Journal of Chemical Health Risks

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JCHR (2024) 14(6), 2015-2030 | ISSN:2251-6727

In Vitro Assessment of the Apoptotic and Anti-oxidant Effects of Pomegranate Extract on Tongue Squamous Cell Carcinoma Cell Line (In Vitro Study)

Mohamed Ahmed Ibrahim, Sherif Farouk El-Gayar, Maii Ibrahim Ali.

(Received: 16 September 2024 Revised: 11 October 2024 Accepted: 11 December 2024)

KEYWORDS

Oral Squamous Cell Carcinoma, Pomegranate Extract, Cetuximab, NRF2.

ABSTRACT:

Background: Oral squamous cell carcinoma is a very dangerous type of cancer, especially those appearing on the tongue. Recently, natural compounds are considered an important method of anticancer drug therapy. Pomegranate extract (PE) has a wide range of pharmacological and therapeutic aspects including reactive oxygen species (ROS) scavenging, anti-inflammatory and anti-hypertensive effects. Moreover, the anticancer effects of PE have been studied in many cancers such as human bladder and breast cancer and showed cytotoxic and anti-proliferative effects against these cancer cell lines.

Aim: To identify the possible anti-cancer and antioxidant effects of PE on Tongue Squamous Cell Carcinoma cell line and compare it with the chemotherapeutic drug Cetuximab and their combination together.

Methods: Tongue squamous cell carcinoma cell line was divided into four groups; group I: untreated control group, group II: treated with pomegranate extract, group III: treated with Cetuximab and group IV: treated with a combination of Pomegranate extract and Cetuximab. Determination of the anticancer and antioxidant effects of these agents on the tongue squamous cell carcinoma cell line was done using Methyl Thiazole Tetrazolium assay, Nuclear Morphometric Analysis, Microscopic Examination, Polymerase Chain Reaction and Immunofluorescence.

Results: PE succeeded to decrease the percentage of viable and proliferating cells. Also, it increased the levels of caspase-3 and Nrf2 expression. Moreover, combination of PE with Cetuximab produced better effects at markedly lower doses than when used individually.

Conclusion: Pomegranate Extract has a strong anticancer effect against squamous cell carcinoma compared to that of Cetuximab. Moreover, combining PE with Cetuximab has synergistic effect.

Introduction: Oral squamous cell carcinoma (OSCC) is the most frequent malignancy in the oral cavity. Unfortunately, the prognosis and survival of OSCC patients is still poor, about 50% of patients die within 5 years from recurrent or metastatic tumor. According to data of the Global Cancer Observatory (GCO), there were 377,713 cases of OSCC in the world in 2020, most of them in Asia. However, the protocols of OSCC treatment depend on surgical intervention, chemotherapy and radiotherapy, which have numerous side effects. Most of which are attributed to the non-specific nature of cytotoxic drugs, where normal and neoplastic cells are equally affected (Mody et al., 2021; Odell et al., 2021 and Romano et al., 2021).

Moreover, a lot of studies shows that natural compounds are helpful in managing cancer with minimal side effects compared to chemotherapy. Among natural compounds, polyphenols are the largest group of phytochemicals present in plants, especially seeds and leaves. Luckily, several studies have shown that consumption of foods rich in polyphenols, is linked to decreased risk of cancer, cardiac problems and neural disorders (Zhang et al., 2021 and Guo et al., 2022).

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JCHR (2024) 14(6), 2015-2030 | ISSN:2251-6727

Pomegranate is one of the paradise fruits mentioned in the Holy Quran. It is mentioned three times, two times in chapter of Cattle and another time in chapter of Merciful. That's why it has always been a focus of medical research since ancient people, that Ibn Sina, the great physician on pomegranates says pomegranates are fruits that have all the medicinal features. They can block bleeding, cure wounds and its skin is useful for treating liver inflammation (Farhangi et al., 2014 and reza Afroogh, 2019).

Interestingly, all pomegranate parts, the leaves, barks and roots, have a high concentration of elements that have are used in medical applications. On the molecular levels pomegranate and it's components can effectively affect a wide variety of signaling pathways associated with tumorigenesis, angiogenesis and metastasis. It regulates inflammatory cytokines, cell adhesion molecules, apoptotic proteins and growth factors (Sharma et al., 2017 and Cicero et al., 2019).

