THE HARMONIOUS INHIBITORY EFFECT OF NATURAL DRUG AMYGDALIN (VITAMIN B17) AND CHEMOTHERAPY (CISPLATIN) ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE

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ABSTRACT

BACKGROUND: Oral squamous cell carcinoma (OSCC) represents the majority of all cancers of the head and neck. In recent times, the development of antitumor drugs has been transformed from cytotoxic drugs to ameliorate the selectivity of the drug, overcoming multidrug-resistant using natural alternatives. Amygdalin is one of the most common non-conventional anticancer drugs. Cisplatin has been used for the treatment of numerous human cancers. Recently combination therapy is the treatment of choice for cancer patients for the reason that it may enhance the efficiency of the combined agents and decreases their toxicities by decreasing the dose required for therapeutic benefits.

AIM: This study was organized to investigate the cytotoxic effect of both amygdalin and cisplatin on OSCC, compare between two effects on OSCC, and investigate the synergistic effect on cytotoxicity of both drugs on OSCC.

METHODS: The cytotoxic effect was determined using methyl tetrazolium assay, microscopic examination, and statistical analysis (ANOVA).

RESULTS: Our findings showed that the cytotoxic effect of both drugs on HNO97 cells was dose-dependent. Microscopic examination showed that apoptotic criteria became more apparent in cisplatin/amygdalin mix-treated HNO97 group. Our statistical analysis results revealed significant differences in statistical analysis among mean values of NAF of 50 cells in each group, with lower mean values of NAF of mix 48 group (p < 0.0001).

CONCLUSION: Both Amygdalin and cisplatin have anti-cancer effect on HN097 separately and their combination induced harmonious inhibitory effect on HNO97 cells.

KEYWORDS: OSCC, amygdalin, cisplatin, apoptosis, ic50.
INTRODUCTION

Cancer is an important public health problem in many parts of the world, and oral cancer is among the 10 most common cancer worldwide. According to the International Agency for Research on Cancer of the World Health Organization (IARC-WHO), cancer rates were expected to increase from 10 million new cases in 2000 to 15 million in 2020 around the world.\(^1\)

Oral squamous cell carcinoma (OSCC) represents 95% of all cancers of the head and neck, and over the last decade, its incidence had increased by 50%.\(^2\,3\) Tobacco and alcohol use are traditionally the greatest risk factors for oral cancer in the western world.\(^4\)

Recently the development of antitumor drugs has been transformed from cytotoxic drugs to ameliorate the selectivity of the drug, overcoming multidrug-resistant development of a new drug with low toxicity and high specificity. So, oncologists and oncology researchers shifted toward natural methods to reduce the toxic effects of the drugs that were used to treat such type of cancer.\(^5\)

Recent studies have shown that amygdalin (vitamin B17) is a natural plant extract that has an anti-carcinogenic effect on several types of cancers. Amygdalin is found in considerable quantities in members of the Rosacea family including apricot, peaches, and almond.\(^6\)

Amygdalin is called bitter apricot, laetrile, and almond. It induces apoptotic cell death in cells by down regulating the excretion of P53 (tumor suppressor gene) so could be used as an anticancer drug\(^7\,9\). It is composed of two molecules of glucose, one molecule of Benz aldehyde, and one molecule of hydro cyanide. Studies have shown that the Benz aldehyde in amygdalin can induce an analgesic effect and the hydro cyanide in amygdalin can induce an anti-cancerous effect. Moreover amygdalin has the ability to decompose carcinogenic substances in the human body, killing cancer cells, blocking the nutrient source of tumor cells, inhibiting cancer cell growth,\(^10\,11\) and can also reduce the incidence of prostate cancer.\(^12\) Cervical cancer,\(^13\) liver cancer,\(^14\) bladder cancer,\(^15\) and SCC in the buccal pouch of hamsters,\(^16\) by promoting apoptosis.

