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ORIGINAL ARTICLE

Additive anticancer effects of chrysin and low dose cisplatin in human malignant glioma cell (U87) proliferation and evaluation of the mechanistic pathway

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Summary

Purpose: *To evaluate the anticancer effect of chrysin and its additive combination with low-dose cisplatin in human glioma (U87) cancer cells and to study its underlying mechanism.*

Methods: *Inverted phase and fluorescence microscopic studies were done to demonstrate the effect of chrysin and its combination with cisplatin on cellular morphology and apoptosis. Annexin V-FITC assay was used to quantify the extent of apoptosis in chrysin and chrysin+cisplatin treated cells. Flow cytometry using propidium iodide (PI) as a staining agent was used to study the effect of chrysin and its combination with cisplatin on cell cycle phase distribution.*

Results: *The results showed chrysin brought about a potent and dose-dependent antiproliferative effect in human glioma cancer cells. However, the combination of chrysin with low dose cisplatin led to a much higher growth inhibitory effects indicating an additive effect between the two compounds. The combined effect of chrysin and cisplatin also gave rise to a greater apoptosis induction as well as cell cycle arrest in comparison to the treatment by chrysin or cisplatin alone. Fluorescence microscopy as well as inverted phase contrast microscopy also revealed that the combination of chrysin plus cisplatin resulted in greater apoptosis induction as well as cell morphology alterations. Combination treatment of chrysin and cisplatin resulted in greater percentage of cells in early as well as in late apoptotic stages. The combination effect was also seen in mitochondrial membrane potential loss.*

Conclusion: *Chrysin additively potentiates the antiproliferative, cell cycle arrest and apoptotic activity of cisplatin in human glioma cancer (U87) cells.*

Key words: *anticancer, apoptosis, chrysin, flow cytometry, glioma*

Introduction

Malignant gliomas are primary brain cancers comprising about 60-75% of all tumors of the central nervous system (CNS) [1]. Most of these malignant tumors are classified as high-grade tumors (grades III and IV) when diagnosed. Malignant glioma, being highly infiltrative, most frequent and morphologically very heterogeneous, is considered to be highly aggressive and deadly. Primary malignant CNS tumors represent ~2% of all cancers but account for a inconsistent rate of morbidity and mortality. They are the foremost cause of death from solid tumors in children and the third leading cause of death from cancer in adolescents and adults aged 15–34 years [2]. The present treatment approach consists of surgery followed by postoperative radiotherapy and chemotherapy. Despite these advances, the patient survival in gliomas remains poor (around 13-14 months), mainly because of inherent radio- and chemoresistance of these tumors [3-6]. Therefore, the compounds that exhibit anticancer activity against glioma are the key candidates to be used against CNS tumors.

Presently, most cancers are treated by using

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drug combination therapies. Most of the known anticancer drugs like cisplatin, paclitaxel, gemcitabine, oxaliplatin, 5-fluorouracil etc exert serious side effects. One way to reduce the side effects and enhance the efficacy of these drugs is to identify new chemical agents preferably from natural sources that can be combined with these known anticancer drugs [7]. Recently, it has been reported that various natural compounds combined with different anticancer drugs led to enhanced anticancer effect in different cancer types. This enhancement can mainly occur through additive effects where a combination of two compounds produces more potent effect than the two compounds separately. Since the combination produces an enhanced effect, it results in less side effects because of less drug dose used [8-10].

In the present study, we evaluated the antiproliferative effect of chrysin and its combination with cisplatin against human glioma cancer (U87) cells by studying the effect on cellular morphology, apoptosis, cell cycle arrest and mitochondrial membrane potential.

Methods

Chemicals

Chrysin flavonoid was purchased from Sigma (St. Louis, MO, USA) with a purity of 98%, as measured by reverse-phase high-performance liquid chromatography.The compound was dissolved in dimethyl sulfoxide (DMSO) prior to use. Deionized water was used in all experiments. Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin-streptomycin were obtained from Hangzhou Sijiqing Biological Products Co., Ltd, China. MTT [3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC-Propidium Iodide (PI) Apoptosis Detection Kit was purchased from Beyotime Institute of Biotechnology, Shanghai, China. RNase A and PI were purchased from Guangzhou Geneshun Biotech Ltd. China. All other chemicals and solvents used were of the highest purity grade. Cell culture plasticware was bought from BD Falcon (USA). Hoechst 33258 and cisplatin were purchased from Sigma (St. Louis, MO, USA).

Cell line and drug treatment

The U87 human glioma cell line was obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 mg/ml streptomycin, 50 IU/ml penicillin, and 2 mM glutamine (Beyotime Institute of Biotechnology) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell proliferation assay

Briefly, U87 human glioma cells were plated at a density of $1x10^5$ cells per well in 96-well plates overnight and then treated with 0, 10, 20, 40, 80 and 120 μM chrysin and 0.5, 1.0 and 2.0 μM cisplatin or their combination (40 μM/l chrysin plus 1.0 μM/l cisplatin) for 48 hrs. Fifty microliters of MTT solution (5 mg/ml in PBS) were added to each well and the cells were cultured for another 3 hrs at 37°C. The medium was completely removed and 160 μl DMSO were added to solubilize MTT formazan crystals. The plates were then agitated and the optical density (OD) was determined at 570 nm (OD570) using an ELISA plate reader (Thermo Molecular Devices Co., Union City, USA). The experiment was repeated three times.

