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"Effect of Eucalyptus Oil and 5-Fluorouracil on Head and Neck Squamous Cell Carcinoma Cell Line" In-Vitro Study

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Abstract:

Oral cancer is a very aggressive malignant tumor which is considered the sixth most common malignancy worldwide. Chemotherapy still the main treatment for malignancy to improve the survival rate and quality of life for patients. 5-fluorouracil considered as a standard chemotherapy regimen in cases of recurrent or metastatic head and neck squamous cell carcinoma. However, the resistance of malignant cells to chemotherapy remains a major obstacle in cancer treatment. Recently, exploring natural products with anticancer properties become a very growing area of study to open the gate for new treatment modalities. Eucalyptus oil consists of many chemicals that have anticancer effect, especially alpha-pinene and beta-pinene. Many other chemicals present in eucalyptus oil are still under study.

Aims and objectives:

The objective of the present studywas to evaluate the possible cytotoxic effect of eucalyptus oil and 5-fluorouracil on head and neck squamous cell carcinoma cell line and to compare the effect of eucalyptus oil and 5-fluorouracil on the expression of the apoptotic marker (Caspase-3) in head and neck squamous cell carcinoma cell line.

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Methods:

Human tongue squamous carcinoma cell line (SCC-15) was used in this study.Different concentrations of 5-fluorouracil and eucalyptus oil were prepared and applied for 24 and 48 hours on the used cell line. The yellow tetrazolium salt (MTT) assay was used for evaluation of cytotoxicity ofeach5-fluorouracil and eucalyptus oil.The quantitative sandwich immunoassay ELISA technique was used for the assessment of caspase-3 expression in the treated cell line.

Results:

MTT assay revealed that 5-fluorouracil and eucalyptus oil showed a significant cytotoxic effecton SCC-15cell line. The ELISA assessment of caspase-3 expression revealed that there was a statistical increase in caspase-3 expression in SCC-15cell line treated with5-fluorouracil than eucalyptus oil.

Conclusion:

Both 5-FU and eucalyptus oil are cytotoxic to OSCC but 5-FU is more effective in activation of apoptotic pathways.

Key words :

5-FU, Eucalyptus oil, caspase-3,SCCcell line

Review:

Oral cancer is a very aggressive malignant tumor which is considered the sixth most common malignancy worldwide. The exact cause of oral cancer remains unknown, there are many factors responsible for occurrence of this type of malignancy, including tobacco products, alcohol consumption, fungal infection, viral infection, nutritional deficiency, syphilis and genetic factor[1].

Among head and neck cancer oral squamous cell carcinoma is considered the most common malignancy with high recurrence rate. Recently the incidence of oral squamous cell carcinoma has been increased. Every year more than200,000 new cases of oral cancer are reported worldwide[2]. There are many treatment modalities available for oral squamous cell carcinoma such as radiotherapy, chemotherapy, molecular targeted therapy and surgery[3]. Chemotherapystillthemaintreatment for malignancy to improve the survival rate and quality of life for patients. 5-fluorouracil considered as a standard chemotherapy regimen in cases of recurrent or metastatic head and neck squamous cell carcinoma[4]. However, the resistance of malignant cells to chemotherapy remains a major obstacle in cancer treatment. Drug resistance occurs due to prolonged use of chemotherapy by many mechanisms such as gene mutation, DNA methylation and histone modification[5]. So, the patients are gradually showing resistance to widely used chemotherapeutic drugs, such as 5-fluorouracil, doxorubicin, cisplatin and campothecin[6]. 5-fluorouracil is one of the antimetabolite cytotoxic drugs which are cell-cycle specific and affect cells only when they are in the S-phase or getting divided. As the cancerous cells divide more rapidly compared to the normal cells, they take up 5-FU more quickly than the normal cells, and hence is more toxic to them[7].5-FU treatment is associated with different side effects, ranging from mild to severe[8,9].

Recently, exploring natural products with anticancer properties become a very growing area of study to open the gate for new treatment modalities. Essential oils considered as important sources of natural products, there are many essential oils that have anticancer properties and one of them is eucalyptus essential oil which is extracted from eucalyptus tree. Eucalyptus is native to Australia, then after the world wars it has been spread all over the world because of its medical uses[10]. Traditionally, eucalyptus oil have many medical uses, it is used to alleviate respiratory tract infection, rheumatoid arthritis, cough,

muscle pain, tuberculosis, sinusitis and cancer. Moreover, it is used topically for ulcers, burns and wound healing[11].

