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Terpinen-4-ol induces apoptosis and antiproliferation in glioblastoma cells via modulation of p53 and VEGF

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Abstract
Glioblastoma is an invasive tumor arising from astrocytes of brain. It has devastating effects and poor prognosis. It tends to be malignant and aggressive with distinct histopathological features. Many therapies including surgical resection, chemotherapy, and radiotherapy are in practice to treat cancer, but it is associated with a lot of adverse side effects. Terpenen-4-ol is a monoterpene derivative of essential oil which has several anti-inflammatory, bactericidal and antifungal activities, and is safe as compared to other treatment options available. In this study, we observed anticancer effects of terpinen-4-ol against glioblastoma cell line, cell viability in all groups of cells evaluated via MTT, morphology, and ELISA of p53 for the estimation of apoptosis, VEGF for the evaluation of angiogenesis, and antioxidant assay. The results demonstrated the anticancer effects of terpinen-4-ol against the glioblastoma SF767 cell line, showing reduced cell viability, increased apoptosis via p53 activation, decreased angiogenesis through VEGF inhibition, and enhanced antioxidant activity with decreased glutathione levels. The current study demonstrated that terpinen-4-ol treatment can cause apoptosis in SF767 glioblastoma cells via maintaining p53 and VEGF expression.

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Introduction

Cancer is a threatening term also called as neoplasia. It is in fact abnormal growth of cells. Cancer cells continue to divide in an uncontrolled manner and may influence surrounding organs and tissues leading to metastasis, which may prove fatal in the long run (Zetter, 1998; Bielenberg and Zetter, 2015). Although it is considered to be a modern world disease, its path can be traced in ancient times (Huebner et al., 2023). It is a complex disease which may affect the body, from disrupting its physiology at cellular level to influencing its complex functions even its metabolic activities (Seyfried et al., 2010).

Glioblastoma is most aggressive and a devastating brain tumor having poor prognosis (Nam and De Groot, 2017). According to WHO classification of tumors, grade IV is the highest grade which can be specified to any tumor, so glioblastoma is considered as grade IV tumor (Urbańska et al., 2014). It

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falls in this category based on its histopathologic features which are necrosis and endothelial proliferation (Agnihotri et al., 2023). “Secondary Glioblastoma” is a classic clinical term which is designated to those tumors that arise from previously diagnosed cases. The treatment of glioblastoma includes surgical intervention, radiotherapy and alkylating chemotherapy (Nam and De Groot, 2017; Lah et al., 2020). Adult GBM is one of the deadly malignancies faced by mankind (Davis, 2016).

Several treatment modalities are already in use to treat cancer; these include surgical treatment, chemotherapy and radiotherapy (Mrugala, 2013; Desai and Bhushan, 2017; Janjua et al., 2021). Conventional cancer treatments like chemotherapy and radiotherapy have certain cytotoxic effects. Some naturally occurring compounds are in use because of the minimum threat that they pose (Erices et al., 2018; Abid et al., 2020). Terpinen-4-ol is a monoterpene derivative which is a component of aromatic oils of *Melaleuca alternifolia* (Hart et al., 2000). Studies have shown that it has antioxidant and anti-inflammatory effects, as well as certain insecticidal, and fungicidal effects (Morcia et al., 2012), and bactericidal and antiviral effects (Wu et al., 2012; Shapira et al. 2016). Studies have shown its antitumor effect as well. It is used in combination with standard chemotherapy or other agents (Shapira et al., 2016). Terpinen-4-ol exerts antitumor effect by different mechanisms such as apoptosis induction via P53 and by limiting VEGF (Wu et al., 2012). Similarly, p53-dependant apoptosis was also observed in human melanoma cells (Calcabrini et al., 2004; Banjerdpongchai and Khaw-On, 2013; Shapira et al., 2016; Nakayama et al., 2017).

