

Cytotoxicity and radiosensitizing effect of wogonin, an extract from *Scutellaria litwinowii*, in head and neck squamous cell carcinoma line HN5Zahra Nariman Asli¹, Mahnaz Nourbakhsh², Seyed Hadi Mousavi^{3,4}, Solmaz Pourgonabadi^{5,6}, Azar Fanipakdel⁷

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Type of article: Original**Abstract**

Background: Radiotherapy is one of the most prominent techniques used in cancer therapy. However, efforts for finding new effective radiosensitizers in improvement of radiotherapy efficacy have been continued yet. Wogonin is a natural flavonoid from *Scutellaria litwinowii* with known cytotoxic effects in some cancer cells.

Objective: To determine the potential of radiosensitizer effects of wogonin in a head and neck cancer cell line (HN5).

Methods: This study was carried out in Omid Hospital and Herbal Medicine Research Center (Mashhad, Iran). HN5 cells were cultured in DMEM medium and incubated with different concentrations of wogonin (25-500 µg/ml) and were exposed to (2-10 Gray [Gy]) γ -rays. Cell viability was quantified by MTT assay in wogonin cytotoxicity assay after 24, 48 and 72 hours and in coincident radiation and wogonin therapy after 72 hours. Data were analyzed by performing one-way ANOVA in SPSS (V.16). P-value ≤ 0.05 was considered significant.

Results: Wogonin decreased cell viability in HN5 cells as a time- and concentration-dependent manner. Wogonin could also enhance radiation-induced toxicity. For instance, while cell viability percentage in separate administration of wogonin (100 µM) and radiation (2 Gy) was significantly decreased to 53.98 \pm 2.76 and 74.97 \pm 1.05 respectively (p<0.001), wogonin 100 µM concurrent with 2 Gy radiation decreased the percentage of cell viability to 33.29 \pm 1.76 (p<0.001).

Conclusion: Concurrent use of wogonin and radiation increased radiation sensitivity and cell death in HN5 cells. Therefore, wogonin is a good radiosensitizer candidate for more study.

Keywords: Cytotoxicity; HN5 cell line; Radiosensitizing; Squamous cell of head and neck; Wogonin

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1. Introduction

Head and neck cancer is the sixth most common cancer in the world (1). This cancer accounted for about 4.9 percent of all cancers in the world in 2012. Between 2003 and 2009, 25,925 cases of Head and neck cancer have been recorded in Iran. Trend of age-standardized rate of head and neck cancer in Iran increased from 4.8 cases per 100,000 in 2003 to 7.4 in 2009 (2). Head and neck squamous cell carcinomas (HNSCC) are the most common subtypes of cancers emanated from this region. SCCs are about 90% of head and neck tumors (3). Chemoradiotherapy is the standard treatment of head and neck cancer (4). Chemotherapy as a systemic treatment for cancer has side effects in normal tissues and suffers from short-term efficacy because of its drug resistance (5). Radiotherapy is regarded as one of the most important therapeutic methods for tumor treatment. This field is undergoing rapid improvement nowadays. Scientists attempt to enhance the sensitization of tumor cells and protection of normal cells with the use of radiosensitizers and radioprotective agents, respectively (6). Large numbers of natural compounds have shown cytotoxic effects in a variety of cancers either alone or in combination with radiation. Wogonin (5, 7-dihydroxy-8-methoxyflavone), a flavonoid-like chemical compound from *Scutellaria* roots, is one of these herbal components that has been demonstrated to induce apoptosis in different cancer cells and has anti-tumor activity (7-11). For example, anticancer activity of wogonin has been reported in various cancers, such as: leukemia (8), lung (9), prostate (12), breast (11) and Hela cell lines (13). The antitumor functions of these flavones are largely due to their ability to scavenge oxidative radicals (8). Furthermore, It has been mentioned that concurrent use of herbal antioxidants and radiotherapy, increases radiosensitivity in tumor cells (14). Base on wogonin antioxidant and anti- tumoral activities (15), in the present study, we investigated cytotoxic and radiation sensitizer effects of wogonin in head and neck cancer cell lines.

2. Material and Methods

This study was carried out in Omid Hospital and Herbal Medicine Research Center (Mashhad, Iran) in 2014, after the ethics committee of Mashhad University of Medical Sciences approved the ethics of the research (IR.MUMS.REC.1392.95).

