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Fennel Extract Inhibit Growth and Induce Apoptosis of Tongue Squamous Cell Carcinoma by Inhibiting Bcl-2

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Aim: The objective of this study is to investigate the effect of fennel extract on tongue squamous cell carcinoma cell line and to assess the expression of the Bcl-2 gene in tongue squamous cell carcinoma cells treated with fennel extract.

Materials and Methods: The HNO-97 cell line of tongue squamous cell carcinoma was acquired from the Cell Culture Department NAWAH-Scientific in Cairo, Egypt. Fennel seed extraction was performed at NAWAH-Scientific in Cairo, Egypt. Cells' cytotoxicity was measured using the MTT assay. The expression of the Bcl-2 gene was assessed by western blotting technique. Cell death mode was identified through microscopic examination as well as image morphometric analysis. The outcomes underwent statistical testing through analysis of variance (ANOVA) test and Post Hoc test (Bonferroni).

Results: The MTT test indicated that the HNO-97 cells displayed sensitivity to fennel extract, showing an IC_{50} of 2.80 mg/ml. Bcl-2 gene expression first decreased and then increased at post-IC_{50} concentration, as determined by western blotting. The mean values of NAF decreased initially and then increased at post-IC_{50} concentration, according to image morphometric analysis. The P-value was less than 0.05 so, the results were considered significant.

Conclusion: IC_{50} concentration of fennel extract has the best cytotoxic effect on the HNO-97 cell line of tongue squamous cell carcinoma.

Keywords: Squamous cell carcinoma, Fennel extract, Western blotting technique, Bcl-2 gene, Apoptosis.

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Introduction

Worldwide, cancer caused nearly 10 million deaths in 2020, establishing its position as one of the top causes of mortality on a global scale.[1] Squamous cell carcinomas (SCC) account for more than 90% of cases of oral malignancies.[2] There are other different tumors that can occur also in the oral cavity such as the tumors of salivary glands, lymphomas and melanoma.[3]

Alcohol, cigarettes, and betel quid using has been significantly linked to oral cancer, with the greatest incidences of oral cancer being associated with these substances. Globally, 72% of head and neck carcinoma cases were linked to tobacco or alcohol use, and 35% of these cases were attributed to the combined use of tobacco and alcohol.[4] Most commonly, cancer is indicated by an ulcer or sore that does not heal and may cause bleeding or discomfort.[5]

Treatment options of caner include radiation, chemotherapy, surgery, or a combination of these. Post-surgical monitoring and follow-up are usually the next steps in the process of treatment.[6] Currently, the main treatment in the case of oral squamous cell carcinoma (OSCC) is surgical resection, with radiation and chemotherapy serving as the primary adjuvant therapies. Although there are multimodality treatments available, the 5-year overall survival rate remains around 60%. Additionally, the mutilation caused by these treatments affects the quality of life.[7]

Throughout history, researchers have carried out numerous investigations to extract novel bioactive substances from plants to combat cancer. Medicinal plants have yielded diverse secondary metabolites, such as terpenoids, flavonoids, polyphenols and alkaloids which have been isolated for their anti-cancer properties. These substances act in a variety of mechanisms to prevent the growth and division of cancerous cells.[8]

The antioxidant properties of natural herbs are gaining increased attention because of the rising demand for them as medicinal ingredients. These herbs include cumin, flaxseed, ginger, cardamom, black seeds, and linseeds.[9]

Among these herbs, fennel (Foeniculum vulgare) is highly important and is used in the medicinal, dairy, healthcare, and cosmetic industries. This aromatic herb belongs to the Umbelliferae family, resembles dill, has yellow flowers, and has both oral and medicinal applications.[10]

Hepatocellular carcinoma cells were shown in a study to exhibit reduced viability, induced apoptosis, and effective inhibition of cell migration when exposed to a 75% ethanol extract obtained from the seed of F. vulgare.[11]

According to a different study, the ethanolic extract of *F.vulgare* seeds effectively decreased the proliferation of the cells of lung cancer both in vivo and in vitro. This was achieved mainly by the release of Cytochrome C, a reduction in the potential of mitochondrial membrane, and suppressing Bcl-2 protein expression. Furthermore, in lung cancer cells, fennel extract significantly reduced the formation of colonies and the migration of cells.[12]

Although some F.vulgare extracts have demonstrated promising anticancer potential thus far, it is unclear how these extracts affect oral cancer and what molecular mechanisms explain their actions.