The cell viability ratio was calculated by the following formula:

Inhibitory ratio (%) = (OD control – OD treated) / OD control × 100%.

Cytotoxicity was expressed as the concentration of the drug inhibiting cell growth by 50% (IC_{50} value).

Phase contrast microscopic study of additive-apoptotic effect of chrysin and cisplatin

U87 human glioma cells ($1x10^5$) were seeded in 6-well plates overnight and then treated with 40 μM chrysin, 2.0 μM cisplatin or their combination for 48 hrs. Control cells treated with 0.1% DMSO alone were also included. The morphological changes were observed under inverted light microscope (Olympus, Center Valley, PA, USA) after 48 hrs. The images were captured at x 200 magnification.

Fluorescence microscopic study of additive-apoptotic effect of chrysin and cisplatin

U87 human glioma cells ($1x10^5$) were seeded in 6-well plates overnight and treated with 40 μM chrysin, 2.0 μM cisplatin or their combination for 48 hrs. After drug treatment for 48 hrs, cell apoptosis was determined by the Hoechst staining kit according to the manufacturer's instruction. The cells were fixed with 4% polyoxymethylene and then incubated in Hoechst solution for 10-15 min in the dark. The staining images were recorded using a UV fluorescence microscope (Olympus, Olympus Optical Co., LTD, Tokyo, Japan) using UV filter at x 200 magnification to detect morphological evidence of apoptosis.

Cell cycle analysis of chrysin and cisplatin combination by flow cytometry

The additive effect of chrysin and cisplatin in U87 human glioma cells was further studied by flow cytometry demonstrating the effect of this combination on cell cycle analysis. U87 human glioma cells ($1x10^6$) in a 60 mm dish were treated with 40 μM chrysin, 2.0 μM cisplatin or their combination for 48 hrs. The cells were collected by trypsinization and washed twice with

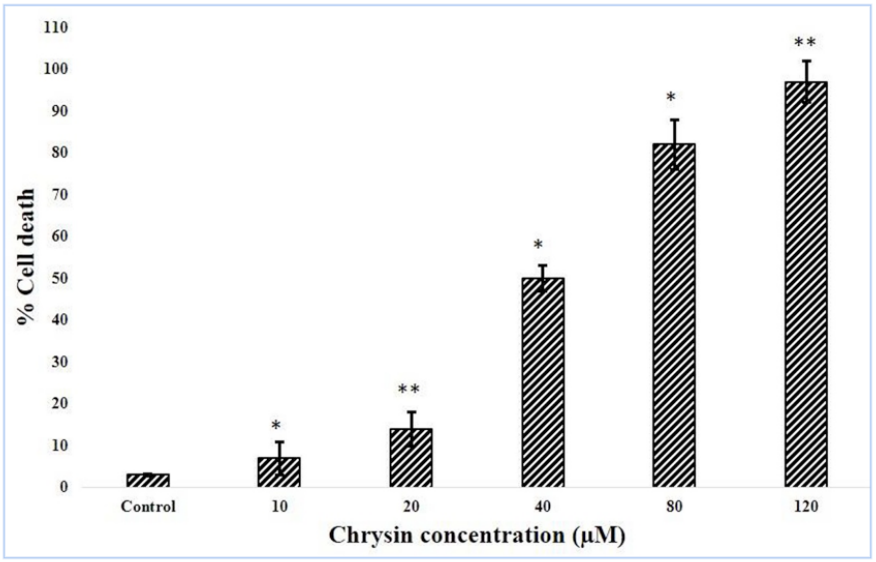

Figure 1. Growth inhibitory effect of chrysin against human glioma (U87) cancer cells at different concentrations. *p<0.05 vs control group. **p<0.01 vs control group.

PBS (Sigma Chemical Co.). Cells were incubated in 50% ethanol at -20°C overnight and then treated with 50 µg/ml RNase A, and stained with 20 µg/ml of PI. Finally, the stained cells were analyzed by using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Annexin V/PI flow cytometric analysis of additive-apoptotic effect of chrysin and cisplatin

To verify the additive enhancing effect of chrysin and cisplatin combinations, the apoptotic induction caused by chrysin+cisplatin in U87 glioma cancer cells was studied using the Annexin V-FITC and PI apoptosis kit (Bestbio, Shanghai, China). U87 human glioma cells ($1x10^6$) were seeded in 6-well plates overnight and then treated with 40 µM chrysin, 2.0 µM cisplatin or their combination for 48 hrs. Cells were collected by centrifugation (15000xg) and washed three times with cold PBS. The staining was performed according to the manufacturer's instructions. Cells grown in media containing an equivalent amount of DMSO without any drug served as control. After 48 hrs of incubation, the cells with different treatments were harvested and the final samples were measured on a FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA) equipped with Cell Quest 3.3 software.

Effect of chrysin and cisplatin combination on mitochondrial membrane potential loss (ΔΨm)

Mitochondrial membrane potential (ΔΨm) loss induced in U87 human glioma cancer cells by chrysin and cisplatin combination was measured by Rhodamine-123 dye (Zouping Mingxing Chemical Co., Ltd.). U87 human glioma cells ($1x10^5$) were treated with 40 µM chrysin, 2.0 µM cisplatin or their combination for 48 hrs and mitochondrial membrane potential was measured by flow-cytometer. Rhodamine-123 (20 mM) was added 2 hrs before the end of the experiment. Then, the cells were washed with PBS and incubated with PI (20 µg/ml) for 20 min. Finally the cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistics

All statistical analyses were done using SPSS® software (version 19.0) and were conducted by one-way analysis of variance (ANOVA) and Tukey test. Data were expressed as mean± standard deviation (SD) and p values <0.05 were considered significant.