2.1. Wogonin

Collection, extraction, isolation and purification of wogonin from *Scutellaria litwinowii* roots were carried out as described previously (13). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 at 25 °C using deuterated chloroform (CDCl₃) as a solvent and tetramethylsilane (TMS) as an internal standard (13).

2.2. Reagents and chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Triton X-100 were purchased from sigma chemical company (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY) and

2.3. Cells and Cell culture

A human squamous cell carcinomas cell line, HN5 (Pasteur Institute, Tehran, Iran), was cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin antibiotic. HN5 cells were grown in monolayers in 25-cm² flasks and were preserved in a humidified 5% CO₂ and 95% air atmosphere at 37 °C.

2.4. Irradiation

The cultured cells were exposed to 2 -10 Gy doses of γ radiation from a Cobalt 60 unit (Theraton 780, Canada). The dose rate was 120 centigray (CGy)/min. Distance from source (SSD) was 80 cm. Field size was 9×5 cm².

2.5. Determination of cytotoxicity of wogonin on HN5 cells

The cell viability of cultured cells was determined by MTT reduction assay. In brief, HN5 cells were cultured at an initial density of 5000 cells/well on flat-bottomed 96-well culture plates and allowed to grow for 24 hours followed by treatment with wogonin (25-500 μ M). After passing the desired time (24 h, 48 h, 72 h) the medium was removed and cells were labeled with MTT solution (5 mg/ml in PBS) for 4 hours. The resulting formazan product was solubilized with DMSO (100 μ l). Absorbance was measured at 570 nm (620 nm as a reference) in an ELISA reader (16).

2.6. Determination of cytotoxicity of wogonin in combination with radiation

At the first step, the effect of continuance or elimination of wogonin during radiotherapy was determined. For this purpose, two groups of HN5 cells were incubated with various concentrations of wogonin (25-500 μ M) in 96 well

plates for 48 h. Then after wogonin elimination in one group, both groups received a 2 Gy radiation dose. Cell viability was determined by MTT method after 66 h, letting irradiated cells enter cell cycle and complete apoptosis. At the next step, for determination of cytotoxicity of wogonin in combination with different doses of radiation, HN5 cells pretreated with wogonin (100 μ M) for 6 h, without any wogonin elimination, were irradiated with γ -rays during exponential cell growth in 96 well plates using a Cobalt-60 unit with five doses (2-10 Gy) of γ -rays (17). Cell viability was evaluated 66 hours after radiation by MTT test (18). In both experiments, the control group did not receive any radiation. Cells were kept in DMEM with 10% FBS and 1% streptomycin and penicillin antibiotic during all γ -ray exposures.

2.7. Statistical analysis

Data were expressed as mean \pm SEM (standard error of mean). The test was performed using one-way ANOVA followed by a Bonferroni test for several comparisons in SPSS 16 (SPSS Inc., Chicago, Illinois, USA). A probability level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity of wogonin on HN5 cells

For determination of wogonin cytotoxicity, HN5 cells were incubated with various concentrations of wogonin (25-500 μ M) for 24, 48 and 72 hours (Table 1). The results displayed that wogonin could decline cell viability in HN5 malignant cells in a time- and concentration-dependent manner (Figure 1). Wogonin concentration of 50 μ M could reduce cell viability after 48 and 72 hours significantly ($p < 0.05$), while higher doses of 100-500 μ M reduced cell viability at all times significantly (Up to 75% cell death). Morphological changes of HN5 cells after treatment with different concentrations of wogonin confirm the results of the MTT assay (Figure 2).

Table 1. Cytotoxic effect of wogonin on HN5 cells

Wogonin concentration	%Cell viability after 24 h	%Cell viability after 48 h	%Cell viability after 72h
	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM
Control	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00
25 μ M	92.11 \pm 3.18	86.21 \pm 5.73	75.28 \pm 7.24
50 μ M	87.40 \pm 2.84	69.33 \pm 4.42	69.67 \pm 4.43
100 μ M	71.73 \pm 2.47	66.84 \pm 4.46	53.99 \pm 2.76
500 μ M	52.43 \pm 0.79	37.02 \pm 1.76	24.77 \pm 1.36