The purpose of this study is to detect the effect of the ethanolic extract of *fennel* on HNO-97 squamous cell carcinoma cell line of tongue and to evaluate Bcl-2 gene expression in fennel extract-treated squamous cell carcinoma cells.

Materials and Methods

The present study is designed depending on previous studies on the effect

of fennel extract on different types of cell lines.

Material

Cell line

HNO-97 squamous cell carcinoma cell line (code number: 300129) was obtained from Cell Culture Department NAWAH-Scientific in Cairo, Egypt. The cell line was maintained in an incubator with humidified CO2 using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cell line was cultivated in 96-well plate or tissue culture flasks (SPL-Korea).

Fennel Seeds Extraction

The extract was prepared at NAWAH-Scientific in Cairo, Egypt. 650 g of fennel seeds were quickly cleaned of dust and contaminants with a single rinse in distilled sterile water. After letting them dry, they were ground into a powder. The final powder was soaked in 75% ethanol at 4 °C for 48 hours, and the solution that was left over after filtering through paper was the leach solution. After being transferred to a rotary evaporator, the leach solution was concentrated and evaporated at low pressure and low temperature until a dark green extract was produced. A 50 mg/ml ethanolic extract of fennel seeds was created by dissolving the extract once more in 75% ethanol and storing it at 4 °C.

Methods

Cytotoxicity assay

Methyl Thiazol Tetrazolium (MTT) assay is a method for quantitatively measuring the proliferation of cells using a colorimetric approach. It involves the use of a water-soluble yellow tetrazolium salt known as 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, which is converted into an insoluble purple formazan complex through the cleavage of its tetrazolium ring in the mitochondria by lactate dehydrogenase (LDH).

The cell membrane is impermeable to the formazan product and therefore it accumulates in the cells that are healthy. Using spectrophotometric techniques, the resultant intracellular purple formazan crystals can be solubilized and measured.

Since mitochondrial reductase enzymes are required for this reduction to occur, the quantity of viable (alive) cells can be directly correlated with conversion. The MTT cell proliferation assay quantifies the rate of cell proliferation; in contrast, a decrease in cell viability occurs when metabolic processes result in necrosis or apoptosis.

Changes in the rate of cell proliferation may be accurately quantified since there is a linear relationship between the quantity of cells and the signal generated for each kind of cell.

Twenty-four hours after treatment, the viability of HNO-97 SCC cells treated with various fennel extract concentrations was assessed. HNO-97 SCC cells were seeded in 96-well culture plates for the MTT test, and they were exposed to different concentrations of fennel extract for a whole day.

The treated well was filled with 50µL of a stock solution containing 0.5 mg/ml of MTT, and it was incubated for 4 hours at 37ºC. Following the careful removal of the treatment medium from each well, 50µL of dimethyl sulfoxide (DMSO) was given to each well in order to dissolve the purple formazan crystals. The cells were then rinsed with PBS.

The Dynatech MR5000 spectrophotometer (Dynatech Laboratories, Inc., Chantilly, VA) was used to measure the absorbance at 570 nm. The 570 nm absorbance readings were compared to a large number of remaining live cells. The means of separate experiments were used to

determine the results. The following formula was used to get the viability percentage:

$$\text{Viability percentage} = \frac{\text{X Absorbance of test dilution}}{\text{X Absorbance means of control}} \text{ x } 100$$

The effective calculated value was the IC_{50} of fennel with the value of 2.80 mg/ml.

Western Blotting technique

Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins can be divided into different sizes. Both are commonly used in conjunction with western blotting (immunoblotting) to ascertain whether a target protein is present and/or how abundant it is in a sample that contains a complex combination of proteins.

This method loads all of the protein in each sample and uses an electric current to electrophoretically separate the proteins by allowing them to move over the gel matrix.

The proteins must first be negatively charged and denatured by exposure to a detergent like SDS in order for them to migrate through the gel. To identify proteins of interest, a molecular weight marker that yields bands of known size is employed.

By passing an electric current through the gel to cause the proteins to migrate out of it and onto the membrane, the protein components can be transferred to a polyvinylidene fluoride (PVDF) membrane once they have been sufficiently separated.

In order to identify a particular protein on the membrane, first a primary antibody against the protein is added, forming a protein-antibody complex. Next, a secondary antibody is added, which attaches to the complex via its antibody side.

