Cytotoxicity and oxidative stress effects of eugenol, phenazine and eugenol-phenazine cocrystals

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UNIVERSITY OF ZAGREB
FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

GRADUATE THESIS

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Marija Filipović-Grčić

CYTOTOXICITY AND OXIDATIVE STRESS EFFECTS OF EUGENOL, PHENAZINE AND EUGENOL-PHENAZINE COCRYSTALS

This study was carried out in the Laboratory for Biology and Microbial Genetics at the Department of Biochemical Engineering at the University of Zagreb Faculty of Food Technology and Biotechnology under supervision of Ksenija Durgo, PhD, and Adrián Rojas Sepulveda, PhD.

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CYTOTOXICITY AND OXIDATIVE STRESS EFFECTS OF EUGENOL, PHENAZINE AND EUGENOL-PHENAZINE COCRYSTALS

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Abstract: This study investigates the cytotoxicity and oxidative stress induced by eugenol, phenazine, and their cocrystals, commonly used in food packaging and household products. While eugenol and phenazine are known for their antimicrobial and antioxidant properties, their potential to cause oxidative stress and cytotoxicity upon migration into human tissues raises safety concerns. This research evaluates the effects of these compounds on various human cell lines, including HaCaT, Cal27, Hep G2, and AGS, using the Neutral Red assay for cell viability and the DCFH-DA assay for reactive oxygen species (ROS) generation. By analysing the impact of these compounds and their cocrystals at environmentally relevant concentrations, this study shows that eugenol, phenazine and their cocrystals display cell specific and concentration-response dependent cytotoxicity. The most sensitive cell line was Cal27, and the most resistant cell line was HepG2. Eugenol and phenazine did not influence on free radical formation nor antioxidant effect at nontoxic concentrations. Cocrystals damaged cells significantly at all concentration range so it was not possible to bring any conclusion about antioxidant/prooxidant nature. All investigated compounds showed prooxidative effect on plasmid φX174 RI DNA, indicating the prooxidative nature of these compounds. Further research is needed to fully understand the mechanistic pathways involved, especially regarding the eugenol-phenazine cocrystals. The concentrations of compounds that migrate into the food is essential to be determined including their effect on the cells of digestive system.

Keywords: eugenol, phenazine, cocrystals, cytotoxicity, oxidative stress

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CITOTOKSIČNI I OKSIDACIJSKI UČINCI EUGENOLA, FENAZINA I EUGENOL-FENAZIN KOKRISTALA

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Sažetak: Ova studija istražuje citotoksičnost i oksidativni stres izazvan eugenolom, fenazinom i njihovim kokristalima, koji se obično koriste u pakiranju hrane i kućanskim proizvodima. Dok su eugenol i fenazin poznati po svojim antimikrobnim i antioksidativnim svojstvima, njihov potencijal da izazovu oksidativni stres i citotoksičnost nakon migracije u ljudska tkiva izaziva zabrinutost u pogledu sigurnosti. Ovo istraživanje procjenjuje učinke navedenih spojeva na različite ljudske stanične linije, uključujući HaCaT, Cal27, HepG2 i AGS, koristeći Neutral Red test za preživljenje stanica i DCFH-DA test za stvaranje reaktivnih kisikovih vrsta (ROS). Analizirajući utjecaj ovih spojeva i njihovih kokristala u ekološki relevantnim koncentracijama, ovaj eksperiment pokazuje da eugenol, fenazin i njihovi kokristali imaju citotoksično djelovanje specifično za pojedine stanice i ovisno o koncentraciji. Najosjetljivija stanična linija bila je Cal27, a najotpornija HepG2. Eugenol i fenazin nisu utjecali na stvaranje slobodnih radikala niti pokazali antioksidativni učinak u netoksičnim koncentracijama. Kokristali su značajno oštetili stanice u svim rasponima koncentracija tako da nije bilo moguće donijeti zaključak o njihovoj antioksidativnoj/prooksidativnoj prirodi. Svi ispitivani spojevi pokazali su prooksidativni učinak na DNA plazmida φX174 RI, što ukazuje na prooksidativnu prirodu ovih spojeva. Potrebna su daljnja istraživanja kako bi se u potpunosti razumjelo djelovanje istaživanih spojeva, posebno eugenol-fenazin kokristala pri čemu je bitno odrediti koncentracije u kojima spojevi migriraju u hranu te njihov učinak na stanice probavnog sustava.

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Contents

1. INTRODUCTION

The study of natural and synthetic compounds for therapeutic or food-preserving applications has gained substantial attention due to their potential to modulate biological processes, including oxidative stress and cytotoxicity. Compounds used in the manufacturing and preservation of food packaging (or that end up in household products, pharmaceuticals, cosmetics or skin-care products) can leach into food, subsequently posing health risks upon ingestion. Among these compounds, eugenol and phenazine, known for their antimicrobial and antioxidant properties, have attracted attention due to their widespread use. However, the potential cytotoxicity and prooxidative effects of these substances, especially when they migrate into human cells, remain areas of concern that warrant thorough investigation.

Eugenol, a naturally occurring phenolic compound primarily found in clove oil, has been widely studied for its diverse pharmacological properties, including antioxidant, anti-inflammatory and antimicrobial activities. However, its ability to induce oxidative stress in cells raises questions about its safety when migrating into human tissue.

Phenazine, on the other hand, is a nitrogen-containing compound with potent antimicrobial properties and an ability to generate reactive oxygen species. This makes it a compound of interest for inducing oxidative stress, a mechanism that can be exploited for therapeutic purposes such as cancer treatment. However, like eugenol, the potential cytotoxicity of phenazine warrants careful examination.

Moreover, the development of eugenol-phenazine cocrystals presents an intriguing approach to potentially modulate the properties of these compounds. Cocrystallization can affect the release, stability, and interaction of these substances, potentially influencing their migration from food packaging materials into food products and, by extension, into human cells. The effect of these cocrystals on cellular oxidative stress and cytotoxicity, particularly in cell lines relevant to human health such as HaCaT (keratinocytes), Cal27 (oral squamous carcinoma), HepG2 (hepatocytes) and AGS (gastric adenocarcinoma), is of particular interest.

This study aimed to determine the cytotoxicity and oxidative stress effects of eugenol, phenazine, and eugenol-phenazine cocrystals using the concentrations expected to be migrate from the environment into human cell models. The cell viability in response to exposure of these compounds was determined using the Neutral Red assay, meanwhile DCFH-DA and plasmid φX174 RI DNA assays were used to measure the generation of ROS in different cell lines and to evaluate oxidative stress and protective effects on DNA, respectively. The outcomes of this research provided valuable insights into the safety of using eugenol, phenazine and their cocrystals in food packaging, with implications for consumer health and regulatory guidelines.

2. LITERATURE REVIEW

2.1. BIOACTIVE COMPOUNDS

Bioactive compounds are diverse metabolites produced by almost all types of living organisms including plants, animals, algae, fungi, and bacteria. These compounds include metabolites such as antibiotics, toxins, alkaloids, food grade ingredients, growth factors, and phenolics. They have extremely important biological activities, such as being antioxidants and antimicrobials, having anti-inflammatory, antifouling, anticancer (antitumor), and antiallergenic properties, and acting as enzyme inhibitors (Kumar and Naraian, 2019). According to Kumar and Naraian (2019), the plant raw materials containing bioactive compounds have been used for treatment of various diseases since the ancient times, while in recent years they have attracted a wide community of researchers, toxicologists and nutritionists, to extract these compounds for their potential applications in the food, chemical and medical industries. Additionally, microbes can sense, adapt and respond to their environment quickly due to their ability to generate unique secondary metabolites, many of whom are highly potent and utilized for the survival of microbes (Zhang et al., 2005). Examples of bioactive compounds include eugenol and phenazine compounds.

2.1.1. Eugenol

Eugenol ($C_{10}H_{12}O_2$; phenylpropanoid) is an aromatic liquid belonging to the group of phenols and is the most important component of clove oil (*Syzygium aromaticum*). It is a clear to pale yellow liquid with an oily consistency and spicy aroma. Pictured in Figure 1, this compound is partially soluble in water, well soluble in organic solvents and has low chemical stability. It is sensitive to oxidation and various chemical interactions. When orally administered, it is rapidly absorbed by various organs and metabolized in the liver (Ulanowska and Olas, 2021).

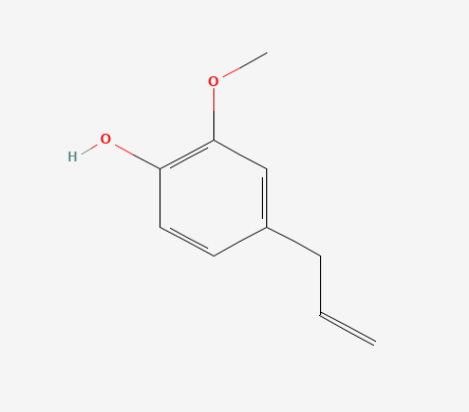

Figure 1. Chemical structure of eugenol (NCBI, 2024a).

Eugenol is a well-known and well-studied compound. It was first isolated in 1929 as a volatile

compound from *Eugenia caryophyllata* (clove), and commercial production began in the USA in 1940, with wide use in dental practice to relieve pain caused by dentinal hypersensitivity (Tammannavar et al., 2013). Additionally, it is used in combination with zinc oxide as a pulp capping agent, temporary filling and root canal sealer (Atsumi et al., 2001). Since it has demonstrated various antioxidant, analgesic, antimutagenic, antiallergic, and anti-inflammatory properties (Marchese et al., 2017), it is considered a generally safe compound at low concentration. Having multidirectional action, it is commonly used in pharmaceuticals, food, cosmetics, and as a local antiseptic and analgesic. It is also a common ingredient in household products, such as soaps, perfumes, skin care products, and fragrance. Furthermore, it can be used as a preservative to protect food from microorganisms as well as a pesticide and fumigant (Ulanowska and Olas, 2021; Batiha et al., 2020; Mohammadi et al., 2017; Pavithra, 2014; Tammannavar et al., 2013). The Joint Food and Agriculture Organization/WHO Expert Committee on Food Additives found that the maximum allowable daily intake of eugenol is 2.5 mg/kg body weight for humans.

While this compound is known to scavenge free radicals, inhibit the generation of reactive oxygen species (ROS), and protect the function of microbial DNA and proteins among other things, this is true for lower doses. Despite these important properties, eugenol can cause irritation and allergy in dependence of concentration, time of exposure and environmental factors (Navarro et al., 2019). A pro-oxidative effect can occur at higher concentrations, resulting in the formation of free radicals (Ulanowska and Olas, 2021). Additionally, eugenol was found to induce apoptosis in human promyelocytic leukaemia cells (HL-60) through a mechanism dependent on ROS and mitochondria, suggesting that it could have apoptosis-inducing chemotactic properties (Yoo et al., 2005). A study by Kozam and Mantell (1978) showed that eugenol caused denaturation of cytoplasmic proteins, loss of cell boundaries, swelling and cell necrosis of oral mucous membranes suggesting that it should not be used in any packs, or other capacities in which it might contact soft oral membranes. This is why there is a need to further study eugenol, its presence in the environment and how it circulates and ultimately affects humans.

On the other hand, because of the safety status, widespread acceptance by consumers and multipurpose use due to its wide array of biological effects, eugenol is used for development of active packaging materials. That is why it is incorporated, together with phenazine, into cocrystals, and the goal of this study is to determine the safety of cocrystals compared to eugenol and phenazine on their own.

2.1.2. Phenazine

Phenazines are a large group of heterocyclic, coloured, and nitrogen-containing compounds of biological and chemical origin with great stability in natural environments. The position and type of functional groups in the phenazines' molecules dictate their chemical, physical and biological properties that encompass antibiotic, antifungal, insecticidal, antitumor, antimalarial, and antiparasitic activities. This wide bioactivity spectrum is conferred by the high redox activity of phenazine molecules and their ability to reduce molecular oxygen to reactive oxygen species (Serafim et al., 2023; Guttenberger et al., 2017). Phenazines are produced by many Gram-positive (e.g. *Streptomyces*) and Gram-negative bacteria (e.g. *Pseudomonas*), or by archaeal *Methanosarcina* species, as secondary metabolites at late growth stages and high cell densities. It has been demonstrated that phenazine-producing organisms exhibit a longer lifespan in the natural environment than their non phenazine-producing counterparts (Laursen and Nielsen, 2004). Since they are secondary metabolites and as such present in nature, more is known about phenazine derivatives than phenazine alone. For instance, pyocyanin is antiproliferative in human cells (Sorensen et al., 1983), while 1-hydroxyphenazine interferes with cellular respiration by acting as an electron acceptor, preventing ATP generation (Stewart-Tull and Armstrong, 1972). Other derivatives can produce ROS *in vitro* (Davis and Thornalley, 1983), and some may penetrate cellular membranes and intercalate DNA due to their planar structure and hydrophobicity (Laursen and Nielsen, 2004). As such, phenazine derivatives are used as medicine and pesticides, but due to their non-selective DNA binding and antiproliferative effect on human cells, they may present toxic risks. Ou et alč (2020) studied the degradation, adsorption and leaching of phenazine-1-carboxamide (PCN) in the agricultural soil, and found that this compound is easily degraded, has high adsorption affinity and a risk of contamination in soils containing low organic matter and low clay content is high.