One of the most frequently used chemotherapeutic agents is Cetuximab (Cetux). It is routinely used in the treatment of several cancers including breast, lung, gastric, ovarian, thyroid, multiple myeloma, sarcoma and pediatric cancers. It has been proposed to exert its action through intercalation into Deoxyribonucleic acid (DNA) with production of free radicals destroying the DNA and cellular proteins. Unluckily, like almost all chemotherapeutic drugs, it has some toxic (Chen et al., 2018 and Yonesaka et al., 2019).

Cetuximab, an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, is an efficient anti-tumor therapeutic agent that inhibits the activation of EGFR. Over 5 days cetuximab treatment in a head and neck squamous cell carcinoma cell line decreased cell motility and enhanced G1 phase cell arrest in the central region of the colonies. It significantly decreases phosphorylation of retinoblastoma (Rb), S-phase kinase associated protein 2 (Skp2), and the phosphoinositide 3 kinase-mammalian target of rapamycin (Akt-mTOR) proteins and accumulation of Cyclin-dependent kinase inhibitor 1B (p27Kip1). These results elucidate the cetuximab-dependent inhibition of cell migration, resulting in high cell density-related stress and persistent cell-cycle arrest at G1 phase culminating in autophagy (Ranee Mehra et al., 2011; Taylor et al., 2015; Trivedi S et al., 2016 and Agarwal et al., 2019).

Thus, the current study aimed to assess the effect of PE as compared to Cetuximab and their combination on the tongue squamous cell carcinoma cell line.

Materials and Methods:

Cell line and culture

Cell line of human tongue squamous cell carcinoma (HSC-3), was purchased from American Type Culture Collection (ATCC)* through Innovation Lab, VACSERA, Cairo, Egypt. PE powder was purchased from Bulk Supplements Company (Address: 7511 East Gate Road, Henderson, NU89011 U.S.A), through www.Amazon.com. Cetuximab (Erbitux) was purchased from Sigma Aldrich. It was stored in liquid nitrogen container at (-196 ℃).

Study design and treatment protocol

Cell line of human tongue squamous cell carcinoma HSC-3 was sub-cultured, then divided into 4 groups, Group 1 was the control group, Group 2 was subjected to Cetuximab, Group 3 was subjected to Pomegranate Extract and Group 4 was subjected to Pomegranate Extract and Cetuximab combination. In each group, the percentage of viability of OSCC cells was assessed using MTT Assay. Furthermore, anti-oxidant effect through NRF2 gene expression measurement was also done by means of PCR. And finally, apoptosis indicator caspase 3 was measured using direct immunofluorescence.

Measurement of Cell Viability by MTT essay:

Before the MTT assay, one ml of cells (50,000 to 100,000 cells/ml) were plated into each well of 96-well culture plate for 24 hours. Then, the cells were incubated for 24 h in CO2 incubator. Pomegranate extract and cetuximab were separately added for cell line with serial dilutions (1mg/ml,100μg/ml, 10μg/ml, 1μg/ml and 0.1μg/ml), respectively and then washed by PBS twice. Cells were continuously examined under the inverted phase microscope, and then incubated with medium containing 0.5 mg/ml MTT in CO2 incubator at 37 ℃ for 4 h. Absorbance at 570 nm was measured for each well using a microplate reader (BioTek, Flx 800). The results were interpreted and the half maximal inhibitory concentration (IC 50) was estimated.

Microscopic examination:

The steps of cell maintenance and subculture protocol were repeated, but the cells were dispensed in a

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25 ml total volume to get a larger quantity of cells for cytological examination. Microscopic examination was done using a digital video camera (C5060, Olympus, Japan) attached to a light microscope (BX60, Olympus). The photomicrographs were assessed for the presence of morphological criteria of apoptosis.

Nuclear morphometric analysis:

The photomicrographs were analyzed using Image J software ver 1.27z (NIH, USA). Following automatic correction of the brightness and contrast of images, they were converted into 8-bit grayscale type, and phase color coding of the area of interest was done automatically. The color threshold was adjusted to select the HSC-3 cell nuclei. For method standardization for all analyzed images, efforts were made to minimize the operator guided in favor of the automatic threshold throughout this step. The nuclei surface area and circularity were automatically measured, and the nuclear area factor (NAF) was calculated using the formula: NAF = Circularity × Object area.

