

RESEARCH ARTICLE

Mumio Potentiates Temozolomide by Inducing Apoptosis and Ferroptosis in Glioblastoma Cells

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Abstract: Introduction: Glioblastoma multiforme is an extremely deadly, aggressive primary brain tumor, distinguished by poor prognosis, and the rapid development of resistance to temozolomide might be the cause of low survival. Novel adjunctive strategies are needed to enhance therapeutic efficacy. Mumio (Shilajit), rich in fulvic acid and bioactive compounds, has shown antioxidant and anticancer effects, but its role in glioblastoma remains unclear. This study evaluated its anticancer potential and its ability to enhance temozolomide efficacy.

Methods: Mumio extract was tested in U87 (sensitive) and LN-18 (resistant) glioblastoma cell lines. Cell viability and clonogenicity were assessed by MTT and colony formation assays. Fluorescent staining evaluated mitochondrial function, reactive oxygen species, and lipid accumulation. Western blotting analyzed apoptotic (PARP, Caspase-3, PTEN), ferroptosis-related (NRF2, FTH1), and Akt signaling proteins. Combination treatments were also examined.

Results: Mumio reduced cell viability in a dose- and time-dependent manner, with stronger effects in U87 cells. It increased reactive oxygen species, disrupted mitochondrial function, and induced apoptosis, as shown by increased cleaved PARP and Caspase-3. Ferroptosis-related changes, including reduced FTH1 in LN-18 cells, were observed. Mumio also enhanced temozolomide-induced cytotoxicity and reduced clonogenic survival.

Discussion: These findings suggest that Mumio exerts anticancer effects through oxidative stress-mediated apoptosis and modulation of ferroptosis pathways. Its ability to enhance temozolomide efficacy, particularly in resistant cells, indicates a potential role in overcoming chemoresistance.

Conclusion: Mumio triggers apoptosis in glioblastoma cells and induces changes consistent with ferroptosis, while also chemosensitizing cells to temozolomide, suggesting its potential use alongside standard therapy.

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1. INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive and deadly primary brain tumor with a median survival of 12-15 months even when treated with a combination of maximal surgical resection, radiotherapy, and temozolomide (TMZ) chemotherapy [1]. Despite the use of advanced treatment strategies, resistance to TMZ continues to limit the therapeutic success in glioblastoma. This resistance is often associated with hypomethylation of the O6-methylguanine DNA methyltransferase (MGMT) promoter, leading to increased MGMT expression. Consequently, tumor cells can efficiently repair TMZ-induced DNA damage, thereby diminishing the overall efficacy of the treatment [2]. Chemotherapeutic agents, such as cisplatin, irinotecan, bevacizumab, and EGFR, generally provide limited clinical benefit and are frequently associated with substantial toxicity [3]. Since GBM exhibits highly aggressive

behavior driven by pronounced inter- and intramolecular level heterogeneity, and consequently responds poorly to standard therapies, it is vital to develop new treatment strategies to overcome resistance.

Mumio is a dark brown to almost black, tar-like mineral material that has been utilized commonly in traditional medicine in South and Central Asia, Russia, and several parts of the Middle East [4, 5]. The term “mumio” or “mumiyo”, often translated as “mountain tears,” comes from its slow release from high-altitude cracks and caves in the Himalayas, Altai, and Caucasus mountains, while “shilajit” comes from Sanskrit and is usually translated as “derived from rock” [5, 6]. Its complex structure is composed primarily of humic substances, including fulvic acid, humic acid, and humin, as well as amino acids, polyphenols, fatty acids, and numerous other organic acids [7, 8]. It was prescribed for different conditions historically, including bone fractures, osteoarthritis, kidney stones, anemia, neurological disorders, and so on. It has also been believed to enhance the efficacy of other medications [8].

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Modern pharmacological scrutiny has indicated that fulvic acid and dibenzo-a-pyrones, two main components of Mumio, can reduce oxidative stress and support memory, suggesting possible benefits for neurodegenerative diseases [9]. Mumio also exhibits anticancer properties *via* several mechanisms. By acting on urokinase-type plasminogen activator (uPA) and chemokine signaling pathways [10], it can limit migration and invasion in oral cancer, suppress epithelial-mesenchymal transition (EMT) and metastasis in breast cancer [11], and trigger apoptosis in bladder cancer cells [12]. Studies in animals also suggest that Mumio has strong potential for neuroprotective effects, such as reducing brain swelling and modulating blood-brain barrier permeability [13].

Even though all these promising biological effects of Mumio are well known, it has not been investigated in GBM, notably in TMZ-resistant disease, creating a clear gap in the current literature. Mumio may represent a promising approach for GBM treatment, as it has bioactive content that consists mostly of fulvic acid, which has a small molecular size and is able to pass the blood-brain barrier [14]. Also, redox-modulating activity of Mumio among other cancer types suggests a potential for TMZ-resistant GBM [15]. In this study, Mumio was examined in both TMZ-sensitive U87-MG (ATCC HTB-14) and TMZ-resistant LN-18 (ATCC CRL-2610) glioblastoma cell lines, assessing cell survival, oxidative stress, mitochondrial function, and lipid metabolism to evaluate its potential as an alternative therapy for GBM.

2. MATERIALS AND METHODS

2.1. Preparation of Mumio Extract and Content Analysis

Mumio stock solution (20 mg/mL; TR-34-K-099663, Altei, Turkey; this commercially available tablet contains only Mumio) was prepared from commercially available 200 mg ALTEI Mumio tablets in 10 mL of sterile distilled water, followed by centrifugation and filtration of the supernatant through a 0.22 μ m syringe filter under a Class II laminar flow cabinet. Batch-to-batch variability was not assessed, and all batches were assumed to be equivalent. The Mumio application dosages (50, 100, 250, 500, and 750 μ g/mL) were prepared by diluting the stock solution (20 mg/mL in dH₂O) into cell culture medium for subsequent cellular experiments. The application dosages were selected based on literature values used in other cancer cell lines [10, 12, 16].

Mineral content was examined by ICP-OES (Agilent 5800, KUTMANLAB, Gebze Technical University), after microwave-assisted acid digestion (2 mL H₂O₂, 5 mL HNO₃) of 0.21–0.9 g ground samples. The samples were then diluted and filtered. Macro- and micro-minerals were determined using certified reference materials for accuracy. The chemical bond structure was analyzed by FT-IR spectroscopy (550–4000 cm⁻¹, 32 scans) to identify the bond structures of Mumio.

Antioxidant capacity was determined using total phenolic content (TPC), Trolox equivalent antioxidant capacity (TEAC), and total antioxidant capacity assays (DPPH). TPC was determined by the Folin–Ciocalteu method: 20 mg/mL homogenized samples were sonicated, centrifuged, and the supernatant reacted with Folin reagent and Na₂CO₃; absorbance was measured at 760 nm, and results were expressed as mg gallic acid equivalent (GAE)/100 g [17]. Radical scavenging activity was evaluated using the DPPH assay, which measures absorbance at 515 nm after incubation with a DPPH solution. The inhibition percentage was calculated as described by Brand-Williams *et al.* (1995) [18].

