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ORIGINAL ARTICLE

Inhibitory and Cytotoxic Activities of Chrysin on Human Breast Adenocarcinoma Cells by Induction of Apoptosis

Saeed Samarghandian[1], Mohsen Azimi-Nezhad[1,2], Abasalt Borji[1], Malihe Hasanzadeh[3], Farahzad Jabbari[4], Tahereh Farkhondeh[5], Mohammad Samini[3]

[1]Department of Basic Medical Sciences, Neyshabur University of Medical Sciences, Neyshabur, [2]Department of Medical Genetics, School of Medicine, Mashhad University of Medical Sciences, [4]Allergy Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, [5]Department of Immunogenetic and Cell Culture, Immunology Research Center, School of Medicine, [3]Department of Gynecology Oncology, Woman Health Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

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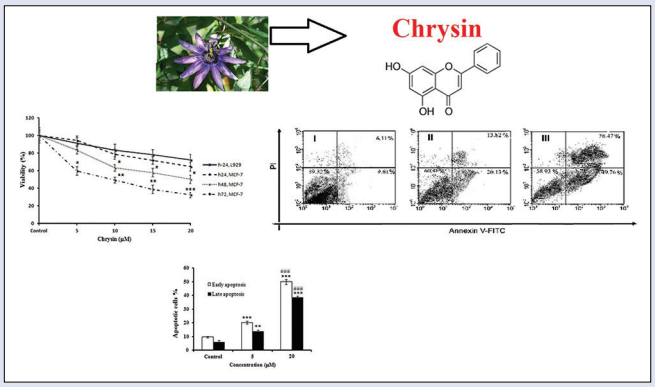
ABSTRACT

Objectives: Chrysin, an active natural bioflavonoid found in honey and many plant extracts, was first known for its antioxidant and anti-inflammatory effects. The fact that antioxidants have several inhibitory effects against different diseases, such as cancer, led to search for food rich in antioxidants. In this study, we investigated the antiproliferative and apoptotic effects of chrysin on the cultured human breast cancer cells (MCF-7). **Materials and Methods:** Cells were cultured in Roswell Park Memorial Institute medium and treated with different chrysin concentrations for three consecutive days. Cell viability was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The percentage of apoptotic cells was determined by flow cytometry using Annexin V-fluorescein isothiocyanate. **Results:** The MTT assay showed that chrysin had an antiproliferative effect on MCF-7 cells in a dose- and time-dependent manner. The 50% cell growth inhibition values for chrysin against MCF-7 cells were 19.5 and 9.2 μM after 48 and 72 h, respectively. Chrysin induced apoptosis in MCF-7 cells as determined by flow cytometry. Chrysin inhibits the growth of the breast cancer cells by inducing cancer cell apoptosis which may, in part, explain its anticancer activity. **Conclusion:** This study shows that chrysin could also be considered as a promising chemotherapeutic agent and anticancer activity in treatment of the breast cancer cells in future.

Key words: Apoptosis, chrysin, honey, human breast cancer cells, lung cancer, viability

SUMMARY

- Chrysin had an antiproliferative effect on human breast cancer cells (MCF-7) cells in a dose- and time-dependent manner
- Chrysin induced apoptosis in MCF-7 cells, as determined by flow cytometry
- Chrysin inhibits the growth of the breast cancer cells by inducing cancer cell apoptosis
- Chrysin may have anticancer activity.



Abbreviations used: Human breast cancer cells (MCF-7), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate-buffered saline (PBS), normal fi broblast mouse (L929).

Correspondence:

Dr. Malihe Hasanzadeh,
Department of Gynecology Oncology,
Women Health Research Center,
Mashhad University of Medical Sciences,
Mashhad, Iran.
E-mail: samarghandians@mums.ac.ir

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INTRODUCTION

Breast cancer is the main health problem in women. It is the one of major causes of death of middle age (40–50-year-old) women.[1] About 23% of total cancer diagnoses and 14% of deaths in women are related to breast cancer; therefore, incident rate of breast cancer is 1 out of 35 people in Asia and one out of eight in the United States.[2-5] Recently, it looks that there is a critical need for amelioration in detection, diagnosis, and treatment of breast cancer. Unlucky, progression of resistance to chemotherapeutic materials is an important obstruction in the treatment of breast cancer.[6] On the other hand, the present treatments (surgery, chemotherapy, and/or radiotherapy) are involved by critical side effects mainly loss of appetite, hair loss, decreased resistance to infections, weakness and fatigue, weight gain and bleeding, premature menopause, and development of tumor resistant.[7] In addition, major cytotoxic treatments mainly target dividing cells both normal and malignant cells that makes meaningful morbidity and limited clinical benefits of the patients. Thus, discovering novel and effective treatments against breast cancer is now-a-days a scientific challenge. Enhanced focus has been currently attended to naturally compounds as novel strategies.[8]

