

Comparison of Cytotoxic Effect of Sunflower Seed (*Helianthus Annuus* L.) and Flax Seed (*Linum Usitatissimum* L.) Extracts on OSCC (KB) Cell Line - An Invitro Study

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ABSTRACT

Introduction: Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity. Conventional treatments such as surgery, chemotherapy, and radiotherapy often cause significant side effects, highlighting the need for alternative approaches. Sunflower (*Helianthus annuus* L.) and flax (*Linum usitatissimum* L.) seeds contain bioactive compounds including antioxidants, lignans, and essential fatty acids with potential anticancer properties, making them promising candidates for oral cancer prevention and therapy.

Aim: 1. To determine the cytotoxic activity of sunflower, flax, and their combination seed extracts on KB cell line. 2. To compare the cytotoxic activity of sunflower, flax, and their combination seed extracts on KB cell line.

Materials & methods: Ethanolic extracts of sunflower seeds, flaxseeds, and their equal-volume combination were prepared by cold maceration and diluted in 0.1% DMSO to concentrations from 15.6 to 1000 µg/ml. KB cells were cultured, treated with the extracts, and cytotoxicity was assessed by MTT assay after 24 hours, with cell viability calculated relative to DMSO and untreated controls; Pearson correlation, one-way ANOVA, and Bonferroni post hoc tests ($p \leq 0.05$) were used for analysis.

Results: All extracts caused a dose-dependent decrease in KB cell viability. The IC₅₀ values were 69.67 µg/ml for sunflower, 24.96 µg/ml for flax, and 15.6 µg/ml for the combination, with viability ranking as: combination < flax < sunflower. Conclusion: Sunflower and flaxseed ethanolic extracts show significant cytotoxic effects on KB (OSCC) cells, with the combination extract being the most potent, supporting their potential as complementary nutraceutical agents in OSCC management.

Keywords: Anticancer, Flax seeds, MTT, OSCC, Sunflower seeds

INTRODUCTION

Oral squamous cell carcinoma (OSCC) ranks as the 16th most common cancer globally with India carrying a disproportionately high burden, accounting for over 30% of all cancers in the country.¹ This concerning trend is mainly driven by the extensive tobacco use, betel quid chewing, poor oral hygiene, dietary deficiencies and compounded by socio-economic challenges and limited access to early diagnosis and treatment, resulting in high incidence and mortality.² OSCC is typically treated through a combination of surgery, radiation therapy, chemotherapy, targeted therapy, or immunotherapy, depending on the cancer's stage and spread. These treatments, while effective in fighting the cancer, often come with side effects such as pain, fatigue, difficulty in swallowing, mouth sores, dry mouth, and weakened immunity. As a result, there is an increasing demand for alternative and complementary therapies that are effective, affordable, and have minimal side effects.³

Herbal products have been utilized for centuries in both Eastern and Western medicine for their therapeutic properties. Natural plant-derived compounds have attracted

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significant attention in cancer research due to their anti-inflammatory, antioxidant, and anticancer effects. Among these, dietary seeds such as sunflower seeds and flax seeds are rich in phytochemicals, including lignans, flavonoids, phenolic acids, and omega-3 fatty acids, all of which exhibit potential anticancer properties.^{4,5,6}

The Asteraceae family, which includes the sunflower (*Helianthus annuus*), is one of the largest plant families and is widely studied for its unique chemical components and their medicinal values. Sunflower seeds are known for their high content of vitamin E, selenium, and bioactive compounds like chlorogenic acid, which have shown anti-proliferative and apoptotic effects against various cancer cell lines.^{7,8} Flaxseeds (*Linum usitatissimum*), belonging to the Lineaceae family, have gained attention as a functional food by establishing its importance in the world's food chain. Native to India, flaxseeds were historically consumed as both food and medicine. They are particularly valued for their high lignan content, notably secoisolariciresinol diglucoside (SDG), as well as omega-3 fatty acids like alpha-linolenic acid (ALA), which are known to influence cancer pathways, including cell cycle arrest and apoptosis induction.^{9,10}

However, while individual components of sunflower and flax seeds have documented anticancer effects, direct comparative evidence of their cytotoxicity alone and in combination on OSCC cell lines is limited. This study addresses this research gap by investigating and comparing the cytotoxic effects of ethanolic extracts from sunflower seeds, flaxseeds, and their combination on the KB (OSCC) cell line. These findings aim to clarify their potential as natural, dietary-based adjuncts for oral cancer management and contribute to the development of nutraceutical-based therapy strategies.