2.2. Demonstration of the Cellular effect of Mumio in *in vitro* GBM Models

TMZ-resistant LN-18 (ATCC CRL-2610) and TMZ-sensitive U87-MG (ATCC HTB-14) glioblastoma cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplement-

ed with 10% fetal bovine serum (FBS, Pan Biotech) and 10 U/mL penicillin/streptomycin (Gibco) at 37°C in a humidified 5% CO₂ incubator (Binder).

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (5 mg/mL in PBS, BioFroxx) [12]. Cells were seeded at 5×10^3 cells/well in 96-well plates, treated with Mumio extract (50–750 μ g/mL) for 24, 48, or 72 hours, and, where indicated, co-treated with TMZ (200 μ M) [19]. After MTT incubation (4 h), formazan crystals were solubilized in DMSO (dimethyl sulfoxide, BioFroxx), and absorbance was measured at 570 nm to determine relative viability using a microplate reader (BioTek).

Colony formation was evaluated by seeding 2.5×10^3 cells/well in 6-well plates, treating with Mumio extracts at the same concentrations used in the MTT assay for 24 h, and then culturing in fresh medium for at least 10 days. Colonies were fixed with a fixation solution (acetic acid:methanol, 1:3), then stained with 0.5% crystal violet, and imaged under light microscopy [20].

Fluorescence microscopy was used to assess cell viability, dead cells, reactive oxygen species (ROS), lipid droplets, and nuclei. Cells (3×10^4 /well, 24-well plates) were treated with Mumio and/or TMZ, then stained with 0.5 μ g/mL PI and 4 nM DiOC₆ (3,3'-hexyloxacarbocyanine iodide). Stained cells were imaged with fluorescence microscopy at 536/617 nm (red, PI for identification of dead cells) and 488/525 nm (green, DiOC₆ for determination of viable cells). Oxidative stress (reactive oxygen species) was visualized with 5 μ M H₂DCFDA, and lipid peroxidation was determined *via* BODIPY (boron–dipyrrromethene) 493/503 staining. Quantification of ROS, MitoTracker, and BODIPY levels (integrated density) was performed in ImageJ, with measurements obtained from at least 50 cells per replicate across two biological replicates from fluorescence images. Cell fluorescence analysis (CTCF) was performed using Equation 1 after fluorescence images were converted to 8-bit, and the background and cells were measured in ImageJ.

$$\text{CTCF} = \text{Integrated Density} - (\text{Area of Selected Cell} \times \text{Mean Background Fluorescence}) \quad \text{Eq. (1)}$$

2.3. Total Protein Isolation and Immunoblotting

Cells (1×10^6 cells/well in 6-well plate) were incubated for 24 h at 37°C, 5% CO₂, then treated with optimized concentrations of Mumio and/or TMZ. Afterward, the cells were harvested, washed with PBS, and lysed in M-PER buffer (Mammalian Protein Extraction Reagent, Thermo Fisher Scientific) on ice for 20 minutes. Lysates were clarified by centrifugation (13,200 rpm, 20 min, 4°C), and protein concentration was determined by the Bradford assay [21].

For immunoblotting, 30 μ g protein per sample was mixed with Laemmli buffer (Bio-Rad), denatured at 95°C for 5 min, and separated on 12% SDS-PAGE gels. Proteins were transferred to 0.45 μ m PVDF membranes using a semi-dry transfer system. Membranes were blocked with 5% non-fat milk in TBST for 1 h, then incubated overnight at 4°C with primary antibodies (1:1000; PARP (#614301, BioLegend), Caspase 3 (#9662, Cell Signaling Technology (CST)), AKT (#ab179463, Abcam), NRF2 (#12721, CST), FTH1 (#4393, CST)). Following two washes with TBS-T and a final wash with TBS (each for 10 min), the membranes were incubated overnight at 4°C with HRP-conjugated secondary antibodies (1:1000; CST, #7074 and #7076). Subsequently, the membranes were rewashed in the same manner, developed using an enhanced chemiluminescence substrate, and protein signals were visualized with a ChemiDoc-MP or iBright imaging system using ECL solution [21]. Quantification of Western blot bands was performed using ImageJ software, and signal intensities were normalized to the corresponding control and housekeeping proteins.

2.4. Statistical Analysis

Statistical analysis of each application's effect was performed using GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, California, USA; www.graphpad.com). For two-way ANOVA and Dunnett's test, all are presented as mean $\pm$ SD of at least three biological replicates of 2 technical replicates each. A p -value of < 0.05 was considered statistically significant to indicate meaningful differences. The immunoblot bands were quantified using ImageJ with the Gel Analysis function, and the results were normalized to the loading control [22]. Statistical analyses were performed using two-way ANOVA followed by Tukey's multiple comparison test.

3. RESULTS

3.1. Mumio Content Analysis

The mineral composition of Mumio, determined by ICP-OES, revealed high levels of potassium (87.6 ± 2.84 g/kg), calcium (12.8 ± 0.37 g/kg), magnesium (11.3 ± 0.39 g/kg), and sulfur (11.0 ± 0.36 g/kg) among the macroelements (Table 1). Notably, iron con-

tent was detected in high concentration (714 ± 13.2 mg/kg). Sodium, as a non-essential element, was abundantly found in the Mumio (966 ± 26 mg/kg). ICP results indicated that the Mumio was rich in essential minerals, highlighting its bioactivities.

The FTIR spectrum of the Mumio sample showed a broad absorption band between 3281 cm^{-1} , attributed to O–H and N–H stretching vibrations, indicating the presence of adsorbed water, alcohol, or phenolic structures (Fig. 1). Moreover, this region shows O–H bonds characteristic of phenolic compounds, and N–H vibrations associated with amide II structures [23, 24]. The bands at around 1557 cm^{-1} are characterized by C=C or aromatic ring vibrations, while the peaks near 1401 cm^{-1} could reflect the phenolic groups of C–H bending or O–H bending. The bands at around $1276\text{--}1261\text{ cm}^{-1}$ are characteristic of C–O stretching in alcohols, phenols, or carboxylic acids. The peaks around 1015 cm^{-1} are associated with C–O or C–O–C stretching of polysaccharides or oxygenated functionalities [25]. Peaks between $764\text{--}750\text{ cm}^{-1}$ are typically associated with out-of-plane C–H bending in substituted aromatic rings, supporting the presence of aromatic components in the Mumio sample [23].

Table 1. Mineral composition results of Mumio conducted *via* ICP-OES. Data are presented as the mean $\pm$ standard deviation from at least three independent replicates.