Eucalyptus oil consists of many chemicals that have anticancer effect, especially alpha-pinene and beta-pinene. Many other chemicals present in eucalyptus oil are still under study. According to previous studies eucalyptus extract showed strong cytotoxic effect against some human cancer cell lines[12].

The effectiveness of anticancer drugs is directly related to the maximum apoptotic effect on tumor cells, which is genetically controlled, and at the same time, the minimal necrotic effect of the drug on both neoplastic and normal surrounding cells[13].

Apoptosis which is a programmed cell death can occur in both physiological and pathological conditions. In apoptosis there are many biochemical changes which are noted including; caspases activation , breakdown of DNA and protein, and phagocytosis. Apoptosis has important role in cancer pathogenesis and cancer therapy[14]. Caspases enzymes are considered the engine of apoptosis. Caspases are a family of intracellular cysteine proteases that activated as apoptosis occurred[15]. Caspases not only play an important role during programmed cell death but some of them regulate inflammatory response[16].

The objective of the present study was to evaluate the possible cytotoxic effect of eucalyptus oil and 5-fluorouracil on head and neck squamous cell carcinoma cell line and to compare the effect of eucalyptus oil and 5-fluorouracil on the expression of the apoptotic marker (Caspase-3) in head and neck squamous cell carcinoma cell line.

Material and methods

I. Material

A. Cell line: squamous cell carcinoma cell line SCC-15, supplied from cell culture department VACSERA-EGYPT was used in the present study.

B. **Reagents:**

1- The 5-Fluorouracil which was purchased from (Sigma Aldrich- USA),supplied in a vial containing 1g of 5-Fluorouracil powder.

2- Eucalyptus oil was purchased from Imtenan Company-Egypt supplied in a glass bottle containing 25 ml of eucalyptus oil.

3- The growth medium Dulbecco-s Modified Eagle-s Medium (DMEM) was supplemented with 10 % foetal bovine serum (FBS), Trypsin solution 0.25% in PBS, pH (7.2) was supplied from Sigma Aldrich- USA and2% sodium bicarbonate was from Sigma-Aldrich -USA.

II. Methods:

Determination of IC50 of both drugs : Cells were placed (cells density 1.2 – 1.8 × 10,000 cells/well) in a volume of 100 μl complete growth medium + 100 ul of each 5-FU and Eucalyptus oil per well in a 96-well plates. Cells were treated with serial concentrations of each drugs to be tested. Non-treated control wells were methanol treated. Plates were incubated for 24 and 48 hours at 37ºC. Cultures were removed from the incubator into a sterile work area. Each vial of MTT [M-5655] was reconstituted to be used with 3 ml of medium without phenol red and serum. The values of the IC50 of 5-FU and Eucalyptus oil were 33.55±1.16 ug/ml and 43.7±1.84 ug/ml respectively. After determination of the IC50 value three concentrations of each drug were used to measure the cytotoxic effect of each drug on SCC-15 cell line for 24 and 48 hours.

For the 5-5-FU the following concentrations were used (5-FU1 17uM) (5-FU2 34 uM) and (5-FU3 68 uM). For the Eucalyptus oil (EU1 22 uM) , (EU2 44 uM) and (EU3 88 uM)

Estimation of caspase-3 by ELISA

The level of caspase-3 was measured in cell lysate by the quantitative sandwich immunoassay ELISA technique. The Invitrogen Caspase-3 (active) Human kit was used indifferent both drugs concentrations . A 96-well plate was coated with an antibody specific for caspase-3. Standards and samples

were pipetted into the wells and the protein existing in the sample is attached to the wells via the immobilized antibody. The wells were washed and biotinylated anti-human antibody was applied. After discarding of the unbound biotinylated antibody, horseradish peroxidase enzyme (HRP) conjugated streptavidin was pipetted to the wells. The wells were washed again, a 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was applied to the wells and color developed was attributed to the amount of protein bound. The Stop Solution changed color from blue to yellow, and the intensity of the color is measured at 450 nm.

