

APOPTOTIC AND ANTI-PROLIFERATIVE EFFECTS OF ETHANOLIC NEEM LEAF EXTRACT ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE

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ABSTRACT

Oral squamous cell carcinoma is one of the most common oral cancers, traditional treatments of cancer (surgery, chemotherapy and radiotherapy) have shown to have many side effects and this led to shifting to natural alternatives. Phytochemicals are now showing more appealing future in cancer treatment.

Objectives: To study the effect of Ethanolic Neem leaf extract (ENLE) on oral squamous cell carcinoma (OSCC) cell line cells.

Material and methods: Immunocytochemical staining of oral squamous cell carcinoma cell line treated with three different concentrations of ENLE for different durations was performed and the levels of caspase-3 and Ki67 were evaluated, followed by statistical analysis.

Results: MTT Cytotoxicity Assay showed a progressive decrease in the mean viability percentage in ENLE treated SCC-15 cells as the ENLE concentration increased at both durations. A statistically significant increase was found on investigation of the cytotoxic effect of different concentrations of ENLE at different durations. Caspase-3 results showed an increase in the number of apoptotic cells, with increasing the concentration and duration, when compared with control groups in both durations. Ki-67 results showed a decrease in the number of viable cells, with increasing the concentration and duration, when compared to control groups in both durations.

Conclusion: The assessment of cytotoxicity of ENLE on SCC-15 cell line showed that the ENLE had a cytotoxic effect on the cells when compared to the control cells. This effect increased with increasing both the concentration and duration. Immunocytochemistry (ICC) is an effective sensitive marker for both apoptosis and proliferation.

KEYWORDS: Ethanolic Neem leaf extract, apoptosis, anti-proliferation, immunocytochemistry

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide and poses a significant health threat, with <60% of patients surviving more than five years^{1,2}. These tumors can arise from the tongue, floor of the mouth, buccal mucosa, alveolar surface and hard palate. The most common site is the tongue, which is also one of the worst sites in terms of prognosis³.

Natural plants and their derivatives have been used to prevent and to treat various diseases for thousands of years⁴. There are excellent sources of bioactive components exerting their health beneficial effects, and very often, these sources are material for gourmet food consumptions. Certain bioactive components from the plants have been confirmed for their anticancer activities. There is an estimate that approximately 50-60% of cancer patients in the United States utilize agents derived from different parts of plants or nutrients (complementary and alternative medicine), exclusively or concomitantly with traditional therapeutic regimens such as chemotherapy and/or radiation therapy⁵. The Neem plant is known for its anticancer properties in terms of its preventive, protective, tumor-suppressive, immunomodulatory and apoptotic effects against various types of cancer and their molecular mechanisms⁶.

This study investigates one of the phytochemicals that has been used in many medical fields for centuries. Ethanolic Neem leaf extract (ENLE) was investigated for its effect on tongue SCC (SSC-15) cell line, using different concentrations of ENLE at different durations.

MATERIAL AND METHODS

Preparation of Ethanolic Neem Leaf Extract (ENLE)

The plant leaves, obtained from the Experimental Station of Medicinal Plants, Faculty of Pharmacy, Cairo University, Egypt, were washed with sterilized

distilled water and crushed using a mechanical grinder and weighed in a sterile disposable cup. 25 gm of plant powder were added to 50 ml of 99% ethanol. The mixture was macerated for 1-2 minutes in a temperature not exceeding 45-50°C. The extract was then filtered through muslin cloth for coarse residue filtering. The extraction process was repeated again using coarse residue and 25 ml 99% ethanol. Both extracts were pooled together and filtered through filter paper. The alcohol part was removed from the extract in water bath till the volume about 25 ml. The extract was stored in an air-tight dark container at 4°C till the start of the study.

