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Evaluation of The Efficacy of Resveratrol and Curcumin on The Expression of *Bcl-2, Bax*, and *P53* Genes and GAFP Protein in Rats Model Glioblastoma Multiforme

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Abstract

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor seen in humans. Several epidemiological studies have demonstrated the chemo-preventive function of naturally occurring food components in cancer. Polyphenolic compounds of natural origin possess promising therapeutic potential as drugs for the management of glioblastoma and are being explored as potential anticancer agents. Despite the promising benefits of natural polyphenolic compounds, their effectiveness in treating GBM is restricted by their limited bioavailability and permeability through the blood-brain barrier. Several compounds derived from plants have demonstrated potential as antitumor therapeutic agents by augmenting apoptosis-related pathways and causing cell cycle impairment in tumor cells, including those found in GBM cell lines. According to reports, curcumin exhibits noteworthy antitumor properties. The anti-glioblastoma effects of heterogeneous compounds can be attributed to their ability to upregulate apoptosis and autophagy, promote cell cycle arrest, interfere with tumor metabolism, and inhibit proliferation, neuroinflammation, chemoresistance, angiogenesis, and metastasis. The development of glioblastoma cancer cells was modestly inhibited by the combination of resveratrol and curcumin. We found that the combined treatments resveratrol with curcumin inhibit the *Bcl-2* expression, the reduction of the expression of *Bax* and *P53*.The findings of this study offer substantiation that the administration of resveratrol with curcumin in combination may present a compelling alternative for combating GBM. Nevertheless, further clinical trials are necessary to validate the characteristics of these compounds both *in vitro* and *in vivo*.

Keywords: Glioblastoma multiform; resveratrol; curcumin, animal model

1. Introduction

Glioblastoma (GBM) is a prevalent and aggressive neoplasm of the central nervous system that exhibits a markedly invasive growth pattern, resulting in unfavorable patient outcomes [1, 2], and It is developed from alterations in astrocytes or glial precursors [3]. Glioblastoma typically exhibits distinct traits, including heightened cellular proliferation and swift infiltration of adjacent brain tissue, resulting in an unfavorable prognosis for affected individuals [2]. The treatment of glioblastoma is highly challenging due to the tumor's heterogeneity, its aggressive invasiveness into adjacent tissues, and the presence of the blood-brain barrier [2]. Notably, the occurrence of primary and secondary glioblastoma subtypes is associated with distinct age groups among patients. Specifically, primary glioblastoma is more prevalent in elderly patients, while secondary glioblastoma tends to affect individuals under 45. The histological characteristics of primary and secondary GBMs are typically indistinguishable. However, there are distinct genetic modifications and genomic profiles between the two groups of GBM, indicating

that they originate from distinct genetic pathways [4]. The signaling pathways that are most pertinent to GBM encompass several pathways that are triggered by growth factor tyrosine kinase receptors (TKRs). These pathways include the Ras sarcoma (Ras) pathway, as well as the phosphatidylinositol 3-kinase (PI3K)/ phosphatase and tensin homolog (PTEN)/AKT, retinoblastoma (RB)/cyclin-dependent kinase (CDK) N2A-$p16^{INK4a}$, and the TP53/mouse double minute 2 (MDM2)/MDM 4/CDKN2A-$p14^{ARF}$ pathways, among others [4]. Frequently observed in cases of GBM are mutations in the retinoblastoma, *P53* genes, and hyperactivation of the PI3-kinase pathway [4]. In addition to its role as a crucial mediator in preventing carcinogenesis, the tumor-suppressor protein *p53* plays an essential role as a transcriptional element in the control of apoptosis and cell proliferation [5]. On the other hand, SND1, Glial fibrillary acidic protein (GFAPα), and GFAPδ are three recently found promising biomarkers to improve diagnosis [6]. GFAP is an intermediate filament protein that is characteristic of astrocyte- and neural stem cell-derived gliomas [7].

