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Combinatorial treatment with formononetin and magnolol induces synergistic cytotoxicity and suppresses migration in glioblastoma cells

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ABSTRACT

Glioblastoma multiform (GBM) is a lethal brain tumor with limited treatment options. This study investigated the combined effect of two natural compounds, formononetin (FMN) and magnolol (MAG), on T98G glioblastoma cells, focusing on oxidative stress mechanisms. Cell viability was assessed via MTT assay to determine IC_{50} values and synergy (Chou-Talalay method). Proliferation, migration (wound healing), colony formation, and apoptosis (Hoechst staining) were evaluated. Gene expression (qPCR) of targets like survivin, cyclin D1, and antioxidant enzymes (SOD, CAT, GPX) was analyzed. Enzymatic activities and total antioxidant capacity (TAC) were measured. FMN and MAG synergistically reduced cell survival (CI=0.72 at 80 µM each). Combination treatment significantly inhibited proliferation, migration, and colony formation more effectively than single agents. This impounds downregulated pro-survival genes (survivin, cyclin D1, MMP-9) and upregulated pro-apoptotic Bad. Paradoxically, it also enhanced antioxidant enzyme activity and TAC. Despite this compensatory response, the treatment induced significant nuclear fragmentation indicative of apoptosis. The FMN and MAG combination exerts potent synergistic antiglioblastoma effects by disrupting proliferation and survival pathways, ultimately inducing apoptosis, even while triggering a compensatory antioxidant response. These in vitro findings provide a hypothesis-generating foundation for further investigation of formononetin and magnolol as a potential combinatorial approach for glioblastoma, pending rigorous in vivo validation and blood-brain barrier permeability studies.

Keywords: Metformin, Prostate Neoplasms, Liver Neoplasms, GADD45A protein, DKK1 protein, MTHFD2 protein

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Introduction

Glioblastoma, also known as glioblastoma multiform, is a type of tumor in the adult population that usually develops in the brain and spinal cord. Most patients die within 1 to 2 years of diagnosis without treatment (1, 2).Treatment for glioblastoma includes surgery, chemotherapy, and radiotherapy, but despite many efforts, little progress has been made in controlling the tumor (3).The main challenges in treating glioblastoma are the high invasiveness of the tumor and the difficult access to remove it. Also, the presence of the blood-brain barrier reduces the effectiveness of conventional chemotherapy drugs (4).

A proper equilibrium between oxidants and antioxidants is crucial for cellular viability. Healthy cells generate controlled levels of reactive oxygen species (ROS), which are effectively counteracted by the cell's antioxidant defenses, ensuring the survival and growth of normal cells (5, 6).Cancer cells generate excessive reactive oxygen species (ROS), yet they avoid oxidative damage by adapting to low-oxygen environments and activating mechanisms that repair cellular harm (5, 6).However, when ROS levels in cancer cells exceed a critical threshold, similar to the effects of chemotherapy, they can trigger cellular damage and death. Conversely, suppressing ROS production may also promote apoptosis in tumor cells. In glioblastoma, these dual roles of ROS in either inhibiting or sustaining cell survival are mediated through key signaling pathways, including MAPK/ERK, PI3K/Akt/mTOR, NF-κB/IKK, and JAK/STAT (7-14).

Formononetin is known as a phytoestrogen and is one of the key active compounds in red clover. Previous research has shown that formononetin has antioxidant, antiviral, and cardioprotective properties (15).Research results show that formononetin effectively suppresses cell growth and metastasis and induces apoptosis in cancer cells by inhibiting several key signaling pathways. This compound plays a dual role in regulating the balance of the oxidant-antioxidant system in cancer cells, both by increasing the level of reactive oxygen species (ROS) and stimulating cell death, and by enhancing the activity of the antioxidant system.However, the exact mechanisms of formononetin's effect on cancer cells, especially in the context of glioblastoma, are still not fully understood and require more extensive and detailed research (16).

Magnolol is another compound obtained from the bark of the magnolia tree and has a variety of medicinal properties, including anti-inflammatory, antioxidant, neuroprotective, and anticancer effects. It modulates cell cycle proteins to inhibit tumor growth and protect neurons from hypoxic injury (17). Previous studies have shown that magnolol inhibits growth, proliferation, and metastasis, as well as induces apoptosis in various cancers such as lung, breast, and colon, through various cell signaling pathways (18-20). Reports have shown that magnolol acts as both a pro-oxidant and an antioxidant. This compound induces apoptosis in cancer cells by producing ROS.On the other hand; it protects the cell by increasing antioxidant enzymes. The effect of magnolol on the oxidant-antioxidant system in tumor cells, including glioblastoma, is not very clear and further studies are needed (21).

In this study, we hypothesized that formononetin and magnolol could induce death in glioblastoma cells by altering the level of cellular oxidative stress. Therefore, we investigated the effect of these two compounds, alone and in combination, on the antioxidant system, growth, migration, and apoptosis of glioblastoma cells.

Methods

Cell culture

The T98G human glioblastoma cell line (Pasteur Institute of Iran, Tehran وIran) was cultured in DMEM medium (Gibco, USA). This medium was enriched with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml of Amphotericin-kanamycin (Gibco), 2 mM of L-glutamine, and 1% sodium pyruvate. The cells were kept at a temperature of 37 °C in a humidified incubator with an atmosphere of 5% CO_2. Treatments commenced once the cultures achieved approximately 80% confluence.

