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Cytotoxic Effects of Eugenol and α-Terpineol on the Rainbow Trout Gonadal Cells

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ABSTRACT

Eugenol and α-terpineol are monoterpenes that are widely used in the food, medical and agricultural sectors and are intensively studied. Although the applications of eugenol and α-terpineol have expanded, there are few reports indicating that they have cytotoxic effects depending on concentration and exposure time. Therefore, the cytotoxic effects of eugenol and α-terpineol on the rainbow trout gonadal cells (RTG-2) have been investigated based on different concentrations (18.75-600 µM and 3.125-100 µM, respectively) and exposure time (24 h and 48 h) in this study. According to the Sulforhodamine B (SRB) test, all concentrations of eugenol significantly increased the viability of RTG-2 cells compared to the control group after 24 h of treatment. In the 48 h treatment, all treatments except for the 18.75 µM treatment significantly increased cell viability. However, these increases in viability were lower compared to those observed in the 24 h treatment.The treatments with α-terpineol (≥12.5 µM) had a toxic effect on the RTG-2 cells after 24 and 48 h of treatment, leading to a significant reduction in cell viability. Treatments of α-terpineol (6.25 µM and 3.125 µM) non-significantly increased the viability of RTG-2 cells compared to the control group for 24 and 48 h, respectively. According to the data obtained from the study, it appears that molecular analyses are needed to fully understand the toxic effects of α-terpineol, especially depending on concentration and exposure time.

Öjenol ve α-Terpineol'ün Gökkuşağı Alabalığı Gonadal Hücreleri Üzerinde Sitotoksik Etkileri

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ÖZ

Öjenol ve α-terpineol gıda, tıp ve tarım sektörlerinde yaygın olarak kullanılan ve üzerinde yoğun olarak çalışılan monoterpenlerdir. Öjenol ve α-terpineolün uygulamaları yaygınlaşmış olsa da konsantrasyona ve süreye bağlı olarak sitotoksik etkilere sahip olduklarını gösteren az sayıda rapor bulunmaktadır. Bu nedenle, bu çalışmada gökkuşağı alabalığı gonadal hücreleri (RTG-2) üzerinde öjenol ve α-terpineol'ün konsantrasyona (sırasıyla 18,75-600 µM ve 3,125-100 µM) ve süreye (24 ve 48 saat) bağlı olarak sitotoksik etkileri araştırılmıştır. Sülforhodamin B (SRB) testine göre öjenolun bütün konsantrasyonları, RTG-2 hücrelerinin canlılığını 24 saat'lik uygulamadan sonra kontrol grubuna kıyasla önemli ölçüde arttırdı. 48 saatlik maruziyette ise 18,75 µM'lık uygulama hariç diğer uygulamalar hücre canlılığını önemli ölçüde arttırdı. Ancak canlılıktaki bu artışlar, 24 saatlik uygulamadakilere kıyasla daha düşüktür. α-terpineol uygulamaları (≥12,5 µM) RTG-2 hücrelerine 24 ve 48 saatlik maruziyette toksik etkide bulunarak hücre canlılığında önemli bir azalmaya yol açmıştır. α-Terpineol (6,25 µM ve 3,125 µM) uygulamaları RTG-2 hücrelerinin canlılığını kontrol grubuna kıyasla sırasıyla 24 ve 48 saat için anlamlı olmayan bir şekilde arttırdı. Çalışma elde edilen verilere göre, özellikle α-terpineol'ün konsantrasyona ve süreye bağlı olarak toksik etkilerinin tam olarak anlaşılabilmesi için moleküler analizlere ihtiyaç duyulduğu görülmektedir.

1. Introduction

Eugenol ($C_{10}H_{12}O_2$) is a volatile monoterpene phenolic aromatic compound found in essential oils obtained from most plant species (Ulanowska and Olas, 2021). Eugenol, which is recognized as a non-mutagenic and safe molecule by the World Health Organization (WHO), is used in various industrial sectors including medicine, pharmaceuticals, dentistry, food sweetening, agriculture, and cosmetics (Nisar et al., 2021). Eugenol's anti-inflammatory, antimicrobial, analgesic, antioxidant, neuroprotective, antidiabetic, and antitumor properties have been scientifically determined. In addition, based on the detection of eugenol's roles in cellular functions, such as triggering apoptosis and stopping the cell cycle, its effectiveness as a therapeutic molecule is being intensively investigated (Zhao et al., 2022; Racea et al., 2023a). Eugenol's therapeutic properties are associated with free radical scavenging (Carvalho et al., 2023). However, it has also been stated that eugenol causes structural and functional damage to tissues in a dose-dependent manner (Carvalho et al., 2022).