Macroelement Concentrations (g.kg ⁻¹)							
Mumio	Ca	K	Mg	P	S	-	-
	12.8 ± 0.37	87.6 ± 2.84	11.3 ± 0.39	0.84 ± 0.02	11.0 ± 0.36	-	-
Microelement Concentrations (mg.kg ⁻¹)							
Mumio	B	Cu	Fe	Mn	Ni	Zn	Mo
	101 ± 2.8	9.57 ± 0.42	714 ± 13.2	54.4 ± 0.98	2.49 ± 0.16	26.2 ± 0.46	0.98 ± 0.05
Non-essential Element Concentrations (mg.kg ⁻¹)							
Mumio	Al	Cd	Co	Na	Pb	-	-
	271 ± 7.3	0.09 ± 0.01	0.51 ± 0.00	966 ± 26	0.34 ± 0.08	-	-

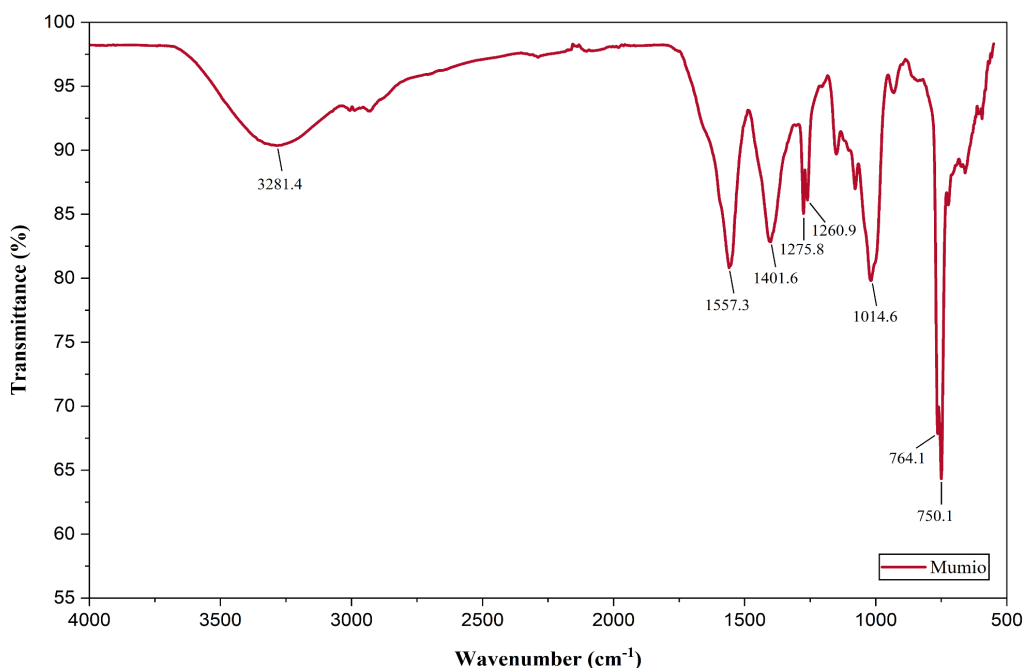


Fig. (1). FTIR results of Mumio for $550\text{--}4000\text{ cm}^{-1}$ with 32 scans to identify the bond characteristics. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

Fig. (2) shows the relation between the DPPH free radical inhibition percentage (%) and Mumio concentration (mg/mL). The EC₅₀ value, the concentration of a sample that produces 50% inhibition, for Mumio was 0.29 mg/mL, indicating marked antioxidant activity even at low concentrations.

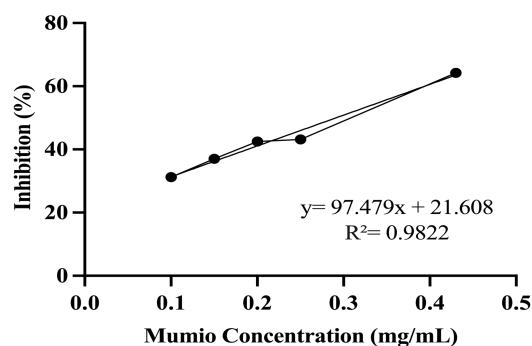


Fig. (2). DPPH assay results, showing that the percent inhibition of free radicals based on Mumio concentration increases (mg/mL). (A higher resolution/colour version of this figure is available in the electronic copy of the article).

TPC of Mumio was calculated using a gallic acid standard curve obtained by the Folin-Ciocalteu method (Fig. S1). The TPC was calculated to be 117.06 mg GAE/100 g fw in 0.5 mg/mL Mumio extract (Table 2).

3.2. Investigation of the Cellular Effects of Mumio in *in vitro* GBM Models

U87 and LN-18 glioblastoma cell lines were used to assess the effects of Mumio extract using the MTT assay at concentrations of 0, 50, 100, 250, 500, and 750 µg/mL. Treatment periods were set to 24, 48, and 72 hours. The effects of Mumio on cell viability were analyzed in a time- and dose-dependent manner. Statistical analysis was performed using two-way ANOVA and Dunnett's comparison test by GraphPad 9.2.0 software (Figs. 3A and B).

With 24-hour application of 50 µg/mL Mumio in U87 cell lines, cell viability was significantly decreased ($p < 0.0001$) compared to the non-treated control group. In the TMZ-resistant LN-18 cell line, treatment with Mumio at 250 ($p < 0.01$) and 500 µg/mL ($p < 0.05$) resulted in a significant reduction in cell viability after 24 hours compared with the control, and this reduction became more pronounced over time. Further experiments were conducted with a 24-hour treatment period based on MTT assay results.

To assess the long-term effects of Mumio on cell viability and clonogenic potential, a colony formation assay was performed using the same concentrations as in the MTT assay. Compared with the control groups, the colony-forming potential of both cell lines did not change qualitatively with Mumio applications (Fig. 3C). Based on the MTT and colony formation assay results, 250 and 500 µg/mL Mumio application dosages were selected for further experiments, because the minimum effective dose for LN-18 was 250 µg/mL, and for U87, cell viability decreased by more than 20% at 250 µg/mL and 500 µg/mL.

To assess the proliferative effect of Mumio, cells were treated with optimized concentrations (250 µg/mL and 500 µg/mL) select-

ed from MTT and colony formation assays for 24, 48, and 72 hours, and viable cells were counted for the cell survival assay (Figs. 3D and E) after each application time.

In U87 cells, both application dosages significantly reduced cellular proliferation over time. No significant change was observed at 24 hours ($p > 0.05$); however, by 48 hours, the 500 µg/mL Mumio caused a notable reduction in cell number ($p < 0.01$), and by 72 hours, both the 250 µg/mL and 500 µg/mL doses led to a significant decrease ($p < 0.0001$, Fig. 3D). In LN-18 cells, Mumio treatment also reduced cell numbers after 24-hour application. Although no significant difference was observed at 24 hours, the 500 µg/mL dose significantly suppressed proliferation after 72 hours ($p < 0.001$) (Fig. 3E). These results indicate that Mumio has anti-proliferative effects on the U87 and LN-18 glioblastoma cell lines, as assessed by a cell survival assay.

