ORIGINAL RESEARCH

Cytotoxic Effects of *Nigella sativa* on Oral Squamous Cell Carcinoma: An In Vitro Study

Imran Mohtesham[1], Riaz Abdulla[1], Vidya Pai[2], Ashwini Prabhu[3,4], Pavan SR[3], Ranajit Das[5]

Abstract

Background: Cancer remains and continues to be the second leading cause of mortality globally, with Oral Squamous Cell Carcinoma (OSCC) ranking as the sixth most common cancer worldwide. Regardless of advancements in surgery, chemotherapy, and radiation therapies, the management of OSCC remains a huge challenge because of the limitations and side effects of cancer therapy. That said, there is a continuing need for effective and alternative therapies. Medicinal plants known for their rich phytochemical properties offer a promising road for developing novel anticancer agents. *Nigella sativa* (*N. sativa*), or black cumin, a medicinal plant belonging to the family Ranunculaceae, has demonstrated countless pharmacological properties on periodontal and gingival diseases, oral mucositis, dental caries, oral ulcerations and candidiasis. Current studies have explored and demonstrated its potential anticancer, cytotoxic and antimicrobial properties, particularly in the context of oral health.

Objective: This study was designed to investigate the cytotoxic efficacy of ethanolic extract of N. saliva on oral squamous cell carcinoma cells in vitro.

Methodology: The cytotoxic potential of the ethanolic extract of *N. sativa* was evaluated using an MTT assay, while the induction of apoptosis was confirmed using acridine orange-ethidium bromide (AO-EB) dual fluorescence staining. The cytocompatibility of the extract on the normal fibroblast cells was assessed on L929 fibroblasts.

Results: The study indicated that the extract exerted significant anticancer effects on OSCC cells with an IC^{50} value of 99.03 µg/mL. the cytotoxic effect of the ethanolic extract of *N. sativa* showed a dose-dependent cytotoxic effect on CAL 27 cancer cell lines. The maximum cytotoxic effect was observed at 250 µg/mL, where 85.43% of the cells were found to be non-viable, indicating that at higher concentrations, the extract has a substantial toxic effect on the oral cancer cells. The effect of the ethanolic extract of *N. sativa* seeds on inducing cytotoxicity via apoptosis on CAL27 oral cancer cell lines was further investigated using the AO-EB differential staining technique. The results showed that the untreated control cells displayed bright green fluorescing nuclei, indicating the viable nature of the cells. Cells treated with the ethanol extract of *N. sativa* seed showed yellow fluorescence, indicating the induction of early apoptosis. The cytocompatibility of the ethanolic extract of *N. sativa* was evaluated on L929 mouse fibroblast cells (normal cell line) using the MTT assay under identical conditions as for CAL27 cells (seeding density 5000 cells/well, 24 h treatment with extract concentrations 12.5–250 µg/mL. In contrast to the significant dose-dependent cytotoxicity observed in CAL27 cancer cells, the ethanolic extract of *N. sativa* showed minimal cytotoxicity on L929 normal fibroblast cells across the tested concentration range (cell viability remained >85% even at 250 µg/mL), indicating good cytocompatibility and selective toxicity toward oral cancer cells.

Conclusion: The results of the present study suggest that the ethanolic extract of *N. sativa* seeds could be a valuable, effective and natural alternative to be used as a cytotoxic agent. its potent cytotoxic effect against oral cancer. Future research should be conducted on optimising and standardising the ethanolic extract of *N. sativa* seeds for clinical applications, thereby exploring its mechanisms of action, and also by testing its efficacy in human trials.

