

Apoptosis-Mediated Anticancer Activity of Geraniol Inhibits NF- κ B, MAPK, and JAK-STAT-3 Signaling Pathways in Human Thyroid Cancer Cells

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ABSTRACT

Aim: Thyroid cancer accounts for 3.8% of the total endocrine cancer and is the fifth most diagnosed cancer among Indian women. Geraniol possesses pharmacological properties, including anti-inflammatory, anti-cancer, and neuroprotective properties. The anti-cancer activity of geraniol on TPC-1 cells was assessed in this study. **Materials and Methods:** The anticancer activity of geraniol in TPC-1 cells was evaluated by MTT test, ROS, DAPI, PI, and AO/EB staining. mRNA and expression were assessed by qPCR and western blot. **Results:** The geraniol has shown cytotoxic effects toward TPC-1 cells and showed an IC₅₀ value of 25 μ M, which decreases cell viability and inhibited cell proliferation. It also increased the accumulation of intracellular ROS, which induces apoptosis in the TPC-1 cells due to the dissipation of membrane potential and disruption of mitochondria. There is a substantial reduction in the mRNA level expressions of Bcl-2, Cyclin D1, c-Myc, COX-2, TNF- α , NF- κ B, IL-6, and surviving. It also upregulated the Bax and caspase-3 mRNA expression suggesting cell death-mediated apoptosis. **Conclusion:** We report that geraniol effectively inhibited the JAK-2, STAT-3, and ERK signaling pathways depicting geraniol as a potential therapeutic molecule for thyroid cancer treatment.

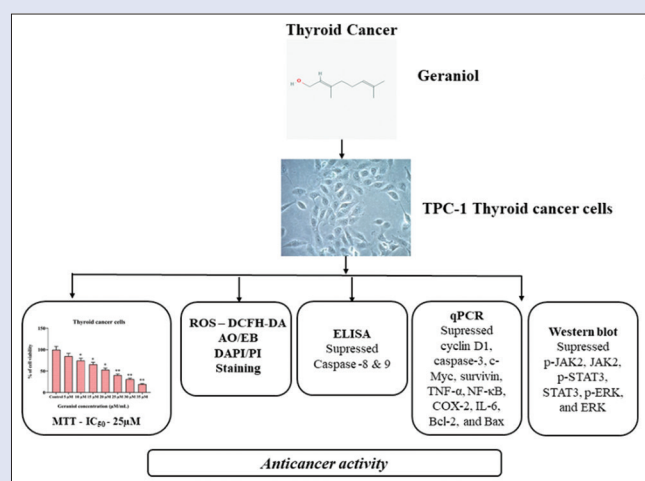
Key words: Apoptosis, cell proliferation, geraniol, MAPK signaling, thyroid cancer

SUMMARY

- Geraniol caused a marked increase in the expressions of caspase-8 and -9.
- Geraniol also suppressed cyclin D1, Bcl-2, c-Myc, survivin, TNF- α , NF- κ B, COX-2, and IL-6 mRNA expressions.
- Geraniol exerted anticancer effects by suppressing JAK2, STAT3, and ERK in TPC-1 cells.

Abbreviations used: DTC: Differentiated thyroid cancer; MTC: medullary thyroid cancer; Bax: Bcl-2-associated X protein; ROS: Reactive oxygen species; ATC: anaplastic thyroid cancer; AO/EB: Acridine orange/Ethidium bromide; TC: Thyroid cancer; DAPI: 4',6-diamidino-2-phenylindole; Bcl-2: B-cell lymphoma 2; PI: Propidium iodide; TNF- α : Tumor Necrosis

Factor- α ; DCFH-DA: 2',7'-dichlorofluorescein diacetate; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; COX-2: Cyclooxygenase-2; IL-6: Interleukin 6.



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INTRODUCTION

Thyroid cancer (TC) is the ninth utmost cancer of overall malignancy and the fifth most diagnosed cancer among Indian women.^[1] It accounts for 3.8% of total endocrine cancers. Over the past decades, TC has been increasing among people as new diagnostic methods developed, such as imaging techniques that can detect thyroid nodules.^[2] Differentiated thyroid cancer (DTC) corresponds to 90% of all diagnosed with TC, followed by the most frequent medullary thyroid cancer (MTC) (10%) and anaplastic thyroid cancer (ATC) (1%).^[3] Papillary thyroid carcinoma is the greatest kind under DTC and follicular cancers.^[4] Over the decade, there is about a 48% and 62% increase in TC incidence among men and women in India, respectively. Although standard treatment methods exhibited a good prognosis and novel treatment methods for TC treatment, very few were proven to be efficient in advanced or metastatic

cancers.^[5] The food and drug administration (FDA) has approved many advanced therapeutic drugs, and several others were under clinical trials and investigation to treat TC.^[6]

