„VICTOR BABEȘ” UNIVERSITY OF MEDICINE
AND PHARMACY TIMISOARA
FACULTY OF DENTAL MEDICINE
DEPARTMENT I

RACEA ROBERT-COSMIN

PhD THESIS

RECOVERY INTERVENTIONS AND TREATMENT IN ORAL PATHOLOGY

Scientific Coordinator:
PROF. UNIV. DR. RUSU LAURA-CRISTINA

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SUMMARY

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Introduction

Cancer is the disease of the 21st century that affects the lives of millions of people, often resulting in fatal events. It is a leading cause of death worldwide, accounting for an estimated 9.9 million deaths in 2020 alone. The most common types of cancer worldwide are lung, breast, colorectal, prostate, and stomach cancer. Oral and oropharyngeal cancer are types of cancer that affect the mouth and throat. Oral cancer refers to cancer that affects the lips, gums, tongue, and other tissues inside the mouth, while oropharyngeal cancer refers to cancer that affects the back of the throat, including the base of the tongue and the tonsils. Oral and oropharyngeal cancer are relatively uncommon compared to other types of cancer, but they can be serious and potentially life-threatening if not detected and treated early.

Several risk factors have been associated with an increased risk of developing oral and oropharyngeal cancer, including: tobacco use, alcohol consumption (people who smoke and drink alcohol together have an even higher risk of developing these types of cancer), Human papillomavirus (HPV) infection (certain strains of HPV have been linked to an increased risk of oropharyngeal cancer), age (the risk of developing oral and oropharyngeal cancer increases with age, with most cases occurring in people over the age of 55), sun exposure (prolonged exposure to sunlight can increase the risk of lip cancer), poor oral hygiene [1,2].

The treatment for oral and oropharyngeal cancer depends on the stage and location of cancer, as well as the overall health of the patient. Treatment options may include surgery, radiation therapy chemotherapy, and immunotherapy. The big disadvantage of current treatments is the lack of selectivity, high toxicity, and often resistance or ineffective. Due to this phenomenon, it is imperative to identify safe alternatives, with a high degree of selectivity in terms of anticancer treatment.

Eugenol (Eug) (4-allyl-2)-methoxyphenol is the main component of cloves (*Syzygium aromaticum* (L.) (Family *Myrtaceae*), an important plant in traditional medicine [3]. A number of therapeutic properties are associated with eugenol, including antioxidant, anti-inflammatory, antimicrobial, analgesic, and antitumor properties [4]. In terms of eugenol's antitumor properties, it has been demonstrated to be beneficial in several kinds of cancer, including oral cancer, lung cancer, skin cancer, breast cancer, or colorectal cancer. It has been demonstrated that Eug induces cellular apoptosis in cancer cells by causing a decrease in the mitochondrial membrane

potential and an increase in the production of reactive oxygen species [5]. To date, however, the biological mechanisms behind Eug's antitumor effects are not fully understood.

The purpose of this study is to shed light on the safety profile of the materials and compounds used in the prevention and treatment process for oral diseases.

In order to meet the goal, the following objectives were pursued:

- in vitro cytotoxicity assessment of Eug on squamous cell tongue carcinoma;
- in vitro characterization of the cytotoxic profile at the level of osteosarcoma, oropharyngeal and colorectal carcinoma cells;
- evaluation of two types of ceramics at the level of the Detroit 562 pharyngeal cancer cell line in order to confirm the applicability of in vitro methods in the dental field
- comparative analysis of other natural compounds in terms of in vitro antitumor activity on squamous cell carcinoma
- evaluation of the biological effects of ozone gas, used in dentistry, on the tooth enamel remineralization process and on morphology and confluence of human primary gingival cells: keratinocytes and fibroblasts.

Results

The first study analyzed the cytotoxicity of Eug on human gingival fibroblasts (HGF) and of squamous cell carcinoma cells (SCC-4). Thus, after 72 h of stimulation with 5 different concentrations of eugenol, 0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1 mM, the MTT (3-(4,5 dimethylthiazol2-yl)-2,5- diphenylte- trazolium bromide) assay was performed. Figure 1 shows a concentration-dependent decrease in viability in both cell lines.