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Utilization of antioxidants has been related to the several preventative effects against different diseases such as cancer, aging, inflammatory disorders, coronary diseases, and neurological degeneration[9-14] and led to search for natural foods rich in antioxidants. One such food includes honey,[15,16] which has a long tradition of use in folk medicine for various purposes and has also been referred to extensively in the medical literature.[17,18] Its major ingredients are fructose and glucose and it also including amino acids, proteins, carbohydrates, minerals, vitamins, water, and enzymes. Honey indicates a broad spectrum of therapeutic aspects, containing antifungal, antibacterial, wound healing, and anti-inflammatory.[19-21] Gribel' and Pashinskii showed that honey possessed antitumor and also antimetastatic effects in the different strains of mouse and rat tumors.[22] It was also currently elucidated that honey could make apoptosis in cancer cell lines.[9,17]

Some minor ingredients of honey are confirmed to have antioxidant aspects[23] including polyphenol compounds.[24,25] Thus, it is possible that the antitumor aspects of honey are relation to its polyphenols. With the development of extraction methods for various polyphenols, it is possible to study and concentrate the polyphenolic ingredients extracted from honey rather than crude honey itself. Phenolic ingredients can be separated into several different types based on their structure: Consisting flavonoids and phenolic acids. Flavonoids (the most important polyphenolic class) are natural antioxidants that exhibit a wide range of biological effects, including antithrombotic, antibacterial, anti-inflammatory, antiallergic, and vasodilatory actions.[26] As natural products, flavonoids are considered as healthy, safe, and easily obtained in normal diet, making them proper treatment for cancer chemoprevention or associated material in clinical treatment. Chrysin (5,7-dihydroxyflavone) is a natural phytochemical flavonoid and biologically active flavone extracted from honey, vegetables, propolis, and fruits [Figure 1].[27,28] Chrysin, especially, has been detected as indicating a broad range of pharmacological aspects, such as anti-oxidation, anti-inflammatory properties, and promotes cell death by perturbing cell cycle progression.[17,29] The present study was aimed to investigate the cytotoxic and apoptotic effects of chrysin against breast cancer cell line.

MATERIALS AND METHODS

Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amerso (USA). Roswell Park Memorial Institute (RPMI) medium 1640 was purchased from Gibco BRL (Grand Island, NY, USA). Annexin V-fluorescein isothiocyanate (FITC) was obtained from Invitrogen Corporation (USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH, Austria. Chrysin (5,7-dihydroxyflavone) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals were of the highest, commercially available quality.

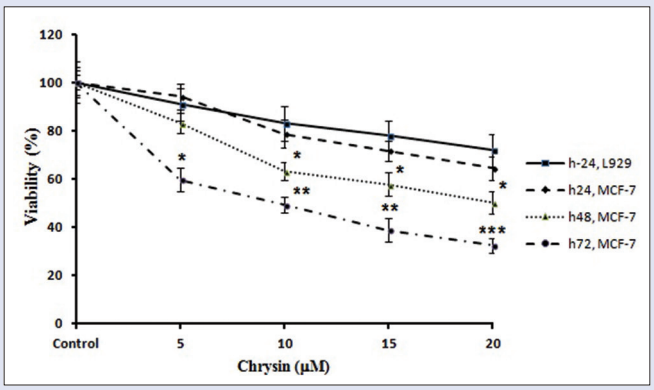

Figure 1: Exposure of A549 and L929 cells for 24, 48, and 72 h with different concentration of chrysin (5, 10, 20, and 30 µM)

Cell culture

The human breast cancer cell line (MCF-7) and normal fibroblast mouse (L929) cell (as control) were obtained from the Pasteur Institute of Iran and cultured in RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The MCF-7 cells were cultured in a CO_2 incubator (MCO-17AI, Sanyo Electric Co., Ltd., Japan) at 37°C in a humidified atmosphere enriched by 5% CO_2 and subcultured every 3–4 days. The malignant and nonmalignant cells were cultured in Dulbecco's modified Eagle's medium with 5% (v/v) FBS, 100 units/ml penicillin and 100 µg/ml streptomycin.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay

