

# Olea Europaea Leaf Extract Attenuates Temozolomide-induced Senescence-associated Secretion Phenotype in Glioblastoma

**Short title: OLE reduces SASP factors in GB**

Melis Erçelik<sup>1</sup>, Berrin Tunca<sup>1</sup>, Seçil Ak Aksoy<sup>2</sup>, Çağla Tekin<sup>1</sup>, Gülçin Tezcan<sup>3</sup>

<sup>1</sup>Department of Medical Biology, Faculty of Medicine, Bursa Uludag University, Bursa, Turkey

<sup>2</sup>Inegol Vocation School, Bursa Uludag University, Bursa, Turkey

<sup>3</sup>Department of Fundamental Sciences, Faculty of Dentistry, Bursa Uludag University, Bursa, Turkey

## Corresponding Author Information

Berrin Tunca

btunca@uludag.edu.tr

+902242954161

<https://orcid.org/0000-0002-1619-6680>

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## ABSTRACT

**Objectives:** The purpose of this study is to investigate the effect of *Olea europaea* leaf extract (OLE) on senescence and senescence-associated secretory phenotype (SASP) caused by temozolomide (TMZ) in Glioblastoma (GB).

**Materials and Methods:** A Senescence  $\beta$ -Galactosidase assay and a colony formation assay were used to determine the effects of OLE, TMZ, and OLE+TMZ on cellular senescence and aggressiveness of GB cell lines T98G and U87MG. mRNA expression levels of P53, a senescence factor, IL-6, MMP-9, and NF- $\kappa$ B1 as SASP factors and Bcl-2 and Bax as senolytic markers were assessed using RT-qPCR. Cells were double-stained with Acridine Orange (AO) and Propidium Iodide (PI) to observe the cell morphology.

**Results:** TMZ increased the senescence rate of GB cells ( $p < 0.001$ ). Besides, OLE+TMZ reduced the proportion of senescent cells ( $p < 0.001$ ) and their capability to form colonies compared to TMZ-only treated cells. In addition, OLE+TMZ co-treatment elevated the mRNA expression levels of MMP-9, IL-6, NF- $\kappa$ B1, p53, and the Bax/Bcl-2 ratio compared to TMZ-only treatment. Especially in U87MG cells, involvement of OLE in TMZ treatments led to an increase of more than six times in the Bax/Bcl-2 ratio compared to TMZ-only, which resulted in the induction of the apoptosis-like morphological features ( $p < 0.0001$ ).

**Conclusion:** Collectively, our findings showed the inhibitory effect of OLE on TMZ mediated SASP-factor production in GB and, accordingly, its potential contribution to elongate the time of recurrence.

**Key Words:** Glioblastoma, *Olea europaea* leaf extract, Temozolomide, Senescence, SASP

## INTRODUCTION

Cellular senescence has been recognized as an essential tumor suppressor mechanism with its ability to the cessation of cell division.<sup>1,2</sup> On the other hand, recent studies evidenced a

contrasting effect of cellular senescence by promoting tumor growth with stimulating growth factors, matrix proteases, and proinflammatory protein, which are described as senescence-associated secretory phenotype (SASP)<sup>2,3,4</sup>. Some chemotherapeutic agents, such as 5-fluorouracil, gemcitabine, doxorubicin, irinotecan, and methotrexate, were shown to induce cellular senescence.<sup>5,6,7,8</sup> It appeared that chemotherapeutic drug-induced aging could be beneficial with its cell proliferation inhibitory effect. However, chemotherapy-induced senescent (TIS) cells give the tumor the ability to a future relapse by producing proinflammatory and matrix-destroying molecules known as SASP, which alters the tumor cells' metabolism in a way that reveals an aggressive cell phenotype.<sup>9</sup> Therefore, the effect of chemotherapy-induced cellular senescence on tumor progression could be two-sided and might be a reason for acquired therapy resistance and tumor recurrence.

Glioblastoma (GB) is one of the deadliest cancer types, and the DNA-methylating drug Temozolomide (TMZ) is the most widely used chemotherapy agent in the treatment of GB. TMZ was shown to induce senescence by the specific DNA lesion O6-methylguanine (O6MeG), which fails recognition of DNA damage, activation of DNA damage response pathway, and arrest of cells in the G2-M phase.<sup>10,11</sup> Considering the potential of GB cells to escape and become drug-resistant after TMZ administration due to being arrested in senescence, involvement of an additive agent with anti-senescence features in TMZ therapy is expected to reduce the risk of senescence-related symptoms and tumor relapse.<sup>12</sup> The therapeutic effect of various natural compounds, such as quercetin, fisetin, and curcumin, and their analogs in age-related diseases were explained with their senescence cell killing and senolytic effects.<sup>13</sup> Similarly, Oleuropein aglycone was reported to modulate angiogenesis on senescent fibroblasts.<sup>14</sup> An individual study showed that Oleuropein exhibits senescence inhibitory features by retaining proteasome function during replicative senescence in human embryonic fibroblasts.<sup>15</sup> Our previous studies indicated that *Olea europaea* leaf extract (OLE), which is known to contain a high amount of oleuropein, increases the therapeutic features of TMZ in GB.<sup>16,17,18,19</sup> Although the effect of oleuropein on cellular senescence was described in fibroblasts; its effect was not described in GB. In addition, one of our previous studies indicated that OLE consists of several additional bioactive components, such as secoiridoids, triterpenes, and flavonoids, in trace amounts, and these minor compounds in OLE could play critical anticancer roles.<sup>17</sup> However, the effect of OLE on TMZ-induced SASP expressions and its effect on tumor progression in GB remains unknown. Therefore, in this study, we investigated the effect of OLE on senescent cells induced by TMZ in GB cell lines with different TMZ sensitivity using in-vitro functional analyses. Our findings identified that OLE attenuates TIS cell-promoted expression of proinflammatory SASP factors and attenuated aggressive characteristics in GB cells, independent from their TMZ sensitivity.

