https://doi.org/10.31925/farmacia.2024.5.4 **ORIGINAL ARTICLE**

EUGENOL - A NATURAL ALTERNATIVE IN DENTISTRY: AN *IN VITRO* AND *IN OVO* BIOSAFETY ASSESSMENT

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Abstract

Oral pathologies and dental problems, in general, have become a public health problem in recent years, with an alarming spread. Dental problems can occur at any age and risk factors are varied, including genetic susceptibility, smoking, or drinking alcohol. Eugenol (EUG) is a compound of natural origin intensively explored for its multitude of biological properties such as analgesic, anti-inflammatory, anesthetic, antidiabetic, neuroprotective, antimicrobial, or anticancer. In addition, EUG is also recognised to be found in dental and oral care products, where it has been shown to inhibit tooth decalcification. The purpose of the present study was to evaluate EUG (10, 25, 50, 75 and 100 µg/mL) *in vitro* for the biosafety on two healthy cell lines (HaCaT and JB6 Cl 41-5a) and also for its irritant potential *in ovo*. The results showed that EUG is a compound that does not induce cytotoxicity on HaCaT cells (cell viability being above 70% at all tested concentrations), but sensitizes JB6 Cl 41-5a cells in a dose-dependent manner, with cell viability reaching up to about 55%. According to the *in ovo* experiment, EUG was included in the category of non-irritant agents following the HET-CAM assay.

Rezumat

În ultimii ani, afecțiunile orale și, în general, problemele stomatologice au devenit o problemă de sănătate publică, răspândindu-se alarmant. Problemele dentare pot apărea la orice vârstă iar factorii de risc sunt variați incluzând susceptibilitatea genetică, fumatul sau consumul de alcool. Eugenolul (EUG) este un compus de origine naturală explorat intens pentru multitudinile de proprietăți biologice pe care le deține precum cele analgezice, antiinflamatorii, anestezice, antidiabetice, neuroprotectoare, antimicrobiene sau anticanceroase. În plus, EUG este recunoscut și pentru faptul că este regăsit în componența produselor de îngrijire dentară și orală, unde s-a demonstrat că inhibă decalcifierea dinților. Scopul prezentului studiu este evaluarea EUG (10, 25, 50, 75 și 100 µg/L *) in vitro* în vederea biosiguranței asupra două linii celulare sănătoase (HaCaT și JB6 Cl 41-5a) dar și a potențialului iritant *in ovo*. Rezultatele obținute au demonstrat că EUG este un compus ce nu induce citotoxicitate asupra celulelor HaCaT (viabilitatea celulară fiind peste 70% la toate concentrațiile testate), însă sensibilizează celulele JB6 Cl 41-5a într-o manieră dozo-dependentă, viabilitatea celulelor ajungând până la aproximativ 55%. Conform experimentului *in ovo,* EUG a fost inclus în categoria agenților non-iritanți în urma testului HET-CAM.

Keywords: eugenol, cytotoxicity, biosafety, stomatology

Introduction

Phytocompounds play an essential role in increasing the quality of life for patients suffering from various diseases. In recent years, with the development of scientific research, more and more botanical resources have proven to be important tools in health promotion. Dental hygiene is a primary factor in human health, and using compounds of natural origin is a valuable alternative to synthetic compounds [5] also, oral health can be affected at all ages, with a multitude of risk factors including medications, genetic susceptibility or infection with human immunodeficiency virus [14].