The core structure of phenazines is a pyrazine ring (1,4-diazabenzene) exhibiting two annulated benzenes (Guttenberger et al., 2017). Its structure is depicted in Figure 2.

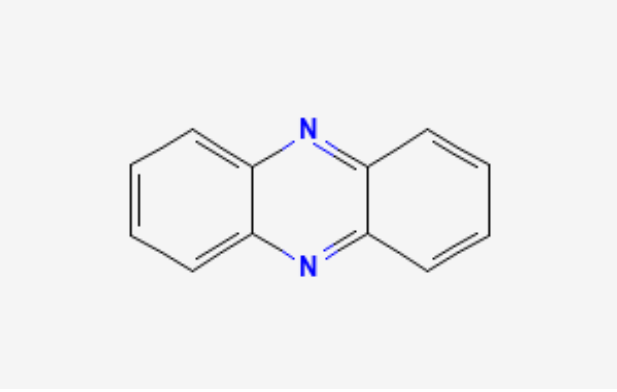

Figure 2. Chemical structure of phenazine (NCBI, 2024b)

According to the research conducted by McGuigan and Li (2013), phenazine has different

effects on HepG2 and T24 cell lines. A cytotoxic, concentration-dependent reduction of proliferation occurs in HepG2 cell, which may enter a cytostatic state at elevated phenazine concentration, but no genotoxic response was observed. On the other hand, T24 cells showed a minor cytotoxic antiproliferative effect, but did experience significant cytotoxicity at concentrations which were not cytotoxic.

This study aims to determine the cytotoxic and oxidative effects of phenazine (1,4-diazabenzene), focusing on its potential biological impacts. Additionally, the study seeks to determine whether phenazine exhibits protective effect on plasmid DNA under oxidative stress conditions. By understanding these effects, the research aims to shed light on phenazine's potential therapeutic and biochemical properties, which need to be further studied.

2.2. COCRYSTALS

The term cocrystal has been used to describe crystalline materials with two or more different molecules in the same crystal lattice. They are highly orientated three-dimensional assemblage of molecules in solid-state. These organised structures are controlled by symmetry and long-range intermolecular interactions that ultimately define their fundamental physical properties (Gunawardana and Aakeroy, 2018).

Cocrystals can be tailored to enhance drug product bioavailability, stability and solubility (Buddhadev and Garala, 2021) and so cocrystallization can be a valuable tool for designing new solid materials with desired physical and chemical properties for specific applications. Most cocrystal research are related to improving drug properties, however, in recent years, there has been an increasing interest in cocrystallization of different compounds, such as polyphenols, known for their biological relevance for food products (Dias, Lanza and Ferreira, 2021). The emergence of packaging materials loaded with natural active ingredients has become a notable strategy for extending the shelf life of food (Abdollahzadeh et al., 2021). These materials offer advantages such as gas selective permeability, anti-microbial activity, barrier properties and environmental friendliness. Essential oil-based cocrystals (such as eugenol-phenazine cocrystals) offer advantages such as good thermal stability, controllable release rates, enhanced solubility and a streamlined preparation process (Song et al., 2024).

The eugenol-phenazine (EU-PHE) cocrystals were prepared by manually grinding equimolar quantities of eugenol and phenazine in an agar mortar for approximately 20 minutes, yielding a yellow powder (Rojas et al, 2024). Their structure is depicted in Figure 3.

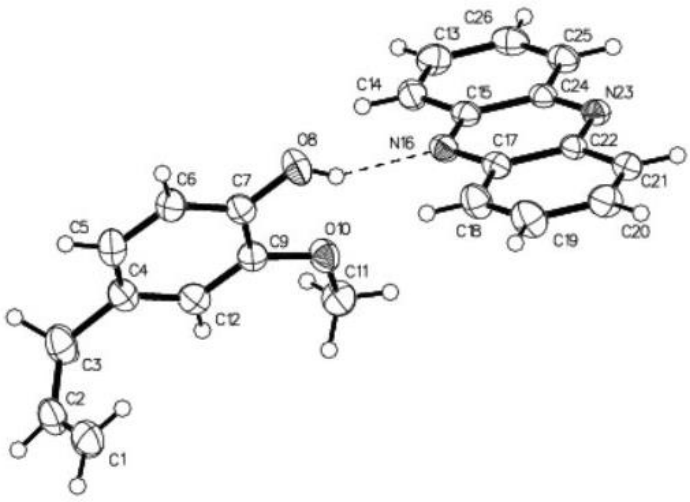

Figure 3. Chemical structure of eugenol-phenazine cocrystals (Mazzeo et al., 2019).

The effects of cocrystals, such as eugenol-phenazine cocrystals warrant further research beyond the scope of this study. While the current findings provide preliminary insights, a more comprehensive evaluation is necessary to fully understand their therapeutic potential, stability, bioavailability and interaction with biological systems.

2.3. HUMAN CELL LINES

In vitro human cell lines are essential tools in various fields of research, including drug discovery, toxicology and biomedical research, making them critical resources for studying compounds like eugenol, phenazine and eugenol-phenazine cocrystals. The use of human cell line models offers several advantages: they provide a renewable resource, and the availability of extensive multiomic data (e.g., genomics, epigenomics, transcriptomics, proteomics and metabolomics) facilitates the investigation of molecular mechanisms. In the context of this study, human cell lines allow for the evaluation of cytotoxicity and oxidative effects. Moreover, high-throughput assays enable efficient measurement of phenotypes such as cytotoxicity, growth rates and oxidative stress response to eugenol, phenazine and their cocrystals. However, there are also limitations associated with the use of human cell lines. The use of human cell lines cannot fully replicate the complexities of the human microenvironment and drug pharmacokinetic effects, which are crucial for understanding clinical responses. Gene expression profiles in these models are not identical with primary tissues, possibly affecting the interpretation of results. Variations such as new mutations and changes in cell line characteristics may occur over time, underscoring the importance of further validation and biomarker confirmation while conducting research (Niu and Wang, 2015). Despite these limitations, human cell lines remain a valuable model for initial testing and understanding the

biological effects of eugenol, phenazine and eugenol-phenazine cocrystals.

Cancerogenic cell lines are commonly used to evaluate the potential of compounds for anticancer activity. The use of cancer cell lines in this study can help determine whether eugenol, phenazine or their cocrystals induce cytotoxicity selectively in malignant cells. Given that phenazine and its derivatives are known to have anticancer properties, it is essential to assess the impact of phenazine as a structural part of the cocrystals on the viability and growth of cancer cells. In contrast, healthy cell lines are crucial for evaluating the safety profile of these compounds and are used to determine whether these bioactive compounds are selective for cancer cells or if they also harm normal cells. This is an essential aspect of drug development, as compounds with high cytotoxicity in cancer cells but low toxicity in healthy cells are ideal candidates for further development.

Use of human keratinocytes (HaCaT) can help evaluate the safety of the researched compounds in healthy epithelial cells, while cells of oral cancer (Cal27), stomach cancer (AGS) and liver cancer (Hep G2) can provide valuable information on effects of these compounds on cancerogenic, gastrointestinal tract.

2.4. CYTOTOXICITY ASSAYS

Cell viability is the number of living cells within a certain population. Measuring the number of proliferating cells is used as a vital indicator of the cell survival or death, usually in response to chemical agents or compounds. The effects of tested compounds can be classified as either cytotoxic - which refers to being toxic and killing the cells - or cytostatic, which is defined as inhibiting cell growth without necessarily inducing cell death. Proliferation and cytotoxicity assays are used for different types of biological studies, for screening the response of the cells to various types of compounds and chemical agents. Various assay methods are available, each relying on different cellular functions, including cell membrane permeability, dye uptake, metabolic activity, cell adherence, ATP production, enzyme release, and DNA synthesis (Adan et al., 2016). The assay used in this study is the Neutral Red method, a quantitative approach that assesses cell viability by measuring lysosomal activity, which is indicative of living, adherent cells. The principle behind this method is that viable cells actively uptake and retain the Neutral Red dye within their lysosomes, while damaged or dead cells do not. The amount of dye retained is proportional to the number of viable cells, allowing researchers to distinguish between viable, damaged or dead cells.

According to the ISO guidance (ISO 10993-5:2009), a sample is considered potentially cytotoxic if its signal is reduced to less than 70% of the blank control (Shafiee et al., 2021). The Neutral

Red assay is particularly suited for adherent cells, making it a reliable tool for assessing cell viability in this study and determining the cytotoxicity of eugenol, phenazine and eugenol-phenazine cocrystals.

2.5. OXIDATIVE EFFECTS ASSAYS

Oxidative stress occurs when there is an imbalance of free radicals and antioxidants, resulting in cellular damage. It is involved in the process of aging and the development of various chronic diseases. Monitoring oxidative stress can be achieved by measuring the products of oxidative damage or by assessing the ability of cells to withstand oxidation. Free radicals, particularly reactive oxygen species (ROS), are highly reactive because of their unpaired electrons, and most of them are unstable and short-lived. Subsequently, ROS can initiate cellular damage by modifying proteins, lipids and DNA, leading to a decline in cell health and viability. There are multiple external sources of free radicals such as UV-photolysis, radiation, ozone, pharmacological agents, pesticides and smoking (Palmieri and Sblendorio, 2007). To counteract their damage, antioxidants either prevent, inhibit or reduce oxidative processes and because of this, understanding the antioxidant properties of these compounds is crucial. Various analytical methods have been developed to evaluate the antioxidant properties of compounds (Kotha et al., 2022).

In this study, ROS production is of particular interest, as eugenol and phenazine are known to have oxidative effects. For this purpose, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is applied to assess the effects of eugenol, phenazine and eugenol-phenazine cocrystals on ROS production of four different cell lines. This method provides insight into how these compounds modulate oxidative stress, either by inducing ROS generation or by acting as antioxidants to mitigate oxidative damage.

3. EXPERIMENTAL PART

3.1. MATERIALS

3.1.1. Biological test systems

As a biological test system, human cell lines from *American Type Culture Collection* (ATCC, USA) were obtained. Plasmid φX-174 RF I was obtained from Promega, USA.

3.1.1.1. Cell lines

In this research, four continuous human cell lines were used: Hep G2, AGS, Cal 27 and HaCaT. Three of them are cancerogenic cell lines (Hep G2, AGS and Cal) and one is a healthy cell line (HaCaT).

Hep G2 is a cell line exhibiting epithelial-like morphology that was isolated from a hepatocellular carcinoma of a white 15-year-old male, with liver cancer (Figure 4). The cells demonstrate decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to oxidative stress. The cells are preserved at temperatures lower than -130 °C in liquid nitrogen vapor (ATCC, 2024).

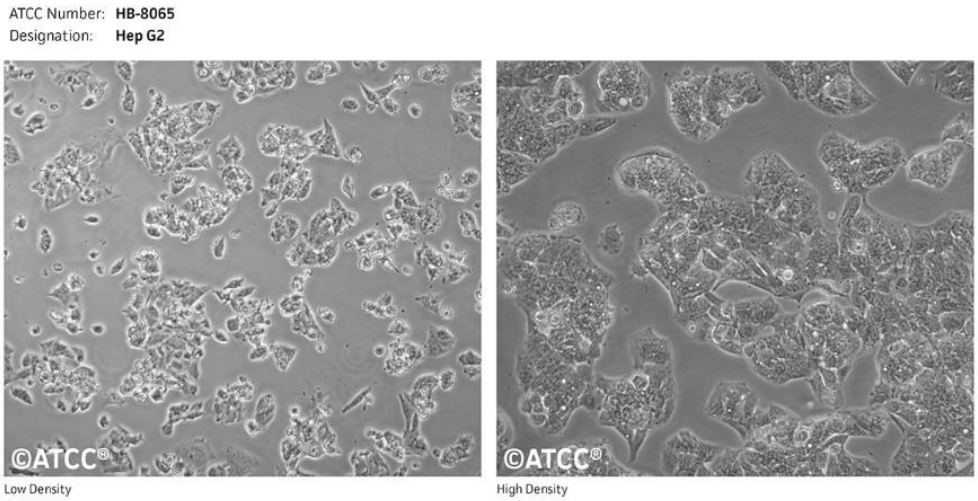

Figure 4. Cell line HepG2 (ATCC, 2024a)

AGS is a cell line exhibiting epithelial morphology that was isolated in 1979 from the stomach tissue of a 54-year-old, white, female patient with gastric adenocarcinoma. This is a hyper diploid human cell line, with modal chromosome number of 49, occurring in 60% of cells (ATCC, 2024).

