ORIGINAL ARTICLE

IN VITRO, ANTI-PROLIFERATIVE, APOPTOTIC AND ANTI-OXIDATIVE ACTIVITIES OF TERPINEN-4-OL

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ABSTRACT

Objectives: The primary bioactive ingredient in tea tree oil is terpinen-4-ola naturally occurring monoterpene with a wide range of biological activity. Our goal is to investigate the anticancer effects of terpinen-4-ol at different concentrations as well as its mode of action on human brain cell cancer lines.

Methods: The study was designed as an in vitro RCT, from March 2020 to April 2022 and carried out at the University of Lahore's IMBB Department in Lahore, Pakistan. Four groups received study treatment: Terpinen-4-ol (experimental group), cisplatin (positive control), negative control (normal cell line), and vehicle control (DMSO). The cryovials were thawed before the cells were cultivated in T75 flasks. Terpinen-4-ol was administered at varying concentrations, Dead Cells Detection (Trypan Blue Assay) and Live Cells Detection (Crystal Violet Assay) were used to quantify its lethal effects via cytotoxicity and cell viability. VEGF angiogenesis factors and enzyme activity were calculated. The ELISA kit was utilized to assess apoptosis and inflammation. Statistical analysis was done using Graph Pad Prism. One-way analysis of variance (ANOVA) with bonferroni comparison within groups was used to analyze the data. $P \leq 0.05$ was considered statistically significant.

Results: Cancer cell proliferation is significantly inhibited by terpinen-4-ol in a dose-dependent manner. The treated cell lines displayed more apoptotic alterations than the control group. The P-value was less than 0.05.

Conclusion: Terpinen-4-ol dramatically slows down the growth of cancer cell lines. Cell death induction is one potential molecular mechanism for its action.

Key words: Terpinen-4-ol (TP-4-ol), apoptosis, Trypan blue and Crystal Violet assay

How to cite this article: Ramzan S. In Vitro, Anti-Proliferative, Apoptotic and Anti-Oxidative Activities of Terpinen-4-Ol. Pak Postgrad Med J 2025;36(2): 85-89

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Received: February 21,2025; Revised: June 27,2025
Accepted: June 29,2025

DOI: https://doi.org/10.51642/ppmj.v36i02.762

INTRODUCTION

Cancer is a disease having fatal effects; its path can be traced in prehistoric times as well. 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.[1, 2] Its infiltrating nature can be attributed to oncogenic properties which either hinder the apoptosis or any defect in p53 signaling pathway, as a result it is unable to perform its function normally, in addition VEGF keeps on promoting angiogenesis which further causes spread of cancer.[3, 4]

Traditional (herbal) medicines have become more popular worldwide due to least side effects. Different forms of these herbal medicines have become most common supply for primary health care of about 75-80% of the world's population estimated by the World Health Organization (WHO) .[5, 6]

It is well known that biological components including proteins, lipids, and DNA can react with reactive oxygen species (ROS), such as oxygen radicals and their reactive byproducts, causing damage to cells and tissues [7]. The ability of ROS to trigger autophagy and apoptosis is widely recognized. The regulation of both autophagy and apoptosis depends on the redox environment of the cell. ROS may be the cause or result of alterations in the death pathways in the mitochondria. The electron transport chain's mitochondria are where ROS are often produced. ROS damage mitochondria and are linked to the apoptotic process in the mitochondria. Apoptotic cell death is frequently closely linked to elevated ROS levels.[8] Glutathione or N-acetylcysteine are examples of radical scavenging compounds that can be used as a pretreatment to demonstrate the causal function of ROS. Angiogenesis is a key factor in the progression of cancer. Nutrients and oxygen are taken by tumor cells through newly formed blood vessels. Therefore, by controlling angiogenesis and making nutrients, depriving cancer cells is a promising way to combat cancer.[9] Among many growth factors, VEGF is the main factor of angiogenesis. In many researches, plant extracts have been used to counter VEGF. Extracts also lowered the VEGF level thus, suppressing angiogenesis. Terpinen-4-ol is a monoterpene derivative of essential oil (tea tree oil) which have many anti- inflammatory, bactericidal and antifungal activities and is safe as compared to other treatments available.[10] Terpinen-4-ol is a compound having antitumor effects against certain cancer cells. It can be used as a potential treatment option.[11] Terpinen-4-ol exerts antitumor effect by different mechanisms that are still not clear and is a subject of interest for new researchers. This study is planned to investigate the anticancer effects of terpinen-4-ol at different concentrations as well as its mode of action on human brain cancer cell lines.