Oral diseases are spreading alarmingly nowadays, and the need for oral preventive hygiene practices and discouraging harmful practices such as smoking or alcohol consumption is growing. Also, it is a key point for patients to understand that bioactive natural compounds are able to positively modulate the composition of the oral microbiota [6]. Botanical-based medicine is currently used by about 80% of the global population, especially by people living in rural areas, and the present studies have focused on investigating the efficacy of several types of natural products for dental diseases, recognised for their economic benefits and antimicrobial properties that

serve to protect against the development of dental caries, a major public health problem [3, 11, 15, 37]. A large number of phytocompounds remain untested or insufficiently tested, resulting in a lack of knowledge about their mechanism of action, potential side effects, interactions, or contraindications [15] information of interest to progress in the herbal pharmaceutical industry. Eugenol (EUG) is a natural compound, a phenylpropene, an allyl chain-substituted guaiacol, widely explored for its biological effects and has been reported to be present in several plant families including *Zingiber officinale* (ginger) or *Eugenia caryophyllata* (clove). EUG or 4-allyl-2-methoxyphenol possesses anti-microbial, anti-inflammatory, analgesic, antidiabetic, neuroprotective and anticancer effects, which outlines a versatile profile of the botanical compound that arouses interest [18, 19, 27, 33]. In stomatology, EUG is recognised for anaesthesia, analgesia [29] and anti-inflammatory effects, being successfully applied in combination with zinc oxide (ZOE) as a coating material in pulpotomy techniques on primary teeth, where the affected portion of the pulp coronary tissue is removed and the combination between EUG and ZOE is placed. Besides this use, EUG can also be found in dental and oral care products, where it has been demonstrated to inhibit decalcification of teeth caused by various apple acidic beverages, and in addition, it can promote remineralization [23, 34, 36]. However, EUG also has reported side effects such as contact dermatitis, local irritation, or allergic reactions that may be present even at low doses [21, 32].

In light of the above, the present study frames the investigation of EUG concerning its cytotoxic action on HaCaT - human immortalised keratinocytes and neonatal BALB/c epidermal cells - JB6 Cl 41-5a, taking into account the possible side effects on skin and the development of its application in the domain of dentistry. The *in vitro* evaluation is complemented by the *in ovo* exploration of the irritant potential of the compound by the HET-CAM method. The safety profiling may point the product toward future directions of research and use in the medical-pharmaceutical field.

Materials and Methods

Specific reagents

Eugenol, phosphate-buffered saline (PBS) and the MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) kit were supplied from Sigma Aldrich Merck KgaA (Darmstadt, Germany), while Dulbecco's Modified Eagle's medium (DMEM), Eagle's Minimum Essential Medium (EMEM-30-2003™), foetal bovine serum (FBS-30-2020™), penicillin/streptomycin antibiotic mixture and trypsin-EDTA solution were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The Lactate Dehydrogenase Cytotoxicity kit and Hoechst 33342 dye were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Cell culture and treatment regimen

In this study, the cell lines used were HaCaT (300493)-human immortalised keratinocytes from CLS Cell Lines Service and JB6 Cl 41-5a - mouse epidermal cells (CRL-2010™, ATCC, Manassas, VA, USA). The cells were grown in the specific culture medium DMEM supplemented with 10% foetal bovine serum and a 1% mixture of penicillin/streptomycin for HaCaT cells and EMEM supplemented with 5% foetal bovine serum and a 1% mixture of penicillin/streptomycin for JB6 Cl 41-5a cells. During the experiments, the cells were maintained under standard conditions of 37°C and 5% CO_2.

For biological analyses, eugenol was dissolved in 0.5% DMSO to obtain a final stock solution of 1 mg/mL. The test solutions of 10, 25, 50, 75 and 100 μg/mL in the culture medium were prepared from the stock solution.

Cellular viability evaluation

The cellular viability was investigated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method in both cell lines. 48 hours after the treatment with EUG in 10, 25, 50, 75 and 100 μg/mL, the culture medium was replaced with a fresh one, and 10 μL of MTT was added to each well and incubated for 3 hours. After this period, 100 μL of MTT solubilizing solution was added in each well, and then the absorbance was read at 570 nm using a Cytation 5 device.

Cell confluence and cell number investigation

To explore the influence of EUG treatment (10, 25, 50, 75 and 100 μg/mL) on HaCaT and JB6 Cl 41-5a cells' confluence and number, representative photos were captured using the objective 4x on the Lionheart FX automated microscope. Next, each image was automatically analysed using the Cell Analysis Tool by the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA) following the supplier's instructions.