Fluorescence staining in U87 cells showed that Mumio treatment qualitatively reduced cell viability, increased reactive oxygen species (ROS) levels, and lowered mitochondrial activity, especially at the higher dose of Mumio, while lipid accumulation was not affected (Figs. S2A and C). These results indicated that Mumio increased ROS levels in U87 glioblastoma cells, thereby reducing cell viability. In LN-18 cells, 24-hour Mumio treatment at 500 µg/mL increased lipid droplet accumulation and ROS levels. However, despite this rise in oxidative stress, mitochondrial activity was similar to that of the control group (Figs. S2B and D).

To identify the combination effects of TMZ and Mumio, the TMZ application concentration was optimized with the MTT assay (Fig. S3), and a dose of 200 µM was selected for combined treatment with Mumio.

Five Mumio concentrations (50, 100, 250, 500, and 750 µg/mL) were applied to both cell lines in combination with 200 µM TMZ. The MTT assay showed that 50 µg/mL Mumio concentration caused a 50% reduction in cell viability in the U87 cell line ($p < 0.0001$) (Fig. 4A). Additionally, for the LN-18 cell line, combined treatment with TMZ and 50 µg/mL Mumio resulted in a significant reduction in cell viability compared with the control ($p < 0.0001$) (Fig. 4A). The colony-forming capacities of both cell lines after TMZ and Mumio treatment inhibited the colonization ability of the treated cells compared to the control groups (Fig. 4B).

Based on the MTT and colony formation results, optimized dosages (50 µg/mL and 100 µg/mL Mumio with 200 µM TMZ) were used in both cell lines for 24, 48, and 72 hours in the cell survival assay. In both cell lines, no significant reduction in cell viability was observed at 24 hours ($p > 0.05$). However, after 48 and 72 hours of treatment, both 200 µM TMZ + 50 µg/mL Mumio and 200 µM TMZ + 100 µg/mL Mumio statistically decreased cell viability compared to the control group in U87 ($p < 0.0001$) (Fig. 4C). In TMZ-resistant LN-18 cells, cell proliferation was inhibited by both treatment conditions at 48 ($p < 0.05$) and 72 hours ($p < 0.01$) compared to the non-treated control group (Fig. 4D).

Quantitative analysis for ROS and mitochondrial levels of both cell lines was performed using ImageJ software (Figs. 5A-D), based on the data presented in Fig. (S4). In Fig. (5A), U87 cells treated with 200 µM TMZ showed a slight enhancement in ROS levels compared to the control. 200 µM TMZ + 50 µg/mL Mumio and 200 µM TMZ + 100 µg/mL Mumio slightly increased ROS production in U87 cell lines compared to the control group, although the increase was not statistically significant compared to

Table 2. Total phenolic content of 0.5 mg/mL Mumio extract in water, which was calculated using a gallic acid standard curve.

-	-	Extract Concentration (mg/ mL)	Standard Used	Total Phenolic Content (mg GAE/ 100 g fw)
Mumio extract	Water	0.5	Gallic acid	117.06 ± 0.005

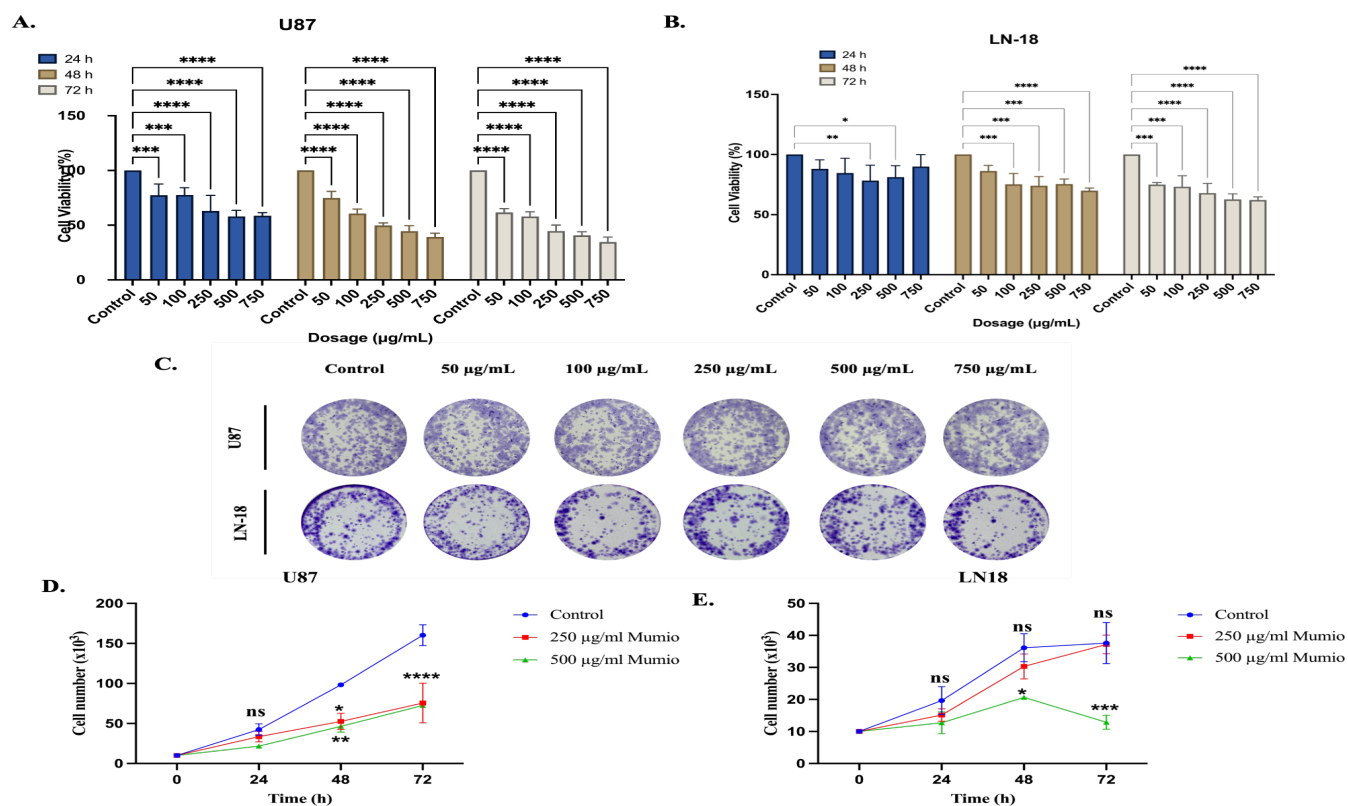
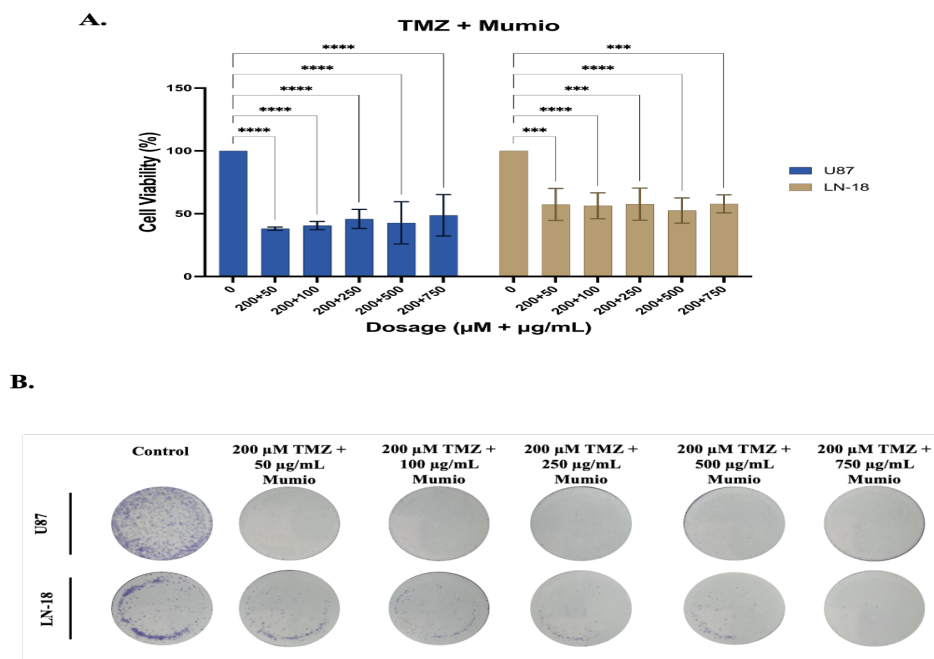