Herbal and natural compounds included in the diet have received a lot of attention in recent years for their potential in treating glioblastoma [3]. Turmeric, or curcumin, is a polyphenol found naturally in the rhizome of the plant *Curcuma longa*. It was first used as a culinary spice in Europe in the 14th century [2]. The bioactive curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) found in Indian curry spice turmeric have garnered a lot of attention in the last few years. Various *in vitro*, *in vivo*, and clinical investigations have shown that curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione), a lipophilic polyphenol, may operate as an anticancer, antimicrobial, anti-inflammatory, and anti-aging agent [8]. Curcumin has been shown to have chemo-preventive, anti-proliferative, anti-invasive, and antiangiogenic properties, which may make it useful in the treatment of neoplasms such as glioma [2]. Curcumin has been shown to increase sensitivity to chemotherapy and radiation in a number of tumor types, including glioma, according to a number of studies. By altering the activity of transcription factors and controlling the expression of genes involved in malignant transformation and cell survival, curcumin is able to exert its effects via several methods [2]. On the other hand, resveratrol is a natural compound found in red grapes and other plants [9]. Resveratrol, along with some naturally occurring analogs like viniferins, pterostilbene, and piceid, is readily available in a regular diet and has numerous health-enhancing properties [10]. One such phytoconstituent is resveratrol (RVT), which has shown promising results in cancer therapy [11]. It has been studied for its potential health benefits, including its ability to act as an antioxidant and anti-inflammatory agent [9]. Some research suggests that RVT may also have anti-cancer properties and could help protect against heart disease [12]. Luo *et al*. have recently reported that curcumin has the potential to inhibit proliferation and induce apoptosis in GBM cell lines [13]. The study conducted by Alkahtani and colleagues demonstrated that the utilization of curcumin in conjunction with the PDT approach resulted in the stimulation of ROS production and subsequent demise of T98G cells through the mediation of NF-κB and Nrf2-dependent MMP2 and MMP9 axis pathways in glioblastoma malignancies [3]. Also, resveratrol has been demonstrated to trigger apoptosis by blocking the PI3K/Akt/

/mTOR pathway [14]. RVT and curcumin together have subsequently gained a growing scientific attention, leading to inquiry on its biological activity, and to several articles. Therefore, this study will focus on expression of some genes in apoptosis approach to treatment of glioblastoma multiform in rat model.

