https://doi.org/10.31925/farmacia.2025.1.18 **ORIGINAL ARTICLE**

QUERCETIN-BIOSAFETY SCREENING FOR FUTURE APPLICATIONS IN THE DENTAL FIELD: AN *IN VITRO* AND *IN OVO* INVESTIGATION

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Abstract

As conventional treatment causes a series of adverse effects, people tend to opt for safer alternatives for oral health, such as natural oral hygiene products. Quercetin (QUE) is a phytocompound that has garnered attention for its antimicrobial, anti-inflammatory, antioxidant, and anticancer properties, offering several applications in dentistry. Therefore, the present study investigates QUE (20 - 100 μM) *in vitro* on a human gingival fibroblast cell line (HGF), but also *in ovo* on the chorioallantoic membrane in terms of irritant potential and impact on blood vessel formation (angiogenesis). The assessments demonstrated that the presence of QUE (20 - 80 μM) does not affect cell viability, and at the highest concentration tested, the viability percentages reach approximately 85%. Complementarily, QUE (20 - 80 μM) did not alter the cell morphology and the appearance of nuclei, whereas the 100 μM dose slightly decreases cell confluency and induces minor dysmorphologies in cell nuclei. Additionally, QUE was classified as a non-irritant (IS = 0.070) and did not alter blood vessel formation. These findings support QUE's biosafety and potential use in dental treatments.

Rezumat

Întrucât tratamentul convențional provoacă o serie de efecte adverse, oamenii tind să opteze pentru alternative mai sigure, cum ar fi produsele naturale de igienă orală. Quercetina (QUE) este un fitocompus care a atras atenția datorită proprietăților sale antimicrobiene, antiinflamatorii, antioxidante și anticancerigene, oferind mai multe aplicații în domeniul stomatologiei. Prin urmare, studiul de față investighează QUE (20 - 100 μM) *in vitro* pe linia celulară de fibroblaste gingivale umane (HGF), dar și *in ovo* pe membrana corioalantoică în ceea ce privește potențialul iritant și impactul asupra formării vaselor de sânge (angiogeneză), pentru aplicații viitoare în sănătatea orală. Evaluările au demonstrat că prezența QUE în intervalul 20 - 80 μM

nu afectează viabilitatea celulară, iar la cea mai mare concentrație testată procentele de viabilitate ajung la aproximativ 85%. În mod complementar, morfologia celulară și aspectul nucleelor nu sunt alterate în intervalul de doză 20 - 80 μM, în timp ce doza de 100 μM scade ușor confluența celulară și induce dismorfologii minore în nucleele celulare. În plus, QUE a fost clasificat ca neiritant (IS = 0,070) și nu a afectat formarea vaselor de sânge. Aceste rezultate confirmă biosiguranța QUE și potențiala sa utilizare în tratamentele dentare.

Keywords: dentistry, quercetin, gingival fibroblasts, biosafety, cytotoxicity

Introduction

Oral health is one of the most concerning health issues, affecting almost half the global population. Over the last few decades, the number of oral health-related diseases has increased by 1 billion. These dental problems can have consequences not only for patients' physical health but also for their aesthetic appearance, as well as their mental and social well-being. Some categories of patients are more likely to develop dental problems, such as children, the elderly, people with special needs, and those with low socio-economic status. The World Health Organization (WHO) continuously raises awareness and implements strategies to educate the population, regardless of their income, about the importance of oral health. It also promotes cost-effective preventive treatments and interventions [49]. One of the most encountered dental problems is tooth decay, with a prevalence of 2.3 billion people having caries in permanent teeth [40]. If this issue remains unresolved, it could lead to periodontitis and even tooth loss. One of the approaches to overcoming and preventing this disease has been implementing products with natural compounds as an alternative to conventional treatments. Natural products have gained interest due to their lower incidence of side effects; for instance, while chlorhexidine is considered the gold standard antiseptic in dentistry for its broad applications, it is often accompanied by adverse effects such as teeth and tongue staining, unpleasant taste, xerostomia, irritation, ulceration, and, in severe cases, anaphylaxis [18]. Natural oral hygiene products have become popular because of their lower cost and various bio-properties. Although plants have been used since ancient times for maintaining good oral health, in recent years, newer extraction techniques have made easier ways to isolate the phytocompounds and implement them in multiple formulations for several purposes [7, 13, 34]. Quercetin (QUE) (Figure 1) is one of the compounds that has sparked interest in dental care in recent years. It is one of the most widely distributed polyphenols from the flavonoid class that can be found in various fruits (tomatoes, grapes, cranberries, apples, bananas), vegetables (garlic, onion, eggplant, potatoes), nuts, plants (capers, dill, coriander, fennel), flowers, and leaves [14, 15, 45].

QUE possesses antimicrobial, antioxidant, anti-inflammatory, and anticancer properties [6] and has been utilised in the treatment of neurodegenerative disorders [21], obesity, diabetes [51], cardiovascular diseases [52], allergic reactions, inflammation [24], and arthritis [26].

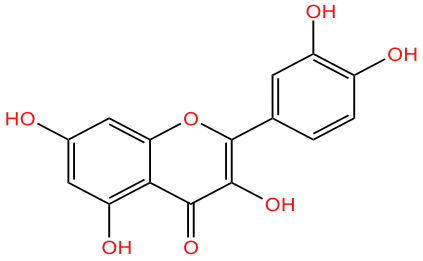

Figure 1.
The chemical structure of quercetin; The figure was made with KingDraw

In dentistry, QUE is an excellent antiseptic with multiple benefits because of its hydroxyl groups, which interact with bacterial cellular proteins and disrupt the cytoplasmic membrane structure. It can also stop the production of nucleic acid and interact with the quorum sensing system, which are two other ways it might be useful in biofilm formation. It acts against Gram-positive and Gram-negative bacteria, some drug-resistant strains, fungi, viruses, and various parasites. QUE's wide spectrum is a fundamental trait for its prophylactic and curative use in oral care [16, 33]. QUE has proven beneficial in periodontitis [1], which is the primary cause of tooth loss, affecting 10% of the global population [48]. Also, it was observed that QUE is useful in the treatment of periodontitis due to its ability to decrease the levels of p21 and p53 (cell cycle and apoptosis regulators that are involved in the inflammatory process) *in vitro* and increase NRF2 both *in vitro* and *in vivo* [48]. In general, QUE is classified as an agent with limited adverse effects, but considering the new approaches and hypotheses for its clinical applications, an in-depth biosafety evaluation is a current priority. In this sense, it is crucial to study its action on healthy cell lines *in vitro*, *in ovo*, and, if possible, in clinical studies to establish its safety in oral care [4, 35, 38, 39]. In light of the above, the present study aims to investigate QUE in dentistry and pharmacy regarding its *in vitro* biosafety on human gingival fibroblast (HGF) cell lines. Human gingival fibroblasts represent one of the most used healthy cell lines that are used to analyse the safety of various compounds for dental use [21]. The irritant potential and activity on angiogenesis by *in ovo* methods are also presented to complete the safety