Gene expression of Nuclear Erythroid Factor 2 (Nrf2) in cultured cells using Quantitative Real-time PCR (qPCR):

Cells disrupted and homogenized by bead-milling in a guanidine–thiocyanate–containing lysis buffer. Disruption and homogenization for tissue was performed using the Tissue Ruptor II (Qiagen, Hilden, Germany). Then, the mixture is centrifugated for 20 minutes at 4000rpm. Finally, the cell supernatant is collected for RNA extraction. Ethanol was added to Tissue homogenate; the sample is loaded onto RNeasy Mini spin column.

The RNA extraction & purification was performed using RNeasy Mini kit, cat no: 74104, Qiagen, Hilden, Germany. The reverse transcription step was performed by the Quantitect Reverse Transcription Kit, cat. No: 205310, (Qiagen, Hilden, Germany). The reaction mix was incubated for 15 min at 42°C, then it was incubated for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. The reverse-transcription reactions were placed on ice, and then real-time PCR was performed directly. The Nrf2 gene expression level was amplified from mRNA using QuantiTect primer assay [Hs_NFE2L2_1_SG QuantiTect Primer Assay, cat no: 249900, ID: QT00027384 (Qiagen, Germany) and QuantiTect SYBR Green PCR Kit cat no: 204141 (Qiagen, Germany) and Hs_ACTB_1_SG QuantiTect Primer Assay (β-actin) cat no: 249900, as housekeeper gene.

All samples were analyzed using the 5 plex Rotor Gene PCR Analyzer (Qiagen, Germany).

Measurement of Caspase 3 protein expression by Immunofluorescence:

Cells were fixed with warm 4% formaldehyde. Then, the cells were immune stained with mouse anti-Caspase 3 Monoclonal Antibody (CPP32 4-1-18), cat no: MA1-16843 primary antibody (Invitrogen; ThermoFisher Scientific, Hilden; Germany), incubated overnight at 4 °C. The cells were washed with PBS and incubated with Goat Anti-mouse IgG H&L secondary antibody-Alexa Flour 488 (Invitrogen; ThermoFisher Scientific, Hilden; Germany).

The specimens were immediately examined or stored at 4 °C protected from light for long term storage. The microscopic examination was performed by LABOMED Trinocular fluorescent phase contrast microscope model TCM400 microscope, and the Atlas 16MP Cmos USB Camera with PixelPro 3.0 software (LABOMED, USA) The IF staining intensity was scored using a Fortier system: 0, no staining; 1+, weak; 2+, moderate; and 3+, strong. In brief, the H-score of each sample was calculated as the sum of each intensity (0-3) multiplied by the percentage of positive cells (0-100%). The score ranged from 0-300. The median value of the H-score was calculated.

Statistical methods:

All data were collected and subjected to statistical analysis. Statistical analysis was performed by Statistical Package for Social Sciences (SPSS). Microsoft office excel was used for handling and graphical presentation of data. Quantitative variables were described by Mean, Standard Deviation (SD), Standard Error (SE), the Range (maximum-minimum) and 95% confidence interval of the mean. One way analysis of variance (ANOVA) first used, followed by Dunnett t test for multiple comparisons between treated groups vs the control groups. The ρ values were considered as follows:

ρ value ≥ 0.05, non-significant.

ρ value ≤ 0.05, significant.

ρ value ≤ 0.001, highly significant.

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JCHR (2024) 14(6), 2015-2030 | ISSN:2251-6727

Results

Cell viability and cytotoxicity determination:

The cell viability percent showed gradual decrease with increasing doses of the proposed treatments (Pomegranate extract, Cetuximab and Combination). The values at different concentrations were shown in table (3) and plotted in figs (17, 18 and19) respectively. Regarding IC50, both Pomegranate extract and Cetuximab showed gradual decrease with increasing doses, as shown in table (3) and fig (20).