## **MATERIALS AND METHODS**

### *Cell culture and reagents*

Human GB TMZ-resistant T98G cells and TMZ-sensitive U87MG cells, and control HUVEC cells (it was used to determine the drug cytotoxicity) were obtained from Medical Biology Department, Faculty of Medicine, Bursa Uludag University (Bursa, Turkey). All cell lines were maintained in a Dulbecco's Modified Eagle's Medium-F12 (DMEMF12; HyClone, Utah, USA) containing L-glutamine supplemented with 10% fetal bovine serum (FBS, BIOCHROME, Berlin, Germany), 1 mM sodium pyruvate, 100 mg/ml streptomycin, and 100 U/ml penicillin. All cells were incubated in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. The standardized OLE (05.06.2007, 10-00014-00- 015-0) was kindly provided by Kale Naturel (Edremit-Balıkesir, Turkey) and prepared as described in our previous study.<sup>19</sup> An Agilent 1200 HPLC system (Waldbronn, Germany) identified 19.419 mg/ml of oleuropein in the phenolic compounds of the OLE fractions in 280 nm of wavelength (Fig. 1A).<sup>19, 20</sup> TMZ

was provided by Sigma, USA. 19.419 mg/ml of oleuropein was detected in the standardized OLE used in this study

#### *Investigation of cell cytotoxicity and proliferation*

The cytotoxicity of OLE and TMZ and their effect on cell proliferation were assessed using a cell proliferation kit (WST-1, Roche Applied Sciences, Mannheim, Germany) as described previously.<sup>18</sup> The inhibitory concentrations at which 50% of the cells die (IC<sub>50</sub>) were selected to treat cells for the all experiment set up. The possible additive, antagonist, and synergistic effect of OLE on TMZ treatment was calculated using a web-based tool, SynergyFinder (version 2.0).<sup>21</sup>

#### *Apoptosis induction assay (Fluorescent Microscopy; Acridine Orange (AO) /Propidium Iodide (PI) Double Staining)*

The effect of OLE on morphological features of T98G and U87MG cells was assessed by AO/PI, a double-fluorescent dye staining method.<sup>22,23</sup> The morphology of the cells was analyzed with an inverted fluorescent microscope (Olympus, Tokyo, Japan). Cells with intact green nuclei were considered viable; Cells with dense green chromatin condensation areas in the nucleus were considered early apoptotic, cells with dense orange chromatin condensation areas were considered late apoptotic, and cells with intact orange nuclei were considered secondary necrotic.<sup>22,23</sup>

#### *Evaluation of tumor aggressiveness*

The CellMAX™ Colonogenic Assay Kit (BioPioneer, USA) was used to determine the effect of OLE on colony formation in T98G and U87MG cells. The blue-colored colonies were counted under an inverted microscope under 20x magnification.

#### *Detection of Senescence-Associated β-Galactosidase*

The rate of senescence in GB cell lines were detected using a Senescence β-Galactosidase Staining Kit (9860, Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's instructions. The blue-colored cells were visualized and counted under light microscopy using a 10x magnification.

#### *Examination of the expression levels of Senescence factors*

Total RNA was isolated using Zymo Research RNA Isolation Kit (Thermo Fisher Scientific, Glasgow, UK). RNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Beckman Coulter, California, USA). RNA samples with a total concentration between 200 and 400 ng/μl were selected for cDNA synthesis (High-Capacity cDNA Reverse Transcription Kit; Thermo Fisher Scientific, Glasgow, UK). RT-qPCR analysis was performed using TaqMan Gene Expression Assays specific to the genes related to senescence factors [p53(DM02154335-g1)], SASP factors [IL-6 (Hs00174131\_m1), MMP-9 (Hs00957562\_m1) and NF-κB1 (Hs00428211-m1)] and senolytic effect [Bcl-2 (Hs00608023\_m1) and Bax (Hs00180269\_m1)] Expression results were normalized to the expression of a housekeeping gene GAPDH (Hs00957562\_m1). The threshold cycle (Ct) for each RNA expression was determined using the StepOne Real-Time PCR System (Applied Biosystems, Warrington, UK). The 2-ΔCt method calculated the fold change in RNA expression.

#### *Statistical analyses*

One-Way ANOVA was utilized to evaluate the findings of the WST-1 assay, and independent T-test analyses evaluated the difference in the number of cell colonies. The independent T-test analyzed the findings of the Senescence-Associated β-Galactosidase assay. Independent T test evaluated the findings of RT-qPCR. Data are presented as mean ± SE. Significance was established at a value of p < 0.05. All Statistical analysis was performed using the IBM SPSS

Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA). The findings were interpreted as graphs using the GraphPad Prism 6 (GraphPad Software Inc, San Diego, Calif).

## RESULTS

### *OLE inhibits the proliferation of GB cells*

Previously determined effective OLE and TMZ concentrations were confirmed using current OLE extract in laboratory conditions. OLE, TMZ, and their co-treatment reduced tumor cell proliferation in GB cell lines, T98G and U87MG. Similar to our previous findings, the effective doses of OLE+TMZ treatments were determined as 2 mg/ml OLE and 400  $\mu$ M TMZ for T98G cells while they were 1 mg/ml OLE and 300  $\mu$ M TMZ for U87MG during 24h of incubation time (Fig. 1B, a-f).<sup>24</sup> In addition, non-of the applied concentrations of OLE caused a notable cytotoxic effect on a non-tumor endothelial cell line, HUVEC (Fig. 1B,g).<sup>24</sup>

Therefore, to investigate the effect of OLE and TMZ on senescence and aggressiveness of GB cell lines, 2 mg/ml OLE and 400  $\mu$ M TMZ were used for T98G, while 1 mg/ml OLE and 300  $\mu$ M TMZ were used for U87MG cells for the remaining in-vitro analysis. According to the findings, while OLE + TMZ treatment showed an additive effect in T98G (SC:-8.253) and U87MG (SC:-2.078) (Fig. 1C) and increased the number of cells with apoptotic morphology compared to TMZ-only treatment, suggesting that OLE provokes cell death independent from TMZ sensitivity of GB cells (Fig. 2).