Bright-field cell morphology evaluation

The activity of EUG (10, 25, 50, 75 and 100 μg/mL) on cell morphologies was examined by taking the representative photos 48 hours after the treatment of the cells under brightfield illumination (20× magnification) on the Lionheart FX automated microscope. The obtained images were processed in the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Cytotoxicity assessment via Lactate Dehydrogenase Leakage (LDH Assay)

To further explore the potential of EUG (10, 25, 50, 75 and 100 μg/mL) on the cells, the LDH assay was carried out 48 hours after the treatment. After the incubation period, 50 μL of culture medium containing

released LDH was transferred into a new 96-well culture plate and combined with 50 µL of the reaction mixture, and the plates were incubated for 30 min. 50 µL of the stop solution was added to each well after the 30 minutes of incubation and the absorbance was read at 490 nm and 680 nm using Cytation 5 device.

Evaluation of irritant potential via HET-CAM assay

For the *in ovo* experiment - the HET-CAM test, the eggs were prepared in a specific incubator at standard conditions of humidity and temperature according to the protocols of the specialised literature [14]. On the 10-day of incubation, the HET-CAM irritation test was performed using 1% sodium dodecyl sulfate-SDS as positive control and ultrapure distilled water (H_2O) as negative control. The substance of interest was EUG at the highest concentration tested in previous *in vitro* experiments (100 µg/mL). Samples were applied directly over the chorioallantoic membrane using a pipette in a volume of 600 µL/ well and for 5 minutes signs of lysis (L), coagulation (c), or haemorrhage were monitored. After the 5 minutes, the irritation score or IS was calculated according to the specific formula in the literature.

$$\mathbf{IS} = \mathbf{5} \times \frac{\mathbf{301-H}}{\mathbf{300}} + \mathbf{7} \times \frac{\mathbf{301-L}}{\mathbf{300}} + \mathbf{9} \times \frac{\mathbf{301-C}}{\mathbf{300}}, \quad (1)$$

where, "IS" is the index that measures the changes that occur following the application of the samples to the vessels (H – haemorrhage, L – vascular lysis and C – coagulation). According to the results obtained from the calculation, the samples were classified as non-irritant, irritant and severe irritant [9, 14].

The representative photos were captured at T0 – before the application of the samples and T5 - 5 minutes after the application with the Discovery v.8 stereomicroscope (SteREO Discovery.V8 stereo-microscope from ZEISS Jena, Germany) and analysed with the ZEN core 3.8 software.

Statistical analysis

All the data are stated as the mean values ± standard deviation (SD) of three independent experiments. GraphPad Prism (GraphPad Software, software version 9.4.0 for Windows, San Diego, CA, USA) was the statistical program used in the current research. One-way ANOVA and Dunnett's multiple comparison post-test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, were performed to compare the groups.

Results and Discussion

According to the literature, the mouth is the visible gateway to the rest of the body showing what is occurring inside. Systemic diseases can have a negative impact on oral health and this idea can also be reversed, so poor oral health can lead to other diseases [17]. EUG is found in many essential oils and natural herbs and is recognised in the stomatological field for its analgesic, antibacterial and anti-inflammatory properties. The purposes for which it is most frequently used are toothache, pulpitis and oral care. However, the therapeutic effect of EUG against dental caries has not been studied in detail and requires much more research. Besides, the fact that it also has certain side effects on the human body increases the need for biosafety evaluations [26, 39]. The current study is based on the assessment of EUG cytotoxicity in two cell lines of healthy origin, namely HaCaT immortalised keratinocytes and JB6 Cl 41-5a epidermal cells, previously used in other research based on dental agents [13] and complementary to the *in vitro* experiments, the irritant potential was also examined by the HET-CAM method *in ovo*, being an agent that can directly come into contact with mucous membranes.