Keywords: *Nigella sativa*, Oral squamous cell carcinoma, Anticancer, Apoptosis, in vitro

Introduction

There is a continuing interest in the medical fraternity to use medicinal plants for treating diseases. The perceived safety when compared to modern medicine is the main reason for its use. *Nigella sativa (N. sativa),* which is known for its historical and medicinal significance, is one of the most researched medicinal plants. *N. sativa* is native to South and Southwest Asia and cultivated in regions such as the Mediterranean and South Europe. Cancer has raised concern in the global scenario regarding its management, which continues to pose challenges despite humongous advancements in modern medicine. *N. sativa* has shown promise in various in vitro and in vivo studies for its anti-proliferative, pro-apoptotic, antioxidant, cytotoxic, and anti-metastatic effects, particularly against cancer cell lines.[3]

Oral cancer remains a significant global public health concern. It ranks among the top 10 most common cancers

Department and Institution Affiliation: [1]Dept. of Oral Pathology, Yenepoya Dental College, Yenepoya (Deemed to be University), Mangalore, India; [2]Dept of Microbiology, Yenepoya Medical College, Mangalore; [3]Division of Cancer Research and Therapeutics (CaRT), Yenepoya Research Centre, Yenepoya (Deemed to be University), Deralakatte, [4]Division of Cell and Molecular Biology, Central Research Laboratory, Srinivas University, Mangalore 574146, India, [5]Division of Data Analytics, Bioinformatics and Structural Biology (DABS), Yenepoya Research Centre, Yenepoya (Deemed to be University), Deralakatte

Corresponding Author: Riaz Abdulla, Professor, Head, Dept. of Oral Pathology, Yenepoya Dental College, Yenepoya (Deemed to be University), Mangalore, India Email: rizdent@yahoo.com.

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worldwide, and despite advancements in research and therapeutic strategies, survival rates have shown limited improvement in recent years. Oral cancer is a malignancy that develops in the oral cavity, including the lips. It is predominantly classified as oral squamous cell carcinoma (OSCC), as approximately 90% of cancers in the oral region arise histologically from squamous cells. OSCC exhibits varying degrees of differentiation and has a notable tendency to metastasise to regional lymph nodes.[1]

Oral cancer is two to three times more common in men than in women across most ethnic groups. Globally, cancers of the oral cavity and pharynx are often reported together, making them collectively the sixth most common cancer worldwide.[2]

Lifestyle change and economic development have largely contributed to the increasing number of cancer patients, making cancer rank among the top three causes of death in rural and urban areas. The incidence is still lower in many western countries while it has skyrocketed in India in recent decades. In India, global mortality rates for ovarian and breast cancer are among the highest. In the Indian scenario, nearly 40% of cancer cases, such as lung and oral cancers, are totally preventable. A major contributing factor to both cancers is the use of tobacco, mainly in the chewable form. Oral cancer comprises malignancies affecting the gingiva, lip, palate, cheek lining, floor of the mouth, or tongue.[3,4]

Oral cancer is largely preventable, with smoking and alcohol being significant risk factors in nearly 90% of cases. Combined, these factors have a synergistic effect, significantly increasing the risk of developing the disease.[5] Additionally, its burden imposes sizable economic and social costs on patients and their families, exacerbating the impact on communities and the healthcare system.[5,6]

In 2007, the International Agency for Research on Cancer (IARC) determined that there is substantial evidence to classify tobacco smoke as carcinogenic, notably linking it to cancers of the oral cavity and pancreas. Smokers are three times more likely to develop oral cancer compared to non-smokers. Additionally, individuals who quit smoking for four years reduce their risk of developing oral cancer by 35% compared to those who continue smoking, and the risk becomes comparable to life-long non-smokers after 20 years of cessation.[117] Besides, with exposure to second-hand smoke, the risk of oral cancer increases by 87% in comparison to those who have neither smoked nor been exposed to such environments.[8]

Oral cancer, particularly OSCC, is a primary public health concern in India, accounting for a significant proportion of cancer cases and deaths. The incidence and mortality rates of oral cancer in India are among the highest in the world, primarily attributed to cultural practices, socioeconomic factors, and lifestyle habits such as tobacco use, betel nut chewing, and alcohol consumption. According to the Global Cancer Observatory, one-third of the global burden of oral cancer is contributed by India, with an estimated 135,929 new cases and 75,290 deaths recorded annually (2020). The main reasons for oral cancer in India include the use of smokeless tobacco products, betel quid consumption, smoking, and alcohol. Tobacco consumption, mainly in the form of smokeless products such as gutkha and khaini, contains carcinogenic substances such as nitrosamines, which are directly implicated in oral carcinogenesis.[9,10]

