








Biosafety and Selective Cytotoxicity of Kojic and Ellagic Acids in Salivary Gland Carcinoma: A Preclinical Perspective

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ABSTRACT: Salivary gland carcinomas (SGCs) are rare, aggressive tumors with high histopathological diversity and resistance to conventional therapies. The need for novel therapeutic approaches has drawn attention to natural compounds with antitumor potential. Objective: This study aims to investigate the in vitro and in ovo cytotoxic and safety profiles of two natural agents, kojic acid (KA) and ellagic acid (EA), on human submandibular salivary gland carcinoma (A253) cells and human immortalized keratinocytes (HaCaT), as well as to assess their irritant potential via the HET-CAM assay. The cytotoxicity and morphological changes of A253 and HaCaT cells were evaluated using the MTT assay and brightfield microscopy. The HET-CAM assay was applied to evaluate the irritant effects of the compounds in ovo. Both KA and EA reduced A253 cell viability in a dose-dependent manner, with the highest cytotoxicity observed at 100 μ M. In contrast, HaCaT cells maintained high viability and exhibited no notable morphological alterations post-treatment, supporting the selectivity of the compounds. HET-CAM scores for both KA and EA fell within the non-irritant range (IS=0.07), further confirming their biosafety. KA and EA exhibit promising antitumor activity against A253 salivary gland carcinoma cells, with minimal toxicity toward normal epithelial cells and no significant irritation potential. These findings justify further investigation of these compounds for their potential use as adjuvant agents in the treatment of salivary gland carcinoma (SGC).

KEYWORDS: Kojic acid, ellagic acid, salivary gland carcinoma, cancer.

Introduction

Cancer, the disease characterized by uncontrolled cell growth and division, remains among the leading causes of death worldwide, which makes it a major health issue on a global scale [1].

Efforts to discover effective cancer treatments are focused on developing novel approaches such as targeted therapies and immunotherapy, as well as on optimizing conventional treatments, including chemotherapy, radiotherapy, and their combinations [2].

Researchers are also investigating the potential use in oncology of drugs already employed in the treatment of other diseases,

whose safety profiles have been previously established, which may facilitate their faster introduction into clinical practice [3].

However, the unsatisfactory selectivity and toxicity of most of these options require further study in order to discover new molecules with more advantageous pharmacological profiles [4].

In the last decades, natural compounds, due to their lower toxicity, have attracted a high level of importance to research.

Compounds of natural origin represent a potentially important therapeutic option, especially since many of these compounds have been shown to be effective in various types of cancer by affecting the process of uncontrolled cancer cell proliferation and metastasis;

therefore, they achieved a favorable effect in cancer regression [5].

Kojic acid (KA) is a natural compound mainly produced as a secondary metabolite by some fungal species such as *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus tamarii*, and *Aspergillus parasiticus* [6].

The main use of KA is in the cosmetic and pharmaceutical industries, especially in whitening products such as creams and soaps, due to its depigmenting action by inhibiting tyrosinase activity; as a consequence of this, the melanin formation is decreased [7].

Other common uses of KA are as a UV-protector agent, next to its various applications in the food industry [8].

Besides these well-known uses, studies indicate that KA has other pharmacological benefits, including its antioxidant activity and the antibacterial effect that seems to be caused by the bacterial membrane disturbing [9,10].

It was documented that KA presents anti-inflammatory, antiviral, and antifungal actions in addition to its antihyperglycemic effect [11].

The anticancer potential of KA was evaluated *in vitro* on multiple cell lines where the antitumor activity was demonstrated [12].

Ellagic Acid (EA) is a natural compound that belongs to the ellagitannins [13].

Many plants have ellagic acid as a bioactive phytocompound; for instance, high concentrations are found in Rosaceae family species such as the fruits of *Rubus ursinus*, *Rubus chamaemorus*, and *Robus idaeus*.

Another rich source is the fruit of *Punica granatum* [14].

EA is characterized with a high antioxidant activity, which could be the reason for several of its reported biological actions [15].

In dermatology, EA has many applications, exhibiting favorable effects in enhancing skin quality and reducing sebum secretion [16].