When an enzyme reacts with its substrate, the secondary antibody is usually bound to the enzyme and causes luminescence. Bio-Rad Imager (Biorad USA) measures the luminescence brightness, which is directly proportional to the amount of protein that reacted with the antibody.

Microscopic examination using Hematoxylin and Eosin staining

Following treatment with varying concentrations of fennel extract pre-, IC-, and post-IC-, HNO-97 SCC cells were fixed on slides. After rehydrating the fixed slides with alcohol at progressively higher concentrations (100%, 90%, 75%, and 50%), they were given a 5-minute soak in distilled water.

After three minutes of immersion in filtered hematoxylin stain, the slides were twice cleaned with distilled water. After five seconds of immersion in filtered eosin stain, the slides were cleaned with distilled water.

Each group's thirty tiny fields were photographed under an oil immersion microscope at a power of 1000X. A digital video camera (Canon, Japan) mounted on an Olympus BX60 light microscope was used for this. After that, the computer system received the images for analysis. The selection of fields was based on which cells were most likely to be apoptotic. The photomicrographs were analyzed to see whether the morphological markers of apoptosis were present.

Nuclear Morphometric Analysis

(Image J, 1.27z, NIH, USA) was used as image analysis software to examine the photomicrographed fields. The photos were first automatically adjusted for contrast and brightness. After that, the corrected photos were transformed into an 8-bit grayscale format, and the targeted area's phase color coding was automatically completed.

Subsequently, the HNO-97 cell nuclei were selected by adjusting the color threshold. Throughout this step, efforts were made to decrease the operator guided in favor of the automatic thresholding in an attempt to

standardize the procedure for all processed images.

Lastly, an automated measurement was made of the nuclei's surface area and circularity. The following formula was used to determine the nuclear area factor: nuclear area factor = circularity x object area. After that, the data was collected in a Microsoft Excel document. (Microsoft Office 2010®)

Statistical Analysis

Using the statistical program for Social Science (SPSS 26.0) Windows software, the mean values of the nuclear area factor (NAF) of the treated cells with different concentrations of fennel extract were evaluated statistically. Analysis of variance (ANOVA) was used and a Bonferroni Post Hoc Multiple Comparisons Test was also done.

Study Design

In the present study, we have four groups:

Group I: control group.

Group II: HNO-97 squamous cell carcinoma will be treated with the pre-IC_{50} concentration of fennel extract.

Group III: HNO-97 squamous cell carcinoma will be treated with the IC50 concentration of fennel extract.

Group IV: HNO-97 squamous cell carcinoma will be treated with the post-IC_{50} concentration of fennel extract.

Ethical Statement

The Research Ethics Committee of Minia University, Egypt submitted its approval for the current study on July 25, 2023. Committee approval number: 98.

Results

Cytotoxicity Assay (MTT Assay)

The MTT test was utilized to investigate the cytotoxic impact of fennel on the viability of HNO-97 SCC cells. According to the data, when the concentration of fennel was reduced from 25 mg/ml to 0.4 mg/ml, the mean viability percentage of the fennel-treated cells increased in comparison to the control cells as shown in Table 1.

The drug's concentration, which lowers absorbance to half that of the control, was used to get the IC_{50} value. The IC_{50} value of fennel was 2.80 mg/ml.

Table 1: The viability percentage of HNO-97 SCC cells after treatment with different concentrations of fennel extract

Conc. mg/ml	25	12.5	6.25	3.12	1.56	0.8	0.4
Viability %	10.74	20.14	26.15	40.14	71.42	78.38	99.20

Western Blotting technique

The expression of Bcl-2 gene in HNO-97 SCC cells that were treated with different concentrations of fennel extract was studied using a western blotting test.

Data obtained revealed that the expression of Bcl-2 protein in HNO-97 SCC cells treated with pre IC_{50} concentration of fennel decreased in comparison to control cells and there was a much greater decrease in cells treated with IC_{50} concentration of fennel. Surprisingly, there was an increase in protein expression with the use of post IC_{50} concentration with the lowest protein expression recorded at IC_{50} concentration as shown in Table 2.