MTT assay

The cytotoxic impacts of formononetin and magnolol on T98G Glioblastoma cells were assessed using the MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay. The experimental groups consisted of five categories: formononetin, magnolol, combination of formononetin

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and magnolol, blank control, and solvent control. Formononetin and magnolol were each dissolved in dimethyl sulfoxide (DMSO) to prepare 100 mM stock solutions and then diluted in culture medium to the indicated final concentrations. The final concentration of DMSO in all treatment groups (including single-agent and combination groups) did not exceed 0.1% (v/v). The solvent control group received culture medium containing 0.1% DMSO (v/v) without any compounds. Cells were plated in a 96-well plate at a density of 8×10^3 cells per well and incubated overnight. The following day, the cells were treated with varying concentrations of the compounds (0-160 µM) in triplicate for duration of 24 h. The cytotoxicity of the compounds was evaluated using the MTT Cell Proliferation Kit (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the established protocol. The absorbance (A) of each well was measured at 570 nm with a microplate reader (Awareness Technology, Palm City, FL, USA). The survival rate was calculated using the formula: SR (%) = (A Treatment / A Control) × 100%. The IC_{50} values were determined utilizing GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

Analysis of combined drug effects

The combination effect of Formononetin and Magnolol on T98G cells was assessed using Chou-Talalay method to describe combination index (CI) (22, 23). The data obtained from MTT assay were transformed to fraction affected values (Fa) in a range of 0 (100% cell survival) to 1 (0% cell survival). To assess Fa values CompuSyn program (ComboSyn Inc., Paramus, NJ, USA) was used. Afterwards, the CI values were used to determine the relationship between the components. A CI bigger than 1 represents for antagonism, a CI equals to 1 show an additive effect and a CI less than 1 indicates a synergism effect.

Cell growth assay

The effects of formononetin and magnolol on cell growth were evaluated through the trypan blue staining technique. T98G cells were maintained in a 6-well plate at a concentration of 2×10^5 cells per well and treated with IC_{50} doses of formononetin, magnolol and formononetin and magnolol in combination for a span of 5 days. Each day, the cells were harvested via trypsinization, and the cell suspension was utilized for the staining process. The cells were stained with 0.4% trypan blue. Cell counting was performed using a hemocytometer and an inverted microscope (Nikon Instrument Inc., Melville, NY, USA). The viability percentage of the cells was calculated according to the formula: (N Treatment /N Control) ×100.

Quantitative real-time PCR (qRT-PCR)

The qPCR technique was utilized to explore the effects of formononetin and magnolol on gene expression. Total cellular RNA was extracted using the RNA extraction kit (Parstous, Tehran, Iran) after a 24 h treatment of cells with formononetin and magnolol, alone and in combination. Complementary DNA was synthesized from the extracted RNA using oligo-dT primers and the Easy cDNA Synthesis Kit (Parstous). The qPCR method was employed to quantify the expression of various genes using SYBR Green qPCR Master Mix (Parstous, Tehran, Iran) in the Light Cycler 96 System (Roche). The total volume of the qPCR reactions 10 µl of SYBR Green, 6 µl of distilled water, 1 µl forward primer, 1 µl reverse primer and 2 µl of template cDNA. The specific sequences of primers for each gene are shown in Table 1.

The qPCR reactions were initiated with a pre-incubation step at 95 °C for 10 min, after which 35 cycles were conducted. The first step of each cycle consisted of a 15 sec incubation at 95 °C, followed by an annealing step at 55 °C for 40 sec, and a final extension step at 72 °C for 35 sec. The results were analyzed using the comparative $2^{-(\Delta\Delta Ct)}$ method (24, 25), with β-actin regarded as the internal control. The amplification efficiency for each primer pair was determined by constructing standard curves using serial 5-fold dilutions of cDNA (ranging from 1:1 to 1:625). Efficiency (E) was calculated as $E = (10^{(-1/slope)} - 1) \times 100\%$. Following each qPCR run, melt curve analysis was performed by gradually increasing the temperature from 60 °C to 95 °C at a ramp rate of 0.1 °C/s with continuous fluorescence acquisition.

Wound healing

To examine the impact of formononetin and magnolol on cell migration and invasion, a wound healing assay was conducted. T98G cells were seeded in a 6-well plate (5×10^5 cells per well), and a consistent scratch was created on the bottom of the wells using the tip of a micropipette. Subsequently, the cells were treated with IC_{50} concentrations of formononetin and

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magnolol, either individually or in combination, and then incubated for duration of 24 h. The medium was then replaced, and cell migration was assessed using an optical microscope at 24 and 48 h after treatments.

Table 1. The sequence of primers used in real-time PCR

Genes	Sequence (5'→3')	Primer length (base)	Tm (°C)
β-actin FW	GACATCCGCAAAGACCTGTA	20	**57.62**
β-actin RV	GGAGCAATGATCTTGATCTTCA	22	**56.22**
CAT FW	TGGAGCTGGTAACCCAGTAGG	21	**62.7**
CAT RV	CCTTTGCCTTGGAGTATTTGGTA	23	**60.2**
SOD FW	GGTGGGCCAAAGGATGAAGAG	21	**62.7**
SOD RV	CCACAAGCCAAACGACTTCC	20	**61.5**
GPX FW	CAGTCGGTGTATGCCTTCTCG	21	**62.5**
GPX RV	GAGGGACGCCACATTCTCG	19	**62.4**
Survivin FW	ACCGCATCTCTACATTCAAG	20	**55.26**
Survivin RV	CAAGTCTGGCTCGTTCTC	18	**55.11**
Cyclin D1 FW	CAATGACCCCGCACGATTTC	20	**59.87**
Cyclin D1 RV	CATGGAGGGCGGATTGGAA	19	**58.87**
Bad FW	CCCAGAGTTTGAGCCGAGTG	20	**58.6**
Bad RV	CCCATCCCTTCGTCGTCCT	19	**57.4**
MMP-9 FW	AGACCTGGGCAGATTCCAAAC	21	**62**
MMP-9 RV	CGGCAAGTCTTCCGAGTAGT	20	**61.3**

FW: Forward, RV: Reverse

Colony formation assay

T98G glioblastoma cells were seeded into 6-well plates at a density of 2×10^4 cells per well in triplicate and incubated overnight. The next day, the cells were treated with IC_{50} concentration of formononetin and magnolol, both alone and in combination, for 24 h. The culture was then replaced with fresh medium, and the cells were incubated for a week. A fixing solution of methanol/acetic acid (1:7) was applied to the colonies, followed by staining with crystal violet (0.5%). The colonies were photographed and evaluated for the colony formation ratio. ImageJ software (NIH, USA) was used to automate counting, and particles with an area corresponding to ≥ 50 cells were counted.