Cisplatin, cisplatinum, or cis-diamminedichloroplatinum (II), is a world-famous chemotherapeutic drug. It has been used for the treatment of numerous human cancers including bladder, head and neck, lung, and testicular cancers.\(^17\) Its mode of action is cross-linkage with the purine bases on the DNA to form DNA adducts\(^18\), interfering with DNA repair mechanisms through induction of P53 signaling and cell cycle arrest, down regulation of proto-oncogenes and anti-apoptotic proteins, and activation of both intrinsic and extrinsic pathways of apoptosis.\(^19\,21\)

Other platinum-containing anti-cancer drugs such as carboplatin, oxaliplatin are preferably used because of drug resistance and undesirable side effects such as severe kidney problems, allergic reactions, decrease immunity to infections, gastrointestinal disorders, bone marrow problems, hemorrhage, and hearing loss, especially in younger patients.\(^17\)

Combination therapy of cisplatin with other natural anti-cancer extracts such as Amygdalin drugs has been utilized as incoming therapeutic strategies for many human cancers to overcome drug resistance and reduce the toxicity of chemotherapy drugs.\(^22\)

Apoptosis is a genetic program of cell death, being initiated by various physiologic and pathologic stimuli that function primarily to eliminate altered cells that are useless or harmful in a living organism.\(^23\) Oral carcinogenesis correlates with the progressive accumulation of genetic mutations in molecules that regulate apoptosis.\(^24\,25\) The morphological characteristics of apoptosis include cell shrinkage, cytoplasmic vacuolation, and
condensation of nuclear chromatin, which is called pyknosis. Apoptosis depends on cysteine proteases called caspases. Caspase-3 is a member of the cysteine protease family and plays an important role in the regulation of programmed cell death (apoptosis) suggesting its use as a prognostic marker for cancers.  

The research aimed to investigate the cytotoxic effect of amygdalin and cisplatin on oral Squamous cell carcinoma, Compare between two effects on oral Squamous cell carcinoma, and investigate the synergistic effects on cytotoxicity of both drugs on oral Squamous cell carcinoma.

MATERIALS AND METHODS

Cell culture

Human oral squamous cell carcinoma (HNO97 cell line) was obtained from Cell Culture Department-VACSERA-EGYPT in the form of a frozen vial from cell lines services (California, USA)

Cell culture protocol

HNO97 (tongue SCC) were established from surgical tissue samples of SCCHN patients, then were grown as a single layer culture on a flask containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% (V/v)* fetal bovine serum (FBS), (100IU/mL) of penicillin, (100IU/mL) of streptomycin and finally, cells were preserved in a humidified incubator which contained 5% CO₂ at 37°C [Jouan – France]. HNO97 cells were divided into four groups: a control group of untreated HNO97 cells, a group of Amygdalin treated HNO97 cells, a group of cisplatin-treated HNO97 cells, and a group of a mix of Amygdalin/cisplatin-treated HNO97 cells. These groups were cultured under high-efficiency sterilization conditions. The last three groups were treated according to standard protocols.

Reagents

Cisplatin is a well-known chemotherapeutic drug, is slightly soluble in water, and is soluble in dimethylprimanide and N, N-dimethylformamide, and has chemical formula [Pt.(NH₃)₂Cl₂].

Cisplatin was obtained from Sigma Aldrich Products (St, Louis, MO, USA), was dissolved in 0.5% dimethyl sulfoxide DMSO and enoxaparin sodium in phosphate-buffered saline PBS, and all manipulations with cisplatin and enoxaparin sodium were performed under subdued lighting.

Amygdalin is a natural drug extracted from apricot kernels ≥ 99%, Cat# A6005, Sigma- Aldrich, Empirical Formula [C₆H₁₄N₂O₃], was dissolved in 0.5% dimethyl sulfoxide DMSO.

Methyl Thiazol Tetrazolium [MTT] Assay Protocol

Cell viability and cytotoxic activity of cancer drugs was measured using MTT assay (3-(4,5 dimethylthiazol diphenyltetrazolium bromide) assay which is a sensitive, quantitative and reliable colorimetric method, it’s based on the ability of mitochondrial lactate dehydrogenase enzyme (LDH) in living cells to convert into the water-soluble substrate.

Briefly, cells were seeded in 96-well plates and treated with 2 folds serially diluted test materials starting from 100µg. At 37c. After 24 hrs. Cells were administered the treatments diluted with DMSO, and incubated for 24-48 hrs.

To measure cell viability at 24hrs and 48hrs, 50µl MTT solution was added directly to each well, and the plates were incubated for 4 h. Solubilization buffer (50 µl) DMSO was then added to each well without removing the medium to dissolve formazan crystals and stop this reaction. The plates were then incubated overnight at 37c.

For the reading, we used microplate reader (scanning multi-well spectrophotometer) ELISA plate reader (BioTek- EL800-USA) was used at a
wavelength of 570nm. The result obtained indicates the optical density, since the darker the color obtained, the greater the MTT metabolism of the cells under study. Consequently, a higher optical density results in less toxicity of the extract tested. The data obtained were analyzed using the Master plex 2010 Fit program.

