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# EFFECT OF ASPARTAME ON VIABILITY, MORPHOLOGY AND MIGRATION IN ORAL SQUAMOUS CELL CARCINOMA CELL LINE: IN VITRO STUDY

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## *ABSTRACT*

**Objectives:** The purpose of this study was to investigate the effects of different concentrations of aspartame (ASP) on oral squamous cell carcinoma (OSCC) cell lines' morphology, migratory potential, and cell viability.

**Methods:** MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay was used to assess cell viability, while a microscopic evaluation of the cells' morphology was done under bright field illumination and photographed at 48 hours post-treatment with ASP. Lastly, in vitro wound healing "Scratch assay" was conducted to evaluate cell migration changes after ASP treatment.

**Results:** Our results revealed significant differences in cell viability at high concentrations (10 and 100 µg/mL) when compared to unstimulated cells. While low concentrations (0.01 µg/mL) showed no significant difference. Regarding morphological changes in OSCC cells our results recorded that the lowest concentration of ASP (0.01µg/mL) did not cause any alteration, while the cells stimulated with 0.1, 1.0, 10, and 100µg/mL, significant changes were noticed. As for cell migration capacity, ASP IC50 caused a notable decrease in OSCC cell line invasion and cell migration.

**Conclusion**: According to our findings, High concentrations of ASP caused decreased cell viability, several morphological changes as well as decline in migration capacity in OSCC cell lines.

**KEYWORDS:** Aspartame, OSCC, Artificial sweetener, Cytotoxicity, Sugar substitutes

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## INTRODUCTION

The contemporary upsurge of public health awareness has prompted the sugar substitution with either natural or artificial sweeteners that belong to a substantial class of additives that enhance the effect of sugar in taste and boost food flavor, though impacting very little to energy intake[1,2]. High intensity sweeteners are chemical compounds that can be natural, synthetic or semi-synthetic which are used instead of sugar in foods, drinks, and various oral medications [3,4].

In 1965, scientist James M. Schlatter made the coincidental discovery of aspartame (ASP), a semi-synthetic sweetener, while investigating potential anti-ulcer medications [3,4]. The Food and Drug Administration (FDA) authorized ASP as an artificial sweetener in 1981[5], It is included in over 6000 products, including chewing gum, yoghurt, non-alcoholic drinks, and even pharmaceutical items like multivitamins and sugar-free cough drops. Since the FDA approved it, ASP has gained popularity as a sweetener that can be used to manage glycemic levels in obese and diabetic patients undergoing weight reduction programs, as well as a dietary ingredient in healthy people [6].

The distinct flavor and strong sweetness of ASP which is 200–300 times sweeter than sucrose are the reasons behind its uniqueness [7]. It acquires its sweetness from the G protein coupled receptor family T1R class, which includes T1R2 and T1R3 [8]. Furthermore, studies have indicated that aspartame has antipyretic, analgesic, and anti-inflammatory properties [1].

L-phenylalanine and L-aspartic acid, two amino acids that are combined by methyl ester linkages to form ASP, being considered as sources of phenylalanine (which should be taken into consideration for those who have phenylketonuria) [9]. When it's dry, ASP is stable, and but not after it's heated for an extended period [10]. Following oral intake, it is absorbed by the intestinal lumen and hydrolyzed into phenylalanine (50%), aspartic acid, an excitatory amino acid (40%), and methanol (10%) [11].

The FDA set the ASP safe consumption level at 50 mg/kg in 2005, however the European Food Safety Authority set it at 40 mg/kg in 2006 [10].

According to previous studies, long-term exposure to ASP might result in neurological conditions such headaches, eye issues like impaired vision, sleeplessness, nausea, hearing problems, low energy, hyperactivity, personality changes, memory loss, and speech impairment [12,13].

