Anti-oral squamous cell carcinoma, DNA damage, and apoptotic induction of *Nectaroscordum tripedale* essential oil

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Abstract

In recent years, advancements in cancer research have led to the identification of numerous bioactive compounds derived from natural sources, particularly plants, many of which exhibit promising antitumor properties. For centuries, plants have been the main source of discovery of various medicines.Among these, essential oils and their constituents have attracted considerable scientific interest due to their potent anticancer effects. Much research is being conducted around the world to discover natural compounds that can inhibit or prevent the process of cancer.The current study explores the anticancer activity and underlying mechanisms of essential oil extracted from *Nectaroscordum tripedale (N. tripedale* EO) on oral squamous cell carcinoma (SCC)

lines.After extraction of the essential oil, its chemical profile was characterized using gas chromatography-mass spectrometry (GC-MS), which identified Germacrene-D as the predominant component, accounting for 32.3% of the oil's composition. The cytotoxicity of *N. tripedale* EO was assessed using the MTT assay on both human oral cancer cells (KB) and normal human gingival fibroblasts (HGF1). The half-maximal cytotoxic concentration (CC50) was determined through probit analysis.Further evaluation focused on the oil's effect on apoptosis-related genes, revealing a marked upregulation of caspase-3 and Bax, alongside a downregulation of Bcl-2, in both HGF1-RT1 and KB cell lines following treatment with the oil at ½ CC50 and CC50 doses. Additionally, DNA synthesis activity was found to decrease in a dose-dependent manner across both cancerous and normal cells.Collectively, these findings highlight the potential of *N. tripedale* essential oil as an effective anticancer agent, capable of inducing apoptosis, reducing cellular viability in malignant cells, and suppressing DNA replication.

Keywords: *Nectaroscordum tripedale*, oral cancer, apoptosis, cytotoxicity

1. Introduction

Cancer, commonly referred to as a malignant neoplasm or tumor, encompasses a broad spectrum of diseases characterized by uncontrolled cell proliferation and the potential to invade or spread to distant organs (1). According to the World Health Organization (WHO), cancer remains a leading global cause of mortality, with approximately 7.6 million deaths annually—a number projected to rise beyond 11 million by the year 2030 (2).

Among the various cancer types, oral cancer ranks as the eighth most prevalent in men and the fifteenth in women (3). This category includes malignancies of the lips, tongue, oral mucosa, gingiva, floor of the mouth, hard and soft palates, tonsils, salivary glands, and regions such as the oropharynx, nasopharynx, and hypopharynx. Over 90% of these oral cancers are diagnosed as squamous cell carcinomas (SCC), while the remaining cases comprise salivary gland neoplasms,

sarcomas, lymphomas, and metastases from other primary sites like the lungs, breast, prostate, and kidneys (4). Histologically, SCC originates from dysplastic epithelium and is marked by the presence of infiltrative malignant epithelial clusters (5).

Conventional treatments for cancer include surgical intervention and chemoradiotherapy, both of which are associated with significant side effects (6). Radiation therapy may lead to xerostomia, mucosal sensitivity, rampant dental decay, and dysphagia. On the other hand, chemotherapy can cause mucositis, gastrointestinal disturbances, immunosuppression, and general systemic toxicity. In advanced-stage cases requiring extensive surgery, patients often face functional impairments in speaking, mastication, and swallowing (7). Despite notable progress in multimodal treatment strategies, the five-year survival rate for SCC remains suboptimal, ranging from 50% to 59% (8). Given these limitations, alternative approaches, including traditional and herbal medicine, have gained increased attention worldwide for their role in disease prevention and complementary therapy (9-11).

Among these, essential oils derived from plants have been extensively explored for their anticancer properties. These oils are rich in bioactive constituents such as monoterpenes, sesquiterpenes, oxygenated derivatives, and phenolic compounds. Their anticancer potential is linked to mechanisms including anti-mutagenic and anti-proliferative activities, enhancement of immune surveillance, induction of detoxifying enzymes, and antioxidant effects (12).

Nectaroscordum tripedale, a perennial species in the Amaryllidaceae family native to Central Asia, has been recognized for its medicinal value. It is characterized by a tall, sturdy stem (50–90 cm), bearing an umbrella-like inflorescence composed of around 30 bell-shaped flowers. Its foliage, reminiscent of garlic, emits a strong, distinctive odor (13,14). Biochemically, the plant is notable for its cysteine-rich profile, containing compounds such as O-phthaldialdehyde (OPA),

(+)-S-(1-butenyl)-L-cysteine sulfoxide, its γ-glutamyl derivatives, and other related sulfur-containing metabolites (15). Prior studies have demonstrated a range of pharmacological activities for N. tripedale, including antioxidant, antimicrobial, antidiabetic, hepatoprotective, and nephroprotective effects(13, 14, 16). Upon these properties, the current study was designed to evaluate the anticancer potential and molecular mechanisms of *N. tripedale* essential oil (EO) in human oral squamous cell carcinoma (SCC) models.