As a first step in determining the *in vitro* impact of EUG on the two cell lines, the MTT assay was performed to assess cell viability 48 hours after the EUG treatment (10, 25, 50, 75 and 100 µg/mL). Previously, EUG was also tested in the same dose range for safety on JB6 Cl 41-5a cells [12]. The MTT test is a colorimetric procedure based on the transformation of MTT into formazan by living cells [25]. Results of MTT analysis (Figure 1) showed that EUG decreases viability in both cell lines in a dose-dependent stepwise manner. EUG acted more intensely on JB6 Cl 41-5a cells showing that they are more sensitive to the compound compared to HaCaT cells, thus at the highest concentration tested (100 µg/mL) the results showed a percentage of 55% viable cells for JB6 Cl 41-5a, while for HaCaT cell viability did not decrease below 78%.

Further, the EUG cytotoxicity was investigated by analysing cell confluency and cell number 48 hours after treatment. The results obtained (Figure 2) for both assays were in agreement with the MTT assay results, with the percentages decreasing in a concentration-dependent manner, and JB6 Cl 41-5a cells appeared to have a higher sensitivity to EUG. The lowest values were detected for both analyses at the highest concentration tested of 100 µg/mL, for HaCaT cells the cell confluence reached 78% and the cell number reached 82%, while for JB6 Cl 41-5a the cell confluence reached 58%, whereas the cell number attained 57%.

According to the literature by ISO Standard 10093 - 5:2009, a compound is cytotoxic if it decreases cell viability by more than 30% [14], in this context only the situation of JB6 Cl 41-5a cells is remarkable, where the percentages reach up to about 55% treated with the highest concentration of EUG (100 µg/mL) at the viability test. In accordance with the previously described standard, HaCaT cells exhibited no signs of cytotoxicity upon EUG treatment at any of the concentrations used in the assays.

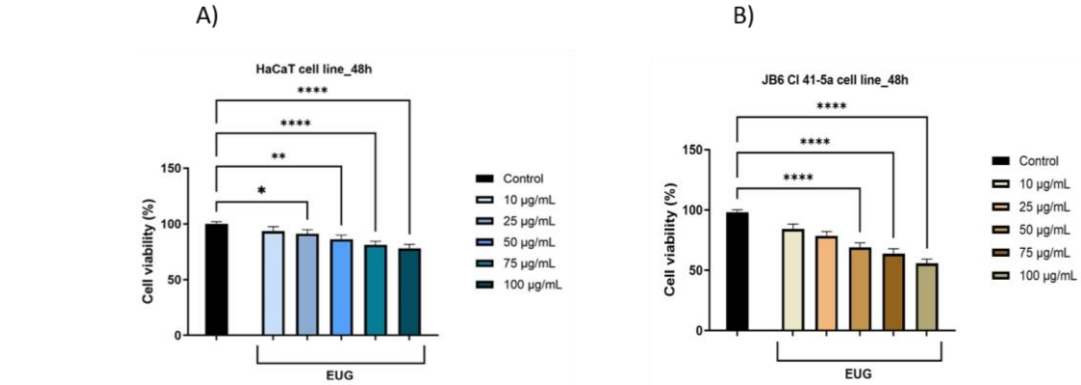

Figure 1.

In vitro assessment of EUG (10, 25, 50, 75 and 100 µg/mL) impact on the viability of HaCaT and JB6 Cl 41-5a cells after 24 h of treatment by applying the MTT test

To identify the statistical differences between the control (untreated) and the treated group, the one-way ANOVA analysis was performed, followed by Dunnett's multiple comparisons post-test (*p < 0.05, **p < 0.01 and ****p < 0.0001).