Cell Culture

Cells of SCC-15 cell line were obtained from Cell Culture Department VACSERA-EGYPT. Cells, imported from the "American Type Culture Collection (ATCC)" in the form of a frozen vial, were cultured in complete culture medium (obtained from Sigma) in a 6 well plate. Cell monolayers were briefly washed with 3 ml PBS. 2.0 to 3.0 ml of trypsin/EDTA solution were added to a 6 well plate and cells were observed under an inverted microscope until a cell layer was dispersed (usually within 3 to 5 minutes). 6.0 to 8.0 ml of complete growth medium were added to deactivate the action of trypsin. The cell suspension was transferred to the centrifuge tube with the medium and centrifuged at approximately 125 xg for 5 times in order to obtain a cell pellet. The supernatant was discarded and the cell pellet was re-suspended in fresh growth medium, where appropriate aliquots of the cell suspension were added to new culture vessels. Cultures were incubated at 37°C for 24 hours in an incubator.

Determining IC50 of ENLE:

SCC-15 cell line cells were placed (cells density 1.2 – 1.8 x 10,000 cells/well) in a volume of 100 ul complete growth medium + 100 ul of ENLE per well in a 96-well plate for 24 hours before the MTT assay. Each test included a blank containing complete

medium without cells. Cells were treated with a serial of concentrations (0.39, 1.56, 6.25, 25 and 100 $\mu\text{g}/\text{m}$) of ENLE to be tested. Untreated control cells were methanol treated. Plates were incubated for 24 at 37°C . Cultures were removed from the incubator and MTT cytotoxicity assay (kit was obtained from Sigma) was performed in laminar flow hood (safety cabinet), for reconstitution of MTT [M-5655] vial, with 3 ml of medium or balanced salt (PBS) solution without phenol red and serum. Reconstituted MTT was added in an amount equal to 10% of the culture medium volume subtracted from the 450 nm measurement. The data recorded from the MTT assay revealed that IC_{50} dose of ENLE in 24 hours post treatment was 42.968 ± 1.82 $\mu\text{g}/\text{ml}$.

Groups and Subgroups

Letter C was used as a code for the control group. The codes N1, N2 and N3 were used for the ENLE concentration less than IC_{50} (25 $\mu\text{g}/\text{ml}$), equal to the IC_{50} (42.968 $\mu\text{g}/\text{ml}$) and higher than IC_{50} (100 $\mu\text{g}/\text{ml}$) respectively. For the duration of application, T1 and T2 were used for 24 and 48 hours, respectively.

Immunocytochemical Staining and Analysis:

Cells of SCC-15 were transferred to the wells of a chamber slide. Cells were then allowed to grow to confluence with the addition of fresh media and washed thoroughly 5 times for 2 minutes each in phosphate buffered saline (PBS). Cells were fixed in 4% formaldehyde /10min at room temperature and rinsed 5 times for 2 minutes each in PBS. The cells were incubated with 0.25-0.5% Triton X-100 in PBS for 10 minutes to permeabilize the membranes. Cells were rinsed 3 times for 5 minutes each in PBS. Endogenous peroxidase was blocked by incubating in 3% H_2O_2 in PBS for 10-30 minutes. Again, the cells were rinsed 3 times for 5 minutes each in PBS. The enzymatic action was blocked with 5% Bovine serum albumin (BSA) for 1 hour. Blocking buffer was then removed from the wells.

Caspase-3 and Ki-67 antibodies were diluted to 1:500 in 1% BSA. Antibodies were added to each

well and incubated for 24 hours at room temperature. The wells were rinsed 3 times for 5 minutes in PBS. Secondary antibody was diluted in 1% BSA diluent. Secondary antibody solution was added into each well and incubated for 1 hour at room temperature. Again, the wells were rinsed 3 times for 5 minutes in PBS. Streptavidin-HRP was added to each well and incubated for 30 minutes at room temperature. The wells were washed 3 times for 5 minutes in PBS on an orbital shaker. DAB solution (3,3'-Diaminobenzidine) which is a derivative of benzene most often used in immunohistochemical (IHC) staining as a chromogen, was added to each cell well.

After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3, 3'-diaminobenzidine, the substrate of horseradish peroxidase. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA) for 30 minutes at room temperature. Once the cells started turning brown, they were washed 2 times for 5 minutes in PBS on the shaker. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium. Each slide was microscopically analyzed and enumerated the percentage of the positively stained cells semi-quantitatively. Counting of the immunocytochemical (ICC) stained cells was carried out using (Image J.V software version 1.37v).