There is disagreement over the potential for carcinogenesis associated with ASP use. The findings of the epidemiological studies do not establish a clear connection between ASP and an increased risk of cancer in people [14], whereas, numerous investigations conducted on animals revealed a significant frequency of dose-dependent malignancies, including hematological tumors [15].

Consuming artificial sweeteners was identified as the third risk factor for colorectal cancer in a case control research conducted in 2014 by Mahfouz and associates [16]. Similarly, at dose levels similar to those that humans may be exposed to, a variety of findings from rat models in Ramazzini Institute indicated that ASP was linked to increased risks of several malignancies (lymphomases, leukemias, hepatocellular, and alveolar/bronchiolar carcinomas). Despite the fact that these conclusions have generated controversy [17], Recently, more information was released that confirms the Ramazzini Institute's initial conclusions about tumor identification. This implies that the carcinogenicity of ASP needs to be reevaluated. Numerous in vitro research have examined the toxicity of ASP, and their findings have indicated that it may be carcinogenic potentially via means of angiogenesis, inflammation, DNA damage promotion, and apoptosis inhibition pathways [18].

There were roughly ten cancer bioassays found, seven of them found that long-term, high-dose exposure to ASP did not cause cancer [19]. Swiss mice and Sprague-Dawley rats were found to have higher incidences of hematological, liver, lung, and peripheral nerve malignancies in the other three studies [20].

The International Agency for Research on Cancer, a division of the World Health Organization. Where determining the etiology of cancer is one of its main functions. Categorized ASP as "possibly carcinogenic to humans" As per Group 2B in 2023 [21].

Despite the fact that ASP has been a part of diets for more than 30 years, there continues to be great concern in investigating its toxicity, especially pertaining to oncogenic risk. Consequently, this study aims to investigate the potential role ASP in OSCC by applying an in vitro assessment to its effect at various concentrations on OSCC cell line's cell viability, morphology, and migratory capabilities.

## METHODS

### *1. Preparation of stock solution of ASP*

ASP in powder form was purchased from Sigma. A stock solution of 100$\mu$g/mL was prepared by reconstitution the 0.1grm in the appropriated volume of 1 mL of DMSO, followed by sonication for 5 seconds, aliquoted and stored at –20°C until use. For every experiment, the test compound's final concentrations were produced by diluting the stock with the medium. The carrier solvent (0.1% DMSO) was added to the control cells.

### *2. Cell proliferation assay*

A day prior to carrying out the experiment, a culture plate with 96 wells was used to seed the OSCC cells. $8\times10^3$ cells per well, Cells were seeded in 200 $\mu$L of Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine serum (FBS).

Streptomycin (10mg), 1% penicillin G sodium (10.000 UI), and amphotericin B (25µg) were added as supplements. (***Gibco,Thermosientific,Germany***). Culture plates were kept in an environment with 5% $CO_2$ at 37°C. for 24 hours to attach cells. The following day, repeated concentrations of ASP (0.01–0.1, 1.0–10, and 100 $\mu$mol/mL) were produced for the treatment of cells. Furthermore, the carrier solvent (0.1% DMSO) was used for the control cells. While for positive control, cisplatin, was utilized at a concentration of 10.0 $\mu$mol/mL. The cells were maintained for 48 hours at 37°C with 5% CO2. [22].

Following the incubation period, the cell cytotoxicity assay was carried out using the ***Vybrant® MTT Cell Proliferation Assay Kit***, cat no: M6494 (***Thermo Fisher, Germany***). A 100$\mu$L of media was replaced by new media. Each well received twenty $\mu$L of *4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT) solution (1mg/mL). For four hours the plates were kept in a 37 °C environment with 5% CO2. Lastly, the MTT solution was discarded, and 100 µL of sodium dodecyl sulfate with hydrochloric acid (SDS-HCL) was added to each well. Using a spectrophotometer (***ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA***), the optical density at 570 nm was measured to assess the vitality of the cells.