Results

Additive effect of chrysin and low-dose cisplatin on human glioma (U87) cell proliferation

In order to examine the combined effect of chrysin and cisplatin on the cell proliferation of U87 cancer cells, the cells were treated with varying concentrations of chrysin and cisplatin for 48 hrs and the cell viability was evaluated by MTT assay. It was seen that both chrysin and cisplatin brought about a growth inhibitory effect on these cells in a dose-dependent manner (Figure 1). Based on these findings, we were able to select a moderate dose (40 µM chrysin+2.0 µM cisplatin) for combination treatment. The combination experiment showed that, in comparison to treatment by chrysin or cisplatin alone, combination of these two drugs inhibited cancer cell growth

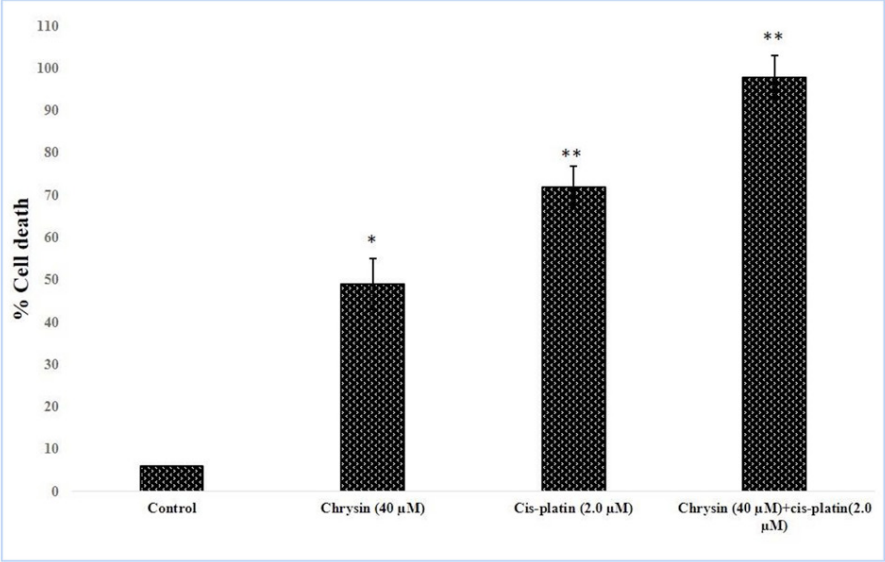

Figure 2. Additive effect of chrysin and cisplatin on human glioma (U87) cancer cell proliferation.*p<0.05 vs control group. **p<0.01 vs control group.

much more considerably (Figure 2). Thus, chrysin flavonoid could enhance the anticancer potential of cisplatin in glioma cancer cells.

Evaluation of the additive-apoptotic cell death induced by chrysin and low-dose cisplatin combination by phase contrast and fluorescence microscopy

The apoptotic cell death induced by chrysin and cisplatin combination was evaluated by inverted phase contrast microscopy. After incubation with 40 µM chrysin, 2.0 µM cisplatin or their combination for 48 hrs, the glioma cells were investigated by inverted phase contrast microscopy for demonstrating the mode of action of this combination. Compared with single drug treatment, apoptotic cells considerably increased with the combination treatment of chrysin and cisplatin (Figure 3A-D). Decrease in the cell population was seen with the onset of combination treatment. Figure 3B represents 40 µM chrysin, Figure 3C shows 2.0 µM cisplatin, while Figure 3D shows the effect of their combination. Figure 3A represents control cells without drug treatment.

The apoptosis-inducing effect of chrysin, cisplatin and their combination was further assessed by Hoechst 33258 staining using fluorescence microscopy. Following treatment with 40 µM chrysin, 2.0 µM cisplatin or their combination (chrysin, 40 µM+cisplatin, 2.0 µM) for 48 hrs, the cells were investigated by fluorescence microscopy. Chromatin condensation, nuclear fragmentation and DNA damage were identified in these treated cells (Figure 4A-D). As compared to single drug treatment with chrysin (Figure 4B) or cisplatin (Figure 4C) alone, their combination produced much more potent apoptotic effect (Figure 4D). Figure 4A represents control cells without drug treatment.

Additive effect of chrysin and low-dose cisplatin on cell cycle phase distribution in human glioma cancer cells (U87)

The effect of chrysin, cisplatin and their combination on the cell cycle phase distribution in U87 human glioma cancer cells is shown in Figure 5 A-D. The cells (U87) were treated with 40 µM chrysin, 2.0 µM cisplatin or their combination for 48 hrs and then analyzed by flow cytometry to evaluate cell cycle phase distribution after drug treatment. As can be seen from the Figure 5, chrysin (Figure 5B), cisplatin (Figure 5C) as well as their combination (Figure 5D) induced a sub-G0/G1 cell cycle arrest. The percentage of sub-G0/G1 population increased in the order chrysin<cisplatin<chrysin+cisplatin. The percentage of sub-G0/G1 population (apoptotic cells) increased from 2.4% in the control (untreated cells) to 8.2, 17.5 and 45.2% in cells treated with 40 µM chrysin, 2.0 µM cisplatin and chrysin (40 µM)+cisplatin (2.0 µM), respectively. The highest percentage of sub-G0/G1 population (48.98%) could be seen in the combination treatment (Figure 6).