MATERIALS AND METHODS

Collection of plant material and authentication:

The seeds chosen in this study to evaluate the cytotoxic effect against oral squamous cell carcinoma cell lines are extract

of Sunflower seeds (*Helianthus annuus*) - Sample 1, extract of Flax seeds (*Linum usitatissimum*) - Sample 2 and Combination of extract of sunflower and flax seeds - Sample 3. The seeds were procured from the local organic store and ethical approval (IHEC-CDCRI/2024/STU-0062) was obtained.

Preparation of crude herbal extract:

The collected seeds were washed, weighed and shade dried. Cold maceration method for extract preparation where 5g of each sample was macerated separately in 50 ml of ethanolic solvent in a conical flask and left at room temperature for 72 hours. Following this, the solutions were filtered using sterile filter paper No. 1. For combination extract, an equal amount of filtrate obtained from both the seed extracts were mixed. The filtrate was further evaporated using a rotary mantle heater until a thick paste was obtained. The obtained paste was then diluted using the vehicle 0.1% Dimethyl sulfoxide (DMSO) in the following concentrations 15.6 µg/ml, 31.2 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml.

Cell lines:

KB (OSCC) cell line was procured from National Center for Cell Sciences (NCCS, Pune, India) and cultured in Minimum essential media (MEM) containing 0.1% streptomycin, 0.1% penicillin G and 0.1% amphotericin B, 10% Fetal bovine serum & L- glutamine. The cells were maintained at 37°C, 95% air, and 5% CO₂ atmosphere at a pH of 7.2 -7.4. The monolayer culture of KB cells at 80% confluence were trypsinized and seeded at the required density for the experiments.

MTT cytotoxicity assay:

MTT assay was used to determine the cell viability following treatment with extracts of sample 1,2 and 3 at different con-