Currently, there is an alarming global interest in the use of medicinal herbs or plants for treating various diseases, as they are considered safer and more economical compared to modern allopathic medicines. Various salubrious and pharmacologically active compounds have been isolated from black seeds, such as thymoquinone (TQ), thymol, and thymohydroquinone.[11]

Nigella sativa has been extensively studied for its biological activities. It is an annual flowering plant in the family Ranunculaceae, also called black cumin or black seed, native to South and Southwest Asia and cultivated in several countries in the Mediterranean region, South Europe, Syria, Turkey, India, Pakistan, and Saudi Arabia.[14-19]

The anticancer potential of *N. sativa* has generated considerable research interest because of its ability to induce apoptosis, inhibit cellular proliferation, and suppress angiogenesis in cancer cells. *N. sativa* is believed to exert its cytotoxic effects via multiple mechanisms, such as modulation of apoptotic pathways, inhibition of cell cycle progression, and suppression of nuclear factor kappa B (NF-κB) and other signalling pathways.[20-23]

The current paper evaluates the cytotoxic and apoptotic activities of the ethanol extract of *N. sativa* in vitro against an oral squamous cell carcinoma cell line. A normal L929 cell line was also included to study the selectivity and safety of the *N. sativa* extract on normal cells.

Materials and Methods:

N. sativa seeds were collected from the local herbalist from local stores. The seeds were authenticated by a Taxonomist. The seeds were identified, cleaned, dried, mechanically powdered, extracted with absolute ethanol and evaporated with a rotary evaporator to render the extract alcohol-free. The extract was stored in a domestic refrigerator at 4◦C for further analysis. Human oral squamous cell carcinoma cells (CAL27) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; ATCC CRL-2095). L929 mouse fibroblast cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. For the apoptosis detection assay, Acridine orange and ethidium bromide stains were procured from HiMedia Laboratories Pvt. Ltd., India.

Evaluation of cytotoxic efficacy of *Nigella sativa* extract on oral squamous cell carcinoma cells:

Cells and Culture conditions

Human oral carcinoma cells (CAL27) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum and 1 % antibiotic-antimycotic solution. They were incubated at 37ºC under 5% CO_2, sub-cultured upon attaining 70% confluence and used for the experiments after three consecutive passages.

Cytotoxicity assessment using Methyl Thiazolyl Tetrazolium (MTT) assay

Cytotoxicity of the compounds on the CAL27 cells was evaluated using MTT assay (Mosmann, 1983).[24] Cells were

seeded at a density of 5000 cells/well in 96 well microtiter plates and incubated at 37ºC under 5% CO_2 in a humidified atmosphere for 24 h. *Nigella sativa* extract was added to the cells at concentrations of 12.5, 25, 50, 100 and 250 µg/mL and incubated further for 24 h. 100 µL of MTT solution (1 mg/mL) was added to the wells and incubated for 4h. Formazan crystals were solubilized in DMSO and absorbance was recorded at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech). (Figures 1 and 2)

Apoptosis detection using acridine orange-ethidium bromide dual staining

The effect of *N. sativa* extract on inducing cytotoxicity via apoptosis was further investigated using the AO-EB differential staining technique. CAL 27 cells were seeded onto 24 well plates at a density of 20,000 cells per well and incubated at 37°C and 5% CO_2 conditions. After adherence, they were treated with selected concentrations of the extract and further incubated for 24 h. Cells were stained using a mixture of acridine orange: ethidium bromide (1:1) for 15 min at 37°C under dark. Excess stain was removed by PBS wash, cells were overlaid with PBS and images were captured using a fluorescence imager at 20X magnification under green and red channels.

Results

The cytotoxic efficacy of the ethanolic extracts of *N. sativa* seeds on oral cancer cells was determined using the MTT assay. The oral cancer cell line CAL27 was used for the study.