SEM: Standard Error of Mean

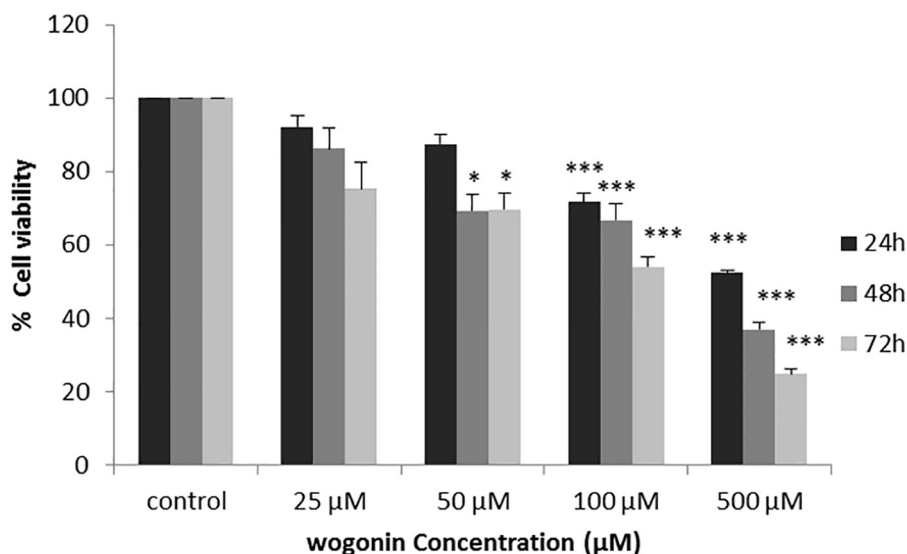


Figure 1. Wogonin cytotoxicity on HN5 cells. HN5 cells were incubated with various concentrations of wogonin (25-500 μ M) for 24, 48 and 72 hours. After these times, the cell viability was determined by MTT assay; * $p < 0.05$, *** $p < 0.001$)

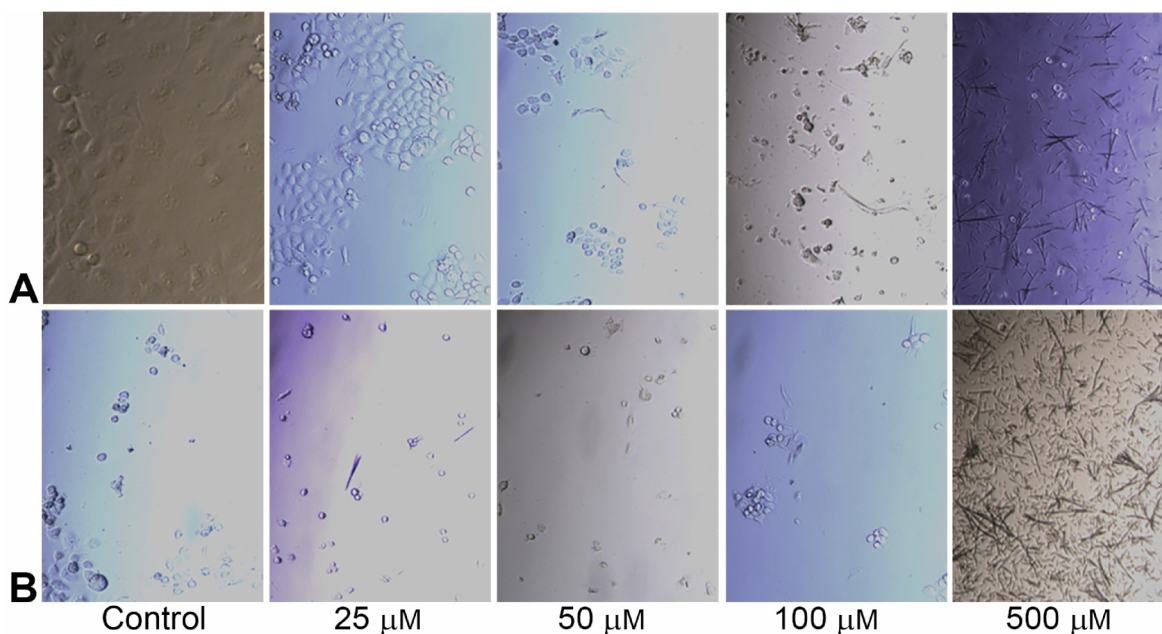


Figure 2. Morphological changes of HN5 cells after treatment with different concentrations of wogonin alone (A) or with exposure to 2 Gy radiation dose (B). HN5 cells were incubated with various concentrations of wogonin (25-500 μM) for 72 hours (A). In combination therapy, HN5 cells were incubated with different concentrations of wogonin (25-500 μM) for 6 hours. Then the cells received 2 Gy radiation dose and incubated more 66 hours (B).