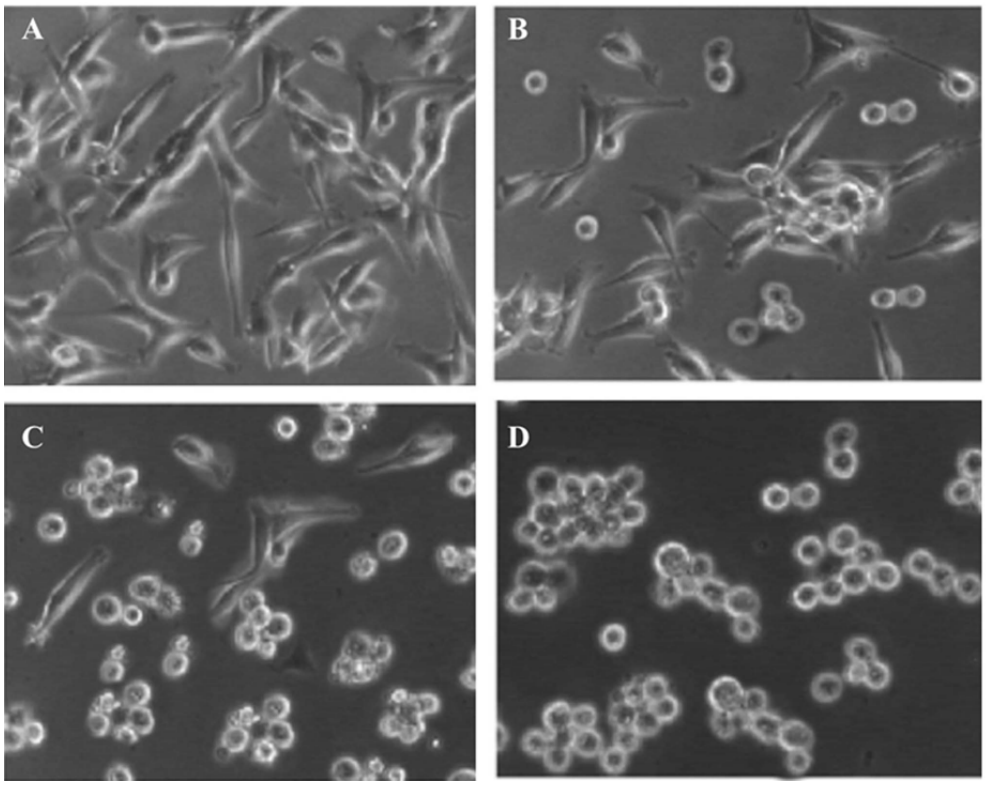

Figure 3. Additive apoptotic effect of chrysin and cisplatin observed by inverted phase contrast microscopy in human glioma (U87) cancer cells. The cells were treated with 40 µM chrysin (**B**), 2.0 µM (**C**) cisplatin or their combination (**D**) for 48 hrs. (**A**) shows the control (untreated) group. The cells became rounded and contracted on treatment with chrysin, cisplatin and more convincingly in combination treatment (**D**) (magnification x200).

Additive apoptotic effect of chrysin and low-dose cisplatin combination in human glioma cells (U87)

The effect of the combination treatment on apoptosis induction in these cells was further demonstrated by Annexin V/PI staining. Human glioma cells (U87) were treated with 40 µM chrysin, 2.0 µM cisplatin or their combination for 48 hrs and then analyzed by flow cytometry to evaluate apoptosis (Figure 7A). As can be seen in Figure 7 B and C, chrysin and cisplatin alone induced apoptosis (early apoptotic cells 6.72% and 9.21%; and late apoptotic cells 3.85% and 15.62%) compared to the control cells (3.87% and 4.27%). Compared with individual treatment, the percentage of early and late apoptotic cells in the combination treatment of chrysin (40 µM) +cisplatin (2.0 µM) was significantly much higher (44.7% and 8.2%) (Figure 7D). Figure 7B is the graphical representation of the effect of chrysin and cisplatin combination on the percentage of early and late apoptotic cells.

Additive effect of chrysin and low-dose cisplatin on the mitochondrial membrane potential loss (ΔΨm) in human glioma cells

The event of early apoptosis is mostly associated with the loss of mitochondrial membrane potential resulting from the disruption and rupture of the mitochondrial membrane. Flow cytometry in combination with rhodamine-123 fluorescent cationic dye were used to evaluate the effect of chrysin, low-dose cisplatin and their combination on the mitochondrial membrane potential loss in human glioma cells. Rhodamine-123 is a fluorescent probe which can cross into the mitochondria and indicate the change in mitochondrial membrane potential (ΔΨm) [11]. The results showed (Figure 8 A-D and Figure 9) that the combination of chrysin (40 µM) and low-dose cisplatin (2.0 µM) led to potent loss of membrane potential as compared to individual treatment as well as to the control.