The current treatment for TC patients includes thyroidectomy, radioactive iodine, external beam radiation therapy, and targeted

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biological therapies.^[6,7] Among elderly individuals, they are more prone to develop resistance against radioactive iodine therapy, making it challenging to treat cancer.^[8] The tyrosine kinase inhibitors against resistance radioactive iodine therapy have widely proven to increase the survival rate. Hence, individual therapy is required for the individuals susceptible to toxicity. Thus, to overcome the limitations of conventional modalities, novel therapies are needed to treat TC effectively.^[9]

Monoterpene is a secondary metabolite phytochemical present in plants as essential oils in various herbs. Monoterpenes have reported having potent antioxidant, anti-inflammatory, and anticancer activities. It also has been reported to have chemoprotective effects.^[10] Geraniol is a monoterpene isolated from various fruits and herbs. It exerts various pharmacological properties, including anti-inflammatory,^[11] anti-microbial,^[12] anti-ulcer,^[13] and neuroprotective,^[14] properties. It also reported that it has potential effects on various types of cancer, including breast cancer cells MCF-7,^[15] colon cancer Caco-2 cells,^[16] pancreatic cancer MIA PaCa-2 cells,^[17] skin cancer cells,^[18] and prostate cancer PC-3 cells,^[19] and A549 human lung adenocarcinoma cells.^[20] Thus, geraniol is envisaged as a potential candidate for TC.

The work's current focus was to evaluate the geraniol anticancer activity against TPC-1 TC cell lines to study the underlying mechanism responsible for the anticancer activity.

MATERIALS AND METHODS

Chemicals and antibodies

Geraniol (Purity, 97%) and other chemicals were procured from Sigma Aldrich Chemicals (USA). Dyes employed in the trial were purchased from Hi-Media, USA. Antibodies were acquired from Cell Signaling Technology, USA. This research was approved by Affiliated Hospital of Hebei University animal ethical committee, Approved No. HUAH-27462.

Cell culture

Human TC cells TPC-1 were procured from Peking Union Cell Resource Center (Beijing, China) and cultured in DMEM with 10% FBS along with the antibiotics (Penicillin and streptomycin) and maintained at the optimal temperature of 37°C for 24 h. Monolayered growth of the cells was observed, and they were further passages with 0.25% Trypsin.

Measurement of MTT assay

Geraniol's effect on cell viability TPC-1 was assessed using an MTT assay. Approximately 5 × 10⁴ cells were seeded for the 96-well plate and kept for 24 h under incubation. Following incubation, the TPC-1 cells were treated with geraniol (5-45 μM/mL) for 24 h. Afterward, the cells were introduced with 0.5 mg/ml of MTT in PBS for 2 h. To dissolve the formazan crystal formed, about 100 μL of DMSO was added and read at 570 nm with an ELISA reader (Thermofisher, USA).

Evaluation of intracellular ROS

ROS production in TPC-1 cells was confirmed by DCFH-DA staining, which can be oxidized fluorescent dichlorofluorescein (DCF) due to ROS generated inside the cell. Before treating the cells with different geraniol (20 and 25 μM/mL), about 1 × 10⁶ cells/well were inoculated and incubated for 24 h. Furthermore, the cells were incubated with 10 μL of DCFH-DA for 2 h. The fluorescence was measured using a fluorescence microscope (Nikon, Eclipse TS 100, Japan) at 530 nm.

Determination of morphological apoptotic changes by AO/EB staining

Apoptotic changes in the morphology of TPC-1 cells were evaluated using AO/EB staining. About 3 × 10⁴ cells/well cultured in a 6-well plate

and treated with geraniol of different concentrations (20 and 25 μM/mL) for 24 h and followed fixation using methanol and acetic acid (glacial). Also, treated cells AO/EB were left for 30 min, washing with PBS.

Determination of morphological apoptotic changes by DAPI and PI staining

TPC-1 cells were grown in a 6-well plate in serum-free media overnight. The cells were added with different concentrations of geraniol (20 and 25 M/mL) for 24 h before being stationary with paraformaldehyde (4%) and permeabilized with 0.1% Triton X-100 subjected to DAPI staining and PI (1 mg/mL) was examined under fluorescence microscopy (Olympus, Japan).