Fig. (3). MTT assay results of (A) U87 and (B) LN-18 glioblastoma cell lines after 24, 48, and 72 h Mumio application with different concentrations (0, 50, 100, 250, 500, 750). Each group represents the mean of four technical replicates across three independent biological experiments, and error bars indicate the standard deviation. Statistical analysis was conducted using a two-way ANOVA followed by Dunnett's multiple-comparison test at each time point, comparing each group with the control. (C) Colony formation assay results of U87 and LN-18 cell lines after 24 h Mumio application at the same concentrations as the MTT assay. The experiment was performed three times, and a representative image is presented. Cell survival assay results of (D) U87 and (E) LN-18 cell lines at 24, 48, and 72 hours after treatment with Mumio at 250 µg/mL and 500 µg/mL, compared with the control group. Each value represents the mean of two technical replicates and two biological replicates. Statistical analyses were conducted using a two-way ANOVA followed by Dunnett's multiple-comparison test, comparing each time point with its control. ns, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (A higher resolution/colour version of this figure is available in the electronic copy of the article).



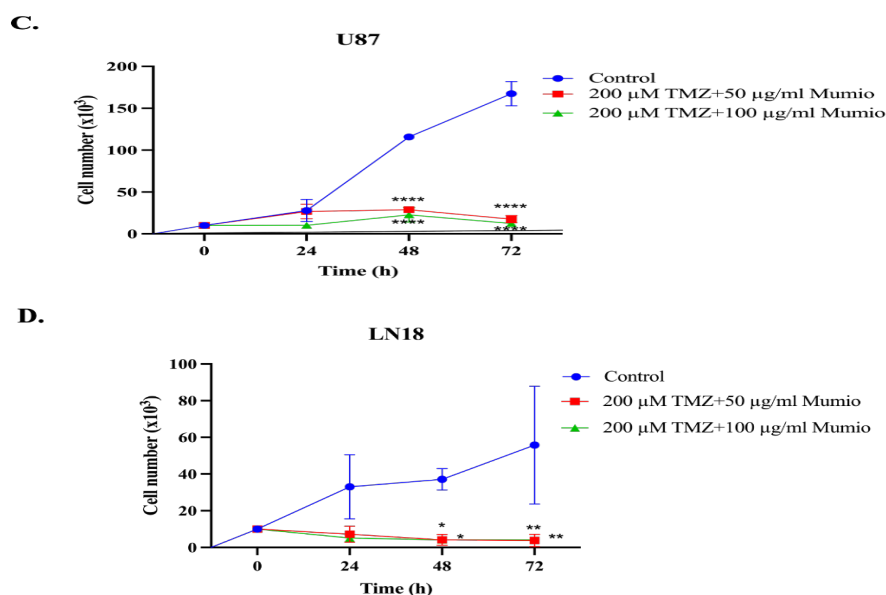


Fig. (4). (A) Relative cell viability based on the MTT assay results of U87 and LN-18 cell lines after 24-hour treatment with 200 μM TMZ with increasing concentrations of Mumio (50, 100, 250, 500, and 750 $\mu\text{g/mL}$) for 24 h. Results are expressed as a percentage relative to the non-treated control group (0 $\mu\text{g/mL}$). Each bar represents the mean of four technical replicates from three independent biological experiments; error bars indicate standard deviation. Statistical analysis was performed using two-way ANOVA followed by Dunnett's multiple comparison test. (B) Colony formation assay of U87 and LN18 cells treated with 200 μM TMZ in combination with increasing concentrations of Mumio (50–750 $\mu\text{g/mL}$) for 24 hours. A representative image from three independent experiments is shown. Cell survival assay results of (C) U87 and (D) LN-18 cells treated with 200 μM TMZ in combination with 50 or 100 $\mu\text{g/mL}$ Mumio for 24, 48, and 72 hours. Each value represents the mean of two biological replicates, each performed in two technical duplicates. Statistical analyses were conducted using a two-way ANOVA followed by Dunnett's multiple-comparison test, comparing each time point with its control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

TMZ alone. Notably, 500 $\mu\text{g/mL}$ Mumio reduced ROS levels compared with the control ($p < 0.01$). Quantitative analysis of mitochondrial activity (Fig. 5C) showed that 200 μM TMZ alone resulted in a modest decrease in MitoTracker signal compared with the control group ($p < 0.0001$). The combinations of 200 μM TMZ + 50 $\mu\text{g/mL}$ Mumio further increased ($p < 0.0001$), and 200 μM TMZ + 100 $\mu\text{g/mL}$ Mumio reduced mitochondrial signal relative to control ($p < 0.0001$). Treatment with Mumio alone at 250 and 500 $\mu\text{g/mL}$ slightly increased mitochondrial activity compared with the control ($p > 0.05$).

In LN-18 cell lines, ROS levels in the combination groups showed a significant difference compared to the control ($p < 0.0001$) (Fig. 5B). TMZ alone applied to the LN-18 groups showed a significant increase in ROS levels compared to the control ($p < 0.0001$). The 500 $\mu\text{g/mL}$ Mumio applied group induced a significant reduction in ROS production ($p < 0.0001$) (Fig. 5B). The combination treatments yielded the lowest ROS levels. For mitochondrial activity, the quantitative analysis is shown in Figure 5D. TMZ alone increased the MitoTracker signal compared with the control group ($p < 0.0001$). Both combination treatments (200 μM TMZ + 50 $\mu\text{g/mL}$ Mumio and 200 μM TMZ + 100 $\mu\text{g/mL}$ Mumio) further enhanced mitochondrial signals compared with the control, as determined by statistical analysis ($p < 0.0001$). Mumio alone at 500 $\mu\text{g/mL}$ also caused a marked increase in mitochondrial activity ($p < 0.01$), whereas 250 $\mu\text{g/mL}$ Mumio produced a moderate increase ($p > 0.05$).