### *OLE inhibits GB tumor aggressiveness*

OLE, TMZ, and their co-treatment led to a significant decrease in the number of tumor colonies of both T98G and U87MG cells compared to untreated cells (Fig. 3). The number of cell colonies was 216 in untreated T98G cells, while it decreased to 38 after OLE-only, 98 after TMZ-only, and 36 after OLE+TMZ treatments ( $p<0001$ , Fig. 3A). In addition, the number of colonies was 235 in untreated U87MG cells, and OLE-only, TMZ-only and OLE+TMZ treatments decreased it to 49, 64, and 46, respectively ( $p<0.001$ , Fig. 3B), suggesting that OLE-only treatment tended to reduce the number of colonies than that of TMZ-only treatment in both T98G and U87MG cells. In addition, the co-treatment of OLE + TMZ reduced the colony formation capacity of cells compared to TMZ-only treated T98G cells. The OLE + TMZ treatment 2.84-fold reduced the number of colonies formed by T98G cells compared to the number of colonies after TMZ-only treatment ( $p<0.001$ ). Moreover, after alone and complementary usage of OLE, the colonies' size was smaller than those of untreated and TMZ-only treated T98G cells. Interestingly, in U87MG, while alone usage of OLE reduced the size of colonies compared to untreated cells, it did not affect the size of TMZ treated cells (Fig. 3).

### *OLE reduces the senescence caused by TMZ*

Untreated senescent cells were 0.8% in T98G and 3.1% in U87MG cells, respectively (Table 1). TMZ-only treatment caused a significant increase in the number of senescent cells in both T98G and U87MG cells compared to those of untreated cells (The rate of senescent cells after TMZ treatment in T98G cells: 47%,  $p<0.001$ ; in U87MG cells: 13.4%,  $p<0.001$ ). Although OLE-only treatment resulted in an increased number of senescent cells in both T98G and U87MG cells, this increase was lower than that after TMZ-only treatment (OLE-only treatment, 11% induced senescent cells in T98G cells and 5.1% in U87MG cells). Likewise, while OLE+TMZ treatment resulted in an increase in the number of senescent cells compared to untreated cells (in T98G cells: 30%,  $p<0.001$ ; in U87MG: 4.5%,  $p<0.001$ , compared to untreated cells), the rate of this increase was lower than that of caused by TMZ-only treatment ( $p<0.0001$ ; Fig. 4). These findings showed that the involvement of OLE in the TMZ treatment reduced the senescence provoking capacity of TMZ (Fig. 4).

### *OLE suppresses the expression of SASP and senescence factors in GB cells*

TMZ-only treatment increased the mRNA expression levels of SASP-related genes, IL-6, NF- $\kappa$ B1, MMP-9, compared to untreated cells (TMZ induced IL-6: 2.03-fold;  $p=0.003$ , NF- $\kappa$ B1:

3.26-fold;  $p < 0.001$  and MMP-9: 6.14-fold;  $p < 0.001$ ; Fig. 5). In contrast, OLE-only treatment slightly increased MMP-9 (1.54-fold,  $p < 0.001$ ) and did not affect NF- $\kappa$ B1 and IL-6. In addition, a co-treatment with OLE+TMZ significantly attenuated the mRNA expression of these genes (OLE+TMZ reduced IL-6: 1.81-fold;  $p = 0.017$ , NF- $\kappa$ B1: 2.35-fold;  $p < 0.001$  and MMP-9: 3.07-fold;  $p < 0.0001$  compared to TMZ-only treatment; Fig. 5). Similarly, TMZ-only treatment significantly induced the mRNA expression of IL-6 (5.73-fold;  $p < 0.0001$ ), NF- $\kappa$ B1 (8.83-fold;  $p < 0.0001$ ), and MMP-9 (6.00-fold;  $p < 0.001$ ) in U87MG cells, compared to untreated cells. In addition, OLE-only treatment decreased the expression of IL-6 (2.22-fold;  $p < 0.003$ ) and NF- $\kappa$ B1 (3.1-fold;  $p = 0.001$ ) and did not affect MMP-9 compared to untreated cells. A co-treatment with OLE+TMZ significantly reduced the expression of these genes compared to TMZ-only treatment (OLE+TMZ reduced IL-6: 14.3-fold;  $p < 0.0001$ , NF- $\kappa$ B1: 13.23-fold;  $p < 0.0001$  and MMP-9: 2.21-fold;  $p < 0.001$ ; Fig. 5). These findings showed that one of the mechanisms of OLE to reduce GB tumor aggressive phenotype is by reducing the expression of TMZ-induced SASP factors.

Beside to the reducing effect on SASP factors, OLE+TMZ significantly reduced the expression level of p53, a senescence factor, while it induced the ratio of Bax/Bcl-2, a senolytic effective factor compared to TMZ-only in both T98G and U87MG cells (OLE+TMZ reduced p53: 1.27-fold;  $p = 0.002$ , and Bcl-2: 1.45-fold;  $p = 0.002$  while it induced Bax: 2.02-fold;  $p = 0.0007$  and Bax/Bcl-2: 1.3-fold  $p > 0.05$  in T98G and it reduced p53: 4.66-fold;  $p < 0.0001$ , and Bcl-2: 1.47-fold;  $p = 0.0001$  and induced Bax: 7.75-fold;  $p < 0.0001$  and Bax/Bcl-2: 6-fold;  $p < 0.0001$  in U87MG compared to TMZ) (Fig. 6). This data provides evidence of the diverting effect of OLE to apoptosis on senescent GB cells due to TMZ treatment.