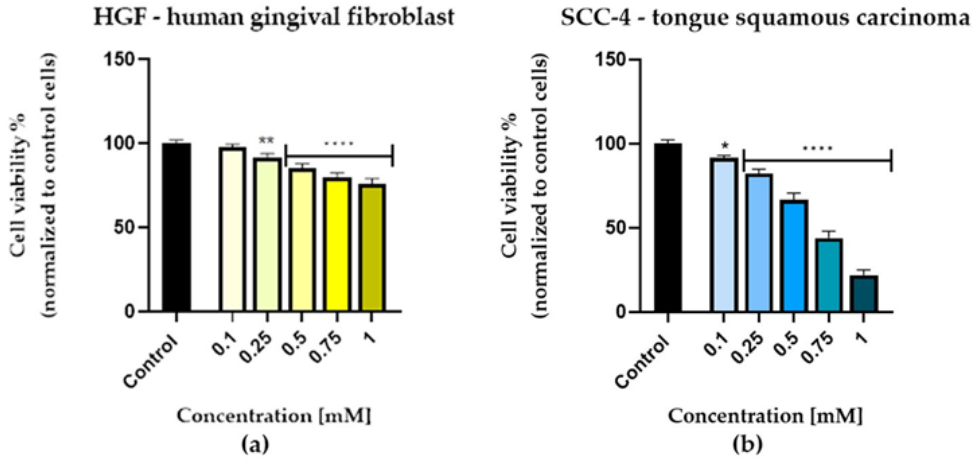

Figure 1. In vitro cell viability evaluation of eugenol (0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1 mM) in HGF (human gingival fibrolasts) (a) and in SCC-4 (tongue squamous cell carcinoma) (b). MTT colorimetric assay was performed after 72 h treatment. Results are presented as viability percentages (%) normalized to control (DMSO-treated cells).

The healthy human gingival fibroblasts were not significantly affected by the 0.1 mM concentration, but a discrete dose-dependent loss of viability was seen at higher concentrations (0.5 mM, 0.75 mM, and 1 mM). In the SCC-4 cell line, the 72 h treatment produced a concentration-dependent decrease in viability compared with the control cells. Consequently, the viability of cells did not decrease significantly at the lowest concentration tested (0.1 mM), with the viability being approximately 91% in this case. In contrast, the higher concentrations resulted in a marked reduction in cell viability, with the most significant effect observed at 1 mM, where the cell viability reached approximately 19% (Figure 1b).

Using fluorescence microscopy, nuclear and cytoskeletal changes in human gingival fibroblasts and squamous carcinoma cells were analyzed after treatment with 0.5 mM Eug. The untreated HGF cells showed no nuclear or cytoskeletal changes under fluorescence microscopy, while in the Eug-treated cells, a slight nuclear condensation was noticed. Eug-treated SCC-4 cells stained with DAPI showed signs of chromatin condensation and fragmentation inside the nuclei. The organization of actin fibers suffered significant changes after treatment with the 0.5 mM concentration of Eug. In the cells stained with rhodamine phalloidin, condensation of actin fibers, especially at the periphery and cell rounding, was noticed. Morphological evaluation was conducted in comparison with control cells where no significant changes were seen (Figure 2).

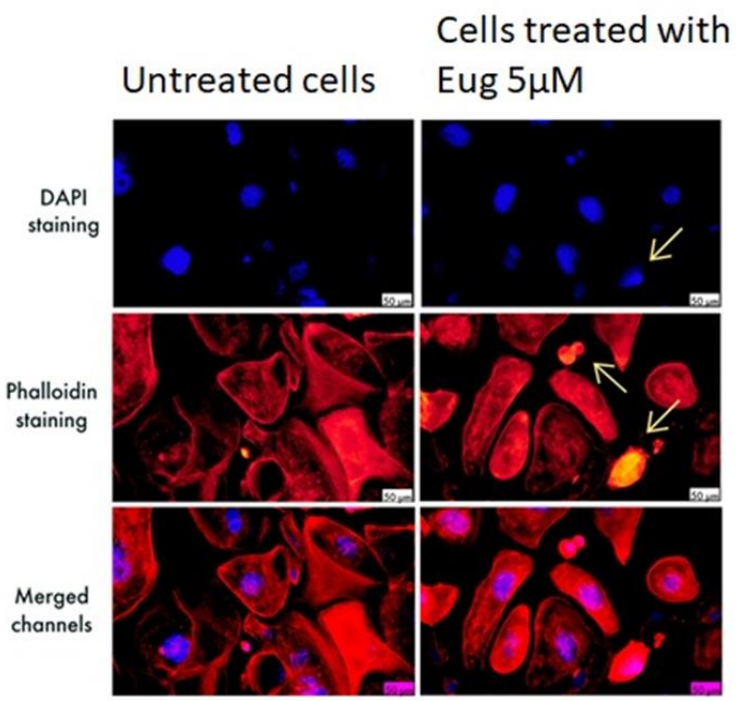

Figure 2. SCC-4 cells visualized by fluorescence microscopy, after treatment with Eug 0.5 mM. The impact of Eug at the level of: nuclei—DAPI staining (blue) and F-actin fibers—rhodamine phalloidin (red). Nuclear fragmentation, chromatin, and peripheral actin fiber condensation can be seen in the treated cells, marked with arrows.

In tongue squamous carcinoma cells, the sub-toxic dose of Eug, 0.5 mM, significantly increased the expression of mRNAs for the pro-apoptotic markers—Bax and Bad, the most

significant increase was recorded for the expression of Bax. Furthermore, Eug had no significant effect on the expression of Bcl-2, an anti-apoptotic gene (Figure 3).