α-Terpineol ($C_{10}H_{18}O$) is the first monocyclic monoterpenoid isomer of terpinols (Negreiros et al., 2023). There are reports on the anti-inflammatory, antiproliferative, antitumor, antiulcer, antibronchitis, antimicrobial, antimutagenic, blood pressure-lowering, antidiarrheal, anticonvulsant, and sedative activities of α-terpineol (Sales et al., 2020; Chen et al., 2023). α-Terpineol is extensively used as a flavoring agent in foods, a fragrance in cosmetics and cleaning products, and as a suspension solvent in the production of paints and fuel cells. Additionally, it has uses in the insecticide and miticide sectors, aromatherapy, and pharmaceutical industry (Sales et al., 2020; Chen et al., 2023). However, concentration- and time-dependent toxic effects of α-terpineol have been demonstrated in various model organisms and cancer cells. These toxic effects have mostly been associated with plasma membrane degradation, ROS production, lipid peroxidation, and mitochondrial degradation (Agus, 2021; Negreiros et al., 2023).

Considering the use of eugenol and α-terpineol in food, medical, and agriculture treatments, data on their potential toxicity is very crucial. Fish cells are widely used *in vitro* toxicology studies. The rainbow trout gonadal cells (RTG-2) from the *Oncorhynchus mykiss* have advantages such as the ability to metabolize toxic compounds, not requiring an exogenous metabolic system, and showing higher sensititivity than mammalian cells (Yurdakök-Dikmen et al., 2018). The RTG-2 cells have been successfully used for cytotoxicity testing of several materials (Çiçek, 2023). To the best of our knowledge, the concentration-dependent cytotoxic effects of eugenol and α-terpineol have not been investigated on the RTG-2 cells. Thus, the present study aimed to determine the dose and exposure time dependent cytotoxic effects of eugenol and α-terpineol on these cells.

2. Materials and Methods

2.1. Materials

The 10th passage of RTG-2 cells (Registration No: 95121808) has been supplied from the ″Türkiye Şap Enstitüsü″ (Ankara, TURKIYE). Eagle's Minimal Essential Medium (EMEM) (30–2003™, ATCC, USA), eugenol (Cat No: W246700, Sigma Aldrich, USA), Penicillin-Streptomycin (Pen/Strep) (P4458, Sigma, USA), α-terpineol (Cat No: 432628, Sigma Aldrich, USA), trichloroacetic acid solution (Cat No: T0699, Sigma Aldrich, USA), sulforhodamine B (SRB) dye (Cat No:230162, Sigma Aldrich, USA), fetal bovine serum (FBS) (Cat No: S1620–500, Biowest, France), acetic acid (Cat No:695092, Sigma Aldrich, USA), and tris base (Cat No: T6066, Sigma Aldrich, USA) were purchased.

2.2. The preparation of the RTG-2 cells and eugenol and α-terpineol treatments

The growth solution for the RTG-2 cells consisted of 1% Pen/Strep, 10% FBS, and 89% EMEM. The cells were grown in an incubator (at 23.7 °C), without CO_2 respiration (Çiçek, 2023). 1.2 M of eugenol and 0.2 M of α-terpineol stock solutions were prepared using a medium as a solvent. Eugenol treatments (600, 300, 150, 75, 37.5, 18.75 µM) and α-terpineol treatments (100, 50, 25, 12.5, 6.25, 3.125 µM) were prepared from stock solutions (Ghodousi-Dehnavi et al., 2021; de Menezes Dantas et al., 2022; Alipour et al., 2022). 3×10^4 and 2.5×10^5 of the RTG-2 cells (16-18th passages) per well were seeded for the 24 h and 48 h treatments in a sterile cabinet, respectively. Eugenol and α-terpineol treatments were added to the RTG-2 cells (n = 6). The cell viability analysis was conducted after 24 h and 48 h of treatment.