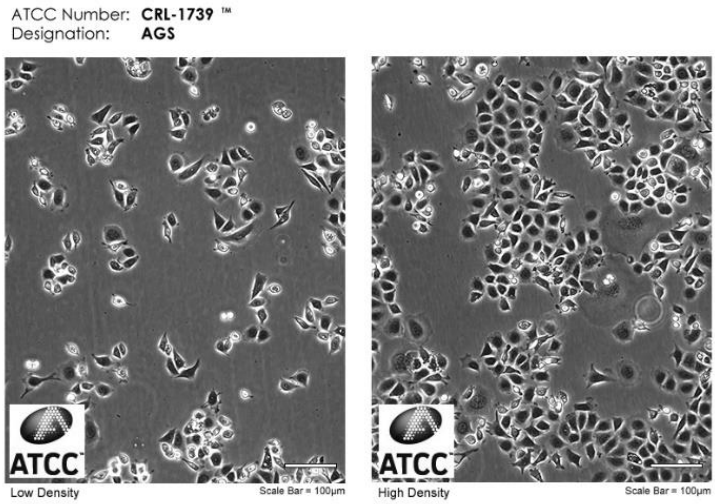

Figure 5. Cell line AGS (ATCC, 2024b).

Cal27 cells are epithelial cells isolated in 1982 from tissue taken prior to treatment from a 56-year-old, white male, with a lesion in the middle of the tongue. These cells are aneuploidic with a modal number of 43 (ATCC, 2024).

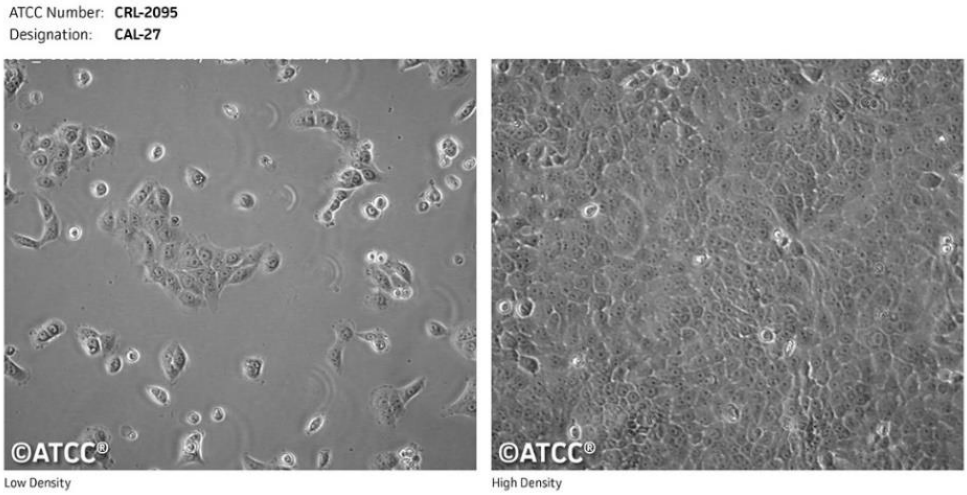

Figure 6. Cell line Cal 27 (ATCC, 2024c)

The spontaneously immortalized HaCaT cell line is derived from adult human epidermal cells and retains the capacity to proliferate and undergo differentiation. They are susceptible to apoptosis and their sensitivity to apoptosis-inducing agents are extensively studied, particularly in context of cytotoxic agents (Cytion, 2024).

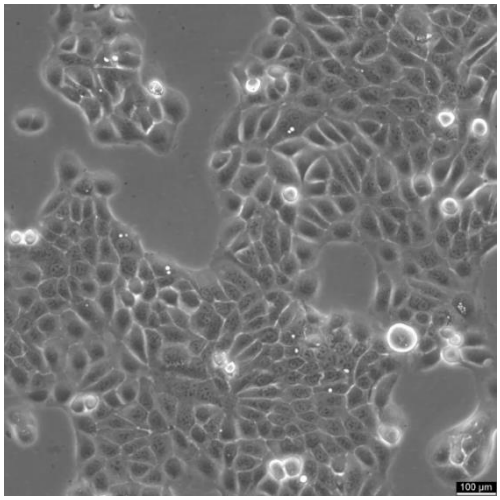

Figure 7. Cell line HaCaT (Cytion, 2024)

All the cell lines are cultured in monolayers in T-flasks incubated at 37 °C in an atmosphere with 5% CO_2 and 95% relative humidity. The cells grow in a completed RPMI medium (*Roswell Park Memorial Institute*) with 10% foetal bovine serum (FBS). After forming a monolayer, the medium is removed and discarded, with each cell line handled separately. The excess serum is washed away with 0.25% trypsin solution ensuring that the monolayer is completely covered, and the cells undergo a trypsinization process to detach from the bottom of the T-flasks. The detached cells are then treated again with medium and serum by repeated pipetting over the surface bearing the monolayer to prevent degradation and forming a single-cell suspension, with cells rounded up as a result (Freshney, 2010).

The next step is counting the cells in a Bürker-Türk counting chamber to determine the number of cells in 1 ml of suspension. The counting chamber consists of four big squares while each of them has 16 smaller squares. The volume of each big square is 10^{-4} ml, and the number of cells in 1 ml is calculated by dividing the average number of cells with the volume of the big square. The next step is to prepare the working suspension with the desired concentration of cells (10^5 cells/ml) which will be used for seeding the cells into transparent (for cytotoxicity assay) and black plates (for prooxidative/antioxidative assay) with 96 wells. The plates are then incubated for 24 hours at 37 °C in an atmosphere with 5% CO_2 and 95% relative humidity.

3.1.1.2. DNA model system

Supercoiled plasmid φX-174 RF I DNA was used as a model of DNA macromolecule which is susceptible to oxidative damage. It was the first DNA virus discovered to have a single-stranded, circular genome. The DNA strand packaged into the virion is termed the ''plus'' strand. After entering the cell, the plasmid is used as a template for minus-strand synthesis, producing

double-stranded DNA (Thermo-Fischer, 2024). This plasmid is commonly used for determining the restrictive enzymes activity and their ability to nick DNA on specific site, but also this plasmid can be used for determining prooxidative activity of investigated compounds. A cuvette with the original solution of plasmid was taken from the refrigerator and using TE (Tris-EDTA) buffer, a solution with required concentration of plasmid and buffer was prepared. Optimisation of the plasmid was performed to determine the appropriate amount of ultraviolet light that would cause supercoiled DNA to break into the relaxed form.

3.1.2. Chemicals

- 2',7' - dichlorodihydrofluorescein diacetate (DCFH-DA), *Sigma Aldrich*, Canada
- Demineralised water
- Dimethyl sulfoxide (DMSO), *Merck*, Germany
- Ethanol (C_2H_5OH), *Kemika*, Croatia
- Ethidium bromide ($C_{21}H_{20}BrN_3$), *Sigma Aldrich*, Canada
- Ethylenediaminetetraacetic acid (EDTA), *Kemika*, Croatia
- Eugenol (EU, 98%), *Sigma Aldrich*, Canada
- Eugenol-phenazine cocrystals
- Foetal bovine serum (FBS), *Capricorn Scientific GmbH*, Germany
- Glacial acetic acid (80% (v/v)), *Kemika*, Croatia
- Low melting point agarose (LMP), *Invitrogen*, UK
- Neutral red (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride), *Feinchemie K.-H. Kallies KG*, Germany
- Phenazine (Phe, 99%), *Sigma Aldrich*, Canada
- Potassium dihydrogen phosphate (KH_2PO_4), Riedel-De Hean, Germany
- RPMI 1640 medium, *Capricorn Scientific GmbH*, Germany
- Sodium chloride (NaCl), *Carlo Erba Reagents*, France
- Sodium hydrogen phosphate (Na_2HPO_4), *Gram-mol d.o.o.*, Croatia
- Sodium hydroxide (NaOH), *Kemika*, Croatia
- Tris, *Invitrogen*, UK
- Trypsin, *Capricorn Scientific GmbH*, Germany

3.1.3. Solutions

3.1.3.1. Tested samples

Eugenol, phenazine, and eugenol-phenazine cocrystals were dissolved in DMSO to make a stock concentration of 60 µM. The added amounts are listed in Table 1.

Table 1. Composition of the samples.

Component	Amount	DMSO (µl)
Eugenol	5 µl	495
Phenazine	0.0054 g	500
Eugenol-phenazine cocrystals	0.01 g	500

3.1.3.2. Phosphate buffer

Phosphate buffer (PBS) was used for diluting different solutions and washing out the cells. Composition of phosphate buffer is listed in table 2.

Table 2. Composition of phosphate buffer.

Compound	Amount
NaCl	8.00 g
KCl	0.20 g
$Na_2HPO_4 * 2H_2O$	1.15 g
K_2HPO_4	0.20 g
Distilled water	Up to 1000 ml

3.1.3.3. Neutral Red

Neutral Red dye was used for staining the cells when determining the percentage of survival with Neutral Red method. The composition of the concentrated solution is listed in Table 3.

Table 3. Composition of stock Neutral Red (5 mg/ml) solution.

Component	Amount
Neutral Red dye	50 mg
Ethanol	10 ml

The composition of working Neutral Red solution is listed in Table 4.

Table 4. Composition of working Neutral Red solution.

Component	Amount
Concentrated Neutral Red solution (5 mg/ml)	0.1 ml
RPMI 1640 medium	9.9 ml

3.1.3.4. Extraction solution

Extraction solution was used during the Neutral Red method for extraction of the accumulated dye out from the cells. The composition of the solution is listed in table 5.

Table 5. Composition of extraction solution.

Component	Amount
Ethanol	50,00%
Distilled water	49,00%
Glacial acetic acid	1,00%

3.1.3.5. 2',7' - dichlorodihydrofluorescein diacetate (DCFH-DA)

DCFH-DA was used for determination of the prooxidative/antioxidative effects of samples. The composition of the solution is listed in Table 6.

Table 6. Composition of DCFH-DA stock solution (2 mM).

Component	Amount
DCFH-DA	2 mg
DMSO	2 ml

Concentration of working solution is 50 µM and it was prepared by dilution of the stock solution in PBS as shown in table 7.

Table 7. composition off DCFH-DA working solution (50 µM).

Component	Amount
DCFH-DA	500 µl
PBS	19.5 ml

3.1.3.6. TRIS-EDTA (TE) buffer

TE buffer was used to prepare reaction mixtures in experiments where the supercoiled plasmid molecule was used as a test system. The composition of the TE buffer is listed in Table 7.

Table 7. Composition of TE buffer

Component	Amount
TRIS (tris(hydroxymethyl)aminomethane)	1.00 ml
EDTA (ethylenediaminetetraacetic acid)	200.00 µl
Distilled water	Up to 100 ml

3.1.3.7. TAE buffer

TAE buffer was used as an electrophoresis buffer. The composition of the buffer is listed in Table 8.

Table 8. Composition of TAE buffer

Component	Amount
Tris	48,40 g
EDTA	3.70 g
Acetic acid	11.40 ml
Distilled water	Up to 1000 ml

3.1.3.8. Ethidium bromide

Ethidium bromide was used as the intercalating dye to visualise the supercoiled and coiled plasmids that formed during the electrophoresis. The composition of the solution is listed in Table 9.

Table 9. Composition of ethidium bromide

Compound	Amount
Ethidium bromide	75.00 µl
Distilled water	7500 ml

3.1.4. Laboratory equipment and devices

- Analytical balance 1712 Mp8, SilverEdition, UK
- Automatic pipettes of 20, 200 and 1000 µL
- Beaker of 20 mL
- CO_2 controlled atmosphere incubator, Brouwer CH, Switzerland
- Eppendorf cuvettes of different volumes
- Erlenmeyer flasks of different volumes
- Extensions for automatic pipettes
- Falcon™ tubes
- Glass tubes
- Inverted biological microscope XDS-1, OPTIKA Microscopes, Italy
- Measuring flasks of different volumes
- Microtiter plate reader, Cecil Instruments Ltd, England
- Microtiter plates with 96 wells
- Multi-channel automatic pipette
- Parafilm
- Pipettes of 1, 2, 5, 10, 20 and 25 mL
- Pipetting gun
- Stands for cuvettes and test tubes
- System for horizontal electrophoresis, Bio-Rad USA
- T-bottles

- Türken-Bürk counting chamber
- Writing markers

3.1.5. Computer programs

Multiple computer programs were used for data analysis, electrophoresis results analysing and statistical data processing. They are as follows: Microsoft Excel 2021 (Microsoft Corporation, USA), GelAnalyzer 23.1.1 (available at www.gelanalyzer.com) by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc and JASP 0.19. (University of Amsterdam, Netherlands).

3.2. METHODS

3.2.1. Determining the cytotoxicity by applying Neutral Red method

To determine if eugenol, phenazine and eugenol-phenazine cocrystals have cytotoxic effect on human cell lines, Neutral Red method was used. It is based on the ability of viable cells to incorporate and bind the supravital dye Neutral Red (3-amino–7–dimethylamino–2-methylphenazine hydrochloride). This weakly cationic dye penetrates cell membranes by non-ionic passive diffusion and concentrates in the lysosomes, where it binds by electrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix (Nemes et all, 1979). The dye is then extracted from the viable cells using an acidified ethanol solution, and the absorbance of the solubilized dye is quantified using a spectrophotometer, measuring the absorbance at 540 nm (Repetto et al., 2008). When the cell dies or the pH gradient is reduced, the dye cannot be retained (Filman, 1975). Consequently, the amount of retained dye is proportional to the number of viable cells, making it possible to distinguish between viable and damaged or dead cells (Repetto et al., 2008).