Discussion

Considerable attention has been paid to identify naturally occurring bioactive molecules and their related synthetic analogues to prevent the development and progression of cancer. A large number of natural foods and their products have

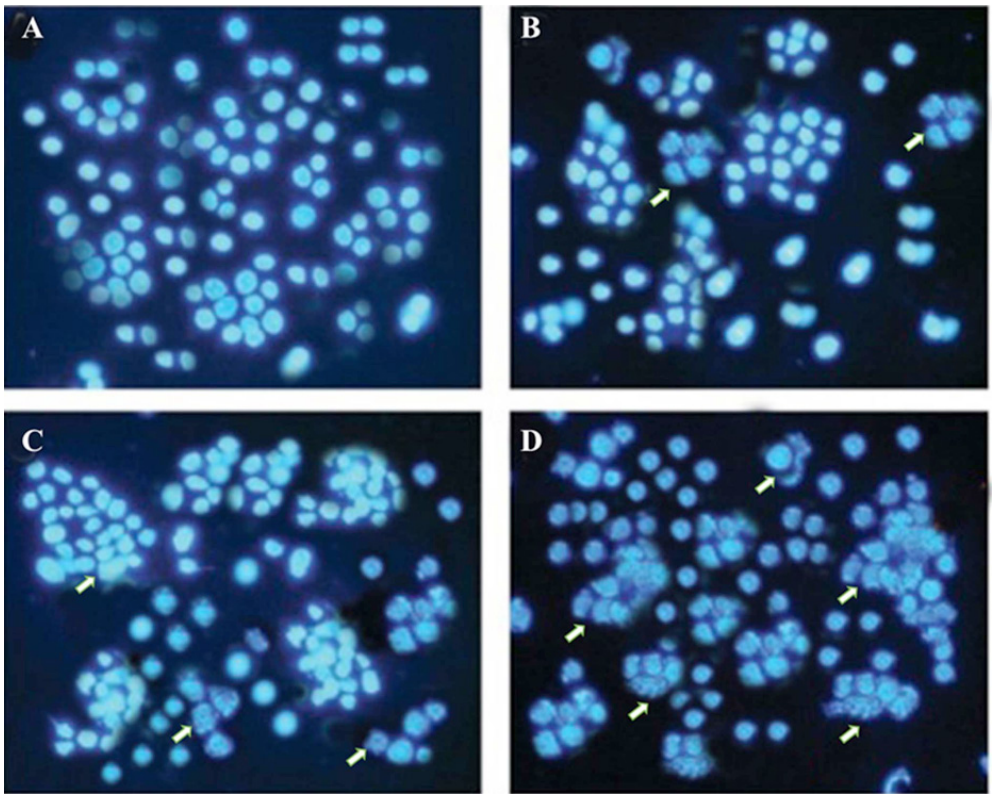

Figure 4. Additive apoptotic effect of chrysin and cisplatin observed by fluorescence microscopy in human glioma (U87) cancer cells. The cells were treated with 40 μM chrysin (**B**), 2.0 μM cisplatin (**C**) or their combination (**D**) for 48 hrs. (**A**) shows the control (untreated) group. Treated cells exhibited chromatin condensation and nuclear fragmentation which was much more eminent in the combination treatment. Arrows indicate the chromatin condensation and nuclear fragmentation (magnification x200).

the ability to induce apoptosis in several cancer cells. It has been reported that almost all stages of the tumor development process can be affected by compounds and extracts from medicinal plants [12]. More than 60% of anticancer drugs currently in clinical use are either natural products or their semisynthetic derivatives. Phenolic compounds are an important group of compounds that occur in plants and comprise at least 8,000 different known structures. Phenolic compounds can be divided into at least 10 different types based on their structure, among which flavonoids are an important class. Flavonoids are a diverse family of natural phenolic compounds usually found in fruits and vegetables. Flavonoids are normally safe and are linked with little toxicity, making them perfect contenders for cancer chemopreventive agents [13]. Chrysin (5, 7-dihydroxyflavone) is a natural and biologically active flavone extracted from many plants. Chrysin has been recently reported to exhibit a high number of bioactive effects including anti-inflammatory, anti-oxidant and anti-tumor effects. It has also been reported to suppress tumor angiogenesis *in vivo* [14,15]. Chrysin has also been reported to inhibit proteasomes which play a key role in regulating vital cellular processes like apoptosis and cell cycle [16]. In a vital study, some authors claimed that chrysin sensitizes tumor necrosis factor-alpha-induced apoptosis in human cancer cells through inhibition of nuclear factor-kappaB [17].

It has been reported that chrysin has a low oral bioavailability, but this does not exclude the occurrence of local pharmacological effects of chrysin, especially in the intestine. The low bioavailability of chrysin has been linked with its extensive presystemic intestinal and hepatic sulphation and glucuronidation [18]. However, the bioavailability problem can be easily solved by combining chrysin with bioavailability enhancers such as piperine, quercetin, and zinc supplements [19].

In view of the published literature, the primary objective of the present study was to determine whether the combined treatment of chrysin with cisplatin is more effective against the human gli-