Viability percentage = (Mean Optical Density of treated cells/ Mean Optical Density of untreated cells) × 100

The data generated were used to plot a dose-response curve that determines the extract concentration capable of killing 50% of the cell population tested, indicating **IC50 (inhibitory concentration)**.

Briefly in this research, we work on four groups of HNO97 cells: control group, amygdalin -treated HNO97 group, cisplatin -treated HNO97 group, and amygdalin /cisplatin mix-treated HNO97 group. The cytotoxicity effect of Amygdalin, Cisplatin, and Amygdalin /Cisplatin mix on HNO97 cell line in vitro was evaluated for 24 & 48 hrs. incubation.

**TABLE (1):** Concentrations of Amygdalin-treated cells, Amygdalin / Cisplatin mix– treated cells, and Cisplatin –treated cells after 24 hrs.

<table>
<thead>
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<th>conc.µg/ml</th>
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<th>Cisplatin</th>
<th>Mix</th>
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**TABLE (2):** Concentrations of Amygdalin-treated cells, Amygdalin / Cisplatin mix– treated cells, and Cisplatin –treated cells after 48 hrs.

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<th>Cisplatin</th>
<th>Mix</th>
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Microscopic examination

Slides Preparation

The same steps of cells maintenance and subculture protocol were repeated but the cells were dispensed in 25 ml total volume to have a larger quantity of cells sufficient for cytological examination. Pelleted cells were re-suspended in PBS and a part (50μL) was dispensed on the clean ethanol washed glass slide, air-dried, and fixed using methanol as a preparatory step for cytological examination.

Hematoxylin and Eosin Staining

- The fixed slides were rehydrated in descending concentrations of alcohol (100%, 90%, 75%, then 50%), then washed in distilled water for 5 min.
- The slides were immersed in filtered hematoxylin stain for 3 min then washed with distilled water twice.
- The slides were immersed in filtered eosin stain for 5 seconds then washed with distilled water.
- Dried slides were immersed in xylene, mounted with Canada balsam then coverslips were placed and left to dry.

Photomicrography and Cytological Evaluation

Slides were photomicrographed at the power of x1000 oil. The photomicrographed fields analyzed using image analysis software (Image J, 1.27z, NIH, USA). Nuclear area factor (NAF) was calculated using the formula:

\[ \text{NAF} = \text{Circularity} \times \text{Object area}. \]

Statistical analysis:

Results were expressed as mean ± standard deviation SD. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 22.0 window software using One-way Analysis of Variance (ANOVA) and Bonferroni Post Hoc multiple comparisons test to determine the significance of differences between groups. With P < 0.05 deemed as statistically significant.

RESULTS

[MTT] cytotoxicity Assay:

Data obtained showed that the cytotoxicity was time-dependent. The mean viability percentage of treated cells is inversely proportional to drug concentration.

In 24hrs incubation, the mean viability percentage of treated cells decreased as the drug concentrations increased. The half-maximal inhibitory concentration (IC_{50}). Values were in order 402.87 μg/ml, 14 μg/ml, 196.50µg/ml for Amygdalin-treated cells, Cisplatin –treated cells, and Amygdalin / Cisplatin mix–treated cells as shown.

In 48hrs incubation, the mean viability percentage of treated cells decreased as the drug concentrations increased. The half-maximal inhibitory concentration (IC_{50}). The half-maximal inhibitory concentration (IC_{50}) values were in order 381.60 µg/ml, 11,19 µg/ml, 189 µg/ml for  Amygdalin-treated cells, Cisplatin –treated cells, and Amygdalin / Cisplatin mix–treated cells as shown in figure (1).

Microscopic examination

Control cells of HNO97 line

Microscopic examination showed that control HNO97 cells were almost rounded with minimum folding in the cellular and nuclear membranes. Cells showed criteria of malignancy such as hyperchromatism, nuclear pleomorphism, poikilokarynosis, and increased mitotic activity with abnormal mitotic figures, increased nuclear-cytoplasmic ratio, anisocytosis, and anisonucleosis.

Only few cells among control cells showed criteria of apoptosis, mainly confined to nuclear shrinkage and peripheral chromatin condensation.
Treated HNO97 cell line:

HNO97 treated cells showed nuclear morphological modifications that correspond to the morphological parameters of apoptosis. These criteria were obvious in cisplatin-treated HNO97 cells and amygdalin-treated HNO97 cells, but they became more apparent in cisplatin/amygdalin mix-treated HNO97 group.