2. Materials and Methods

Experimental design

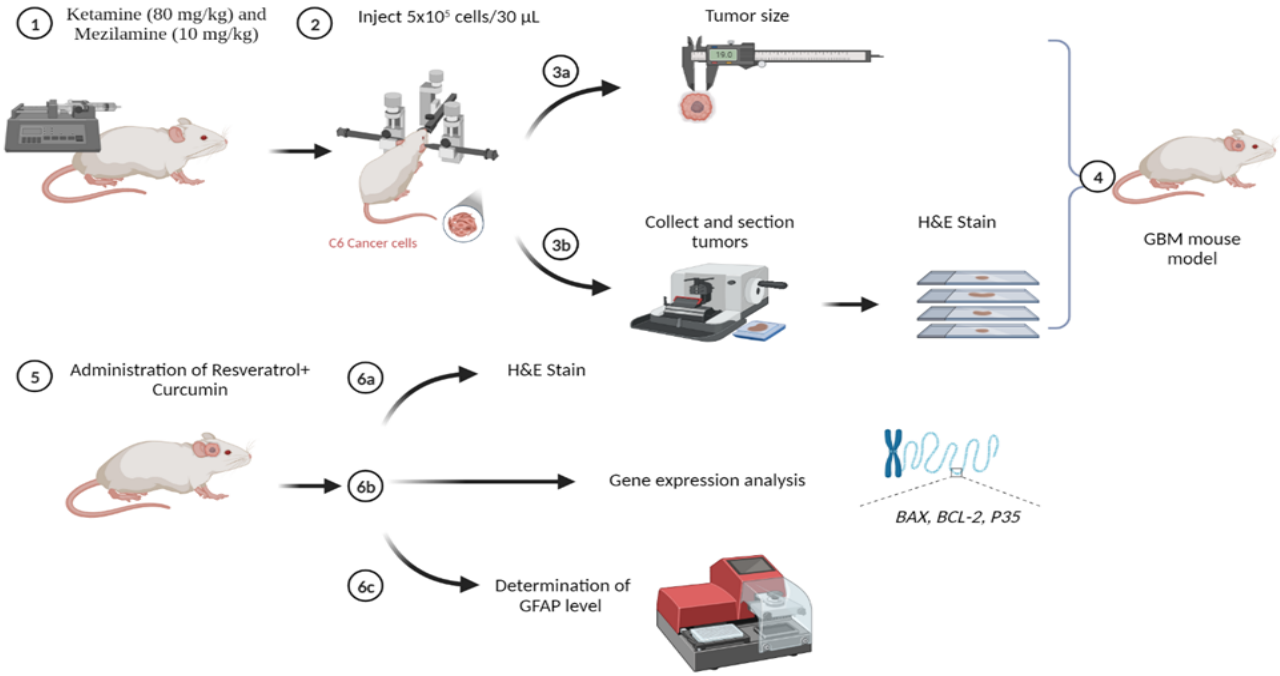

Fig.1.Shematics of experimental design

2.1. Animals

We obtained 40 male Wistar rats weighing 200-250g each from a reputable breeder, the Pastor Institute of Iran. Normal cages with 22±2°C, 55±5% humidity, and a 12-hour light/dark cycle housed the Wistar rats.

2.2. Animal model

GBM in male Wistar rats was produced by stereotactic implantation of C6 glioma cells into the brain. C6 glioma cells were grown and injected into the right frontal cortex of mice using a stereotaxic device to establish a glioblastoma animal model. After creating a skin incision in the back of the skull and extracting the periosteum using an infusion pump, the mice were sedated with ketmine (80 mg/kg) and mezilamine (10 mg/kg). At a depth of 2.5 mm, $5x10^5$ cells/30mL were injected. The mice were killed after the tumor had developed, and the tumor size was assessed using a digital caliper. Tissue processing and hematoxylin and eosin staining were carried out, followed by histological inspection for confirmation. It is vital to emphasize that this technique requires comtence in animal management and neurosurgery, as well as ethical issues.

2.3. Histopathology

The brain tissue obtained was fixed in 10% neutral buffered formalin (NBF, pH=7.26) for 48 hours before being processed and paraffin-embedded. Slices that were 5 µm thick were made and stained with hematoxylin and eosin (H&E). Two sections from each animal in each group were examined. An impartial (blinded) pathologist conducted histological research using an Olympus BX51 light microscope (Olympus, Japan). Different samples were examined for histological changes such as acute and chronic inflammatory response, liquid necrosis, hemorrhage, and/or hyperemia.

2.4.RNA extraction and cDNA synthesis

Total RNA was extracted from brain tissue samples using a total RNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's protocol [18]. To ensure purity, the extracted RNA was treated with DNase (Thermo Fisher Scientific), and its concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). Subsequently, complementary DNA (cDNA) was synthesized from the extracted RNA using a cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran).

To determine the specificity of the primers (Table 1) towards cDNA, a PCR experiment was conducted using cDNA, a -RT control, and water as templates. The PCR amplicons were subsequently separated by electrophoresis on a 2% agarose gel. If the primers exclusively bind to cDNA, a distinct DNA band of the expected size should be observed only in the reaction containing cDNA [18].

