https://doi.org/10.31925/farmacia.2023.2.8 **ORIGINAL ARTICLE**

EUGENOL: *IN VITRO* CHARACTERIZATION OF THE CYTOTOXIC PROFILE AT THE LEVEL OF COLORECTAL CARCINOMA CELLS

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Abstract

In the present study, the main objective was to evaluate the cytotoxic potential of eugenol on colorectal carcinoma cells - HCT-116, as well as on healthy human keratinocytes - HaCaT. Eugenol has been evaluated for its impact on cell viability, morphology, nuclei, and actin filament structure and organization. According to the findings of this study, eugenol is not associated with a significant decrease in cell viability at the level of keratinocytes. Aside from that, no significant morphological changes were observed, and the nuclei and actin filaments were organized similarly to those of control cells. Conversely, in the case of tumour cells, eugenol reduced their viability in a dose-dependent manner. Moreover, the concentration of 0.5 mM induced morphological changes at the level of actin filaments and nuclei characteristic of apoptosis (rounding of cells, condensation of chromatin, reorganization of actin filaments). These results provide evidence for the possibility of eugenol being an effective anti-tumour agent. However, it is necessary to conduct further research in order to elucidate the biological mechanisms involved.

Rezumat

Scopul principal al studiului a fost evaluarea potențialului citotoxic al eugenolului la nivelul celulelor de carcinoma colorectal - HCT-116, precum și la nivelul celulelor sănătoase de keratinocite umane - HaCaT. Impactul eugenolului a fost evaluat în ceea ce privește viabilitatea celulară, morfologia, precum și efectul asupra structurii și organizării nucleilor și filamentelor de actină. Rezultatele studiului au indicat faptul că la nivelul keratinocitelor, eugenolul nu determină o scădere marcantă a viabilității celulare. În plus, nu au fost observate modificări morfologice semnificative, iar nucleii și filamentele de actină au prezentat o organizare similară cu cea a celulelor control. Pe de alta parte, în cazul celulelor tumorale, eugenolul a determinat scăderea doză-dependentă a viabilității celulare. În plus, concentrația de 0.5 mM a determinat modificări morfologice și la nivelul filamentelor de actină și nucleilor caracteristici apoptozei (rotunjirea celulelor, condensarea cromatinei, reorganizarea filamentelor de actină). Toate aceste rezultate indică faptul că eugenolul poate fi considerat un potențial agent antitumoral. Cu toate acestea, sunt necesare studii suplimentare pentru elucidarea mecanismelor biologice.

Keywords: eugenol, colorectal cancer, actin filaments, nuclei, cell morphology

Introduction

Although remarkable progress has been made in the field, colorectal cancer (CRC) continues to pose a major threat to the health of the global population. Consequently, it is the third most common type of cancer diagnosed worldwide and the second most lethal type of cancer [1]. From a physiopathological point of view, CRC is a disorder characterized by abnormal and rapid proliferation of glandular epithelial cells in the colon and rectum. An important concern is

the increasing number of new cases being diagnosed every year, and epidemiological studies estimate that the incidence of CRC will double by 2035 [2]. There are multiple risk factors contributing to the occurrence of this disease, including environmental factors such as diet and lifestyle, genetic factors, or various pathologies such as chronic inflammation or Crohn's disease [3].

In the case of CRC, chemotherapy and surgery are the traditional first-line therapies. Nevertheless, in patients with metastases, antitumor therapy led to an unfavourable prognosis [4]. Furthermore, in patients undergoing surgical intervention, metachronous metastases were found in over 20% of cases [5]. At present, anti-tumour chemotherapy can be administered as mono-therapy, primarily using fluoropyrimidine, or as an associated therapy, which includes a combination of several active agents. However, the use of a single agent in therapy presents an unfavourable effect that involves the acquisition of resistance to the therapy and, ultimately, the failure of the therapy, whereas combined therapy is associated with a number of severe adverse reactions at the systemic level [6]. Several new therapeutic strategies have been introduced in the field of antitumor therapy over the past few years, including targeted therapy [6], gene therapy [7], immunotherapy [8], adoptive T-cell therapy [9], cytokine therapy [10] and complement inhibition [11].

In light of the numerous disadvantages associated with conventional therapy, one approach to CRC could be the use of compounds that are naturally derived. The results of studies conducted at the population level have demonstrated that more than 80% of individuals use products of natural origin as their first option for therapeutic treatment. Furthermore, plant compounds are attracting increasing interest from researchers in the field due to their multiple therapeutic properties in a variety of medical areas, such as malignant diseases and inflammation [12]. Natural products are therefore considered to be potential therapeutic or prophylactic options for the treatment of CRC [13]. A testament to the effectiveness of natural products can be found in the fact that, at present, approximately 50% of the antitumor treatments among them are direct or indirect derivatives of plants [14]. Consequently, the discovery and introduction of plant compounds in antitumor therapy marked a new phase in cancer treatment and prevention. Clinical trials are currently underway for numerous phytocompounds that are being tested for their ability to treat CRC, such as berberine, curcumin, and silymarin [15-17]. Natural compounds have the advantage of interfering with a variety of pathways involved in the processes of metastasis, apoptosis, invasion, or angiogenesis. Since natural compounds have complex biological mechanisms of action, it is more difficult to establish resistance in tumour cells to natural compounds [18].

