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Effects of Chrysin plant flavonoid on proliferation and apoptosis of gastric cancer cell line (AGS)

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ABSTRACT

Introduction: Chrysin is a natural flavonoid antioxidant which its role in tumor cell death has been reported. The aim of this study was to investigate the mechanism of Chrysin effect on proliferation and apoptosis of gastric cancer cell line (AGS).

Methods: Cells were cultured and treated with different concentrations of Chrysin (90, 80, 70, 60, 50 µM) for 48 h and evaluated for cell viability. To examine the cytotoxic effect of drug in inducing apoptosis, staining with fluorescein isothiocyanate (FITC) and propidium iodide (PI) was used. The cells were treated for 48 h with different concentrations of Chrysin (50 of 90 µM) and examined for the morphological changes. The one-way analysis of variance (ANOVA) and the Excel software were used for data analysis.

Results: Different concentrations of Chrysin significantly inhibited the growth and proliferation of AGS cells ($p<0.05$). The IC_{50} dose was determined to be 67.5±0.66. Apoptosis induced by 50 and 70 µM of Chrysin was significantly greater than in untreated cells ($p<0.05$). Cells treated with high concentration of Chrysin (90 µM) showed more prominent growth inhibition and cell shrinkage compared to cells treated with the lower concentrations of Chrysin.

Conclusion: The results of this study showed that Chrysin effects on AGS cell line were significantly high and dose-dependent and might be helpful in the treatment of gastric cancer. Chrysin may therefore be considered a potential candidate for both cancer prevention and treatment. Further investigation is needed to validate the contribution of chrysin in tumor therapy *in vivo*.

Implication for health policy/practice/research/medical education:
Chrysin might be considered as a potential candidate for cancer prevention and treatment. Further investigation is needed to validate the contribution of chrysin in tumor therapy *in vivo*.

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Introduction

Stomach cancer is one of the most important causes of cancer-related deaths worldwide and a major cause of cancer death in Asia (1). Treatment options for these patients include chemotherapy and surgery. But, therapeutic effects of chemotherapeutic drugs are not good enough and these drugs have many side effects (2). In recent years, a lot of researches have been done on drugs that might have protective or therapeutic effects against stomach cancer (3). Various flavonoids have been found to inhibit the development of cancer. Epidemiological studies have also shown that consumption of dietary flavonoids in fruits and vegetables may help reduce the incidence of cancer. This issue has been confirmed on *in vivo* and *in vitro* studies (4). Several mechanisms of action have been proposed for the effect of flavonoids including carcinogens inactivation, inhibition of angiogenesis and elimination of drug resistance or a combination of these mechanisms. Damage to healthy cells and development of cellular resistance to anticancer drugs restrict the use of chemotherapy drugs. All of these suppose the use of flavonoids for future clinical studies (5). Chrysin is a natural flavonoid compound extracted from honey, plants and propolis and been shown to possess anti-

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inflammatory and antioxidant properties (6). The potential apoptotic effect of chrysin has been reported in human cervical cancer, esophageal squamous cancer, malignant glioma, leukemia, breast, prostate and colon cancers, in vitro (7). The aim of this in vitro experimental study was to investigate the antiproliferative and apoptotic effects of Chrysin on gastric cancer cell line (AGS).

Materials and Methods

Cell culture and MTS assay

The AGS cell line (Pasture Institute, Iran) was cultured in DMEM medium and supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and glutamine. The cells were harvested from the culture medium using trypsin (0.25%)-EDTA (0.1%). After washing, the number of viable cells was determined by trypan blue dye exclusion using a hemocytometer. Cells with viability greater than 95% were used for the experiment. MTS assay test was performed to determine the effect of chrysin in AGS cells. IC_{50} value which is the drug concentration required to kill 50% of the cells was obtained, too. Briefly, appropriate numbers of cells (10,000 cells) were cultured in 96-well plates. After 24 h incubation, different doses of Chrysin (90, 80, 70, 60, 50 µM) were added to each well. At least three wells were allocated for each concentration. Wells with cells but without drugs served as controls and 3 wells were used to assess the drug-free control. After 48 h, MTS test was performed. Cell Titer 96® AQueous One Solution Cell Proliferation Assay Kit was used for MTS assay according to the manufacturer's protocol. For this purpose, 20 µl of MTS working solution were added to each well. When MTS solution was absorbed by living cells a formazan product that had an absorbance maximum at 450 nms was produced, thus optical density (OD) would be the number of cells per well. The absorbance (optical density, OD) of formazan was measured using spectrophotometry. IC_{50} value was calculated as the sample concentration that caused 50% cell death as compared to the control cells (no drug added).