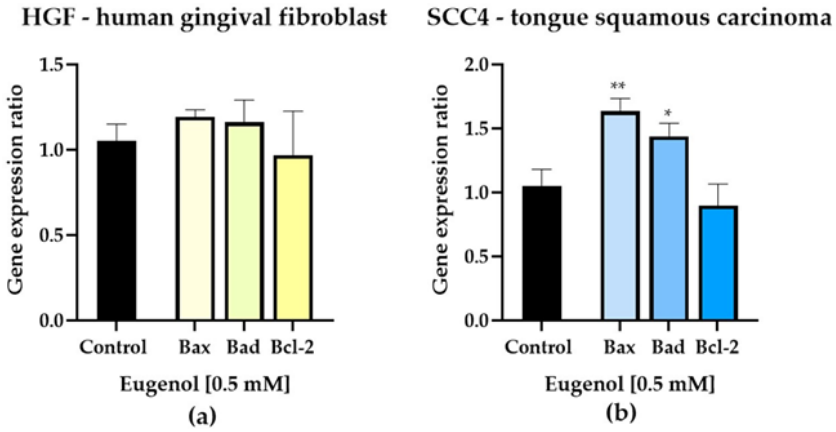

Figure 3. Relative fold change expression of mRNA of proapoptotic (Bax and Bad) and anti-apoptotic (Bcl-2) markers in human gingival fibroblast (HGF) (a) and in tongue squamous carcinoma cells (SCC-4) (b) 72 h after exposure to Eug 0.5 mM. mRNA expression levels normalized to 18 S expression.

The next chapter was foccused on in vitro and in ovo evaluation of Eug on osteosarcona, oropharyngeal and colon cancer, in order to confirm the aplicability of in vitro tests in dental field. Thus, to investigate the potential of in vitro antitumor activity of Eug on SAOS-2 and Detroit 562 cell lines, an MTT assay was performed at the end of the 72 h treatment. According to the results (Figure 4), Eug exerted a concentration-dependent cytotoxicity against both types of cancer cells.

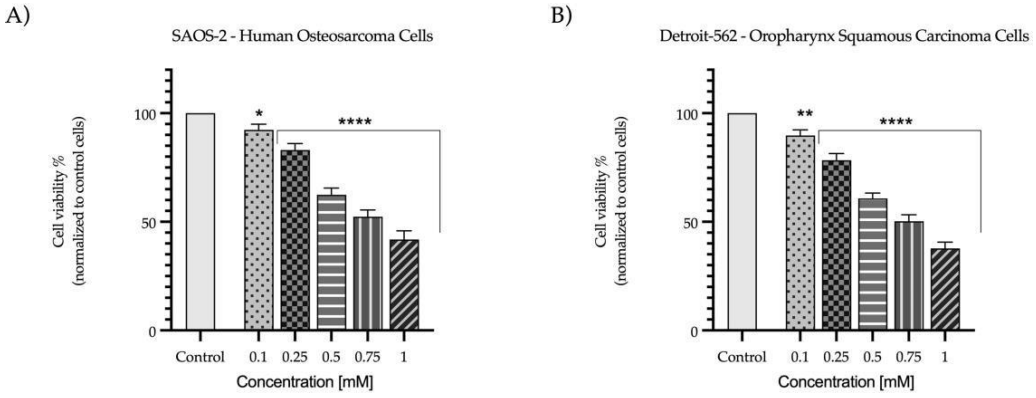

Figure 4. In vitro cell viability evaluation of Eug (0.1, 0.25, 0.5, 0.75, and 1 mM) in: (A) SAOS-2 (human osteosarcoma cells) and (B) Detroit-562 (oropharynx squamous carcinoma cells).

To evaluate the potential cell-death mechanism related to the cytotoxicity of Eug on SAOS-2 and Detroit-562 cancer cells, DAPI staining was performed to highlight the morphological changes occurring at the nuclear level at 72 h post-treatment. Comparatively, the nuclei of the SAOS-2 and Detroit-562 cells exposed to Eug (0.5 and 1 mM) presented an apoptotic-like appearance, being fragmented and massively condensed. Consequently, a significant elevation in the apoptotic index (to 25% for SAOS-2 cells and 30% for Detroit-562 cells) was determined compared to the control (Figure 5 and 6).