To analyze the expression of levels of *BAX, BCL, P35* and *GAPDH* genes, amplification of corresponded genes was performed using Rotor-Gene Q (Qiagen, Germany) thermocycler. The reaction mixture of 20 µL contained 1 µL of each cDNA sample, 10 µL SYBR green qPCR master mix 2X (Ampliqon, Denmark), and 0.5 µL of each primer [19]. The final volume was adjusted by adding RNase/DNase-free water. The thermal cycling conditions consisted of an initial denaturation of 10 min at 95℃ followed by 40 cycles of 95℃ for 30 s, 60 ℃ for 30 s, 72℃ for 30 s and a final extension step at 72℃ for 5 min. The melt curve analysis was performed for each gene to rule out nonspecific amplifications. Relative expression level of each gene was compared to the *GAPDH* gene, and results were analyzed using the $2^{-\Delta\Delta Ct}$ method incorporated into the relative expression software (REST).

2.5.Determination of GFAP level in GMB tissue

To detect and quantify levels of endogenous GFAP protein, the GFAP ELISA kit from ZellBio GmbH, Germany was used in this investigation. The ELISA plate wells were precoated with a GFAP-specific antibody, and after incubation with the coated antibody, GFAP protein in the samples was absorbed onto the plate. To detect the adsorbed GFAP protein, a peroxidase (HRP)-conjugated antibody specific for GFAP was applied following thorough washing. A tetramethylbenzidine (TMB) reagent and a sulfuric acid solution were used to inhibit color development. The resulting color intensity, which is proportional to the amount of bound protein, was measured at 450 nm and 630 nm wavelengths.

2.6. Statistical analysis

The data were presented as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism Version 8.3, using one-way ANOVA Dunnet's test and *t*-test. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Hematoxylin and eosin staining

Figure2 displays a cross-section of the rat brain that has been stained with hematoxylin and eosin dyes.

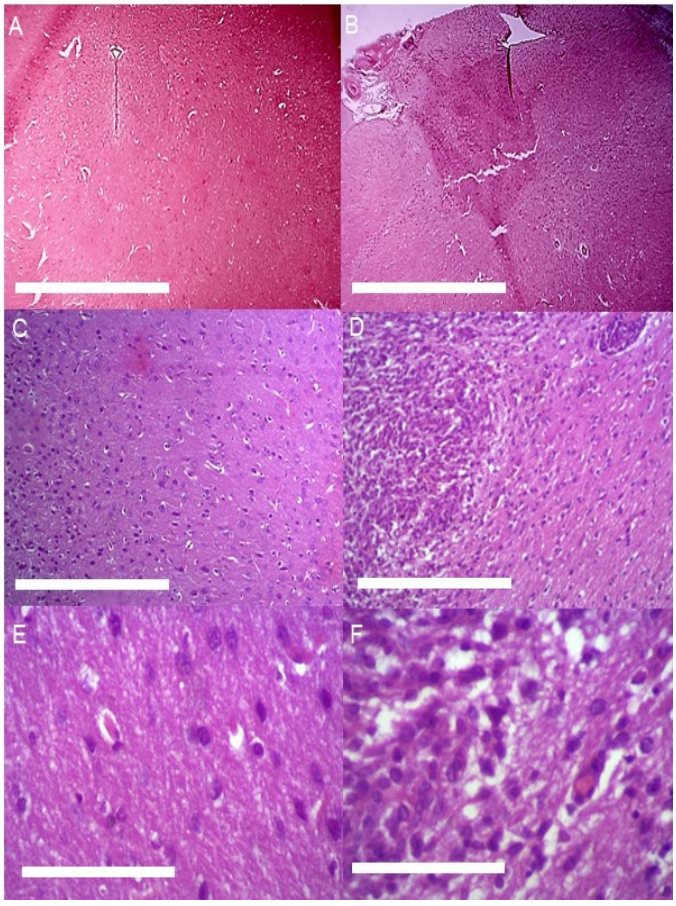

Fig. 2. displays Hematoxylin & Eosin (H&E) staining and tissue samples from rats in different experimental groups. (a) Control group (magnification: ×400, scale bar: 200 µm); (b) RVT+Cur group (magnification: ×400, scale bar: 200 µm); (c) Control group (magnification: ×400, scale bar: 100 µm); (d) RVT+Cur group (magnification: ×100, scale bar: 100 µm); (e) Control group (magnification: ×400, scale bar: 20 µm); (f) RVT+Cur group (magnification: ×400, scale bar: 20 µm).