P53-induced apoptosis has a role in certain human diseases (Amaral et al., 2010). In cancer, ischemia, atherosclerosis, and certain neurodegenerative diseases, the abnormality in apoptosis is linked with p53 abnormality (Martikainen et al., 1990; Abrams et al., 1993; Gudkov and Komarova, 2003; Christophorou et al., 2006; Amaral et al., 2010).

In view of the earlier-mentioned reports, our primary objective was to appraise if terpinen-4-ol has profound effects on SF767 glioblastoma cells, and if it has a potential to induce apoptosis via activating the p53 and exerting anti-angiogenic effects through modulation of VEGF levels.

Materials and Methods

Study design

The treatment was divided into three principal groups: negative control being the normal cell line, and positive control being cisplatin, and terpinen-4-ol (experimental group)

Culturing of SF767 cell line

The cryovials were thawed in a T75 flask and stored in high glucose Dulbecco's modified Eagle's medium (DMM) supplemented with 10% fetal bovine serum (FBS) with streptomycin and penicillin in a humidified incubator at 37 °C and 5% CO_2. The medium was changed every 2-3 days. DMEM without FBS was used for the treatment (Maqbool et al., 2019).

MTT assay

Following the procedures as outlined elsewhere (van Meerloo et al., 2011; Maqbool et al., 2019), MTT assay was used to assess the cell viability. Briefly, the assay was performed in triplicate for each treatment. After the treatment, the cells were washed with PBS and incubated with 100 µL serum-free DMEM and 25 µL MTT solution at 5 mg/mL concentration for 2 h. The purple color formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm. Percent viability was calculated by a protocol described elsewhere (Hadi et al., 2020).

Morphology assay

The GBM SF767 cell line after successful culturing was incubated with IC50 dose of compound for 24 h. The cell morphology of the treated cells was observed under a phase contrast microscopy (Ahmad et al., 2014).

ELISA for P53, and VEGF

The Bioassay Technology Laboratory ELISA kit was used to evaluate angiogenesis and apoptosis. All the reagents, standard solutions and samples were prepared as instructed. An aliquot (50 µL) of the standard was added to the standard well. The sample (40 µL) was added to the sample well, followed by adding 10 µL of P53 (apoptosis) and VEGF angiogenesis antibody. Then an aliquot (50 µL) of streptavidin-HRP was added to the sample and standard well (not blank or control well). The mixture was mixed well, sealed with a sealer and incubated for 60 minutes at 37 °C. After incubation,

the plate was washed 5 times with wash buffer. For washing, 50 µL each of the substrate solution A and substrate solution B were collectively added. Just after 10 minutes, the optical density was measured at 450 nm by adding the stop solution.

Glutathione reductase (GSH) assay

The method described by Shamim and Rehman (2015) was performed for GSH assay. A reaction mixture was prepared by mixing 20 mM KH_2PO_4 buffer (pH 7.5), 40 mM EDTA, and 10 mM oxidized glutathione. After the incubation of the cell line with the treatment, the secretomes obtained from the diverse experimental groups were infused into the reaction combination. Thereafter, 20 mM NADPH was added and the absorption was recorded at 340 nm using a spectrophotometer.

Statistical analysis

Statistical analysis was done using the Graphpad Prism. The data were further analyzed by one-way ANOVA intra-group comparisons using the Bonferroni test at $P \leq 0.05$ to assess statistical significance of different variables.

Results

MTT Assay

Various doses of terpinen-4-ol were tested to evaluate the cell viability of glioblastoma cells. The MTT assay showed a reduction in proliferation in the treated groups compared to the control group, in a dose-dependent manner. The absorbance values obtained demonstrated a distinct trend, with a decrease pattern in response to varying treatment concentrations. These findings suggest a significant biological effect of the treatment on the metabolic activity of the cells, substantiating its potential as an anti-proliferative agent as shown in **Table 1** and **Figure 1**.