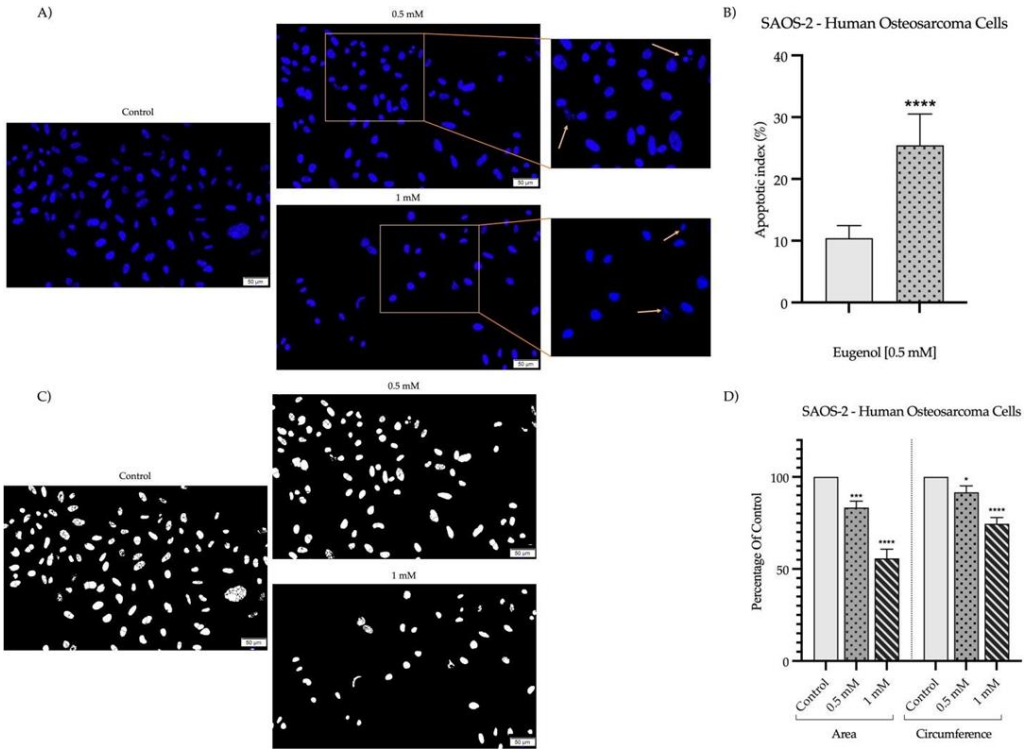

Figure 5. (A) SAOS-2 nuclei stained with DAPI after 72 h treatment with Eug (0.5 and 1 mM). The yellow arrows indicate signs of apoptosis. The scale bars represent 100 µm. (B) Apoptotic index (AI) determination in DAPI-stained SAOS-2 cells following 72 h treatment with 0.5 mM Eug. (C) ImageJ analyses of nuclear morphology. (D) As compared to control cells, Eug stimulation (0.5 and 1 mM) led to a decrease in the area and circumference of the nuclei

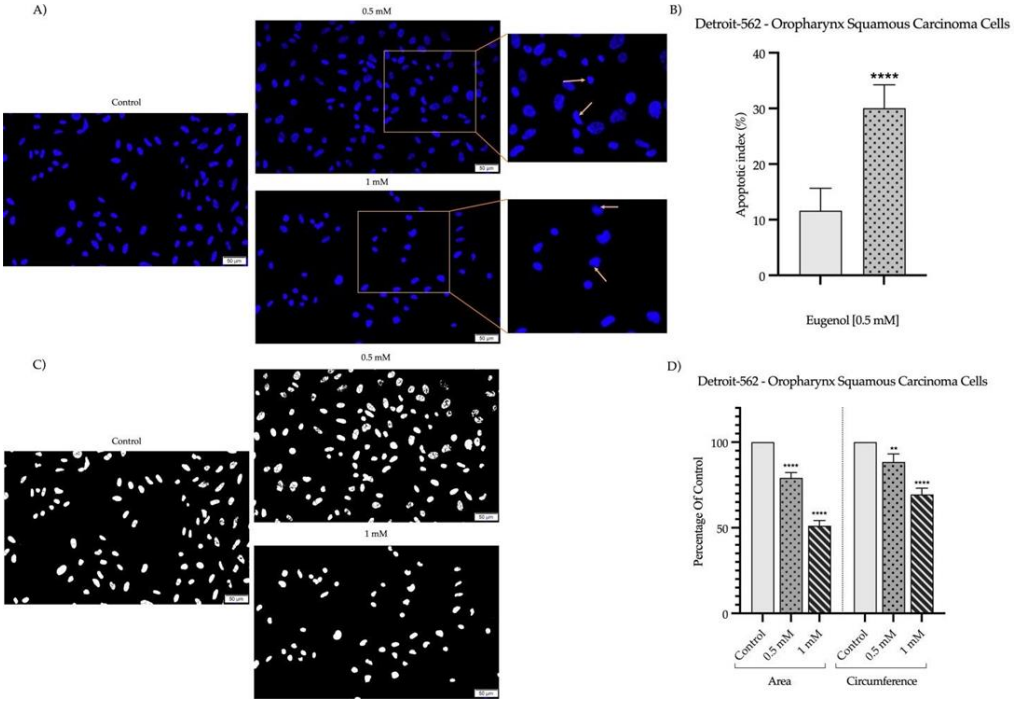

Figure 6. (A) Detroit-562 nuclei stained with DAPI after 72 h treatment with Eug (0.5 and 1 mM). The yellow arrows indicate signs of apoptosis. The scale bars represent 100 µm. (B) Apoptotic index (AI) determination in DAPI-stained Detroit-562 cells following 72 h treatment with 0.5 mM Eug. (C) ImageJ analyses of nuclear morphology. (D) As compared to control cells, Eug stimulation (0.5 and 1 mM) led to a decrease in the area and circumference of the nuclei

The ability of Eug 0.5 mM to induce apoptosis in SAOS-2 and Detroit-562 cancer cells was also evaluated by applying a kinetic annexin-based method. As presented in Figure 7, the

treatment with Eug 0.5 mM induced a time-dependent increase in the luminescence signal, which preceded the increase in fluorescence signal (indicating membrane integrity loss due to secondary necrosis), thus, showing an apoptotic response in both treated cell lines following treatment. In addition, Eug caused vascular lysis and coagulation at the vascular level but only to a limited extent (Figure 8).