2. Materials and Methods

2.1 Ethical Approval

This experimental protocol was reviewed and approved by the Ethics Committee of Lorestan University of Medical Sciences, Khorramabad, Iran, under the ethics code IR.LUMS.REC.1402.244.

2.2. Plant Collection and Identification

Aerial parts of *Nectaroscordum tripedale* were harvested in May 2022 from mountainous regions surrounding Khorramabad, located in western Iran. Following botanical authentication, a voucher specimen was deposited at the Herbarium of Razi Herbal Medicines Research Center under accession number 1402244. The plant material was air-dried and stored in light-protected containers until further processing.

2.3. Essential Oil Extraction

The essential oil was extracted from the dried aerial parts of *N. tripedale* using a Clevenger-type apparatus via hydrodistillation for 2 hours. The resulting oil was dried over anhydrous sodium sulfate to remove moisture and subsequently stored at 4 °C in sealed vials until analysis and bioassays were performed (17).

2.4. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

To identify the chemical composition of the essential oil, GC-MS analysis was conducted using a gas chromatograph (model 7890A) coupled with a mass spectrometer (model 5975A). Components were identified by comparing their retention indices and mass spectra with reference compounds and data from the NIST library. Quantification of individual constituents was achieved by integrating the peak areas in the chromatograms.

2.5. Cell Culture Conditions

Human normal gingival fibroblasts (HGF1) and oral squamous carcinoma cells (KB) were procured from the Pasteur Institute of Iran. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Merck, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures were maintained at 37 °C in a humidified incubator with 5% CO□.

2.6 MTT Cytotoxicity Assay

The cytotoxic potential of *N. tripedale* essential oil was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay (18). Cells were seeded into 96-well plates at a density of approximately 7,000 cells/well and allowed to adhere for 24 hours. Following incubation, the culture medium was replaced with serial dilutions of the essential oil (3.125–200 µg/mL), and cells were exposed for 48 hours. After treatment, 10 µL of MTT solution (Sigma-Aldrich, Germany) was added to each well, followed by 4 hours of incubation. Next, 150 µL of DMSO was added to solubilize formazan crystals, and absorbance was measured at 570 nm using a microplate reader. The 50% cytotoxic concentration (CC50) was determined via probit analysis

using SPSS software version 25.0. The selectivity index (SI) was calculated as the ratio of CC50 in normal cells to CC50 in cancer cells.

2.7. Gene Expression Analysis of Apoptosis Markers

To assess the impact of the essential oil on apoptotic pathways, the expression levels of *caspase-3*, *Bcl-2*, and *Bax* genes were quantified using Real-Time PCR. Total RNA was extracted from both untreated and treated HGF1 and KB cells using a commercial RNA isolation kit (Qiagen, USA), according to the manufacturer's instructions. Cells were detached with trypsin, pelleted, and subjected to RNA extraction, followed by cDNA synthesis using a complementary kit (Qiagen, USA).

PCR amplification was conducted using synthesized cDNA, gene-specific primers (Table 1), and Maxima™ SYBR Green Master Mix (Fermentas, USA). The thermal cycling conditions were as follows: initial denaturation at 96 °C for 7 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 56 °C for 30 seconds, and extension at 72 °C for 30 seconds. Gene expression changes were quantified using the 2^-ΔΔCt method, with β-actin serving as the internal control. Analysis was performed using IQ™5 software (Bio-Rad, Hercules, CA) (19).

Table 1. Sequence of primers used for Real-Time PCR in this study

Gene	Sequences (5´ to 3´)	References
Bax	F: GGCTGGACACTGGACTTCCT R: GGTGAGGACTCCAGCCACAA	(19)
Bcl-2	F: CATGCCAAGAGGGAAACACCAGAA R: GTGCTTTGCATTCTTGGATGAGGG	
Caspase-3	F: TTCATTATTCAGGCCTGCCGAGG R: TTCTGACAGGCCATGTCATCCTCA	
β-actin	F: GTGACGTTGACATCCGTAAAGA R: GCCGGACTCATCGTACTCC	

