Babol University
Of Medical Sciences

IJMCM, Spring 2026, VOL 15, NO 2

International Journal of Molecular and Cellular Medicine

Journal homepage: www.ijmcmed.org

ORIGINAL ARTICLE

Synergistic effects of Temozolomide and Cannabidiol on the Wnt/β-Catenin Signaling Pathway in Glioblastoma

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ARTICLE INFO

Received: 2026/01/7
Revised: 2026/03/24
Accepted: 2026/06/22

ABSTRACT

Glioblastoma (GBM) remains the most aggressive brain tumor, characterized by rapid proliferation and resistance to temozolomide (TMZ). Emerging evidence suggests that the Wnt signaling through stabilization of β-catenin and expression of the non-canonical ligand Wnt5a, has been implicated in the maintenance of GBM. Cannabidiol (CBD) has emerged as a potential adjuvant that may modulate these pathways to overcome chemoresistance. Therefore, we determined human U87-MG cells treatment with different concentrations of CBD and TMZ determined in 24, 48, and 72hours. Cell viability, combination index, $IC_{50}$and IC_5 were assed with MTT assay. Protein levels of β-catenin, Wnt5a, and AXIN1 were evaluated using western blotting and immunofluorescence assay. Treatment with CBD in combination TMZ significantly reduced U87-MG cell viability in a time and dose dependent manner. The combination index analysis indicated a synergistic interaction between CBD and TMZ, particularly at 48 and72 hours. IC_{50}values decreased significantly enhanced co administration compared to mono therapy, suggesting enhanced chemosensitivity. Western blotting and IF results revealed that down regulation of β-catenin and Wnt5a by increasing AXIN1 expression, supporting modulation of canonical and non-canonical Wnt signaling pathways. These findings demonstrate that CBD potentiates the cytotoxic effects of TMZ in glioblastoma cells by interfering with Wnt/β-catenin signaling pathway that may represent a promising adjuvant strategy to improve therapeutic outcomes in GBM.

Keywords: Glioblastoma multiform, Temozolomide, Cannabidiol, adjuvant therapy, Wnt/β-catenin signaling, β-catenin, Wnt5a, AXIN1

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Cite this article: Samiee L, et al. Synergistic effects of Temozolomide and Cannabidiol on the Wnt/β-Catenin Signaling Pathway in Glioblastoma. International Journal of Molecular and Cellular Medicine. 2026; 15-1368-1381. DOI:10.22088/IJMCM.BUMS.15.2.1368

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[DOI: 10.22088/IJMCM.BUMS.15.2.1368]

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Introduction

Glioblastoma (GBM) is an extremely aggressive primary brain tumor characterized by rapid cellular proliferation, diffuse invasiveness, and poor responsiveness to standard therapeutic approaches (1). Although surgical resection, radiotherapy, and chemotherapy have advanced, the median survival of patients with GBM remains very limited, typically under two years after diagnosis (2). Temozolomide (TMZ), an oral alkylating agent, represents the cornerstone of standard chemotherapeutic treatment for GBM. Despite initial clinical effectiveness, the rapid onset of chemoresistance arising from complex cellular and molecular adaptations severely limits long-term patient outcomes, highlighting an urgent need for innovative chemosensitizing strategies (3). Cannabidiol (CBD), a non-psychoactive constituent of *Cannabis sativa*, has been identified as a promising anti-cancer agent due to its anti-inflammatory, pro-apoptotic and anti-proliferative properties (4,5). Recent evidence suggests that CBD can modulate key oncogenic pathway networks in GBM, including the Wnt/β-catenin pathway, which operates as a crucial driver of tumor development, cancer stem cell maintenance, and therapy resistance (6).

The Wnt/β-catenin signaling pathway plays a pivotal role in controlling cell growth, differentiation and survival; its dysregulation in GBM is heavily associated with enhanced tumor progression, and resistance to apoptosis (7). Within this pathway architecture, several key regulators dictate downstream signaling thresholds and subsequent therapeutic responses. β-Catenin, the principal mediator of canonical Wnt signaling, accumulates and translocates into the nucleus, where it stimulates the transcription of genes associated with proliferation, survival, and TMZ resistance (8,9). Conversely, this signaling cascade is attenuated by an intracellular destruction complex, wherein AXIN1 serves as a critical structural scaffold required to physically capture and mark β-catenin for degradation (10). Furthermore, upstream ligands such as Wnt5 act as secreted extracellular factor that interacts with cell surface receptors such as Fizzled and ROR to regulate downstream Wnt/β-catenin and non-canonical signaling (11).

Emerging preclinical evidence suggests that CBD can actively influence this regulatory network (7-9,12-14). CBD has been shown to enhance TMZ efficacy in glioblastoma cells by inhibiting autophagy and inducing apoptosis, highlighting its potential as a potent chemosensitizer (15). Moreover, preclinical evidence indicates that CBD can suppress β-catenin nuclear localization and upregulate its negative regulators, including AXIN1, further supporting its role as a topological modulator of the pathway (16).

Consequently, the combining CBD and TMZ may exert a multi-level modulatory influence on the Wnt/β-catenin axis, as reflected by coordinated changes in upstream ligands and regulatory scaffolds. Although these findings do not establish direct molecular targeting, the concurrent modulation of Wnt5a, AXIN1, and β-catenin suggests a potential strategy for attenuating baseline TMZ resistance. Because these molecular regulators directly shape TMZ responsiveness, investigating their behavior in a controlled cellular model is essential for understanding the mechanisms underlying chemoresistance.

The U87-MG cell line, a well-established human GBM model, displays dysregulated Wnt/β-catenin activity and therefore provides a suitable platform for assessing how targeted modulation of this pathway influences TMZ sensitivity (12). Findings obtained from U87-MG cells offer mechanistic insights that extend beyond this model, clarifying how alterations in Wnt5a, AXIN1, and β-catenin contribute to therapeutic resistance in GBM and informing broader strategies for pathway-directed intervention (13).

This study aimed to investigate the combined effects of CBD and TMZ on the Wnt/β-catenin signaling pathway in human GBM (U87-MG) cells. We hypothesize that the co-administration of CBD and TMZ can cooperatively modulate this signaling axis at multiple regulatory nodes, thereby overcoming TMZ resistance and enhancing therapeutic efficacy. By elucidating the precise molecular interactions between these compounds and key Wnt/β-catenin regulators, this study provides new insights into the potential of CBD as an effective adjuvant therapy for GBM, paving the way for future translational research and clinical applications.