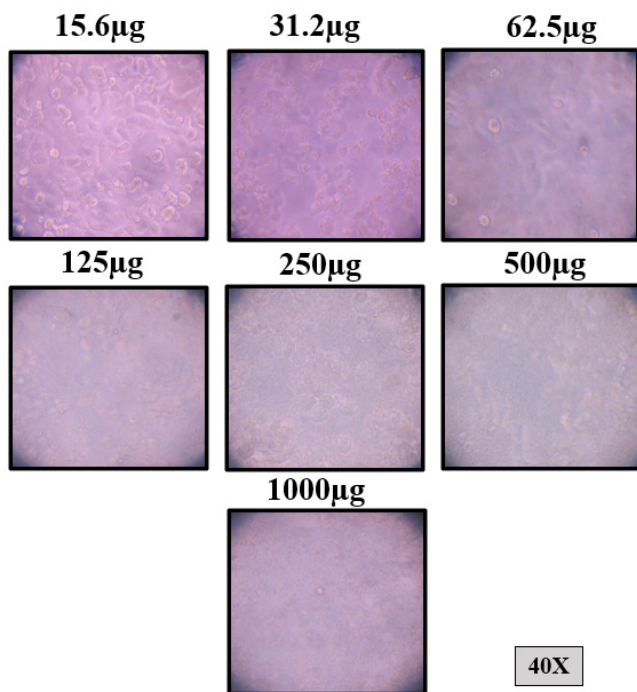


Fig. 1: Various concentrations of Sunflower seed extract on KB cell line

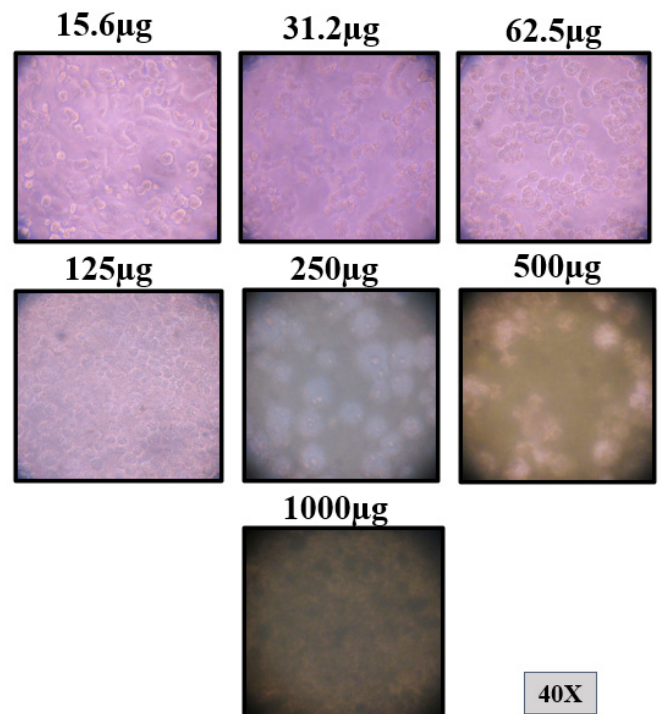


Fig. 2: Various concentrations of Flax seed extract on KB cell line

centrations. The workflow involved the following sequential steps:

- KB cells (1×10^5 cells/well) were seeded in 96-well plates with 0.2 ml of MEM, incubated for 72 hours at 37°C in 5% CO₂ to allow proper cell attachment and formation of a stable monolayer.
- After 72 hours, the medium was removed and replaced with fresh medium containing the test extracts (Samples 1, 2, and 3) at concentrations of 15.6, 31.2, 62.5, 125, 250, 500, and 1000 µg/ml for 24 hours under standard incubator conditions.
- KB cells maintained in culture medium alone served as the untreated control and those treated with 0.1% DMSO served as the vehicle control.
- After 24 hours of exposure, 20 µl of 5 mg/ml MTT reagent was added to each well. And incubated in dark for 4 hours to allow formazan crystal formation.
- The supernatant was carefully removed without disturbing the formed formazan crystals and 200 µl of DMSO was added to each well to solubilize the crystals.
- The optical density was measured at 540 nm using a microplate reader and percentage cell viability was calculated using the formula (% cell viability = A540 of treated cells / A540 of control cells × 100%). Dose-response curves were plotted to determine the IC50 values for each extract.

Statistical analysis:

The cell viability data for KB cell lines treated with sunflower seed extract, flax seed extract, and their combination were recorded in Microsoft Excel and analyzed using SPSS software (version 23). Pearson’s correlation coefficient assessed the relationship between extract concentration and cell viability. Levene’s test evaluated homogeneity of variances for one-way

ANOVA group comparisons. Standard one-way ANOVA with Bonferroni post-hoc tests identified intergroup differences when homogeneity was met (Levene statistic = 2.168, p = 0.143). Welch’s ANOVA was used as a robust alternative if variances were unequal. IC50 values were calculated from dose-response curves. A p-value ≤ 0.05 indicated statistical significance for all analyses.

RESULTS

The KB cells treated with ethanolic extracts of sunflower seeds, flax seeds and their combination at concentrations ranging from 6.25µg/ml to 1000 µg/ml showed an inverse relationship between the percentage of viable cells and the concentration of the extract. Both the DMSO control and the untreated control groups exhibited 100% cell viability, consistent with expectations for non-cytotoxic conditions.

The relationship between the extracts and the average cell viability of KB cell lines was assessed using Pearson correlation analysis. The results revealed a strong negative correlation, indicating that as the concentration of seed extracts increased, the average cell viability decreased. The p-value obtained was less than the significance threshold of 0.05, suggesting that this correlation is statistically significant (Table 1). When viewed under an inverted microscope, the cellular morphology of the KB cells treated with varying concentrations of each extract,

Table 1: Correlation between the concentration of the extracts and average cell viability on KB cell line (N=35):

Concentration of extracts (µg/ml)	Pearson Correlation coefficient	P value
Concentration of Sunflower seed extract	-0.759	0.04*
Concentration of Flax seed extract	-0.651	0.011*
Concentration of Combination seed extract (Sunflower and Flax seeds)	-0.650	0.014*

*Pearson correlation, P-value ≤ 0.05 – statistically significance

Table 2: Comparison of average cell viability of the extracts on KB cell line (N=105):

Type of seed extract	n	Average cell viability (%) (SD)	F statistic	p-value
Sunflower seed extract	35	40.44 (3.15)	867.13	< 0.0001*
Flax seed extract	35	23.20 (2.22)		
Combination seed extract (Sunflower and Flax seeds)	35	16.84 (1.79)		