## DISCUSSION

Cellular senescence arrests the cell cycle by the phosphorylation of p53 and the expression of p21.<sup>2,3,4</sup> One of our previous studies showed that TMZ-only treatment induces mRNA expression of P53 in GB tumors.<sup>19</sup> Confirming our previous findings, TMZ-only treatment induced P53 expression in T98G and U87MG cell lines in this study. Although the well-described function of p53 is activating apoptosis,<sup>25</sup> recent studies highlighted its regulatory role in cellular senescence via DNA damage responses (DDR) activation.<sup>26,27,28</sup> Studies evidenced that P53 could stimulate apoptosis as a response to overwhelming stress, while it could stimulate senescence as a response to less severe damage by failing to induce pro-apoptotic factors and leading to over-expression of the pro-survival gene BCL-2.<sup>29,30,31</sup> A very recent study of Aasland and colleagues linked TMZ-induced P53 expression to initiation of senescence in O6-methylguanine DNA-methyltransferase (MGMT) expressing GB cell lines.<sup>11</sup> Although MGMT is expressed in T98G cells, it is absent in U87MG.<sup>32</sup> In this study, after TMZ-only treatment, P53 and BCL2 expressions were induced in these cell lines. Unlike apoptosis, P53-initiated cellular senescence produces diverse bioactive factors SASP.<sup>33</sup> Activation of SASP requires a nuclear factor kappa B (NF- $\kappa$ B), and C/EBP $\beta$  pathways-mediated sustained DNA damage response. NF- $\kappa$ B regulates the production of IL-6, which plays a role in the maintenance and propagation of the SASP response in the tumor microenvironment.<sup>34,35</sup> Besides, NF- $\kappa$ B leads to transcriptional activation of proinflammatory cytokines and Matrix metalloproteinases (MMPs), such as MMP-9, a key modulators of tumor aggressiveness, in GB.<sup>36,37</sup> The findings of this study showed that TMZ-only treatment induced the expression levels of NF- $\kappa$ B, IL-6, and MMP9. In addition, SA- $\beta$ -gal activity, which mostly detected in senescent cells,<sup>38</sup> was induced after TMZ-only treatment in both cells, suggesting that TMZ-induced P53 dependent cellular senescence may be independent from MGMT in GB.

Conversely, while OLE-only did not affect P53 and slightly induced BCL2 in MGMT expressing T98G cells, it reduced P53 and had a higher capacity to induce BCL2 in MGMT

methylated U87MG cells. These findings indicate that the level of MGMT expression affects the expression of P53 in GB. According to our previous studies, OLE induces a methylation level of MGMT while reducing P53 and promoting apoptosis.<sup>39</sup> Similarly, in this study, the complementary usage of OLE with TMZ increased the Bax/Bcl-2 ratio, which is widely used to predict the cells undergoing apoptosis.

Evidences showed that cellular senescence promotes tumor aggressiveness and drug resistance.<sup>40</sup> However, in this study, OLE reduces the colony-forming ability of GB cells compared to TMZ-only treated cells, suggesting that OLE promotes apoptosis rather than senescence. Supporting this, based on the current findings, OLE did not affect NF- $\kappa$ B and IL-6 in T98G cells and reduced the expression of these genes in U87MG cells. In addition, upon senescence inducing by TMZ, OLE reduced the senescence markers, including NF- $\kappa$ B, IL-6 and MMP-9, and  $\beta$ -Galactosidase staining in both GB cell lines independent of MGMT expression level. Therefore, these findings provide evidence of the effect of OLE on directing TIS-GB cells to apoptosis.

### **CONCLUSIONS**

This study uniquely showed that the enhancing effect of OLE on TMZ sensitivity in GB tumors might be associated with its attenuating capacity to TMZ-induced senescent. Advanced studies are recommended for a better understanding of the effecting mechanism of OLE on TIS and to exhibit the potential of OLE to be a complementary therapy as a senolytic agent for GB patients.

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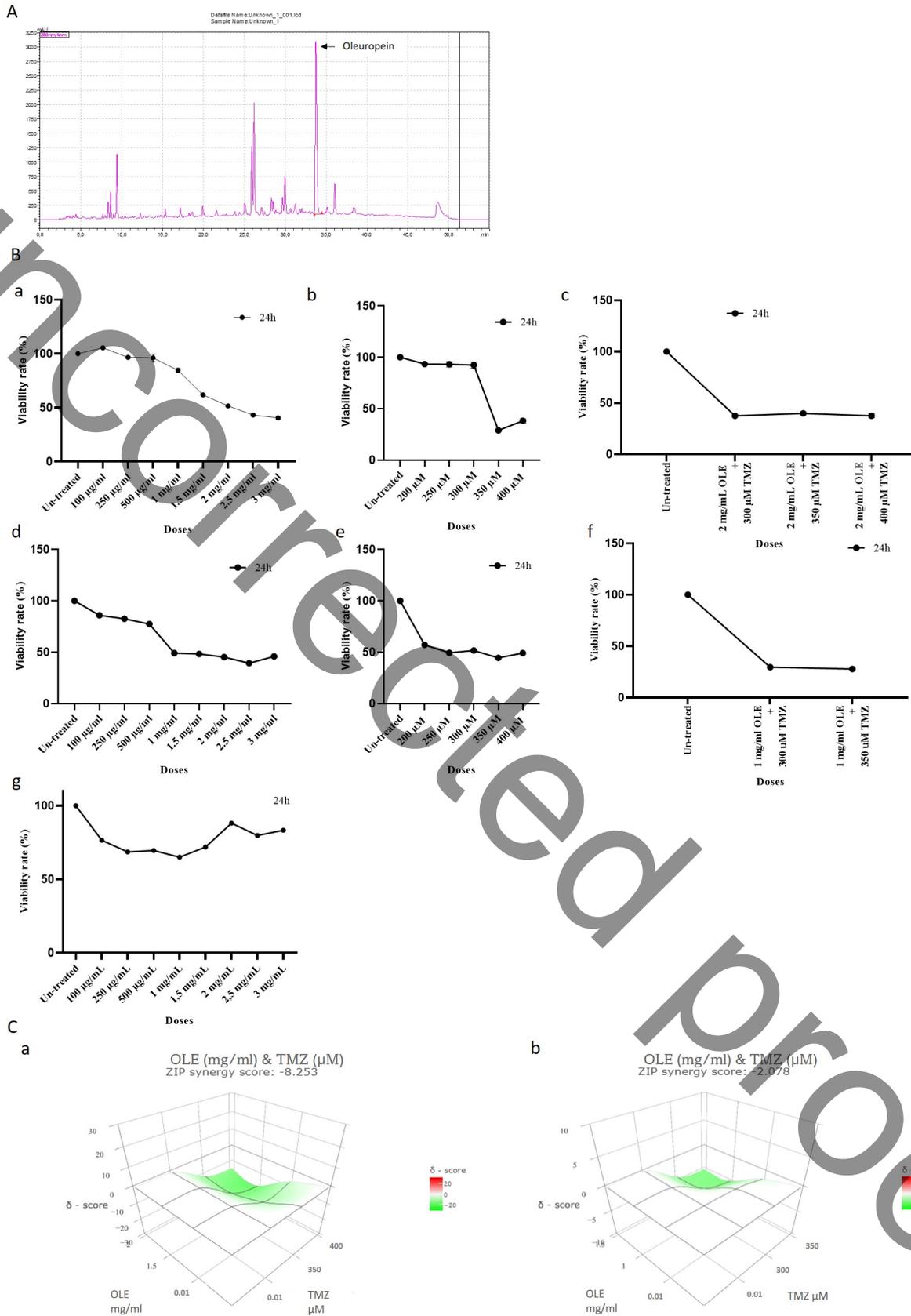
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Table 1. The effect of the combination of OLE, TMZ and OLE + TMZ on cell aging in T98G and U87MG cells