Methods

Reagents and Antibodies

Ca Cannabidiol (CBD) with ≥99% purity (HPLC) was obtained from Tocris Bioscience (Bristol, UK). The compound was dissolved in ethanol (50 mM initial

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concentration) and stored at -20°C until use. The required CBD concentrations were prepared by dilution in serum-free tissue culture media, ensuring that ethanol concentrations remained below 0.05%. Temozolomide (TMZ) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and ethanol were supplied by Merck Millipore (Darmstadt, Germany). Trypan blue and Trypsin-EDTA 10X were obtained from Sigma Aldrich (St. Louis, MO, USA). High-glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were sourced from Gibco (Thermo Fisher Scientific, Waltham, MA, USA).

Phosphate-buffered saline (PBS) was supplied by Biowest (Nuaillé, France). Primary antibodies against Axin1 (sc-293190), β-catenin (sc-7963), Wnt-5a (sc-365370), and β-actin (sc-47778), along with the secondary antibodies anti-rabbit IgG-HRP (sc-2357) and mouse anti-mouse IgGκ BP-HRP (sc-45051) were purchased from Santa Cruz Biotechnology (Dallas TX, USA).

Cell Line and Culture Conditions

The U87MG human glioblastoma cell line (Uppsala 87 malignant glioma) was sourced from the National Cell Bank of Iran, housed at the Pasteur Institute of Iran. Cells were cultured in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin and maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air. When cultures reached approximately 80% confluence, cells were washed with PBS and detachment using Trypsin-EDTA, followed by 5 minutes of incubation at 37°C to ensure complete detachment. Trypsin was neutralized with complete medium, and cells were centrifuged for 5 minutes before reseeding. After centrifugation, cells were resuspended in fresh complete medium and counted using a hemocytometer. Cells were treated with CBD and TMZ after passage three. All experiments were performed using cells between passages 3-8 to ensure consistency and minimize phenotypic variation.

MTT Assay

The MTT assay was performed to evaluate cell viability following treatment with CBD and/or TMZ. Briefly, 10,000 U87-MG glioblastoma cells were seeded per well in 96-well flat-bottom plates and incubated overnight to allow cell adhesion. Cells were then treated with various concentrations of CBD (2.5 to 160 μM) or TMZ (8.1 to 520 μM) for 48 hours. Additionally, a combined treatment with CBD (2.5 μM) and TMZ (65 μM) was performed under the same conditions. All treatments were performed in triplicate. After drug exposure, 10 μl of MTT solution (final concentration: 0.5 mg/mL) was added to each well, and the cells were incubated for 4 hours at 37°C in the dark condition.

Following incubation, the culture medium was gently removed, and 100 μL of pure DMSO was introduced into each well to solubilize the formazan crystals. The optical density (OD) was measured at 570 nm using a microplate reader. Cell viability percentages were calculated using the following equation: % of cell viability = (absorbance of treated cells/absorbance of control cells) × 100

The IC_{50}values of CBD and TMZ were determined using GraphPad Prism 8.

Median Effect Analysis

To characterize the pharmacological interaction between CBD and TMZ, the Chou-talalay median-effect method was applied (17). Dose-response data obtained from the MTT assay were used to generate the median-effect plot for each drug, from which the madian-effect dose (Dm), slope (m), and linear correlation coefficient (r) were derived. These parameters describe the potency and the shape of the dose response curve and serve as the basis for quantitative drug-interaction analysis. for combination treatments, the fraction affected at each dose level was calculated and entered into CompuSyn Software (Biosoft, Cambridge, UK).The software computes the combination index (CI) providing a quantitative evaluation of the interactions between CBD and TMZ. CI values were interpreted as follows: synergism was defined by CI < 1, additivity by CI = 1, and antagonism by CI > 1.

To evaluate drug interactions across different effect levels, three fixed-ratio combination designs were used: (i) an IC_{50}-based ratio derived from the individual IC_{50} values of TMZ and CBD, (ii) combinations using the IC_5 of TMZ with IC_{50} and sub-IC_{50} concentrations of CBD, and (iii) combinations using the IC_5 of CBD with IC_{50} and sub-IC_{50}

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concentrations of TMZ. CI-Fa plots were generated automatically by CompuSyn to visualize the interaction profile across the full range of effects.

Protein Extraction and Western Blotting

Protein extraction was performed using RIPA lysis buffer supplemented with a protease and phosphatase inhibitor cocktail. Following treatment, U87-MG cells were collected by scraping, and the lysates were clarified by centrifugation. Protein concentrations were quantified using the Bradford assay (Thermo Scientific), with absorbance measured at 595 nm on a BioTek microplate spectrophotometer. Equivalent amounts of protein (40 µg per well) were resolved by SDS-PAGE using a Mini-PROTEAN electrophoresis system (Bio-Rad).

Proteins were transferred onto PVDF membranes. The membranes were incubated in 5% non-fat dry milk prepared in TBST (Tris-buffered saline containing 0.1% Tween-20) for 1 hour at room temperature. The primary antibodies against Axin1, Wnt5a, and β-catenin were applied at a 1:300 dilution and incubated with the membranes overnight at 4°C. Following TBST washes, the membranes were exposed to HRP-linked secondary antibodies (1:1000) for 1 hour at room temperature.

Signal development was achieved with enhanced chemiluminescence (ECL) reagents and recorded using a gel documentation system. Protein expression levels were quantified by densitometric analysis in ImageJ software, and β-actin was used as the loading control.

Immunofluorescence assay (IF)

To examine protein localization, U87-MG cells were seeded onto glass coverslips and allowed to attach overnight. A 4% paraformaldehyde solution was used to fix cells for 20 minutes at room temperature. Following fixation, permeabilization was performed using 0.1% Triton X-100 for 10 minutes and the cells were then blocked with 5% bovine serum albumin (BSA) for 1 hour. Primary antibodies for β-catenin and Wnt5a (1:300) were incubated overnight at 4°C. after PBS washes, HRP-linked secondary antibodies (1:1000) were added for 1 hour at room temperature. The reaction was developed using 3, 3′-diaminobenzidine (DAB), and images were acquired using an Olympus IX73 inverted fluorescence microscope. Fluorescence intensity values were analyzed in ImageJ software.

Statistical Analysis

Data are shown as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA). One-way ANOVA was used to assess differences among groups, followed by Tukey's post hoc test for multiple comparisons. A p-value < 0.05 was taken as a statistically significant.

Results

The effect of TMZ on the survival rate of U87-MG cell line

Cell viability of U87-MG cells under TMZ treatment was measured using the MTT assay at 24, 48 and 72 hours (Figure 1). Treatment with TMZ resulted in a significant decline in cell viability compared with the control, exhibiting a clear dose- and time-dependent response. At 24 hours, high concentrations of TMZ (32 -520 µM) significantly decreased cell viability, with survival rates ranging from 85.17% to 44.11%. After 48 hours, viability significantly declined, ranging from 93.56% at low doses to 12.8% at high doses. By 72 hours, a significant reduction was evident across all concentrations, with survival rates ranging from 65.32% to 2.68%, confirming the cytotoxic effect of TMZ on U87-MG cells.