Table 2: The Bcl-2 gene expression in HNO-97 SCC cells following treatment with varying fennel extract doses

S	Compound		Western blotting	
	Code	Conc.	OD Bcl-2	β-actin
1	GII-pre IC50/HNO97	1.40	0.577	√
2	GII-IC50/ HNO97	2.80	0.324	√
3	GII-post IC50/ HNO97	5.16	0.469	√
4	control HNO97	0	0.861	√

Microscopic examination using Hematoxylin and Eosin staining

The control group of HNO-97 SCC cells as well as the cells treated with various fennel extract concentrations were observed under an electron microscope.

I-Control

The majority of HNO-97 SCC cells had hyperchromatic, nearly spherical nuclei, and their cellular outlines were nearly regular and devoid of folding. Of the control cells, only a small percentage displayed signs of apoptosis, which were primarily limited to chromatin condensation and nuclear shrinkage. (Fig.1A)

II-Pre IC_{50}

Many cells exhibited morphological changes that corresponded with apoptotic morphological criteria 24 hours after being treated with pre-IC_{50} concentration of fennel. These included peripheral chromatin condensation and shrinking of the nucleus and cells. (Fig.1B)

III- IC_{50}

Numerous cells exhibited apoptotic alterations, including cellular and nuclear shrinkage, membrane blebbing, peripheral chromatin condensation, irrigular cell membrane, apoptotic bodies, and necrotic debris, 24 hours after treatment with IC_{50} concentration of fennel. (Fig.1C)

IV- Post IC_{50}

Following 24 hours of treatment with fennel at a post-IC_{50} concentration, certain cells experienced swelling, necrosis, and burst membranes. Other cells displayed aberrant cell membranes after apoptosis. Apoptotic bodies, membrane blebbing, and nuclear and cellular atrophy are also visible. (Fig.1D)

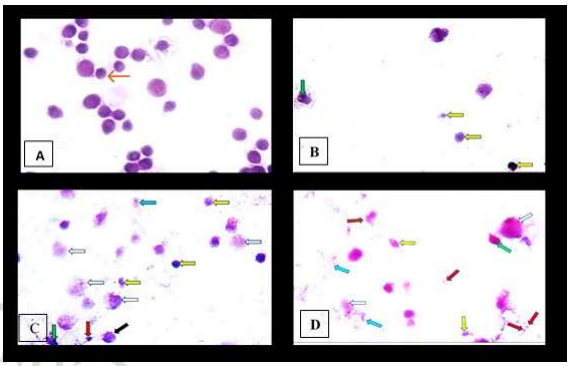

Figure 1: A: A photomicrograph of cancer cells showing cellular and nuclear pleomorphism with hyperchromatic nuclei (orange arrows) (H and E, Original magnification 100X, oil), **B:** A photomicrograph displaying nuclear and cellular shrinkage in apoptotic cells (yellow arrow). Peripheral chromatin condensation (green arrow) is visible in some cells (H and E, original magnification 100X, oil), **C:** A photomicrograph illustrating apoptotic cells with apoptotic body (red arrow), uneven cell membrane (black arrow), peripheral chromatin condensation (green arrow), and cellular and nuclear shrinkage (yellow arrow). Note the presence of necrotic debris (blue arrow) and a torn cell membrane (white arrow) in the necrotic cell (H and E, original magnification 100X, oil), **D:** A photomicrograph of swollen necrotic cells with ruptured cell membrane (white arrow), necrotic debris (blue arrow), apoptotic cells with cellular and nuclear shrinkage (yellow arrow), membrane blebbing (brown arrow), peripheral chromatin condensation (green arrow), and apoptotic bodies (red arrow) (H and E, Original magnification 100X, oil).

Nuclear Morphometric Analysis

When HNO-97 SCC cells treated with Pre IC_{50} concentration and IC50 concentration were compared to control cells, the recorded results showed a decrease in the mean values of NAF. It was only at post-IC_{50} concentration that the mean of NAF rose as illustrated in Table 3.

Table 3: The calculated nuclear area factor means of HNO-97 SCC cells following fennel extract treatment at several doses

Group	Control	Pre IC_{50}	IC_{50}	Post IC_{50}
NAF	15574.18	4389.783	2945.536	13269.75

Statistical Analysis

There was a significant difference in the NAF mean between the control group, pre-IC_{50}, IC_{50}, and post-IC_{50} groups, according to the ANOVA test as clarified in Table 4. Additionally, the Post Hoc (Bonferroni) test compared the NAF mean between these groups as shown in Table 5.