METHODS

This study, carried out at CRIMM and IMBB department of University of Lahore, Pakistan. We examined the effects of terpinen-4-ol on the SF767 cell line. Four groups participated in the experiment: the experimental group (terpinen-4-ol), the positive control (cisplatin), the negative control (normal cell line), and the vehicle control (DMSO). Dulbecco's Modified Eagle Medium (DMEM) with high glucose, streptomycin, penicillin, and 10% fetal bovine serum (FBS) were used to cultivate SF767 cells in T75 flasks. The flasks were kept in a humidified incubator at 37°C and 5% CO_2, with medium changes occurring every two to three days. Cell morphology was examined using phase contrast microscopy after a 72-hour treatment with DMEM devoid of FBS and the compound's IC50 dosage [12].Terpinen-4-ol was administered at varying concentrations(25,50 &100). Dead Cells Detection (Trypan Blue Assay) and Live Cells Detection (Crystal Violet Assay) were used to quantify its lethal effects via cytotoxicity and cell viability. After incubation, washing, and substrate addition, the color changed from blue to yellow, which was detected at 450 nm. Three separate experiments were statistically analyzed, and the mean ± SEM was used to express the results. Using an ELISA kit and conventional procedures, inflammation and apoptosis were assessed.[13]

Anti-oxidative Enzymes, Glutathione Reductase (GSH) and Superoxide dismutase (SOD) Assay

Glutathione reductase (GSH) was performed by Shamim,[14] in a 96 well plate with a reaction mixture of 200µL in each well. A reaction mixture was prepared by mixing 20 mM KH2PO4 buffer (PH 7.5), 40 mM EDTA, and 10 mM oxidized glutathione. Secretomes obtained from different experimental group of post treatment of cell lines were added to the reaction mixture. In the end, of 20 mM NADPH was added, and absorbance was taken using a spectrophotometer at 340 nm. For SOD, the reaction mixture was prepared by mixing the secretome of different experimental groups of post-treatment on SF767 cells with 100mH KH2PO4 buffer (pH 7.8), 0.1mM EDTA, 13mM methionine, 2.25 mM nitro-blue tetrazolium chloride (NBT), 60µM riboflavin. Its optical density was measured at 560nm by spectrophotometer.

STATISTICAL ANALYSIS

Graph Pad prism was used to perform statistical analysis. The data was analyzed using one- way analysis of variance (ANOVA) with bonferroni comparison within groups. Group means were compared by one-way ANOVA and Bonferroni's test was used to identify differences between groups. $P \leq 0.05$ was considered statistically significant.

RESULTS

Cytotoxic effect of terpinen-4-ol on human brain cancer cells (Trypan Blue Assay)

Using trypan blue as a detecting agent for live and dead cells, I washed cells with PBS and then observed it under microscope. Cells that were stained were dead.

Cell viability effect of terpinen-4-ol on human brain cancer cells (Crystal Violet Assay) This assay is performed to check viability of SF767 cell line using crystal violet staining. When SF767 were treated with TP4O doses of 50µM and 100µM. The cells treated

were less viable as compared to control

Table.1: Effect of Terpinen-4-ol on Cell Death in Human Brain Cancer Cells (Trypan Blue Assay, Number of dead cells)

Sr.no	Groups	Mean ± Standard Deviation
1.	Control C	34.67 ± 5.033
2.	PC100	48.33± 3.512
3.	25	35.33 ± 7.572
4.	50	52.00 ± 2.646
5.	100	63.33 ± 3.055

Table.2: Effect of Terpinen-4-ol on Cell Viability in Human Brain Cancer Cells (Crystal Violet Assay, Number of alive cells)

Sr. No.	Groups	Mean ± Standard Deviation
1.	Control C	1.087± 0.03288
2.	PC 100	0.8333 ±0.02082
3.	25 T4-ol	1.150 ± 0.08718
4.	50 T4-ol	0.7533± 0.04163
5.	100 T4-ol	0.5700 ± 0.07211

The antioxidant potential using the glutathione reductase (GSH) assay shows the free radical scavenging activity. GSH activity was more in experimental groups were treatment was applied as compared to control groups.

The absorbance of Superoxide Dismutase was also observed and it showed the increased antioxidant potential particularly against high doses of Terpinen-4-ol applied.

ELISA for VEGF: VEGF angiogenesis antibody was added in 96-well plate and absorbance was checked afterwards, it showed that TP4O have inhibited the tumor angiogenesis

DISCUSSION

It is believed that natural medicines are much safer as compared to synthetic drugs, drawing the attention of humans toward natural medicines as phytotherapeutic agents and phytopharmaceuticals products [15]. This fact has led to a resurgence of scientific interest in the biological effects of natural products. The second-hand metabolites produced in medicinal plants under biotic and abiotic conditions have been proved useful in the treatments of various human diseases.[16] Recent reports have cited that plants and their components could act as a tumor suppressor and apoptotic inducers in cancerous cells.[17]