Hoechst staining

The impact of formononetin and magnolol on the apoptosis of T98G cells was evaluated through Hoechst staining. The cells were plated in a 12-well plate at a density of 1×10^5 cells per well and incubated for duration of 24 h. Following this, the cells were subjected to treatment with IC_{50} concentration. After another 24 h of incubation, the cells were rinsed with PBS twice and subsequently fixed with 3.7% formaldehyde, followed by staining with Hoechst 33342 (5 µg/ml) for 30 min. Next, morphological alterations indicative of apoptosis was examined under a fluorescent microscope.

Oxidative stress

We next investigated the effect of formononetin and magnolol on the activity of important antioxidants enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). Moreover, the effect of these compounds on total antioxidant capacity (TAC) was explored. In each experimental group, 1×10^6 cells were grown in 25 cm² flasks. After 24 h incubation, the cells were treated with the IC_{50} concentrations of the compounds, either alone or in combination. Twenty-four hours after treatment, the enzymatic activities and total antioxidant capacity were measured using specialized assay kits, following the manufacturer's instructions (Arman Biotech, Tehran, Iran).

Statistical analysis

Quantitative results are shown as the mean ± standard deviation (SD), with at least three separate

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experiments performed for each measurement. Differences between groups were assessed using a one-way ANOVA, and individual group comparisons were made using Student's t-test. All statistical analyses were done with GraphPad Prism software (version 5.0, GraphPad, San Diego, CA, USA). Results with a *p*-value of 0.05 or lower were considered statistically significant.

Results

Formononetin enhanced the cytotoxic effect of magnolol in glioblastoma cells

To determine if formononetin could enhance the cytotoxic effect of magnolol in glioblastoma cells, a combination treatment of formononetin and magnolol was executed on T98G cells. The results obtained from the MTT assay revealed that treatment with formononetin and magnolol individually led to a significant reduction in the cell survival rate in a dose-dependent manner when compared to the blank control (Figure 1 A).

Moreover, the combination treatment further lowered the cell survival rate in comparison to the treatments with formononetin or magnolol alone ($p<0.05$). The IC_{50} values for formononetin and magnolol after 24 h of treatment were 29.3 and 73.8 µM in T98G cells. The combination treatment with formononetin and magnolol resulted in a marked decrease in the IC_{50} value relative to the individual treatments. These data suggest that formononetin can enhance the cell survival inhibition ability of magnolol in glioblastoma cells.

The synergistic effect of formononetin and magnolol on glioblastoma cancer cells was observed

To determine if the effect of formononetin and magnolol on cell survival is the cause of their synergistic interaction, we executed a combination index analysis based on the non-constant method of Chou-Talalay. The CI-Fa plot revealed that the combination of formononetin (0-160 µM) with magnolol (0-160 µM) on glioblastoma cells resulted in a synergistic interaction (CI<1). Notably, the significant synergistic effect was observed after 24 h of treatment, particularly with a combination of 80 µM formononetin and 80 µM magnolol (CI=0.72), with a Fa level of 0.92 in the T98G cell line (Figure 1 B).

Formononetin enhanced the inhibitory effect on cell proliferation caused by magnolol

We subsequently analyzed the effects of formononetin and magnolol on the proliferation of glioblastoma cells. The cell proliferation assay revealed that, when compared to the control groups, the viability of cells in the formononetin, magnolol, and combination groups significantly decreased in a time-dependent manner. In T98G cells, the viability after 24 h of treatment was recorded at 63. 6%, 71.1%, and 61.7% for the formononetin, magnolol, and combination groups, respectively, which further declined to 19.5%, 27.4%, and 18.4% by the end of the experiment ($p<0.05$; Figure 1 C). These findings indicate that the combined effect of the two agents on inhibiting cell proliferation is more effective than their individual actions.

Formononetin and magnolol altered the gene expression pattern in T98G cells

In order how investigate how formononetin and magnolol influence the expression of genes pertinent to the cell cycle, cell survival, and apoptosis, qPCR was utilized. In our study, all RNA samples had A260/A280 ratios between 1.9 and 2.1, indicating high purity. Following DNase I treatment, the absence of genomic DNA contamination was confirmed by no-reverse transcriptase controls (-RT controls) , which showed no amplification (undetected or $Ct \geq 35$). All primer pairs exhibited amplification efficiencies between 90% and 105% (slope range: -3.2 to -3.4) with correlation coefficients (R^2) greater than 0.99. Melt curve analysis confirmed a single product for each amplicon, and no template controls (NTC) remained negative. For all genes, a single sharp peak was observed at the expected melting temperature (Tm), confirming specific amplification and the absence of primer-dimer formation or non-specific products. The results demonstrated that treatment with formononetin and magnolol, whether separately or in combination, significantly affected the expression of MMP-9, CAT, survivin, cyclin D1, SOD, and GPX genes in comparison to control groups ($p< 0.05$, Figure 2).

Twenty-four hours exposure of the cells to the IC_{50} of these agents led to a decrease in the expression of survivin, cyclin D1, and MMP-9 ($p<0.05$), while the expression of SOD, CAT, GPX, and Bad increased ($p<0.05$). Additionally, there was no notable difference

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in gene expression between the blank control and solvent control groups (p>0.05).