Table 4: Descriptive statistics of ANOVA test for the means of nuclear area factor of the SCC cells treated with different concentrations of fennel extract

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5267003403.659	3	1755667801.220	15.977	.001
Within Groups	25054145495.739	228	109886603.051		
Total	30321148899.398	231			

Table 5: Comparison of nuclear area factor means between the groups according to the Post Hoc (Bonferroni) test.

(I)group	(J) group	Mean Difference (IJ)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
control	pre IC50	20544.0310	6483.0526	.001	2641.57624	13513.54183*
	IC50	13870.3188	5251.8752	.001	1619.11035	9561.09700*
	post IC50	10681.5998	453.9943	.025	1921.41677	5567.79703*
pre IC_{50}	Control	-6483.0526-	-20544.0310-	.001	2641.57624	-13513.54183-*
	IC50	3194.2992	-11099.1888-	.855	2685.25682	-3952.44482-
	post IC50	-286.9240-	-15604.5656-	.037	2877.66020	-7945.74479-*
IC_{50}	Control	-5251.8752-	-13870.3188-	.001	1619.11035	-9561.09700-*
	pre IC50	11099.1888	-3194.2992-	.855	2685.25682	3952.44482
	post IC50	1279.1899	-9265.7899-	.270	1981.04052	-3993.29997-
post IC_{50}	Control	-453.9943-	-10681.5998-	.025	1921.41677	-5567.79703-*
	pre IC50	15604.5656	286.9240	.037	2877.66020	7945.74479*
	IC50	9265.7899	-1279.1899-	.270	1981.04052	3993.29997

Discussion

The most prevalent type of cancer affecting the head and neck region is SCC, which causes about 25% of cases to occur in the oral cavity, most commonly affecting the tongue.[13] Tongue cancer has been thought to be associated with elderly men who have smoked and consumed alcohol for a long time. However, now it is increasing among patients under the age of 45 and in young women. Tongue cancer prognosis in both young and old people is debatable.[14]

Since tongue SCC is the most common form of oral cancer and has a high mortality and morbidity rate, it was used in the current study. Nowadays, the treatment of choice involves surgical removal, along with either chemotherapy or radiotherapy before or after the surgery. Widening the resection margins during surgery can cause aesthetic and functional issues for the patient, so the patient will require reconstructive surgery.[15]

Phytochemicals derived from plants are essential for the development of drugs and the treatment of cancer by regulating the molecular pathways linked to cancer growth and progression.[16]

Fennel (*F.vulgare*) holds great importance and is employed in the healthcare, cosmetic, food, and pharmaceutical industries. It is recognized as one of the most potent medicinal herbs worldwide and is valuable for addressing gastrointestinal issues due to its antibacterial, anti-inflammatory, antispasmodic, analgesic, and diuretic properties. Moreover, its antioxidant and anti-ulcer effects make it beneficial for treating neurological disorders.[17]

Research on animals and a few clinical studies indicate that long-term usage of *F.vulgare* is safe. Fennel is a versatile vegetable that may be eaten in various ways, including raw in salads and snacks, stewed, boiled, grilled, or baked, and even used to make herbal beverages.[18]

In the present study, ethanolic fennel extract was used to evaluate its effect on HNO-97 tongue SCC. The present findings are corroborated by studies on other cancer cell lines because, as far as known, no study on the impact of ethanolic fennel extract on tongue HNO-97 SCC has been published in English literature.

In this study, 24h post-treatment with fennel extract, HNO-97 SCC cells shown a reduction in cell viability that was dose-dependent and the IC_{50} value was 2.80 mg/ml. The cytotoxicity of ethanolic fennel extract as an anticancer agent against breast cancer cells, hepatocellular carcinoma cells, colon cancer cells, cervical cancer cell lines and lung carcinoma cells was demonstrated by Suleiman and Helal's findings, which were in line with the outcomes. The cytotoxicity increased with increasing concentration of ethanolic fennel extract.[19]

The current results match those of Ke, Zhao, et al., which showed that ethanolic fennel extract dramatically decreased lung cancer viability in a dose- and time-dependent manner.(12) Furthermore, the results of this study results align with Rahamouz-Haghighi, Asadi, et al. who demonstrated the impact of *F.vulgare* ethanol seed extract on the in vitro proliferation of colorectal cancer cells. The cells exposed to varying concentrations of the ethanol extracts showed a notable reduction in cell viability.[20]

In the current work, the effect of fennel extract on inducing apoptosis was assessed by histologically comparing cancer cells treated with and without the extract. After being exposed to fennel concentrations at pre-, IC_{50}, and post-IC_{50} for 24 hours, HNO-97 SCC cells exhibited necrotic and apoptotic alterations. Compared to pre-IC_{50} and IC_{50} concentrations, necrotic alterations were more noticeable in the post-IC_{50} concentration. Apoptotic alterations, on the other hand, were more noticeable in IC_{50} concentration and included nuclear and cellular shrinkage, peripheral chromatin condensation, membrane blebbing, uneven cell membrane, and apoptotic bodies.