Table 1. Mean proliferation as absorbance values at varying levels of terpinene-4-ol ± standard deviation

Groups	Mean ± SD
Control C	0.9433 ± 0.04509
PC 100	0.6000 ± 0.05000
25 µM	0.8767 ± 0.03786
50 µM	0.6433 ± 0.04933
100 µM	0.4607 ± 0.03580

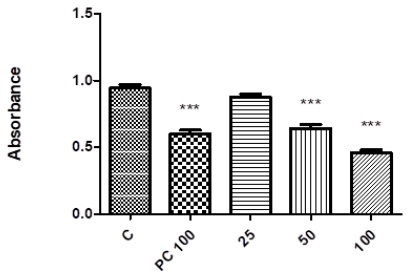

Figure 1. Anti-proliferative activity (MTT absorbance) of terpinen-4-ol. Values are indicated as the mean ± SD where * indicates level of significance at $P \leq 0.001$.**

Morphology assay

The morphological analysis of SF767 cells was carried out by the Floid cell imaging station (**Figure 2**). There had been considerable change in morphology of the cell line at varying levels of TP4O.

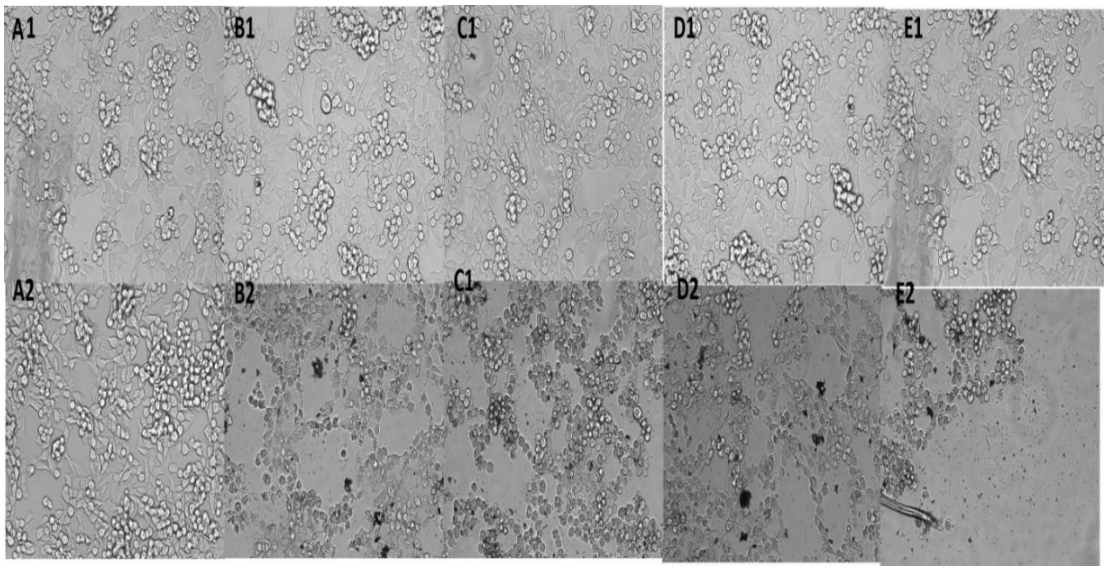

Figure 2. Morphology of five groups of SF767. A1 and A2 are control, B1 and B2 as positive control, C1 and C2 each 25 µM, D1 and D2 each 50 µM, E1 and E2 100 µM

ELISA for P53

Terpinen-4-ol caused SF767 to undergo more apoptosis as detected with ELISA. It was evident that as the concentration of terpinen-4-ol increased the degree of apoptosis increased markedly in the cancer cell line. In the cell line treated with TP4O, the amount of p53 increased consistently with increase in the level of TP4O, which is an important component of apoptosis as shown in **Table 2** and **Figure 3**.

ELISA for VEGF

The VEGF angiogenesis antibody was added in a 96-well plate and absorbance was noted afterwards. TP4O application inhibited the tumor angiogenesis as shown in **Figure 4** and **Table 3**.