The effect of CBD on the survival rate of U87-MG cell line

The U87-MG cell viability under CBD treatment was evaluated with the MTT assay at 24,48 and 72 hours (Figure 2). Treatment with CBD resulted in a significant decline in cell survival compared with the control. At 24 hours, cell viability markedly decreased at high concentrations of the CBD (10 to 160), with survival rates ranging from 91.57 to 37.9%. After 48 hours, viability significantly decreased, reaching 94.67% at low concentration and 15.44% at high concentration. By 72 hours, an enhanced reduction was observed across all concentrations, with survival rates ranging from 73.74% to 6.3%.

The effect of co-administration of TMZ and CBD on the survival rate of U87-MG cell line

To identify the optimal combined concentration where TMZ and CBD exert a synergistic effect on reducing U89-MG cell viability, three different steps of combined MTT assays were performed: In the first step

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of the combined MTT assay, a fixed ratio of 1:2.67 was established based on the independent IC_{50} values obtained for TMZ and CBD (43.89 µM and 45.33 µM, respectively).

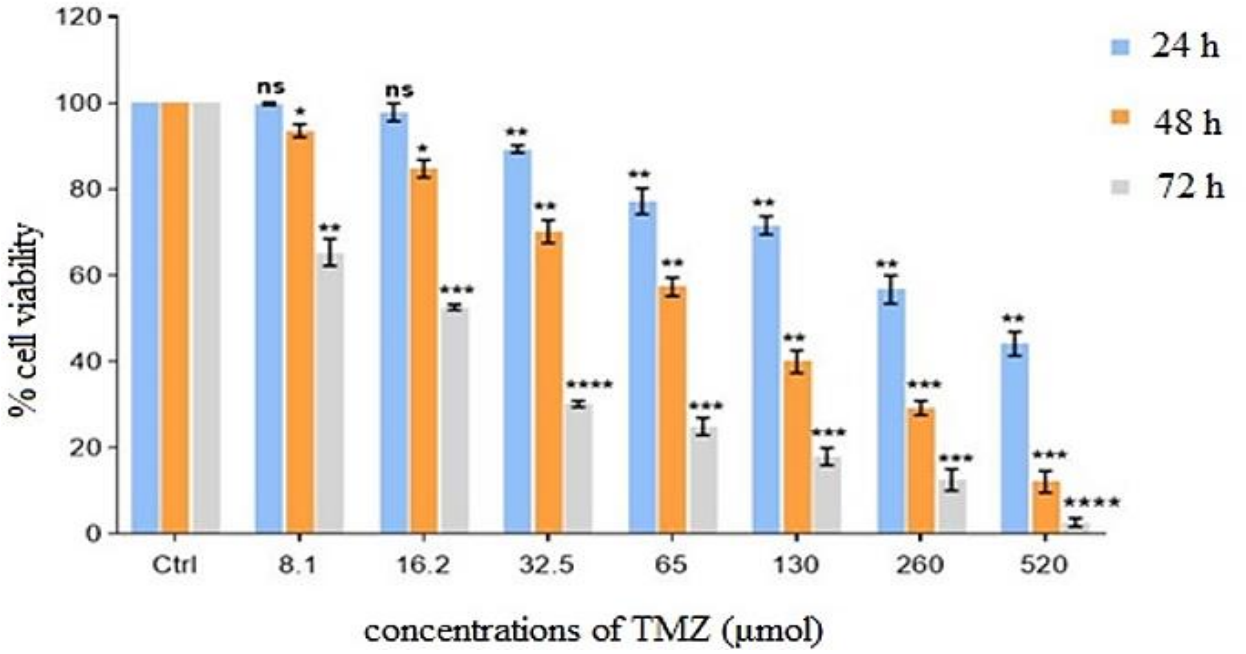

Figure 1. Effect of TMZ on the survival rate of the U87-MG cell line. Cells in the control group received an equal volume of drug-free culture medium. Data represented as mean ± SEM. Statistical significance was determined relative to the control group (*P<0.01, **P< 0.001, and ***P <0.0001).

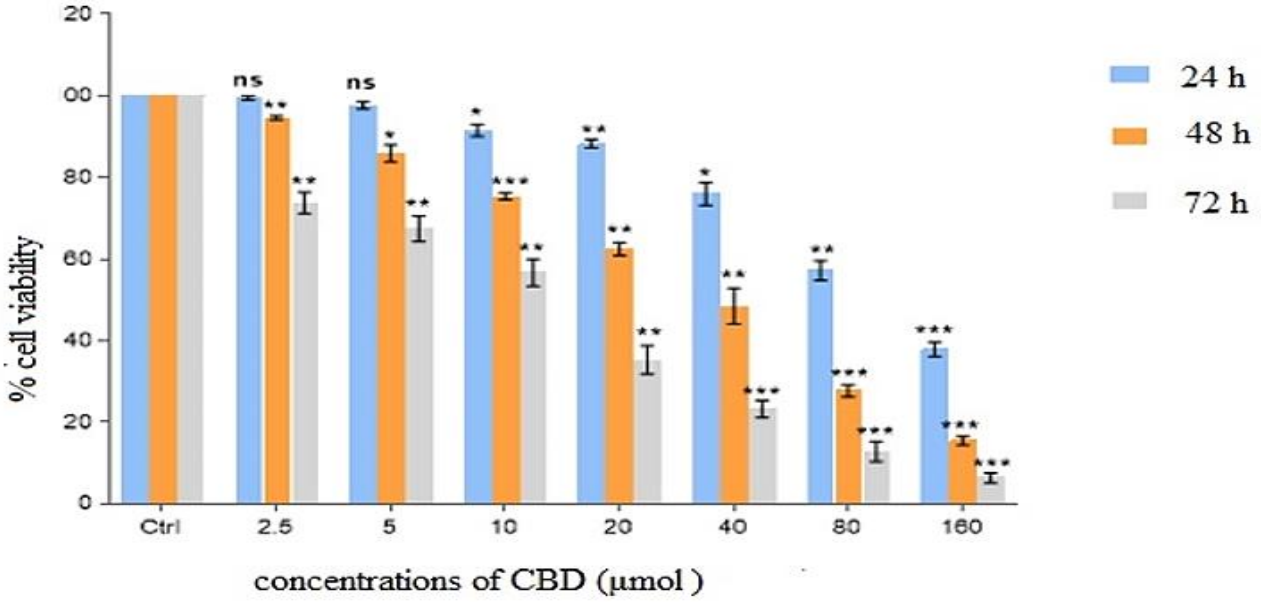

Figure 2. The effect of CBD on the survival rate of U87-MG cells. Cells in the control group recieved an equal volume of drug-free culture medium. Data are represented as mean ± SEM. Statistical significant was determined relative to the control group (*P<0.01, **P< 0.001, and ***P <0.0001).