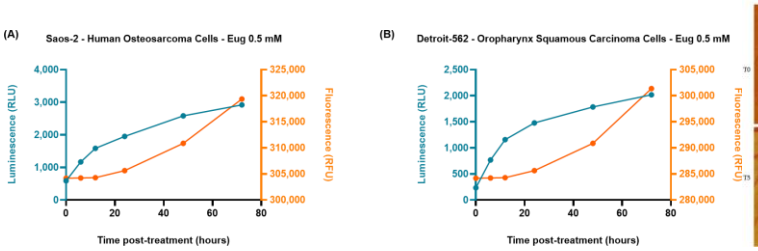

Figure 7. Saos-2 (A) and Detroit-562 (B) cells were treated with Eug 0.5 mM and the RealTime-Glo™ Annexin V Apoptosis/Necrosis Assay reagent.

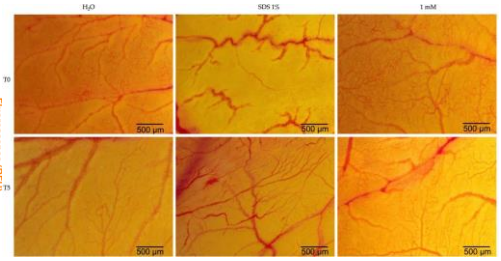

Figure 8. Analysis of the irritant potential of eugenol at a concentration of 1 mM by the HET- CAM method.

An important aspect is the fact that products used in dentistry, in smaller or larger quantities, can reach the gastro-intestinal tract, thus in the following study, Eug was tested in colorectal cancer cells HCT-116. So it was noticed the fact that, Eug decreased the percentage of viable cells according to the concentration tested. Therefore, at the lowest concentration tested (0.1 mM), a significant decrease in cell viability of approximately 88% was observed. The most significant effect was observed at a concentration of 1 mM, when the percentage of viable cells decreased to approximately 34% (Figure 9A).

On the other hand, cell apoptosis is characterized by a series of morphological changes such as cell contraction, the appearance of apoptotic bodies, cell rounding. All these changes were also observed in the present study, indicating that Eug can induce apoptotic-like effect. The effect of Eug 0.5 mM on the structure of the nuclei and actin filaments of HCT-116 cells was examined in order to obtain a more detailed understanding of potential targets and biological mechanisms (Figure 9B).

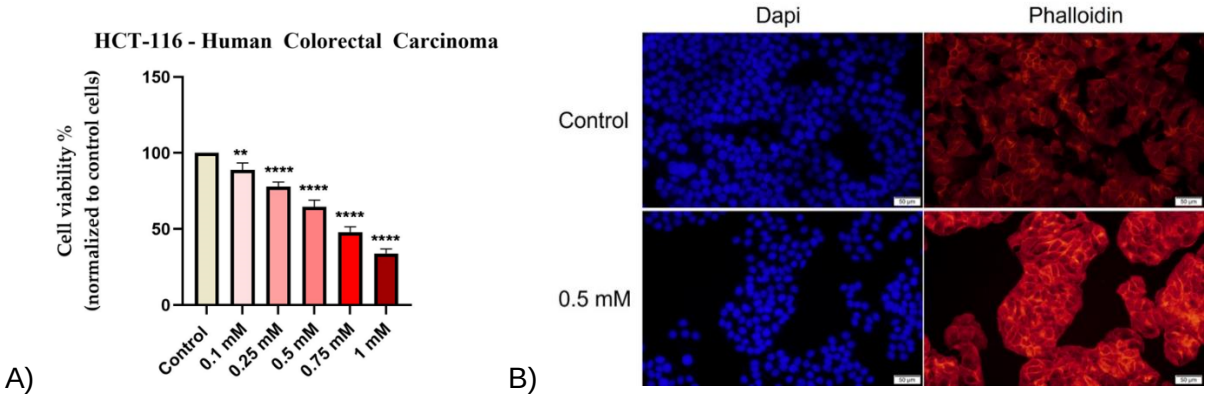

Figure 9. A) In vitro assessment of the cytotoxic effects of Eug (0.1, 0.25, 0.5, 0.75 and 1 mM) at the level of human colorectal carcinoma – HCT-116 after 72 hours of treatment. B) The impact of Eug 0.5 mM in HCT-116 cells on:

nuclei—DAPI staining (blue) and F-actin fibers—Phalloidin (red). The pictures were taken using 20× objective at a scale bar of 50 µm.

At a concentration of 0.5 mM of Eug induced chromatin condensation and a reduction in nuclei size within colorectal carcinoma cells. Additionally, apoptotic bodies were observed. As well as this, Eug determined a series of changes that occur at the level of actin filaments, such as their condensation and reorganization into peripheral rings.