The migration rate of glioblastoma cells was diminished by formononetin and magnolol

To evaluate the effect of formononetin and magnolol in cancer cell migration and metastasis, a wound healing assay was performed. The results indicated that treatment with the IC_{50} of these compounds reduced migration in T98G cells when compared to the blank control and solvent control groups (Figure 3). The effect of combination treatment on inhibition of cell migration was greater than single treatment. No apparent differences were observed in the control groups

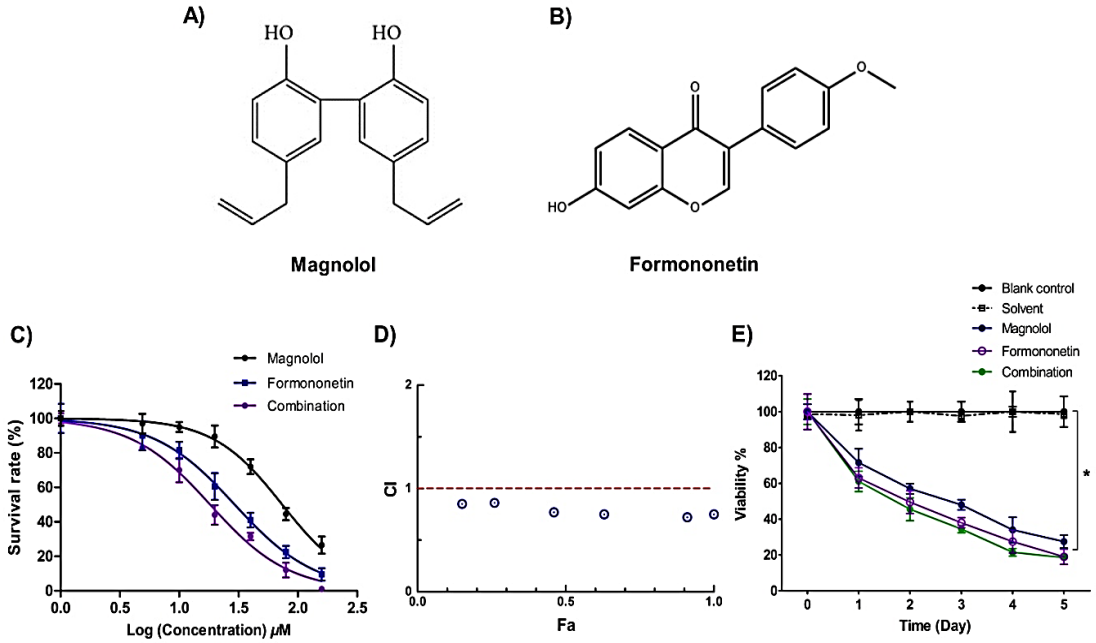

Figure 1. Effects of magnolol and formononetin on glioblastoma cell survival and proliferation. (A-B) Dose-response curves of cell viability after 24 h treatment with magnolol (A) or formononetin (B). (C) Cell survival curves generated from MTT assay data. (D) Combination index (CI) values calculated using CalcuSyn software, demonstrating synergistic effects (CI < 1). (E) Growth inhibition curves over time. Data represent mean ± SD of three independent experiments (*p<0.05 vs. control).

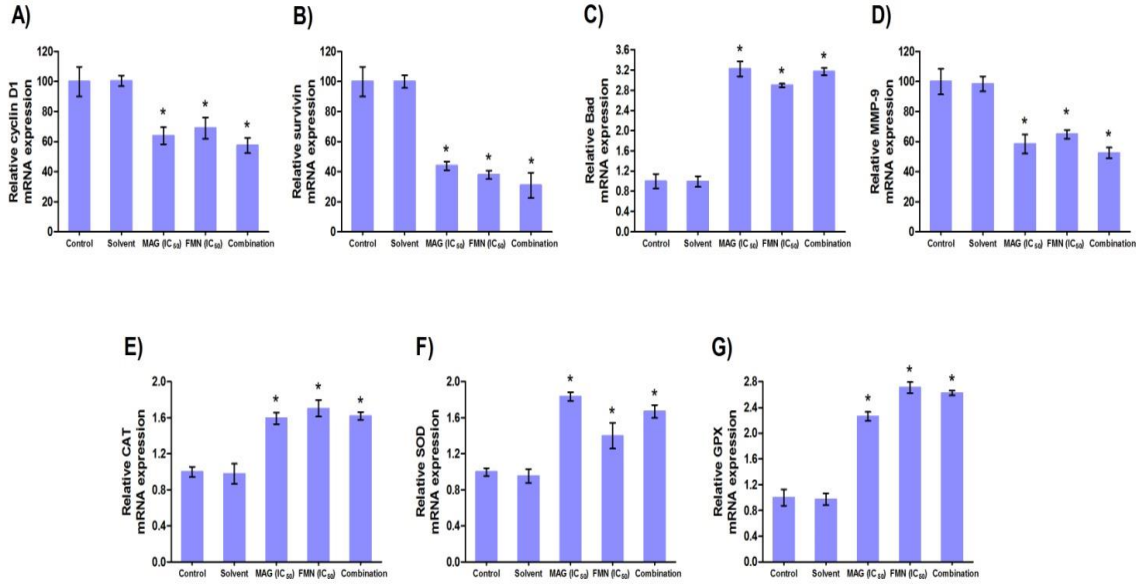

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Figure 2. The modifications in gene expression within T98G glioblastoma cells subsequent to treatment with magnolol and formononetin. A 24-hour exposure to the compounds at IC_{50} concentrations was followed by an analysis of relative mRNA expression levels for the genes (A) cyclin D1, (B) survivin, (C) Bad, (D) MMP-9, (E) CAT, (F) SOD, and (G) GPX. The gene expression was determined via quantitative reverse transcription PCR (RT-qPCR), employing the $2^{(-\Delta\Delta Ct)}$ method for calculation. Findings are displayed as the mean ± standard deviation from three separate experimental replicates. $^{*}p<0.05$ versus the control groups. MAG: magnolol; FMN: formononetin.