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Using this constant ratio, three concentrations below the IC_{50} were selected and tested. The results showed that although the combined drugs decreased U89-MG cell survival in a dose-dependent manner (Fgure 3.A), the interaction between TMZ and CBD at all tested concentrations with this fixed ratio was antagonistic (CI>1) (Figure 3.B). In the second step of the combined MTT assay, U89-MG cells were treated with a variable concentration (IC_{50} and sub-IC_{50}) of CBD in combination with a fixed IC_5 concentration of TMZ. Although these findings indicated dose-dependent reduction in cell survival (Figure 3.C), the interaction between all tested combinations remained entirely antagonist (CI > 1) (Figure 3D).

In the third step of the combined MTT assay, U87-MG cells were treated with a variable concentration (IC_{50} and sub-IC_{50}) of TMZ combined with a fixed IC_5 concentration of CBD (2.5 µM). The findings revealed that cells treated with these combined concentrations exhibited a significantly lower survival rate compared to the untreated control group, displaying a clear dose-dependent response.

Notably, the results indicated that the interaction between TMZ and CBD was additive (C = 1) only at the specific combination of 2.5 µM CBD and 1.8 µM TMZ, while all other concentration combinations showed a synergistic interaction (CI < 1) (Figure 3.E). The maximum synergistic effect (CI = 0.37) was achieved when the baseline IC_{50} concentration of TMZ (43.89 µM) was combined with the IC_5 of CBD (2.5 µM), leading to a 76% inhibition of cell growth. (Figure 3F).

Based on these outcomes, the final concentration selected for subsequent downstream molecular experiments were determined to be 65 µM of TMZ and 2.5 µM of CBD. This specific concentration was chosen due to it achieved effective cell inhibition within the desired IC_{50} range while maintaining a robust and desirable synergistic interaction (CI=0.69).

Expression β-Catenin following Co-Administration of TMZ and CBD on U87-MG cell line

To investigate β-Catenin protein expression, we performed IF-saining and western blot assay (Figures 4 A-D). In IF-staining revealed that both CBD group (P = 0.0004) and TMZ group (P = 0.0001) significantly reduced β-Catenin expression compared to the control group. Moreover, β-Catenin levels were significantly lower in the CBD+ TMZ group compared to CBD group (P < 0.0001). Semi-quantitative Western Blot analysis confirmed these findings: TMZ reduced β-Catenin to 29% of control group, while CBD decreased levels to 36%. Notably, co-administration of CBD and TMZ suppressed β-Catenin expression to 15% of control, consistent with a synergistic inhibitory effect on WNT/ β-Catenin signaling.

Expression Wnt5a following Co-Administration of TMZ and CBD on U87-MG cell line

To determine Wnt5a protein expression, we used IF-staining and western blot analysis (Figures 5 A-D). Treatment with CBD (P < 0.0001) and TMZ (P = 0.0002) resulted in a significant reduction of Wnt5a levels compared with the control group. Moreover, Wnt5a expression significantly decreased in the CBD+TMZ group compared to the both control group (P < 0.0001) and TMZ group (P = 0.04). Furthermore, the TMZ group exhibited a significant reduction in Wnta5 expression compared to CBD group (P = 0.04).

Western Blot analysis confirmed that TMZ reduced Wnt5a expression to 49% of the control level, while CBD decreased it to 45%. Moreover, the combined CBD+TMZ treatment suppressed Wnt5a expression to 15% of control, administered a synergistic inhibitory effect on WNT/ β-Catenin signaling pathway.

Expression AXIN1 following Co-Administration of TMZ and CBD on U87-MG cell line

To evaluate expression AXIN1 protein expression, we used IF-staining and western blot analysis (Figures 6 A-D). IF intensity of AXIN1 significantly increased in CBD (P < 0.0001), TMZ (P = 0.0002), and TMZ+CBD (P < 0.0001) groups compared to control group. Moreover, the CBD+TMZ group exhibited a significant increase in AXIN1expression compared to TMZ group (P = 0.04).

Western Blot quantification confirmed these findings, demonstrating a significant induction of AXIN1 protein expression following treatment. Relative to control group (fold=1, 100%), AXIN1 levels were elevated in the TMZ group (fold= 83.9, 839) and CBD group (fold= 68.3, 683%). Co administration of CBD and TMZ elevated AXIN1 levels (fold=126.9, 1269%) and consistent with an additive effect.