3.2. The effect of the continuance or elimination of wogonin during radiotherapy

Investigation on the effect of the continuance or elimination of wogonin during radiotherapy revealed that at all concentrations of wogonin (25-500 μM) radiation along with the drug, reduced cell viability more significantly. In high concentrations of 100 and 500 μM, the effects of presence of wogonin on cell viability were significant ($p < 0.001$) in both situations (Figure 3).

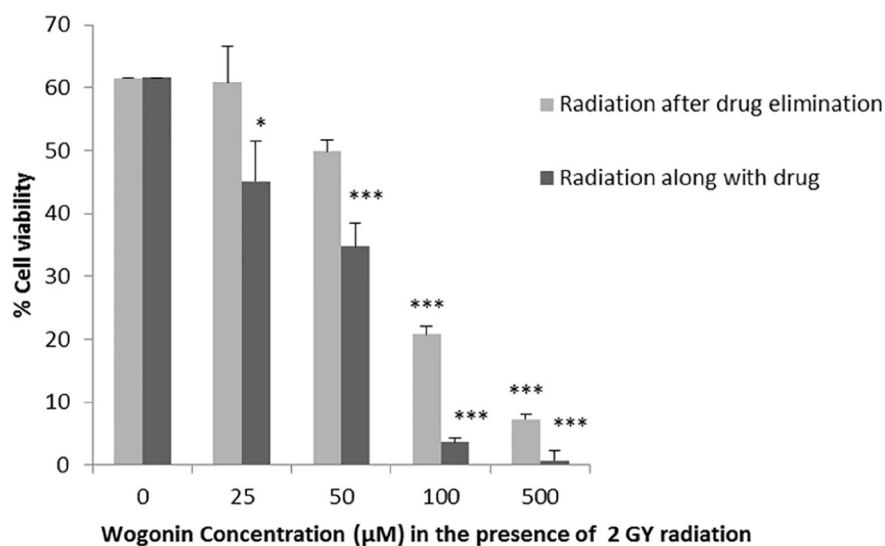


Figure 3. The effects of wogonin elimination during radiotherapy. HN5 cells were incubated with various concentrations of wogonin (25-500 μM) for 48 hours. Then after wogonin elimination in one group, both groups received 2Gy radiation dose. After a further 66 hours' incubation, cell viability was determined by MTT assay; * $p < 0.05$, *** $p < 0.001$)

3.3. Effects of coincident radiotherapy and wogonin on cell viability HN5

For investigation into the effects of different doses of radiotherapy and wogonin on cell viability HN5, the concentration of 100 μM of wogonin was chosen based on previous steps (Figure 3). The cells were incubated with

100 μM of wogonin for 6 hours. Afterward it was affected by 2-10 Gy γ -rays and then incubated for 66 hours. As shown in Figure 4, wogonin could sensitize cells to radiation-induced toxicity. At lower dose of radiation (2-4 Gy) the radiosensitizer effects of wogonin was more observable and these combination therapies reduced cell viability significantly ($p < 0.001$ and $p < 0.01$ respectively). In other words, wogonin increased radiation sensitivity and cell death. The effects of combination therapy compared to either wogonin or radiation alone have been better exhibited in Figure 5. As one can see, combination therapy of 100 μM wogonin and 2 Gy radiation (compared to each of wogonin or radiation alone) reduced cell viability significantly ($p < 0.01$ and $p < 0.001$ respectively).