### *3. Calculation of $IC_{50}$ of ASP on OSSC cells*

Following the completion of the cell proliferation assay, the percentage of cell viability, which reflects the cytotoxic impact of successive Magnifier doses at each test, was calculated. The relationship between the normalized response and the log dose (inhibitor) was represented by the plotting of the XY curve. Using linear regression analysis, the best-fit point was identified.

Calculation of half-maximal stimulatory concentration ($IC_{50}$) using the ***GraphPad prism software, Prism 9, version 9.1.0(221)***. based on

cellular metabolic activity concentration-response curves that were standardized to untreated OSCC cells, the IC50 for each group was determined.

### *4. Cell Morphology*

After 48 hrs. of OSCC cells treatment with ASP dissolved in PBS at 0.01, 0.01, 1.0, 10, and 100µg/mL, bright field illumination was used to examine the cells and photographed using the ***Labomed inverted microscope (Labomed, USA) and Vega Digital Camera*** live-cell imaging software (***Labomed, Los Angeles, USA***), and ***LC-6 USB3.0 Colorful CMOS Digital Cameras, Labomed, Los Angeles, USA.***

### *5. In vitro wound healing "cell migration assay"*

To assess the effect of ASP on OSCC cell's migration, an In vitro wound healing "Scratch assay" was conducted. As a primary step, the cells were cultured to form a confluent cell monolayer. At this stage, the monolayer depicts the tissue's in vivo state prior to injury. After the cells have become confluent, a cell-free gap in the monolayer is performed by mechanical scratching (scratch wound) The cells were treated with the $IC_{50}$ of ASP (15.66µg/mL) at 48 hrs. [23].

Furthermore, for the control cells, the carrier solvent (0.1% DMSO) was also utilized. After being incubated for 48 hours at 37 °C in 5% CO2, the invasive effect of the treated cells was assessed using an optical microscope. At the end of incubation time, the relative wound density "ratio of the occupied area in the gap to the total area in the initial gap," wound area ($\mu$m) "cell-free area" and the Gap width ($\mu m^2$) "average distance between the edges of the gap" were measured over time and data are expressed as a percentage. The ratio of the occupied area in the gap to the total area in the tested group was normalized to the initial gap. The analysis was performed using the live-cell imaging software (***Labomed, Los Angeles, USA***), and ***LC-6 USB3.0 Colorful CMOS Digital Cameras, Labomed, Los Angeles, USA.***

### Statistical analysis

Statistical data analysis was carried out using GraphPad Prism version 8.0.0 for Windows, which was created by San Diego, California-based GraphPad Software. Results were presented as mean ± standard deviation (SD). Dunnett's post-test was used after the statistical differences between the control and treatment groups were determined using a one-way ANOVA. Moreover, the Unpaired t-test was employed to assess differences between two independent groups (** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

## RESULTS

### 1. Assessment of ASP Cytotoxicity in a dose-dependent manner

The cytotoxic effect of ASP on OSCC was assessed by exposing cells to increasing concentrations (0.01, 0.1, 1.0, 10, and 100 µg/mL) for 48 hrs. Results revealed the most pronounced decrease in viable cell percentage observed at higher concentrations (10 and 100 µg/mL): 76.7% and 49.3%, respectively (**Table 1 and Fig. 1**). Conversely, the lowest concentration (0.01 µg/mL) showed no significant impact on cell viability ($p > 0.05$). Notably, at a 0.1 µg/mL concentration, ASP exhibited a mild yet significant difference in cell viability compared to unstimulated cells ($p = 0.02$).