Statistical analysis

Data was coded and entered using the statistical package SPSS version 21. Data was tested for normality using KS test and distribution curve. Data was summarized using mean and standard deviation and standard error of the mean for quantitative variables. Comparison between groups was done using unpaired t-test when comparing two groups and analysis (ANOVA) with multiple comparison post hoc test (Tukey) when comparing more than

2 groups in normally distributed quantitative variables. P values less than or equal to 0.05 were considered as statistically significant.

RESULTS

MTT Cytotoxicity Assay Results

Cytotoxic effect of ENLE was determined 24 and 48 hours post treatment. Recorded data revealed that the cytotoxicity effect of ENLE extract on SCC-15 cell line was relatively proportional to ENLE concentrations at both durations.

There was a progressive decrease in the mean viability percentage in ENLE-treated SCC-15 cells in relation to control cells as the ENLE concentration increased from N1 to N3 (60.70, 50.01 and 43.87 respectively) at 24 hours post treatment and (57.77, 48.72 and 40.51 respectively) at 48 hours post treatment. (Fig. 1, 2)

Immunocytochemical Results

Caspase-3 Results:

There was an increase in the number of apoptotic cells (immunopositive), with increasing the concentration and duration, when compared to control groups in both durations. For both durations, the CT1 and CT2 groups showed immuno-negative viable SCC-15 cells, arranged in colonies,

polygonal in shape with regular membranes, regular nuclei and increased nuclear cytoplasmic ratios. SCC-15 cells treated with ENLE showed increased number of immunopositive apoptotic cells with signs of apoptosis (shrunken cells, shrunken nuclei, irregular cell membranes, blebbing of cells and peripheral chromatin condensation) that increased with increasing the dose, being highest in N3T1 and N3T2 groups (100ug of ENLE) and decrease number of immuno-negative viable cells. (Figs. 3, 4, 5)

Ki-67 Results:

There was a decrease in the number of viable cells (immuno-positive), with increasing the concentration and duration, when compared to control groups in both durations. For both durations, the CT1 and CT2 groups showed immuno-positive viable SCC-15 cells, arranged in colonies, polygonal in shape with regular membranes, regular nuclei and increased nuclear cytoplasmic ratios.

SCC-15 cells treated with ENLE showed decreased number of immuno-positive viable cells, and increased number of apoptotic cells (immuno-negative) with signs of apoptosis that increased with increasing the dose, being highest in N3T1 and N3T2 groups (100ug of ENLE). (Figs. 6,7,8)

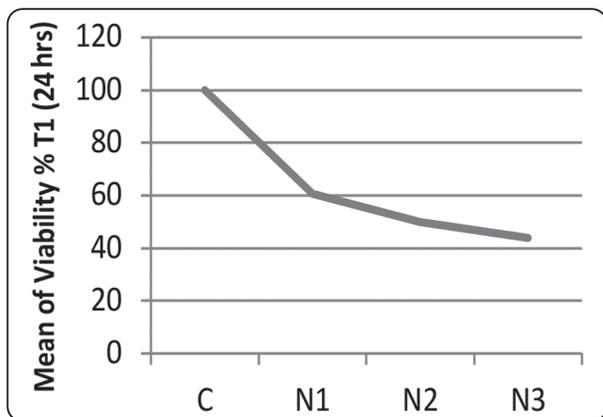


Fig. (1): Mean plot of viability percentage at 24 hours post treatment (T1) for different ENLE concentrations

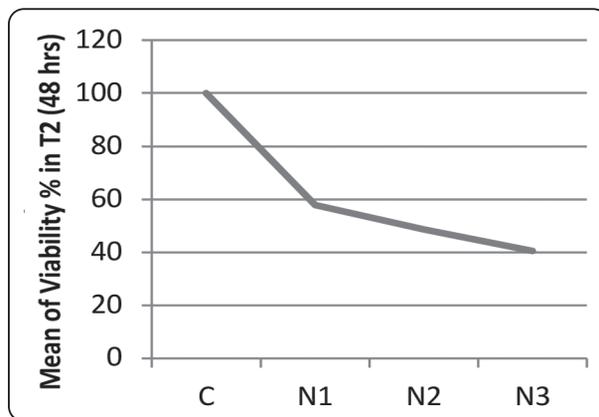


Fig. (2): Mean plot of viability percentage at 48 hours post-treatment (T2) for different ENLE concentrations