These criteria included peripheral condensation of chromatin against the nuclear membranes, irregularities in the nuclear and the cellular membranes, membrane blebbing, nuclear shrinkage (Pyknosis), cellular shrinkage, and nuclear fragmentation (Karyorrhexis).

In addition to apoptotic criteria, some cells revealed nuclear alterations that resembled the morphological hallmarks of necrosis such as nuclear and cellular swelling, increased eosinophilia of the cytoplasm, and cell membrane rupture leads to loss of membrane integrity with amygdalin-treated HNO97 cells, cisplatin/amygdalin mix-treated HNO97 cells and increased with cisplatin-treated HNO97 cells.

In amygdalin-treated HNO97 cells, cisplatin-treated HNO97 cells, and cisplatin/amygdalin mix-treated HNO97 cells, the presence of secondary necrotic cells with both apoptotic and necrotic characteristics such as nuclear fragmentation and cytoplasmic swelling were detected.

Statistical analysis:

ANOVA and Bonferroni Post Hoc multiple comparison tests revealed significant differences in statistical analysis among mean values of NAF
Fig. (2): A photomicrograph of control HNO97 cell line showing: increased mitotic activity (green arrow), increased nuclear/cytoplasmic ratio (yellow arrows), peripheral chromatin condensation (blue arrows), nuclear fragmentation (grey arrows), anisocytosis & anisonucleosis (pink arrows), and abnormal mitotic figure (red arrows) (H & E 1000x oil).

Fig. (3): A photomicrograph of HNO97 cell line 24 hours treated with amygdalin showing: peripheral chromatin condensation (blue arrow), membrane blebbing (yellow arrow), secondary necrotic cell (white arrow), nuclear shrinkage (Pyknosis) (purple arrow), and nuclear fragmentation (red arrows) (H & E 1000x oil immersion).

Fig. (4): A photomicrograph of HNO97 cell line 48 hours treated with amygdalin showing: peripheral chromatin condensation (blue arrow), membrane blebbing (yellow arrow), necrotic tissue (green arrow) and nuclear fragmentation (red arrows), secondary necrotic cell (white arrow) (H & E 1000x oil immersion).

Fig. (5): A photomicrograph of HNO97 cell line 24 hours treated with cisplatin showing: peripheral chromatin condensation (blue arrows), secondary necrotic cell (white arrow) and necrotic cell (green arrow). (H & E 1000x oil immersion).
of 50 cells in each group (control HNO97 cells, Amygdalin-treated cells, Cisplatin –treated cells, and Amygdalin / Cisplatin mix–treated cells after 24 & 48 hrs) and the difference is statistically significant (p value<0.0001) as shown in table (1).

**TABLE (3): ANOVA test for the mean values of NAF ± stranded deviation of different groups**

<table>
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<tr>
<th>Group</th>
<th>N</th>
<th>NAF Mean±SD</th>
<th>Standard error</th>
<th>P-value</th>
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<tr>
<td>Control</td>
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<td>0.170±0.067</td>
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<tr>
<td>Amygdalin 24</td>
<td>50</td>
<td>0.068±0.088</td>
<td>0.012</td>
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</tr>
<tr>
<td>Amygdalin 48</td>
<td>50</td>
<td>0.064±0.107</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Cisplatin 24</td>
<td>50</td>
<td>0.095±0.076</td>
<td>0.011</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cisplatin 48</td>
<td>50</td>
<td>0.051±0.039</td>
<td>0.005</td>
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</tr>
<tr>
<td>Mix 24</td>
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<td>Mix 48</td>
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DISCUSSION

Cancer is the second leading cause of death worldwide, and exposure to risk factors plays an important role in the biology and burden of many cancer types.\(^{(29)}\)

OSCC, which affects the epithelial layer of the oral cavity, is a common malignant tumor of the head and neck with low survival rates and high risks of recurrence.\(^{(30)}\) Clinically, OSCC includes lip cancer, which accounts for the majority of OSCC, and intra-oral cancer, which mainly affects the tongue.\(^{(31)}\)