2.3. Sulforhodamine B test

The Sulforhodamine B (SRB) assay was proposed in other studies to examine the cytotoxicity of compounds with redox potential in adherent cells (Shakil et al., 2022; Işık and Çiçek, 2024). Therefore, SRB assay was used to assess the cytotoxicity of eugenol and α-terpineol treatments due to their involvement in redox reactions, and because the RTG-2 cells are adherent cells (Bezerra et al., 2017; Agus et al., 2022; Işık and Çiçek, 2024). Briefly, cells were fixed with 100 µL of cold 10% trichloroacetic acid and holded at + 4 °C for 1.5 h. The plates were washed with distilled water (dH_2O) five times and were air-dried. Then, the fixed cells were dyed with SRB staining dye by adding 50 µL of a 0.4% (w/v) 1% acetic acid solution and incubated for 30 min in the dark. Later, the plates were washed with a mixture of 5% acetic acid (in a 5:1 ratio) to remove the free staining dye. After the air-drying process, 150 µL of 10 mM Tris base was added to each well, and the plates were agitated at 150 rpm for 10-20 min. The absorbance values were read using a microplate reader (EpochTM, BioTek, USA) at 564 nm (Vichai and Kirtikara, 2006; Shakil et al., 2022).

2.4. Data analysis

The study data were analyzed using the GraphPad Prism 9 software (GraphPad Software, Inc., California, USA). Treatments were assessed using one-way analysis of variance and Dunnett's multiple comparison test. All throughout were stated as mean ± SD. Analysis was performed in triplicate (n=6).

3. Results and Discussion

The SRB test revealed that all doses of eugenol significantly enhanced the cell viability of the RTG-2 cells compared to the control group after 24 h of treatment. At 24 h of treatment, eugenol concentrations of 18.75, 37.5, 75, 150, 300, and 600 μM resulted in cell viabilities of 123.11%, 145.60%, 124.57%, 150.99%, 180.90%, and 186.47% relative to the control group (100%) for RTG-2 cells. These treatments of eugenol resulted in 88.48%, 122.35%, 125.81%, 141.49%, 173.61%, and 168.67% cell viability after 48 h of treatment, respectively (Figure 1).

Reports on the concentration-dependent cytotoxic effects of eugenol are scarce. High concentrations of eugenol (≥3 mmol/L) have been reported to be cytotoxic to primary oral mucosal fibroblasts. This effect is associated with intracellular glutathione and ATP depletion. However, it has also been stated that low concentrations of eugenol (<1 mmol/L) have a protective effect on cell viability by inhibiting xanthine oxidase activity and lipid peroxidation. It has been reported that eugenol treatment (83 μM) significantly increased the metaphase II rate, first polar body extrusion, cumulus cell expansion, cytoplasmic maturity, viability, glutathione level, division rates, embryo development, expanding blastocyst and blastomere number in bovine oocytes (de Oliveira et al., 2021). It has been demonstrated that treatment with eugenol (50 μM) increased the viability of healthy human keratinocytes (HaCaT) cells over time (Aburel et al., 2021). In addition, it has been stated that eugenol (0.1 mM) did not cause a significant decrease in HaCaT cell viability after 72 h of treatment (Racea et al., 2023b). Some studies have highlighted that the concentration of eugenol matters in inducing cytotoxicity. Besides reports stating that it can only induce cytotoxicity at concentrations in the mM range, there are also some studies showing cytotoxicity at concentrations in the μM range (Bezerra et al., 2017). In this study, eugenol was seen as the only concentration (18.75 μM) that reduced cell viability compared to the control group at 48 h. This effect may be attributed to the dual nature of eugenol, which can act as both an antioxidant and a pro-oxidant agent in response to oxidative stress (Bezerra et al., 2017).