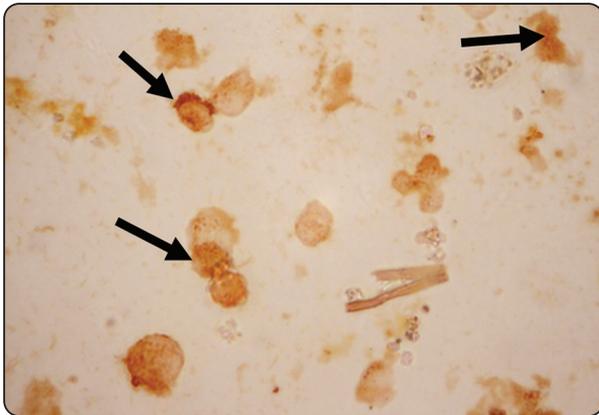


Fig. (3): Photomicrograph showing SCC-15 cells treated with 25ug of ENLE for 24 hours (N1T1), showing a few immuno-positive apoptotic cells, with some morphological signs of apoptosis. (Caspase-3, original magnification x100, oil).

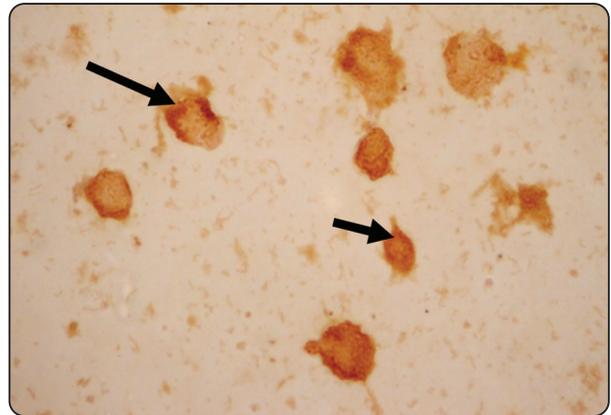


Fig. (4): Photomicrograph of SCC-15 cells treated with 42.968 ug of ENLE for 24 hours (N2T1), showing many immuno-positive cells with signs of apoptosis. (Caspase-3, original magnification x100, oil).

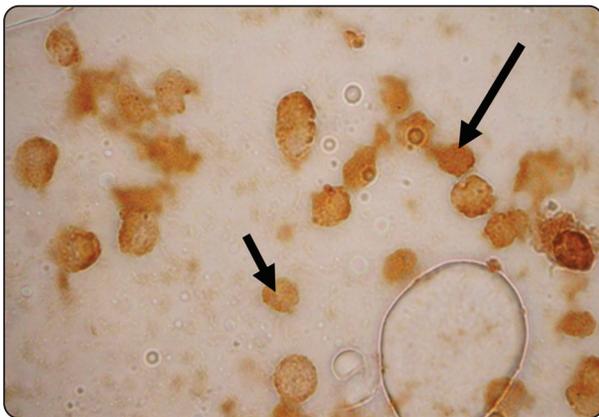


Fig. (5): Photomicrograph of SCC-15 cells treated with 100 ug of ENLE for 48 hours (N3T2), showing immuno-positive cells with signs of apoptosis. (Caspase-3, original magnification x100, oil).



Fig. (6): Photomicrograph of untreated SCC-15 cells cultured for 24 hours (CT1), showing immunopositive colonies of viable cells, polygonal in shape, with regular cell membranes and regular nuclei. (Ki67, original magnification x100, oil).