TABLE (1). Comparison between different concentrations of ASP for the OSCC cell viability after 48 hours

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **OSCC vs. ASP (0.01)** | 2.650 | -1.222 to 6.522 | 0.2306 |
| **OSCC vs. ASP (0.1)** | 6.043 | 2.172 to 9.915 | 0.0029 |
| **OSCC vs. ASP (1.0)** | 10.95 | 7.082 to 14.82 | <0.0001 |
| **OSCC vs. ASP (10)** | 24.19 | 20.32 to 28.06 | <0.0001 |
| **OSCC vs. ASP (100)** | 51.62 | 47.75 to 55.49 | <0.0001 |

**Dunnett's multiple comparisons test (ANOVA)*

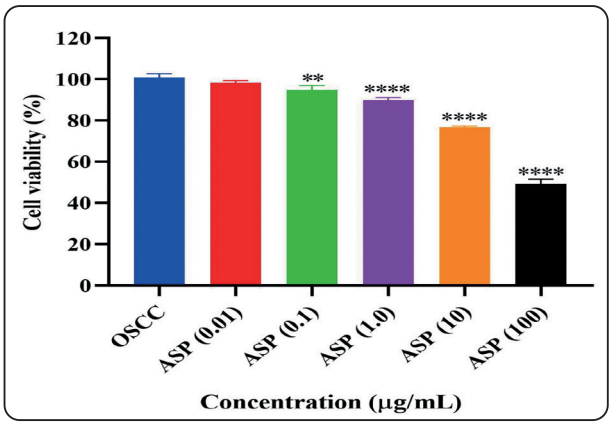


Fig. (1) In vitro assessment of the cytotoxic effect of serial concentrations of ASP on OSCC cells after 48 hours (** $p < 0.01$, and **** $p < 0.0001$).

**2-Morphological changes in OSCC cells.**

High concentrations of ASP induced morphological changes in OSCC cells. The lowest concentration of ASP (0.01µg/mL) did not alter the OSCC cells' confluence, their shape or their capacity to adhere to the culture plate, as for the OSCC cells stimulated with 1.0, 10, and 100µg/mL, Notable alterations were observed: a lower confluence than in the control group, multiple floating round cells, shrinkage or size reduction, Nuclear fragmentation or condensation, membrane blebbing as well as protrusions, and cytoplasmic vacuolations. However, no significant morphological changes were detected in OSCC treated with 0.1 µg/mL of ASP compared to control cells (unstimulated) (**Fig. 2,3**)

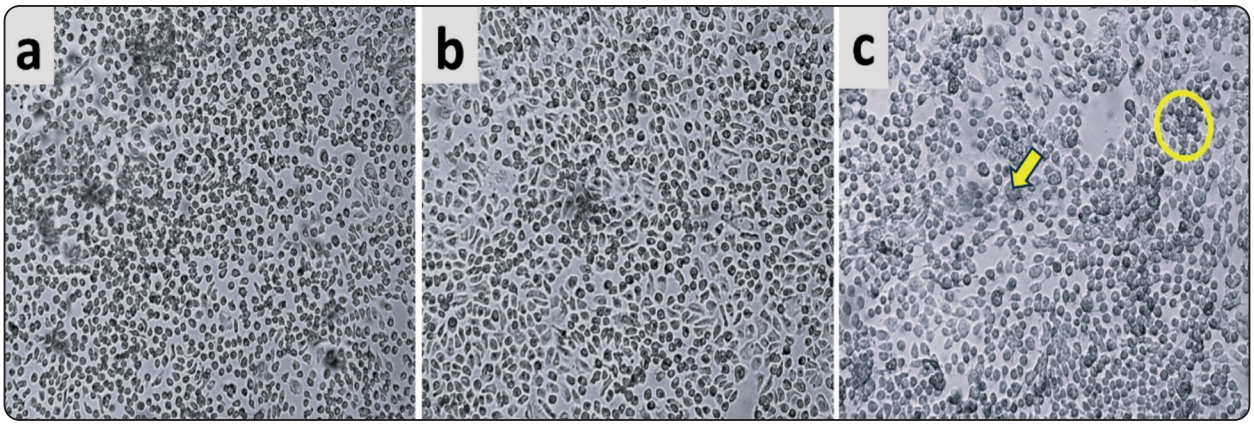


Fig. (2). Morphological changes induced by ASP solution in OSCC cells: (a). Control and b, c, cells were stimulated with increasing concentrations (0.01,0.1) µg/mL of ASP in PBS for 48 h. Nuclear fragmentation or condensation (yellow arrow), and detached floating cells (yellow circle).