In the secound part of the same chapter it was analyzed the safety profile of dental materials in order to confirm the applicability of in vitro protocols. Ceramic materials have been shown to be effective in bone reconstruction due to their biocompatibility, availability, and their close similarity to inorganic bone components [6]. To date, there is little evidence of a link between dental implants and oral carcinomas [7]. Thus, the present study was designed to evaluate two types of ceramics, obtained from Ceramco iC Natural DentineDentsply Sirona (P1) and Ceramco iC Natural Enamel, Dentsply Sirona (P2), respectively at the level of the Detroit 562 pharyngeal cancer cell line. After 72 h of stimulation with five concentrations (5, 10, 25, 50, and 75 µg/mL), the effect of the ceramic samples (P1 and P2) suspended in artificial saliva at three pH values (acidic, neutral, and basic) on pharyngeal cancer cells was determined (Figure 10).

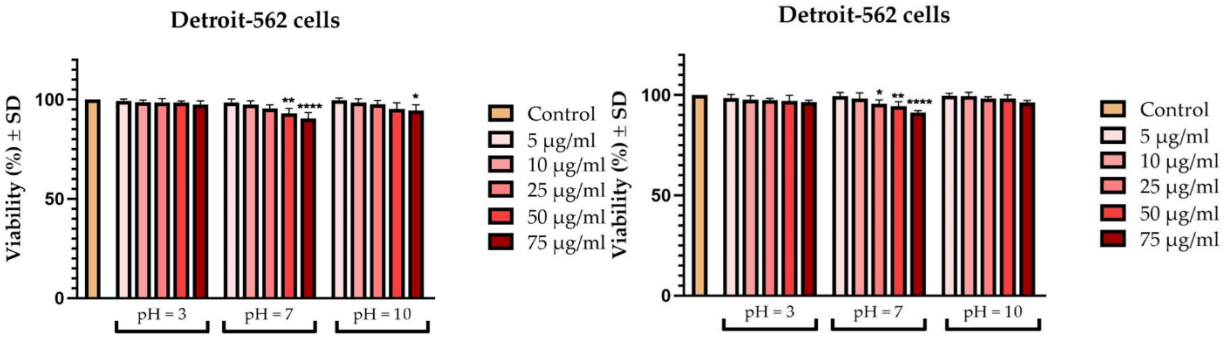

Figure 10. In vitro evaluation of the effect of P1 (left)/P2 (right) (5, 10, 25, 50, and 75 µg/mL) suspended in artificial saliva with acidic pH (pH = 3), neutral pH (pH = 7), and basic pH (pH = 10) at the Detroit-562 cell line level after 72 h of treatment.

To evaluate the influence on the migration of pharyngeal cancer cells, the wound-healing method was applied, in which two concentrations (5 and 75 µg/mL) were suspended in three types of saliva with varying pH values for analysis. The migration of cells was slightly inhibited at all three analyzed pH levels for P1. Among the concentrations tested, 75 µg/mL at pH 7 showed the most potent inhibition of cell migration. P2 also inhibited migration, but this inhibition was less pronounced compared to P1 (Figure 11).

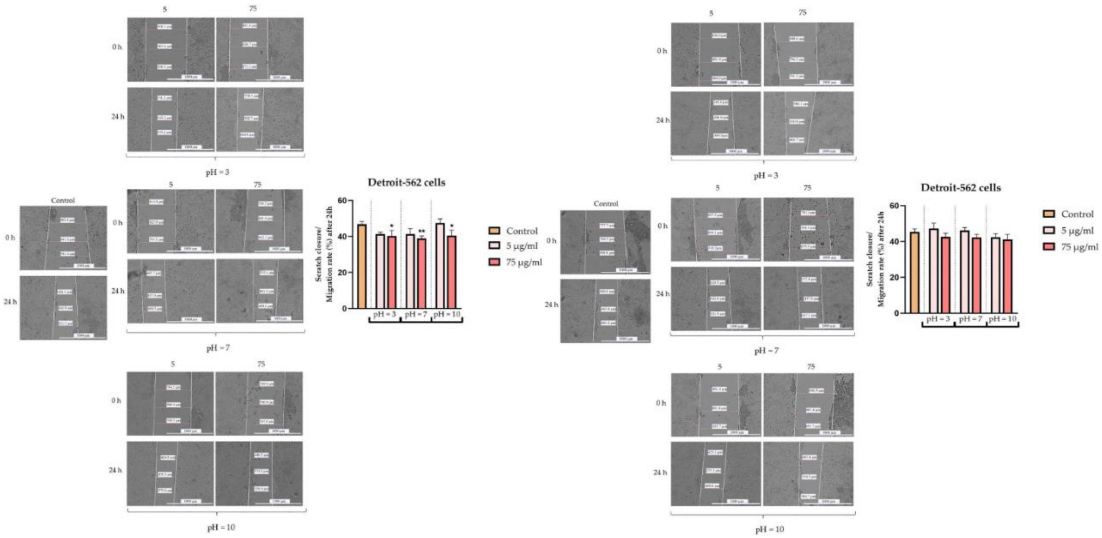

Figure 11. Scratch closure rate of Detroit-562 cells following P1 (left) adn P2 (right) treatment (5 and 75 µg/mL) in artificial saliva with three types of pH (acid, neutral, and basic). The bar graphs are presented as wound closure percentage after 24 h compared to the initial surface.