profile of QUE. The *in ovo* bioassays were also applied on the basis that dental treatments come into direct contact with gingival tissues. Thus, vascular changes induced by their contact with the chorioallantoic membrane may serve as indicators of potential irritation of the oral mucosa [23].

Materials and Methods

Reagents and Instruments

Quercetin, the phosphate-buffered saline (PBS), and the MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl-tetrazolium bromide) kit were obtained from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). Fibroblast Basal Medium (ATCC PCS-201), Fibroblast Growth Kit-Low serum (ATCC PCS-201-04), penicillin/streptomycin/amphotericin B (PCS-999-002™), and the dimethylsulfoxide (DMSO, 4-X™) were purchased from American Type Culture Collection (ATCC) Manassas, VA, USA. Hoechst 33342 dye was procured from ThermoFisher Scientific (Waltham, MA, USA). Cytation 5 (plate reader) and Lionheart FX (automated microscope) were provided by BioTek Instruments Inc. (Winooski, VT, USA), while the SteREO Discovery.V8 stereomicroscope was obtained from ZEISS (Jena, Germany).

Preparation of the samples

For the *in vitro* evaluation of QUE, test samples (concentrations of 20, 40, 60, 80, and 100 µM) were prepared by diluting the stock solution (QUE in DMSO) in the culture medium specific to the cell line used. The final concentration of DMSO in each test sample did not exceed 0.5%.

Cell Culture Conditions

The study was conducted using the HGF primary human gingival fibroblasts (PCS-201-018™; ATCC, Manassas, VA, USA). HGF cells (passage 15) were cultured in the specific fibroblast growth medium supplemented with Fibroblast Growth Kit-Low serum and penicillin/streptomycin/amphotericin B (PCS-999-002™). The cell line was maintained at 37°C and 5% CO_2 in a humidified incubator. The cells presented normal morphology and proliferation during all the experiments.

Cell Viability (MTT Test) Evaluation

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) test was used to determine cell viability. The MTT assay was performed following the 24-hour treatment of HGF cells with QUE at varying concentrations (20, 40, 60, 80, and 100 µM). The cells were seeded in flat-bottom 96-well plates at a density of $1x10^4$ cells *per* well and treated with the compound under investigation. After 24 hours, the culture medium was changed with a fresh one, then 10 µL of MTT reagent was added to each well, and the cells were incubated for 3 hours at 37°C and 5% CO_2. After the incubation period, 100 µL of solubilising solution was added, and the plates were maintained for 30 min at room temperature. Finally, the absorbance was measured at 570 and 630 nm using a Cytation 5 device. The protocol was followed, as presented by Gag *et al*. [20].

Bright-Field Cellular Morphology Assessment

The effect of QUE on the morphology of HGF cells was examined by capturing representative images of both controls (untreated cells) and treated cells. These cells were cultured in 96-well flat-bottom plates at a density of $1x10^4$ cells *per* well, and the cell line was maintained at 37°C and 5% CO_2 in a humidified incubator. The cells were treated with QUE 20, 40, 60, 80, and 100 µM for 24 hours. After this period, the cells were imaged under brightfield illumination at 20× magnification using the Lionheart FX automated microscope. The images were subsequently processed using Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Hoechst Nuclear Staining

To investigate further, the effect of QUE on cell nuclei was evaluated using the Hoechst method. Cells were cultured in 12-well plates at a density of $1x10^5$ and treated with QUE (20, 40, 60, 80, 100 µM) for 24 hours and maintained in the incubator at 37°C and 5% CO_2. After this period, protected from light, the medium was first removed from the plate, and the Hoechst solution, obtained by 1:2000 dilution of Hoechst in PBS, was added. The plate was stored for 5 - 10 min, protected from light. Subsequently, the staining solution was removed, and the wells were washed 3 times with PBS. The representative images with the nuclei were taken using a Lionheart FX automated microscope at 20x objective and analysed using Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Based on the following formula [50], the apoptotic index (AI) was calculated:

$$I\ (\%) = \frac{Number\ of\ apoptotic\ cells}{Total\ number\ \ of\ cells} \times 100$$

Fertilized hen eggs preparation for the in ovo experiments

Fertilised hen eggs were used for the chorioallantoic membrane assays. On the first day, they were disinfected with 70% alcohol, dated, and incubated for 3 days. On day 4, a small cut was made on top of the eggs, and about 6 - 7 mL of albumen was extracted with a syringe needle, and then the cuts were covered with adhesive tape. On day five, the upper part of the eggs was removed to visualise the vessels of the chorioallantoic membrane; then, each egg was covered with adhesive tape. The eggs were reintroduced into the specific incubator, which was constantly maintained at an optimal temperature (37°C) and humidity (60%) in the incubator until the time of the experiments [27].

Assessment of the irritant potential via the HET-CAM Method

On day ten of incubation, the irritant potential of quercetin (100 µM) was evaluated by applying a volume of 600 µL of substance to the chorioallantoic membrane. Sodium lauryl sulphate 1% (SLS) was used as a positive control, and distilled water was used as a negative control. For 5 minutes (300 seconds) after the application of the samples, specific signs of lysis (L), haemorrhage (H), and coagulation (C) were monitored. Representative pictures of the membrane were taken before the application of the samples (at T0) and at the end of the treatment (T5). The images were performed using the Discovery v.8 stereomicroscope and the ZEN Core 3.8 software. As a final step, the irritation score (IS) was calculated using the formula:

$$\boldsymbol{IS} = 5x\,\frac{301-H}{300} + 7x\,\frac{301-L}{300} + 9x\,\frac{301-C}{300},$$

IS is a parameter that determines the irritant potential of samples by measuring the time when changes (H - haemorrhage; L - vascular lysis; C - coagulation) occur at the vascular level. Depending on their value, the test samples can be categorised as non-irritant if IS = 0 - 0,9, irritant if IS = 1 - 8,9, severe irritant if IS = 9 - 21 [27]. This experiment was performed in triplicate.