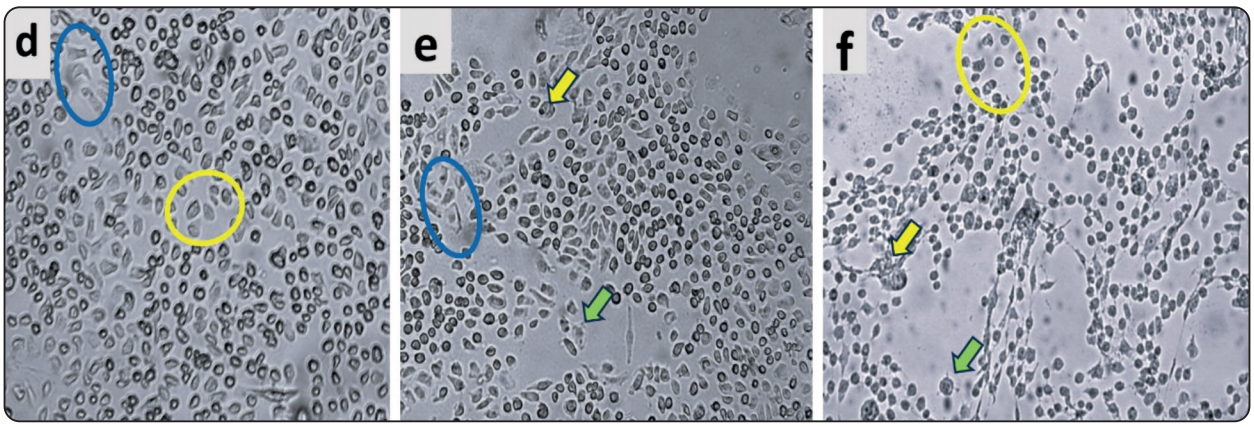


Fig. (3). Morphological changes induced by ASP solution in OSCC cells: d, e, and f cells were stimulated with increasing concentrations (1.0, 10, and 100 µg/mL of ASP in PBS for 48 h. Nuclear fragmentation or condensation (yellow arrow), membrane blebbing or protrusions (green arrow), cytoplasmic vacuolations and cell shrinkage (blue circle), and detached floating cells (yellow circle).

**3- Cell migration assessment.**

In this experiment, cells were treated with the calculated IC50 (15.66 µg/mL) for 48 hrs. The results showed a significant decline in cell migration and invasion at 15.66 µg/mL of ASP ($p < 0.01$). However, control cells exhibited the highest migration rate (**Table 2** and **Fig. 4,5**).

TABLE (2) Descriptive analysis for the OSCC cell migration after treatment with IC50 of ASP for 48 hours

| Cell viability (%) | Relative wound density (%) | wound area ($\mu m^2$) |
|---|---|---|
| OSCC (untreated) | 68.3±12.6 (55.0 – 80.0) | 16.0±5.29 (10.0 – 20.0) |
| OSCC treated with 15.66µg/mL of ASP | 24.0±8.54 (15.0 – 32.0) | 85.0±5.00 (80.0 – 90.0) |
| Statistics (Unpaired t-test) | t: 5.04, p=0.0072 | t: 16.42, p<0.0001 |