Fluorescence staining results indicate that in both U87 and LN-18 cell lines, lipid droplet formation increased based on BODIPY staining results compared to the control group after 24 h application of 200 μM TMZ + 50 $\mu\text{g/mL}$ Mumio and 200 μM TMZ + 100 $\mu\text{g/mL}$ Mumio (Figs. S4A–B). For quantitative analysis, in U87 cells, BODIPY-based quantification of lipid droplet-associated signal (Fig. 5E) revealed that 200 μM TMZ + 50 $\mu\text{g/mL}$ Mumio

and 200 μM TMZ + 100 $\mu\text{g/mL}$ Mumio significantly enhanced BODIPY intensity relative to the control ($p < 0.0001$), indicating increased lipid droplet accumulation. Mumio alone treatments at 250 and 500 $\mu\text{g/mL}$ caused a non-significant reduction and moderate increase compared to the control, respectively ($p > 0.05$). In LN-18 (Fig. 5F), BODIPY intensity was significantly increased with Mumio alone groups (250 and 500 $\mu\text{g/mL}$) compared to the control group ($p < 0.0001$). In combination groups (200 μM TMZ + 50 $\mu\text{g/mL}$ Mumio and 200 μM TMZ + 100 $\mu\text{g/mL}$ Mumio), lipid droplet formation levels were similar to the control group ($p > 0.05$).

3.3. The Expression Profiles of Apoptotic and Ferroptotic Markers were Analyzed following Treatment with Mumio Alone and in Combination with TMZ In Glioblastoma Cell Lines

Cell viability assays and fluorescence staining showed that Mumio treatment was relatively toxic to U87 and LN-18 cells after 24 hours compared to the control. To investigate this effect at the molecular level, western blot analysis was performed using apoptotic markers (PARP, PTEN, and Caspase 3) and a ferroptotic marker (NRF2). β -actin was used as a loading control. ImageJ software was used to quantify changes in expression for each marker [22].

We first examined the effect of Mumio alone on apoptosis- and stress-related proteins in U87 and LN18 cells (Figs. 6A–C). In U87 cells, PARP expression increased markedly (~11-fold, $p < 0.05$) at 250 $\mu\text{g/mL}$ Mumio, whereas a more moderate increase (~2-fold) was observed at 500 $\mu\text{g/mL}$ (Figs. 6A and B). Caspase 3 levels were also significantly elevated at both doses ($p < 0.05$), indicating activation of apoptotic pathways. Similarly, NRF2 expression increased significantly ($p < 0.05$), suggesting induction of antioxidant responses. In LN-18 cells, the response was more dose-dependent.

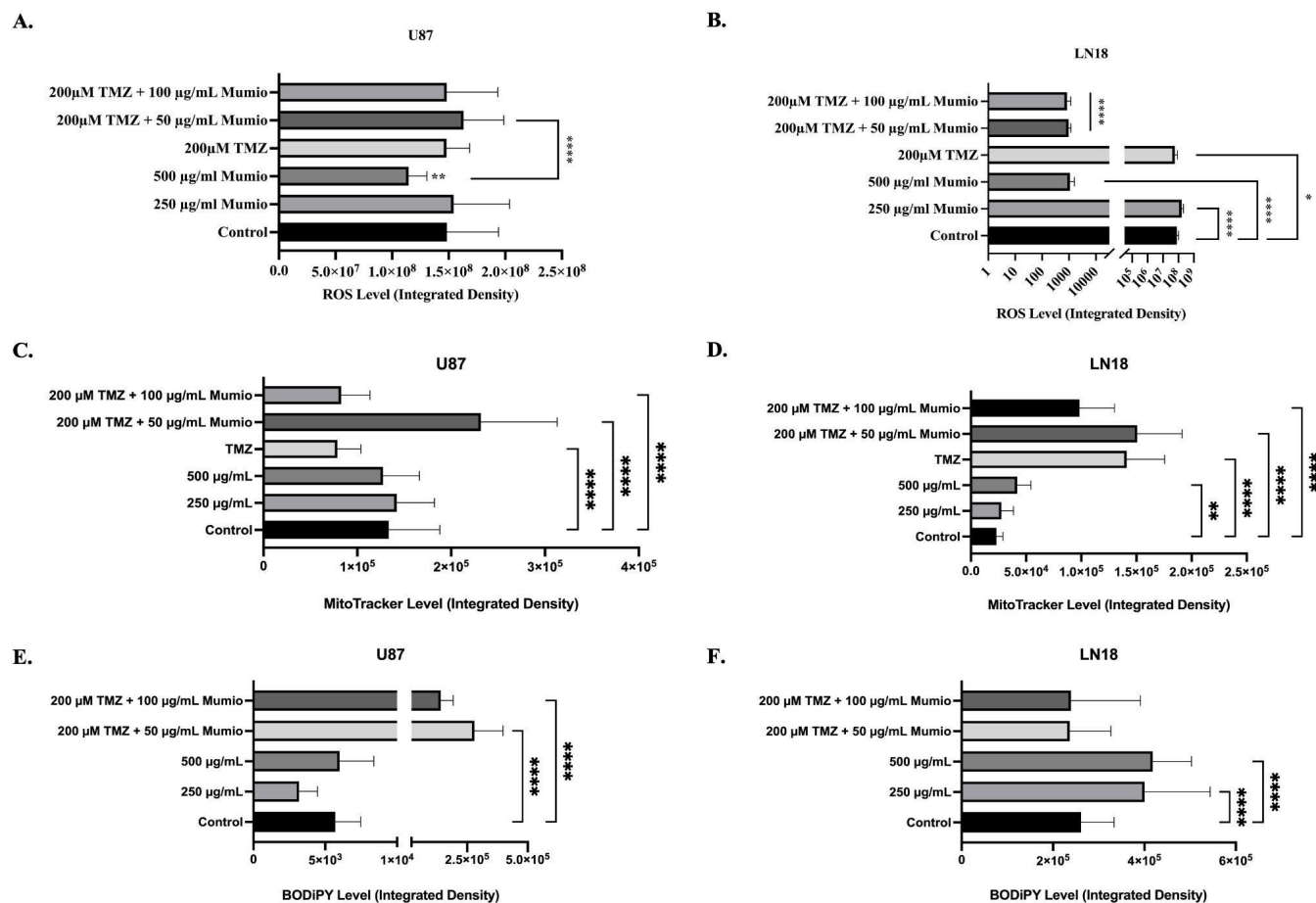


Fig. (5). Effects of TMZ and Mumio on ROS, mitochondrial status, and lipid peroxidation in U87 and LN-18 cells. ROS levels in (A) U87 and (B) LN-18 cells treated for 24 h with 200 μM TMZ alone, 50 or 100 μg/mL Mumio in combination with 200 μM TMZ, Mumio alone (250 or 500 μg/mL), or untreated control. ROS levels were quantified as DCFDA fluorescence integrated density using ImageJ. Data are shown as mean ± SD (2 biological replicates, each with 2 technical replicates; ≥50 cells analyzed per replicate). Statistical analysis was performed using mixed-effects analysis with Tukey's multiple comparison test (GraphPad Prism 9.2.0). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. MitoTracker fluorescence (integrated density) in (C) U87 and (D) LN-18 cells under the same treatment conditions, reflecting mitochondrial activity. Data are presented as mean ± SD (2 biological replicates, each with 2 technical replicates; ≥50 cells analyzed per replicate). Statistical analysis was conducted using ordinary one-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism 9.2.0). ns $p > 0.05$ (not presented any *), * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