In the Middle East, population witnessed a surprisingly accelerated incidence of some cancers, which are often secondary to adverse lifestyle choices and westernization. Despite this, the incidence is far lower than reported figures from Western countries. Several clinical trials are warranted to study the effect of caloric restriction, fasting, and type of food in the Middle East on cancer incidence.\(^{(32)}\) Another recent study showed that Egypt had the lowest mortality crude rate of oral cancer 0.4%.\(^{(33)}\)

Combination therapy of cisplatin with other natural anti-cancer extracts such as Amygdalin drugs has been utilized as incoming therapeutic strategies for many human cancers to overcome drug resistance and reduce the toxicity of chemotherapy drugs.\(^{(34)}\)

In our research, the cytotoxicity effect of Amygdalin, Cisplatin, and Amygdalin /Cisplatin mix on HNO97 cell line in vitro was evaluated for 24 & 48 hrs.

Cell viability and cytotoxic activity of cancer drugs at different concentrations were measured using an MTT assay (3-(4, 5 dimethylthiazol diphenyltetrazolium bromide) assay which is a sensitive, quantitative and reliable colorimetric method, it’s based on the ability of mitochondrial lactate dehydrogenase enzyme (LDH) in living cells to convert into the water-soluble substrate.\(^{(35)}\)

Our obtained data showed that the cytotoxicity was dose-dependent. The mean viability percentage of treated cells is inversely proportional to drug concentration. The (IC50) value of Cisplatin was less than that of Amygdalin and Amygdalin /Cisplatin mix, so Cisplatin had a higher cytotoxic effect on malignant cells with lower concentrations.

Those findings were consistent with Said A. Barakat & et al. 2021, study was undertaken to investigate antitumor and antioxidant effects of vitamin B17 beside platinum-based drugs in Ehrlich ascites carcinoma (EAC) -bearing female rats. Results showed that administration of vitamin B17 alone or in combination with cisplatin or oxaliplatin led to a decrease of tumor markers together with enhancement of antioxidant indicators compared with EAC bearing rats.\(^{(36)}\)

And agreed with D Siresha, Basireddy Siva Reddy & et al.2019 who proved that both almond and apricot extracts showed antitumor activity on KB cell line (Oral cancer cell line). The viability decreased in a dose-dependent manner. The cell viability decreased as the concentration of the extracts increased. Almond exhibited maximum efficacy at 50 µg/mL by killing 78% of cells, whereas apricot required a concentration of 100 µg/mL to attain 82% of activity. Based on the results of this study, amygdalin extracted from the seeds of almonds and apricots showed cytotoxic effect on human oral cancer cell lines.\(^{(37)}\)
At the microscopic and cellular levels, HNO97 treated cells demonstrated a considerable increase in apoptotic cells after 24 & 48 hrs. of amygdalin , cisplatin, and amygdalin /cisplatin mix therapy. These criteria were obvious in cisplatin-treated HNO97 cells and amygdalin -treated HNO97cells, but they became more apparent in cisplatin/ amygdalin mix-treated HNO97 group. In addition to apoptotic criteria, some cells revealed necrotic and secondary necrotic characteristics. Secondary necrosis is beneficial in cancer therapy because it indicates the death of malignant cells.

Our results agreed with El-Desouky A. Mohamed, Fahmi A & et al., 2020, who examined the effect of amygdalin with and without zinc on hepatocellular carcinoma (HepG2) cell line. They proved that amygdalin promotes apoptosis via the intrinsic cell death pathway (the mitochondria-initiated pathway) and cell cycle arrest at G/M. (38)

And agreed with Gómez-Ruiz, Santiago & et al., 2012, who summarized mode of action of cisplatin on the tumor cells and found that the net effect of intracellular interaction of cisplatin with DNA and non-DNA targets is the cell cycle arrest and subsequent death in sensitive clones. There are two type of death signals resulting from cellular intoxication by this drug .Fundamentally, the drug concentration presents the critical point for cell decision to undergo apoptotic or necrotic cell death. Primary cultures of proximal tubular cells isolated from mouse died by necrosis if they were exposed to high doses of cisplatin just for a few hours while apoptotic cell death is often triggered by long-term exposure to significantly lower concentrations. (39)

Finally our statistical analysis results revealed significant differences in statistical analysis among mean values of NAF of 50 cells in each group ,with lower mean values of NAF of mix 48 group that proved the synergistic cytotoxic effect of amygdalin and cisplatin on HNO97cells.(P value < 0.0001).

CONCLUSION

Both Amygdalin and cisplatin have anti-cancer effect on HNO97 separately, and their combination induced harmonious inhibitory effect on HNO97 cells.

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