Also, it is recognized as a depigmenting agent [17,18].

EA displays other pharmacological advantages, like its anti-inflammatory, neuroprotective, microbicidal, antidiabetic, and hepatoprotective effects; even more, there is much evidence that EA promotes the cardiovascular function and protects the body from various types of toxins [19].

Additionally, multiple studies have demonstrated that EA can suppress the growth and multiplication of cancer cells, along with its possible role in cancer prevention [20].

Salivary gland carcinoma (SGC) is an uncommon type of cancer, accounting for approximately 5% of total cases of head and neck squamous cell carcinoma.

Currently, because of the high resistance of SGC to the actual cancer therapies, the treatment options for SGC are limited, so the first-line treatment is surgery followed by chemotherapy and radiotherapy [21,22].

Based on the points mentioned above, this study aims to offer an *in vitro* evaluation of KA and EA on human submandibular salivary gland carcinoma (A253) and human immortalized keratinocyte cells (HaCaT).

The current study was conducted *in vitro* and *in ovo* and aims to evaluate the potential of KA and EA in inhibiting the cell growth on the human submandibular salivary gland carcinoma A253 cell line and the healthy immortalized keratinocyte cell line HaCaT.

Materials and Methods

Kojic acid, ellagic acid, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) kit, FCS (fetal calf serum), and DMSO (dimethyl sulfoxide) were supplied from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany).

Dulbecco's Modified Eagle's medium (DMEM-30-2002TM), McCoy's 5A medium (30-2007TM), trypsin-EDTA solution, and penicillin/streptomycin mixture were purchased from American Type Cell Collection (ATCC Manassas, VA, USA).

The study has the approval of the University of Medicine and Pharmacy "Victor Babes" Ethics Committee (Ethics Approval Nr. 91/04.10.2021 rev 2025).

Equipments

Cytation 5 (plate reader) and Lionheart FX (automated microscope) were obtained from BioTek Instruments Inc. (Winooski, VT, USA).

The SteREO Discovery.V8 stereomicroscope was provided by ZEISS (Jena, Germany).

Preparation of the samples

For the *in vitro* assessment of KA and EA, test samples (KA and EA at 50, 75, and 100 μ M) were prepared by diluting the stock solutions (KA and EA in DMSO) in the culture medium specific to the cell line used. For the *in ovo* evaluation, test samples (KA and EA at 100 μ M) were prepared from the stock solutions (KA, EA in DMSO) by diluting in sterile distilled water.

Cell culture

This study was carried out using a human immortalized keratinocyte HaCaT cell line, procured from CLS Cell Lines Service GmbH (Eppelheim, Germany), and human submandibular salivary gland carcinoma A253 (HTB-41™), purchased from ATCC (American Type Cell Collection) as a frozen vial.

The cells were cultured in their medium, DMEM for HaCaT cells and McCoy's for A253 cells.

The mediums were supplemented with 10% FCS and 1% 100U/ml Penicillin/100µg/ml Streptomycin. The cells were incubated at 37°C and 5% CO₂ in the course of the experiment.

Cellular viability evaluation

The cell viability assessment was performed using the MTT technique.

Three concentrations of KA and EA were evaluated on A253 and HaCaT cells, cultured in 96-well plates (10⁴/well), after 24h of treatment it was added 100µl of fresh media and the MTT reagent at 10µl/well, after 3h of incubation at 37°C and 5% CO₂, 100µl/well of solubilization solution was added, following the incubation of the plate at room temperature for 30min in a dark place, and the absorbance reading at 570 and 630nm by means of Cytation 5.

Morphology assessment

To analyze the impact of KA and EA, on the cellular morphology of HaCaT and A253 cells, a microscopic exposure examination was followed after 24 h of treatment.

The cells were cultured in 96-well plate at a density of 1x10⁴/well.

The morphological modifications were observed using brightfield illumination at x20, using the Lionheart FX automated microscope.