Measurement of caspase-8 and -9 by Enzyme-linked immunosorbent assay (ELISA)

The effect of geraniol on apoptotic proteins, including caspase-8 and -9 production assessed using the ELISA technique in TPC-1 cell lines. The caspase-8 and -9 levels were evaluated through the commercially available ELISA Kit Thermo Fisher Scientific (USA) as per the manufacturer's protocol.

Real-time PCR analysis

The mRNA expressions of the cell signaling regulators, Bcl-2, Bax, cyclin-D1, caspase-3, c-Myc, survivin, TNF-α, NF-κB, COX-2, and IL-6 proteins contributing to the pathogenesis of the cancer were examined by RT-PCR. About 3 × 10⁵ cells cultured until they grow confluence to 80%. Then, treated cells were added with geraniol at 20 and 25 μM/mL for 24 h. The total RNA extraction is done by employing Qiagen RNeasy Mini Kit. As per the manufacturer's instructions, RT-PCR (Applied Biosystems) mixture system was performed. The primers were acquired from Integrated DNA technologies, US, and presented in Table 1. The xperiment was repeated three times and the fold changes were done using the Δct method.

Western blot analysis

The protein levels were detected using western blotting which was carried out using a standard protocol. SDS-PAGE electrophoresis was performed on 50 g of total protein lysate. Protein bands were then

Table 1: List of specific gene primers used for RT-PCR

Gene name	Sense primer (5'-3')	Antisense primer (5'-3')
Cyclin D1	CAGATCATCCGCAAACA CGC	AAGTTGTTGGGGCTCC TCAG
Caspase-3	TGTCGATGCAGCAAACC TCA	GACTTCTACAAC GATCC CCTC
Bax	ACTGAAGCGACTGATGT CCC	CAAAGATGGTCACGGT CTGC
Bcl-2	GAAGTGGGGGAGGATT GTGG	GCCGGTTCAGGTACTC AGTC
c-Myc	CCTCCACTCGGAAGGAC TATC	TGTTGCGCTCTTGACA TTCTC
Survivin	CACCGCATCTCTACATT CAAGA	CAAGTCTGGCTCGTTCT CAGT
NF-κB	ATGGACGATCTGTTTCC CCT	CGGTTTACTCGGCAGAT CTT
Cox-2	TGGGCCATGGAGT GGACTTA	ATGAGCCTGCTGGTTTG GAA
TNF-α	TCTGGGCAGGTCTACTT TGG	TCTTCTCAAGTCCTGCA GCA
IL-6	AAACAACCTGAACCT TCCAAAGA	GCAAGTCTCCTCATTGA ATCCA
β-actin	GGTCACCAGGGCTGCT TTTA	GGATCTCGCTCCTGGAA GATG

seen using an enhanced chemiluminescence kit (Bio-Rad, USA) and quantified using Quantity One software after being transferred to the nitrocellulose membrane.

RESULTS

Cytotoxic properties of geraniol on TPC-1 cells

The MTT assay evaluated the anti-proliferative effect on TPC-1 cells induced by the cytotoxic effects of geraniol. On treating the TC cells with geraniol at varying concentrations (5–45 $\mu\text{M/mL}$) for 24 h, it inhibited the cell proliferation and induced cytotoxicity in a dose-dependent manner, as shown in Figure 1. There was significant damage to the cells after

35 μM and which significantly inhibited the growth in the concentration of 25 μM (IC_{50}). From the results, 20 and 25 $\mu\text{M/mL}$ sub-lethal doses were selected for auxiliary investigation.

Effects of geraniol creation of intracellular ROS in TPC-1 cells

ROS generation inside the cell is a critical factor for the induction of cell apoptosis evaluated through the staining of DCFH-DA. Geraniol acts on ROS generation at the concentrations of 20 and 25 $\mu\text{M/mL}$ in the cancer cells, as shown in Figure 2. The results indicated the enhanced production of ROS in the cell in a dose-reliant way.

Effects of geraniol-induced apoptotic changes by AO/EB staining on TC cells

To explore the efficacy of apoptotic changes in the TPC-1 cancer cells studied using AO/EB staining. The fluorescence in the TC cells was significantly increased in a dosage-reliant mode of geraniol (20 and 25 $\mu\text{M/mL}$), as shown in Figure 3. The results depicted apoptotic cells' presence of bright green color, whereas the control cells showed a distinct nucleus representing the presence of live cells.