The impact on angiogenesis - Chorioallantoic Membrane Assay (CAM)

To assess the compound's impact on angiogenesis on the chick embryo chorioallantoic membrane, 10 µL of quercetin (100 µM) was applied in a plastic ring placed on the membrane between two large blood vessels starting on day 8 of incubation. Vascular changes were monitored daily for 5 days by microscopic photographs using the Discovery v.8 stereomicroscope and the ZEN Core 3.8 software [28]. This assay was performed in triplicate.

Statistical Analysis

Statistical analysis of all data was carried out using GraphPad Prism software version 10.2.3 GraphPad Software, San Diego, CA, USA, www.graphpad.com, and by employing two statistical methods: the one-way ANOVA analysis and Dunnett's multiple-comparison test, respectively. All statistically significant results were marked using "*" (* $p < 0.05$; ** $p < 0.01$).

Results and Discussion

Dental problems are a global burden due to their high incidence (3.5 billion in 2022) and their negative effects on patients' quality of life [49]. Although conventional treatments have been a tremendous help in this matter, they also come with adverse effects, most commonly hypersensibility and irritation [18]. For this reason, natural compounds have grown in scale because they can be just as effective as synthetic products while presenting fewer and milder negative outcomes [1]. Concerning oral health for children, in pedodontics, dental diseases seem to evolve into a public health problem, increasing morbidity. Under these considerations, natural compounds could outline an alternative to prevention or treatment [10, 33]. QUE - a bioflavonoid, is one of those phytocompounds that has gained attention in dentistry in recent years due to its antimicrobial, anti-inflammatory, antioxidant, and antitumoral effects. These properties offer QUE therapeutic applications in oral health, such as reducing inflammation or promoting tissue healing [6].

In light of these premises, we conducted a study to evaluate the biosafety profile of QUE on a healthy cell line (HGF-human gingival fibroblast). Evaluating QUE *in vitro* on human gingival fibroblasts, an experimental model commonly used in other studies to verify the safety of different agents or materials used in dentistry, is a key link to the development of its use in the field and, subsequently, to clinical investigation [3, 43]. Additionally, experiments *in ovo* strengthened and complemented the biosafety research by verifying the irritant potential of the compound under investigation and its effect on angiogenesis.

The first step in evaluating the impact of QUE on healthy human gingival fibroblast cells was to investigate cell viability after 24 hours of treatment using the MTT assay. Figure 2 shows that QUE slightly increased the viability of HGF cells at concentrations of 20, 40, 60, and 80 µM, stimulating the cells' proliferation. However, at the highest concentration (100 µM), it was shown that QUE decreased cell viability to approximately 85%.

Under ISO Standard 10993 - 5:2009, a compound is considered cytotoxic if it causes a reduction in cell viability exceeding 30% [17]. The results indicate that the viability of HGF cells remained over 70%, suggesting that QUE has no cytotoxic effect even at the highest concentration (100 µM). Similarly, it has been previously shown that QUE does not impact cell proliferation in HGFs when used at concentrations of 5, 10, and 20 µM. However, in the same study, it has been demonstrated that with the increase of the concentration, the cell viability and proliferation decreased in a dose-dependent manner [23]. Orihuela-Campos *et al*. proved in a study that QUE at concentrations of 15 µM and 20 µM increased cell proliferation and viability after 48 h of treatment in HGF cells that were previously treated with H_2O_2, by 41%, respectively, 22% after 48 h [42]. Another healthy cell line used to test QUE's safety is HaCaT (Human Keratinocyte Cells). In a study conducted by So Ra Kim *et al*., it was demonstrated by the MTT assay that after 24 hours of exposure at concentrations of 10-80 µM, QUE induced no visible effect on cell viability. In contrast, when HaCaT cells were treated with QUE at 160 µM, the viability of the cells was significantly reduced to about 50% [25].

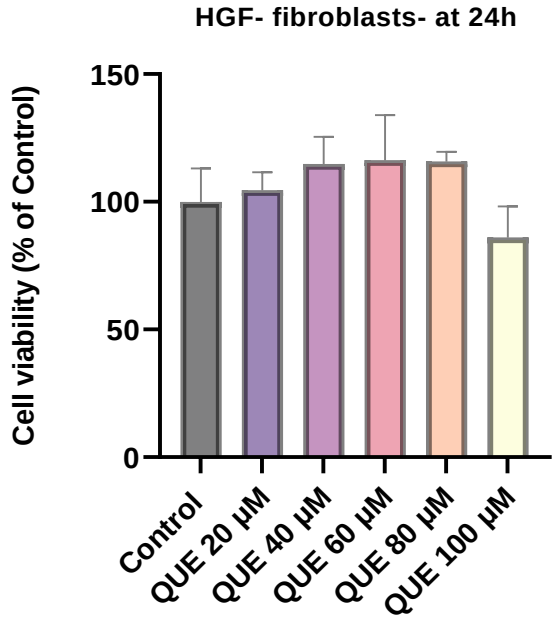

Figure 2.
In vitro cell viability evaluation of QUE (20 µM, 40 µM, 60 µM, 80 µM, and 100 µM) in HGF (human gingival fibroblasts). After 24 h post-treatment, an MTT colorimetric assay was performed. Results are expressed as viability percentages (%) normalised to control (untreated cells). Presented data are expressed as mean values ± SD of three independent experiments performed in triplicate. A one-way ANOVA test was assessed to observe the statistical differences between the control and the treated group, and then Dunnett's multiple comparisons post hoc test was performed.