Assessment of apoptosis

To examine the cytotoxic effect of drug and its mechanisms in inducing apoptosis FITC Annexin V Apoptosis Detection Kit (BD) was used according to the manufacturer's protocol. Cancer cells were added in each well of 6-well plate. After 24 h, cells were treated at dose levels higher and lower than the IC_{50} value of chrysin (50 and 70 µM). After 24 h incubation, cells were rinsed with cold PBS and re-suspended in 100 µl of binding buffer. Then, stained with 5 µl FITC Annexin V and 10 µl Propidium iodide and incubated for 15 min at room temperature in the dark. The cells were examined by flow-cytometer. Cells that are FITC Annexin V positive and PI negative are in early stage of apoptosis; cells that are FITC Annexin V and PI positive are in late stage of apoptosis or already dead, cell that are FITC Annexin V negative and PI positive are considered necrotic.

Morphological study

The morphological changes of the cells and cells death were observed by using a normal inverted microscope. Cells were treated for 48 h with different concentrations of Chrysin (50 of 90 µM) and then morphological changes were examined and recorded.

Statistical analysis

In the present study, AGS cells were divided into two groups: Control group that did not receive any treatment and a group that received a drug. All experiments were carried out in triplicate. All results were expressed as mean ± SEM. Data were analyzed statistically using the SPSS 16 software. The one-way analysis of variance (ANOVA) was used to determine whether there were any significant differences between the means. A probability level of $p<0.05$ was considered statistically significant.

Results

Effects of different concentrations of Chrysin on the cell growth and proliferation

Results of the Chrysin effect on the growth and proliferation of AGS cell was reported as the average of several independent experiments (Figure 1). Different concentrations of Chrysin significantly inhibited the growth and proliferation of gastric carcinoma cells ($p<0.05$). The IC50 dose was determined to be 67.5±0.66.

Inducing apoptosis in cancer cells treated with Chrysin

In control group (untreated) 0.94% necrosis and 2.48% early apoptosis was observed. Induced necrosis, early apoptosis and late apoptosis in AGS cells treated with 50 µM Chrysin respectively were 2.91, 2.21 and 19.3. In cells treated with 70 µM Chrysin they were 1.26, 4.23 and 22.9, respectively, (Figures 2 and 3). Apoptosis induced by 50 and 70 µM of Chrysin was significantly greater than in untreated cells ($p<0.05$).

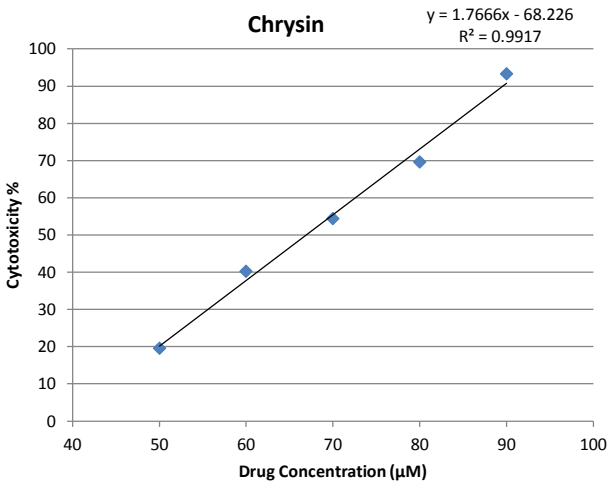

Figure 1. Effect of chrysin on the cell growth and proliferation. The cytotoxicity of AGS was increased significantly with increasing the concentration of chrysin.