Effects of geraniol on apoptotic morphological changes by DAPI and PI staining on TPC-1 cells

Changes in the cell morphology and cell structure were assessed in the DAPI and PI staining, as shown in Figure 4. The cells were incubated for 24 h after post-treatment of geraniol at various concentrations. The treated cells revealed disruption in the cell structure with chromatin condensation, as shown in Figure 4. The untreated control cells showed bright green color indicating the intact viable cells. Apart from the DAPI staining, PI staining showed the nuclei's condensation with apoptotic structures in the cancer cells compared to the untreated control cells.

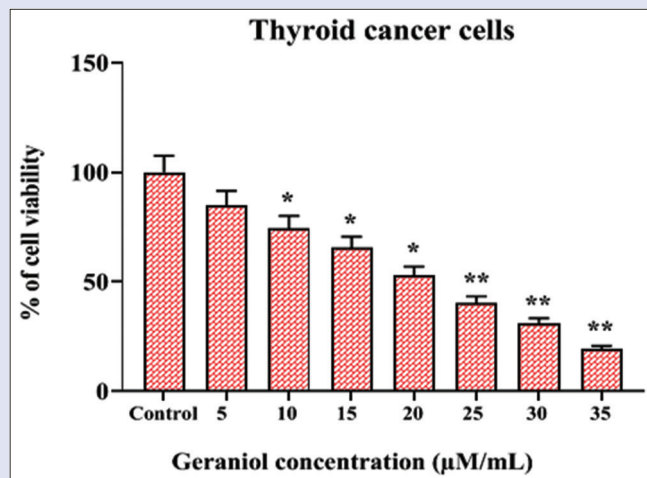


Figure 1: Effect of geraniol on cell viability in TPC-1 cells. Data representing mean $\pm$ SEM of triplicate experiments. ***represents $P < 0.05$

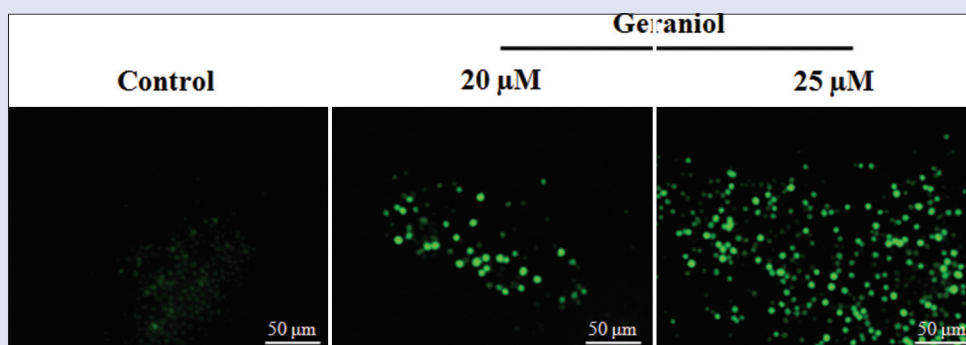


Figure 2: Effect of geraniol on reactive oxygen species generation in TPC-1 human thyroid cells. Scale bar = 50 μm

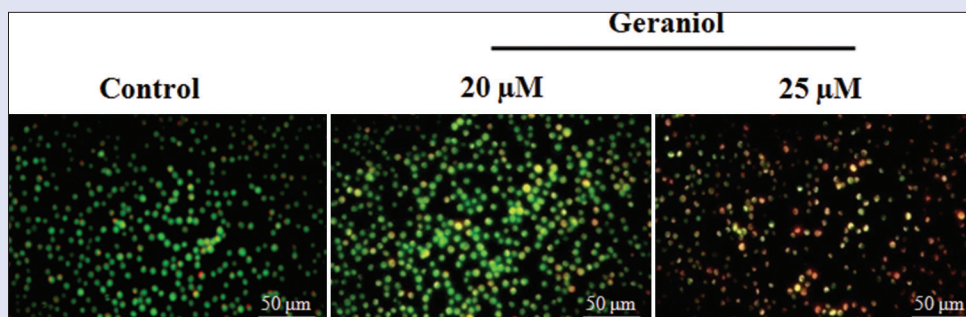


Figure 3: Effect of geraniol-induced apoptotic morphological changes in TC cells. Scale bar = 50 μm

Effects of geraniol on apoptosis-induced caspase-8 and -9 on TC cells

Various results revealed the induction of apoptosis in geraniol-treated cancer cells, as shown in Figure 5. Hence, the cells were evaluated quantitatively for the presence of apoptotic protein, including caspase-8 and -9 through ELISA. These apoptotic proteins have a crucial role in inducing apoptosis in cancer as they are called initiator caspases.^[21] After incubation for 24 h, geraniol-treated cells showed a marked increase in caspase-8 and -9 in a dose-dependent manner, as shown in Figure 5. These results depict the involvement of caspases in apoptosis.