In the control group, the brain tissue appears coherent and uniform, with a uniform distribution of hematoxylin color in the nucleus and eosin in the cell cytoplasm. The rate of neuronal cell death is very low, and the nuclei of living cells are completely round and somewhat transparent, making them clearly visible. Examination of the images from the different groups revealed that the size of the tumor was greater in the control group than in the other groups. Additionally, a high number of cells in the mitosis phase were observed in this group, indicating high cellular activity in the tumor and an increase in angiogenesis and blood supply to the tissue. The amount of apoptosis in this group was lower than in the other groups, and parts of the tumor exhibited necrosis, potentially due to a lack of oxygen in the center of the tumor. The results of the Cur+RVT group showed a significant reduction in tumor size and necrotic tissue compared to the other groups, indicating the effectiveness of the medicine and exercise on cancer cells after 30^{th} days.

3.2. Quantity analysis of *Bax, Bcl2* and *p53* genes

The results showed that resveratrol and curcumin, in combination, significantly increased expression of the tumor suppressor gene *P53*($P<0.001$) and the pro-apoptotic gene *Bax*($P<0.001$). This indicates that the compounds stimulated anti-tumor pathways. Conversely, expression of the anti-apoptotic gene *Bcl-2* was decreased($P<0.001$) (Fig. 3).

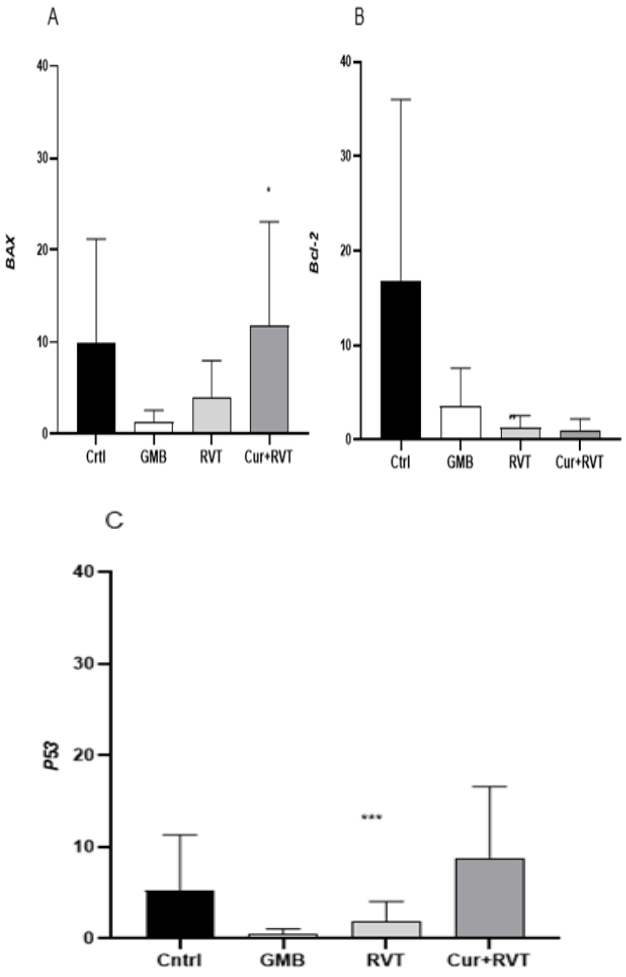

Fig. 3.Comparison of BAX(A), Bcl2(B) and P53(C) mRNA expression after treatment of GBM rats with RVT and Cur+RVT.

3.3. Effects of curcumin and resveratrol on GFAP protein levels

The expression levels of the GFAP protein in different groups are presented in Fig. 4. The results demonstrated that the protein levels in Ctrl and RVT groups are almost identical ($p > 0.05$), whereas an increase is observed in the GMB group without significant changes. Notably, the level of GFAP significantly decreased ($p < 0.05$) in the Cur+RVT group, which was treated with Cur+RVT.