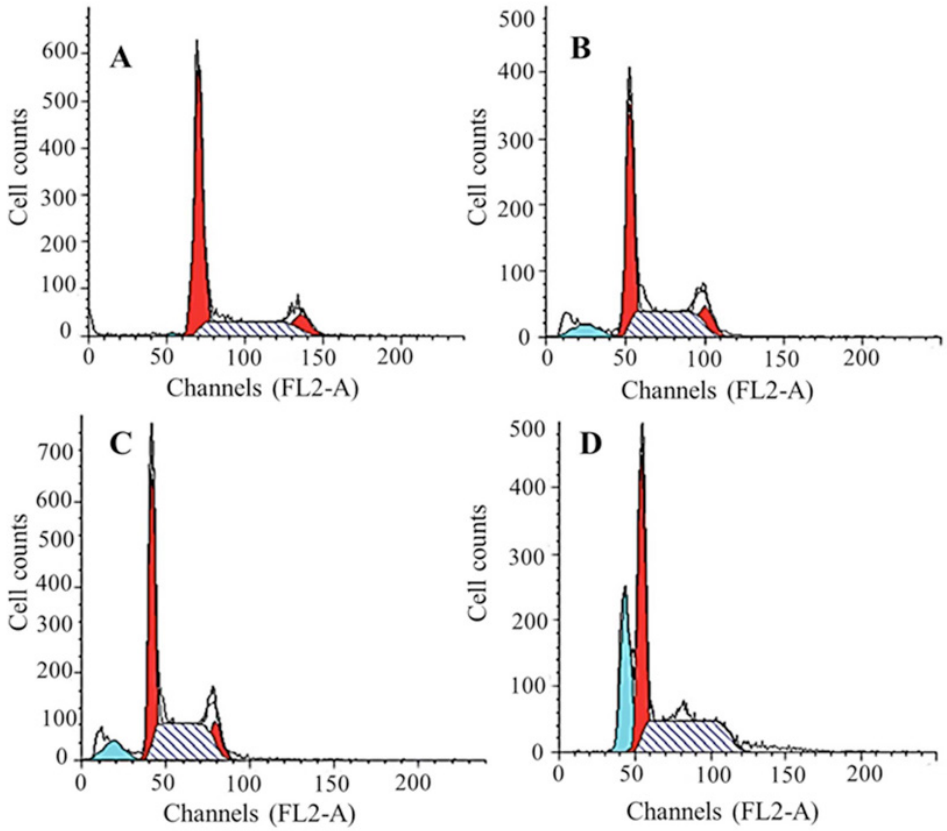

Figure 5. Combined effect of chrysin and cisplatin on the cell cycle arrest in human glioma cells (U78). U87 cells were treated with 40 μM chrysin (**B**), 2.0 μM cisplatin (**C**) or their combination (**D**) for 48 hrs. (**A**) shows the control (untreated) group. Chrysin, cisplatin as well as their combination induced a sub-G0/G1 cell cycle arrest in these cells, but the effect of the combination on the sub-G0/G1 population was much more prominent with combination treatment (**D**) in comparison to chrysin (**B**) or cisplatin (**C**) alone.

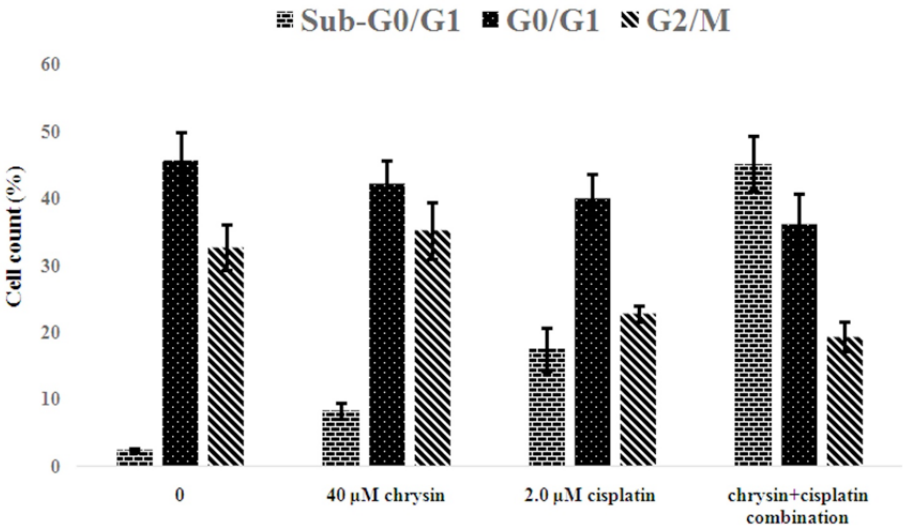

Figure 6. Graphical representation of the cell cycle arrest at sub-G0/G1 phase measured by flow cytometry using propidium iodide as a fluorescent probe. The cell population of sub-G0/G1 was highest in the combination-treated cells as compared to the chrysin or cisplatin treatment alone ($p<0.05$).