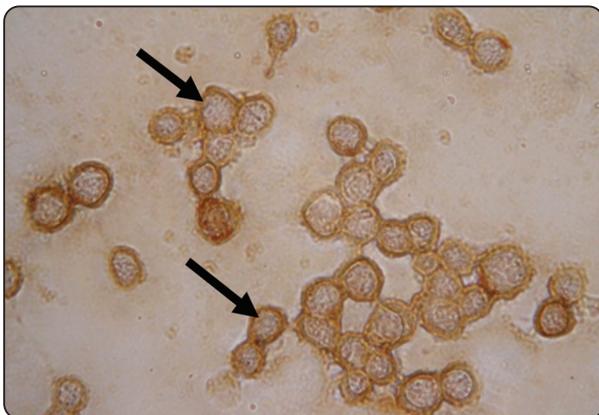


Fig. (7): Photomicrograph of untreated SCC-15 cells cultured for 48 hours (CT2), showing immunopositive viable cells arranged in colonies, polygonal in shape, with regular cell membranes and regular nuclei. (Ki67, original magnification x100, oil).

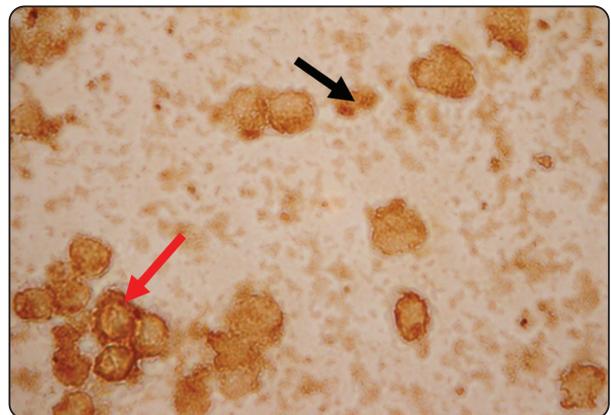


Fig. (8): Photomicrograph of SCC-15 cells treated with 42.968 ug of ENLE for 48 hours (N2T2), showing some immunopositive viable cells (red arrow). Some immunonegative cells, with signs of apoptosis are also seen (black arrow). (Ki67, original magnification x100, oil).

Statistical Results

MTT Cytotoxicity Assay Statistical Results:

There was a progressive decrease in the mean viability percentage in ENLE treated SCC-15 cells as the ENLE concentration increased at both durations (Figs. 9, 10). ANOVA test revealed a highly significant difference for the mean viability percentage between different groups of ENLE-treated SCC-15 cells at both durations (P value = 0.000). Post Hoc comparison (Tukey test) revealed a statistically high significant difference in mean viability percentage between N1 and the other 2 groups N2 and N3 at both durations (P value <0.05). In addition, N2 had a statistically high significant difference with N3 at both durations (P value <0.05).

Caspase-3 Statistical Results:

There was a progressive increase in the mean percentage of apoptotic cells in ENLE-treated SCC-15 cells as the ENLE concentration increased at both durations (Fig.11). ANOVA test revealed a highly significant difference for the mean percentage of apoptotic cells among different groups of ENLE-treated SCC-15 cells at both durations (P value <0.001). Post Hoc comparison (Tukey test) revealed a statistically high significant difference in mean percentage of apoptotic cells between N1 and the other 2 groups N2 and N3 at 24 hours (P value <0.05). In addition, N2 had a statistically high significant difference with N3 at 24 hours (P value <0.05). Similar results were recorded after 48 hours duration (Fig. 11)

Ki-67 Statistical Results:

There was a progressive decrease in the mean percentage of viable cells in ENLE-treated SCC-15 cells as the ENLE concentration increased at both durations (Fig.12). ANOVA test revealed a highly significant difference for the mean percentage of viable cells among different groups of ENLE-treated SCC-15 cells at both durations (P value <0.001). Post Hoc comparison (Tukey test) revealed a statistically