The images were analyzed using Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

HET-CAM (HEN'S EGG TEST-CHORIOALLANTOIC MEMBRANE) assay

In order to evaluate the irritant properties of the samples, the HET-CAM assay was utilized, following a modified protocol based on ICCVAM guidelines (Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM), ICCVAM Recommended Test Method Protocol: Hen's Egg Test-Chorioallantoic Membrane, 2010). Available online: <http://iccvam.niehs.nih.gov/>.

In a controlled environment at regulated parameters of 37 °C and a humidity level of 70%, fertile hen eggs were incubated.

On day four of incubation, 10mL of albumen was removed.

A window was created on the fifth day to observe the embryo vascularization.

The eggs were then sealed with medical adhesive tape.

On the tenth day, the HET-CAM assay was performed by applying the test samples onto the chorioallantoic membrane.

The negative control consisted of sterile distilled water (H₂O_d), while the positive control was sodium dodecyl sulfate (SDS) 1%.

A total of 600µL of both samples and controls were utilized for performing the assay.

Following sample application, vascular responses-hemorrhage (H), lysis of blood vessels (L), and coagulation (C)-were monitored continuously for 5 minutes.

If no reaction was observed within this period, a default time of 301 seconds was assigned, indicating the absence of that vascular response.

Images of the membrane were taken before exposure (T₀) and five minutes after application (T₅) using a Zeiss Discovery v.8 stereomicroscope equipped with an AxioCAM 105 camera.

The images were further analyzed using ZEN Core 3.8 software to assess changes in vascular integrity induced by each sample.

The irritation score (IS) was calculated based on the recorded findings using a specific formula [23]:

$$IS = 5 \times \left[\frac{301 - tH}{300} \right] + 7 \times \left[\frac{301 - tL}{300} \right] + 9 \times \left[\frac{301 - tC}{300} \right]$$

Where tH, tL, and tC represent the time in seconds at which hemorrhage, lysis, and coagulation were first observed.

Lower reaction times indicate faster onset of vascular damage and thus a higher IS value.

Conversely, delayed or absent reactions (values close to 300 seconds) yield lower IS values.

The weighting coefficients (5, 7, and 9) reflect the relative severity of each vascular reaction, with coagulation considered the most critical.

Upon calculating the irritation score (IS), the analyzed samples can be grouped into various categories that reflect their irritant potential.

The classifications include non-irritant samples, which have an irritation score ranging from 0 to 0.9; weak irritants, identified for scores between 1 and 4.9; moderate irritants, which score from 5 to 8.9; and strong irritants, defined by scores from 9 to 21 on the irritation scale

Statistical analysis

The obtained results were statistically analyzed using GraphPad Prism software (version 10.2.3; GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Statistical differences between the control and the groups treated with KA and EA were assessed using one-way ANOVA followed by Dunnett's multiple-comparison test. Results are expressed as mean±SEM (standard error of the mean).

All statistically significant results were marked with “*” (*p<0.05, **p<0.01,

***p<0.001, **p<0.0001), while non-significant differences are reported as p>0.05.

Results

Cellular viability evaluation

For the purpose of evaluating the cell viability of A253 and HaCaT after 24h of treatment, the MTT assay was performed.

The results indicate a decrease in cell viability of A253 in a dose-dependent manner; the highest cytotoxicity was observed at 100µM of Ka (70.81%) and 75µM of EA(66.60%).

On HaCaT cells, an increase in cell viability after the treatment with KA at 50 and 75µM; a slight decrease in cell viability was scored after treatment with 100µM of EA (91,82%).

At 100µM of KA the viability of HaCaT cells was up to 103,92%. Figures 1 and 2 illustrate the viability of A253 and HaCaT cells after 24 hours of exposure to KA and EA at concentrations of 50, 75, and 100µM.