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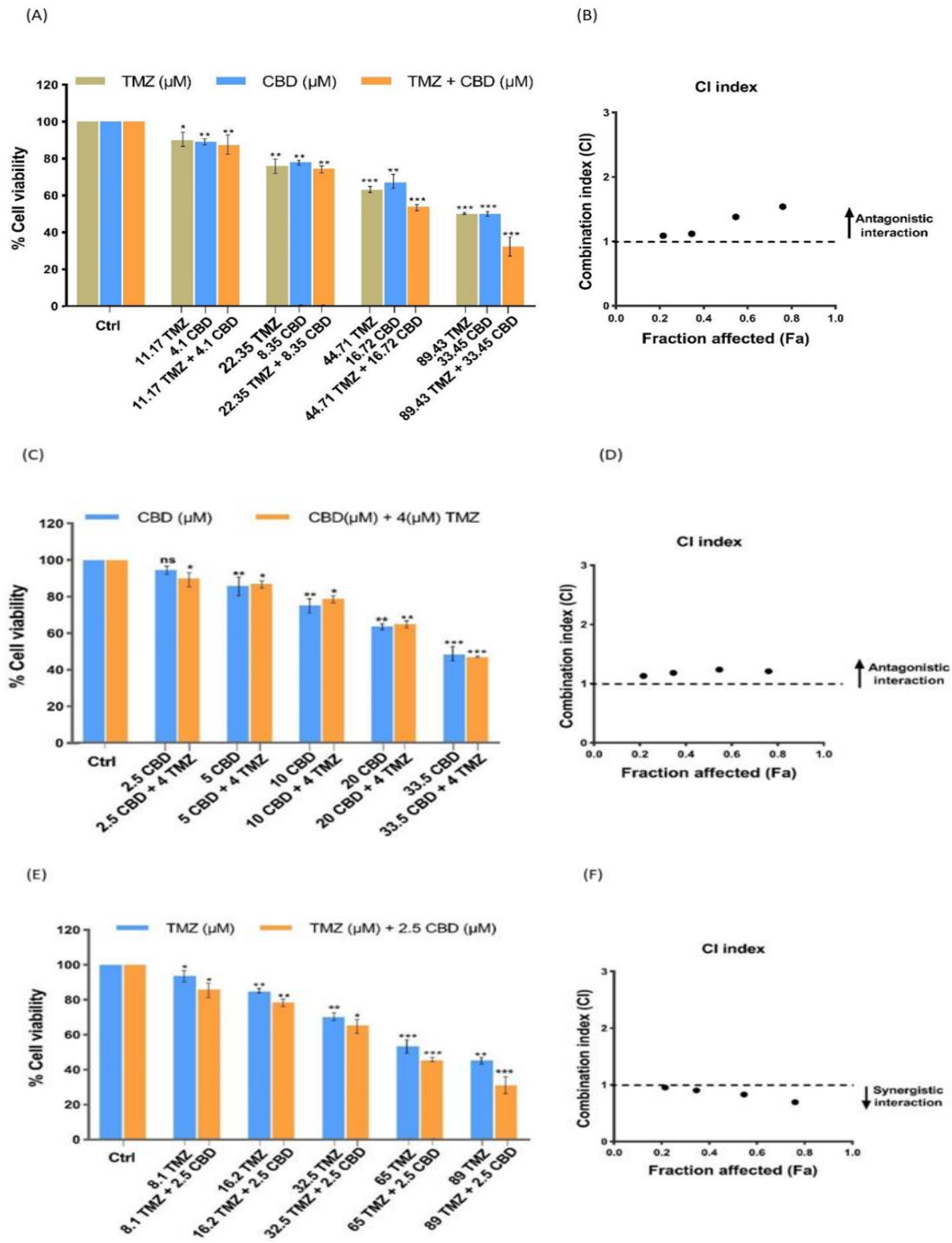

Figure 3. The effect of Co-administration of TMZ and CBD on the survival rate of U87-MG cells following 48 h of treatment. Cells in the control group received an equal volume of drug-free culture medium. step1 (A and B): Fixed-ratio (1:2.67) based on the independent IC_{50} values of TMZ and CBD. Although the combined drugs reduced cell viability in a dose-dependent manner, all tested concentrations exhibited antagonistic interactions (CI>1). step 2 (C and D): Variable CBD concentrations (IC_{50} and sub-IC_{50}) combined with a fixed IC_5 concentration of TMZ. This approach also resulted in dose-dependent reductions in viability; however, all combinations demonestrated antagonistic effects (CI>1). Step 3 (E and F): variable TMZ concentrations (IC_{50}

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and sub-IC_{50}) combined with a fixed IC_5 concentration of CBD (2.5 µM). These combinations significantly decreased cell viability in a dose-dependent manner. CI analysis showed an additive interaction (CI=1) only at 2.5 µM CBD + 1.8 µM TMZ, while all other combinations produced synergistic effects (CI<1). The strongest synergy (CI=0.37) occurred when the IC_{50} concentration of TMZ (43.89 µM) was combined with IC_5 concentration of CBD (2.5 µM). Data are presented as mean ± SEM. Statistical significance was determined relative to the control group (*P<0.01, **P< 0.001, and ***P <0.0001).

