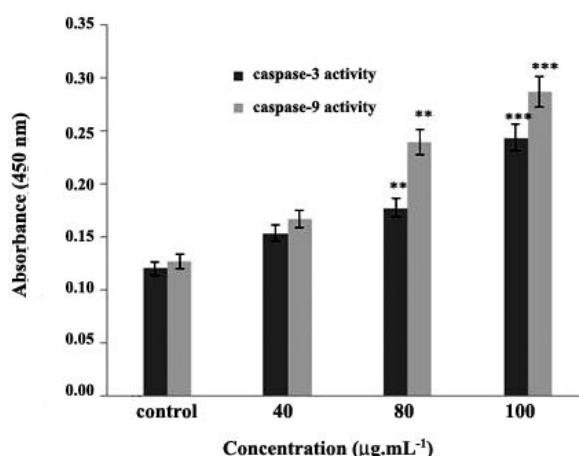


Figure 4. Caspase-3 and Caspase-9 activities in the CT26 cells treated with 40, 80, 100 $\mu\text{g.mL}^{-1}$ of chrysin. Values are expressed as the mean \pm SD of the triplicate experiment. Significant differences were compared with the control at * $p < 0.05$ by t-test

effects in a mouse model of the colon cancer. As it is shown in (Figure 5A), at the terminalexperiment, the oral administration of chrysins at 8 and 10 mg.kg⁻¹ has led to a significant regression in the tumors' volume as compared to the control group treated with the vehicle solution (i.e. PBS).

4.7. Effect of Chrysins on *Sall4* and *Bax* mRNA Expression in vivo

To investigate the involvement of *Bax* in the induction of apoptosis *in vivo* in the CT26 colon cancer cells, cells were treated with 50% inhibitory concentrations of chrysins, then expression of *Bax* family as proapoptotic factors was assessed using RT-PCR. Figure 5B shows that chrysins has up-regulated the level of *Bax* in a dose dependent manner. As well, analysis of *sall4* transcriptional levels showed that treatment with chrysins has down regulated *sall4* mRNA level dose dependently in colon cancer cells.

5. Discussion

In our study, the anticancer efficacy of the chrysins against CT26 colon cancer cells was investigated. Our observation revealed that chrysins possess moderate cytotoxic effect on CT26 cells in a concentration

dependent manner. In addition, the findings from fluorescence microscopy, Annexin-PI and caspase assays showed that the cytotoxicity of chrysins was related to the recruitment of the intrinsic pathway of apoptosis cell death.

Natural products have been used as an alternative medicine from the ancient time and plant-derived compounds were considered as the safe modalities that offer multitude benefits for preventing chronic diseases such as cancer (21, 22). Chrysins is a natural flavonoid presented in many plant extracts, including blue passion flower (*Passiflora caerulea*), honey, and propolis (23). It is a natural, biologically active compound that possesses potent anti-inflammatory, anti-cancer, and antioxidant properties (24).

The pro apoptotic effect of chrysins has been reported in breast carcinoma, cervical cancer, leukemia, lung cancer (NSCLC), and colon cancer *in vitro* (9). A study by Zhang *et al.* has demonstrated that chrysins and tetraethyl bis-phosphoric ester (one of chrysins derivatives) exhibited potential anti-cancer effects in human cervical carcinoma cells (25).

The survey carried out by Shao *et al.* has revealed that chrysins induced growth inhibition and apoptosis in the cultured lung cancer; the A549 cells, along with activation of AMP-activated protein kinase (AMPK) (26). Recently, a synergism between doxorubicin and chrysins administrated cancer cell death has been shown through chemosensitizing these cells to chemotherapy via GSH depletion in the cancer cells (27).

In this study, we have investigated the anti-cancer effect of chrysins against colon cancer cells *in vivo*. CT26 cell line was injected subcutaneously into mice for allograft colon cancer model formation and assessment of the anti-cancer efficacy of chrysins against colon cancer. The tumor volume measurement has indicated that chrysins (i.e., 4, 8, 10 mg.kg⁻¹) dose dependently reduces the tumor volume. In the present study we attempted to further address the anti-cancer potential of chrysins and the involved molecular mechanisms. The *in vivo* results confirmed that regression of mouse CT26 tumor volume under exposure to chrysins was associated with apoptotic death via up regulation of *Bax* mRNA level in concentration of 10 mg.kg⁻¹.