Cell viability was determined using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Briefly, the cells were seeded at a density of 1×10^3 cells/ml in 96-well plates and allowed to attach for 24 h, resulting in log phase growth at the time of drug treatment. Chrysin at different concentrations (5, 10, 20, and 30 mM) was added to the wells for 24, 48, and 72 h. After removing the medium, cells were then labeled with MTT solution (5 mg/ml in phosphate-buffered saline [PBS]) for 4 h at 37°C and the resulting formazan was solubilized with dimethyl sulfoxide (100 µl). Absorbance was measured at 550 nm using an automated microplate reader (Bio-Rad 550). Cell viability was expressed as a percentage of the value for control culture value. The cytotoxic effects of chrysin on MCF-7 cells were expressed as 50% cell growth inhibition (IC_{50}) values (the drug concentration that reduced the absorbance of treated cells by 50% compared to untreated cells). Experiments for each concentration were carried out in triplicate, including untreated cell control and a blank cell-free control.

Assessment of apoptosis by Annexin V-fluorescein isothiocyanate

Apoptotic cell death was measured using an FITC-conjugated Annexin V/propidium iodide (PI) assay kit by flow cytometry. Briefly, 5×10^5 cells were washed with ice-cold PBS, resuspended in 100 ml binding buffer, and stained with 5 ml of FITC-conjugated Annexin V (10 mg/ml) and 10 ml of PI (50 mg/ml). The cells were incubated for 15 min at room temperature in the dark, 400 ml of binding buffer was added, and the cells were analyzed (FACScan, Becton-Dickinson, USA). The MCF-7 cells were gated separately according to their granularity and size on forward scatter versus side scatter plots. Early and late apoptosis was evaluated on fluorescence 2 (FL2 for PI) versus FL1 (for Annexin) plots. Cells stained with only Annexin V were evaluated as being in early apoptosis; cells stained with both Annexin V and PI were evaluated as being in late apoptosis or a necrotic stage.

Statistical analysis

All results were expressed as mean ± standard error mean. Significance was evaluated using ANOVA and Bonferroni's test. A probability level of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of chrysin on cell viability

MCF-7 cancerous cells were incubated with different concentrations of chrysin for 24, 48, and 72 h. The cytotoxicity of chrysin on cell viability was quantitated by MTT assay. Exposure of the MCF-7 cells with chrysin showed significantly high growth inhibitory effects on the breast carcinoma cell line in a concentration- and time-dependent manner ($P < 0.001$). The exposure of MCF-7 cells for 24 h showed no significant result at any concentration of chrysin. However, there were significant decreases in viability for the concentrations of 5, 10, 15, and 20 μM after 48 and 72 h ($P < 0.05$, $P < 0.01$ and $P < 0.001$, Figure 1). The dose inducing IC_{50} against the malignant cell (MCF-7) was determined at being 19.5 ± 0.5 μM and 9.2 ± 0.7 μM at 48 and 72 h, respectively.

Quantification studies for apoptosis by chrysin

To study the roles of chrysin in apoptosis, chrysin was used to setup apoptosis system on the MCF-7 cell line. The MCF-7 cells were treated with concentrations of 5 and 20 μM of chrysin for 48 h. After treatment, the cells were harvested and apoptosis was examined by flow cytometry [Figure 2]. Quantitative analysis using Annexin V/PI assay further showed that the proportion of early stage apoptotic cells (Annexin V+/PI−) increased significantly from 20.13% to 49.76% while proportion of late stage apoptotic cell (Annexin V+/PI+) increased significantly from 13.82% to 38.47% when the cells were treated with the concentrations of 5 and 20 μM of chrysin, respectively [Figure 3]. Apoptosis induced from 5 and 20 μM of chrysin was statistically higher than control and the percentage of the early and late apoptotic cells significantly increased by increasing chrysin concentration ($P < 0.001$), and also the number of the late apoptotic cells versus early apoptotic cells at concentration of 5 and 20 μM of chrysin treated cells were statically significant ($P < 0.01$, $P < 0.001$) [Figure 3].