Table 1indicates that there is a dose-dependent cytotoxic effect of ethanolic extracts of *N. sativa* on CAL 27 cancer cell lines. The maximum cytotoxic effect was observed at 250 µg/mL, where 85.43% of the cells were found to be non-viable, indicating that at higher concentrations, the extract has a substantial toxic effect on the oral cancer cells. (Table 1, Figure 3)

The cytocompatibility of the ethanolic extract of *N. sativa* was evaluated on L929 mouse fibroblast cells (normal cell line) using the MTT assay under identical conditions as for CAL27 cells (seeding density 5000 cells/well, 24 h treatment with extract concentrations 12.5–250 µg/mL. In contrast to the significant dose-dependent cytotoxicity observed in CAL27 cancer cells, the ethanolic extract of *N. sativa* showed minimal cytotoxicity on L929 normal fibroblast cells across the tested concentration range (cell viability remained >85% even at 250 µg/mL), indicating good cytocompatibility and selective toxicity toward oral cancer cells.

Data represented is the cytotoxicity (%)(Mean±SD) of ethanol extracts of *N. sativa* seeds at various concentrations (12, 25, 50, 100, 250 µg per mL). The concentration of the substance was tested in µg per mL. Tests were done using the MTT assay. Statistical test used: one-way ANOVA followed by Tukey's Multiple Comparisons. Level of significance: **p*<0.05 was considered significant, *p*>0.05 was considered non significant (ns).

Table 2 presents a pairwise comparison of the cytotoxic effects of the ethanolic extract of *N. sativa* seed on CAL27 oral squamous carcinoma cells at various concentrations (12.5–250 µg/mL), demonstrating statistically significant differences across all tested concentrations (Table 1). One-way ANOVA followed by Tukey's post hoc test revealed that each concentration showed a highly significant difference ($p < 0.0001$) as compared with the others. This indicates that the cytotoxic response was dose-dependent, with higher concentrations producing progressively greater cytotoxic effects on CAL27 cells.

Table 1 Cytotoxicity of ethanolic extracts of *Nigella sativa* seeds on CAL27 oral cancer cell line

Concentration (µg/mL)	Cytotoxicity(%) *p value*
12.5	4.19 ± 2.00
25	24.64 ± 1.98
50	45.84 ± 1.15 <0.0001
100	72.77 ± 1.37
250	85.43 ± 1.53

*Data represented is the cytotoxicity (%)**(Mean ± SD)** of ethanol extracts of N. sativa seeds at various concentrations (12.5, 25, 50, 100, 250 µg per mL**)**. The concentration of the substance tested in µg per mL. Tests were done using the MTT assay. Statistical test used: one-way ANOVA followed by Tukey's Multiple Comparisons. Level of significance: *p<0.05 was considered significant, p>0.05 was considered non significant (ns).*

Table 2 Pairwise comparison of cytotoxic effects of *Nigella sativa* ethanolic seed extract on CAL27 oral squamous carcinoma cell line

Concentration (µg/mL)		p value
1	2	
12.5	25	<0.0001
	50	<0.0001
	100	<0.0001
	250	<0.0001
25	50	<0.0001
	100	<0.0001
	250	<0.0001
50	100	<0.0001
	250	<0.0001
100	250	<0.0001

The data represented is pairwise comparisons between concentrations, which were performed using one-way ANOVA followed by Tukey's post hoc test. p < 0.0001 indicates a highly significant difference between the compared concentrations.

Figure 3 indicates that there was a dose-dependent cytotoxic effect of the ethanolic extract of *N. sativa*. The (****) represent significant differences in variation between the groups, (12.5, 25, 50, 100, 250 µg/mL) is statistically significant ($p<0.0001$). This suggests a dose-dependent cytotoxicity, i.e., an increase in concentration is associated with an increase in the percentage of cell death.

Apoptosis detection using acridine orange-ethidium bromide (AO-EB) dual staining

The effect of the ethanol extract of *N. sativa* seeds on inducing cytotoxicity via apoptosis on CAL27 oral cancer cell lines was further investigated using AO-EB differential staining technique.