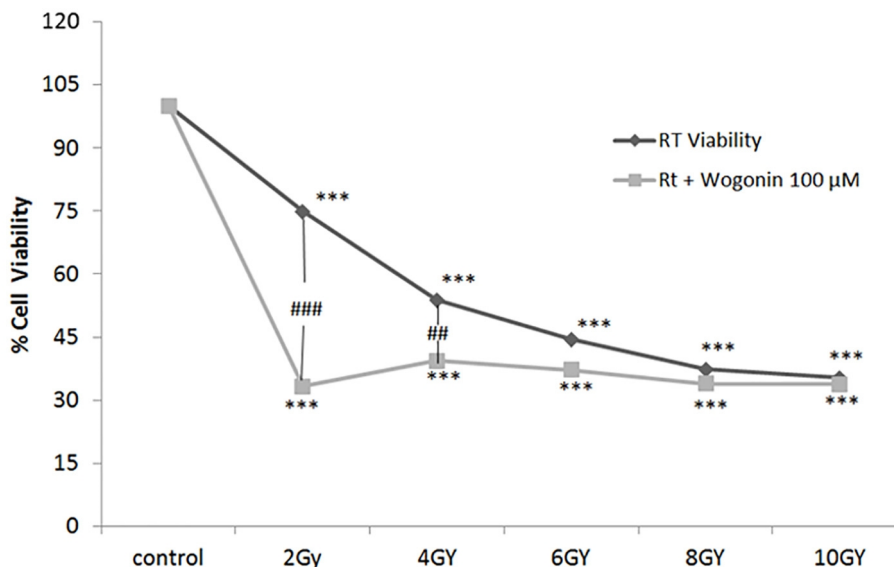


Figure 4. Comparison of the wogonin effects in different radiation doses. HN5 cells were incubated with 100 μM concentration of wogonin for 6 hours. Then the cells received 2-10 Gy radiation dose. After a further 66 hours' incubation, cell viability was determined by MTT method. Statistical differences have been shown as *** $p < 0.001$ when compared with the control group and ## $p < 0.01$ and ### $p < 0.001$ in comparison of two wogonin treated and untreated groups.

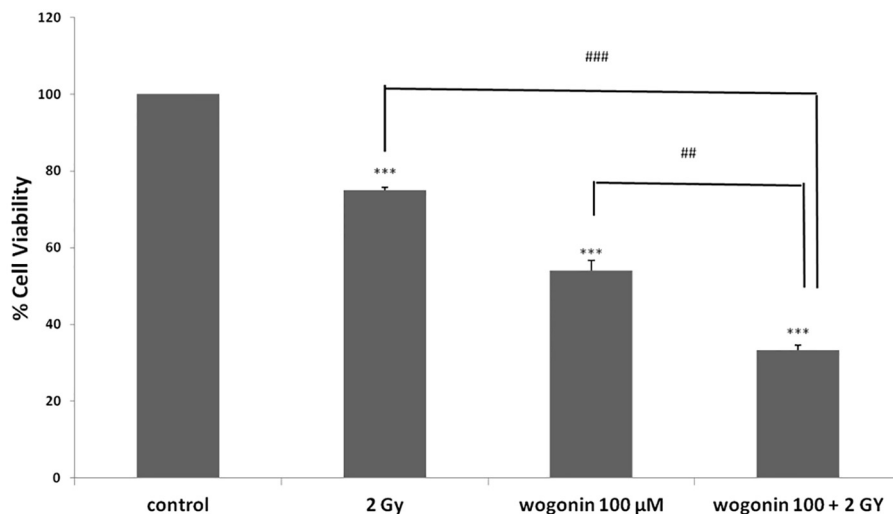


Figure 5. Comparison of the effects of wogonin (100 μM) and 2 Gy irradiation and combination therapy. HN5 cells were incubated with wogonin (100 μM) for 6 hours. Then the cells received 2Gy radiation dose. After a further 66 hours' incubation, cell viability was determined by MTT method. The 2 Gy irradiated group did not receive any wogonin; the wogonin treated group was incubated with 100 μM wogonin for 72 hours (6+66h) without any irradiation. Untreated cells considered as the control group. Statistical differences have been shown as *** $p < 0.001$ when compared with the control group and ## $p < 0.01$ and ### $p < 0.001$ in comparison to the combination treated group.