Cell number (n=3)	Average cell number	Number of aging cells (n=3)	Average number of

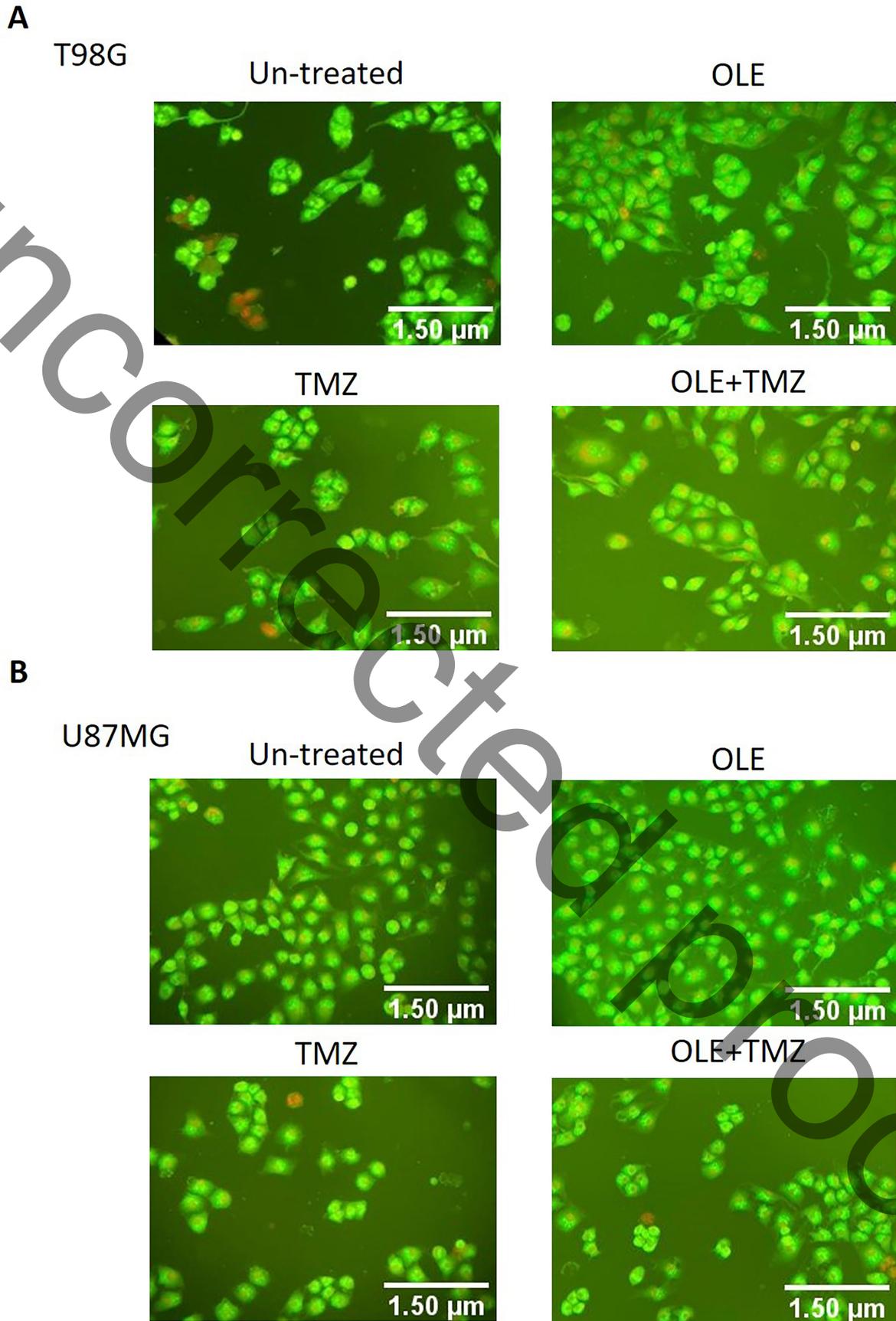
							aging cells (%)	
	I	II	III	I	II	III		
<b>T98G</b>								
Un-treated	121	111	107	113	0	3	0	1 (0.8 %)
OLE	54	67	76	42	7	8	7	7 (11 %)
TMZ	100	104	115	106	42	33	76	50 (47 %)
OLE+ TMZ	75	90	85	83	25	28	22	25 (30 %)
<b>U87MG</b>								
Un-treated	412	418	424	418	10	13	16	13 (3.1 %)
OLE	279	192	234	235	10	14	12	12 (5.1 %)
TMZ	461	454	469	461	62	75	49	62 (13.4 %)
OLE+ TMZ	391	330	398	373	27	17	8	17.5 (4.5 %)



**Fig. 1. A.** The oleuropein content of OLE. The oleuropein concentration of OLE was detected using HPLC/DAD analyses. **B.** T98G and U87MG cells viability rates of determined after treatment with OLE, TMZ, and OLE + TMZ and HUVEC cells viability rates of determined after treatment with OLE for 24 hours. OLE (a), TMZ (b), OLE+TMZ (c) treatment in T98G.