Morphology assays represent essential tools in evaluating the effects of natural agents on cell lines, as they provide insights into changes in cell shape, size, growth, and underlying biological mechanisms, thereby helping to characterise the compound's impact and potential therapeutic applications in oral health. It is also of high value to evaluate the effect of a compound on healthy human-like cells that mimic human tissue and mucosa [16, 29]. Therefore, the next step in investigating the safety of QUE was to analyse cell morphology 24 hours after treatment of human gingival fibroblasts at the same dosages (20, 40, 60, 80, and 100 µM), as seen in Figure 3

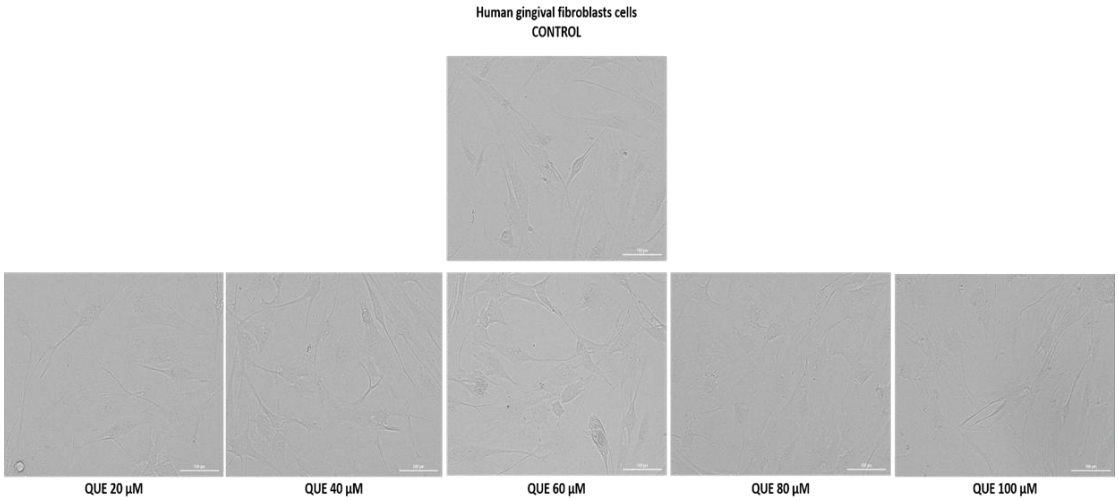

Figure 3.
Representative images illustrating the morphological changes observed in HGF cells after 24 h post-treatment with QUE at 20, 40, 60, 80, and 100 µM. The pictures were taken at a magnification of 20×, and the scale bar indicates 100 µm. Three independent experiments were performed in triplicate.

Any noticeable dysmorphologies were observed in the QUE-treated cells at 20 to 80 µM concentrations. However, at the highest concentration tested (100 µM), a decrease in cell confluency can be observed, aligning with the results previously obtained from the MTT assay.

The general lack of adverse effects of QUE on cells' morphology underlines its safety profile in dental medicine. In the same manner, QUE has been evaluated by Sang Young Seo and colleagues in terms of its impact on cell morphology and at higher concentrations (300 µM) on healthy keratinocytes - HaCaT cell line, as well as on two cancer cell lines. The findings indicated that QUE did not affect the morphology of healthy cells, presenting no dysmorphology, in contrast to cancer cells, where there were numerous changes in cell shape. Also, in the same study, similar results were observed in terms of cell viability, with QUE showing selective behaviour [44].

The following step in exploring the impact of QUE in terms of its cytotoxicity was to analyse the nuclei of HGF cells (Figure 4). After 24 hours of treatment, the results show that QUE gives more prominent cytotoxic effects only at the highest concentration tested, i.e., 100 µM. Thus, at this concentration, the shrinking of nuclei, rounding of their shape, and chromatin condensation can be observed (white arrows). However, these changes occur only occasionally. In the range of 20 - 80 µM, nuclear dysmorphologies are rare and not significantly different from the appearance of the nuclei of untreated cells.

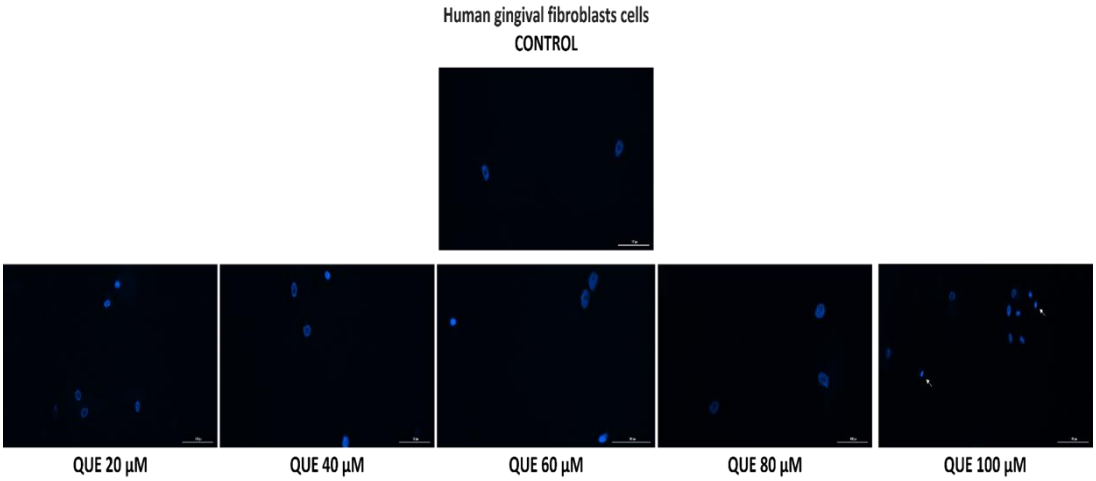

Figure 4.
Representative images presenting the morphological changes observed in nuclei of HGF cells after treatment with QUE at 20, 40, 60, 80, and 100 µM. White arrows indicate changes in the appearance of nuclei. The scale bars indicate 100 µm. Three independent experiments were performed in triplicate.