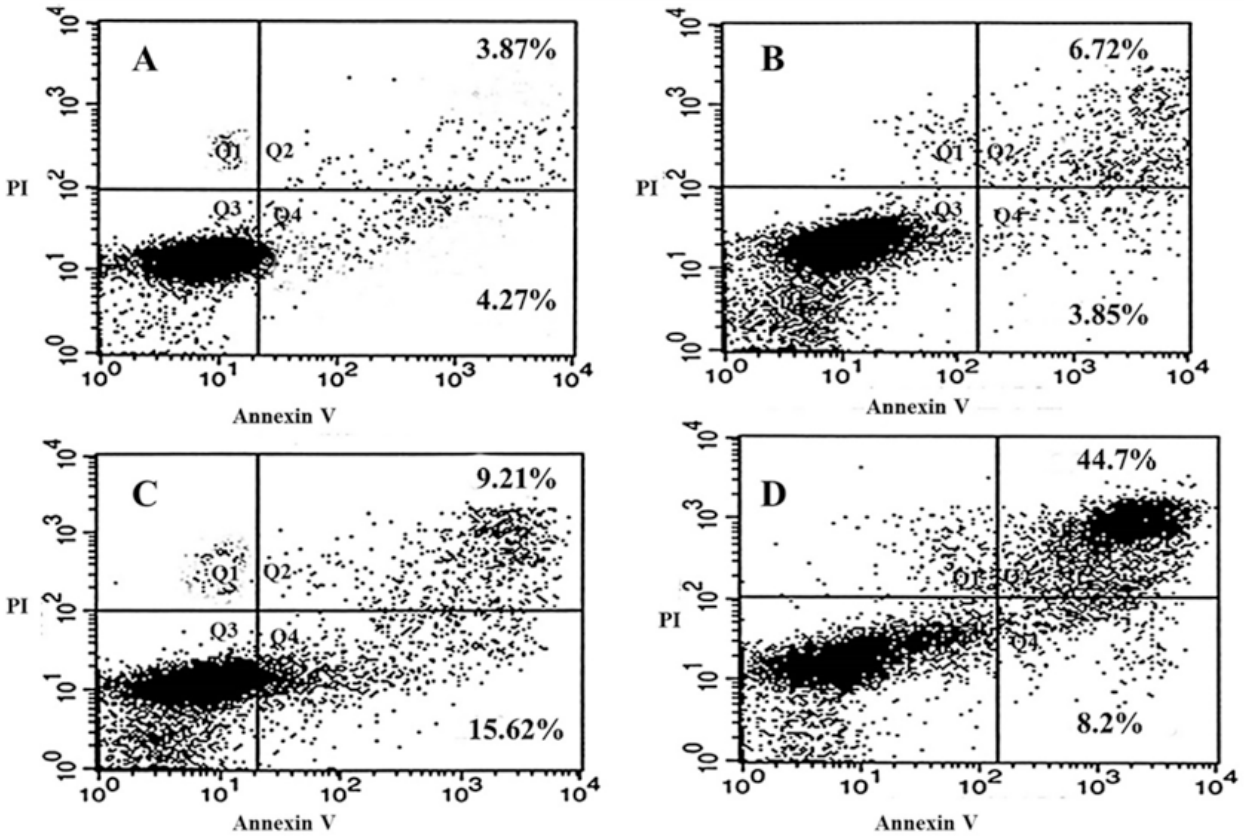

Figure 7A. Combined effect of chrysin and cisplatin on the apoptosis induction in human glioma cells (U87) using Annexin V-FITC assay. The cells were treated with 40 μM chrysin (**B**), 2.0 μM cisplatin (**C**) or their combination (**D**) for 48 hrs. (**A**) shows the control (untreated) group. Normal healthy, early apoptotic, late apoptotic and dead/necrotic cell populations are shown as percentage of total cells in the quadrants Q3, Q4, Q2 and Q1, respectively. Combination treatment induced a potent apoptosis in these cells as compared to chrysin or cisplatin alone ($p<0.05$ vs control group).

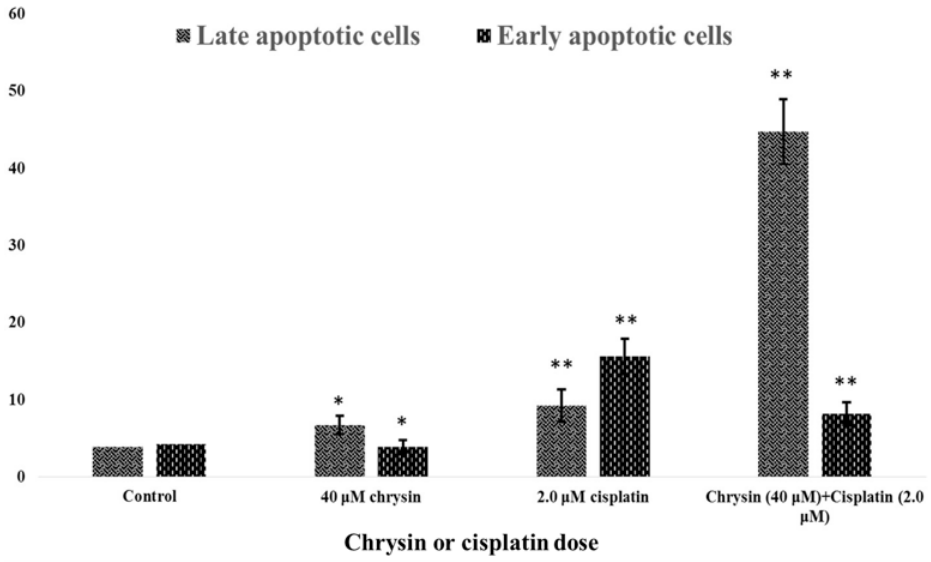

Figure 7B. Graphical representation of the effect of chrysin and cisplatin combination on the percentage of early and late apoptotic human glioma cells. Data are expressed as mean±SD from three independent experiments. *$p<0.05$ vs control group. **$p<0.01$ vs control group.