DISCUSSION

Cancer is a very complex disorder and the occurrence and progression of cancer cells are strongly connected to abnormal intracellular signal transduction system.[30] One of the basic strategies of new cancer treatment is chemotherapy. However, the common anticancer agents currently used for treating different types of cancer have severe side effects. Therefore, in the present time, the recent research has mainly concentrated on herbs and plants which have been studied for being nontoxic and for the treatment and prevention of breast cancer. Thus, it is substantial to screen natural products, either as isolated components or as crude extracts, for apoptotic abilities to detect potential anticancer compounds. Over 60% anticancer drugs recently applied come from natural sources, including marine organisms, plants, and microorganisms,[31] and they offer an opportunity to investigate the molecular mechanisms of tumorigenesis.[32] Significant interest is recently focused on the usage of foods for the protection of human health. Especially, there is severe interest in the function of dietary antioxidants, which can scavenge the free radicals and oxidants responsible for developing and initiating different diseases.[33] The confirmed data that antioxidants have several preventative effects against various disorders, such as coronary diseases, cancer, neurologic degeneration, inflammatory disorders, and aging, have led to a investigate for foods containing antioxidants.

Research on the antioxidant aspects of various beverages, foods, spices, and herbs have been done[13,34] and the number of articles addressing the health-protective and antioxidant characteristics of honey is increasing. Honey has for a long time been used as a natural source of sugars, as well as strong ingredient in traditional medicine, having antitumor, antimicrobial, and antiinflammatory properties.[35] Honey contains a different ingredient including benzoic acid, and cafeic acid, esters, substitute's phenolic acids, flavonoid glycones, and beeswax.[36,37] Some of the illustrated biological activities of honey may be traced to its chemical ingredients.[38,39] The therapeutic and health-protective impacts of honey were previously explained by the existence of various antioxidant ingredients, such as organic acids, flavonoids (such as chrysin), phenolic acids, enzymes, and vitamins.[17,40,41]

In the current study, chrysin could reduce cell viability of the MCF-7 breast cancer cell line. Chrysin-induced cell toxicity is achieved through the inhibition of cell proliferation and induction of apoptosis [Figures 1-3]. Our research showed that chrysin prevented the growth of the MCF-7 cells in a dose-dependent manner. The prevention of cell growth was in agree with that mentioned by Parajuli *et al.*,[5,42] who applied chrysin extracted from the roots, stem, and leaf of various *Scutellaria* plants. According to their research, chrysin at a concentration of 100 μM significantly prevented the growth of MDA-MB-231 cells after 4 days' treatment (about 50.0%, $P < 0.05$).[42] In our study, 20 μM chrysin was sufficient to prevent the growth of the MCF-7 cells during 3 days' treatment (73.9%, $P < 0.05$). A 48 h treatment of chrysin was applied to discover the preliminary mechanisms of the chrysin on the MCF-7 cells because this was the condition under which the induction of early growth inhibition in the MCF-7 cells was

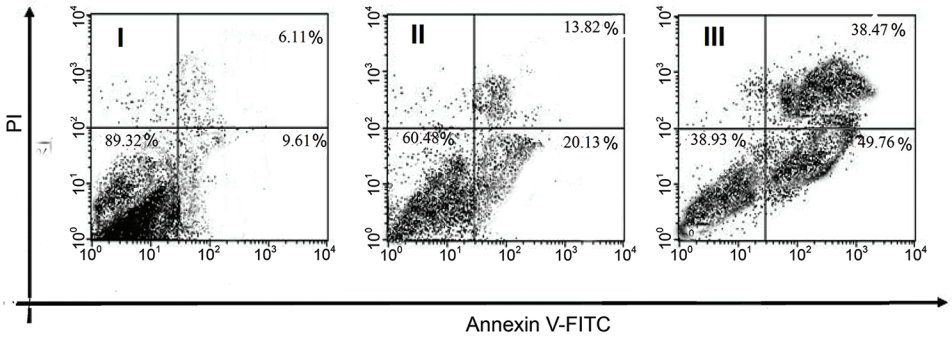

Figure 2: Assessment of apoptosis by Annexin V/propidium iodide on the alveolar human lung cancer cell line (A549). The cells were treated with 20 and 30 μM chrysin for 48 h (symbol II and III) or media (control symbol I), and apoptosis was examined with flow cytometry after Annexin V/propidium iodide double staining. The necrotic cells lost cell membrane integrity that permits propidium iodide entry. Viable cells exhibit Annexin V−/propidium iodide− (symbol a); early apoptotic cells exhibit Annexin+/propidium iodide− (symbol b); late apoptotic cells or necrotic cells exhibit Annexin V+/propidium iodide+ (symbol c)