The obtained results proved that the QUE concentrations ranging from 20 to 80 µM after 24 h of treatment determined no alterations in the nuclei of the human gingival fibroblasts, with no signs of chromatin condensation, apoptotic bodies, nuclear bleaching features, or reduction in the size of nuclei, which are common signs of cytotoxicity to nuclei [31]. In contrast, at the 100 µM dose, it appears in some places that the nuclei present a rounder shape, as well as chromatin condensation is observed. The compaction of chromatin, as well as the morphological transition of the nuclei toward a rounder shape, is representative of nuclear condensation, and it usually reflects cellular stress or signs of apoptosis in the early stages. In this study, the lack of such changes at the dosage interval of 20 - 80 µM suggests that the tested compounds exert no cytotoxicity, maintaining the structural integrity of the nuclei [22]. Hoechst staining is important because it allows clear visualisation of cell nuclei, which helps in assessing nuclear morphology and detecting apoptotic changes. The round shape represents a loss of the characteristic nuclear structure, and it usually reflects the activation of cell death pathways, which may occur in response to toxic agents or unfavourable environmental conditions. Thereby, chromatin condensation and morphological changes, such as cell rounding, are valuable hallmarks that indicate the potential cytotoxic effects of a compound or unfavourable environmental conditions [22]. Thus, by using a healthy cell line, we can ensure a compound's safety by observing how it behaves on cells that mimic human-like tissues. By other staining methods such as DAPI staining and Annexin-V/7-ADD, the impact of QUE (100, 200, and 300µM) was evaluated on skin cancer cells, as well as on HaCaT cells - healthy cells. According to the results, the lowest signs of cytotoxicity were present in HaCaT cells, where at the highest concentration of 300 µM, changes in nuclear shape were observed [44]. After Hoechst

assessed the cell nuclei, they were analysed in order to calculate the apoptotic index and provide a more visible statistical overview of the obtained results. The apoptotic index is the number of apoptotic cells characterised by alterations to nuclear structures, expressed as a percentage of the total number of cells [9].

According to the apoptotic index (%), Figure 5, apoptotic nuclei are more frequently observed among the highest concentration tested (100 µM), as was to be expected following the previous analysis. However, the difference between the cells treated with 100 µM QUE and the control cells (untreated cells) is not very large, considering that in control, the percentage of AI is about 4.5%, and at the highest concentration of QUE analysed, only 11%. Cells treated with QUE 20-80 µM did not display statistically significant changes in the appearance of nuclei compared to control.

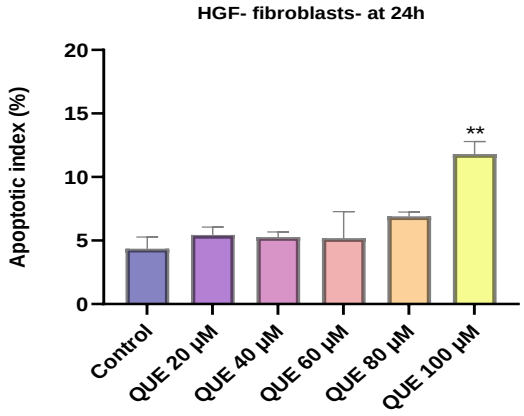

Figure 5.
Calculated apoptotic index in HGF cells treated for 24 h with QUE at 20, 40, 60, 80, and 100 µM. The results represent the mean ± standard deviation of three experiments done in triplicate. One-way ANOVA analysis and Dunnett's multiple comparison post-test were carried out to determine the statistically significant differences between control (non-treated cells) and treatment (** $p < 0.01$).

To better explore the safety profile of QUE, the next step was *in ovo* testing on the chorioallantoic membrane. The chicken chorioallantoic membrane is an extraembryonic vascularised membrane. The tissue composition represents a preclinical model of interest among bioengineers, biochemists, and drug development researchers [37]. The highest concentration of QUE tested *in vitro* was also evaluated for its anti-irritant potential on the chorioallantoic membrane of chicken eggs using the HET-CAM method. Due to its rapidity and simplicity, the HET-CAM assay is advantageous for evaluating the irritant properties of different chemicals, formulations [42], biomaterials, and implants [46]. It also finds application in evaluating compounds with anti-cancer potential on tumour cell lines [19]. The HET-CAM test confers numerous benefits in studies involving angiogenesis, inflammation, drug delivery, and irritation assessment by mimicking potential mucosal utilisation and local toxic reactions caused by a wide range of formulations [16].

For this investigation, the highest concentration tested *in vitro* (100 µM) was selected and tested in parallel with a positive control - sodium lauryl sulphate 1% (SLS), and a negative control - pure distilled water. Possible changes in blood vessels, such as lysis, coagulation, and haemorrhage, were monitored for 5 minutes, and based on the obtained times, the irritation score (IS) was calculated. According to the results (Figure 6 and Table I), QUE showed no signs of specific irritation of the chorioallantoic membrane and was classified as a non-irritating agent (IS = 0.07) along with the negative control, distilled water (IS = 0.07). SLS 1%, used as a positive control, was included in the class of severely irritating substances due to the appearance of the 3 specific signs of irritation (haemorrhage, lysis, and coagulation) in a short period of time, with an IS = 16.337.

QUE has also been studied in nanoemulsions, showing excellent biocompatibility according to the HET-CAM test [14]. Similarly, another study involved the evaluation of eugenol in terms of irritant potential at 1 mM concentration by the HET-CAM method [47]. Eugenol is another botanical compound with numerous functions in the dental area for its analgesic effect as a dental pulp dressing, but it also has beneficial effects in oral cancer and periodontal diseases [30]. However, the results of the assay indicated that eugenol showed a slight irritancy potential with an IS = 1.69 [41]. However, other important unnatural agents with applicability in dental medicine, such as chlorhexidine digluconate, were also examined by this method by

Dinu *et al*., who noticed that at a concentration of 0.02%, the agent caused lysis and coagulation. At a concentration of 0.2%, it additionally induced microhemorrhages, being classified as a severe irritant sample [16].

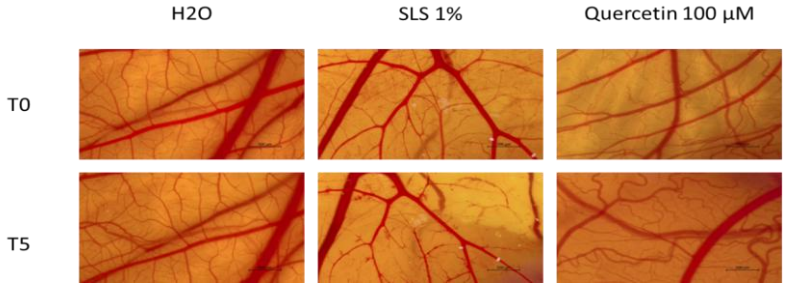

Figure 6.

Representative images of the chorioallantoic membrane vasculature before (T0) and 5 minutes (300 seconds) after (T5) the local application of H_2O (negative control), SLS 1% (positive control), and QUE 100 µM. Scale bars show 500 µm.