Statistical Analysis

1.Data were described as mean (M) and standard deviation (SD) value. Student's t- test was used for comparisons between mean values of two different groups and paired "t" test was used for comparison between 2 different points in time for one group. One-way Analysis of Variance (ANOVA) was used for comparisons between more than two groups. The significance level was set at $P \leq 0.05$.

Results

Statistical Results

A- Cytotoxicity assay results

Table (1): Comparison of the mean viability percentage between different 5-FU ,Eucalyptus oil concentrations and control at T1 and T2 durations.

Concentration	Mean % ± SD	P value
T1 (24hrs)		
5-FU1	53.70 ±3.18	<0.001*
5-FU2	45.02 ±2.67	<0.001*
5-FU3	32.47 ±1.91	<0.001*
T2 (48hrs)		
5-FU1	49.59 ±2.27	<0.001*
5-FU2	38.39 ±2.08	<0.001*
5-FU3	27.34 ±1.61	<0.001*
T1 (24hrs)		
EU1	59.51 ±3.41	<0.001*
EU2	50.58 ±2.63	<0.001*
EU3	40.18 ±2.49	<0.001*
T2 (48hrs)		
EU1	55.63 ±2.95	<0.001*
EU2	47.48 ±2.24	<0.001*
EU3	33.03 ±1.28	<0.001*

*P-values less than 0.05 were considered as statistically significant.

ANOVA and Student's t- test revealed that there was a statistically significant difference between each group and control (P-values less than 0.05).

Table (2): Comparison of the mean viability percentage between the different 5FU concentrations at T1 and T2 time duration

	T1	T2	P value
Concentration	Mean % ± SD	Mean % ± SD	
5-FU1	53.70 ±3.18	49.59 ±2.27	> 0.05
5-FU2	45.02 ±2.67	38.39 ±2.08	< 0.05*
5-FU3	32.47 ±1.91	27.34 ±1.61	< 0.01*

*P-values less than 0.05 were considered as statistically significant.

ANOVA and paired "t" tests revealed that there was no statistically significant difference between (5-FU1T1&5-FU1T2) but there was a statistically significant difference between (5-FU2T1&5-FU2T2) and (5-FU3T1&5-FU3T2) (P-values less than 0.05).

Table (3): Comparison of the mean viability percentage between different Eucalyptus oil concentrations at T1 and T2 durations

	T1	T2	P value
Concentration	Mean % ± SD	Mean % ± SD	
EU1	59.51 ±3.41	55.63 ±2.95	< 0.05*
EU2	50.58 ±2.63	47.48 ±2.24	< 0.05*
EU3	40.18 ±2.49	33.03 ±1.28	< 0.05*

*P-values less than 0.05 were considered as statistically significant.

ANOVA and paired "t" test revealed that there was a statistically significant difference between (EU1T1&EU1T2), (EU2T1&EU2T2) and (EU3T1&EU3T2) .

Table (4): Comparison of SCC-15cells viability between Eucalyptus oil and 5-FU concentrations at T1 and T2

	Eucalyptus oil	5-FU	
Concentration	viability%± SD	viability%± SD	P value
24 hour			
EU1 & 5-FU1	59.51 ±3.41	53.70 ±3.18	<0.01*
EU2 & 5-FU2	50.58 ±2.63	45.02 ±2.67	<0.05*
EU3 & 5-FU3	40.18 ±2.49	32.47 ±1.91	<0.001*
48 hour			
EU1 & 5-FU1	55.63 ±2.95	49.59 ±2.27	<0.05*
EU2& 5-FU2	47.48 ±2.24	38.39 ±2.08	<0.01*
EU3 & 5-FU3	33.03 ±1.28	27.34 ±1.61	<0.05*

*P-values less than 0.05 were considered as statistically significant.

ANOVA and Student's t- tests revealed that there was a statistically significant difference between Eucalyptus oil and 5-FU concentrations at T1 and T2.