*Welch’s ANOVA, P-value ≤ 0.05 – statistically significance

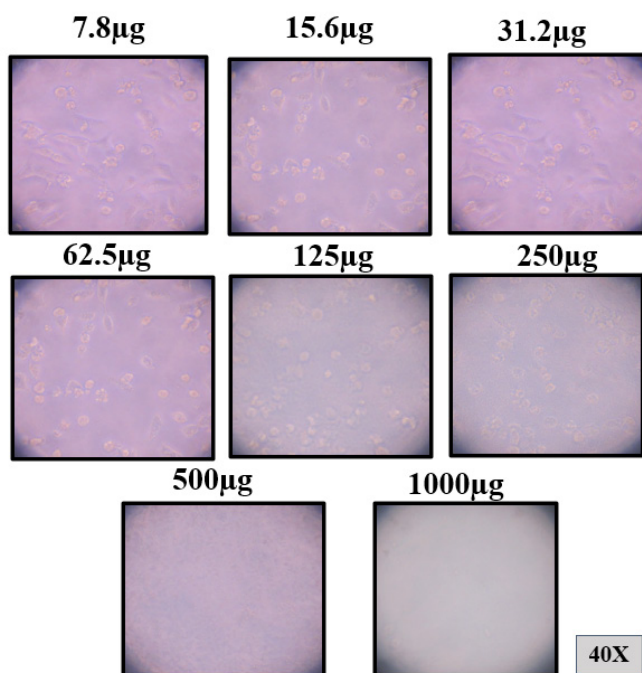


Fig. 3: Various concentrations of Combination seed extract (Sunflower seeds and Flax seeds) on KB cell line

showed alterations such as loss of adhesion and rounder outlines, which became more pronounced with increasing sample concentrations. (Fig 1, 2 and 3). This implies that higher concentrations of seed extracts are associated with reduced cell viability in a dose-dependent manner. The IC50 concentration

of sunflower, flax and combination seed extracts was found to be 69.67µg, 24.96µg and 15.6 µg respectively. On comparison, IC50 infers, combination seed extract is more cytotoxic than flax seed extract and sunflower seed extract (Graph 1).

Table 3: Intergroup comparison of average cell viability of the extracts on KB cell line (N=105):

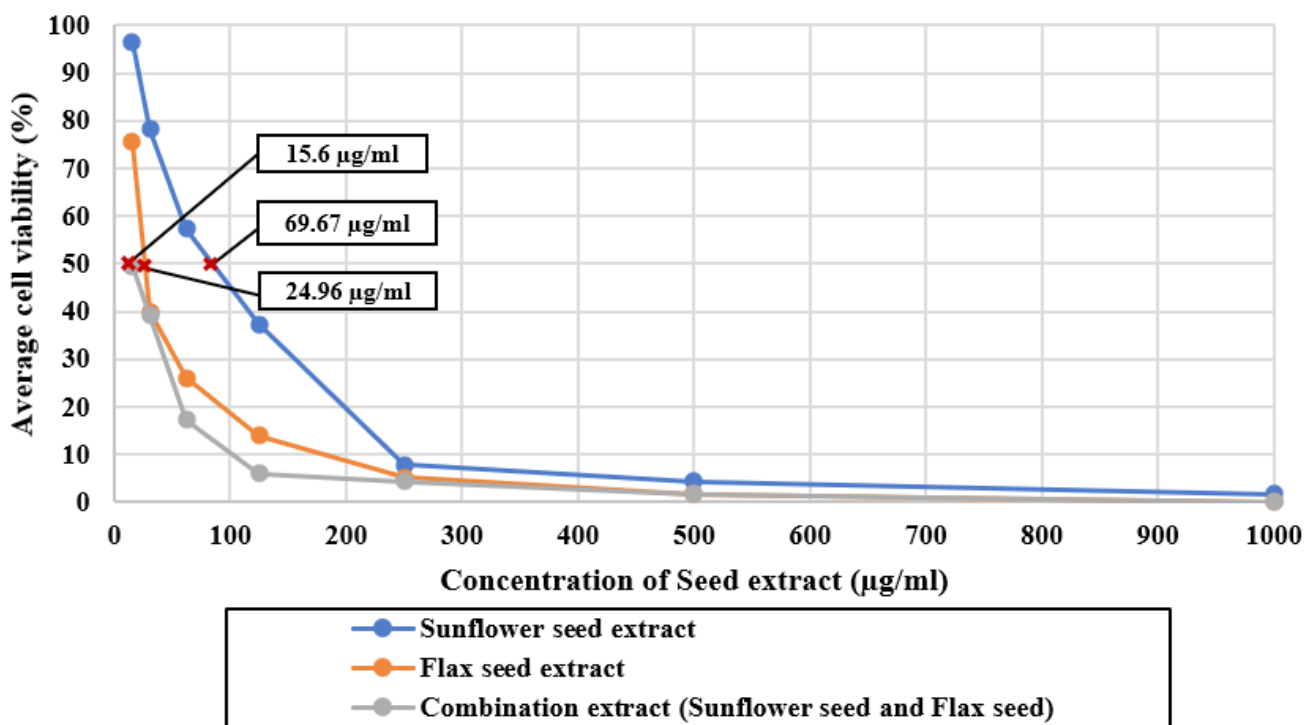
Group		Mean Difference	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Combination seed extract (Sunflower and Flax seeds)	Sunflower seed extract	-23.60	0.026*	-35.04	-15.84
	Flax seed extract	-6.36	0.049*	-9.79	-4.08
Sunflower seed extract	Flax seed extract	17.24	0.034*	8.68	24.19