The protocol used was adapted according to Repetto et al., 2008. Each well of transparent 96-well microtiter plates were filled with 100 µl of cell suspension at concentration of 10^5 cells/ml. Cell lines (Hep G2, AGS, Cal 27 and HaCaT) were seeded at a concentration of 10^5 cells/ml. The cells were then incubated for 24 hours in an incubator at 37 °C with 5% CO_2 atmosphere and 95% relative humidity. After the incubation, cells were treated with test samples in the concentrations of 0.16, 0.3, 1.6 and 3 µM for 24 hours. Concentration range was set according to residual concentrations that can be found in water, soil or food containers (Ou et all., 2020). Subsequently, the medium was removed from the wells and after the washing of the cells with PBS, the working solution of Neutral Red dye was added. The cells were incubated with the dye for one hour at 37°C. After incubation, the dye solution was removed, and the cells were washed with phosphate-buffered saline (PBS). After removing the PBS and drying the plates, extraction solution was added and accumulated neutral red was taken out of the cells in the

form of transparent solution. The intensity of colour was determined by measuring absorbance at a wavelength of 540 nm, with the intensity of the colouring being proportional to cell viability. Each concentration was tested in eight replicates per cell line and each experiment was repeated 3 times. The viability is calculated according to Formula 1.

$$\%(viability) = (\frac{A_{540} nm\ sample}{A_{540}\ nm\ control}) * 100 \qquad [1]$$

With A_{540} marking absorbance value measured at 540 nm.

3.2.2. Determining the oxidative effect using DCFH-DA method

Reactive oxygen species (ROS) are common by-products of normal aerobic cellular metabolism and play important physiological roles in intracellular cell signalling and homeostasis. However, a condition known as oxidative stress (OS) occurs, when ROS overwhelm the cell's ability to readily detoxify them. Excessive amounts of free radicals generated under OS conditions cause oxidative damage to proteins, lipids, and nucleic acids, severely compromising cell health and contributing to disease development. One way to estimate the cellular levels of ROS is using fluorogenic probes (Cohen et al., 2016). Hydrogen peroxide (H_2O_2), hydroxyl radicals (OH-), and peroxyl radicals (ROO-) can be measured in the treated cells after addition of 5-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This membrane-permeable probe diffuses into the cells where it becomes hydrolysed by intracellular esterase to DCFH. The latter remains trapped within the cells and reacts with H_2O_2, generating the fluorescent 2',7'-dichlorofluorescein (DCF). Therefore, the amount of peroxide produced by the cells can be estimated by the fluorescence intensity of DCF by using a fluorescence plate reader, with excitation at 485 nm and emission at 530 nm (Katerji et al., 2019).

The protocol used in this method was adapted from Wang and Joseph (1999), with modifications. Cells were seeded in black, 96-well microtiter plates and each well filled with 100 µl of cell suspensions at concentration of 10^5 cells/ml. Cell plating and treatment were performed as described in the protocol for determining the cytotoxic effects. After 24 hours incubation, the medium was removed from each well, and 0.05 mmol/l solution of DCHF-DA was added. The plates were then incubated for one hour at 37°C. Following incubation, the fluorescence intensity was measured using a fluorescence plate reader. The fluorescence intensity, which is proportional to the formation of reactive oxygen species (ROS), was used to calculate the pro-oxidative activity by comparing the fluorescence intensity of the sample to that of the control. Each concentration was tested in eight replicates per cell line and each experiment was repeated 3 times. The viability is calculated according to Formula 2.

$$ROS\ induction\ \% = \frac{\frac{fluorescence\ (sample)}{viability\ (sample)}}{\frac{fluorescence\ (control)}{viability\ (control)}} * 100 \qquad [2]$$

3.2.3. Determining of the protective effect on DNA due to the action of ROS

This method was adapted from the protocol by Keum et al., (2000) with slight modifications. The exposure of the plasmid to UV radiation and H_2O_2 generates hydroxyl radicals leading to the unwinding of the supercoiled plasmid DNA, into its relaxed form. In this work, φX174 RI circular plasmid DNA was exposed to hydroxyl radicals and examined compounds to determine protective, antioxidative effect of examined compounds, but also plasmid was exposed solely to UV radiation and examined compounds to determine if examined compounds, once they were exposed to UV light can generate hydroxyl radicals which might damage DNA. This structural transition can be effectively monitored using agarose gel electrophoresis.

The initial step involved optimizing the plasmid DNA to ensure consistency and maximize assay sensitivity, which is crucial for accurately detecting ROS-induced changes. Agarose gel electrophoresis was utilized for this purpose. In order to optimize experimental procedure and to determine optimal time of UV exposure each well was loaded with 0.044 µg/ml of plasmid DNA. The first well also contained 18 µl, while wells two through fourteen contained 16 µl of TE buffer each. Additionally, 0.18% hydrogen peroxide (H_2O_2) was added to wells two through fourteen to induce oxidative stress. The fifteenth well served as a standard control. The prepared wells were then exposed to UV radiation for varying duration (2, 4, 6, 7, 8, 9, 10, 15, 17, 20, 25, and 30 minutes) to determine optimal exposure time for φX174 RI plasmid DNA.

To determine prooxidative/antioxidative effect of investigated compounds, concentrations of the investigated compounds and mixtures were adjusted to a total reaction volume of 20 µl. Samples were exposed to UV radiation for 4 minutes at a distance of 50 cm from a 30 W germicidal UV lamp (Philips, The Netherlands). After a 30-minute incubation at room temperature, 1 µl of 6x concentrated loading buffer was added to each sample. The products were analysed by agarose gel electrophoresis (1% gel prepared in 1 x TAE buffer) and visualised using a transilluminator after staining with ethidium bromide solution (0.5 µg/ml). Gel electrophoresis was conducted at 60 V for two hours. The resulting gel images were processed using GelAnalyzer 23.1.1., with results expressed as the ratio of the surface areas of the supercoiled and relaxed bands, statistically analysed against the control sample (plasmid only) according to Formula 3. Each experiment was repeated 3 times.

$$\%\ linearisation\ \left(\frac{SCP}{RCP}\right) = \frac{volume\ of\ SCP}{volume\ of\ RCP} * 100 \qquad [3]$$

4. RESULTS AND DISCUSSION

The effects of eugenol, phenazine and eugenol-phenazine cocrystals were evaluated using a range of assays, including those assessing proliferative and antiproliferative activity, prooxidative/antioxidative effects on human cell lines, and DNA protective capacity. The experiments were conducted on human cell lines derived from gastric epithelial adenocarcinoma (AGS), squamous epithelium of tongue carcinoma (Cal27), hepatocellular carcinoma of the liver (HepG2) and spontaneously transformed aneuploid immortal keratinocyte cell line (HaCaT). Additionally, the circular plasmid φX174 RFI DNA was used as a model macromolecule to evaluate the DNA protective effects of tested compounds.

The chosen concentration range (0.16, 0.3, 1.6 and 3 µM) reflects levels that could realistically be achieved through dietary intake and environmental exposure to the individual compounds and their cocrystals.

Due to the low solubility of these compounds in water, they were prepared by dissolving in dimethyl sulfoxide (DMSO).

Cytotoxic effects on the human cell lines were assessed using the Neutral Red method, which quantifies cell viability by measuring lysosomal activity in adherent cells. Oxidative effects were evaluated using DCFH-DA method, which measures reactive oxygen species (ROS) production. The DNA protective effect of the compounds was examined using the model plasmid and performing agarose gel electrophoresis to assess any protective or damaging effects on DNA structure.

4.1. RESULTS OF DETERMINING THE CYTOTOXIC EFFECTS OF EUGENOL, PHENAZINE AND EUGENOL-PHENAZINE COCRYSTALS

As previously mentioned, cytotoxic effects of eugenol, phenazine and eugenol-phenazine cocrystals were assessed through Neutral Red method, on squamous epithelium of tongue carcinoma (Cal 27), hepatocellular carcinoma of the liver (HepG2), gastric epithelial adenocarcinoma (AGS) and spontaneously transformed aneuploid immortal keratinocyte cell line (HaCaT). Each cell line was exposed to the concentrations of 0.16, 0.3, 1.6 and 3 µM of the compounds for 24 hours.

The results obtained are presented graphically, showing the relations between compound concentrations and cell survival, expressed as a percentage of relative cell viability compared to the control. The graphs include standard deviations of the measurements and highlight statistically significant differences in the results.

HaCaT

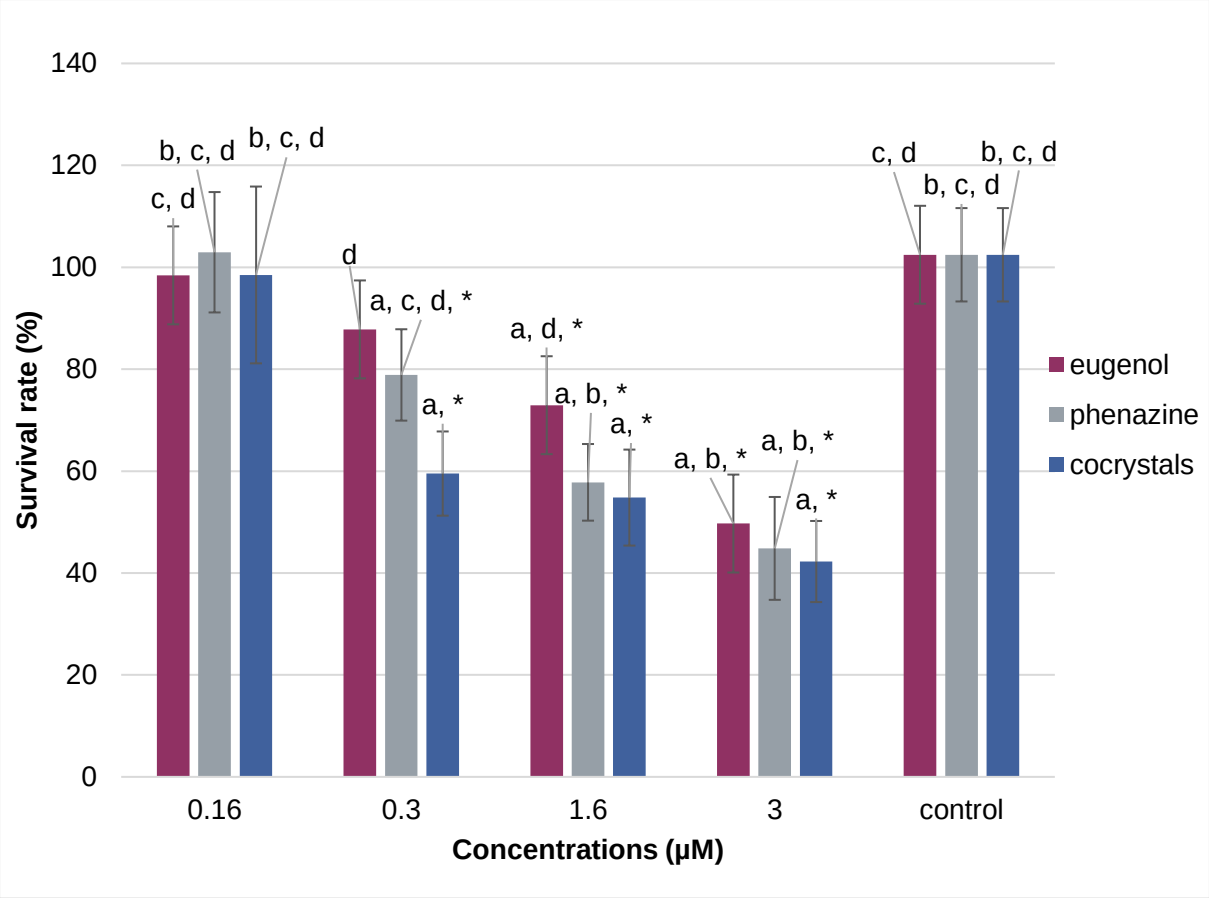

Figure 8. The survival rate of HaCaT cell line treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals, including statistical significance labels.

Statistically significant difference with $p < 0.01$ is denoted as follows: a – significant difference compared to the 0.16 μM concentration; b – significant difference compared to the 0.3 μM concentration; c – significant difference compared to the 1.6 μM concentration; d – significant difference compared to the 3 μM; * - significance in relation to control.

As the concentration increases from 0.16 μM to 3 μM, there is a general decrease in HaCaT cell survival rates, as seen in Figure 8. This trend suggests that higher concentrations of these compounds are more cytotoxic to the cells. Eugenol maintains a high survival rate at 0.16 μM, with no significant reduction compared to the control, while higher concentrations indicate higher cytotoxicity. Similarly, phenazine shows a more pronounced cytotoxic effect than eugenol at concentrations higher than 0.16 μM, with survival rates decreasing more steeply as concentrations increase. At 3 μM, the survival rate drops below 40% indicating strong cytotoxic effect. Eugenol-phenazine cocrystals show similar trend to phenazine alone, with significant cytotoxicity at higher concentrations. The survival rate at 3 μM is low, comparable to phenazine, suggesting that the cocrystals might combine the cytotoxic effects of both components. The reductions of cell viability are significant across the different concentrations and compared to

the control, highlighting the importance of concentration in determining cytotoxic effects.