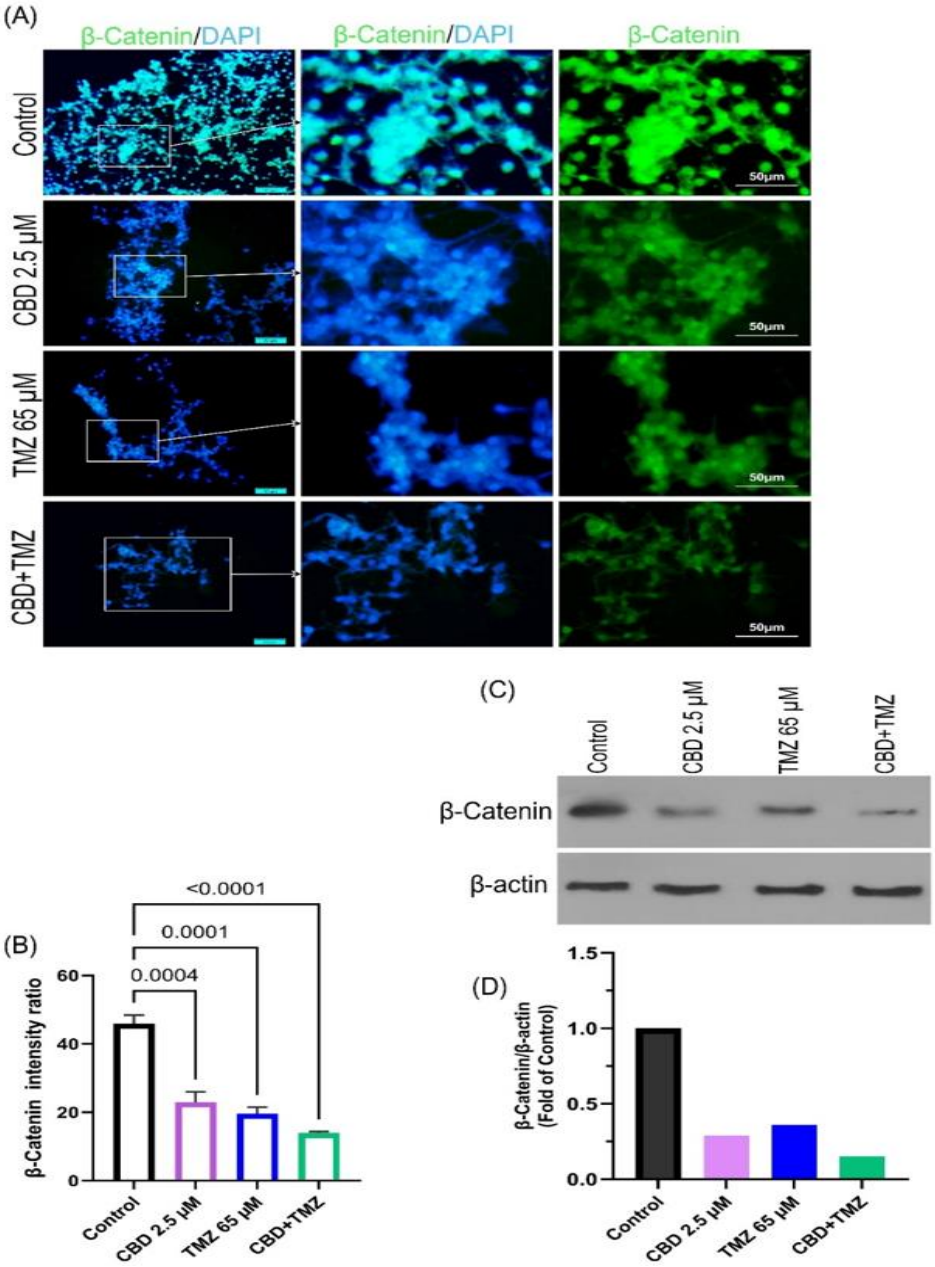

Figure 4. Expression β-Catenin following Co-Administration of TMZ and CBD on U87-MG cells. (A) Immunofluorescence staining of β-catenin, with β-actin used as a reference marker. All images were acquired using an Olympus IX73 microscope under identical exposure settings. Scale bar: 50 µm. (B-D) Western blot analysis was performed to detect the activated (dephosphorylated) form of β-catenin using specific antibodies. Quantification of β-catenin band intensity is shown. Relative protein levels were normalized to the loading control; values are expressed as fold change relative to the control. Data are presented as mean ± SEM.

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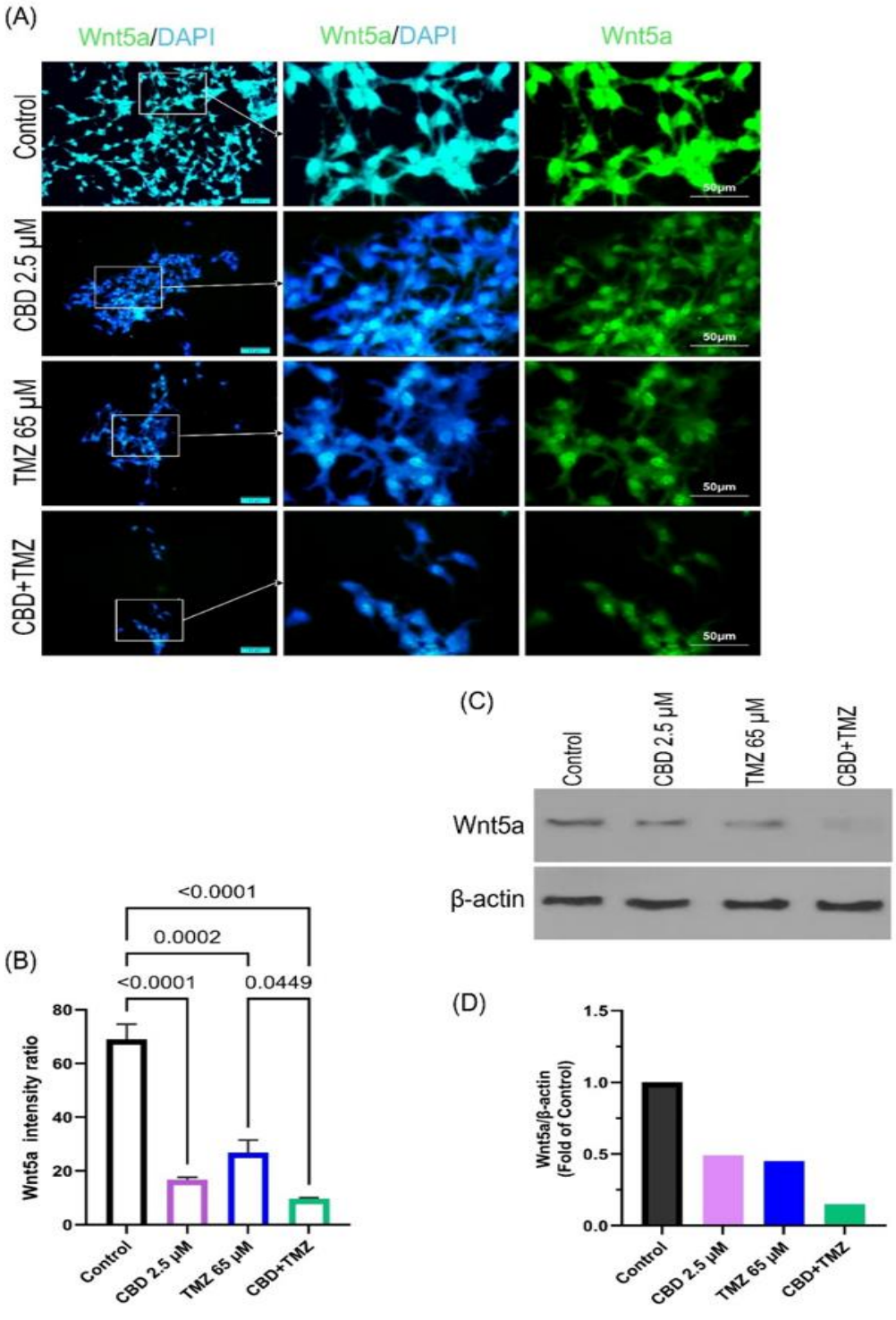

Figure 5. Expression Wnt5a following Co-Administration of TMZ and CBD on U87-MG cell line. (A) Immunofluoresence staining of Wnt5a, with β-actin used as a reference marker. All images were acquired using an Olympus IX73 microscope under identical exposure settings. Scale bar: 50 µm. Quantificantion of Wnt5a fluorescence intensity is shown. (B-D) Western blot analysis was performed to detect the Wnt5a using specific antibodies. Relative protein levels were quantified and normalized to the loading control, and values are expressed as fold change relative to the control. Data represented as mean ± SEM