2.8. Assessment of DNA Synthesis Inhibition

To evaluate the effect of *N. tripedale* essential oil on DNA synthesis, cell treatment was carried out in 96-well plates following the protocol used in the MTT assay. The BrdU (5-bromo-2′-

deoxyuridine) incorporation assay was performed using a commercial ELISA kit (Roche, Germany) as per the manufacturer's instructions (20). Briefly, after 24 hours of EO treatment, 5 µL of BrdU labeling solution was added to each well and incubated for 3 hours. The culture medium was then removed, and 100 µL of Denaturation-Fixation solution was added to each well, followed by incubation at room temperature (21 °C) for 30 minutes. Subsequently, 50 µL of anti-BrdU-POD conjugate was added and incubated for 90 minutes at 21 °C. After washing the wells thoroughly with phosphate-buffered saline (PBS), 50 µL of the substrate solution was added. Absorbance was measured at 405 nm and 490 nm using a microplate reader to determine DNA synthesis levels.

2.9. Statistical Analysis

All experiments were performed in triplicate. Data were analyzed using SPSS software (version 25.0). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. GC-MS Analysis of *N. tripedale* Essential Oil

The chemical composition of the essential oil extracted from *N. tripedale* was determined using gas chromatography-mass spectrometry (GC-MS). As summarized in Table 2, the analysis revealed that Germacrene-D was the most abundant constituent, comprising 32.3% of the total oil content. Other major components included hexadecanoic acid (13.2%) and diphenylamine (10.7%), along with several minor compounds contributing to the overall phytochemical profile.

Table 2. Chemical composition of *N. tripedale/* EO analyzed by GC/MS

No.	Compound	Kovats indexes (KIs)	Percent (%)
1.	β─phellandrene	1028	0.76
2.	n-Nonanal	1087	3.2

3.	E-Caryophyllene	1098	1.2
4.	α─campholenal	1130	1.1
5.	n-Decanal	1190	1.2
6.	2-Decenal	1240	2.3
7.	2,4-Decadienal, (E, E)	1305	7.6
8.	Trans-2-Undecenal	1358	3.8
9.	Germacrene-D	1480	**32.3**
10.	bicyclogermacrene	1488	5.6
11.	Dibutyl disulfide	1493	1.8
12.	Gamma, Cadinene	1496	1.9
13.	α-Farnesene	1506	2.1
14.	Caryophyllene Oxide	1576	4.1
15.	Diphenylamine	1589	**10.7**
16.	Delta,Cadinol	1641	1.8
17.	Heptadecane	1688	1.9
18.	octadecane	1788	2.9
19.	Hexadecanoic Acid	1944	**13.2**
	Total		99.56

3.2. Cytotoxic Activity of *N. tripedale* Essential Oil

As depicted in Figure 1, the MTT assay demonstrated that treatment with *N. tripedale* EO led to a concentration-dependent reduction in cell viability in both KB oral squamous carcinoma cells and normal human gingival fibroblasts (HGF1-RT1) ($p < 0.001$). The half-maximal cytotoxic concentration (CC□□) was calculated to be 58.6 µg/mL for KB cancer cells and 136.4 µg/mL for HGF1-RT1 cells. Based on these values, the selectivity index (SI)—calculated as the ratio of CC□□ in normal cells to CC□□ in cancer cells—was greater than 2, suggesting that *N. tripedale*

EO exhibited selective cytotoxicity against cancerous cells while exerting minimal toxicity on non-cancerous cells.

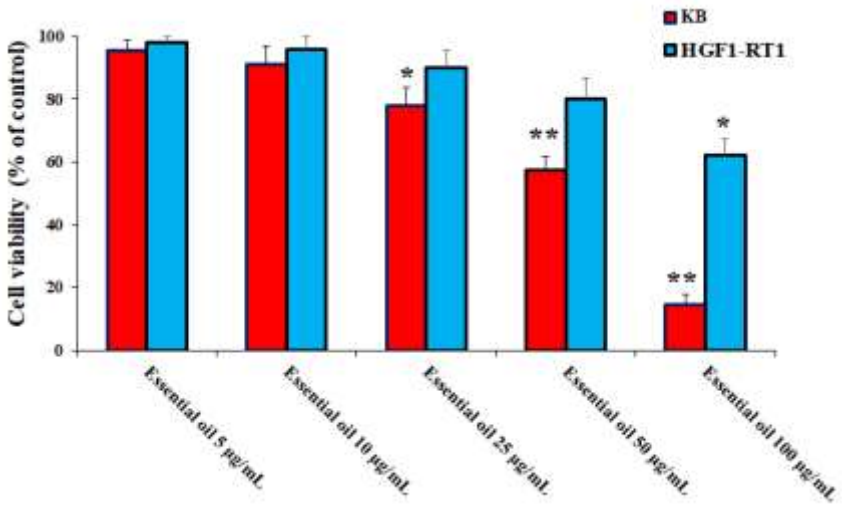

Figure 1. The effect of different concentrations of *N. tripedale* EO on the survival of normal human gingival fibroblast cells (HGF1-RT1) and oral cancer cells (KB mean ± SD). $p<0.05$* and $p<0.001$**