Fig. 1: Multimode microplate reader used for MTT assay

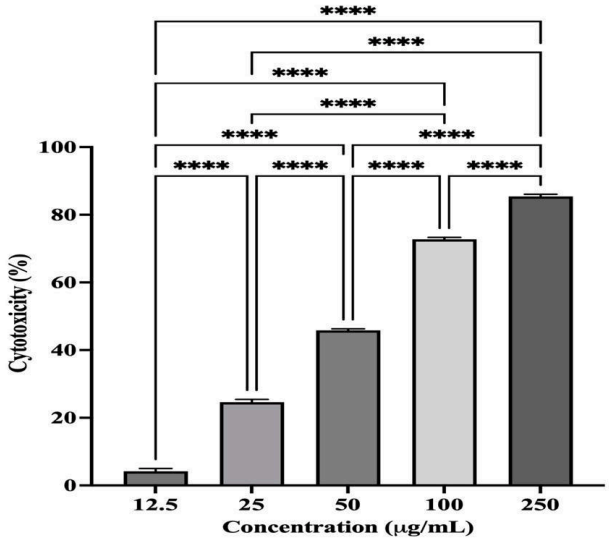

Fig. 3 Dose-dependent cytotoxic effect of the *Nigella Sativa* extract on the CAL27 cancer cell line

*Data represented is as follows: the y axis represents the percentage of cytotoxicity, the x axis represents the concentration of ethanolic extract of N. sativa in µg/mL (12.5, 25, 50, 100, 250). Each bar corresponds to the cytotoxicity level at a specific concentration. Statistical test used: one-way ANOVA followed by Tukey's Multiple Comparisons. Level of significance: *p<0.05 was considered significant, p>0.05 was considered non significant (ns).*

Figure 4 shows that the untreated control cells displayed bright green fluorescing nuclei, indicating the viable nature of the cells. Cells treated with the ethanol extract of *N. sativa* seed showed yellow fluorescence, indicating the induction of early apoptosis.

Discussion

The utilisation of medicinal plants for treating various diseases has played a critical role in the development of new drugs because of their rich phytochemical composition.[1] The medicinal use of plants has been documented in ancient texts from around 3000 BC, while various systems such as Ayurveda, Siddha, and traditional Chinese medicine continue to use herbal therapies to treat various ailments.[2] Medicinal practices were largely plant-based, but with the advent of synthetic drugs, there has been a decline in herbal usage.[3] Despite this paradigm shift, plant-derived compounds have been used in the development of various drugs, such as vinblastine used in treating Hodgkin's lymphoma, which have contributed

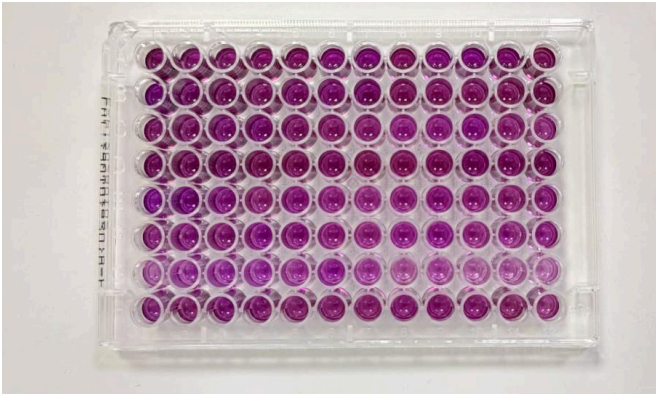

Fig. 2: Representative 96-well microtiter plate from MTT assay.