Table I

Irritation score (IS) was determined following the local treatment of the CAM with H_2O (negative control), SLS 1% (positive control), and QUE 100 µM

Sample	Irritation score	Irritation category
H_2O	0.070	Non-irritant
SLS 1%	16.337	Severe irritant
QUE 100 µM	0.070	Non-irritant

To complete the safety profile of QUE, its angiogenic effect was also evaluated *in ovo*. Angiogenesis is the formation of new blood vessels from preexisting vessels. This process has been studied since ancient times using the chorioallantoic membrane (CAM). This highly vascularised membrane comes in direct contact with the eggshell and is directly involved in the respiratory process [47]. Angiogenesis has a crucial role in wound healing and numerous pathologies such as cancerous tumours, arthritis, or diabetes [43]. The dental pulp is a highly vascularised tissue exposed to various local traumas due to mechanical stress during mastication. Therefore, the pulp is required to generate new blood vessels, a process called endodontic angiogenesis, which is necessary for the long-term support of the newly regenerated pulp [8, 12]. Due to the low cost, speed, and repeatability, the CAM method represents an alternative regarding the studies of drugs and natural compounds on blood vessels but may be limited by the non-specific inflammatory reactions that may occur [36].

To investigate the effects of QUE on angiogenesis, the same concentration of 100 µM as in the HET-CAM test was selected. Both QUE and the negative control - H2O were applied to CAM and evaluated for 5 days (Figure 7). During the monitoring period, QUE did not induce noticeable changes compared to distilled water, which was used as a control. Additionally, blood vessels were well-defined, and vasculature development was normal. For both QUE and the control agent, the vasculature inside the ring attached to the chorioallantoic membrane was similar to the outside ring.

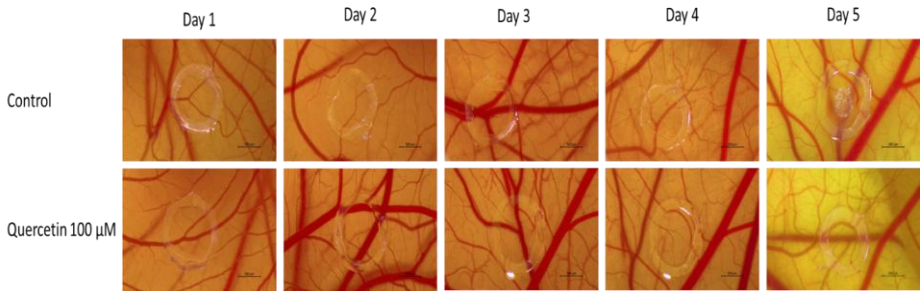

Figure 7.

Assessment of angiogenesis in the chick chorioallantoic membrane subjected to applications of 100 µM QUE and ultrapure distilled water (control). The investigation involved the capture of indicative stereomicroscopic images throughout the experimental period, starting from day 1 to day 5.

The results of our study show that the concentration of 100 µM of QUE did not negatively affect the development of blood vessels during the five days of CAM exposure.

QUE is a phytocompound that has demonstrated its relevance in several studies in treating or preventing several dental diseases [35]. The current study complements the present findings by verifying the safety profile in preclinical, experimental models as relevant as possible for clinical practice, namely human gingival fibroblasts and chorioallantoic membranes. Future directions should turn their attention to *in vivo* biosafety evaluation with transmission to clinical investigations or even its evaluation in targeted delivery systems with the objective of dose optimisation and bioavailability improvement, being known for the low water solubility of QUE [2].

Conclusions

The current study demonstrates that QUE is a promising candidate for advanced research as a natural alternative in dental treatments. QUE was found to be non-cytotoxic on human gingival fibroblasts, with cell viability remaining at 86% even at the highest concentration tested (100 µM). Furthermore, cell morphology and cell nuclei were affected in a reduced manner only at the highest concentration tested. QUE also maintained its behaviour *in ovo*, where it was framed as a non-irritant agent, the blood vessels remaining unchanged after the treatment period. All these findings outline a safe profile for QUE and open the interest of future investigations regarding its potential use in oral health treatments. A future perspective is to test QUE against other antiseptics already used on the market over more extended periods of time to evaluate its efficacy and safety further.

Conflict of interest

The authors declare no conflict of interest.

References

1. Aithal GC, Nayak UY, Mehta C, Narayan R, Gopalkrishna P, Pandiyan S, Garg S, Localized *in situ* nanoemulgel drug delivery system of quercetin for periodontitis: development and computational simulations. *Molecules*, 2018; 23(6): 1-15.
2. Alizadeh SR, Savadkouhi N, Ebrahimzadeh MA, Drug design strategies that aim to improve the low solubility and poor bioavailability conundrum in quercetin derivatives. *Expert Opin Drug Discov.*, 2023; 18(10): 1117-1132.
3. Alleyn CD, O'Neal RB, Strong SL, Scheidt MJ, Van Dyke TE, McPherson JC, The effect of chlorhexidine treatment of root surfaces on the attachment of human gingival fibroblasts *in vitro*. *J Periodontol.*, 1991; 62(7): 434-438.
4. Amirchaghmaghi M, Delavarian Z, Iranshahi M, Shakeri MT, Mozafari PM, Mohammadpour AH, Farazi F, Iranshahy MA, A randomized placebo-controlled double-blind clinical trial of quercetin for treatment of oral lichen planus. *J Dent Res Dent Clin Dent Prospects*, 2015; 9(1): 23-28.
5. Ardelean S, Feflea S, Ionescu D, Nastase V, Dehelean CA, Toxicologic screening of some surfactants using modern *in vivo* bioassays. *Med-Surg J.*, 2011; 115(1): 251-258.
6. Azeem M, Hanif M, Mahmood K, Ameer N, Chughtai FRS, Abid U, An insight into anticancer, antioxidant, antimicrobial, antidiabetic and anti-inflammatory effects of quercetin: a review. *Polym Bull.*, 2023; 80(1): 241-262.
7. Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, Jahurul MHA, Ghafoor K, Norulaini NAN, Omar AKM, Techniques for extraction of bioactive compounds from plant materials: a review. *J Food Eng.*, 2013; 117(4): 426-436.
8. Baru O, Nutu A, Braicu C, Cismaru CA, Berindan-Neagoe I, Buduru S, Badea M, Angiogenesis in regenerative dentistry: are we far enough for therapy? *Int J Mol Sci.*, 2021; 22(2): 929.
9. Bhardwaj S, Wani FA, Evaluating the importance of apoptotic index, mitotic index and turnover index in premalignant and malignant lesions of cervix. *Open J Pathol.*, 2015; 5(2): 29-37.
10. Budala DG, Martu MA, Maftei GA, Diaconu-Popa DA, Danila V, Luchian I, The role of natural compounds in optimizing contemporary dental treatment - current status and future trends. *J Funct Biomater.*, 2023; 14(5): 273.
11. Carrillo-Martinez EJ, Flores-Hernández FY, Salazar-Montes AM, Nario-Chaidez HF, Hernández-Ortega LD, Quercetin, a flavonoid with great pharmacological capacity. *Molecules*, 2024; 29(5): 1000.
12. Caviedes-Bucheli J, Gomez-Sosa JF, Azuero-Holguin MM, Ormeño-Gomez M, Pinto-Pascual V, Munoz HR, Angiogenic mechanisms of human dental pulp and their relationship with substance P expression in response to occlusal trauma. *Int Endod J.*, 2017; 50(4): 339-351.
13. Daliborca VC, Dumitrascu V, Popescu R, Cimporescu A, Vlad CS, Flangea C, Grecu DS, Vágvölgyi C, Papp T, Horhat FG, Gas chromatography-mass spectrometry evidences for new chemical insights of *Momordica charantia*. *Rev Chim.*, 2015; 66(11): 1914-1920.
14. Dario MF, Oliveira CA, Cordeiro LRG, Rosado C, Mariz IFA, Maçôas E, Santos MS, Piedade M, Baby AR, Velasco MV, Stability and safety of quercetin-loaded cationic nanoemulsion: *in vitro* and *in vivo* assessments. *Colloids Surf A Physicochem Eng Asp.*, 2016; 506: 591-599.
15. Dinu S, Dumitrel SI, Buzatu R, Dinu DC, Popovici R, Szuhanek C, Matichescu A, New perspectives about relevant natural compounds for current dentistry research. *Life*, 2024; 14(8): 951.