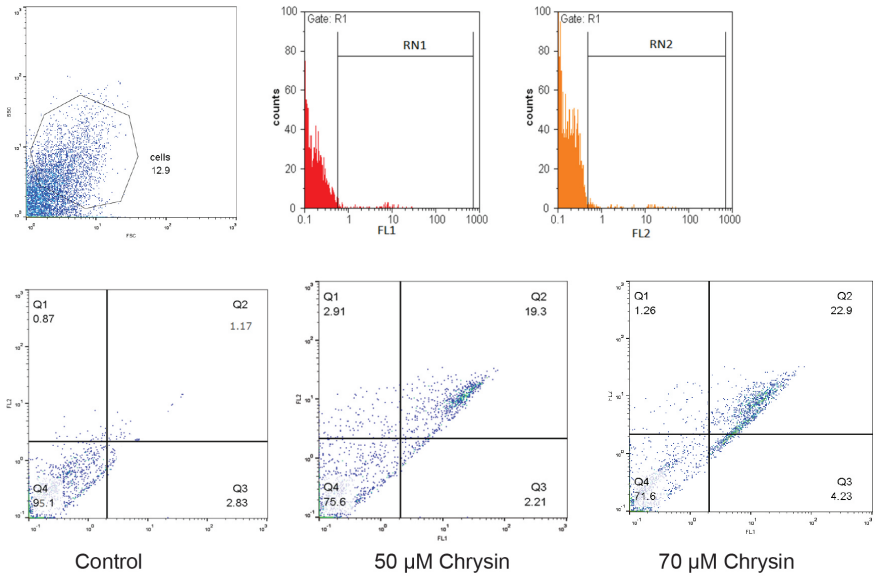

Figure 2. Flow-cytometry analysis using Annexin V-FITC/PI double staining depicting apoptosis. The cells were treated with 50 and 70 µM chrysin for 48 h or media only (control), and apoptosis was examined by flow-cytometry after Annexin V-PI double staining. Necrotic cells lose membrane integrity, permitting PI entry. Viable cells exhibited Annexin V (-)/PI (-); early apoptotic cells exhibited Annexin (+)/PI (-); late apoptotic cells or necrotic cells exhibited Annexin V (+)/PI (+).

Morphological Evaluation

There were no significant morphological changes after 24 h of incubation with the low concentration of Chrysin (50 µM), but after 48 h of incubation with 50 and 90 µM of Chrysin, significant changes in morphology were observed (Figure 4). Cells treated with high concentration of Chrysin (90 µM) compared to cells treated with the lower concentrations showed more prominent growth inhibition and cell shrinkage.

Discussion

In recent years, considerable efforts have been done to identify naturally occurring compound and related synthetic agents that can prevent the development and recurrence of cancer. Several natural compounds have been found to induce apoptosis in various tumor cells and there are clear evidences that these compounds are potent inhibitors of cancer cell proliferation (8). Chrysin is a natural flavonoid extracted from honey, propolis (a wax) and various plants. It has been reported to have potent antioxidant and anti-inflammatory properties and promotes cell death by perturbing cell cycle progression. In order to determine the effect of Chrysin on gastric cancer, its effects on growth and proliferation of human gastric adenocarcinoma cell line were assessed in vitro using a MTS assay test. The results of this study showed that different concentrations of Chrysin markedly inhibit growth and proliferation in AGS cells in a dose-dependent manner. Inhibition of growth in cancer cells by minimum and maximum concentrations of Chrysin (50 and 90 µM) were 19.6 and 93.2, respectively. Our results showed that Chrysin had significant cytotoxic activity against human gastric cancer cells. This is the first report on chrysin-induced cytotoxicity and apoptosis in human gastric adenocarcinoma cell line.