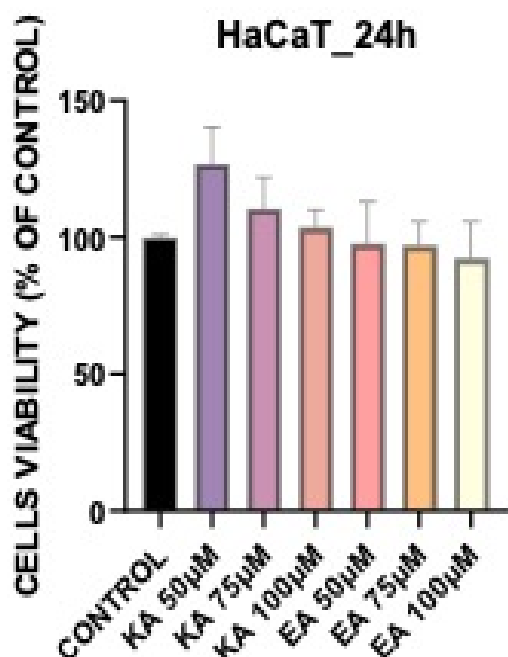


Figure 1. A graphical representation of the viability of HaCaT cells after 24 h of treatment with KA and EA (50, 75, 100µM). The results are expressed as a percentage (%) normalized to control. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison post-test, which revealed no statistically significant differences between the treatment groups and the control (p>0.05).

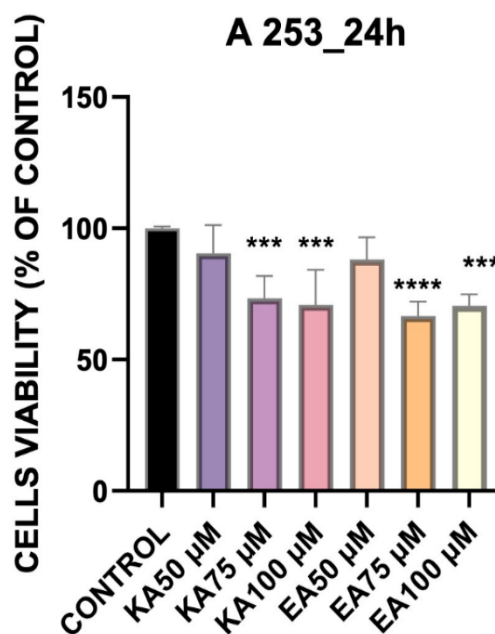


Figure 2. A graphic illustration of cell viability of A253 cells after 24 h of treatment with KA and EA (50, 75, 100µM). The results are shown as a percentage (%) normalized to control. To analyze statistical differences between control and treatment groups, the one-way ANOVA test was followed by Dunnett's multiple comparison post-test. “***” marks statistical significance (*p<0.05, **p<0.01; ***p<0.001; ****p<0.0001).

Morphology assessment

The morphology assessment of HaCaT and A253 reveals no significant modifications of cell shape after 24 h treatment for HaCaT.

On A253, a decrease in cell confluency and a slight change in cell morphology were observed

after treatment with KA and EA at 75, 100µM, cells being more elongated after the KA and EA exposure.

Figures 3 and 4 show the morphological changes in A253 and HaCaT cells after 24-hour treatment with KA and EA at 50, 75, and 100µM.