16. Dinu S, Matichescu A, Buzatu R, Marcovici I, Geamantan-Sirbu A, Semenescu AD, Bratu RC, Bratu DC, Insights into the cytotoxicity and irritant potential of chlorhexidine digluconate: an *in vitro* and *in ovo* safety screening. *Dent J.*, 2024; 12(7): 221.
17. Dinu S, Nicolae Daniel O, Matichescu A, *In vitro* biocompatibility and *in ovo* irritant potential screening of two clear aligners with orthodontic applications. *Farmacia*, 2024; 72(3): 513-520.
18. Dumitrel SI, Matichescu A, Dinu S, Buzatu R, Popovici R, Dinu DC, Bratu CD, New insights regarding the use of relevant synthetic compounds in dentistry. *Molecules*, 2024; 29(16): 3802.
19. Elberskirch L, Le Harzic R, Scheglmann D, Wieland G, Wiehe A, Mathieu-Gaedke M, Golf RAH, von Briesen H, Wagner S, A *HET-CAM*-based vascularized intestine tumor model as a screening platform for nano-formulated photosensitizers. *Eur J Pharm Sci.*, 2022; 168: 1-12.
20. Gag O, Macasoi I, Pinzaru I, Dinu S, Popovici R, Cosoroaba MR, Buzatu R, Cabuta M, Chiriac SD, *In vitro* assessment of the impact of ultraviolet B radiation on oral healthy and tumor cells. *Photonics*, 2023; 10(4): 464.
21. Goyal R, Mittal G, Khurana S, Malik N, Kumar V, Soni A, Chopra H, Kamal MA, Insights on quercetin therapeutic potential for neurodegenerative diseases and its nano-technological perspectives. *Curr Pharm Biotechnol.*, 2023; 25(9): 1132-1141.
22. Haj-Ali D, Dumitrel SI, Trandafirescu C, Manea HC, Lascu A, Dinu S, Macasoi I, Buzatu R, Szuhanek C, Dumitrescu C, Olariu I, *In vitro* and *in ovo* comparative toxicological assessment of self-assembling peptide-based dental repair agents. *Farmacia*, 2024; 72(6): 1376-1385.
23. Huang CY, Ng MY, Lin T, Liao YW, Huang WS, Hsieh CW, Yu CC, Chen CJ, Quercetin ameliorates advanced glycation end product-induced wound healing impairment and inflammaging in human gingival fibroblasts. *J Dent Sci.*, 2024; 19(1): 268-275.
24. Ke X, Chen Z, Wang X, Kang H, Hong S, Quercetin improves the imbalance of Th1/Th2 cells and Treg/Th17 cells to attenuate allergic rhinitis. *Autoimmunity*, 2023; 56(1): 2189133.
25. Kim SR, Lee EY, Kim DJ, Kim HJ, Park HR, Quercetin inhibits cell survival and metastatic ability via the EMT-mediated pathway in oral squamous cell carcinoma. *Molecules*, 2020; 25(3): 757.
26. Liu X, Tao T, Yao H, Zheng H, Wang F, Gao Y, Mechanism of action of quercetin in rheumatoid arthritis models: meta-analysis and systematic review of animal studies. *Inflammopharmacology*, 2023; 31(4): 1629-1645.
27. Macasoi I, Pavel IZ, Moaca AE, Avram S, David VL, Coricovac D, Mioc A, Spandidos DA, Tsatsakis A, Șoica C, Dumitrașcu V, Dehelean C, Mechanistic investigations of antitumor activity of a rhodamine B-oleanolic acid derivative bioconjugate. *Oncol Rep.*, 2020; 44(3): 1169-1183.
28. Magyari-Pavel IZ, Moacă EA, Avram Ș, Diaconeasa Z, Haidu D, Ștefănuț MN, Rostas AM, Muntean D, Bora L, Badescu B, Iuhas C, Dehelean CA, Danciu C, Antioxidant extracts from Greek and Spanish olive leaves: antimicrobial, anticancer and antiangiogenic effects. *Antioxidants*, 2024; 13(7): 774.
29. Marcovici I, Vlad D, Buzatu R, Popovici RA, Cosoroaba RM, Chioibas R, Geamantan A, Dehelean C, Rutin linoleate triggers oxidative stress-mediated cytoplasmic vacuolation in non-small cell lung cancer cells. *Life*, 2024; 14(2): 215.
30. Markowitz K, Moynihan M, Liu M, Kim S, Biologic properties of eugenol and zinc oxide-eugenol: a clinically oriented review. *Oral Surg Oral Med Oral Pathol.*, 1992; 73(6): 729-737.
31. Martelli AM, Zweyer M, Ochs RL, Tazzari PL, Tabellini G, Narducci P, Bortul R, Nuclear apoptotic changes: an overview. *J Cell Biochem.*, 2001; 82(4): 634-646.
32. Matei MN, Dumitru IF, Neagu AI, Maris M, Carp GB, Gabriela T, Manole Palivan CC, Practical aspects of pediatric dentistry. *Rom J Oral Rehabil.*, 2020; 12(3): 259-268.
33. Memariani H, Memariani M, Ghasemian A, Quercetin as a promising antiprotozoan phytochemical: current knowledge and future research avenues. *Biomed Res Int.*, 2024; 2024(1): 7632408.
34. Moghadam ET, Yazdanian M, Tahmasebi E, Tebyanian H, Ranjbar R, Yazdanian A, Seifalian A, Tafazoli A, Current herbal medicine as an alternative treatment in dentistry: *in vitro*, *in vivo* and clinical studies. *Eur J Pharmacol.*, 2020; 889: 173665.
35. Mooney EC, Holden SE, Xia XJ, Li Y, Jiang M, Banson CN, Zhu B, Sahingur SE, Quercetin preserves oral cavity health by mitigating inflammation and microbial dysbiosis. *Front Immunol.*, 2021; 12: 1-23.
36. As MN, Deshpande R, Kale VP, Bhonde RR, Datar SP, Establishment of an *in ovo* chick embryo yolk sac membrane (YSM) assay for pilot screening of potential angiogenic and anti-angiogenic agents. *Cell Biol Int.*, 2018; 42(11): 1474-1483.
37. Nowak-Sliwinska P, Segura T, Iruela-Arispe ML, The chicken chorioallantoic membrane model in biology, medicine and bioengineering. *Angiogenesis*, 2014; 17(4): 779-804.
38. Pandya M, Kalappanavar AN, Annigeri RG, Rao DS, Relative efficacy of quercetin compared with benzydamine hydrochloride in minor aphthae: a prospective, parallel, double-blind, active control, preliminary study. *Int J Dent.*, 2017; 2017(2): 1-6.
39. Pierro FD, Derosa G, Maffioli P, Bertuccioli A, Togni S, Riva A, Allegrini P, Khan A, Khan S, Khan BA, Altaf N, Zahid M, Ujjan ID, Nigar R, Khushk MI, Phulpoto M, Lail A, Devrajani BR, Ahmed S, Possible therapeutic effects of adjuvant quercetin supplementation against early-stage COVID-19 infection: a prospective, randomized, controlled, and open-label study. *Int J Gen Med.*, 2021; 14: 2359-2366.
40. Qin XF, Zi H, Zeng XJ, Changes in the global burden of untreated dental caries from 1990 to 2019: a systematic analysis for the Global Burden of Disease study. *Heliyon*, 2022; 8(9): e10714