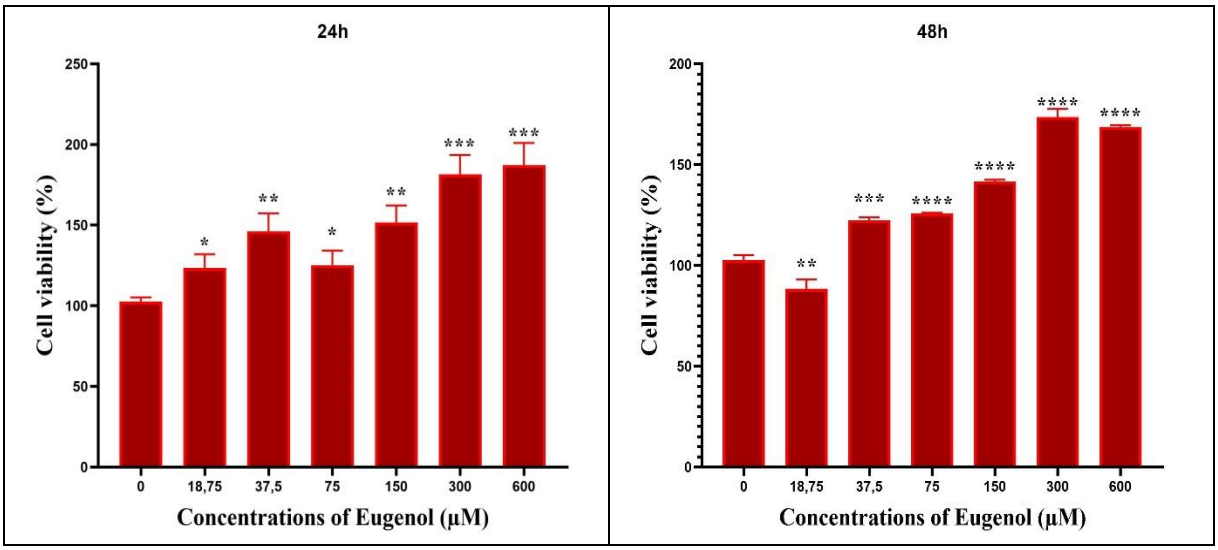

Figure 1. Cytotoxicity of eugenol (18.75-600 µM) on the RTG-2 cells depending-concentration and time. *p<0.05 significant, **p<0.01 very significant, ***p<0.0005, and ****p<0.0001 extremely significant, vs. the control group

Figure 2 demonstrates that both higher and 12.5 µM concentrations of α-terpineol showed highly toxic effects on the RTG-2 cells after 24 h and 48 h of treatment. While 3.125 µM of α-terpineol exhibited a toxic effect after 24 h of exposure, it non-significantly increased cell viability after 48 h of treatment. In addition, 6.25 µM of α-terpineol non-significantly improved the viability of the RTG-2 cells relative to the control group after 24 h of treatment, but decreased the cell viability after 48 h of treatment. 3.125, 6.25, 12.5, 25, 50, and 100 µM of α-terpineol caused 53.39%, 108.12%, 12.41%, 19.11%, 27.26%, and 27.71% of the RTG-2 cell viability compared to the control group (100%) after 24 h of treatment. These treatments of α-terpineol resulted in cell viabilities of 105.90%, 54.97%, 15.41%, 12.51%, 12.05%, and 21.32% after 48 h of exposure.

It has been reported that α-terpineol can inhibit cellular division, have a mutagenic effect with bridge formation and delayed anaphase stages, and have a cytotoxic effect on cells through apoptosis depending on the dose and exposure time (Negreiros et al., 2023). In addition, considering the penetration effects of α-terpineol, the cytotoxicity of α-terpineol can be associated with apoptosis resulting from in cell permeability, increased fluidity, and cell cycle rate. It has been reported that α-terpineol caused changes in cell membrane function and leakage of intracellular materials by destabilizing the cell membrane, showing a toxic effect against antibiotic-resistant bacteria (e.g., *Escherichia coli* O157:H7) (Zengin and Baysal, 2014). In another study, it has been reported that the hydroxyl group of α-terpineol can form glycosidic bonds and hydrogen bonds with PO^{2}- and COO-, transforming the polar head groups of phospholipids into a tight package. This process reduces membrane fluidity, disrupts electron transport in the membrane, precipitates the membrane pH gradient, and accumulates reactive oxygen species (Yang et al., 2023). Zarei et al. stated that α-terpineol (1 mg/mL) inhibited 38% of human breast cancer cells (MCF7) and 18% of human foreskin fibroblast cells (HFF) (Zarei et al., 2022). Chen et al. reported that α-terpineol can induce apoptosis in cancer cells by inhibiting of the NF-κB signaling pathway, causing mitochondrial depolarization,

releasing cytochrome C, and altering the transcriptomic expression of Bcl-2 and Bax genes (Chen et al., 2023).