Table (3): **IC_{50}** concentrations of Cetuximab, Pomegranate and combination **(combination group has the lowest IC_{50} value)**

Study group	Cetuximab	Pomegranate	Combination
IC_{50}	3.98	154.30	0.97

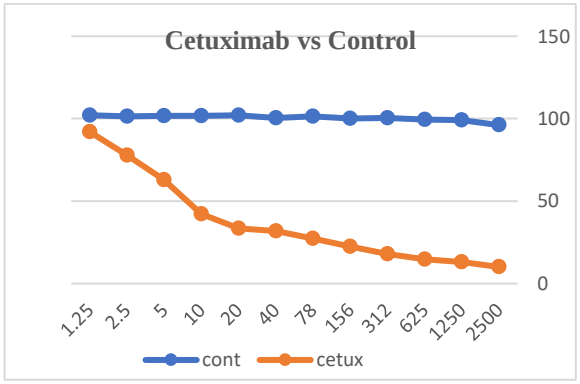

Fig (17): Graph showing viability % of cancer cells in the treated study groups with different concentrations of Cetuximab VS control group.

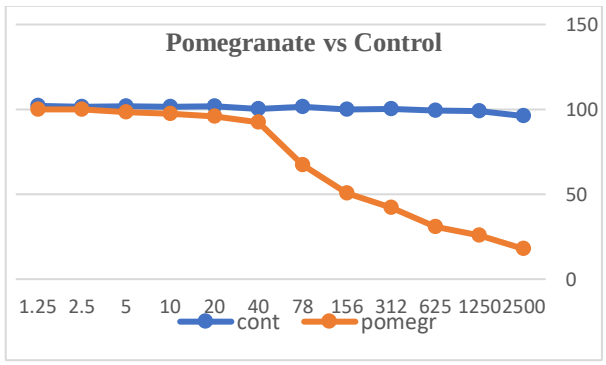

Fig (18): Graph showing viability % of cancer cells in the treated study groups with different concentrations of Pomegranate extract VS control group.

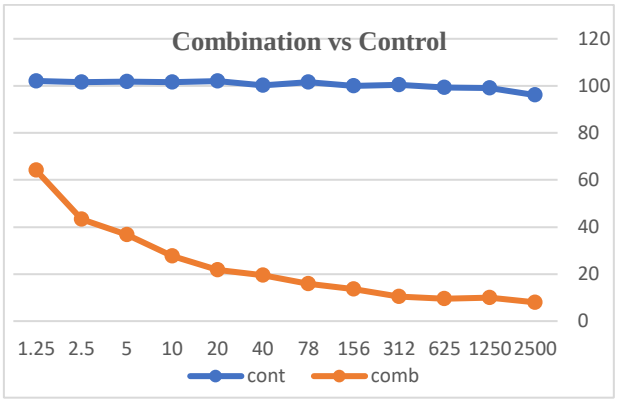

Fig (19): Graph showing viability % of cancer cells in the treated study groups with different concentrations of combination of cetuximab and Pomegranate extract VS control group.

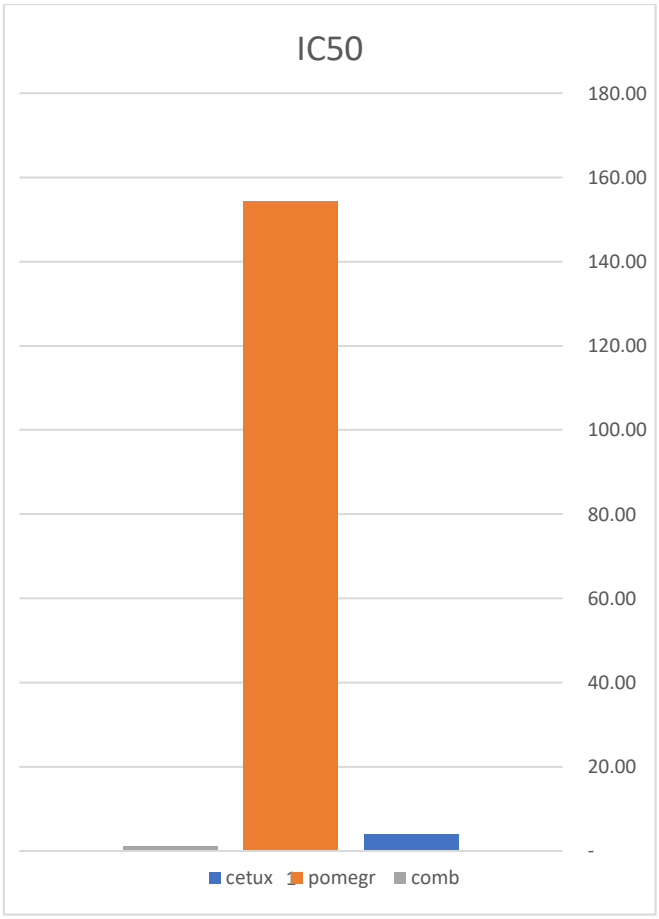

Fig (20): A bar chart showing the different IC50 values for Cetuximab and Pomegranate Extract and Combination of both.