PARP expression increased at 250 μg/mL (~2.3-fold, $p < 0.001$) but decreased below control levels at 500 μg/mL (~0.9-fold, $p < 0.05$) (Figs. 6A and C). As expected, PTEN was not detected in U87 cells, whereas in LN-18 cells it showed a modest but significant increase ($p < 0.001$). Caspase 3 followed a similar trend, increasing at the lower dose but slightly decreasing at the higher dose. In contrast, NRF2 expression was significantly reduced at both concentrations ($p < 0.001$).

The combined effect of TMZ and Mumio on apoptotic signaling was then evaluated (Figs. 6D-F). In U87 cells, combination treatment strongly enhanced apoptosis. Caspase 3 expression showed a pronounced increase (~7-fold, $p < 0.05$), while c-PARP levels were also significantly elevated ($p < 0.05$) (Figs. 6D and E). As expected, PTEN expression was not detected. Akt1/2/3 and FTH1 levels were moderately but significantly increased ($p < 0.05$). In LN-18 cells, a similar but more variable pattern was observed. c-PARP expression increased markedly (~3.9-fold, $p < 0.05$), and Caspase 3 was also significantly upregulated ($p < 0.05$) (Figs. 6D and F). PTEN expression decreased at the lower dose but increased above control levels at 100 μg/mL ($p < 0.05$). Akt1/2/3 levels were elevated, while FTH1 showed a decrease at the lower dose, followed by a significant increase at the higher dose ($p <$

0.05). Overall, densitometric analysis (Figs. 6B, C, E and F) supports the immunoblot data and indicates that Mumio promotes apoptotic signaling both alone and in combination with TMZ. Notably, the magnitude and direction of these effects differ between U87 and LN-18 cells, highlighting cell line-specific responses.

4. DISCUSSION

GBM is the most aggressive and common malignant brain tumor in adults, characterized by high intratumoral heterogeneity, resistance to therapy, and fast progression. Even with traditional treatments such as radiotherapy, surgery, and/or chemotherapy, the median survival time is 12-15 months [1,3]. TMZ is a standard chemotherapeutic agent used in GBM treatment; however, resistance associated with O-6-methylguanine-DNA methyltransferase (MGMT) limits its clinical effectiveness [2]. Other approaches, like cisplatin, bevacizumab, irinotecan, and EGFR inhibitors, give the opportunity to treat GBM, but significant toxicities occur with these treatments. To overcome these bottlenecks, recently, advanced strategies have been initiated to investigate the use of natural or bioactive compounds to improve the efficiency of the traditional treatment techniques.