Impacts of geraniol on apoptosis mediators mRNA expressions in TPC-1 cells

The mRNA gene expressions of the regulatory proteins were assessed in geraniol (20 and 25 μM /L) treated TPC-1 cells. The mRNA expression of the proteins, including cyclin D1, caspase-3, c-Myc, survivin, TNF- α , NF- κB , COX-2, IL-6, Bcl-2, and Bax, were analyzed by RT-PCR, as shown in Figures 6 and 7. The results have exhibited the 0.25-fold downregulation of cyclin D1, Bcl-2, c-Myc, survivin, and 2.5-fold downregulation of TNF- α , NF- κB , COX-2, IL-6 at 20 μM . There was a significant increase in the Bax and caspase-3 mRNA expressions compared to the untreated control.

Effects of geraniol on MAPK/JAK-STAT pathways in TPC-1 cells

Western blot analysis was used to evaluate protein regulation mediating the cell-signaling pathways contributing to the disease progression.

TPC-1 cells were added with geraniol at varying concentrations (15 and 20 μM /mL). p-JAK2, JAK2, p-STAT3, STAT3, p-ERK, and ERK protein expression were evaluated, whereas β -actin was used as an internal standard, as shown in Figure 8. The levels of cell-signaling proteins were significantly suppressed, thus indicating that the involvement of the protein in geraniol anticancer activity induces apoptosis.

DISCUSSION

The incidence of TC has been increasing over the past decade as the improvement in the diagnostic modalities. The metastatic state of cancer and conventional therapy side effects create the demand for novel therapeutic methods for cancer.^[22] The geraniol has been shown to have potential anticancer activities against various cancer types, both *in-vitro* and *in-vivo* models.^[20,23] Cytotoxic effects of geraniol on the TPC-1 TC cells through MTT assay. The geraniol-treated cells exhibited a cytotoxic behavior with a significant decrease in the cell numbers and the IC_{50} concentration of 25 μM . Cytotoxic behavior is attributed to the induction of apoptosis by geraniol. It exhibited potent cytotoxic effects over Colo-205 cells at the concentration of 20 μM and colon cancer cell lines (Caco-2), inhibiting 70% of cell growth.^[23,24] When the A549 human lung adenocarcinoma cells were treated with geraniol, they significantly inhibited cells' proliferation.^[20]

To understand the cell death effect in cancer cells. The geraniol-treated cells were exposed to staining with DCFH-DA, AO/EB, DAPI, and PI. Mitochondria have been the central hub for inducing cell apoptosis, mainly through ROS generation.^[25] ROS generation plays an inevitable role in the apoptosis resulting from the dissipation of membrane potential and disruption of mitochondrial structure. Accumulation

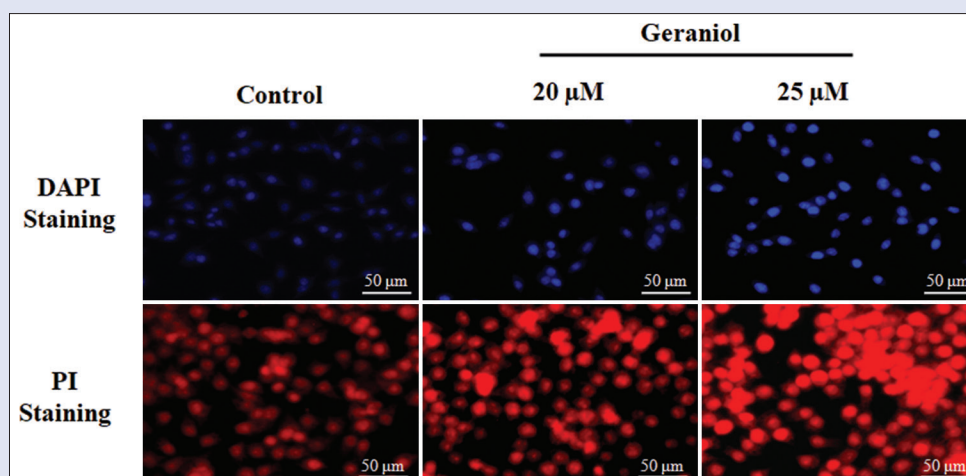


Figure 4: Effect of geraniol-induced nuclear morphological changes in MG-63 cells. Scale bar = 50 μm