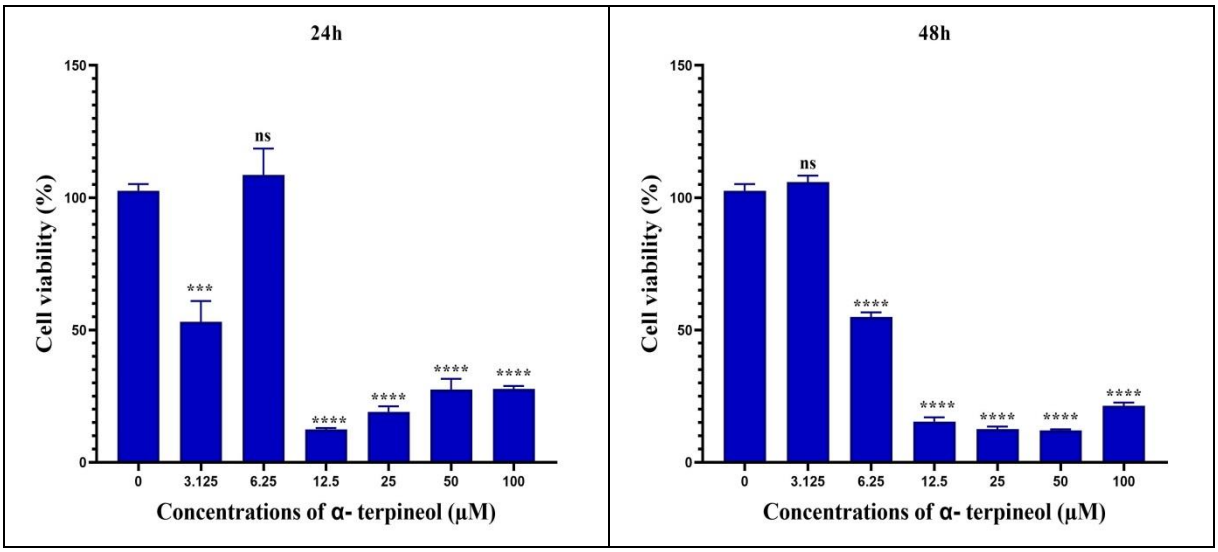

Figure 2. Cytotoxicity of α-terpineol (3.125-100 µM) on the RTG-2 cells depending-concentration and time. p>0.05 not significant (ns),***p<0.0005, and ****p<0.0001 extremely significant, vs. the control group

4. Conclusion

In this study, it was shown that all eugenol treatments (≥37.5 µM) significantly increased the RTG-2 cell viability compared to the control group cell viability (100%) at 24 and 48 h of treatment. In addition, it has been understood that α-terpineol (≥12.5 µM) had a toxic effect on the RTG-2 cells. Based on this data, it can be concluded that eugenol may have the potential to be used as a healing agent, particularly in wound care, while α-terpineol may have the potential to serve as an antibiotic agent or a toxic agent against microorganisms and pests. However, it should not be forgotten that results obtained from *in vitro* studies may differ from responses obtained from *in vivo* models. Therefore, 3D cell culture techniques, which yield results closely resembling in vivo studies, and molecular-based toxicogenomic research that relies on the correlation between concentration and treatment time, are necessary to comprehend the behavior and identify the pathways, reactions, and environmental conditions of eugenol and α-terpineol at the cellular level. Future studies may focus on these compounds and provide the data needed for these two monoterpenes.

Statement of Conflict of Interest

The author declares that there is no conflict of interest regarding the study.

Author's Contributions

The author declares that she has contributed 100% to the article.

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