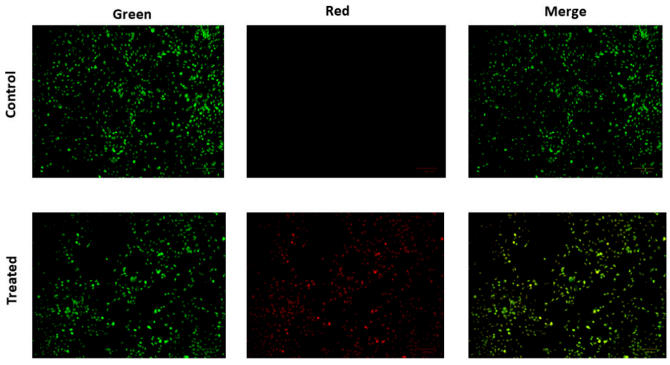

Fig. 4: Apoptosis in CAL27 oral squamous carcinoma cells treated with ethanol extract of *N. sativa*

The data represented is the effect of the ethanolic extract of N. sativa seeds on the CAL27 cell line, indicating cytotoxicity via apoptosis. The test was done using the AO-EB differential staining technique. The untreated control cells showed bright green nuclei, indicating their viability. In contrast, cells treated with Nigella sativa extract displayed yellow fluorescence, which represents the induction of early apoptosis

immensely to modern therapeutics, thereby highlighting the importance of natural products in drug development.[4]

There were huge concerns over the toxic effects of synthetic drugs by the mid-20th century, which sparked a renewed interest in herbal medicines. The World Health Organisation (WHO) encouraged and supported traditional plant-based medicine by promoting its use in healthcare systems. Markedly, nearly 25% of modern medicines are derived from plant sources, and around 80% of the global population relies on herbal remedies for their primary healthcare.[5] One such ubiquitous plant is *Nigella sativa (N. sativa),* often referred to as the "miracle herb of the century", known for its curative and healing properties.

The rationale for the selection of *N. sativa* in the present study is due to its rich phytochemical composition, well-researched and documented pharmacological properties, and scientific, pharmacological, and therapeutic considerations that align with the objectives of evaluating antimicrobial, anticandidal, and cytotoxic potentials in relation to oral health and its management.

N. sativa has shown significant anticancer potential, specifically its cytotoxic and pro-apoptotic effects on various cancer cell lines. The seed extract and its bioactive constituents have been demonstrated to induce apoptosis through mitochondrial and caspase-dependent pathways, regulate key oncogenic signalling cascades (such as p53, NF-κB, and PI3K/AKT), and inhibit tumour proliferation.[6] These properties provide a compelling rationale for investigating *N. sativa*, particularly given the need for safer, plant-derived alternatives or adjuncts to conventional chemotherapeutic agents.

The present study showed cytotoxic effects of the ethanolic extract of *N. sativa* seeds on CAL27 cells in a dose-dependent pattern, with higher concentrations leading to a larger percentage of cell death. In the context of cancer research, these results are significant, as CAL27 is a well-established oral squamous cell carcinoma (OSCC) cell line. The dose-dependent cytotoxicity observed aligns with the findings from earlier studies on the cytotoxic potential of *N. sativa*.

Earlier studies have consistently reported and highlighted the cytotoxic potential of *N. sativa* extracts. Woo et al. demonstrated that *N. sativa* can induce apoptosis in cancer cells by activating multiple signalling pathways such as p53, NF-κB, and PI3K/AKT.[7] The present study demonstrated a similar effect, with induction of apoptosis at higher concentrations (100 and 250 μg/mL), as verified by cytotoxicity rates of 72.77% and 85.43%, respectively. The apoptosis detected using the acridine orange–ethidium bromide (AO–EB) staining technique validates the findings of Woo et al. and other studies that identified *N. sativa* as a potent pro-apoptotic agent.

This dose-dependent cytotoxicity observed in the present study is in line with the study conducted by Roepke et al., who explored the dose-dependent effect of thymoquinone on breast cancer cells.[8] The cytotoxicity and apoptosis observed at higher concentrations in their study were similar to the present findings on CAL27 cells, where 250 μg/mL of ethanolic extract of *N. sativa* seeds exhibited 85.43% cytotoxicity. This highlights the importance of concentration in determining the therapeutic dose of ethanolic extract of *N. sativa* seeds.