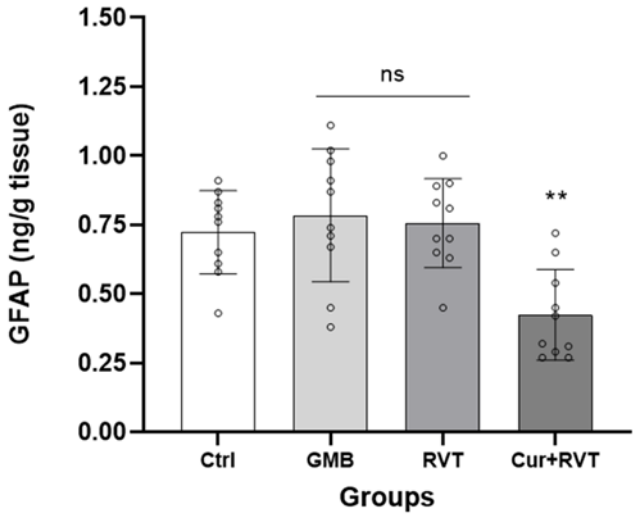

Fig. 4. The expression level of GFAP in brain tissue. Values are expressed as mean ± SD (n = 10); they are substantially different from the Ctrl group. *P* value shows as $^{**}p < 0.01$, ns = no significant.

4. Discussion

The most prevalent kind of primary brain tumor is GBM. There are several repeatable animal experimental models of GBM available, in which aspects of human tumor physiology can be replicated [20]. On the other hand, GBM is low despite improved treatment. GBM patients survived 7.7 to 13.5 months and 7.9 to 10.0 months after surgery and additional chemoradiation. Despite this, the prognosis for surviving GBM remains dismal. Thus, there is an essential need to discover novel chemicals that may control blood brain-barrier (BBB), thus reducing tumor growth and avoiding the emergence of recurring tumors [21]. The application of chemotherapy drugs is often restricted by the resistance of tumor cells and the associated side effects. However, curcumin, a phenolic pigment derived from plants of the zingiberaceae family, such as turmeric, has demonstrated a wide range of pharmacological activities [22]. This compound produced from the spice turmeric, has also been found to have anti-cancer properties by targeting a variety of molecular targets such as *Bcl-2*, *Bax*, and *p53* [23].

targeting a variety of molecular targets such as *Bcl-2*, *Bax*, and *p53* [23]. Also, curcumin has a positive safety profile and its interaction with cell cycle regulators and regulators of oxidative stress make it an attractive possible adjunct to current standard chemotherapeutic agents and radiation in the treatment of glioblastoma [24]. In recent years, several natural compounds have been investigated for their anti-tumor properties, including resveratrol and curcumin [20]. Studies have shown that both resveratrol and curcumin have the ability to induce apoptosis in GBM cells by regulating the expression of genes such as *Bcl*-2, *Bax*, and *p53* [22, 25]. In addition, our study investigated the expression of *Bcl-2*, *Bax*, and *p53* genes, which are involved in the regulation of apoptosis. The administration of curcumin has been shown to enhance the presence of pro-apoptotic proteins, including caspase-3, caspase-7, caspase-8, and caspase-9, which are pivotal in triggering and carrying out the apoptotic cascade. In essence, curcumin has the ability to facilitate the expression of crucial agents that promote programmed cell death [26]. The results of the study demonstrated that the combined treatment of resveratrol and curcumin significantly decreased the expression of GFAP protein. GFAP, an intermediate chain widely expressed in astrocytes, is an essential cytoskeletal protein [27]. High GFAP levels cause brain tissue damage, including nerve damage and neurodegenerative disorders [28]. GFAP is much higher in tumor cells than in normal brain tissue, and it may be used to diagnose and prognose many brain malignancies, including GBM [29]. Other studies suggested that a high GFAPδ/α ratio is associated with a more malignant and invasive behavior of glioma cells [30]. Our results suggest that the decrease in GFAP expression may indicate the anti-tumor effects of resveratrol and curcumin in GBM.