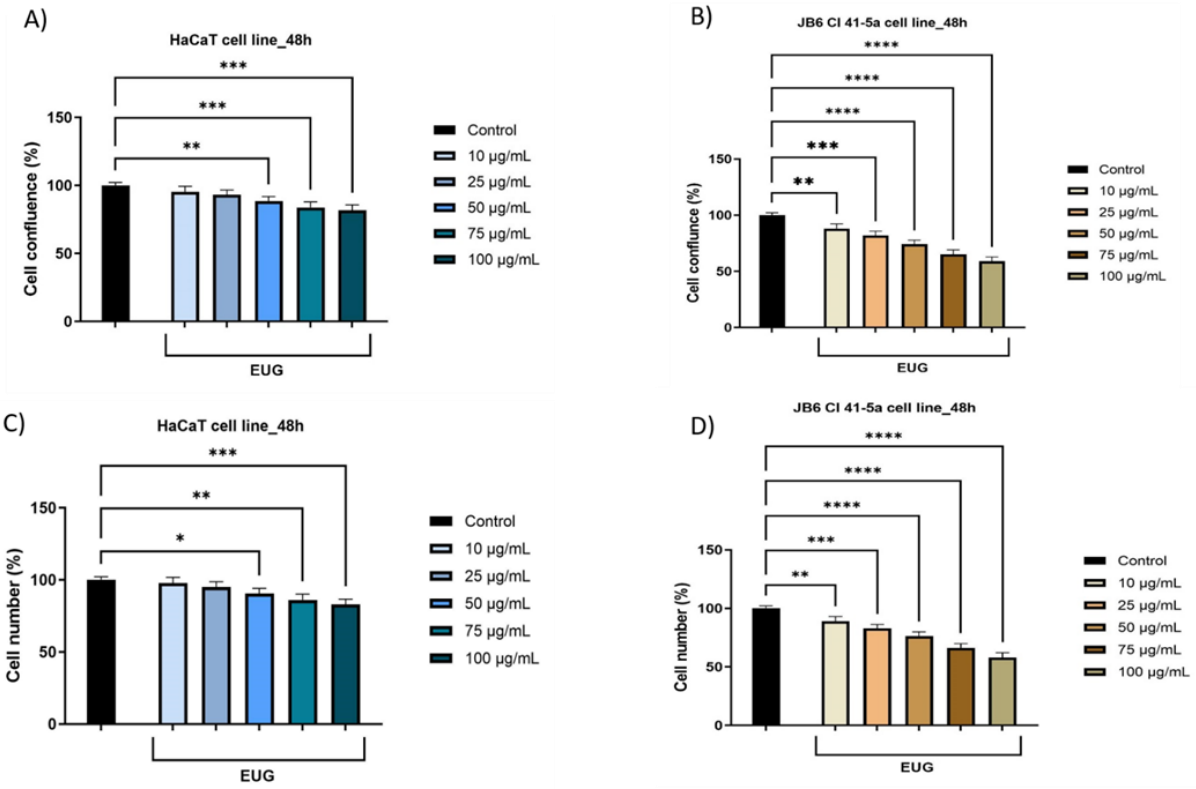

Figure 2.

Graphical representation of confluence and cell number obtained 24 h after treatment of HaCaT and JB6 Cl 41-5a cells with EUG

To identify the statistical differences between the control (untreated) and the treated group, the one-way ANOVA analysis was performed, followed by Dunnett's multiple comparisons post-test (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001).

Similarly, other studies have evaluated the impact of EUG (50 µM) on HaCaT keratinocytes for 24, 48 or 72 hours. After the 24 and 48-hour intervals, the decrease in cell viability was insignificant, and 72 hours after treatment the cells were stimulated by EUG [2]. Also, on a different healthy cell line (HGF-human gingival fibroblasts), EUG 0.1 - 1 mM was explored by Surducan *et al.*, and their results indicated that cell viability decreased dose-dependently up to 76% after 72 hours of treatment [36]. Another study by Racea and coworkers regarding the impact of EUG (0.1 - 1 mM) on HaCaT and HCT-116 cells demonstrated that EUG bypasses HaCaT cell damage. According to the results of MTT analysis after 72 hours of treatment, at 1 mM concentration, the cell viability revealed 70%. The lowest concentration in this study, *i.e.* 0.1 mM induced an insignificant reduction of up to 93% after 72 hours of stimulation [31]. Moreover,

EUG was also investigated for other biological activities including the anticancer activity. A study conducted on MDA-MB-231 and SK-BR-3 breast cancer cells demonstrated that EUG inhibits cell proliferation dose-dependently and time-dependently, making it a promising compound in the fight against cancer [1].