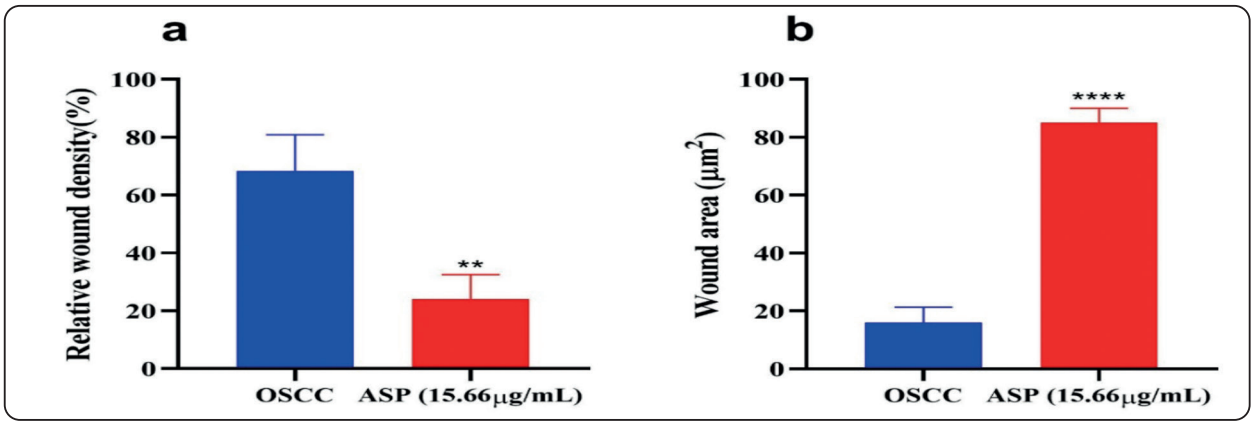


Fig. (4). In vitro assessment of the migration inhibitory effect of ASP (15.66µg/mL) on OSCC cells after 48 hrs. The results are presented as (a) relative wound density (%), and (b) wound area µm2, compared to untreated control cells. Results are expressed as mean values and SD of three measurements (**** $p < 0.0001$).

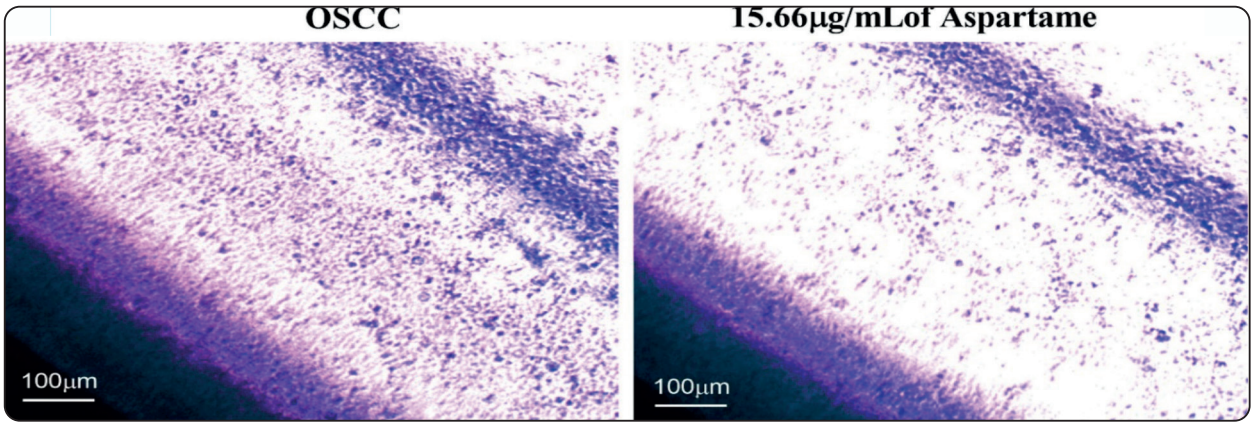


Fig. (5). Photomicrographs illustrate the cytotoxic effect of ASP at the IC50 concentration on the invasion of OSCC cell lines. Untreated OSCC cells showed a narrow gap between the two edges of the scratch, compared to the effect on cells cultured in DMEM supplemented with 15.66 µg/mL of ASP for 48 hours.