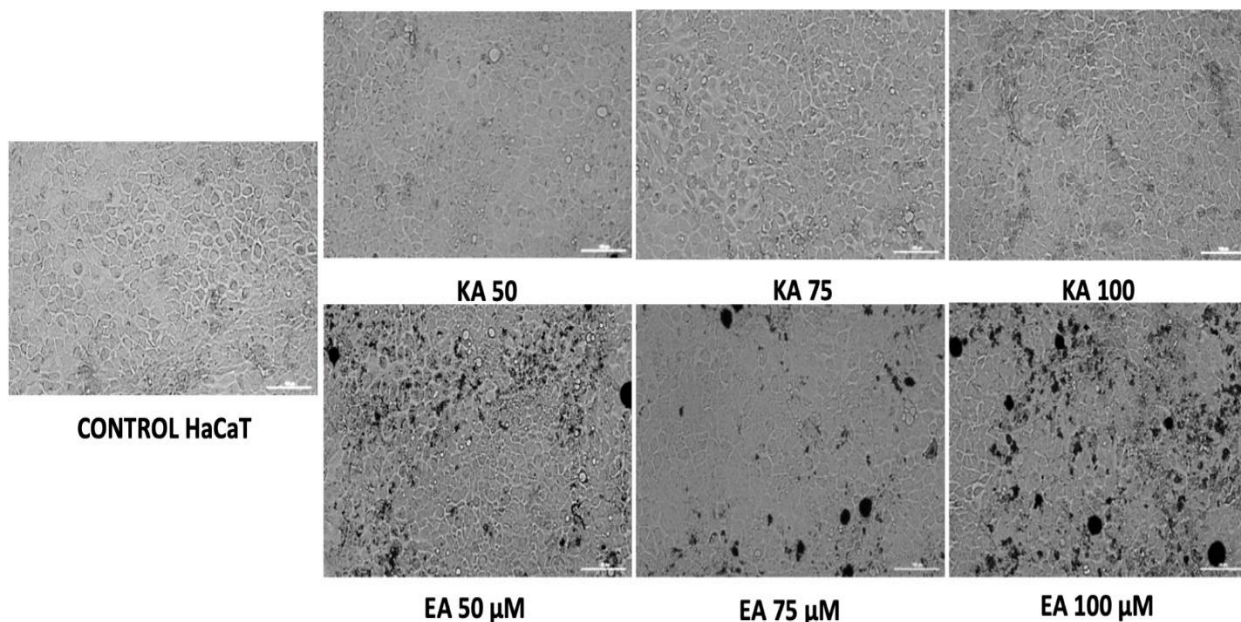


Figure 3. The morphological appearance of HaCaT after 24 h treatment with KA and EA at 50, 75, and 100µM. The scale indicates 100

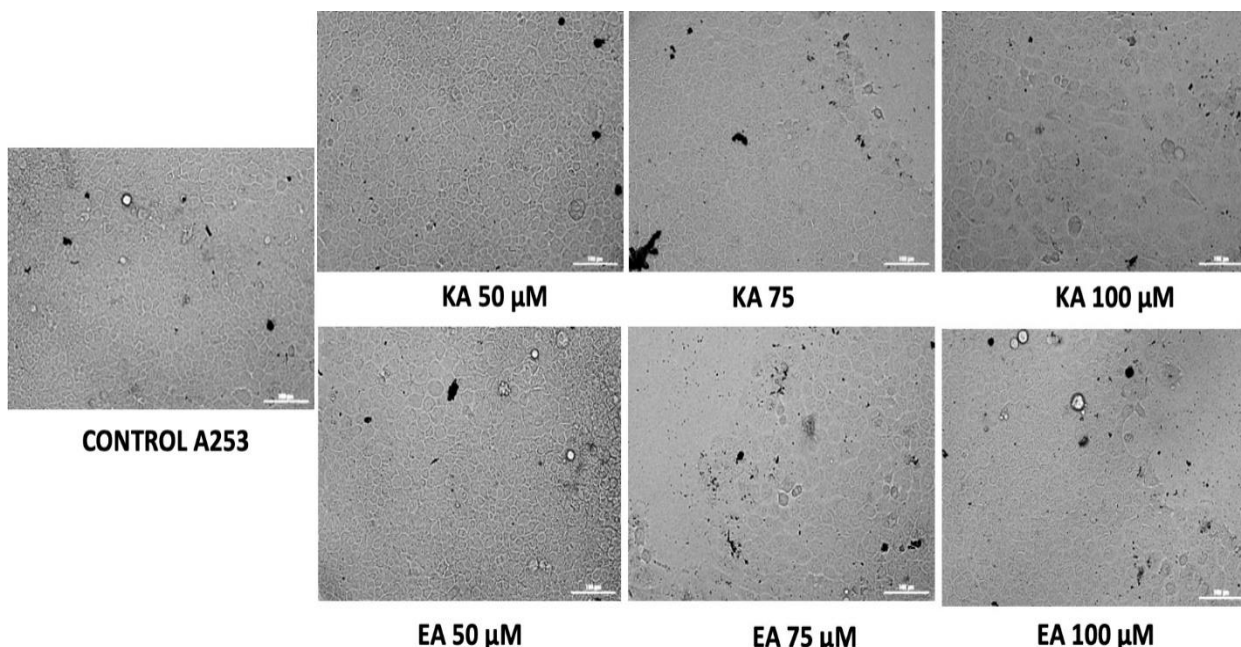


Figure 4. The morphological appearance of A253 after 24 h treatment with KA and EA at 50, 75, and 100µm. The scale indicates 100µm

HET-CAM (HEN'S EGG TEST-CHORIOALLANTOIC MEMBRANE) assay

The HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) assay was employed to evaluate the potential irritant effects of EA (100 μ M), KA (100 μ M), alongside a positive control (sodium dodecyl sulfate 1%) and a negative control (distilled water).