The next study highlithted the impact of the hydroalcoholic extract of *Viscum album* (VAex), which is known to be efficient on oral cancer, on two skin cancer cell lines: skin epidermoid carcinoma – A431, a squamous cancer, as well on a murine melanoma tumor cell line – B164A5 and, also on a healthy human keratinocyte cell line.

Regarding the effect of VAex the most significat effect was observed on A431, where there were observed a decrease in cell viability from 50 µg/mL (approximately 83%). And in this case, the most cytotoxic concentration was 1000 µg/mL, where the viability value was about 39%. VAex manifests a strong effect on the nuclei of squamous skin carcinoma cells (Figure 12).

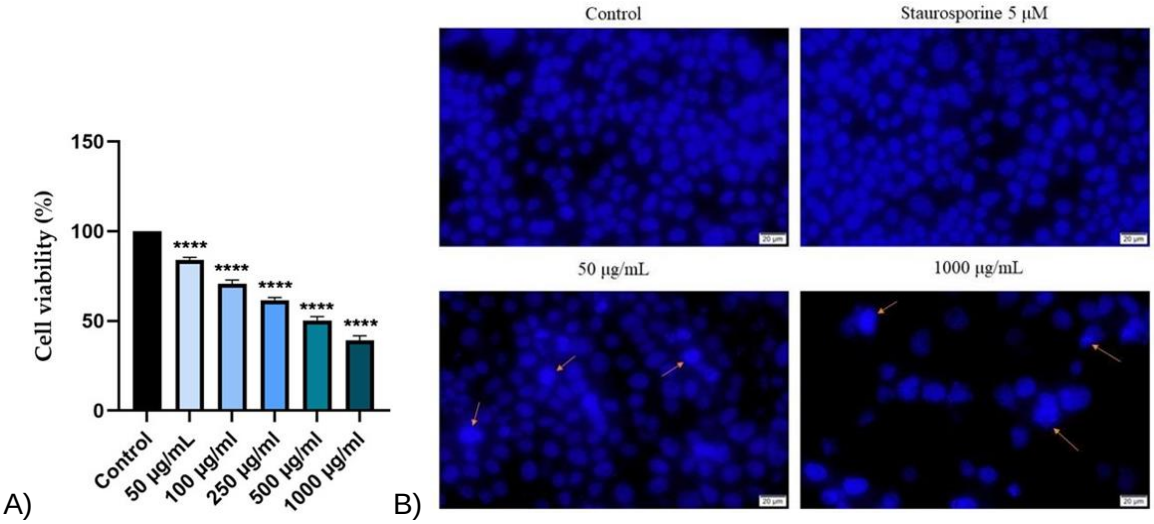

Figure 12. A) In vitro assesment of the effect of VAex (50, 100, 250, 500 and 1000 µg/mL) exerts on the viability of skin epidermoid carcinoma cells (A431) after 24 h of treatment. B) Skin squamous cells carcinoma – A431 nuclei stained with Hoechst 33342 dye after a 24 h treatment with VAex (50 and 1000 µg/mL) Staurosporine (5 µM) was used as the positive control for apoptotic changes at nuclear level. The orange arrows indicate signs of apoptosis.

The last study (starting from the premise that bio-oxidative ozone therapy reduces the number of bacteria), aims to evaluate the impact of gaseous ozone therapy on the morphology and confluence of human primary gingival cells: keratinocytes (PGK) and fibroblasts (HGF), which represent most of the oral cavity resident cells.

A single exposure to gaseous ozone for 20 s induced significant changes in the fibroblasts' morphology and confluence at both 24 and 48 h post-exposure (Figure 16), which were as follows: confluence was very low at 24 h after exposure, and the cells, although still adherent to the plate, displayed a different shape (were much more elongated and had some kind of vesicles on the surface; at 48 h post-exposure to ozone, fibroblasts began to regain their initial morphology, similar to the one exhibited by the control cells. An increase in confluence was also observed compared to those from 24 h exposure, but was still lower than that of control cells. No significant changes in cells morphology and confluence were observed in the case of gingival keratinocytes at this interval. As can be seen in Figures 17, exposure to gaseous ozone for longer periods—40 and 60 s—led to a decrease in the confluence of keratinocytes and even the presence of cells floating in the culture medium. Some changes in morphology similar to those seen in fibroblasts were observed, but were not as intense.