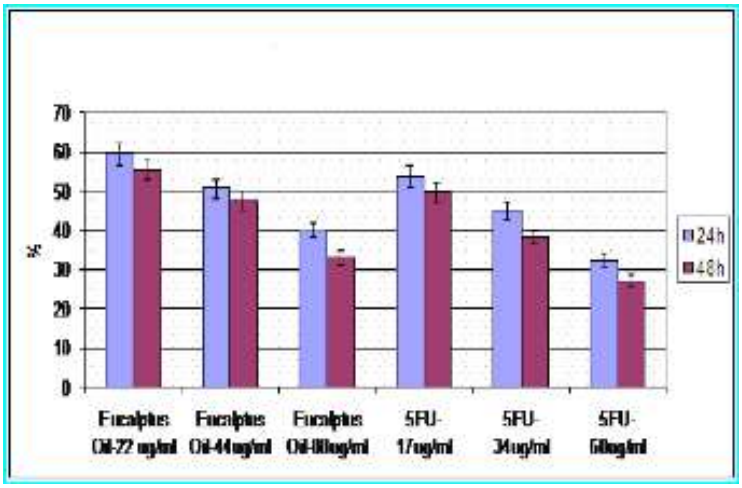

Figure 1: Bar chart representing the difference in cell viability percentage of the different 5-FU and eucalyptus oil concentrations at both durations.

B- ELISA results

Table(5): Comparison of FLD change for caspase-3 between different 5-FU concentrations, Eucalyptus oil concentrations and control at T1 and T2 durations

Concentration	Mean % ± SD	P value
T1		
5-FU1	5.7 ±0.5	<0.001*
5-FU2	7.7 ±0.3	<0.001*
5-FU3	8.8 ±0.3	<0.001*
T2		
5-FU1	8.9 ±0.4	<0.001*
5-FU2	8.9 ±0.4	<0.001*
5-FU3	9.5 ±0.08	<0.001*
T1		
EU1	3.7 ±0.8	<0.01*
EU2	5.5 ±0.6	<0.001*
EU3	6.4 ±0.5	<0.001*
T2		
EU1	4.7 ±0.2	<0.001*
EU2	5.4 ±0.2	<0.001*
EU3	6.7 ±0.4	<0.001*

P-values less than 0.05 were considered as statistically significant.

ANOVA and Student's t- tests revealed that there was a statistically significant difference between each group and control.

Table (6): Comparison of FLD change of caspase -3 between 24 hour and 48 hour for the different 5-FU concentrations

	T1	T2	P value
Concentration	Mean % ± SD	Mean % ± SD	
5-FU1	5.7 ±0.5	8.8 0.4±	< 0.05*
5-FU2	7.7 ±0.3	8.9 ±0.4	< 0.05*
5-FU3	8.8 ±0.3	9.5 ±0.4	<0.05*

P-values less than 0.05 were considered as statistically significant.

ANOVA and paired "t" - tests revealed that there was a statistically significant difference between each concentrations of 5-FU1,5-FU2 and 5-FU3 at T1 and T2 duration.

Table (7):Comparison of FLD change of caspase-3 betweenT1 and T2 for the different Eucalyptus oil concentrations.

	T1	T2	P value
Concentration	Mean % ± SD	Mean % ± SD	
EU1	3.7 ±0.8	4.7 ±0.2	> 0.05
EU2	5.5 ±0.6	5.4 ±0.2	> 0.05
EU3	6.4 ±0.5	6.7 ±0.4	> 0.05

P-values less than 0.05 were considered as statistically significant.

ANOVA and paired "t" - tests revealed that there was a statistically insignificant difference between each concentrations of eucalyptus oil as p value more than 0.05.

Table (8):Comparison between FLD change of caspase-3 of Eucalyptus oil and 5-FU FLD at T1 and T2 durations.

	Eucalyptus oil	5-FU	
Concentration	FLD ± SD	FLD % ± SD	P value
T1			
EU1&5-FU1	3.7 ±0.8	5.7 ±0.5	<0.05*
EU2&5-FU2	5.5 ±0.6	7.7 ±0.3	<0.01*
EU3&5-FU3	6.4 ±0.5	8.8 ±0.3	<0.01*
T2			
EU1& 5-FU1	4.7 ±0.2	8.9 ±0.4	<0.001*
EU2 & 5-FU2	5.4 ±0.2	8.9 ±0.4	<0.001*
EU3 & 5-FU3	6.7 ±0.4	9.5 ±0.08	<0.001*

*P-values less than 0.05 were considered as statistically significant.