The irritation scores (IS) obtained from the assay are as follows: distilled water exhibited an IS of 0.07, indicating a non-irritant profile; SDS, as a strong irritant, yielded an IS of 16.360; KA and EA, both recorded an IS of 0.07, categorizing them as non-irritants;

The results indicate that all tested samples, apart from SDS, fall within the non-irritant

category, which is defined by an irritation score ranging from 0 to 0.9.

This classification is critical as it suggests that both individual compounds do not provoke significant irritation to the chorioallantoic membrane.

The positive control, SDS, demonstrated a significantly elevated irritation score, firmly placing it within the strong irritant category, which ranges from 9 to 21 on the irritation scale.

This stark contrast between the irritant properties of SDS and the tested compounds underscores the safety profile of EA and EA.

Stereomicroscopic images of the CAM under KA and EA treatments at different concentrations are shown in Figure 5.

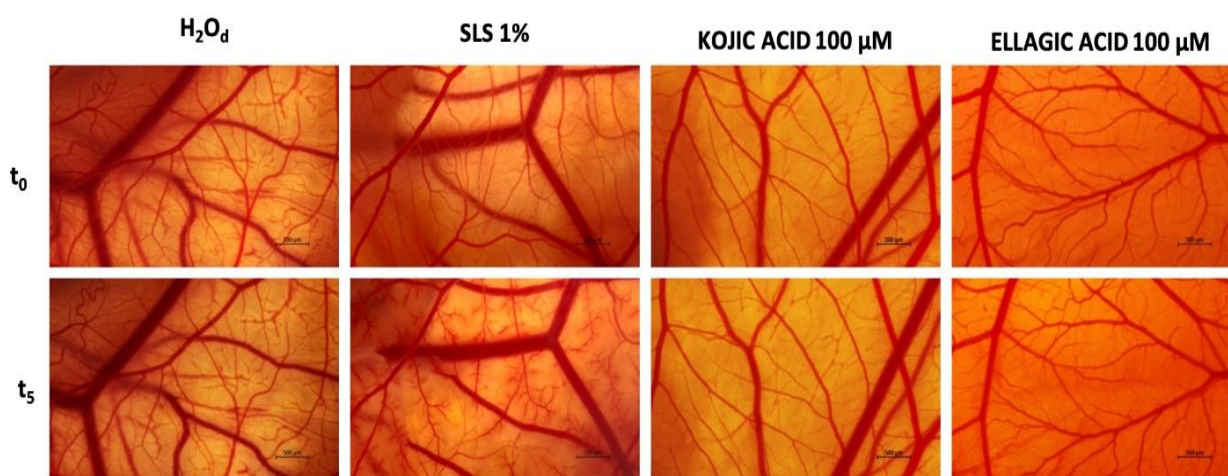


Figure 5. Stereomicroscopic images of CAM for the positive and negative control and KA, EA at 50, 75, and 100 μ M at times t₀ (before application) and t₅ (5 minutes after application).

Discussion

SGCs are among the rarest types of tumors, being represented by 0.5% of total cancer cases worldwide.

According to the WHO, SGCs feature around 24 histological subtypes, each of which is associated with a different clinical profile and prognosis [24].

The etiology of SGCs is diverse and not yet fully elucidated; thus, diet may be a predisposing factor, in addition to radiation exposure or prior presence of benign salivary tumors [25].

Given the diversity of this disease, in addition to the high resistance of these tumors to most current therapies, surgical resection is currently the first choice in most diagnosed cases, a fact that highlights the need for continued research to identify new potential therapies for SGCs [21,22].