T98G cells exhibited a reduced number of colonies following a 24 h exposure to formononetin and magnolol

The ability to form colonies is a fundamental characteristic of cancer cells. To assess the effects of formononetin and magnolol on T98G cells, a colony formation assay was performed. The results revealed that treatment with the IC_{50} of the two compounds suppressed the capacity for colony formation in glioblastoma cells (Figure 3).

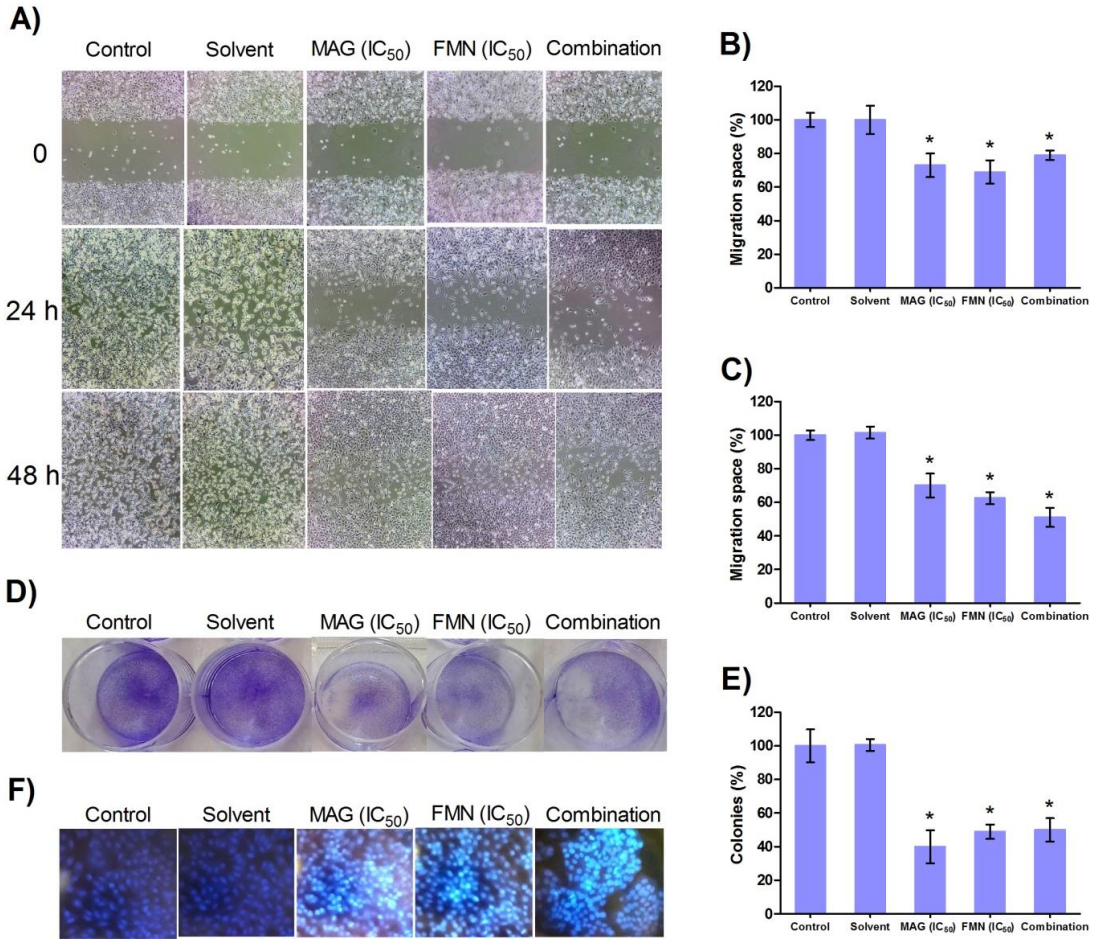

Figure 3. Magnolol and formononetin suppress migration, reduce colony formation, and trigger apoptosis in glioblastoma cells. T98G cells were subjected to treatment with these compounds at their IC_{50} concentrations. The capacity for cell migration was determined by quantifying the areas of wound closure at time points of 24 (A and B) and 48 hours (A and C). The ability to form colonies was evaluated following exposure to formononetin (D and E). Morphological changes indicative of apoptosis within cell nuclei were inspected via Hoechst 33342 staining and fluorescence microscopy after a 24-hour treatment period (F). The presented results are representative of three separate experimental trials. *$p<0.05$ compared to control groups. MAG: magnolol; FMN: formononetin.

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Moreover, a significant reduction in colony formation was observed in combination group. No clear differences were found between the blank control and the solvent control groups

Formononetin and magnolol led to the induction of apoptosis in T98G glioblastoma cells

To investigate how formononetin and magnolol can trigger apoptosis in T98G cells through morphological alterations in the nucleus, Hoechst staining was conducted. As shown in Figure 3, after 24 h of treatment, the cells exposed to formononetin and magnolol displayed enhanced nuclear fragmentation and chromatin condensation in comparison to the blank control group. No significant differences were observed between the apoptotic features of the blank control and the solvent control groups.

Formononetin and magnolol enhanced the activity of the antioxidant enzymes and improved the total antioxidant capacity

The role of formononetin and magnolol in modulating the activity of antioxidant enzymes like SOD, GPX, and CAT was investigated through the use of dedicated kits. The results revealed that the activity of these enzymes was enhanced following a 24 h treatment with the IC_{50} doses of formononetin and magnolol when compared to the control groups. This increase in enzymatic activity subsequently resulted in a rise in the total antioxidant capacity of the cells ($p<0.05$). The effect of combination treatment on antioxidant activity of the cells was greater than single treatment. No significant differences were noted between the blank control group and the solvent control group ($p>0.05$; Figure 4).