The apoptosis seen with yellow fluorescence staining using AO–EB aligns with the study conducted by El-Mahdy et al., which explored the mechanisms of apoptosis induced by *N. sativa* in various cancer cell lines.[9] It is postulated that *N. sativa* triggers intrinsic apoptosis through the mitochondrial pathway, which is characterised by early apoptosis markers. The yellow fluorescence in CAL27 cells treated with the ethanolic extract of *N. sativa* seeds indicates early apoptosis.

The statistical significance (p<0.0001) of cytotoxicity observed between concentrations (12.5, 25, 50, 100, and 250 μg/mL) indicates a dose–response effect. This is in agreement with the findings of Shoieb et al., who demonstrated dose-dependent cytotoxic effects of ethanolic extracts of *N. sativa* seeds on different cancer cell lines.[10] Higher doses of thymoquinone resulted in profound apoptosis, similar to the observations of the present study.

The comparison of the ethanolic extract of *N. sativa* seeds with DMSO (solvent control) highlights the effectiveness of the extract, suggesting that the observed effects are due to the bioactive components of *N. sativa* rather than the solvent. Badary et al. reported the low toxicity of DMSO, supporting the validity of the current findings.[11]

CAL27 cells, derived from human oral squamous cell carcinoma, represent an optimised in vitro model for assessing the therapeutic efficacy of ethanolic extracts of *N. sativa* seeds in oral cancer research. Effenberger et al. demonstrated that *N. sativa* and thymoquinone significantly slowed tumour growth in animal models of oral cancer.[12] The observations from the present study indicate that ethanolic extract of *N. sativa* seeds may be explored as an effective adjuvant therapy in oral cancer management.

The cytotoxicity observed at 250 μg/mL suggests that the ethanolic extract of *N. sativa* seeds possesses potent anticancer properties. These findings indicate that the extract may be considered in developing strategies to combat cancer, either as an alternative or an adjuvant to existing chemotherapeutic agents. The early apoptosis induced at various concentrations highlights the involvement of specific apoptotic pathways. Further studies are necessary to elucidate the exact molecular targets, particularly the role of mitochondrial pathways and apoptosis-related proteins such as caspases and Bcl-2 family members.

While several studies have explored the anticancer potential of *Nigella sativa* (mainly thymoquinone) on various cancer cell lines, this study is unique in the following aspects: firstly, it specifically evaluates the ethanolic seed extract (not isolated thymoquinone) against oral squamous cell carcinoma (CAL27), which is highly relevant to the high burden of OSCC in India and other South Asian countries where betel nut/tobacco-related oral cancers predominate. Secondly, it demonstrates both cytotoxicity (MTT) and apoptosis induction (AO-EB staining) in an oral cancer model. Thirdly, it includes a normal fibroblast (L929) control to establish selectivity, which is often lacking in similar studies. Lastly, the findings support development of a locally accessible, cost-effective natural agent for a cancer with a very high incidence in the Indian subcontinent.

The current study demonstrated that the ethanolic extract of *N. sativa* seeds exhibits robust dose-dependent cytotoxicity on CAL27 oral cancer cells, with marked apoptosis at higher concentrations. Future research is necessary to explore the clinical applicability of these findings, especially in the context of oral cancer therapy.

Conclusion:

The findings underscore the promise of *Nigella sativa* as a natural compound for the development of novel therapeutic strategies for oral cancer. Given the prevalent challenges in managing oral cancer in regions like India, where late diagnosis and socio-economic factors complicate treatment, *N. sativa* offers a potentially safer, economical, and effective alternative. The data suggest that *N. sativa* extract, with its rich phytochemical composition, could be integrated into existing chemo-preventive and therapeutic regimens to enhance treatment efficacy and reduce systemic toxicity associated with conventional chemotherapy.

Future research should focus on the in vivo efficacy and safety of *N. sativa* extract, exploring its mechanisms of action and potential synergistic effects with other chemo-preventive agents. The continued exploration of medicinal plants like *Nigella sativa* could pave the way for more accessible and effective treatments for oral cancer, addressing a critical public health challenge with significant implications for patient survival and quality of life.

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