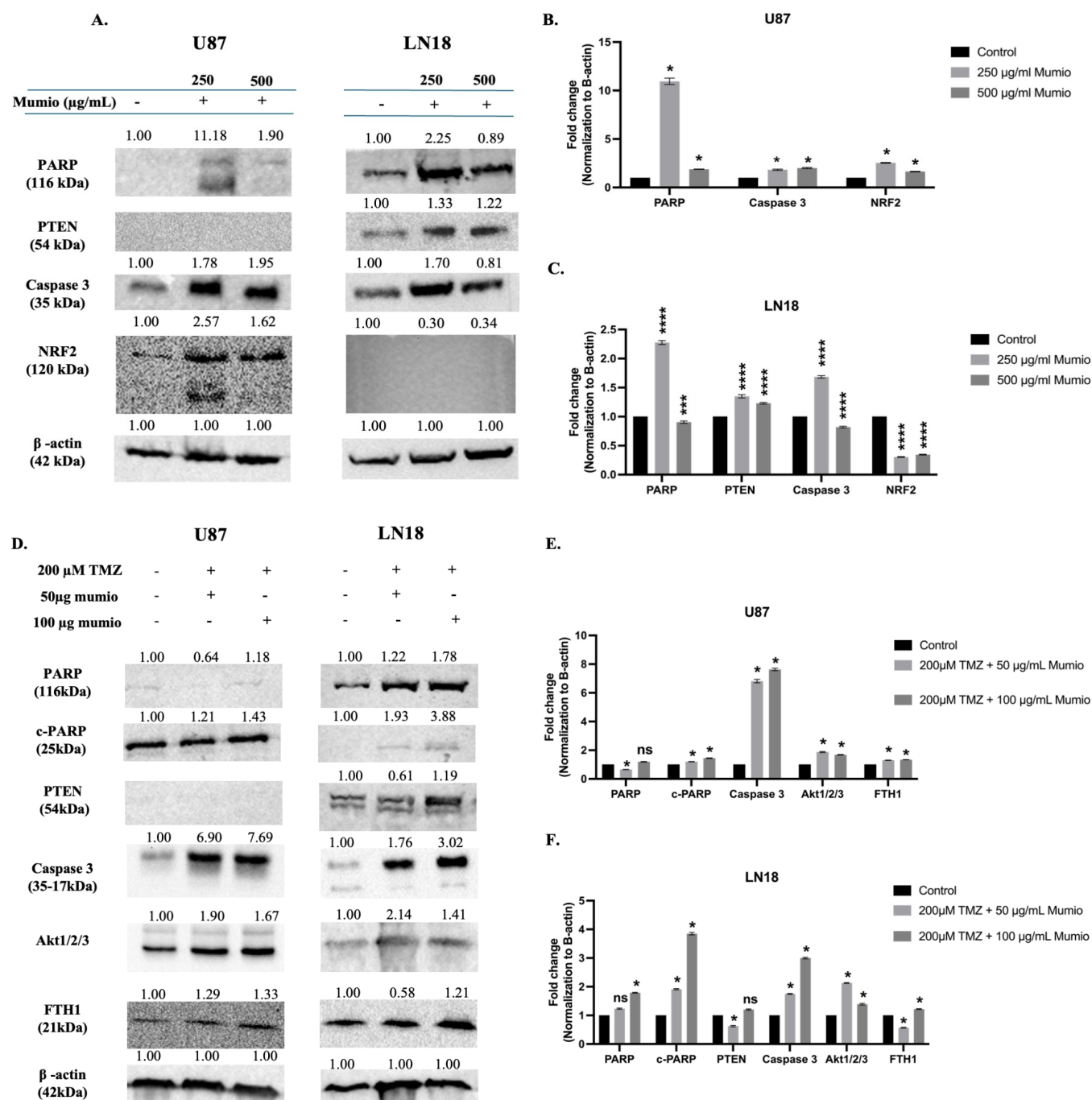


Fig. (6). (A) Western blot analysis of apoptotic markers (PARP, PTEN, Caspase-3) and the ferroptosis-related marker NRF2 in U87 and LN-18 cells following 24-hour treatment with 250 or 500 μg/mL Mumio. (B-C) Densitometric analysis of protein expression in (B) U87 and (C) LN18 cells treated with Mumio is demonstrated. (D) Western blot analysis of apoptotic (PARP, cleaved PARP [c-PARP], PTEN, Caspase-3, Akt1/2/3) and ferroptotic (FTH1) markers in U87 and LN-18 cells after 24-hour co-treatment with 200 μM TMZ and 50 or 100 μg/mL Mumio. (E-F) Densitometric analysis of protein expression in (E) U87 and (F) LN18 cells following combined TMZ and Mumio treatment is shown. β-actin was used as a loading control in all blots. Densitometric analysis was performed using ImageJ; the target protein intensities were normalized to the β-actin and then to their respective control group. Data are presented as mean ± SD. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Prism 9). Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns: not significant, $p > 0.05$. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

In the literature, it is known that as a natural material, Mumio has promising effects in the treatment of musculoskeletal, renal, hematologic, and neurological disorders with the favor of its biological properties such as anti-inflammatory, antioxidant, immunomodulatory, wound-healing, and cognitive-enhancing, anti-cancer effects [4, 5, 11, 12, 26].

In this study, Mumio was examined in GBM cell lines U87 and LN-18, both in the presence and absence of TMZ, for the first time in the literature. The results indicated that cell viability significantly decreased after 50 μg/mL Mumio application in U87 cell lines for 24 h; whereas higher dosages (250 and 500 μg/mL) were required for LN-18 cell lines (Fig. 3). U87 and LN-18 cell lines dif-

fer in molecular properties, such as PTEN, p53, and MGMT status, that influence proliferation, stress responses, and sensitivity to cytotoxic agents [27]. Therefore, Mumio was cytotoxic at lower doses in U87, whereas higher doses were required for LN-18. This dose- and genotype-dependent response aligns with previous reports in bladder and breast cancer models [11, 12]. The resistance of LN-18 cells likely reflects their PTEN and MGMT promoter methylation profile, consolidating the significance of tumor genetics in shaping responses.

The biological effects of Mumio extract were characterized biochemically *via* ICP-OES analyses (Table 1). It is revealed that Fe (iron) levels in Mumio are higher than reported levels in Mumio from India and Pakistan [7, 28]. In the literature, it is suggested that Fe content in Mumio increases with altitude [23, 29], and the current sample used in this article was sourced from the Altai Mountains. A high level of Fe could explain the oxidative stress observed in this study, which led to cytotoxicity.

Antioxidant activities in this study indicated a high radical-scavenging capacity, with a DPPH EC₅₀ of 0.29 mg/mL (Fig. 2). Moreover, the total phenolic content of Mumio was confirmed to include redoxactive phytochemicals.

Mumio alone decreased ROS levels in U87 and LN-18, with an enhancement in U87 and a decrease in LN-18 in combination with TMZ (Fig. 5). Therefore, mumio showed a complex effect on ROS levels in glioblastoma cell lines. In the literature, it was known that anticancer agents could first increase ROS, triggering oxidative damage and cell death, and then induce antioxidant defenses that lower ROS again in surviving cells [30, 31]. Excessive ROS can overwhelm the cell's antioxidant defenses, leading to oxidative DNA damage, lipid peroxidation, and activation of cell death pathways [32, 33]. These results are consistent with previous reports showing that redox-modulating compounds enhance the anti-tumor effect of TMZ in glioblastoma [15, 34]. Therefore, the redox-modulating properties of Mumio may offer a particular advantage when used as an adjuvant to standard chemotherapy.

Apoptotic markers were increased in U87 cell lines after Mumio treatment, indicating that Mumio affects apoptosis, as assessed by markers such as Caspase-3 and PARP [35]. A combination of TMZ and Mumio increased apoptotic markers (Caspase-3 and PARP) more in both cell lines; therefore, Mumio in combination with TMZ could be effective for TMZ-resistant therapies. The high level of Fe in Mumio showed effectiveness in increasing the ferroptotic marker FTH1 in U87, whereas a reduction was observed in LN-18, which has a more resistant biological profile [36].

Depending on cell type and biological properties, Mumio's effectiveness varied. However, this study indicated that Mumio, in combination with TMZ, could be a promising therapy for GBM to overcome TMZ resistance and enhance TMZ's effectiveness.

CONCLUSION

For the first time in the literature, this study showed that Mumio reduces GBM cell viability *via* oxidative stress, apoptosis, and ferroptosis-related pathways. Mumio, in combination with TMZ, helps overcome TMZ resistance, with enhanced cytotoxicity and apoptotic effects. These properties make a potentially promising supplement in GBM treatments. This study is limited by the fact that some assays were performed with only two independent biological replicates (each with technical duplicates), and thus, the results should be interpreted with appropriate caution. Moreover, additional experiments are needed to determine its safety profile, including *in vivo* studies at physiologically achievable doses, toxicological protocols, and examination of additional markers such as GPX4, SLC7A11, and ACSL4 to more thoroughly confirm ferroptosis and further elucidate Mumio's anticancer mechanism.

AUTHORS' CONTRIBUTIONS

The authors declare the following contributions to this work: B.D., Z.D., and A.Ş.M. performed the experimental studies and data analysis. B.D. and Z.D. were responsible for the formal analysis and preparation of the initial draft of the manuscript, whereas E.D.A., P.O.Y., P.U.O., and S.D. provided critical revision and editorial input. The overall study concept and design were developed by P.O.Y., S.D., and E.D.A. All authors examined the findings and approved the final version of the manuscript.

LIST OF ABBREVIATIONS

BODIPY	=	Boron-dipyrromethene
c-PARP	=	Cleaved PARP
DiOC6	=	3,3'-dihexyloxacarbocyanine Iodide
DMSO	=	Dimethyl Sulfoxide
DPPH	=	Total Antioxidant Capacity Assays
Fe	=	Iron
GAE	=	Gallic Acid Equivalent
GBM	=	Glioblastoma Multiforme
LN-18	=	TMZ-resistant GBM Cell Models
M-PER	=	Mammalian Protein Extraction Reagent
MGMT	=	O-6-methylguanine-DNA methyltransferase
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ROS	=	Reactive Oxygen Species
TEA	=	Trolox Equivalent Antioxidant Capacity
TMZ	=	Temozolomide
TPC	=	Total Phenolic Content
U87	=	TMZ-sensitive GBM Cell Models
uPA	=	Urokinase-type Plasminogen Activator

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, (Elif Damla Arisan), upon reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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