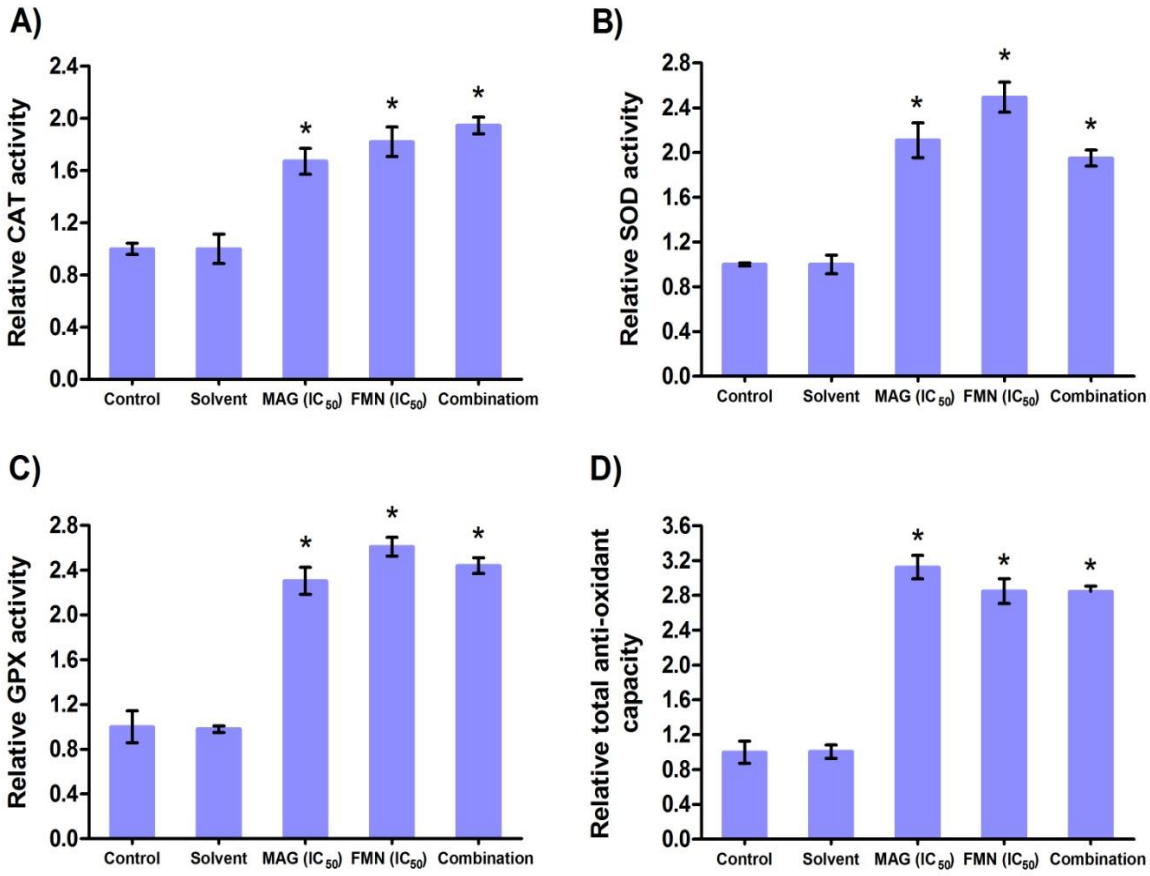

Figure 4. The impact of magnolol and formononetin on the antioxidant defense system in T98G cells. Following a 24-hour treatment period with magnolol and formononetin at IC_{50} concentrations, a significant increase was observed in the activity of the antioxidant enzymes (A) catalase (CAT), (B) superoxide dismutase (SOD), and (C) glutathione peroxidase (GPX). (D) The overall total antioxidant capacity of the cells was also markedly enhanced. The data, expressed as mean ± standard deviation (n=3), demonstrate a statistically significant difference ($^*p<0.05$) when compared to the control groups. MAG: magnolol; FMN: formononetin.

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Discussion

Glioblastoma multiform represents the most aggressive primary brain tumor in adults, with median survival under two years despite multimodal treatment. Its therapeutic resistance stems from invasive growth patterns, molecular heterogeneity, and the blood-brain barrier limiting drug efficacy. Novel therapeutic strategies targeting underlying molecular mechanisms are urgently needed (18, 26). This study investigates two natural compounds, formononetin and magnolol, for their combined effects on oxidative stress modulation in glioblastoma cells, addressing a critical research gap in neuro-oncology.

This study demonstrates potent synergistic cytotoxicity between formononetin and magnolol in T98G glioblastoma cells, with combination index values reaching 0.72 at specific concentrations (80 µM each). The observed IC_{50} reduction (29.3 µM formononetin and 73.8 µM magnolol versus 18.2 µM in combination) reveals substantial enhancement of anticancer effects through combination therapy, potentially allowing lower effective doses and reduced toxicity. These findings align with (19), who emphasized rational drug combinations to overcome therapeutic resistance in solid tumors by targeting complementary pathways. The synergistic suppression of colony formation and cell proliferation corroborates magnolol's established anti-tumorigenic properties in hepatocellular carcinoma, where it inhibits HDAC expression and metastasis (17), but extends its applicability to neuro-oncology through this novel pairing. The superiority of the combination over monotherapy is best demonstrated by dose equivalence. The combination achieved comparable or greater inhibition of proliferation, migration, and colony formation using significantly lower individual doses (≈18–20 µM each) than the IC50 of formononetin (29.3 µM) or magnolol (73.8 µM) alone. Thus, across all functional assays, the combination is more effective because it produces stronger or equal effects at much lower concentrations, reflecting true synergy.