41. Racea RC, Macasoi IG, Dinu S, Pinzaru I, Marcovici I, Dehelean C, Rusu LC, Chioran D, Rivis M, Buzatu R, Eugenol: *in vitro* and *in ovo* assessment to explore cytotoxic effects on osteosarcoma and oropharyngeal cancer cells. *Plants*, 2023; 12(20): 3549.
42. Orihuela-Campos RC, Tamaki N, Mukai R, Fukui M, Miki K, Terao J, Ito HO, Biological impacts of resveratrol, quercetin, and *N*-acetylcysteine on oxidative stress in human gingival fibroblasts. *J Clin Biochem Nutr.*, 2015; 56(3): 220-227.
43. Sabaner MC, Duman R, Vurmaz A, Ertekin T, Effects of topical prostaglandin drops on angiogenesis in an *in ovo* chick chorioallantoic membrane model. *Cutan Ocul Toxicol.*, 2021; 40(1): 54-60.
44. She SY, Ju WS, Kim K, Kim J, Yu JO, Ryu JS, Kim JS, Lee HA, Koo DB, Choo YK, Quercetin induces mitochondrial apoptosis and downregulates ganglioside GD3 expression in melanoma cells. *Int J Mol Sci.*, 2024; 25(10): 5146.
45. Sheiham A, Williams DM, Weyant RJ, Glick M, Naidoo S, Eiselé JL, Selikowitz HS, Billions with oral disease: a global health crisis - a call to action. *J Am Dent Assoc.*, 2015; 146(12): 861-864.
46. Surducan DA, Racea RC, Cabuta M, Olariu I, Macasoi I, Rusu LC, Chiriac SD, Chioran D, Dinu S, Pricop MO, Eugenol induces apoptosis in tongue squamous carcinoma cells by mediating the expression of Bcl-2 family. *Life*, 2023; 13(1): 22.
47. Tahergorabi Z, Khazaei M, A review on angiogenesis and its assays. *Iran J Basic Med Sci.*, 2012; 15(6): 1110.
48. Wei Y, Fu J, Wu W, Ma P, Ren L, Yi Z, Wu J, Quercetin prevents oxidative stress-induced injury of periodontal ligament cells and alveolar bone loss in periodontitis. *Drug Des Devel Ther.*, 2021; 15: 3509-3522.
49. World Health Organization (WHO), Global oral health status report: towards universal health coverage for oral health by 2030: summary of the WHO European region. *Dental Abstracts*, 2022; 57(2): 1-120.
50. Wu J, Chen Z, Liu Q, Zeng W, Wu X, Lin B, Silencing of *Kv1.5* gene inhibits proliferation and induces apoptosis of osteosarcoma cells. *Int J Mol Sci.*, 2015; 16(11): 26914-26926.
51. Yi R, Liu Y, Zhang X, Sun X, Wang N, Zhang C, Deng H, Yao X, Wang S, Yang G, Unraveling quercetin's potential: a comprehensive review of its properties and mechanisms of action in diabetes and obesity complications. *Phytother Res.*, 2024; 38(12): 5641-5656.
52. Zhang W, Zheng Y, Yan F, Dong M, Ren Y, Research progress of quercetin in cardiovascular disease. *Front Cardiovasc Med.*, 2023; 10: 1203713.