The next step in investigating the influence of EUG on the two types of cell lines was to analyse cell morphology 48 hours after treatment. As can be seen in Figure 3A, for HaCaT cells, at the highest concentration tested a shrinking of the cells can be observed in places, but the morphologic appearance is not considerably affected. For JB6 Cl 41-5a cells (Figure 3B), more intense action of EUG can be observed by the presence of debris, shrinkage, or elongation of the cell shape in places at the highest concentrations. Moreover, for JB6 Cl 41-5a cells, a reduction in confluency can also be observed with increasing doses.

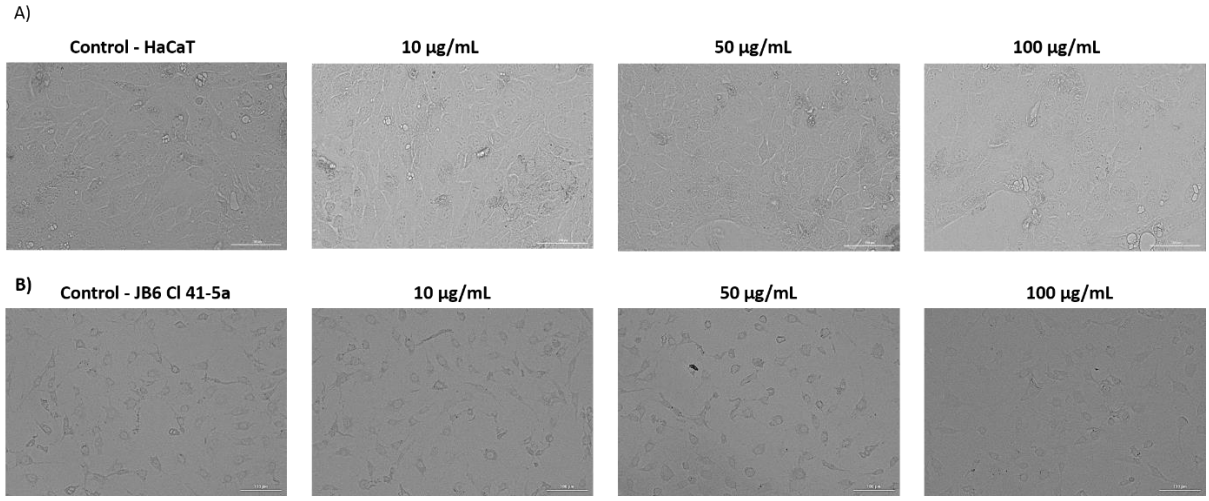

Figure 3.

The morphological aspect of HaCaT and JB6 Cl 41-5a cells after 24 h of treatment with EUG (10, 50 and 100 µg/mL) The scale bars indicate 100 µm

The study of morphology provides key information about cell behavior [35]. Racea *et al*. analysed the morphology of HaCaT cells after 72 h treatment with EUG (0.1 - 1 mM). Similar to the results of the current study, EUG did not cause significant dysmorphology up to a concentration of 1 mM where a rounding of the cells and a decrease in confluency can be observed. In the same study, the number and shape of nuclei were also analysed for the 0.5 mM concentration of EUG at the same exposure time, 72 hours. According to the results, there were minor condensations of chromatin and no major changes on actin filaments [32]. Since EUG possesses multiple biological activities, the anti-tumour function was also evaluated on cancer cell lines on SAOS-2 and Detroit 562 cells concerning morphological changes after 72 hours of treatment with 0.1 - 1 mM EUG. For SAOS-2 cells EUG produced cell detachment from the plaque, cell shrinkage, and a decrease in confluency, the most significant changes being evident at 1 mM concentration. For Detroit 562 cells, it was observed that confluency decreased dose-dependent, but without significant dysmorphology compared to the control [30].