Contrasting with studies reporting formononetin's pro-oxidant lethality in lung cancer (27), our data revealed concurrent upregulation of antioxidant enzymes (SOD, CAT, GPX) and total antioxidant capacity during combination treatment. This paradoxical response parallels with Olivier et al.'s (5) "two faces of oxidative stress" model, where glioblastoma cells activate compensatory antioxidant mechanisms against redox-targeting agents as a survival tactic. However, our observed downregulation of survivin and cyclin D1, despite antioxidant induction, demonstrates successful disruption of pro-survival signaling, contrasting with Kumari et al. (7) observations of ROS exploitation in cancer proliferation and highlighting the combinatorial strategy's efficacy in bypassing redox adaptations.

The potent MMP-9 suppression and significant migration inhibition align with Guoet al.'s (28) identification of migration biomarkers in glioblastoma, suggesting the combination targets core invasive pathways like EMT and matrix remodeling. Unlike conventional temozolomide monotherapy showing limited survival benefits (26), this dual phytochemical approach simultaneously impairs multiple cancer hallmarks including proliferation, apoptosis resistance, and invasion. The absence of solvent control effects reinforces the specificity of the phytochemical actions. While nanocarrier strategies improve drug delivery (4), our combinatorial efficacy at low micromolar concentrations suggests intrinsic bioactivity merit even before delivery optimization.

Notably, the synergistic apoptosis induction via nuclear fragmentation supports Benhar et al.'s (20) framework of ROS-mediated stress kinase activation in cancer cell death. However, the significant antioxidant enzyme elevation contrasts with magnolol's reported pro-oxidant effects in breast cancer, highlighting glioblastoma-specific redox adaptations and the context-dependency of phytochemical mechanisms. This cell-type specificity necessitates further investigation into tumor microenvironment influences on phytochemical efficacy, particularly regarding Nrf2 pathway activation dynamics observed in other neuroprotective contexts but unexplored here. The combination's ability to suppress long-term clonogenic survival suggests potential for reducing recurrence, a major clinical challenge in glioblastoma management post-resection.

The synergistic effects likely involve complementary targeting of redox-sensitive pathways. Formononetin's disruption of PI3K/Akt signaling, evidenced by survivin downregulation, compromises anti-apoptotic defenses, while magnolol's HDAC inhibition impairs DNA repair mechanisms. Concurrent Bad upregulation promotes Bax

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oligomerization and mitochondrial outer membrane permeabilization (MOMP), facilitating cytochrome c release and caspase-9/3 activation despite elevated antioxidant enzymes. The MMP-9 suppression impedes extracellular matrix degradation, explaining the marked migration inhibition.

The paradoxical antioxidant response strongly suggests Nrf2 pathway activation as a compensatory mechanism against phytochemical-induced oxidative stress, consistent with Moloney et al.'s (6) model of cancer redox adaptation. However, it is important to note that while the coordinated upregulation of SOD, CAT, and GPX is consistent with Nrf2 pathway activation, we did not directly interrogate Nrf2 nuclear translocation or its transcriptional activity. Therefore, the precise role of Nrf2 in mediating the observed compensatory antioxidant response remains inferential. Direct measurement of Nrf2 target genes such as NQO1, HO-1, and GCLC, along with confirmation of Nrf2 nuclear accumulation, would be required to establish a causal link between the combination treatment and Nrf2-dependent antioxidant gene expression. Accordingly, we have identified this as a critical direction for future mechanistic investigation. However, the net pro-apoptotic outcome indicates that combinatorial signaling disruption overrides this defense. Specifically, cyclin D1 suppression induces G1/S arrest via Rb protein hypophosphorylation, while survivin inhibition disables IAP-mediated caspase blockade. Mechanistically, magnolol may initiate ROS generation through mitochondrial complex I perturbation, while formononetin simultaneously inhibits NF-κB-dependent survival pathways and modulates JAK/STAT signaling, contributing to Bad activation.

Their combination creates irreconcilable redox stress: antioxidant enzymes neutralize basal ROS but cannot compensate for disrupted pro-survival signaling cascades and direct mitochondrial apoptosis induction. This aligns with Zhang et al.'s (10) findings that ROS-independent JNK/STAT pathway modulation can induce apoptosis despite antioxidant upregulation. The observed gene expression shifts confirm simultaneous induction of oxidative damage responses (antioxidant genes) and apoptotic machinery (Bad), ultimately overwhelming cellular homeostatic mechanisms through multi-target inhibition. The temporal sequence likely involves initial ROS generation by magnolol triggering Nrf2-mediated antioxidant transcription, followed by formononetin's sustained blockade of repair pathways like PI3K/Akt and JAK/STAT, preventing recovery and driving cells towards the apoptotic threshold via intrinsic pathway activation (9, 12). The concurrent upregulation of antioxidant enzymes and induction of apoptosis in our study exemplifies a phenomenon increasingly recognized in cancer redox biology: redox resilience.

This term describes the ability of cancer cells to transiently adapt to oxidative stress by activating Nrf2-driven antioxidant programs, thereby avoiding immediate cell death. However, when oxidative stress exceeds a critical threshold or when compensatory pathways are simultaneously inhibited, this adaptive resilience is overwhelmed, and cell death ensues (5, 29). In summary, the "paradoxical" antioxidant response we observed is not a contradiction but rather a reflection of the temporal and contextual dynamics of redox resilience: the same treatment that triggers an adaptive antioxidant program can, when combined with sustained disruption of survival signaling, ultimately drive the cell past the apoptotic threshold. Direct testing of this temporal model will require time-resolved measurements of ROS, Nrf2 activation, and caspase activity in future studies.