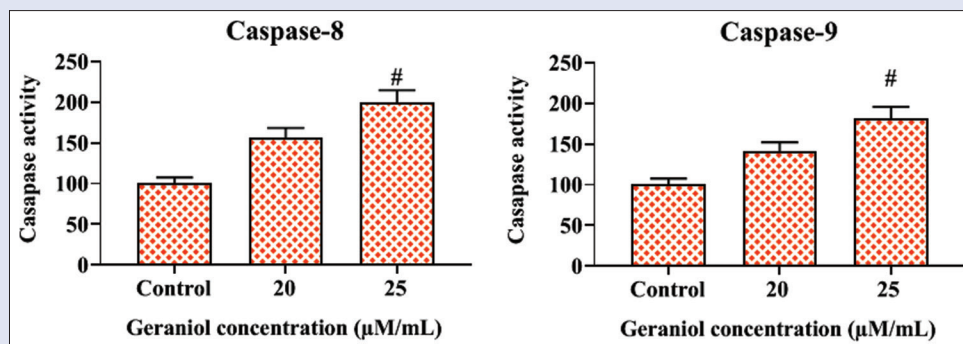


Figure 5: Effect of geraniol on apoptosis-related protein levels in TC cells. Data representing mean $\pm$ SEM of triplicate experiments. #represents $P < 0.05$

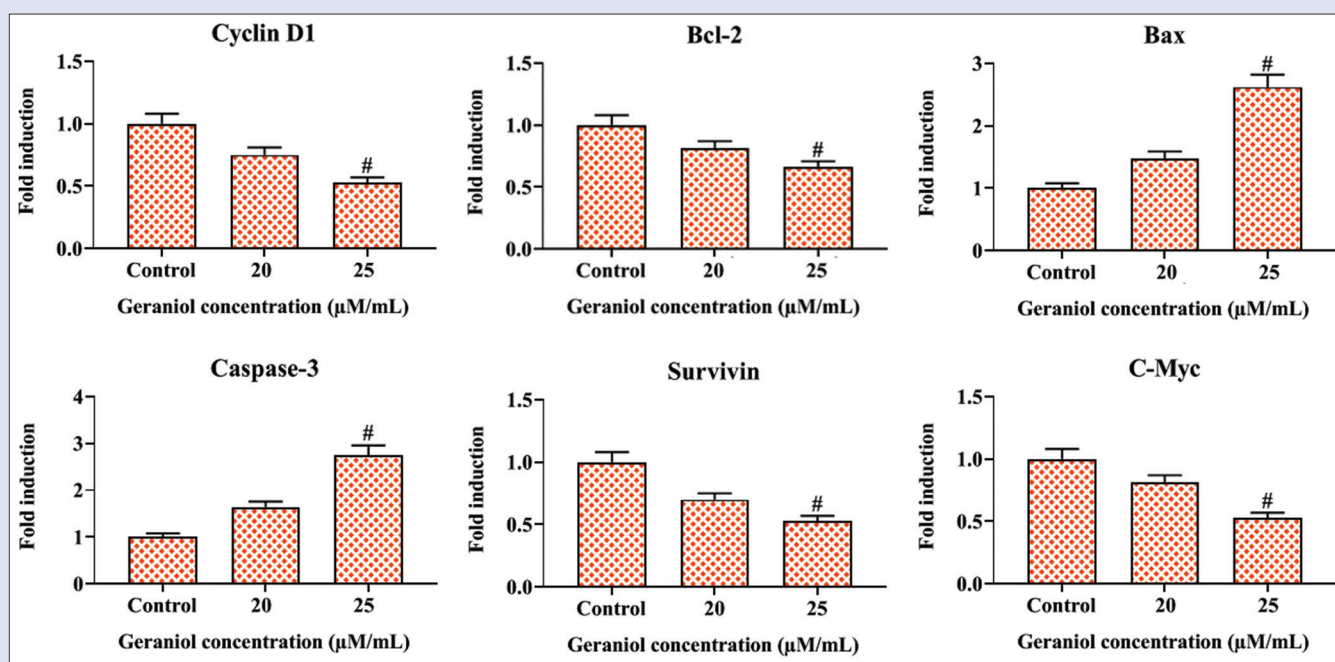


Figure 6: Effect of geraniol on cell proliferation and apoptosis-related and gene regulator protein expressions in TPC-1 cells. Data representing mean $\pm$ SEM of triplicate experiments. #represents $P < 0.05$