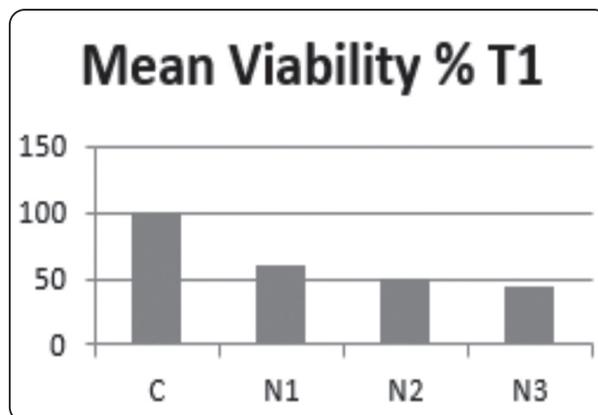


Fig. (9): A bar chart showing the viability percentage with different concentrations of ENLE at 24 hours (T1)

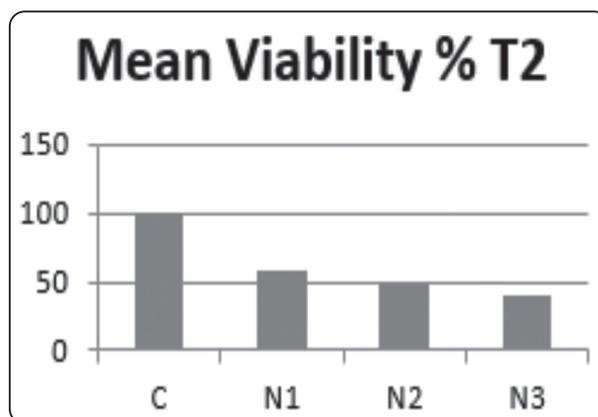


Fig. (10): A bar chart showing the viability percentage with different concentrations of ENLE at 48 hours (T2)

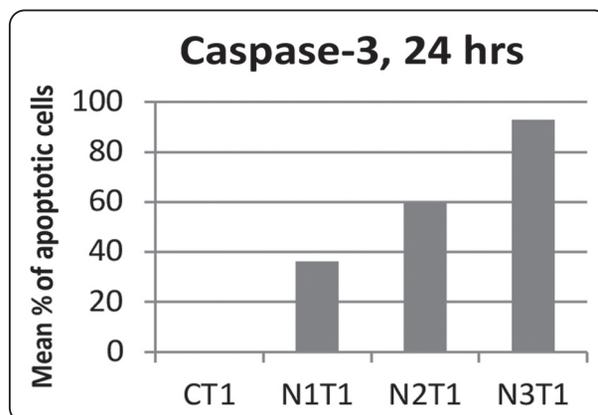


Fig. (11): A bar chart showing the mean percentage of apoptotic cells in different groups in T1 and T2

high significant difference in mean percentage of viable between N1 and the other 2 groups N2 and N3 at 24 hours (P value <0.05). In addition, N2 had a statistically high significant difference compared with N3 at 24 hours (P value <0.05). Similar results were recorded after 48 hours duration (Fig. 12).

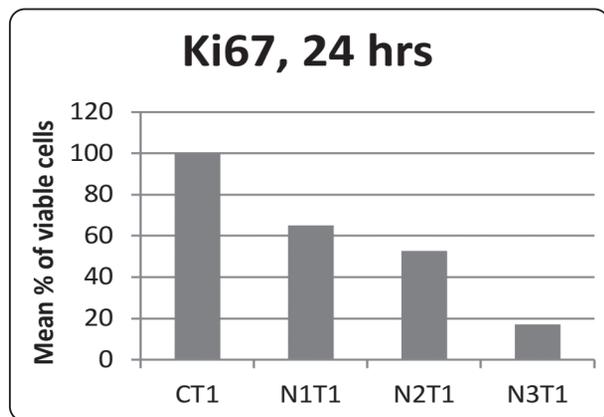


Fig. (12): A bar chart showing the mean percentage of viable cells in different groups in T1 and T2

DISCUSSION

Oral squamous cell carcinomas represent the most frequent of all oral neoplasms, and more than 90 % of all oral neoplasms are estimated to be OSCC⁶. Several studies were performed to prove the effectiveness of combining radiotherapy with/without chemotherapy and/or surgery for treatment of head and neck cancer patients^{8,9}. These treatment modalities cause adverse effects like osteoradionecrosis, xerostomia, trismus, dysphagia, radiation caries and neuropathic pain which can leave patients physically and emotionally disabled and some of these can be lifelong problems in cancer patients¹⁰.