The anti-cancer potential of chrysins has also been reported in a variety of cell lines (28) and animals (29). Other previous studies have shown that chrysins sensitizes apoptosis induced cell death by tumor necrosis factor (TNF), or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in various human cancer cells (30), suggesting the therapeutic potential of the chrysins for treatment of human cancers. Izuta and his

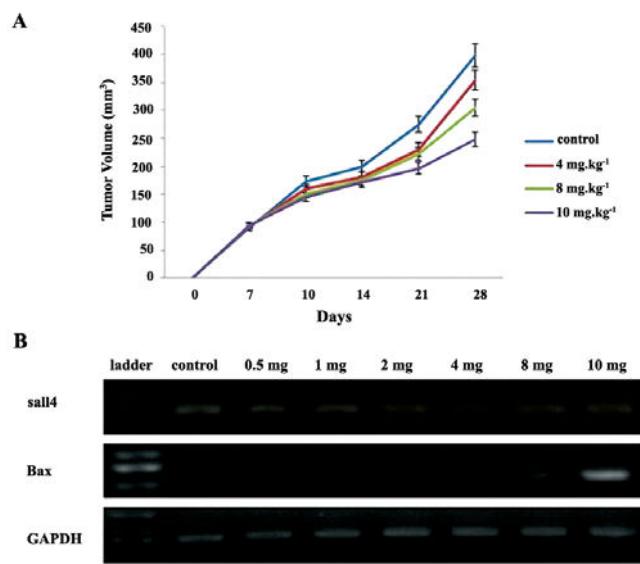


Figure 5. A: Relative variations in the tumors volume within different groups during 28 days. Tumor volumes were measured following to the exposure of mice to the 0.5, 1, 2, 4, 8, 10 mg.kg⁻¹ chrysins and were monitored every day using a digital caliper, and relative variations of the growth inhibition of tumors in different groups were measured. B) Effects of chrysins on *sall4* and *Bax* mRNA levels in CT26 cells. Transplanted BALB/c mice were treated with 0.5, 1, 2, 4, 8, and 10 mg.kg⁻¹ chrysins which demonstrated the involvement of *sall4* expression and pro apoptotic effect of chrysins on CT26 cells

colleagues have found that chrysins triggers caspase-3 activation, mitochondrial membrane depolarization and mitochondrial cytochrome c release (31).

Pal-Bhadra and coworkers have shown the effect of chrysins and its analogues on cell viability and cell cycle analysis by MTT assay and flow cytometry. They elucidated that chrysins possess potent *in vitro* anti-cancer activity via suppression of the cell proliferation, induction G₁ cell cycle arrest, along with the up regulation of p21, in addition to reduction of cyclin D1 and Cdk2 protein levels (32).

Associated with the analysis of sall4 expression; in most human malignancies, SALL4 might be over expressed and potentially used as a diagnostic marker. Similarly, Cao *et al* have reported the over expression of SALL4 protein as a novel diagnostic marker for germ cell tumors such as testis and ovary as well as other metastatic germ cells' tumors (33). Forghani Fard *et al.* (2013) have reported that sall4 is one of the genes involved in the colon carcinogenesis (34).

A well, in this study we assessed whether anti-cancer effect of chrysins on CT26 cells is associated with sall4 expression or not. Hence, RT-PCR analysis was performed and the mRNA profile of sall4 has demonstrated that chrysins exerts cytotoxicity on CT26 colon tumor cells via down regulation of the sall4 expression. However, sall4 down regulation could be considered as a palliative marker for treatment of colon cancer.

In conclusion, we have elucidated that chrysins inhibited the proliferation and induced apoptosis in CT26 colon cancer cells and reduction of tumor volume in animal model. Moreover, human clinical trials are necessary to make it clear whether this flavonoid could be proposed as a possible natural agent for the prevention and treatment of the human colon cancer.

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