To further explore EUG cytotoxicity in more detail, an LDH assay was performed. The results of the LDH test confirmed that the toxic potency of the compound increases with rising doses for both cell lines. For HaCaT cells at the highest concentrations (75 - 100 µg/mL), the percentages reach values between approximately 10 - 15% LDH released, while for JB6 Cl 41-5a cells at the highest concentrations (75 - 100 µg/mL), the values are between 15 and 20%.

The Lactate Dehydrogenase or LDH test is a reliable and sensitive method for assessing cytotoxicity. It is designed to quantitatively detect LDH in serum, tissue, or various cell culture samples. Once LDH levels are elevated it means tissue damage or inflammation [20]. In the current study, for both cell lines, the maximum percentage of LDH released did not exceed more than about 20%.

The dose-dependent cytotoxic effect of eugenol on HaCaT and JB6 Cl 41-5a cells is can be determined by several mechanisms that include induction of oxidative stress, activation of apoptosis, cell cycle disruption, alteration of signalling pathways and mitochondrial dysfunction. At higher doses, these processes become more pronounced, leading to an increase in cell death, mainly through apoptosis and necrosis [7, 28].

Eugenol can arrest the cell cycle arrest in different phases, such as G1/S or G2/M, which is controlled by cell cycle proteins, such as cyclins and cyclin-dependent kinases [10]. Induction of apoptosis has

been demonstrated through intrinsic mitochondrial pathways as well as extrinsic receptors. The intrinsic apoptotic cascade can be triggered by DNA damage and endoplasmic reticulum stress [38].

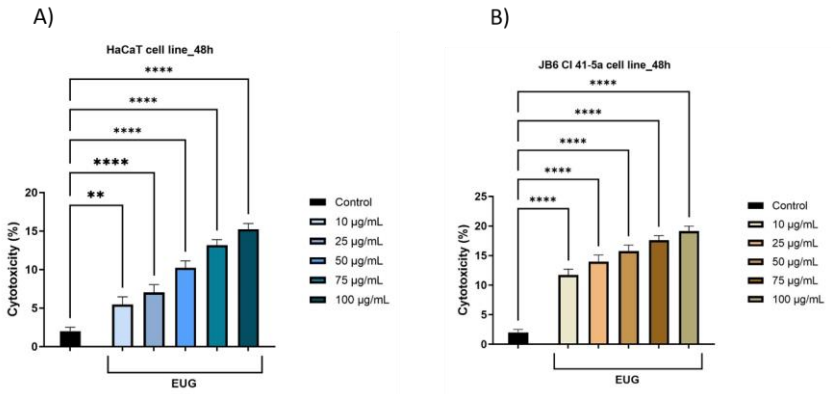

Figure 4.

Graphical illustration of lactate dehydrogenase leakage in (A) HaCaT and (B) JB6 Cl 41-5a cells at 24 h after treatments with EUG (10, 25, 50, 75 and 100 µg/mL)

To identify the statistical differences between the control (untreated) and the treated group, the one-way ANOVA analysis was performed, followed by Dunnett's multiple comparisons post-test (**p < 0.01 and ****p < 0.0001).

The final step in the exploration of EUG was the HET-CAM *in ovo* assay, which has previously been used for various agents or materials used in dentistry [13, 14].

According to the results obtained *via* the HET-CAM assay (Figure 5, Table I), EUG is included in the category of non-irritant samples. As expected for the controls used in the experiment, SDS 1% caused severe damage and is categorised as a severely irritating sample, while H_2O is non-irritating.