Cal27

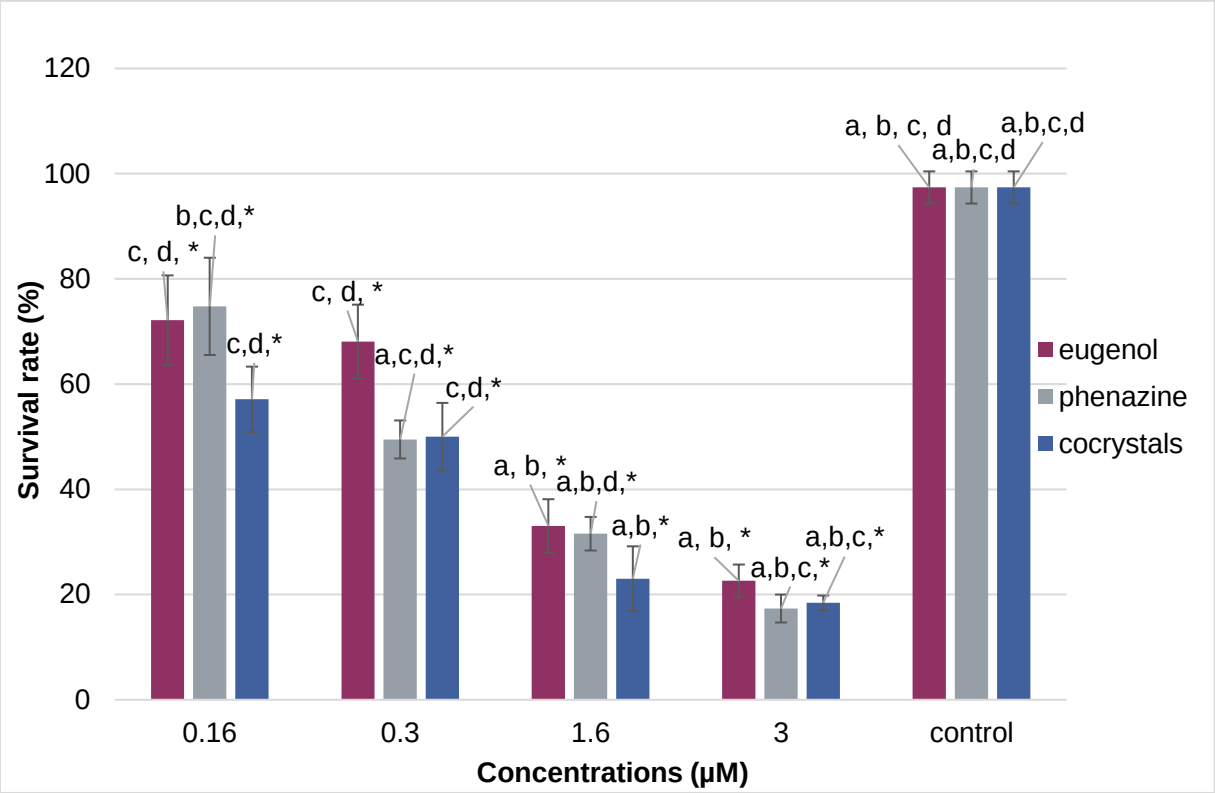

Figure 9. The survival rate of Cal27 cell line treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals.

Statistically significant difference with $p < 0.01$ is denoted as follows: a – significant difference compared to the 0.16 μM concentration; b – significant difference compared to the 0.3 μM concentration; c – significant difference compared to the 1.6 μM concentration; d – significant difference compared to the 3 μM; * - significance in relation to control.

The data seen in Figure 9 reveals a clear concentration-dependent decrease in cell viability for all three treatments of Cal27 cells. At the lowest concentration of 16 μM, cells treated with eugenol and phenazine exhibit a survival rate slightly lower to the control, indicating minimal cytotoxicity at this level. However, eugenol-phenazine cocrystals already show a notable reduction in cell viability, suggesting a stronger cytotoxic effect even at low concentrations. As the concentration increases to 0.3 μM, this trend continues, with further declines in survival rates for all compounds, particularly for phenazine and the cocrystals. Eugenol shows substantial decrease in cell survival, while phenazine and the cocrystals lead to even more significant drops in viability, with survival rates falling well below 30% at 3 μM. The data suggest that the combination of eugenol and phenazine in cocrystals does not mitigate the cytotoxic effects; rather, it appears to enhance them. The control group consistently maintains a high survival rate, underscoring the impact of the treatments. Statistical significance labels

demonstrate that the observed differences in survival rates across different concentrations are consistent.

Hep G2

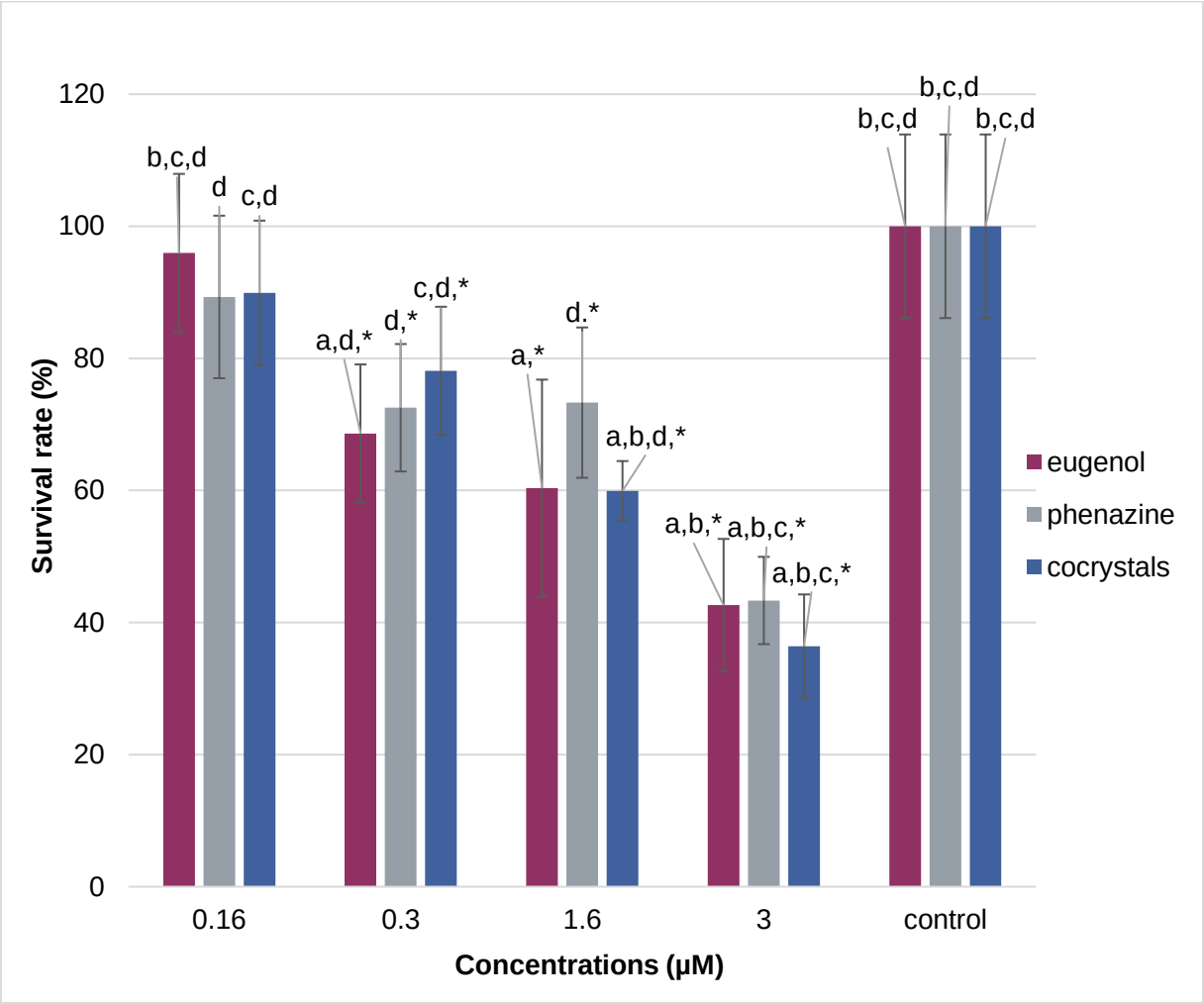

Figure 10. The survival rate of Hep G2 cell line treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals, including statistical significance labels

Statistically significant difference with $p < 0.01$ is denoted as follows: a – significant difference compared to the 0.16 µM concentration; b – significant difference compared to the 0.3 µM concentration; c – significant difference compared to the 1.6 µM concentration; d – significant difference compared to the 3 µM; * - significance in relation to control.

Similar trend to HaCaT and Cal27 is shown in Figure 10 for HepG2 cells. There is a noticeable trend of decreasing cell survival as the concentration of the compounds increases, indicating that higher doses of these compounds exhibit greater cytotoxicity towards HepG2 cells. At the lowest concentration of 0.16 µM, cell survival remains relatively high, with only moderate reductions in viability observed, particularly for phenazine and cocrystals. Eugenol appears to have the lowest impact at this concentration, suggesting it may be less cytotoxic at lower doses. With the increase of concentrations, the viability drops are more prominent, with a consistent

statistical significance difference.

AGS

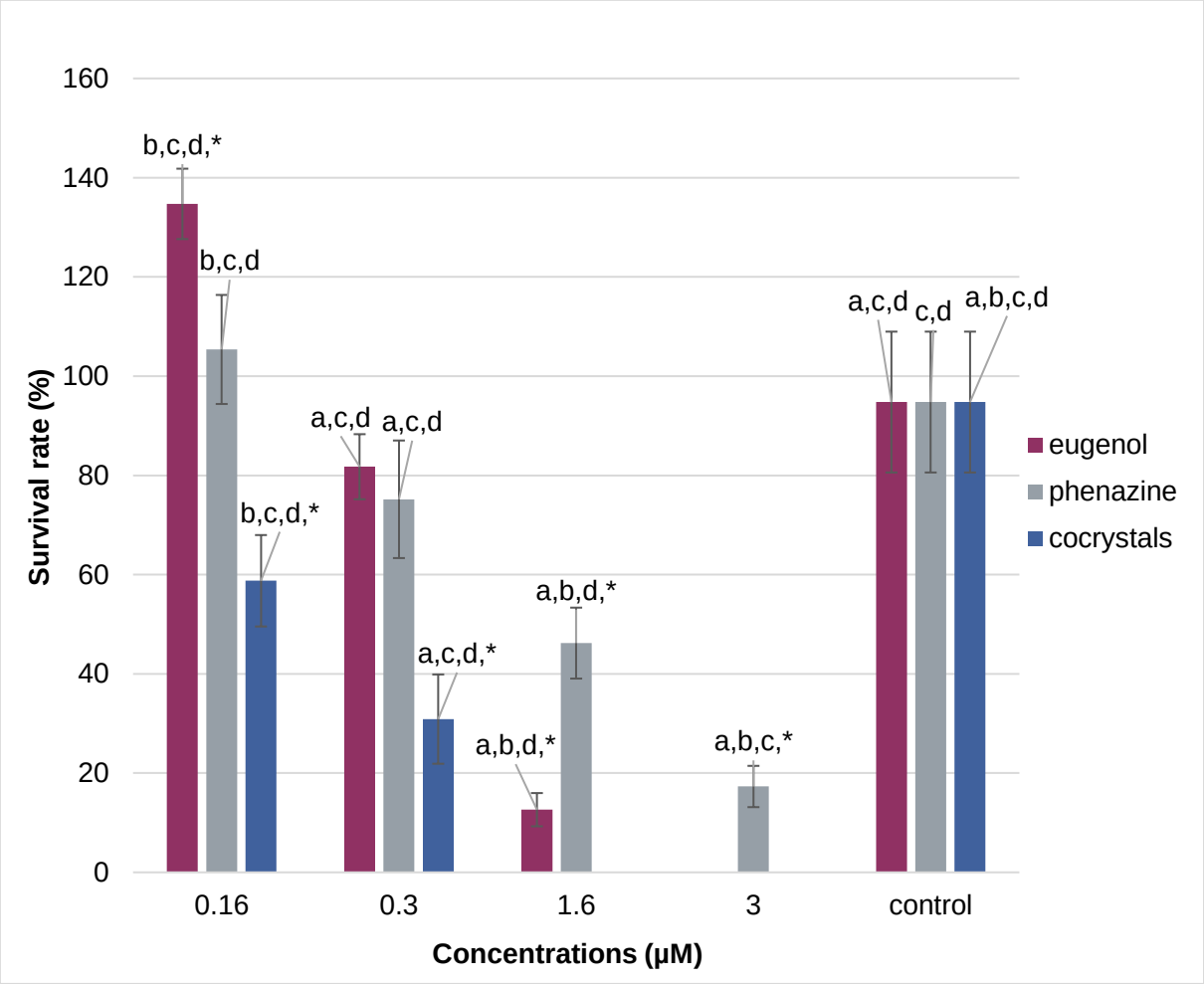

Figure 11. The survival rate of AGS cell line treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals.