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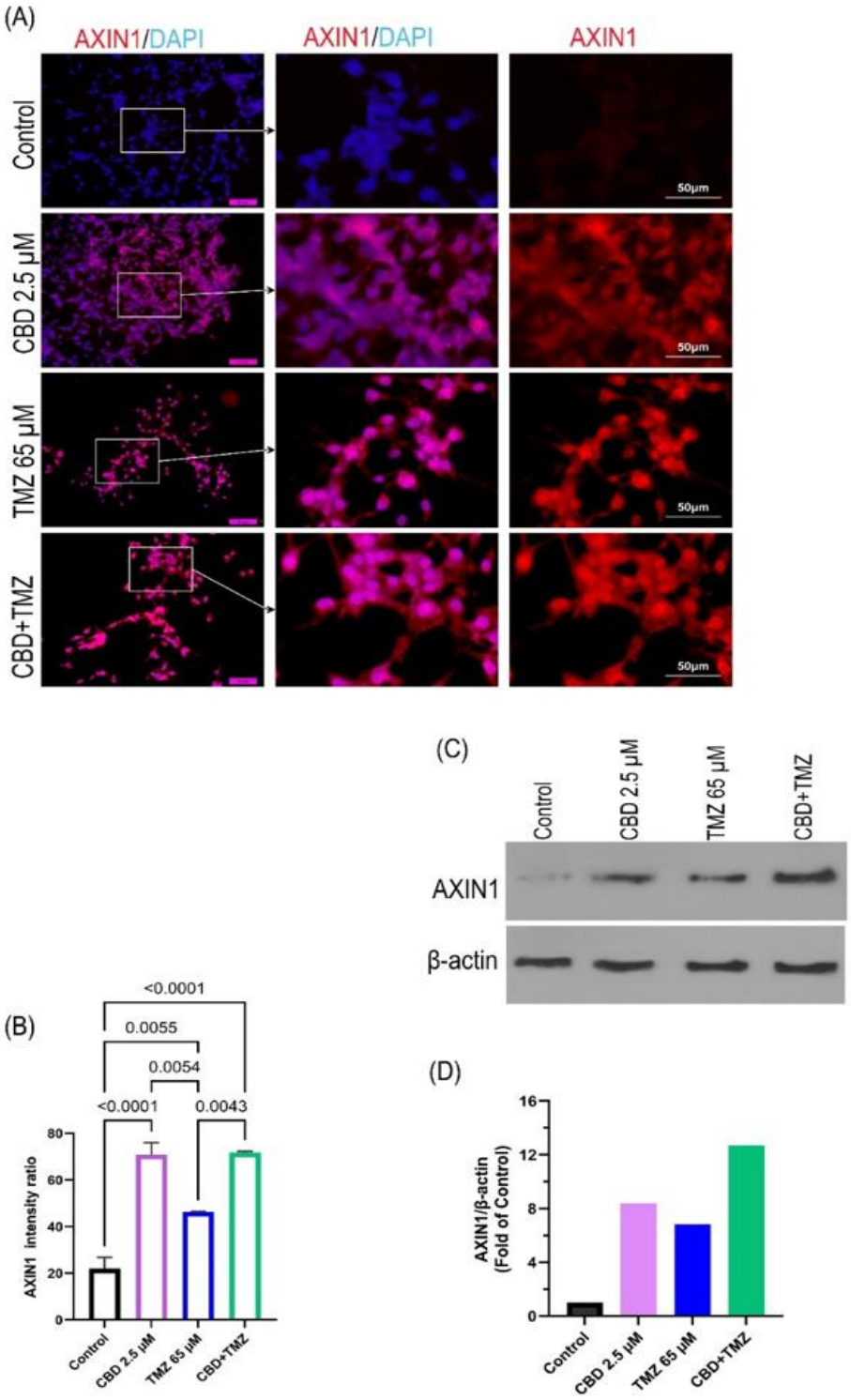

Figure 6. Expression AXIN1 following Co-Administration of TMZ and CBD on U87-MG cell line. (A) Immunofluorescence staining of AXIN1, with β-actin used as the reference marker. All images were acquired using an Olympus IX73 microscope under identical exposure settings. Scale bar: 50 µm. Quantification of AXIN1 fluorescence intensity is shown. (B–D) Western blot analysis was performed to detect AXIN1 using specific antibodies. Relative protein levels were quantified and normalized to the loading control, and values are expressed as fold change relative to the control. Data are presented as mean ± SEM.

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Discussion

In this study, we demonstrated that co-administration of CBD and TMZ produces synergistic cytotoxic effects in U87-MG glioblastoma cells. This enhanced response is associated with coordinated suppression of Wnt5a and β-catenin together with a robust induction of AXIN1. The combined treatment produced a more pronounced reduction in cell viability than either agent alone, suggesting that the enhanced cytotoxicity may be linked to a multi-node disruption of the Wnt/ β-catenin signaling axis.

Glioblastoma represents a highly aggressive brain malignancy, distinguished by rapid growth and therapeutic resistance. The Wnt/β-catenin signaling pathway plays a critical role in promoting tumor growth, invasion, and therapeutic resistance (18). To overcome the limitation of TMZ monotherapy, CBD was incorporated into this treatment. previous studies have revealed that CBD can inhibit Wnt/β-catenin signaling, reduce DNA repair activity and promote apoptosis (19). Consistent with this, Nebissi et al. reported that CBD enhances TMZ responsiveness by increasing drug uptake and promoting apoptosis through TRPV2 activation, supporting the concept that CBD can potentiate TMZ efficacy.

Our results confirmed that TMZ exerts a robust cytotoxic effect on U87-MG glioblastoma cells, consistent with its established role as the standard chemotherapeutic agent for GBM (20). Notably, higher concentrations and longer exposure times (24h, 48h, and 72h) significantly reduced cell viability, confirming the sensitivity of U87-MG cells to TMZ. However, sub-IC_{50}concentration of TMZ revealed limitations in effectiveness, likely due to the persistence of resistant cells mediated by DNA repair mechanisms and survival signaling pathways. Although high doses of TMZ decreased glioblastoma viability, they can also increase systemic toxicity particularly hematologic toxicity (neutropenia, thrombocytopenia, anemia), gastrointestinal intolerance and liver dysfunction (21). Therefore, Combination therapy aimed at enhancing the TMZ efficacy without increasing systemic toxicity represents a promising strategy to improve therapeutic outcomes in glioblastoma.

Our previous study showed that CBD reduced the viability of U87-MG cells in a dose and time dependent manner (22). Consist with earlier reports, CBD impairs glioblastoma cell survival through modulation of apoptosis, autophagy, and oxidative stress (23,24). Importantly, low concentration of CBD inhibited Wnt/β-catenin signaling, which is implicated in glioblastoma progression (25). In our study, Combination Index analysis revealed that the interaction between CBD and TMZ was predominantly synergistic, possibly attenuating DNA repair responses and thereby overcoming partial resistance (26). This aligns with findings from Deng et al.'s study, who demonstrated that CBD enhances TMZ-induced antiproliferative and cytotoxic effects in GBM cells, further supporting the synergistic potential of this combination.