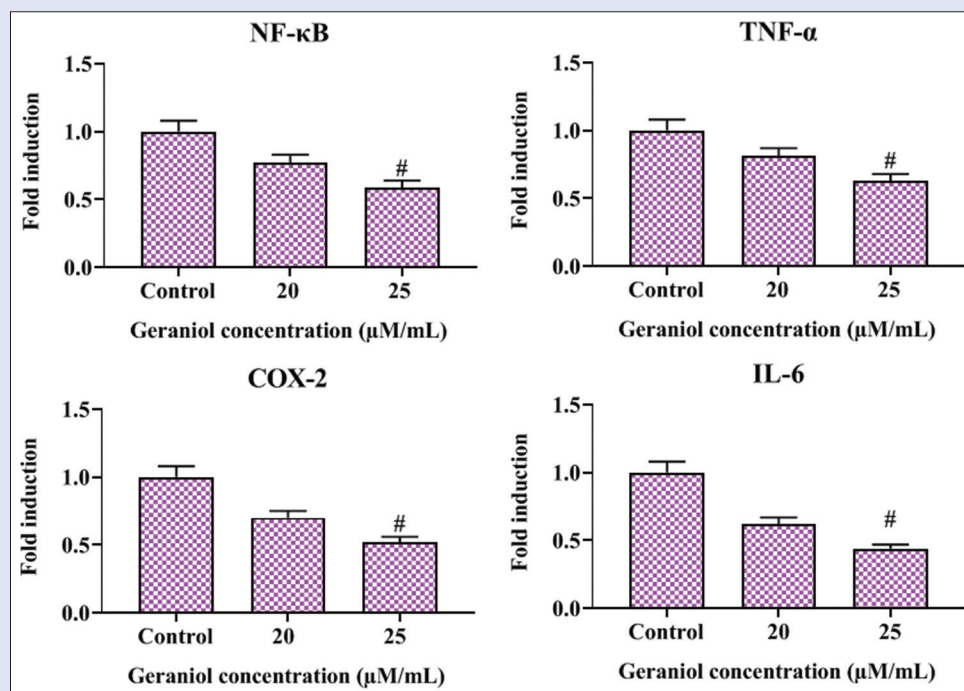


Figure 7: Effect of geraniol on signaling and effector molecule expressions in TC cells. Data representing mean $\pm$ SEM of triplicate experiments. #represents $P < 0.05$

of ROS in the cell causes the activation of pro-apoptotic caspases leading to the sequential activation of other caspases regulated through cytochrome-c.^[26] The DFCHA-DA staining results exhibited a remarkably increased accumulation of ROS inside the cell at the concentration of 25 μ M. Following the ROS assessment, in AO/EB staining, the geraniol-treated cells showed bright fluorescence when treated at the concentration of 25 μ M, exhibiting distinct apoptotic

structures when observed under the microscope. In DAPI and PI staining, after 24 h treatment of geraniol (25 μ M/mL), TPC-1 cells revealed apoptotic morphology with the disruption of cell structure, nuclear, and chromatin condensation.

Initiation and execution of apoptosis were mainly regulated through the members of the caspase protease family.^[27] The caspases are classified based on the function, including (Caspase-2, -8, -9, and -10) and (Caspase-3, -6,