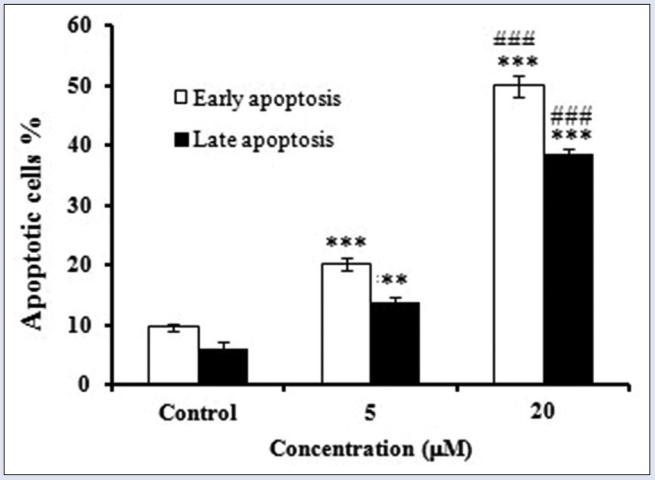

Figure 3: Percentage of cell death based on the assessment of apoptosis by Annexin V/propidium iodide. ***$P < 0.001$, compared with control; ** $P < 0.01$; ###$P < 0.001$, versus the other chrysin concentration

statistically significant [Figure 2]. As 48 h is competent to determine the effect of chrysin on the MCF-7 cells, a longer treatment time did not require for our preliminary optimization research. Certainly, study of Parajuli *et al*. (2009) illustrated that chrysin at a concentration of 100 µM significantly prevented the proliferation of MDA-MB-231 cells after 4 days treatment, but no apoptosis was noted in the research when the mechanism was determined by flow cytometry, demonstrating that the augment of chrysin concentration and treatment time did not raise its outcome on MDA-MB-231 cells.[41,42] In addition, although IC_{50} is the most common representative index of the dose-response curve to determine the half maximal effective concentration of a drug, some other endpoints, such as IC_{20} and IC_{80}, are also necessary to assess the early inhibitory effect of a drug after certain specified exposure time. In the current research, the cytotoxic and proapoptotic effects of chrysin on the MCF-7 cell lines were investigated. To the authors' knowledge, this is the first report on chrysin-induced apoptosis in MCF-7. Our data confirmed that chrysin has cytotoxic activity against the carcinomic human breast cells, which are consistent with previous studies, indicating that chrysin possesses anticarcinogenic and antitumor properties.[9,17] Different studies have also shown the antiproliferative activity of honey on human colon cancer cell line.[42]

The ability to establish tumor cell apoptosis is a critical property of a candidate anticancer drug, which differentiates between toxic compounds and anticancer drugs.[43] Our investigation identified that chrysin has a significant proliferation inhibitory activity against the MCF-7 cells in a dose-dependent manner [Figure 1]. Much effort has been managed toward the research of the effect of chrysin on apoptosis and understanding the mechanisms of action. The apoptosis evoked by chrysin was verified by the Annexin V-FITC [Figures 2 and 3]. In the present research, chrysin evoked apoptosis that was implicated in cell death. Apoptosis is characterized by oligonucleosomal DNA fragmentation, distinct morphologic features, including chromatic condensation, cell and nuclear shrinkage, and membrane blabbing.[44]

The origin of cancer relates to the suppression of apoptotic processes, as well as the magnified cellular proliferation. Apoptosis is a major type of cell death in response to cytotoxic candidate in cancerous situation. It has been shown that many natural agents with anticancer property could induce apoptosis of cancer cells.[45] Furthermore, antioxidant properties of chrysin have been mentioned in numerous papers.[17] Natural antioxidants with their ability to scavenge free radicals can protect the cells from different diseases such as cancer.[46,47]

CONCLUSION

Taken together, this study indicates that chrysin has a potential cytotoxicity and apoptotic properties on the MCF-7 cells. Further studies are needed to fully recognize the mechanism involved in cell death, and chrysin could be considered as promising chemotherapeutic agent in breast cancer treatment.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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