Statistically significant difference with $p < 0.01$ is denoted as follows: a – significant difference compared to the 0.16 µM concentration; b – significant difference compared to the 0.3 µM concentration; c – significant difference compared to the 1.6 µM concentration; d – significant difference compared to the 3 µM; * - significance in relation to control.

AGS cells also exhibit a concentration-dependent response to eugenol, phenazine and eugenol-phenazine cocrystals, similar to the other cell lines. At the lowest concentration of 0.16 µM, AGS cells treated with eugenol and phenazine are shown to have proliferative effect. However, as the concentration increases, there is a notable decrease in cell viability, particularly at 3 µM, where survival rate is non-existent. Phenazine displays a more pronounced cytotoxic effect than eugenol at the lower concentrations (0.3 and 1.6 µM), while at the concentration of 3 µM there is a survival rate well below 20%, suggesting a potent cytotoxic effect. In case of eugenol-phenazine cocrystals, even lower concentrations exhibit significant cytotoxicity, while

concentrations of 1.6 and 3 µM result in cell death.

The results indicated a concentration-dependent decrease in cell viability across all cell lines, with higher concentrations leading to increased cytotoxicity. Specifically, eugenol showed relatively low cytotoxicity at lower concentrations but became more toxic at higher levels. Phenazine exhibited more pronounced cytotoxic effects even at lower concentrations, and the eugenol-phenazine cocrystals showed an enhanced cytotoxicity compared to the individual compounds, suggesting a potential synergistic effect. These findings are consistent with existing literature on the cytotoxic properties of eugenol and phenazine. Eugenol was found to induce apoptosis in human promyelocytic leukaemia cells (HL-60) through a mechanism dependent on ROS and mitochondria (Yoo et al., 2005). According to Fangjun and Zahijia (2018), eugenol may have chemotherapeutic properties against human lung cancer. Similar research on phenazine and its derivatives confirm their strong cytotoxic effects on cancer cells, as McGuigan and Li (2014) have found that this compound has concentration-dependent cytotoxicity on Hep G2 and T24 (colorectal adenocarcinoma) cells. Eugenol-phenazine cocrystals specifically, are not investigated in this aspect but the study of Saha et al. (2014) shows that similar cocrystals exhibit a specific cytotoxic effect on lung cancer cells (A549) depending on the combination and concentration used, which aligns with findings that EU-PHE cocrystals did not mitigate but instead intensified the cytotoxic effects at higher concentrations.

This underscores the importance of considering the concentration and combination of these compounds in therapeutic or food-preserving applications, as their cytotoxic effects can be significant and may pose health risks if not carefully managed.

4.2. RESULTS OF DETERMINING THE PROOXIDATIVE EFFECT OF EUGENOL, PHENAZINE AND EUGENOL-PHENAZINE COCRYSTALS

The DCFH-DA method measures the production of reactive oxygen species (ROS) by evaluating the fluorescence intensity, which is an indicator of oxidative stress. The assay was conducted on four cell lines, and each sample was investigated in four parallels, with results presented as the arithmetic mean of all parallels. The measured parameter was the fluorescence intensity of the sample divided by the fluorescence intensity of the control. Figures 11-14 graphically show the dependence of the fluorescence ratio of samples and control on the concentration of the samples, with the standard deviations of the measurements and the statistically significant relationship of the results.

HaCaT

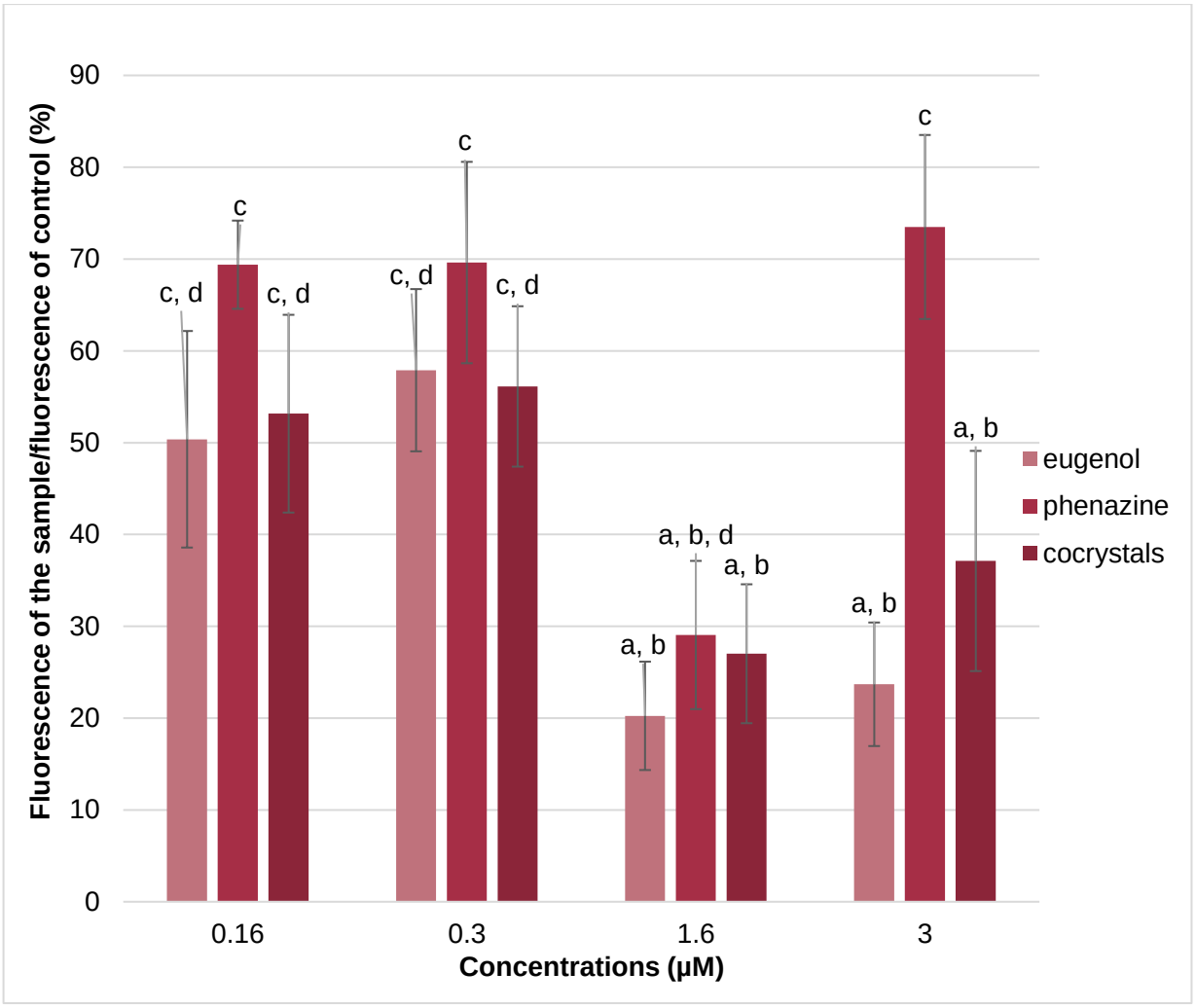

Figure 12. Ratio of fluorescence of the sample and control of HaCaT cell lines treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals

Marks of statistically significant differences are as follows: a – significant difference in relation to 0.16 µM; b – significant difference in relation to 0.3 µM; c – significant difference in relation to 1.6 µM; d – significant difference in relation to 3 µM.

In case of HaCaT cells, at lower concentrations (0.16 and 0.3 µM), phenazine shows higher fluorescence indicating a higher level of ROS compared to eugenol and cocrystals. Eugenol and cocrystals have similar and lower prooxidative effects, but as the concentration rises to 1.6 µM the prooxidative effect of all three compounds drop sharply. This indicates a reduction in ROS generation, which could be connected to lower survival rates of cells at this concentration. At the highest concentration, phenazine shows a significant increase in fluorescence, indicating a strong prooxidative effect, however this result is questionable due to low survival of cells at this concentration. But on the other hand, both eugenol and cocrystals are more effective at reducing ROS than phenazine.

Cal27

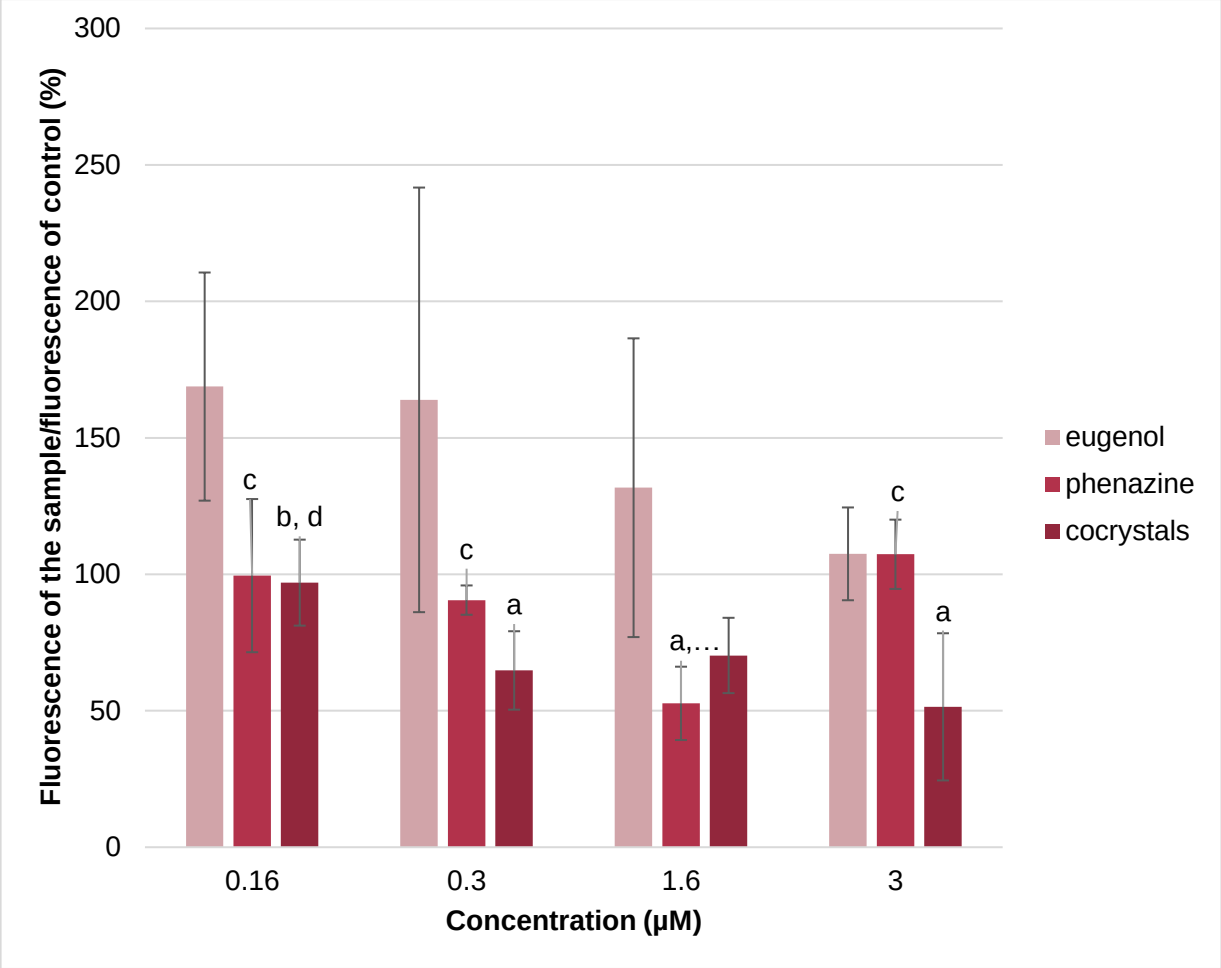

Figure 13. Ratio of fluorescence of the sample and control of Cal27 cell lines treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals.

Marks of statistically significant differences are as follows: a – significant difference in relation to 0.16 µM; b – significant difference in relation to 0.3 µM; c – significant difference in relation to 1.6 µM; d – significant difference in relation to 3 µM.

Effect of eugenol on tongue cells shows that this compound has concentration-dependent prooxidative effect. There is a similar trend with phenazine and cocrystals, except for 3 µM concentration of phenazine which shows antioxidative effect. However, this effect should not be considered as antioxidative since survival rate at these concentrations is very low. It is questionable if DCF is distributed in the cells at all and which percentage of activated molecule gets in the interaction with ROS. Also, it is questionable which percentage of ROS are maintained in the highly damaged cells.