*Bonferroni one-way ANOVA test, P-value ≤ 0.05 – statistically significance

One-way ANOVA revealed highly significant differences in mean cell viability across seed extract groups ($F(2,102) = 867.13, p < 0.0001$), with sunflower extract showing highest viability ($40.44 \pm 3.15\%$), followed by flax ($23.20 \pm 2.22\%$), and combination extract lowest ($16.84 \pm 1.79\%$). Levene’s test confirmed homogeneity (statistic = 2.168, $p = 0.143$), validating Bonferroni post-hoc results: combination vs. sunflower (mean difference = -23.60, $p = 0.026$), combination vs. flax (-6.36, $p = 0.049$), sunflower vs. flax (17.24, $p = 0.034$). These findings demonstrate dose-dependent cytotoxicity, with combination extract most potent ($IC_{50} = 15.6 \mu\text{g/ml}$), supported by morphological changes and strong negative Pearson correlations (all $p < 0.05$) (Table 2).

Intergroup comparisons were conducted to analyse the average cell viability among the samples on KB cell lines using Bonferroni post hoc test. The results revealed significant differences in cell viability between the groups. The combination extract of sunflower and flax seeds showed a mean difference of -23.60 ($p = 0.026$) compared to sunflower seed extract, with a 95% confidence interval ranging from -35.04 to -15.84. Additionally, the combination extract demonstrated a mean difference of -6.36 ($p = 0.049$) when compared to

Comparison of average cell viability of sunflower, flax seed extract and their combination on OSCC (KB) cell lines



Graph 1: Line diagram showing the comparison of average cell viability of Sunflower seeds, Flax seeds and their combination on KB cell line



flax seed extract, with a confidence interval of -9.79 to -4.08. Conversely, the sunflower seed extract had a mean difference of 17.24 ($p = 0.034$) when compared to the flax seed extract, with a confidence interval of 8.68 to 24.19 (Table 3).

Overall, the order of average cell viability among the groups can be represented as: combination extract < flax seed extract < sunflower seed extract. Since all p -values are less than 0.05, these findings indicate statistically significant differences in cell viability between the seed extract groups, suggesting that the combination seed extract is more cytotoxic than both individual seed extracts.

DISCUSSION

The present study demonstrated that the ethanolic extract of sunflower seeds (*Helianthus annuus*) exhibited moderate cytotoxicity against KB cells, with an IC₅₀ of 69.67 $\mu\text{g/mL}$. Smith et al. (2016)¹¹ reported stronger cytotoxicity in colorectal adenocarcinoma Caco-2 cells at 25 $\mu\text{g/mL}$ using LDH assay, a variation likely influenced by differences in assay methods and the intrinsic sensitivity of the cancer cell lines. Similarly, Al-Jumaily et al. (2013)¹² observed an IC₅₀ of 83.2 $\mu\text{g/mL}$ using hexane extract on Rhabdomyosarcoma (RD) and Murine L20B cell lines, which aligns closely with our findings and suggests comparable efficacy despite differences in bioactive compound profiles due to different extracts.