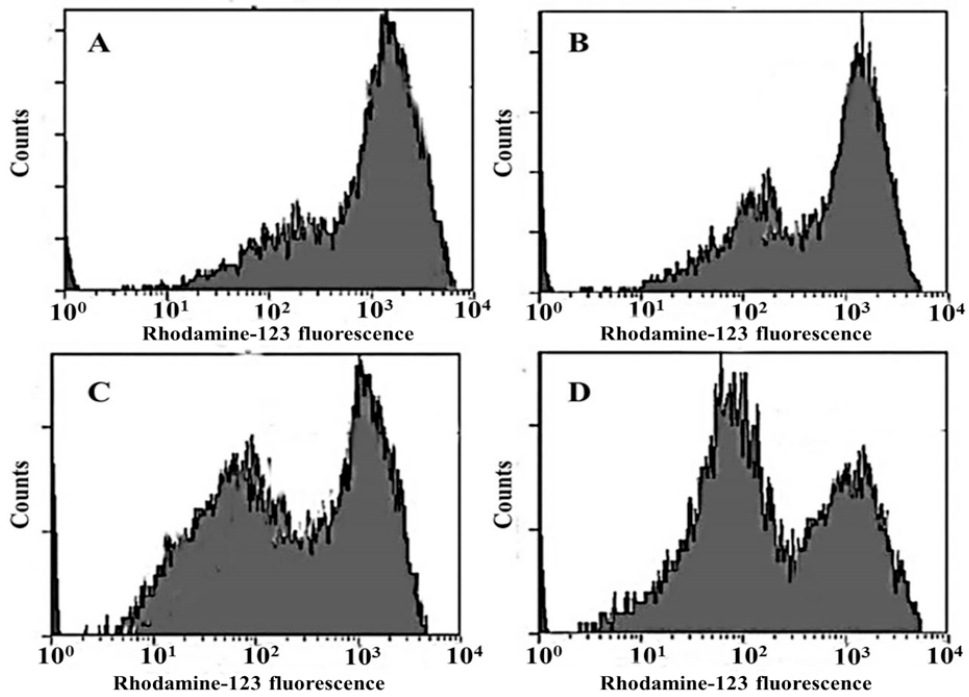

Figure 8. Additive effect of chrysin and low-dose cisplatin combination on the mitochondrial membrane potential loss (ΔΨm) in human glioma cells. Cells were treated with 40 μM chrysin (**B**), 2.0 μM cisplatin (**C**) and combination of chrysin (40 μM)+cisplatin (2.0 μM) (**D**) for 48 hrs and then incubated with Rhodamine-123 dye and examined by flow cytometry. Percentage loss of ΔΨm in the controls (**A**). The percentages of cells in the right section indicate the number of ΔΨm collapsed cells. The number of cells with ΔΨm collapsed cells was maximum in the combination treatment of chrysin and low-dose cisplatin ($p<0.01$ vs control group).

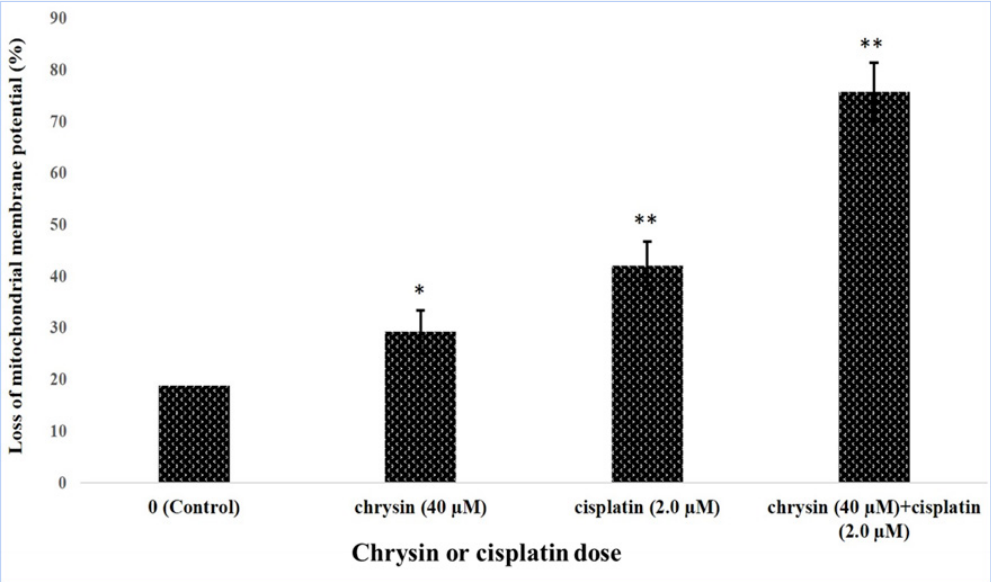

Figure 9. Graphical representation of the loss of mitochondrial membrane potential in glioma cells treated with chrysin, low-dose cisplatin and their combination. Data are expressed as mean±SD from three independent experiments. *$p<0.05$ vs control group. **$p<0.01$ vs control group.

oma cancer cells (U87) than treatment with cisplatin or chrysin alone. The results showed that chrysin could additively enhance the anticancer potential of low-dose cisplatin. The combination of chrysin with cisplatin produced a much more cytotoxic effect as compared to the chrysin or cisplatin alone. The cell morphological studies also indicated that the combination of chrysin and low-dose cisplatin was much more effective in changing the cellular morphology and inducing apoptotic morphological features in these glioma cells. Flow cytometry revealed that the combination protocol was also much more effective in inducing sub-G0/G1 cell cycle arrest as well as mitochondrial membrane potential loss. The percentage of early as well as late apoptotic cells was much higher in the combination treatment in comparison to separate treatment by chrysin or cisplatin alone. These results may help reduce the serious side effects of cisplatin. Chemotherapy is well known to produce serious side effects and this study demonstrates that low doses of cisplatin can be used in conjunction with chrysin to produce potent anticancer effects.

In conclusion, chrysin additively enhances the anticancer efficacy of low-dose cisplatin by inducing apoptosis, cell cycle arrest in sub-G0/G1 phase and mitochondrial membrane potential loss in human glioma (U87) cells.

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