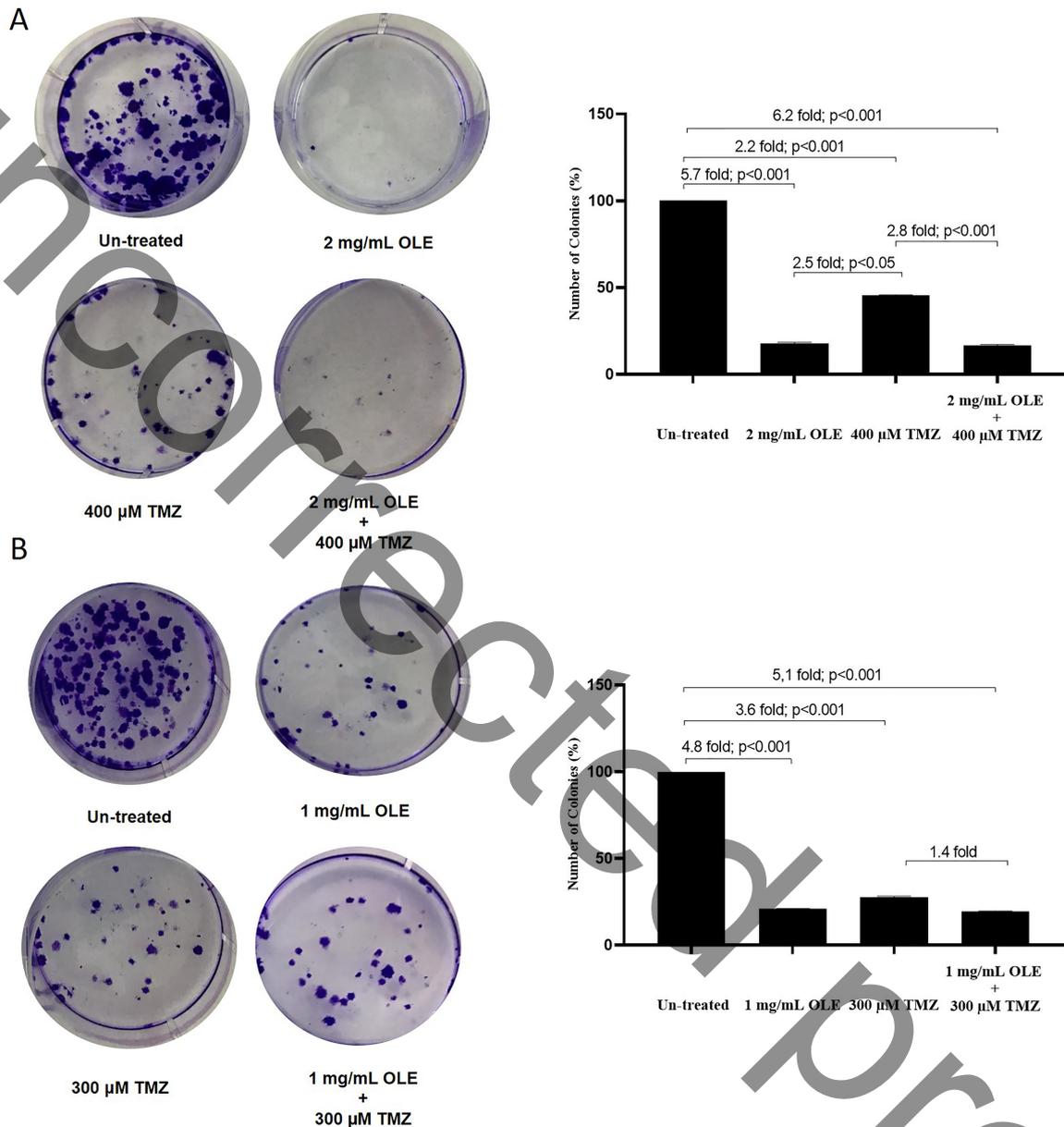
OLE (d), TMZ (e), OLE+TMZ (f) treatment in U87MG. OLE (g) treatment in HUVEC ( $p < 0.001$ , One-sample t-test). C. The combined effect of OLE and TMZ in T98G and U87MG cell lines. The additive effect of OLE and TMZ were detected according to the Zero interaction strength (ZIP) synergy scoring system. The effect of OLE +TMZ in T98G cells was shown in "a", and in U87MG cells was shown in "b".