HepG2

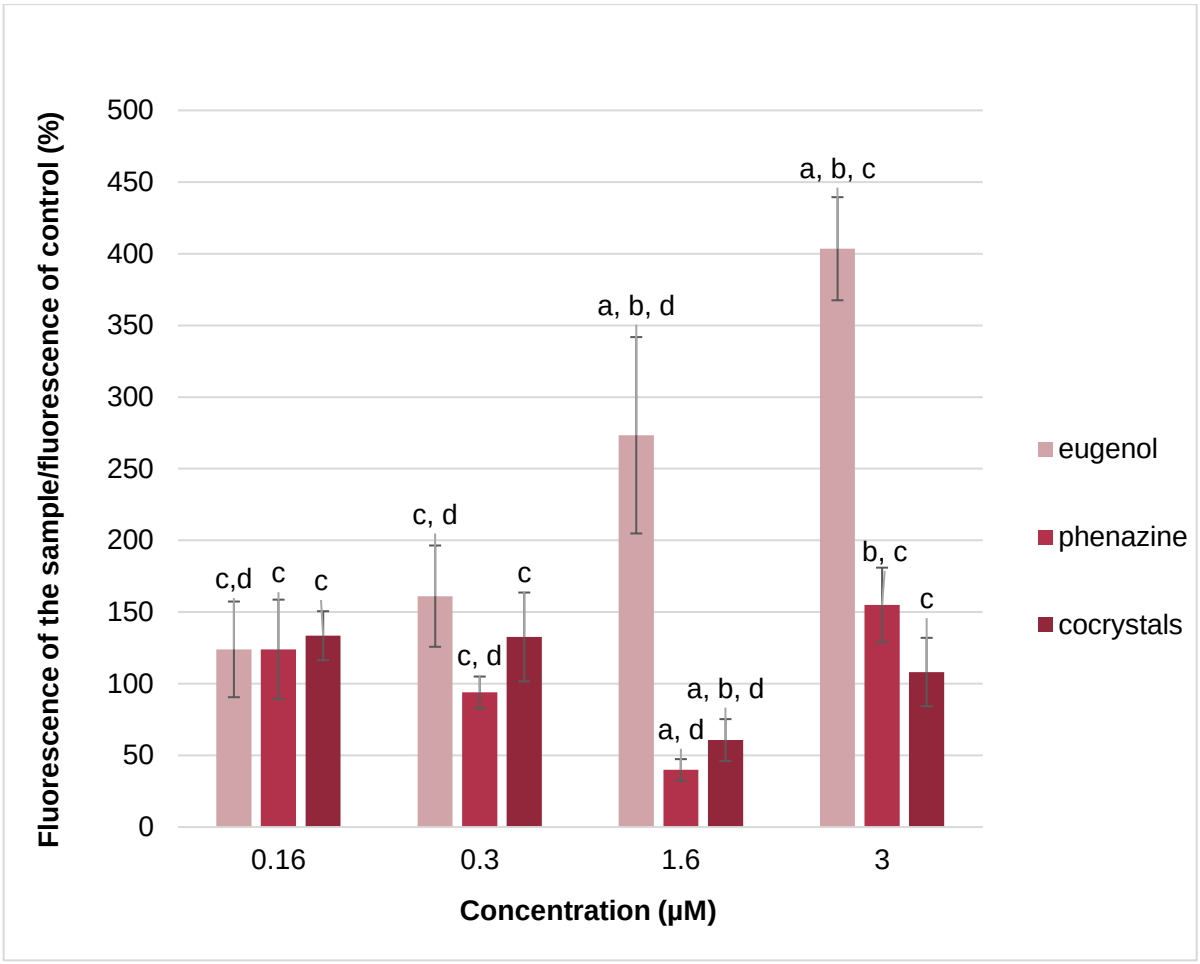

Figure 14. Ratio of fluorescence of the sample and control of HepG2 cell lines treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals.

Marks of statistically significant differences are as follows: a – significant difference in relation to 0.16 µM; b – significant difference in relation to 0.3 µM; c – significant difference in relation to 1.6 µM; d – significant difference in relation to 3 µM.

All three compounds have similar prooxidative effects at the concentration of 0.16 µM on the HepG2 cells, while 0.3 µM shows slight decrease in ROS for phenazine. However, as the concentrations rise, there are different effects of eugenol as opposed to phenazine and eugenol-phenazine cocrystals. Eugenol shows a significant and consistent increase in ROS as the concentrations rise, indicating high prooxidative effects, while phenazine causes the decrease in ROS at 1.6 µM. Similarly to Cal27, these results need to be studied further as survival rates of cells at concentrations of 1.6 and 3 µM for all three compounds are low.

AGS

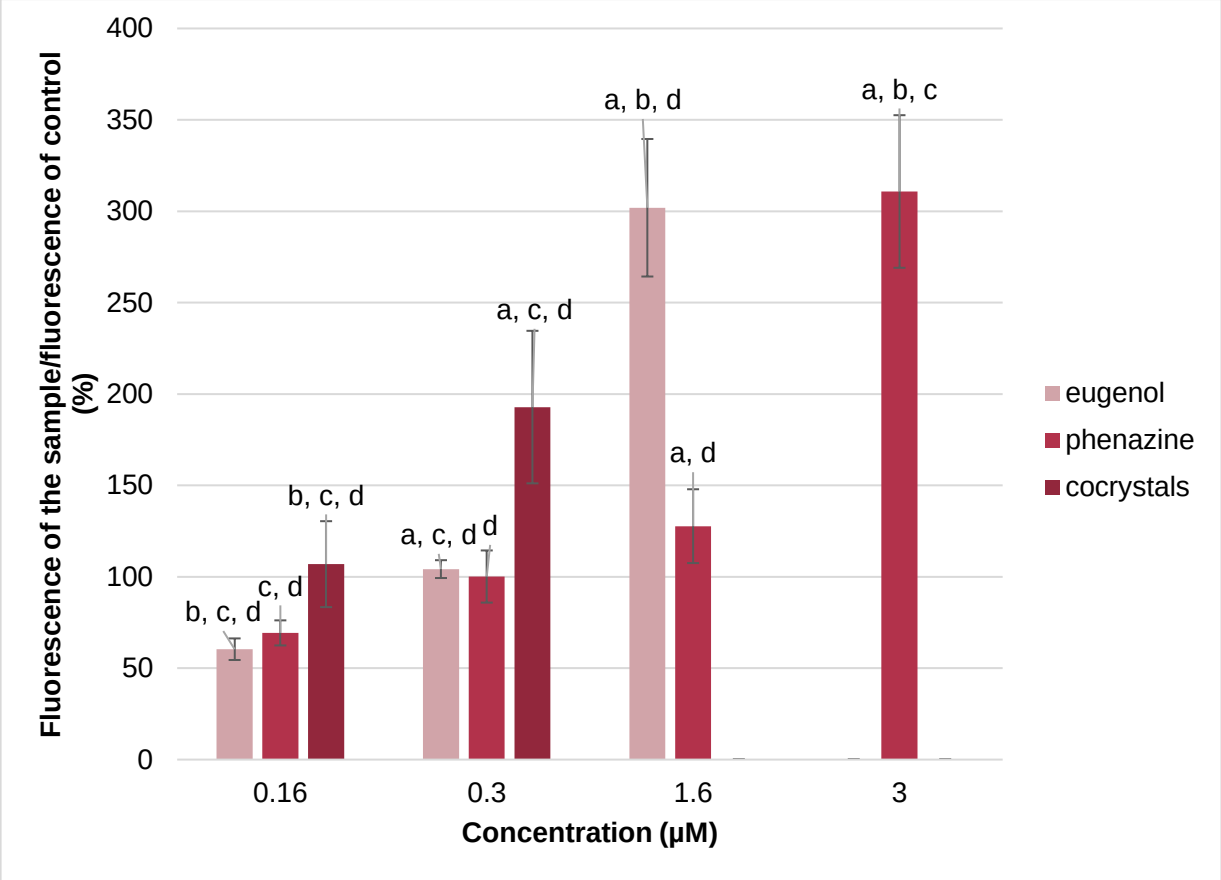

Figure 15. Ratio of fluorescence of the sample and control of AGS cell lines treated with different concentrations of eugenol, phenazine and eugenol-phenazine cocrystals.

Marks of statistically significant differences are as follows: a – significant difference in relation to 0.16 µM; b – significant difference in relation to 0.3 µM; c – significant difference in relation to 1.6 µM; d – significant difference in relation to 3 µM.

Effects of different concentrations of eugenol, phenazine and their cocrystals on the AGS cell lines are depicted in Figure 15. At a concentration of 0.16 µM, eugenol and phenazine show a slight increase in fluorescence, with cocrystals demonstrating the highest increase in fluorescence at this concentration, indicating a strong prooxidative effect. As the concentrations increase, the fluorescence of all three compounds rises, but the most noticeable change is with cocrystals indicating their prooxidative effect.

According to Ulanowska and Olas (2021) and Bezerra et al. (2017) eugenol exhibits dual antioxidant and prooxidant activities, depending on the concentration and environment. At lower concentrations, it tends to act as an antioxidant, while at higher concentrations it may produce reactive oxygen species, leading to cytotoxic effects, as shown by the DCFH-DA assay. Furthermore, these studies suggest that eugenol's cytotoxic effects are closely related to its

ability to induce oxidative stress, which is critical to its potential use in cancer therapy and antimicrobial applications.

While there are no studies focused on phenazine alone, phenazine compound pyocyanin is known for its ability to induce intestinal barrier destruction including inflammation and ROS accumulation in duodenum (Peng et al., 2021), while Savage et al. (1989) reported that twenty-six different phenazine compounds showed spontaneous generation of superoxide by human neutrophils *in vitro*. Therefore, phenazine's cytotoxicity, especially at higher concentrations could be linked to its ROS-generating properties which damage cellular components and reduce cell viability.

Overall, while eugenol tends to increase ROS generation with concentration, studies on phenazine derivatives support its role in inducing oxidative stress and inflammation, which further explains its cytotoxicity in higher concentrations. The cocrystals exhibit a blend of these effects, suggesting they may be more effective in modulating ROS generation, depending on the dose. The results also highlight the need for further studies, particularly at higher concentrations, where low cell survival complicated the interpretation of ROS measurements.

4.3. RESULTS OF DETERMINING THE PROTECTIVE EFFECT ON DNA DUE TO THE ACTION OF ROS

Circular DNA of φX174 RFI exists in both relaxed and supercoiled forms. The relative amounts of these forms were determined through optimisation experiment, which included identifying the necessary time of exposure to UV radiation for consistent results. The results of optimisation process is depicted in Figure 16.

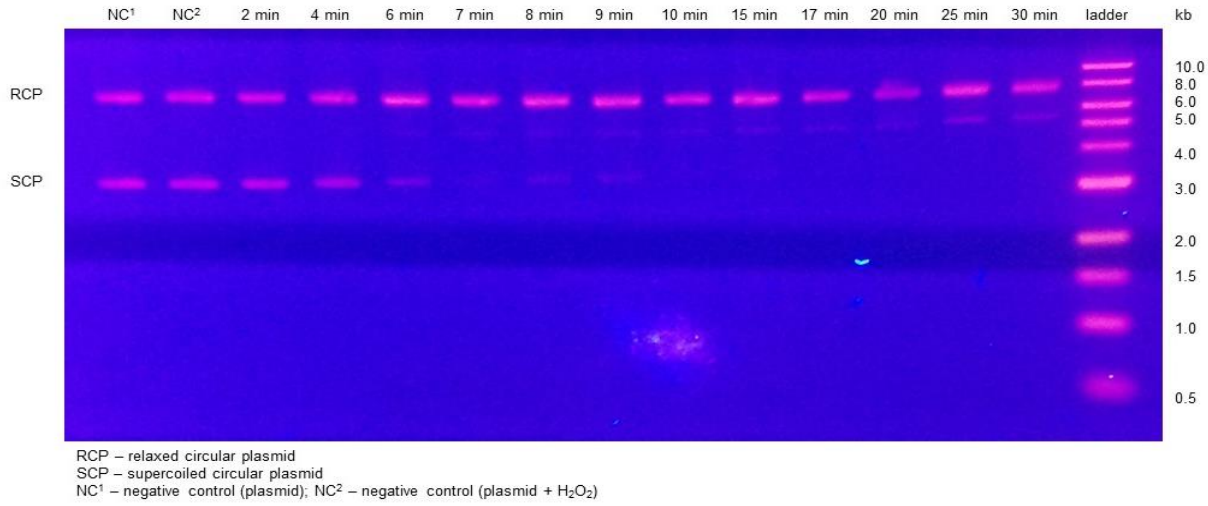

Figure 16. Picture of agaroze gel after optimisation process, oxidative DNA damage induced by UV-photolysis of H_2O_2.

The results of UV-photolysis on the hydrogen peroxide and directly on the DNA molecule with the addition of tested samples are shown in Figures 17 and 18. If DNA damage occurs, the plasmid changes conformation from supercoiled circular (SCP) to relaxed circular form (RCP). When in the relaxed form, the plasmid travels more slowly through the gel, compared to supercoiled form. Both Figure 17 and 18 show that by increasing the concentration of compounds, the amount of SCP drops, meaning that assessed compounds do not have protective effect on DNA.