ANOVA and Student's t- tests revealed that there was a statistically significant difference between eucalyptus oil and 5-FU FLD at T1 as p value less than 0,05 and there was a statistically highly significant difference between eucalyptus oil and 5-FU FLD at T2 duration as p value <0.001.

Discussion

OSCC is the most common malignant neoplasm of the oral cavity and represents about 90% of all oral malignancies. Various life style risk factors for the development of OSCC are well-known, including tobacco products alcohol and betel use[17]. There are many treatment modalities for OSCC. The main modalities are presently applied in the conventional treatment of OSCC are being surgery, radiation and chemotherapy Among anticancer drugs, 5-florouracil is a basic drug for OSCC. After discovery of 5-FU by Heidelberger in 1957, it has been in use for over 50 years [18,19]. 5- FU drug has many side effects ranging from mild to severe [8,9]. Natural plant-derived products are widely applied to treat a broad range of human diseases particularly including cancer. Eucalyptus is a large species of the Myrtaceae family, it is evergreen big tree native to Australia and Tasmania [20]. Since 1850s, it has been effectively introduced into 90 countries worldwide where it is now one of the most important and broadly planted genus[21].

In the following study the effect of both 5-FU and eucalyptus oil on SCC-15cells was investigated using the MTT and ELISA assay. Concerning MTT which is a sensitive and reliable colorimetric assay that measures viability of cells. MTT assay is based on the potential of active mitochondrial dehydrogenase enzyme of living cells to break the tetrazolium rings of the yellow MTT and form dark blue insoluble formazan crystals which is mainly impermeable to cell membranes, leading to its accumulation within healthy cells. The MTT assay is appropriate to measure drug sensitivity in cell lines. The decrease in cell number reveals cell growth inhibition [22].

In this study, Individual treatment of SCC-15 cells with 5-FU and eucalyptus oil resulted in a time and dose dependent decrease in cell viability when compared to untreated control cells. These findings were estimated from the obvious decrease in the viability percentage of the cultured SCC-15 cells as the concentration of the used drugs increased and as the time increased (24 ,48 hrs).

In the present study, the assessment of the effect of 5-fluorouracil on SCC-15 cell line revealed a statistically significant cytotoxic effect on SCC-15 cells when compared to untreated control cells in T1 and T2 durations. This cytotoxic effect was in a concentration and duration dependent manner. These findings were estimated from the obvious decrease in the averages of viability percentages of cultured SCC-15 cells as the 5-fluorouracil concentration increased. The data that confirmed these findings showed that the averages of viability percentages decreased from 53.70 % to 32.47 % after T1 post treatment, from 49.59 to 27.34% after T2 post treatment with 5-fluorouracil. These findings indicated that the higher concentration of 5-fluorouracil was highly toxic to the viable cultured cells. These results were found to be consistent with the results of other studies which reported that 5-FU exhibit anti-cancer property through promoting apoptosis and suppressing cell proliferation of MCF7-Breast and human colon cancer[23,24].

As we compared the viability percentage between different concentrations of 5-fluorouracil we observed a statistically significant difference between (5-FU2T1&5-FU2T2) and (5-FU3T1& 5-FU3T2), these findings indicated that the higher concentration of 5-fluorouracil was highly toxic to the viable cultured cells as the time increased from T1 to T2 durations.

5- fluorouracil often relies on its characteristics to reduce the ability of cancer cells to grow and divide and to induce cell damage and death. 5-FU act on the promotion stage of cancerous cells through the inhibition of cellular proliferation and increased rate of cell death. The cytotoxicity results of 5-fluorouracil in the present study could be explained by the mechanism of action of 5-FU,where it quickly enters the cell and transformed intracellularly to several cytotoxic metabolites FdUMP, FdUTP and FUTP. These metabolites incorporated into RNA and DNA and inhibiting TS, finally leading to cell cycle arrest and apoptosis in cancer cells. FdUTP metabolities is incorporated into DNA strands,

while FUTP incorporated into RNA and finally FdUMP binds to the nucleotide-binding site of TS inhibiting TS and stops completion of DNA synthesis[25].