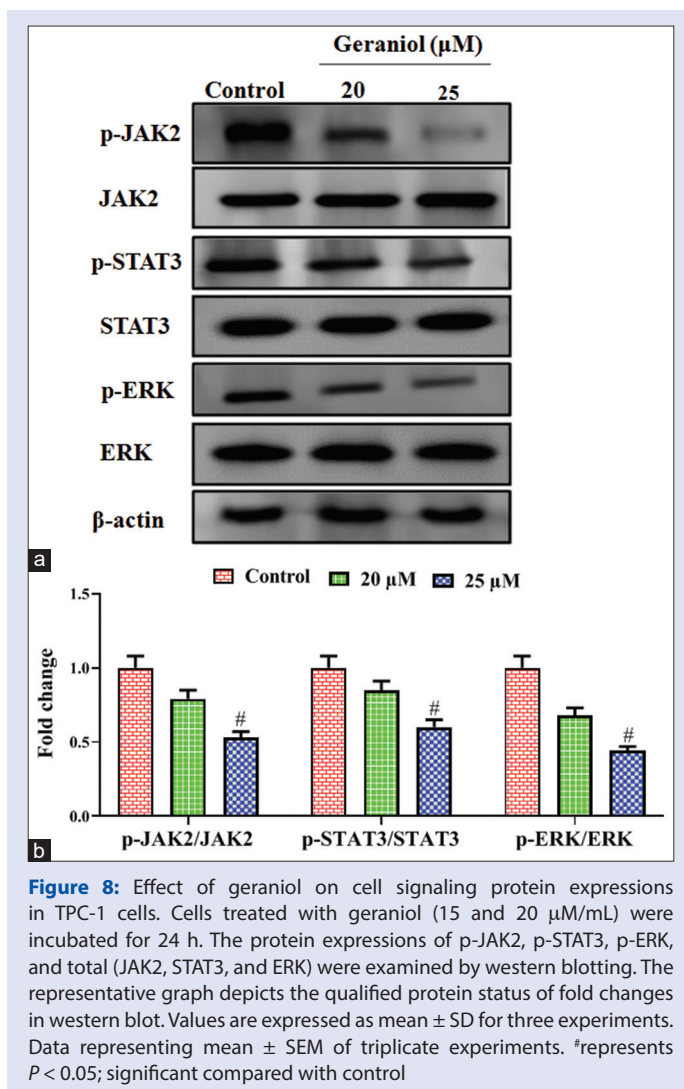


Figure 8: Effect of geraniol on cell signaling protein expressions in TPC-1 cells. Cells treated with geraniol (15 and 20 μM/mL) were incubated for 24 h. The protein expressions of p-JAK2, p-STAT3, p-ERK, and total (JAK2, STAT3, and ERK) were examined by western blotting. The representative graph depicts the qualified protein status of fold changes in western blot. Values are expressed as mean $\pm$ SD for three experiments. Data representing mean $\pm$ SEM of triplicate experiments. #represents $P < 0.05$; significant compared with control

and -7) renowned as initiator and executioner caspases, respectively.^[21] So, we have quantitatively evaluated the levels of caspase-8 and -9 by ELISA. Thus, we demonstrated a two-fold increase in the levels of caspase-9 in the geraniol-treated TPC-1 cells significantly. As emphasized to study the mitochondria-mediated apoptotic pathway, the mRNA expression levels of apoptotic proteins were evaluated by RT-PCR.^[28] The result showed a significant decrease in their levels, revealing the cell cycle halt and stimulation of apoptosis by geraniol in the TPC-1 cells. It also showed a marked increase in the levels of Bax and caspase-3, depicting the apoptosis-induced anticancer activity of the geraniol. To further investigate the molecular pathway behind the progression of the disease and responsible for anticancer activity, the mRNA expression levels of TNF- α , NF- κ B, COX-2, and IL-6 were assessed. Thus, the data showed a marked decrease in the levels of their mRNA expressions as the TPC-1 was treated with 20 μM of geraniol. Geraniol potentially suppressed the levels of apoptotic proteins in PC-3 prostate cancer cells.^[19] The geraniol has also reduced the expression levels of regulatory proteins and upregulated apoptotic proteins in DMBA-treated hamsters.^[29] The cell signaling regulators and the transcription factors were linked to multiple cancer signaling pathways. NF- κ B serves as the focal hub of multiple signaling pathways, which activates multiple signaling cascades that have been downregulated by the geraniol indicating that it can be a potential therapeutic molecule for the treatment of TC.

The Janus kinase JAK/STAT and ERK signaling pathway control critical immune function, differentiation, hematopoiesis, and cell proliferation.^[30,31] Western blot analysis exhibited the potential inhibition of JAK2, STAT3, and ERK with the noticeable lessening in the expression levels of β -actin in a concentration-reliant mode. Elevated levels of these proteins have been reported in most inflammatory conditions and activation, suggesting that the inhibitory action of geraniol against TPC-1 acts as the potential therapeutic candidate for TC.

CONCLUSION

In conclusion, this study established the potential cytotoxic actions of geraniol against TPC-1 cells via ROS accumulation, causing apoptosis. Geraniol-treated cells showed distinct apoptotic structures and downregulated mRNA expressions of apoptosis mediators. It also upregulated the mRNA levels of Bax and caspase-3 in a dosage-reliant way. It was also found to significantly decrease the levels of JAK2, STAT3, and ERK in a concentration-dependent mode. The findings of this study depict geraniol as a possible therapeutic agent for the treatment of TC.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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