Uncorrected proof

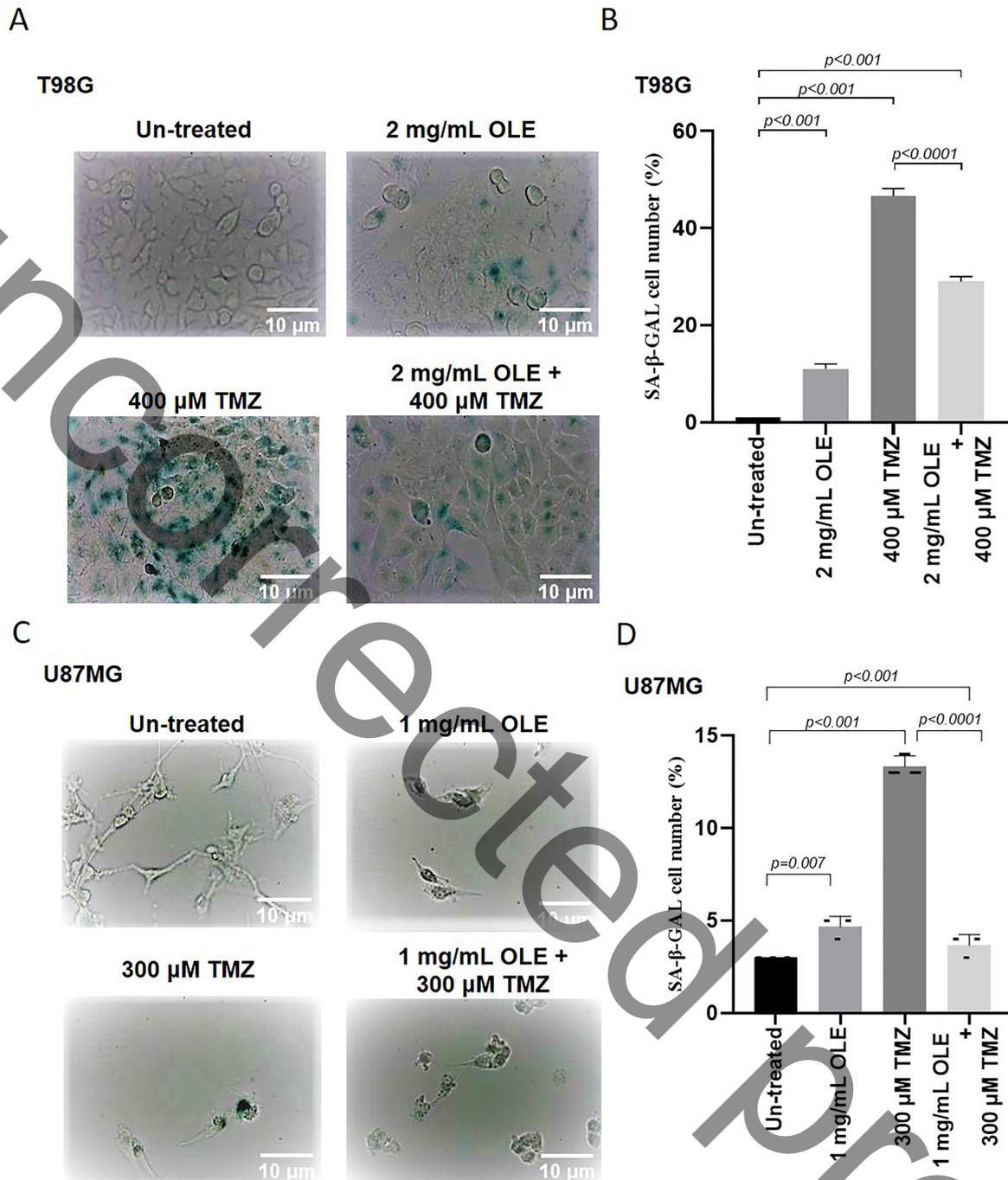


**Fig. 2:** The effect of OLE and TMZ on GB cells morphology. Findings of AO/PI staining showed that OLE and OLE+TMZ increased apoptosis rate in T98G (A) and U87MG cells

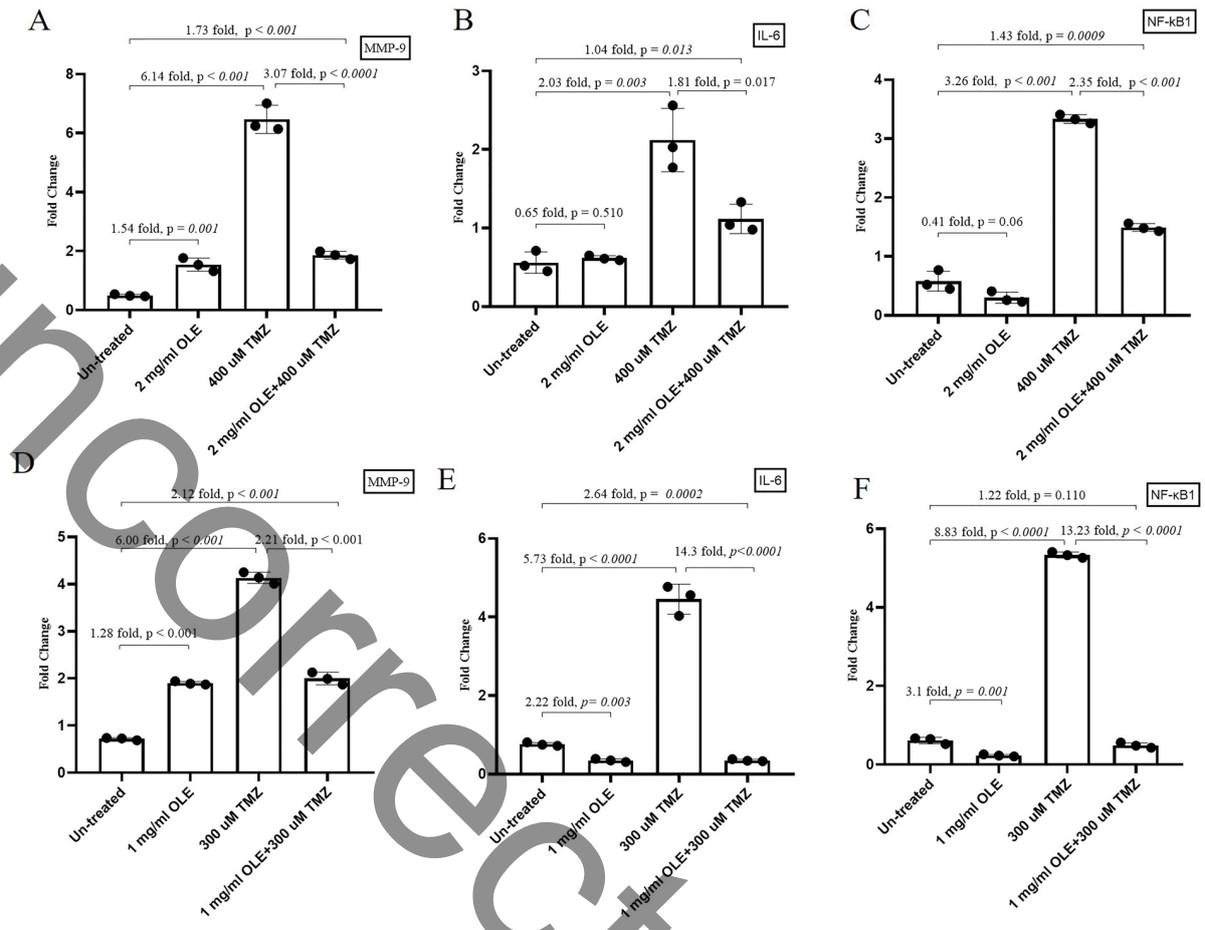
(B). Living cells: Cells with intact green nuclei; Early apoptotic cells: Cells with dense green chromatin condensation areas in the nucleus; Late apoptotic cells: Cells with dense orange chromatin condensation areas; Secondary necrotic cells: Cells with intact orange nuclei.