Table I

Table with the calculated irritation score (IS) for EUG 100 µg/mL *via* HET-CAM method

Sample	IS	Irritation Category
SDS 1%	19.86	Severely Irritant
H_2O	0.06	Non-irritant
EUG 100 µg/mL	0.76	Non-irritant

SDS 1% represents positive control and H_2O was used as negative control

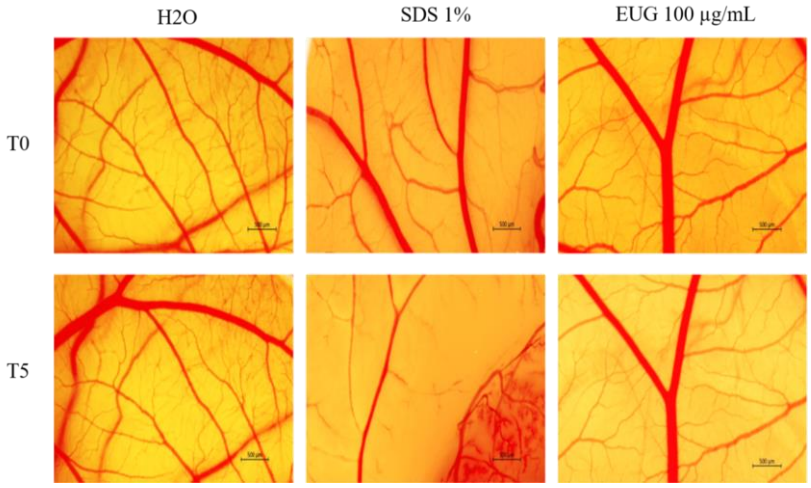

Figure 5.

Stereoscopic images of the HET-CAM method illustrating the initial effect (T0) and at 5 min (T5) after the application of the samples: negative control (H_2O), positive control (SDS 1%) and EUG 100 µg/mL

The scale bars indicate 500 µm.

Following the literature, samples can be divided into several types of categories by performing HET-CAM tests as follows: non-irritant samples if IS = 0 - 0.9, irritant samples if IS = 1 - 8.9; and (iii) severely irritant samples if IS = 9 - 21 [4, 9]. Each investigated parameter follows an action to be performed, such as bleeding for haemorrhage (H), vessel disintegration for lysis (L) and protein denaturation intra- and extra-vascular for coagulation (C) [16].

In a different study, EUG was also evaluated at 1 mM concentration by the HET-CAM method, the results indicating a mild irritation with an IS = 1.69 [30]. In addition, other studies have evaluated materials or agents used in dentistry to check the irritant potential by the HET-CAM method. Chlorhexidine has been evaluated by Dinu and associates and the results indicated that 0.02% chlorhexidine is categorised as irritant, while the 2% concentration is severely irritant [13]. Another study examined the irritant potential for sodium fluoride, xylitol and the combination of the two, compounds with a wide use in dentistry. The highest irritation score was observed for sodium fluoride (IS = 1.4), while for xylitol or combination, the IS was below 1 [8]. As a result, and compared to other substances commonly used in the sector, EUG has potential as a compound that did not show significant signs of irritation.

One of the key aspects in the use of an agent is its compatibility with mucous membranes, so one of the best methods to check this property is the HET-CAM test. It is also important to know a product in terms of irritancy, especially when it can be an attractive alternative, as EUG can be in the dental industry [34].

Over the years the HET-CAM method has proven to be a rapid, economically acceptable and low-cost method that provides useful information concerning embryo-toxicity, teratogenicity, systemic and immuno-pathologic effects, as well as the irritant potential of certain substances [22]. Due to the rich vascular network of CAM, the pharmacotoxicological evaluation of different agents by the HET-CAM method can supplement *in vitro* studies [30].

Conclusions

Considering the above, EUG is a compound of natural origin with potential for dental medicine. Future studies could be directed toward more advanced research on the activities that can help dentists, considering that it is a botanical compound with low adverse effects. Furthermore, additional lines of future investigation may aim to optimise dosing by finding the most effective dose that produces the least damage.

Conflict of interest

The authors declare no conflict of interest.

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