Based on our outcomes, we selected 65 µM TMZ combined with 2.5 µM CBD for subsequent experiments. This concentration falls within the intermediate IC_{50}range of TMZ in U87-MG cells, ensuring biological relevance and substantial inhibition without excessive cytotoxicity. Importantly, clinical pharmacokinetic studies in GBM patients have shown that peak plasma concentrations of TMZ routinely reach approximately 20-100 µM depending on dose and patient variability. therefore, using 65 µM TMZ positions our in vitro model within a translationally realistic therapeutic window, strengthening the relevance of our findings. Lopez-Valero et al. demonstrated that 2.5 µM CBD alone exert negligible cytotoxicity toward glioma-initiating cells (GIC), indicating that this concentration is insufficient to disrupt GIC viability on its own. However, when the same low dose of CBD was incorporated into a THC: CBD mixture and administered concurrently with temozolomide, the combination produced a marked synergistic suppression of GIC proliferation, self-renewal, and tumorigenic potential. Overall, our findings are consistent with accumulating evidence that cannabinoids may influence key signaling pathways implicated in glioblastoma resistance, such as PI3K/AKT, NF κB, and Wnt/β-catenin (9). This suggested that co-administration of TMZ with CBD represent a promising strategy to overcome chemoresistance and improve treatment outcomes in glioblastoma.

β-catenin is the main effector of the Wnt signaling pathway and plays a pivotal role in maintaining glioblastoma stem cells (GSCs), which are highly resistant to therapy and drive recurrence (27). Elevated β-catenin expression has been consistently associated

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with aggressive glioblastoma phenotypes (28). Our finding revealed that increased expression of β-catenin expression in U87-MG cells, supporting its role in enhancing proliferation and invasion. Previous studies demonstrated that β-catenin promotes DNA repair capacity through (MGMT) upregulation, thereby protecting glioblastoma from alkylating agents such as TMZ (29).

Importantly, co-administration of CBD and TMZ significantly decreased β-catenin expression in U87-GM cells suggesting a synergistic inhibitory effect on Wnt/β-catenin signaling.

Previous study showed that CBD sensitized glioblastoma cells to TMZ by inhibiting RAD51 mediated homologous recombination repair (26). Furthermore, recent evidence has highlighted that Wnt/β-catenin signaling contributes not only to chemoresistance but also to immune evasion, as β-catenin upregulates PD-L1 expression and suppresses $CD8^+$ T-cell activity. Therefore, β-catenin inhibition may enhance both chemotherapy sensitivity and anti-tumor immunity (30). In line with our data, quercetin has been shown to inhibit the GSK3 β/ β-catenin pathway, leading to the downregulation of the EMT-associated transcription factor ZEB1 and reduced glioma cell invasiveness.

Wnt5a has been shown to regulate cell motility and invasion, elevated Wnt5a expression associated with enhanced epithelial to mesenchymal transition (EMT) and great migratory potential (31). In our study, Wnt5a expression was significantly reduced following treatment with CBD or TMZ, while the combined treatment showed a greatest decrease. This finding suggested that the combination therapy not only interferes with canonical β-catenin signaling but also attenuates non-canonical Wnt pathways that drive glioblastoma invasiveness. Zhou et al. report that lncRNA H19 promotes glioma proliferation, migration, and angiogenesis by sponging miR-342 to upregulate Wnt5a and activate β-catenin signaling in A172, LN229, and U251 cells and in U251 xenografts, indicating that both genetic and pharmacologic modulation of the Wnt/ β-catenin axis produce convergent oncogenic phenotypes. Also, previous reports showed that the inhibition of Wnt5a reduced tumorigenicity and sensitizes cells to chemotherapy (32,33). Altered AXIN1 expression has been reported in glioblastoma, with reduced levels linked to enhanced pathway activation and tumor aggressiveness (34). In our study, AXIN1 expression was significantly induced following treatment with CBD or TMZ, and most strikingly in the CBD+TMZ group, where levels increased more than 12-fold compared to control. This significant induction suggests that combination therapy not only suppresses oncogenic drivers such as β-catenin and Wnt5a but also reinforces tumor suppressive regulators, thereby amplifying pathway inhibition. Although the combination treatment induces an additive increase in AXIN1, while simultaneously producing a synergistic reduction in Wnt5a and β-catenin, this pattern is entirely consistent with the non-linear regulatory architecture of the Wnt pathway (10,35). Because AXIN1 functions as the absolute rate-limiting scaffold of the destruction complex, even a modest rise in its abundance can markedly enhance complex assembly and β-catenin turnover by accelerating concentration-dependent AXIN polymerization and processive capture (10). When this accelerated clearance of intracellular of β-catenin is coupled with concurrent suppression of upstream activators like Wnt5a, the entire cascade undergoes a threshold-like collapse. under this non-linear framework, minor additive increases at a rate-limiting nodal scaffold effectively amplifying into the synergistic inhibition of downstream oncogenic signaling (10,36).

Our study demonstrates that co-administration of CBD and TMZ exerts a synergistic inhibitory effect in U87-MG glioblastoma cells by coordinately suppressing Wnt5a and β-catenin while markedly inducing AXIN1. This coordination modulation disrupts Wnt/β-catenin signaling at multiple levels, leading to reduced proliferation and invasiveness and overcoming, at least part, therapy resistance. Importantly, the induction of AXIN1 provides a plausible explanation for the observed threshold like collapse of β-catenin signaling, and our data support the concept that CBD act as a chemosensitizer that amplifies TMZ efficacy through both suppression of oncogenic drivers and reinforcement of tumor suppressive regulators. Collectively, these findings suggested that CBD has potential as an adjuvant to TMZ in GBM treatment warrant further preclinical evaluation.

Limitation and future directions:

Despite the promising findings, several limitations should be acknowledged. First, our experiments were

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conducted exclusively in the U87-MG glioblastoma cell line. Although U87-MG is widely used, it does not fully capture the genetic and phenotypic heterogeneity of patient-derived GBM tumors. Validation in additional glioblastoma models, including patient-derived cells and in vivo xenografts, will be necessary to confirm the synergistic interaction and its relevance to tumor heterogeneity. Second, while we demonstrate coordinated modulation of Wnt signaling pathway component, the precise direct molecular mechanisms underlying CBD-mediated AXIN1 induction and subsequent β-catenin turnover remain to be fully elucidated. Future investigation should explain upstream regulators and post-translational mechanisms that mediate this response. Third, while the concentration of TMZ utilized aligns with clinical pharmacokinetic data, future in vivo studies must incorporate pharmacokinetic assessments to evaluate blood-brain barrier (BBB) penetration, metabolic clearance, and potential systemic toxicity. Finally, our study focused primarily on canonical and non-canonical Wnt/β-catenin signaling; future research should explore parallel resistance pathways, such as the PI3/AKT and NF- κB pathways, and determine how these interactions contribute to improved therapeutic outcomes.

Funding

This work was a Master student thesis of in Anatomical Sciences supported by the Babol University of Medical Sciences (Babol, Mazandaran, Iran) with grant number. All experimental procedures were approved by the Ethics Committee of Babol University of Medical Sciences (Approval No: IR.MUBABOL.HRI.REC.1400.039).

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