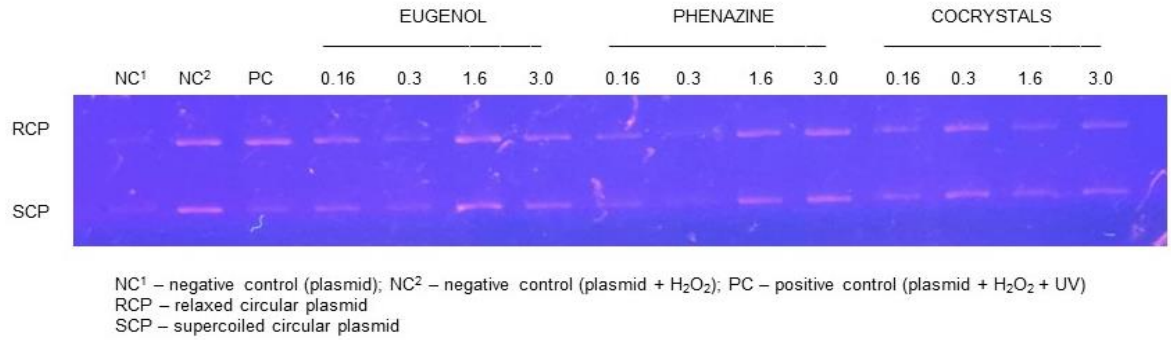

Figure 17. Image of the gel showing the effects of eugenol, phenazine and eugenol-phenazine cocrystals on oxidative DNA damage induced by UV-photolysis of H_2O_2.

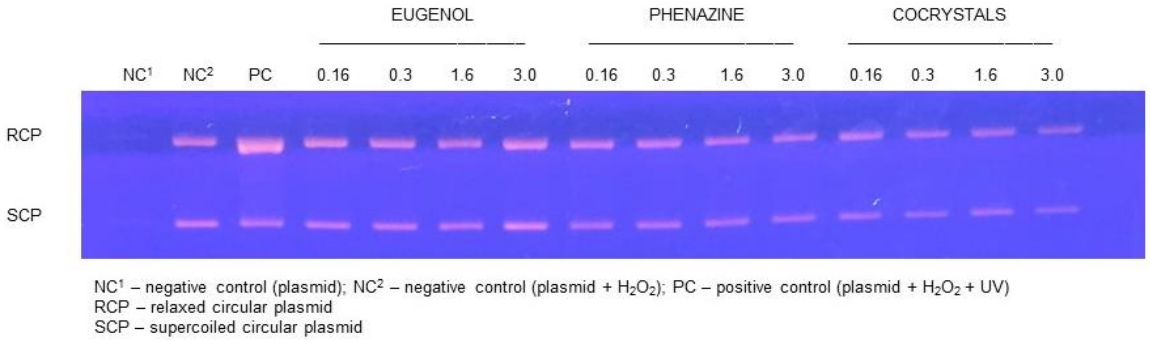

Figure 18. Image of the gel showing the effects of eugenol, phenazine and eugenol-phenazine cocrystals on oxidative DNA damage induced by UV-photolysis of H_2O_2.

The gel images in Figures 17 and 18 illustrate the effect of different concentrations of the compounds (0.16 µM, 0.3 µM, 1.6 µM and 3 µM) on plasmid DNA, with lanes for each treatment and the controls.

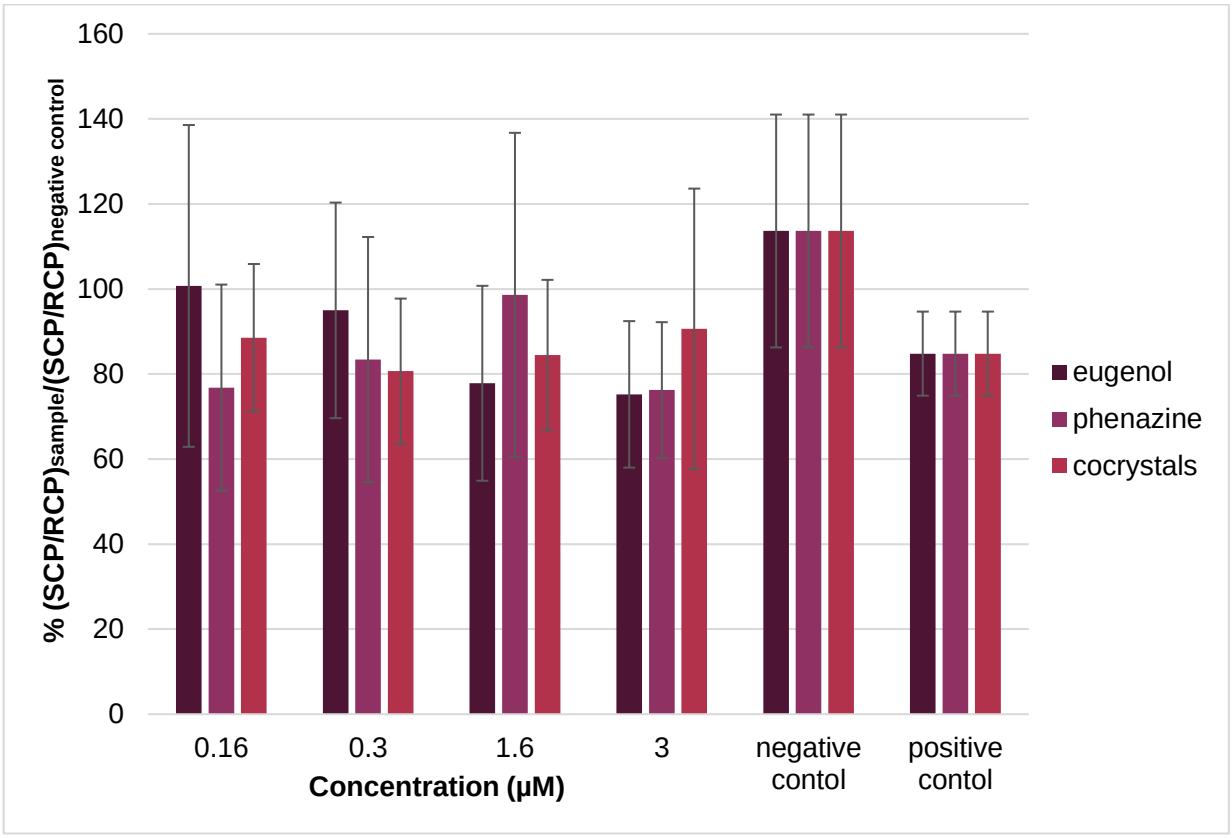

Figure 19. Oxidative effects of eugenol, phenazine and eugenol-phenazine cocrystals in comparison to negative control

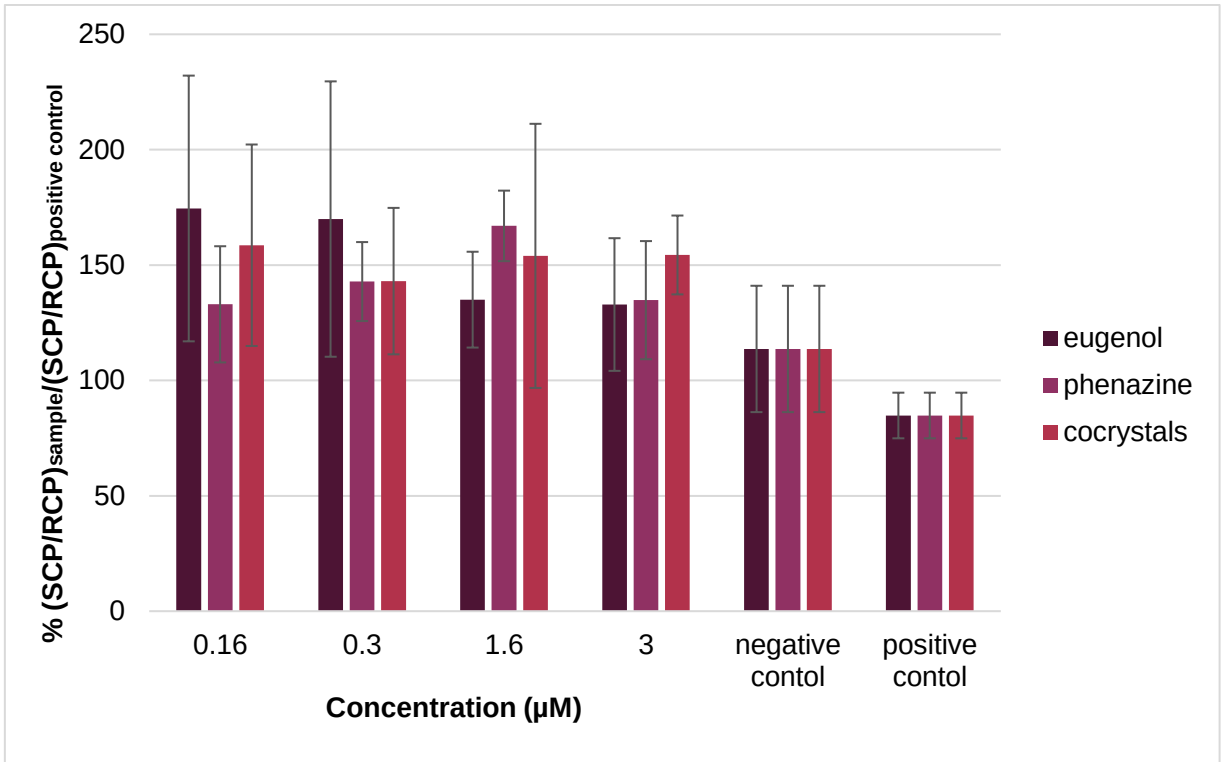

Figure 20. Oxidative effects of eugenol, phenazine and eugenol-phenazine cocrystals in comparison to positive control

At lower concentrations (0.16 µM and 0.3 µM), eugenol demonstrates consistent protection of DNA, as indicated by the predominance of supercoiled plasmid DNA. However, as the concentrations increase to 1.6 µM and 3 µM, there is a slight reduction in the protective effect, suggesting mild DNA damage. This could indicate that eugenol may exhibit some prooxidant activity or reduced efficacy in DNA protection at higher doses. This observation aligns with research conducted by Ma et al. (2021), which demonstrated that eugenol increases the transcriptional activity and expression level of nuclear factor erythroid 2-related factor (Nrf2), a central regulator of cellular responses to oxidative stress, in a dose-dependent manner. Additionaly, eugenol reduced intracellular ROS levels while increasing cellular resistance to hydrogen peroxide in a manner that was dependent on Nrf2.

Phenazine, on the other hand, shows slightly lower DNA protection across all concentrations. This may be attributed to its reactive oxygen species (ROS)-generating capacity, which could lead to DNA damage even at low concentrations. Similarily, investigation of antifungal mechanism of phenazine-1-carboxamide from *Pseudomonas* sp. conducted by Tupe et al (2015) showed antagonistic effect due to their redox activity. Phenazines diffuse across the cell membrane and act as reducing agents, which results in uncoupling of oxidative phosphorylation and generation of toxic intracellular ROS which are harmful to the organism (Turner and Messenger 1986).

The eugenol-phenazine cocrystals appear to provide intermediate protection compared to the individual compounds. At 0.16 µM and 0.3 µM, the cocrystals offer a decent level of protection similar to eugenol, but as the concentrations increase, the protection diminishes. This could suggest that at higher concentrations, the cocrystals may synergize to induce oxidative stress, resulting in reduced protection against DNA damage. The cocrystals behave similarly to phenazine, indicating that combining the two compounds may not mitigate their individual prooxidative effects but could instead exacerbate DNA damage under higher oxidative stress conditions.

To conclude, while eugenol may function as a protective antioxidant at lower doses, its prooxidant potential at higher concentrations, especially when combined with phenazine, highlights the complex and concentration-dependent nature of these compounds in modulating oxidative stress and DNA protection.

5. CONCLUSION

1. Eugenol, phenazine and eugenol-phenazine cocrystals show concentration-response dependent cytotoxicity in all cell lines. The most sensitive cell line was Cal27, but the steepest curve was obtained on AGS cell line. The most resistant cell line was HepG2 on which the lowest concentrations of eugenol and phenazine were not toxic. Cocrystals showed strongest cytotoxic effect on all cell lines applied.
2. Eugenol and phenazine did not influence on free radical formation nor antioxidant effect at nontoxic concentrations. Cocrystals damaged cells significantly at all concentration range so it was not possible to bring any definitive conclusion about their antioxidant/prooxidant nature.
3. In order to determine antioxidant/prooxidant nature of examined compound plasmid was used as test system; from these results it can be concluded that all investigated compound increased prooxidative effect of hydroxyl radicals indicating the prooxidative nature of these compounds.
4. Further research is needed to fully understand the mechanistic pathways involved, especially regarding the eugenol-phenazine cocrystals. Also, it is crucial to determine the concentration of these compounds which migrate into the food and to determine the effect of these concentrations on the cells of digestive system.

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ATCC (2024b) AGS cell line ATCC - American type culture collection https://www.atcc.org/products/crl-1739. Accessed on 2nd of August 2024.

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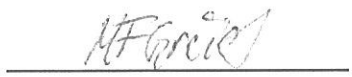
DECLARATION OF ORIGINALITY

I Marija Filipović-Grčić declare that this master's thesis is an original result of my own work and it has been generated by me using no other resources than the ones listed in it.



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