Medicinal herbs and their derivative phytochemicals are being increasingly recognized as useful complementary treatments for cancer. A large volume of clinical studies has reported the beneficial effects of herbal medicines on the survival, immune modulation and quality of life of patients¹¹.

In the present study, the assessment of cytotoxicity of ENLE on SCC-15 cell line showed that the ENLE had a cytotoxic effect on the cells when compared to the control cells. This effect increased with increasing both the concentration and duration. It was estimated from the decrease in the mean viability percentage of SCC-15 cells as the ENLE concentration was increased at different durations. The data showed that the mean viability percentage decreased progressively from 60.70 to 43.87 (24 hours post treatment) and from 57.77 to 40.51 (48 hours post treatment) as the ENLE concentration increased progressively.

These findings were compatible with a study done on two breast cancer cell lines, estrogen dependent (MCF-7) and estrogen independent (MDAMB-231), studies on two prostate cancer cell lines LNCaP (androgen dependent) and PC-3 (androgen independent) and on chronic lymphocytic leukemia showed similar results^{12,13,14}.

After applying ENLE to the SCC cells, in three different dose concentrations (25, 42.968 and 100 ug) and two durations (24 and 48 hours), ICC results showed a generalized increase in the total count of apoptotic cells as evident by caspase-3 immun-expression on increasing the dose concentration and duration, i.e. increased in a dose-dependent manner. Thus, results were highest with 100 ug at 48 hours. Statistical analysis among the 3 groups (N1, N2, and N3) in 24 hours (T1) was statistically significant, where the highest mean percentage was N3T1 of 92.6821%, while the lowest mean percentage was N1T1 of 36.3657%, with a P value (<0.001). Statistical analysis among the 3 groups (N1, N2 and N3) groups in 48 hours (T2) was also statistically significant, where the highest mean percentage was N3T2 of 95.3030%, while the lowest mean percentage was N1T2 of 39.0678%, with P value (<0.001).

These results are compatible with a previous study done on two breast cancer cell lines, estrogen

dependent (MCF-7) and estrogen independent (MDAMB-231). Another study on two prostate cancer cell lines, LNCaP (androgen dependent) and PC-3 (androgen independent), an experiment estimated the effect of ENLE in vitro on C4-2B and PC-3M-luc2 prostate cancer cell lines and in vivo on C4-2B and PC-3M-luc2 prostate cancer xenografts in nude mice. Likewise, a study done on patients with chronic lymphocytic leukemia, after administrating ENLE orally, showed a dose dependent significant increase of apoptosis, defined by high caspase-3 expression and inhibition of Bcl-2^{12, 13, 14, 15}.

On the other hand, upon application of ENLE on the SCC cell line, in three different dose concentrations for two durations, ICC results showed a decrease in the viable proliferating cells as evident by Ki67 immuno-expression on increasing the dose concentration and duration; that is decreased in a dose-dependent manner. Thus, results were lowest with 100 ug at 48 hours. Statistical analysis among the 3 groups (N1, N2 and N3) in 24 hours (T1), showed a statistically significant difference, where the lowest mean percentage was N3T1 of 16.9913%, while the highest mean percentage was N1T1 of 65.0459%, with a P value (<0.001). Statistical analysis among the 3 groups (N1, N2 and N3) in 48 hours (T2) also showed a statistically significant difference, where the lowest mean percentage was N3T2 of 8.4675%, while the highest mean percentage was N1T2 of 61.9394%, with a P value (<0.001).

Thus, we may conclude that ENLE induces apoptosis and inhibits proliferation in SCC cells, and exerts its effect in a dose-dependent as well as time-dependent manner.

CONCLUSION:

Ethanollic Neem Leaf Extract (ENLE) has as cytotoxic effect on tongue squamous cell carcinoma cell line (SCC-15), as it induces apoptosis and

inhibits proliferation of tumor cells. The cytotoxic effect of ENLE on SCC-15 cells is dose and time-dependent, being most effective at a high drug concentration (100ug) for a longer duration (48 hours).

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