Sunflower seeds are known to be rich in polyphenolic compounds, including caffeic, chlorogenic, and ferulic acids, which have demonstrated strong antioxidant and antimutagenic properties. The polyphenols and tannins neutralize free radicals and inhibit the activation of mutagens, contributing to the protective effect against cancer cell proliferation. Additionally, they are rich in selenium, which, in combination with vitamin E, offers a protective effect against oxidative stress and DNA damage linked to cancer development. Selenium is known to enhance the body's antioxidant defences, contributing to cell protection from cancerous transformations.^{7,8,13} These mechanisms are relevant to OSCC, where oxidative stress and chronic inflammation play key roles in carcinogenesis. The moderate cytotoxicity observed suggests that sunflower seed extract may contribute to antiproliferative activity in OSCC but may require combination with more potent agents for enhanced efficacy.

In the present study, the ethanolic extract of flax seeds (*Linum usitatissimum*) showed strong cytotoxicity against KB cells, with an IC₅₀ of 24.96 $\mu\text{g/mL}$, indicating significant anticancer activity. Hu et al. (2019)¹⁴ reported a much higher IC₅₀ (367.28 $\mu\text{g/mL}$) in MCF-7 breast cancer cells, suggesting that variations in extraction methods and cell line characteristics may account for the stronger response observed in our study. Al-Radadi (2021)¹⁵ further demonstrated enhanced anticancer activity using flaxseed-derived gold nanoparticles, with IC₅₀ values of 9.9 $\mu\text{g/mL}$ for breast cancer (MCF-7), 14.5 $\mu\text{g/mL}$ for hepatocellular (HepG-2), and 17.9 $\mu\text{g/mL}$ for colon (HCT-116) cancer cell lines, indicating that nanoparticle formulations may increase the potency of flaxseed compounds by enhancing the bioavailability.

Flaxseeds are rich in lignans particularly high levels of

secoisolariciresinol diglucoside (SDG), a lignan metabolised into enterolignans by the gut and exhibits antiproliferative and anti-estrogenic activity modulating the growth of hormone-sensitive tumours such as breast and prostate cancers.¹⁶ In addition to lignans, Alpha-linolenic acid (ALA), an omega-3 fatty acid has demonstrated anticancer properties through its anti-inflammatory properties by reducing the levels of pro-inflammatory cytokines and inflammatory mediators like TNF- α , IL-6, and COX-2, which are involved in cancer cell growth and survival. ALA can induce cell cycle arrest in the G1 phase, preventing cancer cells from progressing to DNA replication and division.¹⁷ Flax seeds are rich in antioxidants, including polyphenols, such as caffeic acid, ferulic acid, and chlorogenic acid. These compounds play a crucial role in neutralizing reactive oxygen species (ROS), which can damage cellular components like DNA, proteins, and lipids, leading to cancer initiation and progression.^{16,17} This antioxidant activity, combined with the anti-inflammatory effects of ALA and the antiproliferative properties of lignans, contributes to the strong cytotoxicity observed in the present study.

A key finding of this study is the enhanced cytotoxicity of the combination extract, which exhibited an IC₅₀ of 15.6 $\mu\text{g/mL}$, significantly lower than either extract alone. This suggests synergistic interaction between polyphenols, lignans, and omega-3 fatty acids with enhanced antioxidant activity, reducing ROS burden associated with OSCC and improved induction of apoptosis due to complementary mechanisms. While literature on these seeds often centers on breast, colon, or leukemia models, the mechanisms they target such as oxidative stress, chronic inflammation, angiogenesis, and dysregulated apoptosis are highly relevant to OSCC biology. As no previous reports have evaluated a combined flax-sunflower extract in OSCC or other cancers, this represents a novel contribution. This study adds to the body of evidence suggesting that polyphenol-rich plant extracts may modulate OSCC progression. Omega-3 fatty acids and lignans offer complementary anticancer effects and combinations of phytochemicals can yield superior anticancer responses.

CONCLUSION

The present study demonstrated that both individual extracts and their combination exhibit significant anticancer activity, with the combination showing the most potent cytotoxic effects. The synergistic interaction between the bioactive compounds from sunflower seeds and flaxseeds contributed to enhanced cytotoxicity compared to the individual extracts. Future studies should focus on isolating specific bioactive compounds from sunflower and flax seed extracts to determine their individual contributions to cytotoxicity. Furthermore, clinical trials involving OSCC patients are necessary to assess the therapeutic potential and safety of these extracts in real-world settings. Exploring the synergistic effects of these extracts in combination with conventional treatments could also provide valuable insights into their role in nutraceutical Oncotherapy.



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