In this context, a natural therapy approach could be an alternative in SCG management since many naturally derived compounds have been found to be effective against many forms of cancer by targeting molecular mechanisms involved in carcinogenesis, modulation of the tumor microenvironment, and induction of apoptosis [5].

KA, a naturally occurring compound produced by fungi, is primarily recognized for its skin-lightening properties due to its potent inhibition of tyrosinase, an enzyme critical for melanin production.

Additionally, KA has been shown to possess antioxidant properties, contributing to its protective effects against oxidative stress [26-28].

The antiproliferative effect of KA appears to be a result of the suppression of releasing reactive oxygen species and the nuclear factor Kappa B (NF- κ B), which is involved in the cellular inflammatory responses, along with activation of the MAPK pathway [29,30].

EA is a polyphenolic compound found in various fruits and nuts that has garnered attention for its diverse biological activities, including antioxidant, anti-inflammatory, and anticancer properties.

Studies have demonstrated that EA exhibits significant antioxidant activity, as evidenced by its ability to scavenge free radicals in assays such as DPPH and ABTS [31,32].

This high potential as an antioxidant agent makes EA a promising compound for the treatment of various diseases, including cancer [15].

The mechanism of antitumor action of EA is complex; for instance, there is some evidences that EA has the ability to inhibit Casein kinase II (CK2), which plays a key role in cell differentiation and proliferation by modulating several proteins involved in tumor suppression, such as p53 and PTEN, and in tumorigenesis, including c-Myc, c-Jun, and NF- κ B.

In addition, EA regulates several cell cycle proteins such as CDK2, protein kinase C alpha (PKC α), and the vascular endothelial growth factor (VEGF) [33].

In the light of all these mentioned data, in addition to the fact that to date, no study has evaluated the effect of KA and EA on salivary gland tumors.

The first step was to evaluate the cytotoxic activity of KA and EA on both cell lines as well as the safety on the HaCat cell line using the MTT (4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, which is a common method used to evaluate the *in vitro* toxicity based on converting MTT into formazan crystal by viable cells in order to measure the cell viability [34].

According to the results of the current study, HaCat cells showed greater viability compared to the control after treatment with KA, scoring 103.92% viability at 100 μ M.

After the treatment with EA, a slight decrease in cell viability was observed, being 91,82% at 100 μ M.

On A253, the MTT assay revealed an antiproliferative effect in a dose-dependent manner for cells treated with KA, being 70.81% at 100 μ M.

The lowest percentage of viability (66.60%) after EA exposure was recorded at 75 μ M.

Previous studies demonstrate the cytotoxic effect of KA and EA on multiple cell lines.

Mahmoud GA et al. tested KA on HepG2, Huh7 (hepatocarcinoma cell lines), and Mcf7 (breast cancer cell line), where it was confirmed the antiproliferative action on all of these cell lines [12].

The antitumor assessment on A375 (a melanoma cell line) also showed a decrease in cell viability after the treatment with KA [35].

According to Wang F et al., EA can decrease the cell proliferation rate of WM115 and A375 (melanoma cell lines) [33]; this effect might be caused by lowering the expression of the epidermal growth factor receptor (EGFR) [36].

The antitumor potential of EA was also evaluated on other cell lines where the decreasing action on the cells viability and division was observed [37].

Examples of these cell lines are: PANC-1 (pancreatic cancer cell line) [38], HeLa (cervical carcinoma cell line), PC3 (prostate cancer cell line) [39], and HepG2 (hepatocarcinoma cell line) [40].

Considering the MTT assay's results, the second step in this study was to determine the impact of KA and EA treatment on cell morphology.

The morphology assay is a key method for analyzing the effect of natural substances on cell lines since it offers information about changes in cell shape, size, and expansion, contributing to a better understanding of the mechanism of action of tested compounds [41].

When KA and EA were applied to the HaCat cell line at 50, 75, and 100 μ M, no relevant dysmorphologies were noticed.

By contrast, on the A253 cell line, changes in cell shape were observed after the exposure to KA and EA at 75 and 100 μ M; the cells had developed a slightly elongated morphology, and the cell confluency was decreased.