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Morphological Assessment

A. Control group:

This group shows cancer cells with signs of dysplasia such as cellular and nuclear pleomorphism, nuclear hyperchromatism and increased nuclear cytoplasmic ratio.

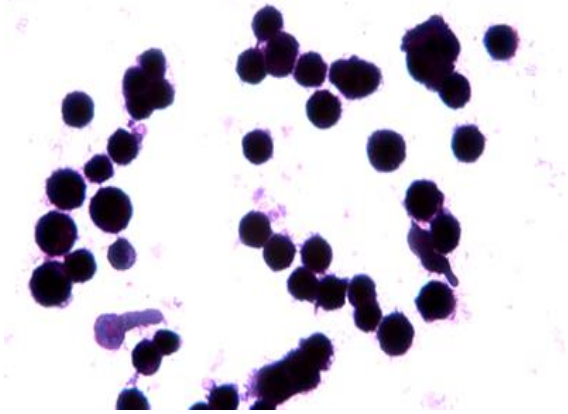

Fig (21): A photomicrograph showing cancer cells with hyperchromatic nuclei, increased nuclear/cytoplasmic ratio, cellular and nuclear pleomorphism, and abnormal mitosis.
(H and E, Original magnification 100X, oil).

B. Cetuximab group: Some cells show apoptosis (cellular and nuclear shrinkage) apoptotic bodies. Other cells show necrosis in the form of swollen cells.

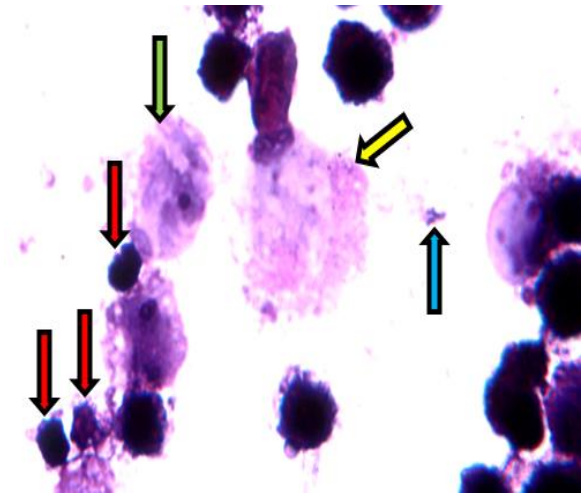

Fig (22): A photomicrograph showing apoptotic cells (red arrows), apoptotic bodies (blue arrows), swollen necrotic cells (green arrows), and necrotic debris (yellow arrow). (H and E, original magnification 100X, oil).

C. Pomegranate extract group: Some cells of this group show apoptosis (cellular and nuclear shrinkage) apoptotic bodies. Other cells show necrosis in the form of swollen cells.

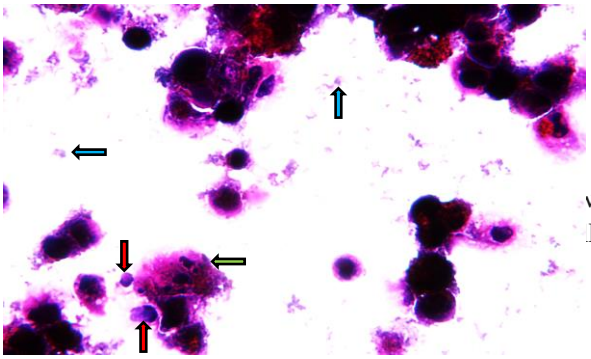

Fig (24): A photomicrograph showing apoptotic cells with peripheral chromatin condensation (red arrows), apoptotic bodies (blue arrows), and swollen necrotic cell (green arrow). (H and E, original magnification 100X, oil).

D. Combination group:

This group shows necrotic swollen cells as well as shrunken apoptotic cells and apoptotic bodies.