As a result of treating lung cells with ethanolic fennel extract, adherent cells shrank and separated from the bottom of the culture plate, both of which are signs of apoptosis, according to the current results in agreement with Ke, Zhao et al. When Hoechst 33258 fluorescent dye staining was used under a fluorescence microscope to visualize DNA condensation or fragmentation in the cell nucleus, these results were verified.[12]

Since nuclear area factor (NAF) is dependent on two morphological criteria related to apoptosis, it can aid in the sensitive and early prediction of morphological changes in cells during this process. The Image J program was used to calculate the NAF by multiplying surface area by circularity.

After exposure to different concentrations of fennel extract for a whole day, the mean values of NAF in control and HNO-97 SCC cells were noted. The ANOVA test revealed that the values showed a significant decrease in NAF mean, compared to control,

when we used pre-IC_{50} and IC_{50} concentration of fennel and started to increase again when post IC_{50} concentration was used with the lowest value at IC_{50} concentration.

These results indicate that the apoptotic changes started to occur with the use of pre IC_{50} concentration and became more obvious when we used IC_{50} concentration. The necrotic changes predominated when the cells were treated with post IC_{50} concentration accounting for the increase in NAF value.

Three subgroups comprise the Bcl-2 family: anti-apoptotic proteins such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bcl-B, and A1/Bfl-1; pro-apoptotic BH3-only proteins such as Bim, Bid, Bad, Noxa, Puma, and Bmf; and multidomain pro-apoptotic proteins such as Bax and Bak.[21]

Bcl-2 protein level is much higher in tumor cells compared to normal cells. Therefore, a new treatment strategy involves inhibiting the anti-apoptotic Bcl-2 protein.[22]

Western blotting test was used in the current work to examine Bcl-2 gene expression in HNO-97 SCC cells treated with various fennel extract doses.

It was found that the expression of Bcl-2 protein in HNO-97 SCC cells treated with pre IC_{50} and IC_{50} concentration of fennel decreased in comparison to control cells. Unexpectedly, there was an increase in protein expression with the use of post IC_{50} concentration.

Along with the present findings, Ke, Zhao, et al. used Western blotting to examine the processes by which EEFS triggered apoptosis. Examination took place on the protein levels of p53, Bak, Bax, Bcl-2, Bcl-xL, Mcl-1, and Survivin. The data obtained showed that in both cell lines, there was an increase in the expression of the tumor suppressor p53 and pro-apoptotic proteins Bak and Bax, but a down-regulation of the anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1, and Survivin. Using qRT-PCR to measure Bcl-2 mRNA levels, these results were validated. EEFS substantially and dose-dependently decreased Bcl-2 mRNA.[12]

So, the current findings of the study proved that the IC_{50} concentration of ethanolic fennel extract induced apoptosis of the tongue SCC HNO-97 cell line through the downregulation of the Bcl-2 gene. Taking into account that working on cell lines does not reveal the effect of the extract on other types of cells nor the tissue interactions which in turn limits the study.

Conclusion

Based on the current study's findings, it can be concluded that ethanolic fennel extract at an IC_{50} concentration causes cytotoxicity in HNO-97 SSC cells, with its effects being mediated by Bcl-2 inhibition. So, it is recommended to use fennel extract in oral cancer treatment.

Declarations

Potential Conflicts of Interest

Conflicts of interest are not disclosed by the writers.

Details of Funding

Assuit University provided funding for this work.

Statement of Ethics

On July 25, 2023, the study was carried out with permission from Minia University's Research Ethics Committee in Egypt. 98 is the committee approval number.

Conflicting Interests

No competing interests are disclosed by the writers.

Accessibility of Information and Resources

This article contains all of the data that were created or examined during the investigation.

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