Angiogenesis is the formation of blood vessels, it helps in vasculature development in embryonic stage and in tissue repair and normal growth later in life.[18] In addition to this it plays a role in cancer by pro-angiogenic factors that help in tumor growth, its progression, and its invasive nature may even cause metastasis.[19] VEGF is a mediator of angiogenesis and is considered as an important pro-angiogenesis factor.[20] It shows that anti- angiogenesis approach is important in anti-cancer approaches. In my study I used VEGF angiogenesis antibody to study its effect and result showed reduced growth of SF767 that was treated with TP4O. Previous studies also show Terpinen-4-ol induced human Leukemic HL-60 cell death occurred by different mechanisms including autophagy and apoptosis.[21] According to present research work, we found the similar results, when cancer and normal cells were treated with T4OL, low level of VEGF was observed thus, inhibiting the angiogenesis in cancer cells while no or very minute effect was observed in normal cells. Similar results have been studied by researchers in colorectal cancers.[22]

LDH is an enzyme released when damage occurs to cell membrane and is estimated by supernatants of cell cultures. In many studies, control cells showed lesser LDH release and cells treated with plant extracts showed higher LDH release.[23] The same results were observed when treated HeLa, HepG2 cell lines with Trepenin-4-ol, LDH level was significantly high but in normal cells, it was not affected.[12, 24]

It is considered that "oxidative stress" is a primary cause of cancer and occurs as a result of dysregulation between the production and demand for oxygen and nutrients. As in the proliferating tumor cells, the demand for oxygen and nutrients increases rapidly and an inadequate, dysfunctional blood supply resulting from tumor angiogenesis.[25] Oxidative stress results in an increased level of ROS but the cancer cell can adjust against ROS levels by using metabolic shift that can save them from apoptosis due to oxidative damage thus rapid proliferation is there.[26] Anti-oxidative enzymes (APOX, CAT, SOD and GSH) affect the proliferation of a cell in a positive way but when these antioxidants are given in compliance with some anti-proliferative therapy, it enhances the efficacy of the therapy by reducing the levels of ROS.[12, 27]

According to a recent study, ROS produced in mitochondria play a part in controlling autophagy. According to it, anti-cancer drug causes cell death in malignant glioma cells as well as mitochondrial damage, ROS generation, and the selective destruction of mitochondria by autophagosomes.[28] According to the current study, terpinen-4-ol could dramatically increase ROS production and autophagic cell death in HL-60 cells, which resulted in the removal of damaged organelles. The autolysosomes, microsomes, and mitochondria may all produce ROS and oxidative damage. Oxidative stress can cause apoptosis. Within pancreatic acinar cells, the oxidative stressor triggers two distinct apoptotic pathways: the classical mitochondrial calcium-dependent pathway, which is

initiated quickly in most cells, and a slower caspase-8-mediated pathway that relies on cathepsin lysosomal activities and is employed when the caspase-9 pathway is inhibited constantly in the pancreatic acinar.[29]
Terpinen-4-ol has antiproliferative and anticancer effects on human nonsmall lung cancer cells.[30] According to our research work, when treated cancer cells (HeLa and HepG2) with terpene OL 4, antioxidants SOD and GSH activities were increased thereby oxidative stress is decreased, while a slight difference was observed in levels of antioxidants between pre-treatment and post-treatment of normal cell lines.

CONCLUSION

The anti-cancer activity of trepenin-4-ol has been investigated which indicates that Trepenin-4-ol showed promising results against the growth of cancer cells. According to our findings, treatment of brain carcinoma cell lines with Trepenin-4-ol induces apoptosis, restricts proliferation and angiogenesis, and enhances the anti-oxidative index in post-treated cells.

THE LIMITATION OF THIS STUDY

We only investigated the effects of trepenin-4-ol in cancer cell lines. A more extensive analysis with a variety of chemotherapeutic agents could have yielded more profound insights into the possible synergistic effects of trepenin-4-ol. Future research should investigate the effects of trepenin-4-ol in conjunction with other anticancer treatments to ascertain whether trepenin-4-ol amplifies their effectiveness. This would provide a more thorough comprehension of its potential as an auxiliary therapeutic approach.

ETHICAL APPROVAL

Ethical approval was granted by the Institutional Review Board of the University of Lahore dated: 26/08/2022.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

FUNDING SOURCE: None

HF: Experimental setup, performed laboratory work, data collection and analysis, drafting the results section.
MZ: Experimental design, statistical analysis, data interpretation, writing the discussion and conclusion.
ZH: Laboratory supervision, methodology, development, critically review
AK: Interpretation of results, revisions to the methods and results sections.
SRM: Conceived the study idea, literature review, manuscript writing.
HM: Sample preparation, data acquisition, reviewed the manuscript.
ZC: Literature review, prepared introduction, manuscript writing
TM: Provided expert input on cancer biology and angiogenesis mechanisms, editing the discussion and conclusion sections.
All Authors: Approval of the final version of the manuscript to be published

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