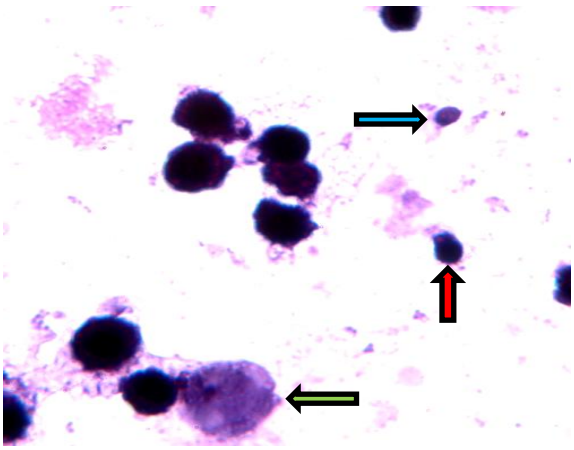

Fig (26): A photomicrograph showing necrotic cells with ruptured cell membrane (green arrows), and shrunken apoptotic cells (red arrows). (H and E, original magnification 100X, oil).

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Measurement of Caspase 3 protein expression by Immunofluorescence:

Evaluation of the apoptosis was done through measuring caspase 3 expression in the study groups, and the results came as shown in the following table.

Serial	Group	Cells stained with CASP3
		H-score
1	control	8
2	control	5
3	control	1
4	PE	12
5	PE	16
6	PE	10
7	Cetux	120
8	Cetux	104
9	Cetux	135
10	Combination	246
11	Combination	228
12	Combination	216

Table (4): Calculation of H-score for CASP3 protein expression in Cancer cells

Control group:

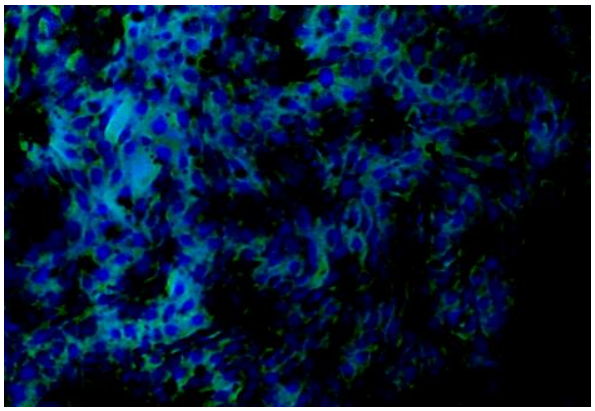

Fig (28): Immunofluorescent picture of caspase 3 expression in control group (magnification 100X)

Cetuximab group:

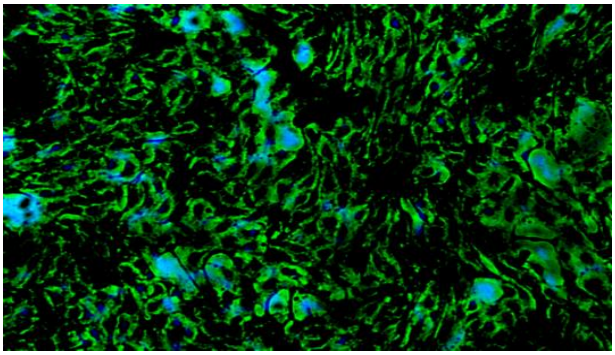

Fig (30): Immunofluorescent picture of caspase 3 expression in Cetux treated group (magnification 100X)

Pomegranate group:

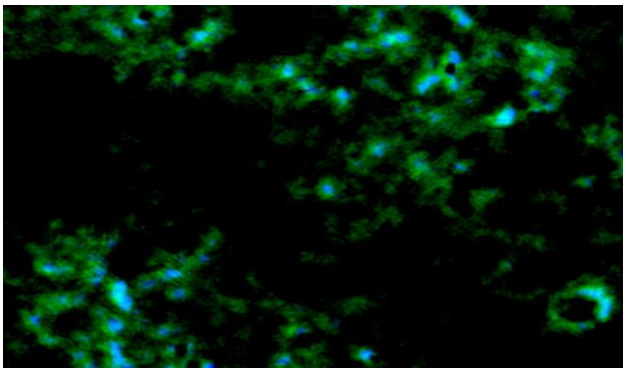

Fig (29) Immunofluorescent picture of caspase 3 expression in PE treated group (magnification 100X)

Combined therapy group:

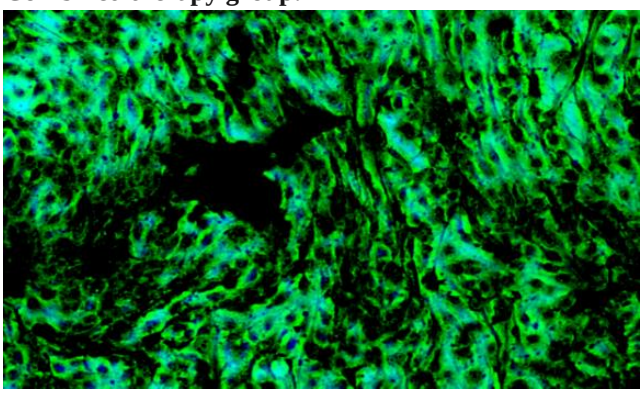

Fig (31): Immunofluorescent picture of caspase 3 expression in Cetux & PE combination treated group (magnification 100X)

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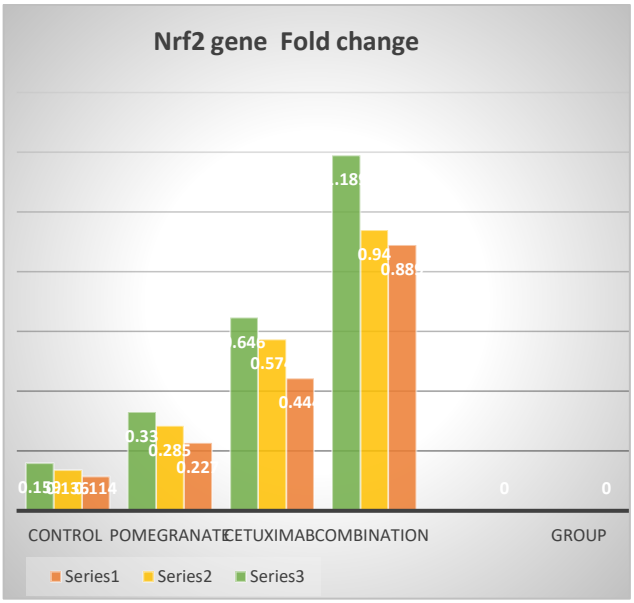
Gene expression on Nuclear Erythroid Factor 2(Nrf2) in cultured cells using Quantitative Real-time PCR (qPCR):

Table (6): showing Nrf2 gene fold changes of different groups of control and treated cells

Group	NFE2L2 fold change (FC)		
Combination	1	2	3
	0.889	0.940	1.189
Cetuximab	1	2	3
	0.444	0.574	0.646
Pomegranate	1	2	3
	0.227	0.285	0.330
Control	1	2	3
	0.114	0.136	0.159



Bar chart showing Nrf2 gene fold changes of different groups of control and treated cell

Discussion

HNSCC is a devastating disease and one of the major public health problems worldwide, accounting for 650,000 new cases and 350,000 deaths every year. It exhibits aggressive behavior with high incidence of secondary primaries (5–7% per year) together with a high incidence of distant metastases. Furthermore, advances in surgical and medical therapies have only resulted in a modest improvement in the mortality rate, which remains at approximately 50% (Gormley et al., 2022).

Certainly, tongue SCC cell line was used because it was reported as a more aggressive subset of OSCC that carried a higher risk for early invasion and metastases owing to its proximity to the underlying neurovascular bundles. Moreover, it has a unique entity, as its clinical presentations might mimic other oral lesions like those of inflammatory or reactive origin, which has often led to misdiagnosis or even delay in diagnosis and treatment, so making the prognosis worse (Migueláñez-Medrán et al., 2019).

Among the fruits of paradise, Mighty Allah has mentioned pomegranate, which is one of the richest sources of polyphenols. Luckily, it is available in the Egyptian market with a relatively low cost. All the different parts of this fruit have health benefits. PE was chosen as the subject of the study, because it is such a promising bioactive phytochemical that have shown very strong anti-inflammatory and anti-oxidant properties and exhibited no apparent toxicity in vivo animal models. The anti-tumor activity of PE has been shown in many cancer models such as colorecta, cervical and lung cancers (Shaikh & Bhandary, 2021 and Wong et al., 2021).