## DISCUSSION

The association of ASP and potential of cancer development is still substantially controversial. According to recent studies, using ASP for longer periods of time and at larger consumption levels has been linked to a higher risk of developing malignancies [13].

Our research reviewed ASP cytotoxicity on OSCC cell lines and revealed significant differences in cell viability at high concentrations (10 and 100 μg/mL) when compared to unstimulated cells. While low concentrations (0.01 μg/mL) showed no significant difference.

These findings were in agreement with several studies as Susan et al, whose findings showed that ASP doses starting at 10 $\mu$M led to a dose-dependent reduction in cell viability of SCC cells, [24].

Additionally, ASP research done on several tumor types revealed analogous results to our findings including Van Eyk, who studied the effect of several artificial sweeteners including ASP on colorectal adenocarcinomas, where exposure to doses as high as 10 mM decreased the cell viability[25]. Additionally, Pandurangan et al. studied the effect of ASP on human cervical cancer cells and found that following 48 hrs of exposure to several concentrations of ASP, cell viability dramatically decreased [13].

Similarly, Cadrici et al, stated that ASP caused human blood cells' viability to decline in a concentration-dependent way [26]. Moreover, Horio et al. reported a severe decrease in cell vitality of PC12 cells (neural cells) at concentrations greater than 1 μg/mL of ASP [27].

These findings were explained by a study that highlighted the ASP induction of substantial alterations in the human colorectal cancer cell's mRNA expression of apoptotic genes, up-regulating of the expression of bcl-2 and tumor suppressor gene p53, indicating that ASP causes apoptosis [13].

Another study done on fibroblasts and endothelial cells revealed that ASP produces more reactive oxygen species linked to cytotoxicity, raises the level of the inflammatory mediator IL-6 and, has a pro-angiogenic impact at low doses via stimulating the MAPK pathway [28].

Regarding morphological changes in OSCC cells our results recorded that the lowest concentration of ASP (0.01μg/mL) did not alter the OSCC cells, as for the cells stimulated with 0.1, 1.0, 10, and 100μg/mL, significant changes were noticed compared to control cells. Compatible results were found by Susan et al in their study which was done on normal keratinocytes as well as OSCC [24].

Additional study done on colorectal carcinoma showed that the HT-29 cells' confluence, ability to stick to the culture plate, or morphology were unaffected by the lowest ASP concentrations. Regarding the cells stimulated with high concentrations showed remarkable alterations: cellular debris, several spherical cells floating around, and a lower confluence in comparison to control cells [18].

The influence of ASP on cell morphology was explained by several studies which suggested that actin filaments mediate adhesion between cells as well as cells and substrates. ASP alter these cellular connections and consequently their morphology [13].

An additional aspect that was investigated in our study was the influence of ASP IC50 (15.66 μg/mL) for 48 hrs on the migratory potential of OSCC cells. The acquired results demonstrated a notable decrease in cell invasion and migration. Similarly, ASP was found to hinder colorectal cancer cells' migration in a another study [18].

Sawadsopanon et al demonstrated similar outcomes in their investigation of human intestinal epithelial cells and provided an explanation of the potential mechanism of ASP on cell migration and proliferation. These results were obtained by Western blotting the expression of regulating proteins, which revealed that integrins Akt, FAK, Cav-1, Rac1-GTP and RhoA-GTP proteins were down-regulated by ASP, inhibiting cell migration [29].

## CONCLUSION

The results of our study regarding influence of ASP at high concentrations in OSCC cells, revealed a reduction in percentage of viable cells, migration capability as well as induction cellular morphological changes. These findings support the cytotoxicity of ASP in OSCC cells at high concentrations; consequently, they may serve as a foundation for future research efforts to clarify the mechanism of action of ASP in OSCC as well as on normal epithelial cells.

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