Concerning the effect of eucalyptus oil onSCC-15 cell line, it revealed a statistically significant cytotoxic effect on SCC-15 cells when compared to untreated control cells at T1 and T2 durations. This cytotoxic effect was in a concentration and duration dependent manner. These findings were estimated from the obvious decrease in the averages of viability percentages of cultured SCC-15 cells as the eucalyptus oil concentration increased. The data that confirmed these findings showed that the averages of viability percentages dropped from 59.51 % to 40.18 % after T1 post treatment, from 55.63%to 33.03 % after T2 post treatment with eucalyptus oil. These findings indicated that the higher concentration of eucalyptus oil was highly toxic to the viable cultured cells. These results were found to be consistent with Doll-Boscardin et al. result who reported that eucalyptus oil has a potent cytotoxic effect on both Jurkat and HeLa cell lines as the in-vitro MTT assay showed that Jurkat and HeLa cell lines were sensitive to the eucalyptus oil suggesting that eucalyptus oil can be used as cytotoxic agent and potential anticancer drug[26].

As we compared the percent viability between different concentrations of eucalyptus oil we observed that a statistically significant between (EU1T1&EU1T2), (EU2T1&EU2T2) and (EU3T1&EU3T2). These findings indicated that the higher concentration of eucalyptus oil was highly toxic to the viable cultured cells as the time increased from T1 to T2 durations.

MTT assay is usually performed to study mitochondrial dehydrogenase activity as a cytotoxic test for a variety of chemical compounds. Eucalyptus oil is potentially effective to change the enzymatic activity of mitochondria and initiate a preliminary injury that leads to cell death. Furthermore, it was also reported that eucalyptus oil can cause damage in the mitochondrial membrane since they provoke depolarization of the mitochondrial membranes by decreasing the membrane potential and also alter the fluidity of membranes which become abnormally permeable. These mechanisms also had contributed to the cytotoxic effect of eucalyptus oil. So, it exhibit cytotoxicity through membrane disruption in cancerous cells.

Among the different components of eucalyptus oil, 1, 8-cineole.It has been shown to have numerous pharmacological effects. The cytotoxic activities against tumor cell lines were previously reported in leukemia cell lines and against human colorectal cancer .The mechanism of cell killing by 1, 8-cineole is not fully understood. Suppression of growth by 1,8-cineole in the leukemia cell lines was mediated by the induction of apoptosis. Survivin is a type of the Inhibitor of Apoptosis (IAP) gene family . Several studies show that high survivin expression in tumors is associated with lower apoptosis index, increased resistance to chemotherapeutic agents and poor patient survival. Survivin was found in 72% OSCC. In tumor cells, survivin accumulates and localizes to the mitochondria , enhancing cell resistance to apoptosis. 1, 8-cineole inhibit survivin expression then mediates the apoptosis and growth arrest in human colorectal cancers by induction of caspase-3 cleavage finally caused apoptosis[27,28].

In the present study, when we compared the cytotoxic effect of 5-FU and eucalyptus oil, there was astatistically significant difference between all concentrations of both 5-FU and eucalyptus oil drugs at T1 and T2. So, the 5-fluorouracil drug caused more cell death when compared to the eucalyptus oil. As a result 5-FU concentrations were more toxic than eucalyptus oil concentrations at both T1 and T2 durations.

In order to explore the exact mechanism by which 5-FU and eucalyptus oil act on SCC-15 cells, in the present study we selected to measure the Caspase-3 level in SCC-15 cells.

Caspase-3 expression has been extensively studied in several cancer cells. The positive

association between its expression and a good prognosis has been demonstrated in several cancers, recommending its use as a prognostic indicator for cancers. In this study caspase-3 was evaluated using sandwich ELISA technique after individual treatment of SCC-15cells with eucalyptus oil and 5-FU. Caspase-3 (central death executioner) activation which is essential for apoptosis to occur, was confirmed by evaluation of caspase-3 gene expression by ELISA. Caspase-3 is the main effecter caspase-3 that cleaves the greater part of the cellular substrates in apoptotic cells.

In the present study it was observed that, the expression of caspase-3 during T1was statistically increased in 5-FU treated cells in comparison to control cells. As FLD change increased from 5.7 to 8.8 at T1 duration, and from 8.9 to 9.5 at T2 duration. So, 5-FU was found to induce the apoptotic activity in comparison to that of the control.