On the other hand, Cetux was used as a reference drug for comparison with PE. It was chosen as being regarded one of the most powerful anti-neoplastic agents that acts against a wide number of tumors including OSCC. Moreover, The National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology also recommended the inclusion of Cetux in the systemic therapy regimen for advanced cases of HNSCC (Angellotti et al., 2023).

Looking at MTT assay data, our results show decreased viability percent of cells with increasing doses of PE as shown in table (3), which indicates potential cytotoxic effect of PE. Similarly, for groups treated with Cetux and Cetux-PE combination. These results came in accordance with the finding of (Asmaa et al., 2015) who stated that PE caused an inhibition in cell proliferation of chronic myeloid leukemia cells mainly by cell cycle arrest. Also, (Fatwa Imanu et al., 2023) stated that PE

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likely has four mechanisms against oral cancer: inhibiting the invasion, migration and growth of cells, increasing apoptosis through regulating antioxidant genes.

IC_{50} values were (**154.30, 3.98, 0.97**) for PE, Cetux and combination groups respectively. IC_{50} of both PE and Cetux showed gradual decrease with increasing doses from 1.25 µg to 2500 µg which indicates that the cytotoxic effect of PE against OSCC cell line could be efficiently exerted with the same efficacy either by a high dose of PE for a shorter time, or reduced dose for prolonged time. This is in line with (Peng et al., 2021)**,** who reported that longer exposure to PE for 72 h decreases more viability to oral cancer cells than that of the 24 h treatment.

In addition, combining both PE and Cetux together resulted in a marked decrease in the IC_{50} value which indicates the synergistic effect of PE when added to Cetux. Which is confirmed also in the study of (Chen et al., 2018), who demonstrated that co-treatment with cetuximab and curcumin exerts synergistic anticancer effects on OSCC cells through the suppression of the EGFR signaling by regulation of the MAPK pathway.

Then, morphological analysis of treated cancer cells revealed that PE exerted its anti-tumor action mainly by leading cancer cells into apoptosis and by causing some necrosis as well. Which was confirmed afterwards through nuclear area factor analysis (**NAF**) that combination treated groups had the lowest NAF values as compared to the control group cells. These results came in accordance with (H. Weisburg et al., 2010) studies who demonstrated that the antiproliferative mechanism of PE against cancer cells was by induction of oxidative stress and the mode of cell death was by apoptosis, as shown by flow cytometry and activation of casapase-3.

On the other side, Immunofluorescence results showed an increase in both cytoplasmic and membranous caspase 3 expression. Where cells treated with Cetuximab or Pomegranate individually showed mild to moderate increase of CASP3 protein expression, while cells co-cultured with combined therapy of showed marked increase in both the percentage of CASP3 positive cells and intensity. Similarly, (Peng et al., 2021) reported that PE was capable of providing antiproliferation and apoptosis effects on oral cancer cells through impaired mitochondrial functioning. And this was associated with downregulating antioxidant gene expression and triggering mitochondrial impairment, causing ATP depletion as well as decreases in mitochondrial mass. This finding agrees with the results of (Alsubhi et al., 2022), who stated that PE induces cell death and apoptosis in small cell lung carcinoma and human colorectal lymph node by boosting caspase 9 and 3 activity.

Lastly, we tried to look more in depth for the underlying molecular mechanism through which PE exerts its anti-tumor effect on cancer cells and PCR results came as follows; PE increased the expression of Nrf2 gene when used alone or in combination with cetuximab, which confirmed that potent PE chemo-preventive action is mainly through the strong antioxidant activity proven by upregulation of such a gene. (Bishayee et al., 2011) examined mechanism-based chemo-preventive potential of PE against hepatocellular carcinoma induced in rats, and their results provided substantial evidence that pomegranate constituents afford chemoprevention of hepatocarcinogenesis possibly through potent antioxidant activity achieved by upregulation of several housekeeping genes under the control of Nrf2 without toxicity.

In summary, the present study clearly demonstrated high success rate and promising PE efficacy against OSCC cells, which was somehow compared to that of the well-known chemotherapeutic drug Cetuximab. This was clear through observing the difference between PE and Cetux treated groups values in terms of cytotoxicity and comparing IC_{50} values for both which indicated comparable qualities. Moreover, apoptotic action through expression of caspase-3 and Nrf2 by PE especially when combined with cetuximab. Collectively, our work supposes that PE can be a useful additive or somehow an adjunctive therapeutic means against OSCC.

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