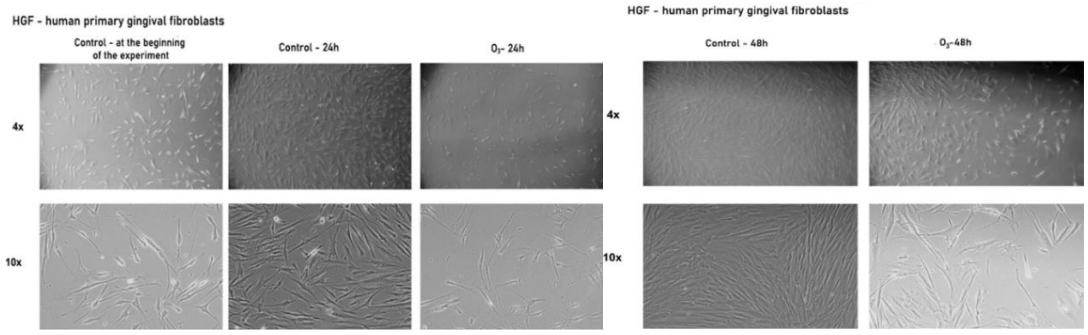

Figure 16. Microscopical aspect of the primary gingival fibroblasts—HGF in culture at 24/48 h post- exposure to ozone gas for 20 s. Pictures were taken with 4× and 10× lenses.

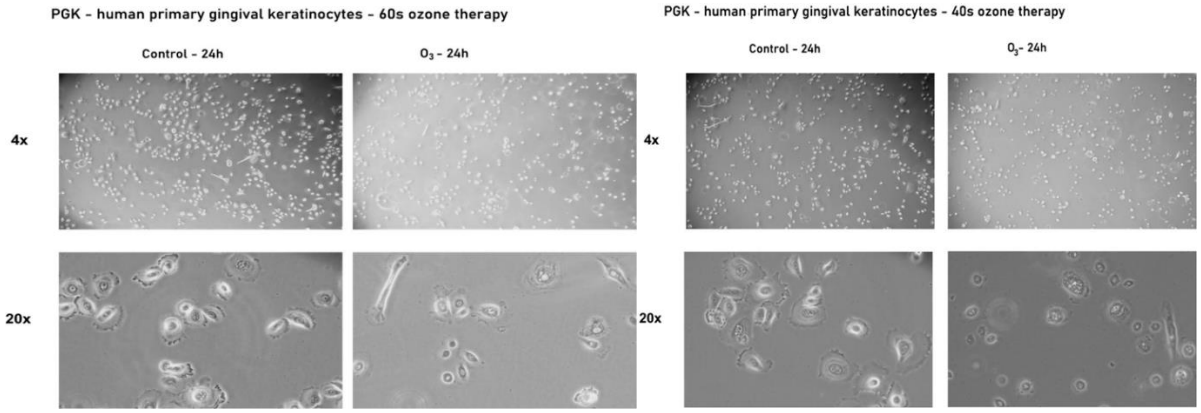

Figure 17. Microscopical aspect of the primary gingival keratinocytes—PGK at 24 h post-exposure to ozone gas for 40 and 60 s. Pictures were taken with 4× and 20× lenses.

Conclusions

The first study focused on evaluating the in vitro cytotoxic effect of Eug, on oral squamous carcinoma cells, as well as at the level of human gingival fibroblasts. According to the results, Eug inhibits tongue squamous carcinoma cells in a dose-dependent manner. Additionally, the cytotoxic effect was associated with the appearance of apoptotic-like changes in cell morphology, as well as in nuclei and cytoskeleton structures. Furthermore, Eug treatment of SCC-4 cells resulted in an increase in the expression of pro-apoptotic markers—Bax and Bad.

In the second research, the cytotoxic potential of Eug was evaluated and analyzed with respect to osteosarcoma, oropharyngeal, and colorectal carcinoma cells. As a result of the studies, Eug inhibits the proliferation of cancer cells in a dose-dependent manner and induced specific apoptotic-like effects. When in vitro protocols were applied on ceramics, results showed that the two ceramic materials tested (P1 and P2) displayed no obvious protumor effects on the pharyngeal cancer cell line Detroit-562.

In the third study, VAex was found to be cytotoxic toward pigmented and non-pigmented melanoma cells in a dose-dependent manner. As a result, 24 hours of stimulation led to a decrease in cell confluence, while, at the same time, morphological changes in the nucleus of the cells were observed, suggesting an apoptotic-like effect. A noteworthy finding of the study was that VAex is more cytotoxic to non-pigmented cells (A431) than pigmented cells (B164A5), highlighting the selectivity in squamous cells.

In the last study, biological ozone therapy, which can be used as a common measure to prevent tooth decay, to take the tooth out from the risk area, to reduce bacterial plaque from pits and fissure, and to create the premises of the enamel remineralization process was tested. Following the bio-oxidative ozone therapy, an improvement in enamel quality with significant changes in the demineralization values of dental tissue was found, from equivalent initial enamel demineralization values to values that corresponded to those of integral enamel, without the risk of a carious process.

In addition, the present study showed that exposure to 20, 40, and 60 s of gaseous ozone determined a cell-type-dependent response, as follows: the gingival keratinocytes were affected by ozone after only 60 s exposure, whereas in the case of gingival fibroblasts, the morphological changes were observed at the shortest interval of exposure— 20 s.

The conducted experimental studies reflect original contributions that mainly complete the existing data regarding the therapeutic profile of naturally occurring compounds with significant importance in dentistry and widen the safety spectrum of both natural compounds and classic ones used in stomatology. In the future, we propose the integration of natural compounds into innovative formulas (nanostructures) to increase their therapeutic potential and their subsequent inclusion in finished formulations, for dental use.

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