4. Discussion

Cancer is a major health difficulty worldwide. Natural compounds have been used to prevent and treat cancer for a long time. Thus they are appropriate for the development of anti-cancer drugs (19). Anti-tumor drugs are known to regulate cell cycle progression, inhibit cell proliferation, and induce apoptosis in cancer cells (20). The two main ways to prevent tumor growth and progression include, induction of cell death and inhibition of cell growth (21, 22). The dried root of *Scutellaria* from the Lamiaceae family is a widely used Chinese herbal medicine that historically is used in anti-inflammatory and anticancer therapy (23-27). The bioactive components of the *Scutellaria* species have been confirmed to be flavones (28). Wogonin is one of the major flavones of *Scutellaria litwinowii* that induces apoptosis in tumoral cells via different pathways (29). The antitumor functions of these flavones are largely due to their abilities to upregulation of intracellular reactive oxygen species (ROS), to attenuate NF- κ B activity to inhibit several genes important for regulation of the cell cycle, to suppress COX-2 gene expression and to prevent viral infections. Wogonin can also target PI3k/AKT and MAPk pathways (30). On the other hand, some studies have been shown that wogonin has tumor-selectivity. This tumor-selectivity might be due to presence of more O_2^- in tumor cells, so wogonin causes more H_2O_2 production in tumoral cells compared to normal cells (8). Susan Shojaee et al. demonstrated *S. litwinowii* root extract protected normal cells against DNA damage caused by H_2O_2 . They noted flavonoids may impart in this effect by scavenging free radicals (31). In an earlier study, Najarani et al. showed that wogonin had an anti-cancer effect on Hela cell line (13). The effects of wogonin as a chemosensitizer has been studied too. Wogonin acted as a chemosensitizer in combination with Etoposide, TNF- α and TRAIL, 5-FU, Cisplatin and Doxorubicin (30). There is no study on the radiosensitizer effect of wogonin.

Concomitant use of chemotherapeutic agents and radiotherapy on radiosensitivity in head and neck cancers has been investigated in some studies. Torres et al. studied the effects of AC480, a pan-HER inhibitor, combined with radiation on head and neck cancer. They found that the drug significantly enhanced the in vitro radio sensitivity of HN-5 cell lines (18). Luka Milas et al. studied the effects of the drug (C225 antibody) when combined with radiation on head and neck cancer. They reported that the drug enhanced the response of these cells to radiation in vivo; the enhancement was attributed to increased radiation-induced apoptosis (32). In another study, Sano et al. showed that vandetanib restores head and neck cancer cells sensitivity to cisplatin and radiation. They found that vandetanib and cisplatin effectively radiosensitized head and neck squamous cell carcinoma both in vitro and in vivo (33).

Since no information is available on the effects of wogonin combined with radiotherapy, in this study we investigated the effects of wogonin in combination with radiotherapy on head and neck cancer cells for the first time. For this purpose, at the first step, the effect of this flavonoid on induction of cell death and inhibition of cell growth on head and neck cancer cells was evaluated. Cells were exposed to increasing concentration of wogonin for 24, 48, and 72 hours, and cell viability was quantified by MTT assay. Our results confirmed that wogonin derived from *Scutellaria litwinowii* has anti-proliferation and anti-carcinogenic activities. Cell viability in the HN-5 cell line decreased after exposure to wogonin in a time- and concentration-dependent manner.

At the next step, the effects of wogonin combined with radiation on HN-5 cell line were determined. Our study confirmed that concomitant use of the drug (wogonin) and radiation on HN-5 cell line increases radio sensitivity and cell death. We demonstrated that at the presence of 2 Gy radiation, cell viability reduced by increasing the dose of the wogonin and keeping it during radiation. The concentration of 100 μ M wogonin was chosen as the best wogonin concentration for future studies because of its acceptable cytotoxic effects. Wogonin in this concentration reduced cell survival significantly, but was not too toxic to hide radiosensitizing effects. On the other hand, the results demonstrated the best radiation dose to reveal the radiosensitizing effect of 100 μ M wogonin was 2 Gy; this effect was not observable by radiation dose increasing, due to high cell cytotoxicity.

Some studies declared that wogonin in the concentration range of 10-100 μ M induces apoptosis in many cancer cells without any toxicity on normal cells (30). The protective effects of wogonin have been demonstrated in some normal cells (31, 34). In this study, we confirmed the cytotoxic effects of wogonin on HN-5 cells in these concentrations. Furthermore, we demonstrated the radiosensitizer effects of wogonin on these cancer cells at 100 μ M concentration. For better commenting on the use of wogonin as a radiosensitizer, we need to study the radioprotective effects of wogonin on normal cells too. Considering the various mechanisms that are mentioned for the effects of wogonin on cancer cells, the diagnosis of the possible mechanisms for the radiosensitizer effects of wogonin should be further investigated.

5. Conclusions

Concurrent use of wogonin and radiation, increased radiation sensitivity and cell death in HN5 cancer cells. Therefore, wogonin can be well considered as a potential sensitizer in cancer treatment. For this purpose protective effects of wogonin on normal cells must be investigated in future.

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Conflict of Interest:

There is no conflict of interest to be declared.

Authors' contributions:

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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