Studies involving the impact of natural compounds on cell viability and morphology of the A253 cell line are relatively limited.

However, a few studies have explored the effect of specific natural compounds on salivary gland carcinoma.

Su-Bin Park et al. evaluated for the first time the antiproliferative potential of the natural lactone Esculetin on the A253 cell line at 50, 100, and 150 μ M.

The results indicate a dose-dependent cytotoxic effect, being approximately 50% at 150µM after 24h of treatment.

The study also indicates that Esculetin exhibits pro-apoptotic activity by increasing the pro-apoptotic proteins like Bax and decreasing the antiapoptotic protein Bcl-2 [42].

Other study conducted by Peng Xiao et al. suggest that artemisinin extracted from the species *Artemisia annua*, when applied to the A253 cell line, reduced the cell viability significantly at concentrations between 10 and 30µM, producing changes in cell morphology and confluence.

These effects seem to be a result of increasing the pro-apoptotic proteins (Bax, Bim, Bad, Bak) and decreasing the anti-apoptotic proteins (Bcl-2, Bcl-XL), additionally to activating caspase-3 and PARP1 [43].

The combination of *in vitro* and *in ovo* assays for determining the toxicity behavior has the advantage of offering further details regarding the safety profile of tested compounds.

The hen's egg chorioallantoic membrane (CAM) method provides valuable data about the inflammatory response and the irritacy potential; thus, the integration of the HET-CAM assay into the assessment of these compounds can facilitate the development of safer and more effective treatments [44].

Therefore, in the next step, the HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) assay was employed to evaluate the potential irritant effects of EA (100µM) and KA (100µM), alongside a positive control (sodium dodecyl sulfate 1%) and a negative control (distilled water).

The findings from the HET-CAM assay provide compelling evidence regarding the irritant properties of ellagic acid and kojic acid.

The irritation scores obtained for distilled water, kojic acid, and ellagic acid, all being 0.07, suggest that these substances do not induce any significant irritation when applied to the chorioallantoic membrane.

This is particularly noteworthy given the increasing interest in the application of these compounds in various pharmaceutical fields, where safety and biocompatibility are paramount.

EA, a natural polyphenol, and KA, a bioactive metabolite derived from fungal sources, exhibit potent antitumor action through the inhibition of cellular proliferation and the induction of apoptosis.

The results obtained from this study reveal that both compounds have significant biological activity

on the A253 cell line and satisfactory safety on the HaCaT cell line.

Notably, this study represents the first exploration of EA and KA on the A253 cell line, an *in vitro* model for salivary gland carcinoma.

These aspects underlie the necessity for further research to identify the molecular mechanisms that produce the cell toxicity on the A253 cell line after applying EA and KA in different concentrations.

The present study assessed the cytotoxic activity of two natural compounds, EA and KA, *in vitro* on the human submandibular salivary gland carcinoma A253 cell line and the healthy immortalized keratinocyte HaCaT cell line, as well as *in ovo* on the chorioallantoic membrane of chicken eggs.

The results demonstrate a cytotoxic action of EA and KA on A253, reducing the cell viability in a dose-dependent manner and inducing dysmorphologies and a decrease in cell confluency.

Conclusions

On the HaCaT cell line, there was no significant cytotoxic effect, which demonstrated their biosafety.

Both EA and KA are the non-irritant effects on the chorioallantoic membrane of chicken eggs.

These findings underscore the promising potential of KA and EA in addressing underexplored types of cancer, such as SCG, highlighting the importance of a more in-depth study of these compounds and their future applications in cancer therapy.

Author Contributions

Conceptualization, F.I. and C.T.; Methodology, D.H.A., S.D. and I.M.; Data analysis, L.C.R. and D.H.A.; Manuscript writing and initial draft preparation, F.I., S.D., D.C.; Manuscript review and editing, C.T. and I.M.; Supervision, L.C.R and C.T. All authors read and approved the final manuscript.

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Conflicts of interest

The authors declare no competing interests

Institutional Review Board

The study was conducted according to the guidelines of the Declaration of Helsinki.

Consent Statement

Not applicable.

Data availability

All data presented in the manuscript are available from the authors upon request.

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