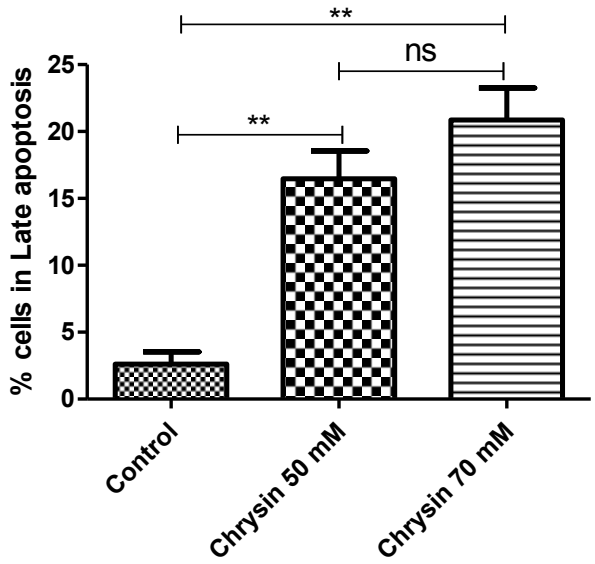

Figure 3. Induction of apoptosis in AGS cells after 48-hour treatment with chrysin. Statistical analysis performed by ANOVA followed by post hoc Tukey,S test. Value of **p<0.001 was assumed statistically significant. (ns: not significant and p>0.05)

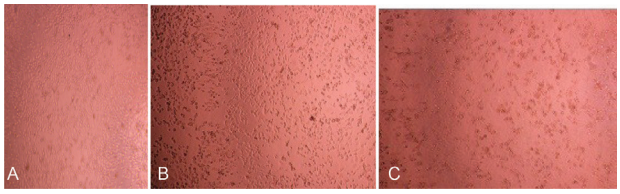

Figure 4. Morphological changes of cells after treatment with different concentrations of chrysin 48 hours post-treatment. A) Normal cells; B) 50 mM; and C) 90 mM. Cells treated with high concentration of Chrysin (90 µM) compared to cells treated with the lower concentrations showed more prominent growth inhibition and cell shrinkage.

Xue *et al* investigated the cytotoxic and antiproliferative effects of Chrysin on prostate cancer cell line which is in agreement with our results. Their results showed that Chrysin exhibited inhibitory effects on angiogenesis in mouse model, the critical step of tumor growth and metastasis (9). Hong *et al* reported the anticancer effect of Chrysin on MDA-MB231 breast cancer cells. In their study, the effect of Chrysin on cells was determined 24 and 48 h after treatment. 24 h after treatment no significant effect was observed, but after 48 h treatment with concentrations of 20 and 40 µM respectively 73.9 and 73.1 viability were observed. Thus, 20 µM Chrysin for 48 h was reported as the effective concentration (4). In Zhang *et al* study anticancer effects of Chrysin on a human esophageal adenocarcinoma cell line (OE33) was investigated and reported IC_{50} value of Chrysin was 90 µM. In our study IC_{50} value of Chrysin was 66 µM and it seems that cytotoxic effects of Chrysin on gastric cancer cells were much more than those on esophageal adenocarcinoma OE33 cells (10).

In the present study, types of cell death (apoptosis or necrosis) induced by Chrysin in AGS cells were determined by flow-cytometry. Chrysin induced early and late apoptosis and necrosis in gastric cancer cells, but the rate of late apoptosis was significantly higher. Thus secondary apoptosis seems to be the major mechanism of tumor cell killing. Therefore, apoptosis plays an important role in the anticancer activity of Chrysin. Zheng *et al* reported that Chrysin induces apoptosis human myeloid leukemia cell lines by induction of caspases and inactivation of Akt (11). In another study, anticancer effects of this drug and its derivatives on human gastric cancer cell lines (SGC-7901) and human colon carcinoma cell lines (HT-29) have been reported (12).

Conclusion

The results of our study showed that Chrysin could inhibit gastric cancer cells. Further researches are needed to fully understand the mechanism of action, in order to use in modern therapies as chemotherapy adjuvant in the treatment of gastric cancer. Because chemotherapy drugs that are used to treat cancer often cause unwanted side-effects, simultaneous use of various compounds derived from medicinal plants may increase effectiveness of other drugs in treatment of cancer.

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Authors' contributions

All authors contributed to research design. NAD performed the experiment and prepared the first draft of the manuscript. HS edited the final version of the paper. All authors read and confirmed the final draft of the manuscript.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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