A key concept that our cross-sectional (24-hour) measurements cannot capture is the temporal shift from adaptation to toxicity. We hypothesize that the sequence of events following FMN+MAG treatment involves: (i) an early phase (0–6 hours) characterized by ROS generation and initiation of Nrf2 nuclear translocation; (ii) a middle phase (6–24 hours) marked by elevated antioxidant enzyme expression and activity (the adaptive "redox resilience" response); and (iii) a late phase (>24 hours) where sustained pro-apoptotic signaling (Bad upregulation, survivin downregulation) overrides the compensatory defense, leading to caspase activation and nuclear fragmentation. This temporal model predicts that measuring ROS or Nrf2 activity at a single time point (e.g., 24 hours) may miss the peak of oxidative insult, instead capturing the residual adaptive response. Such a biphasic pattern has been documented for other redox-modulating agents in glioblastoma (27, 30).

Several factors likely determine whether the net outcome of FMN+MAG treatment is survival or death: (i) the magnitude and duration of ROS elevation; (ii) the efficiency of Nrf2 transcriptional activity; (iii) the

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functional status of downstream pro-apoptotic effectors (e.g., Bad, Bax); and (iv) the integrity of compensatory survival pathways (PI3K/Akt, NF-κB). In T98G cells, the combination appears to strike a balance where antioxidant adaptation occurs but ultimately fails to prevent apoptosis, likely because formononetin simultaneously inhibits Akt-dependent survival signals that would otherwise reinforce the Nrf2 response (16). This multi-target interference with both the oxidative insult and the survival machinery distinguishes combination therapy from single-agent treatments, which may allow cells to fully adapt without reaching the apoptotic threshold.

Key strengths of our study include rigorous assessment of synergistic effects through Chou-Talalay methodology and multi-modal evaluation of proliferation, migration, and redox dynamics. The inclusion of combination therapy demonstrates enhanced efficacy in vitro; however, clinical translatability cannot be inferred without in vivo studies and BBB permeability data. However, exclusive use of T98G cells limits generalizability across glioblastoma subtypes. In vivo validation is absent, particularly regarding blood-brain barrier penetration, a critical therapeutic hurdle. The paradoxical antioxidant response warrants deeper temporal analysis to distinguish adaptive versus cytotoxic phases. Several limitations should be acknowledged.

First, although the upregulation of antioxidant enzymes and total antioxidant capacity indirectly suggests redox perturbation, we did not directly quantify intracellular reactive oxygen species (ROS) levels. Consequently, we cannot definitively conclude whether the observed apoptosis results from ROS accumulation, ROS depletion, or ROS-independent signaling. Second, while the expression pattern of SOD, CAT, and GPX is compatible with Nrf2 activation, we did not assess Nrf2 nuclear translocation, its binding to antioxidant response elements (AREs), or the expression of canonical Nrf2 target genes (e.g., NQO1, HO-1, GCLC). Thus, the involvement of the Nrf2 pathway remains speculative at this stage. Primary human cell validation and Nrf2 pathway interrogation would strengthen mechanistic claims. Pharmacokinetic interactions remain uncharacterized. While Hoechst staining revealed nuclear fragmentation and chromatin condensation—morphological features classically associated with apoptosis—we did not quantitatively confirm apoptosis using gold-standard methods. Specifically, Annexin V/PI flow cytometry (to distinguish early apoptosis, late apoptosis, and necrosis) and western blot analysis of executioner caspases (cleaved caspase-3, cleaved PARP) were not performed. Therefore, we cannot definitively exclude the possibility that other forms of regulated cell death (e.g., necroptosis, autophagy-associated cell death) contribute to the observed cytotoxicity. Our claims regarding apoptosis should be interpreted as suggestive rather than conclusive.

Formononetin and magnolol demonstrate synergistic anti-glioblastoma activity by concurrently disrupting proliferation, survival, and migration pathways while ultimately triggering nuclear morphological changes consistent with apoptosis. Despite triggering compensatory antioxidant responses, the combinatorial treatment overwhelms cellular defenses through multi-target inhibition of redox-sensitive signaling networks. The marked downregulation of survivin, cyclin D1, and MMP-9, alongside impaired clonogenicity, supports further in vitro mechanistic investigation, but does not yet justify translational claims. This dual phytochemical approach represents a promising strategy against glioblastoma's therapeutic resistance, meriting further investigation into blood-brain barrier penetration and in vivo efficacy.

Future Directions

Priority should include orthotopic glioblastoma models evaluating intracranial efficacy and pharmacokinetics. Combination studies with TMZ/radiation are essential to assess clinical relevance. Mechanistic work should identify upstream initiators of the antioxidant response using Nrf2-knockdown models. Development of blood-brain barrier-penetrating formulations (e.g., nanocarriers) is crucial. Biomarker studies should correlate antioxidant enzyme profiles with treatment responsiveness in patient-derived cells. To establish causality between formononetin and magnolol treatment and redox-sensitive signaling, future studies must include: (i) quantitative measurement of intracellular ROS levels at multiple early time points (e.g., 1, 6, 12, and 24 hours post-treatment) using fluorescent probes such as DCFH-DA or dihydroethidium (DHE); (ii) direct interrogation of the Nrf2 pathway via

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immunofluorescence or Western blotting for Nrf2 nuclear translocation, along with qPCR or reporter assays for ARE-driven gene expression (e.g., NQO1, HO-1, GCLC); and (iii) loss-of-function experiments (e.g., Nrf2 siRNA or pharmacological inhibition) to determine whether the observed antioxidant response and apoptosis are Nrf2-dependent. Additionally, orthotopic glioblastoma models are required to assess the in vivo efficacy and blood-brain barrier penetration of this combinatorial regimen, and patient-derived GBM cells should be tested to evaluate inter-tumoral heterogeneity.

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