3.3. Effect of *N. tripedale* EO on Apoptosis-Related Gene Expression

Quantitative real-time PCR analysis revealed a significant upregulation of *caspase-3* and *Bax* gene expression in both KB oral cancer cells and normal gingival fibroblast cells (HGF1-RT1) following treatment with *N. tripedale* EO at concentrations corresponding to ½ CC□□ and CC□□ ($p < 0.05$). Conversely, the expression of the anti-apoptotic gene *Bcl-2* was markedly downregulated in both cell types, with the most pronounced reduction observed at the higher concentration (CC□□) of the essential oil ($p < 0.05$), as shown in Figure 2. These findings suggest that *N. tripedale* EO may induce apoptosis through a caspase-dependent pathway and by modulating the Bax/Bcl-2 regulatory axis.

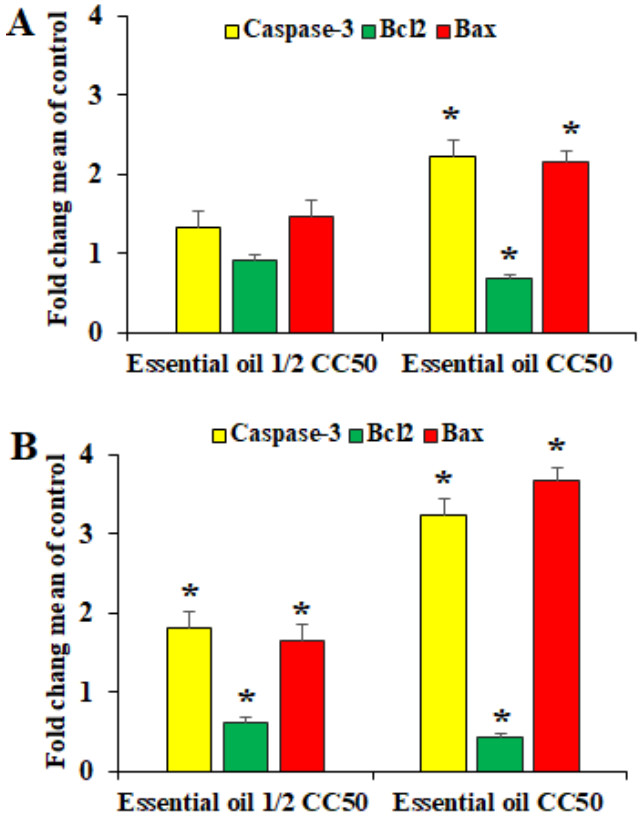

Figure 2. The effect of different concentrations of *N. tripedale* EO on *Caspase-3, Bax / Bcl-2* genes expression in HGF1 (A) and KB. (B) Cells. Mean ± SD (n = 3). *P<0.001.

3.4. Inhibition of DNA Synthesis by *N. tripedale* Essential Oil

The analysis of DNA synthesis using the BrdU incorporation assay revealed a concentration-dependent inhibition of DNA replication in both KB oral cancer cells and normal HGF1 fibroblasts following exposure to *N. tripedale* EO. As shown in Figure 3, treatment at the CC□□ concentration led to a marked suppression of DNA synthesis in both cell types, with a more substantial effect observed in the cancerous cells. These results suggest that *N. tripedale* EO may interfere with cell proliferation by impairing DNA synthesis mechanisms.