Our findings indicated that the tumor volume in Cur+RVT group were significantly lower than that in the Ctrl group ($p < 0.001$).The researchers have demonstrated that curcumin has the potential to hinder the growth of HepG2 cells by increasing the expression of the *Bax* gene and decreasing the expression of the *Bcl-2* gene [31]. *Bax* is a pro-apoptotic factor, meaning it promotes apoptosis, while *Bcl*-2 is an anti-apoptotic factor, meaning it inhibits apoptosis. The expression levels of *Bcl*-2 and *Bax* are closely related to the regulation of apoptosis in cells [22]. Curcumin may inhibit MDM2 protein synthesis or alter its interaction with p53 through the Akt/mTOR signaling pathway and PP2A dephosphorylation [32]. Studies have demonstrated that curcumin can activate the *p53* pathway by causing DNA damage and inhibiting the function of NF-κB, which is a transcription factor that has the potential to repress *p53* gene expression [33], which involved in cellular proliferation, inflammation, and cell survival [23]. Modulation of NF-κB activity by curcumin exerts advantageous effects by increasing the regeneration of muscles soon after trauma [33]. GBM molecular study shows dysregulation of nuclear signaling pathways as *Bax*/*Bcl-2*, PI3K, MAPK, mitogen, and *p53* [34]. Chemo- and radio-resistant TP53 mutations occur in 30-40% of GBM patients [35]. TP53 governs DNA repair, cell cycle arrest, senescence, and apoptosis [36]. Haas-Cogan *et al*. observed increased radio resistance to radiotherapy in GBM cells expressing mutant *p53* [37], while transfection of *p53* in the 9L GBM cell line increased sensitivity to cisplatin [38].

Other study by Mejia-Rodriguez *et al*. discovered that the combined treatments with AZD5363+AZD8542+Curcumin,AZD8542+Curcumin+Resveratrol inhibit the PI3K/AKT and SHH

survival pathways by decreasing the activity of AKT, the reduction of the expression of SMO, pP70S6k, pS6k, GLI1, p21 and p27, and the activation of caspase-3 as a marker of apoptosis [39]. A study by Mukherjee *et al*. (2018) has remarkably demonstrated that curcumin has the ability to induce the repolarization of tumor-supporting M2-like microglia/macrophages towards the tumoricidal M1-like phenotype. In addition, this study has revealed that activated natural killer (NK) cells are recruited intra-GBM as a result of this repolarization [40]. Simultaneously, curcumin has the ability to increase the expression of key tumor-suppressing proteins, such as *p53*, *p21*, and caspase 3 [26, 41]. Hence, in order to obtain a comprehensive understanding of the mechanisms underlying the individual and combined effects of curcumin and resveratrol on GB cell death, additional animal experiments are necessary. These experiments will help confirm and optimize the therapeutic strategy before its potential clinical application. The synergistic effects of resveratrol and curcumin in this context highlight the potential of these natural compounds as adjuvant therapies for GBM treatment. One promising approach to using regenerative medicine to treat GBM is to use stem cells. Stem cells are undifferentiated cells that can give rise to specialized cells, such as neurons and glia. They have been shown to have the ability to repair damaged tissue and suppress tumor growth.

Conclusion

Over the course of the last 20 years, there has been a significant amount of research indicating the advantageous effects of phytochemicals, with a particular emphasis on curcumin, in a diverse array of human diseases, including those that involve the brain such as neurodegenerative diseases (NDDs) and brain tumors, with a specific focus on GBM. In summary, our findings indicate that combining natural polyphenols with anti-cancer properties, such as curcumin and resveratrol, results in greater inhibition of glioblastoma growth and proliferation compared to individual treatments. However, more *in vivo* and *in vitro* studies are required to substantiate the features of these molecules.

Conflict of interest

The authors declare no conflict of interest.

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