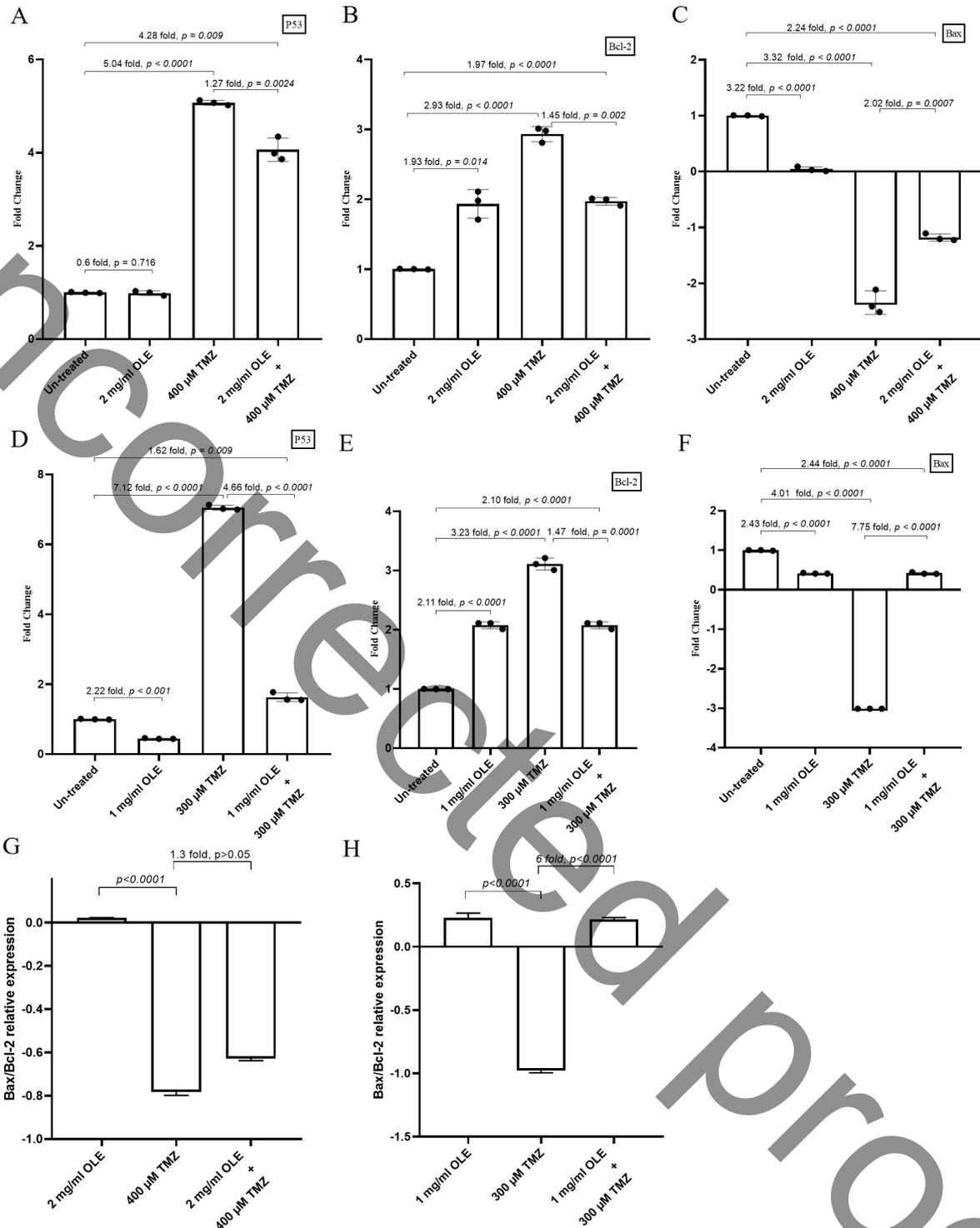
**Fig. 3:** Effects of OLE, TMZ and OLE + TMZ combination on colony formation of GB cells. in T98G cells OLE led 6.25-fold ( $p < 0.001$ ); TMZ: 2.20-fold ( $p < 0.001$ ) and OLE+ TMZ 5.71-fold ( $p < 0.001$ ) (A) in U87MG cells OLE led 4.8-fold ( $p < 0.001$ ); TMZ: 3.60-fold ( $p < 0.001$ ) and OLE+ TMZ 5.1 ( $p < 0.001$ ) (B) fold decrease compared to those of untreated cells. P values were calculated using an independent T test ( $n = 3$ ).



**Fig. 4:** Effects of OLE and TMZ on cellular senescence in T98G (A) and U87MG (B) (A light microscope was analyzed using 20X objectives for T98G cells and 10X objectives for U87MG cells). Comparison of the effects of OLE and TMZ treatments on senescence in T98G (A) and U87MG cells (B).



**Fig. 5:** The effect of T98G and U87MG cells treated with OLE, TMZ, and OLE + TMZ on the expression levels of SASP factors. MMP-9 (A), IL-6 (B), NF- $\kappa$ B1 (C) expression levels in T98G. MMP-9 (D), IL-6 (E), NF- $\kappa$ B1 (F) expression levels in U87MG.



**Fig. 6:** The effect of T98G and U87MG cells treated with OLE, TMZ, and OLE + TMZ on the expression levels of senescence factors and senolytic effect. p53 (A), Bcl-2 (B), Bax (C) expression levels in T98G. p53 (D), Bcl-2 (E), Bax (F) expression levels in U87MG. Bax/Bcl-2 relative expression of T98G (G) and U87MG (H) cells treated with OLE, TMZ, and OLE + TMZ.