Glutathione reductase (GSH) assay

The antioxidant potential measured as the activity of glutathione reductase (GSH) increased consistently with increase in exogenous application of TP4O (Fig. 5 and **Table 4**).

Discussion

Glioblastoma (GBM) being an invasive tumor arising from the astrocytes of the brain and having tendency to become malignant, shows considerable therapeutic resistance (Wu et al., 2021). It also contains tumorigenic cancer stem cells that have properties of self-renewal and show therapeutic resistance (Lathia et al., 2015). During its progression, it can be controlled by many therapeutic techniques including surgical resection, chemotherapy, and radiotherapy (Chu et al., 2024). These therapies are widely used, but due to some limitations and associated undesirable effects, new means have to be developed, one being the use of natural compounds to treat GBM (Zhai et al., 2021).

Numerous natural compounds are widely utilized for therapeutic purposes due to their medicinal properties in treating various diseases (Zhai et al., 2021; Giammona et al., 2024). Many novel drugs have been developed by structurally modifying natural compounds to enhance their efficacy (Yao et al., 2017). In the present study, the natural compound terpinen-4-ol, a monoterpene derivative of tea tree oil, was investigated. Its anticancer and anti-inflammatory potential (Hart et al., 2000), antibacterial (Zhang et al., 2019), insecticidal, fungicidal effects (Morcia et al., 2012) have already been established.

Terpinen-4-ol not only plays an important role in cell death, but it also enhances biological and chemotherapeutic effects of anticancer therapies (Shapira et al., 2016). The authors also showed that it causes a significant growth inhibition of

Table 2. ELISA-tested apoptosis measured as p53 of the cells after application of TP4O

Groups	Mean (absorbance) ± SD
Control C	0.753 ± 0.0153
PC 100	1.257 ± 0.0551
25 µM	0.897 ± 0.0451
50 µM	1.220 ± 0.1000
100 µM	1.417 ± 0.0586

Table 3. Tumor angiogenesis values

Groups	Mean (absorbance) ± SD
Control C	1.225 ± 0.08261
PC100	0.773 ± 0.02517
25 µM	1.223 ± 0.08505
50 µM	0.873 ± 0.03055
100 µM	0.636 ± 0.01528

Table 4. Glutathione reductase activity measured as absorbance of the cells treated with TP4O

Groups	Mean (absorbance) ± SD
Control C	0.801 ± 0.02723
PC 100	1.157 ± 0.06028
25 µM	0.903 ± 0.04726
50 µM	1.163 ± 0.07095
100 µM	1.367 ± 0.02944

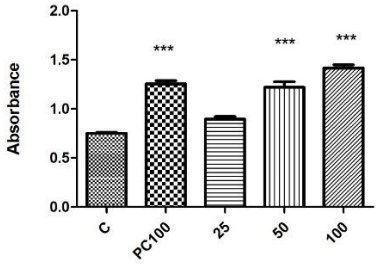

Figure 3. Degree of apoptosis (p53) of the cells after application of TP4O. * level of significance at $P \leq 0.001$**

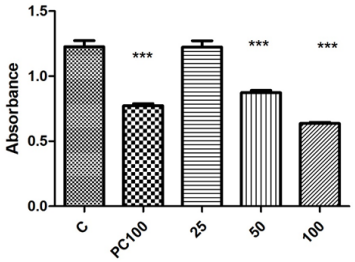

Figure 4. VEGF levels after application of varying doses of TP4O.* means differed significantly from the others at $P \leq 0.001$**

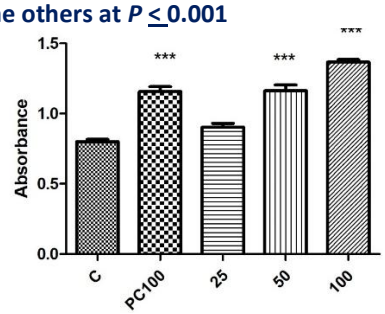

Figure 5. Glutathione reductase activity (GSH) measured as absorbance of the cells treated with TP4O. * means differed significantly at $P \leq 0.001$**

colorectal, pancreatic, prostate and gastric cancer cells in a dose dependent way (Shapira et al., 2016). In the present study, the effect of different doses of TP4O using different assays was observed on the GBM cell line.