Extension of treatment to 48 hours with 5-FU resulted also in a statistical increase in the apoptotic activities. Our findings are in agreement with previous findings that administration of 5-FU for longer time periods is more effective in increasing direct caspase-3 activity on human tumor cells (human oral squamous and Hepatocellular Carcinoma Cell Lines) than the treatment for shorter times. The apoptotic effect 5-FU may be due to the inhibition of the function of RNA or DNA synthesis causing the cytostatic effect accompanied by cell apoptosis. Previous study done by **Iawse et al** and **Okamura et al** evaluating casapse-3 activity, demonstrated that 5-FU significantly increased caspase-3 activity in human oral squamous cell carcinoma cell lines. These results could be explained as 5-fluorouarcil increase the susceptibility of oral squamous cell carcinoma cell lines to the Fas mediated apoptosis through activation of caspase-3[29,30]. 5-FU amplifies Fas receptors(death receptors) expression on cancer cells, and that increased the susceptibility to Fas mediated apoptosis by Fas ligands expressing T cells. As Fas receptors attached to Fas ligands ,Fas-associated death domain protein (FADD) accumulates on the cytoplasmic surface of the receptors. FADD, in sequence, attracts pro caspase-8, an initiator protein, to form the death-inducing signal complex (DISC), Ascaspase-8 activated it activate caspase-3, an effector protein, to start cell destruction.

In the present study it was observed that, the expression of caspase-3 was statistically increased in eucalyptus oil treated cells in comparison to control cells. As FLD increased from 3.7 to 6.4 at T1duration,and from 4.7 to 6.7 at T2 duration. There was a statistically significant difference between EU concentrations and control. So, eucalyptus oil was found to induce the apoptotic activity in comparison to that of the control. According to the previous findings; the examined eucalyptus oil showed high biological activities specially apoptotic activity, and this could be connected with the content of 1,8-cineole (eucalyptol)in eucalyptus oil. Eucalyptol have a tendency to trigger the generation of massive amounts of Reactive oxygen species (ROS), Since ROS impairs the mitochondria, the mitochondrial membrane potential collapses, opening the transition pore through which cytochrome c is released in large quantity. This in turn cleaves and activates caspase-9, which causing the sequential cleavage and activation of caspase-3. Once caspase-3 is activated, the sequence hits the point of no return, leading to the activation of caspase-6 and -7, and finally to apoptosis[31].

On the other hand, as compared different concentration of eucalyptus oil with each other at T1 and T2 ,SCC-15 cells exhibit insignificant difference in caspase-3 expression. So the maximum capase-3 expression was at 24 hour in contrast with MTT assay. That could be explained as one of the disadvantages of the MTT cytotoxicity assay is that it is non-specific assay as it does not differentiate between necrosis and apoptosis[32,33]. Also, it underestimated cellular damage as it detects death only at the later stage of apoptosis when the metabolic activity became clearly reduced since in early stages of apoptosis, little or no ultrastructural changes of cytoplasmic organelles is observed[34]. So the significant

decrease of cell viability percentages in MTT assay after 48 hours despite the insignificant increase of caspase -3 expression may be due to necrotic cell death rather than apoptosis.

In the present study, when we compared the FLD of 5-FU and eucalyptus oil, there was a statistically significant difference between 5-FU and eucalyptus oil groups at T1 and T2. So The 5-fluorouracil drug showed more caspase-3 expression as compared to eucalyptus oil at T1 and T2. As a result 5-FU concentrations had higher apoptotic activities than eucalyptus oil concentrations at both T1 and T2 durations and concentrations.

In the present study, the use of two different techniques MTT assay and caspase-3 expression by ELISA sandwich technique helped in the estimation of cytotoxic and apoptotic effect of 5-FU and eucalyptus oil that might occur with different 5-FU and eucalyptus oil concentrations. Since, MTT assay provided an assessment of cell viability. The ELISA technique facilitated the examination of caspase-3 level.

Conclusion

Based on the results of the present study, it could be concluded that: Both 5-FU and eucalyptus oil are cytotoxic to OSCC but 5-FU is more effective in activation of apoptotic pathways as confirmed by MTT and ELISA.

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