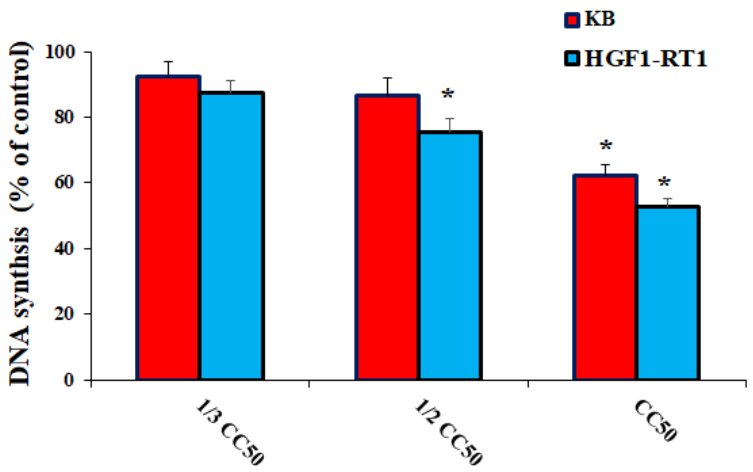

Figure 3. The effect of different concentrations of *N. tripedale* EO on DNA production and synthesis in normal human gingival fibroblast cells (HGF1-RT1) and KB oral cancer cells. Mean ± SD (n = 3). *P<0.001.

4. Discussion

Nectaroscordum tripedale is a medicinal plant known for its diverse array of bioactive compounds, contributing to a range of biological effects such as antioxidant, antimicrobial, anti-inflammatory, and anticancer activities (14,16). In the present study, gas chromatography-mass spectrometry (GC/MS) analysis revealed that Germacrene-D, hexadecanoic acid, and diphenylamine were the major constituents of the essential oil derived from *N. tripedale*. Each of these compounds has been previously associated with pharmacological properties. For instance, Germacrene-D, a sesquiterpene, has demonstrated antimicrobial and anti-inflammatory effects (21). Hexadecanoic acid, a saturated fatty acid also known as palmitic acid, is abundant in plant oils and has been reported to exhibit cytotoxic and anti-inflammatory actions (22). Diphenylamine, a nitrogen-containing aromatic compound, is recognized for its antioxidant properties, which may contribute to its potential antitumor activity (23).

In line with these biochemical profiles, our study demonstrated that *N. tripedale* essential oil exerted cytotoxic effects on KB oral squamous carcinoma cells in a dose-dependent manner, while

maintaining relative safety toward normal human gingival fibroblasts. The calculated selectivity index (SI > 2) further supports the selective toxicity of the essential oil toward malignant cells. These results are consistent with previous findings by Ezatpour et al., who reported the cytotoxicity of *N. tripedale* extracts against leukemic cell lines, with limited toxicity to normal cells (15). Moreover, the low systemic toxicity of *N. tripedale* in vivo has been previously confirmed in animal models, where no significant alterations in liver and kidney function biomarkers were observed (14). Collectively, these findings suggest the potential of *N. tripedale* EO as a relatively safe and natural anticancer agent.

To explore the underlying mechanisms of its anticancer activity, we assessed the expression of key apoptosis-related genes following treatment with *N. tripedale* EO. Notably, exposure to the essential oil resulted in a significant upregulation of the pro-apoptotic genes *caspase-3* and *Bax*, along with downregulation of the anti-apoptotic gene *Bcl-2*. These findings align with the established roles of these genes in the regulation of programmed cell death: caspase-3 functions as a central executioner of apoptosis (24), *Bax* promotes mitochondrial membrane permeabilization, and *Bcl-2* acts as a suppressor of apoptosis by stabilizing mitochondrial integrity (25). The observed gene expression pattern indicates activation of the intrinsic apoptotic pathway, suggesting that *N. tripedale* EO may trigger mitochondrial-mediated cell death in KB cells.

Additionally, our data showed that DNA synthesis was markedly suppressed in both normal and cancer cells treated with the essential oil, with the greatest inhibition observed in KB cells. This inhibitory effect on DNA replication could contribute to reduced cell proliferation and tumor progression, further reinforcing the potential of *N. tripedale* EO as an antiproliferative agent.

Given the limitations of conventional therapies—such as chemotherapy and radiotherapy—which are often associated with adverse side effects and limited specificity, the development of plant-

derived compounds offers an attractive alternative. In this context, our study adds to the growing body of evidence supporting the application of essential oils in cancer treatment. The promising in vitro effects observed for *N. tripedale* EO warrant further investigation in animal models to validate its safety and efficacy under physiological conditions. Ultimately, such studies could pave the way for clinical trials aimed at developing novel, plant-based therapeutics for oral cancers.

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Authors contribution

Concept and design: P.SH. Conduct laboratory tests: M. R., A. S., and A. H.. Acquisition, analysis, or interpretation of data: Z.SH., F. T. Drafting of the manuscript: P. SH. Editing: R. A.

Ethics

The present study was approved by the Ethics Committee of Lorestan University of Medical Sciences, Khorramabad, Iran, with the ethics number IR.LUMS.REC.1402.244.

Conflict of Interest

All authors declare that they have no competing interests with any organization or institution related to this article.

Data Availability

None.

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