The results revealed a significant reduction in cell proliferation in the experimental groups compared to the control group. Furthermore, to study apoptosis and p53 activity, a p53-specific ELISA was conducted. The findings indicated that apoptosis was markedly increased in the TP4O-treated cells, highlighting its potential as an anti-proliferative and pro-apoptotic agent. Similarly, Cao et al. (2021) have shown that TP4O inhibited the proliferation and mobility of pancreatic cancer cells by downregulating Rho-associated coiled-coil containing protein kinase 2. Moreover, earlier Shapira et al. (2016) concluded that TP4O is actively involved as an anti-cancer agent. They further reported that this compound can be used as alone or in combination with several other such compounds for the treatment of cancer.

Angiogenesis relates to the formation of blood vessels as a critical factor for vasculature development in embryogenesis, and it plays an important role in tissue repair and further normal growth later in life (Awan et al., 2017a). It also includes some oncological processes which relate to tumor growth and they can be translated into metastasis (Zetter, 1998; Bielenberg and Zetter, 2015). VEGF (vascular endothelial growth factor) is a signal protein that is an important factor in angiogenesis and pro-angiogenesis (Pérez-Gutiérrez et al., 2023). In the current study, VEGF levels decreased consistently with increase in the level of TP4O, indicating that the tumor angiogenesis was inhibited considerably by VEGF.

Oxidative stress refers to the maladjusted ratio between pro-oxidants and the immune system, causing its dysregulation (Rahal et al., 2014; Vona et al., 2021). It induces the alteration in numerous signaling pathways, which can undermine cellular metabolism and the formation of the ROS (Awan et al., 2017b). Antioxidant enzymes, such as glutathione reductase (GSH), are known to positively influence cell proliferation under physiological conditions. However, in the context of anti-proliferative therapies, these enzymes can act synergistically by reducing ROS levels, thereby enhancing the therapeutic efficacy of anticancer agents (Couto et al., 2016). In this study, the antioxidant potential of GSH in modulating the anti-proliferative effects of TP4O was investigated. Using SF767 cell lines, we observed a significant anti-proliferative activity in the cells treated with TP4O, suggesting that these antioxidant enzymes may contribute to the therapeutic effects by counteracting ROS-mediated cellular damage while supporting the efficacy of the treatment (Deen et al., 2023).

Conclusion

In conclusion, the study demonstrated that terpinen-4-ol exhibits a significant anticancer potential against SF767 cells, with effects such as reduced cell viability, anti-proliferation, decreased angiogenesis, and increased apoptosis.

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Contribution of authors
Planning and conduction of experiment: TM, TM. Conduction of research: RS, MAJ. Data collection, visualization and interpretation: RS, MAJ, TM. Graphical presentation/visualization: TM, TM, RS, MAJ, HA. Statistical analysis: RS, TM, MAJ. Preparation of initial draft: RS, MAJ, TM. Review of initial draft: RS, TM. Proof reading and approval of the final version: TM, TM, RS, MAJ, HA. Revisions and corrections: TM, TM, RS, MAJ, HA.

Ethical approval
This study does not involve human/animal subjects, and thus no ethical approval is required.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with great care during collection and experimental procedures. After completion of the study, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

Supplementary material
No supplementary material is included with this manuscript.

Conflicts of interest
The authors declare no conflict of interest.

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher/Journal management. These are available with the corresponding author (s) and/or with other author(s) as declared by the corresponding author (s) of this manuscript.

Authors' consent
All authors have critically read this manuscript and agreed to publish in IJAaEB.

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It is declared that the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

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