Due to its many biological properties, cloves (*Syzygium aromaticum* L.) (*Myrtaceae*) are regarded as an important plant in traditional medicine. In particular, eugenol (Eug) (4-allyl-2)-methoxyphenol is the main component of cloves [19]. A number of therapeutic properties are associated with eugenol, including anti-oxidant, anti-inflammatory, antimicrobial, analgesic and antitumor properties [20]. In terms of eugenol's antitumor properties, it has been demonstrated to be beneficial in several kinds of cancer, including lung cancer, skin cancer, breast cancer, and colorectal cancer. It has been demonstrated that Eug induces cellular apoptosis in cancer cells by causing a decrease in the mitochondrial membrane potential and an increase in the production of reactive oxygen species [21]. To date, however, the biological mechanisms behind Eug's antitumor effects are not fully understood.

Given the aforementioned considerations, the objective of the present study was to assess the pharmaco-toxicological characteristics of Eug on both the colo-rectal cancer cell line HCT-166 and the healthy human keratinocyte cell line HaCaT. The investigation centred around the evaluation of potential cytotoxic effects, including changes in cell viability and morphology, as well as alterations in the nuclei and actin filaments.

Materials and Methods

Reagents

This study was performed with the following reagents: Eugenol, phosphate saline buffer (PBS), trypsin-EDTA solution, dimethyl sulfoxide (DMSO), foetal calf serum (FCS), penicillin-streptomycin, penicillin-streptomycin-amphotericin, MTT [3-(4,5-dimethyl-thiazol2-yl)-2,5-diphenyltetrazolium bromide], along with Dapi (4′,6-diamidino-2-phenylindole dihydro-chloride dihydrochloride) which were purchased from Sigma Aldrich, Germany). All the reagents were analytically pure and of appropriate quality for use in cell culture.

Cell culture

The cancer cell lines HCT 116 (CCL-247™) were acquired from ATCC and HaCaT (catalog number: 300493) was purchased from CLS Cell Lines Service GmbH as frozen vials. HCT-116 was cultured in McCoy's 5a Medium and HaCaT was cultured in DMEM medium, both of which were supplemented with 10% FCS and 1% penicillin (100 U/mL) - streptomycin (100 µg/mL) mixture. All cell lines were maintained in standard conditions in an incubator at 37°C and 5% CO_2.

Cellular Viability Evaluation

The MTT method was used to determine the cytotoxic potential of the compound on HCT-116 and HaCaT cells. For the assessment of cell viability, cells were cultured in 96-well plates at a ratio of 1 x 10^4 cells *per* well. After reaching a confluence of approximately 90%, the cells were stimulated with five concentrations of

eugenol (0.1, 0.25, 0.5, 0.75 and 1 mM) for a time interval of 72 hours. Following this period of time, the medium was replaced with fresh medium, MTT reagent (10 µL/well) was added, and the cells were then incubated for three hours. The cells were then maintained at room temperature for 30 minutes in a solubilization solution (100 µL/well). Finally, the absorbance was measured at a wavelength of 570 nm, using the Cytation 5 device (BioTek Instruments Inc., Winooski, VT, USA).

Cellular Morphology

After stimulation with the five concentrations of Eug for a period of 72 hours, cell morphology was evaluated in order to better understand the cytotoxic effects of Eug. To determine cell morphology, photos were taken of the cells under bright field illumination using an Olympus inverted microscope IX73 (Olympus, Tokyo, Japan). After the images were acquired, they were analysed using cellSens Dimensions v.1.8 Software (Olympus, Tokyo, Japan).

Immunofluorescence staining

A study was conducted to examine the impact of Eug (0.5 mM) on the nuclei and actin filaments of HaCaT and HCT-116 cells in order to gain a better understanding of the potential biological mechanism of action. This was achieved by cultivating the cells in 12-well plates, at a density of 1 x 10^5 cells *per* well. Once the cells had reached a suitable confluence, they were stimulated with 0.5 mM Eug for 72 hours. Following this interval, the cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for one hour at room temperature. Following permeabilization with Triton X 2%, they were incubated with DAPI for 15 minutes for nuclei visualization and with Rhodamine Phalloidin for 20 minutes in order to examine the actin filaments. The photographs were taken with an Olympus IX73 inverted microscope equipped with a DP74 camera and analysed using Olympus CellSens v.1.18 software (Tokyo, Japan).

Statistical analysis

The findings of the present study were presented as mean ± standard deviation (SD). To assess the statistical distinctions between the groups, the one-way ANOVA test was employed, followed by Dunnett's multiple comparison post-test. The statistical analysis was conducted using GraphPad Prism version 9.4.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The outcomes that exhibited significant statistical differences are denoted with asterisks (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$).

Results and Discussion

Globally, colorectal cancer remains one of the greatest threats to health, representing the second leading cause of death from cancer. A primary cause of death is the migration of tumour cells from the site of the primary tumour to neighbouring organs, such as the liver or lungs [22]. Thus, the therapeutic strategy for advanced stages of colorectal cancer (CRC) persists as a prominent research topic in the field of oncology [6]. The microbiome has been identified as a potential risk factor for the onset and progression of colorectal cancer (CRC), and has also been found to significantly impact the response to different systemic therapies [23]. Compounds that are derived from natural sources have been suggested as a possible treatment for CRC. One of these compounds is eugenol, which has attracted the attention of researchers because of its wide range of therapeutic actions, including anti-inflammatory, antioxidant, antimicrobial and antitumor properties [24-27]. It has been demonstrated in recent studies that Eug and its derivatives exhibit antitumor effects both *in vitro* and *in vivo* because they interfere with pathways essential to the development and proliferation of tumour cells, as well as induce apoptosis and cell cycle arrest [28]. Starting from these premises, the aim of the current study was to evaluate the cytotoxic effect of Eug at the level of viability, morphology and nucleus and actin filament structure of colorectal carcinoma cells - HCT-116, as well as at the level of healthy human keratinocyte cells - HaCaT. Regarding human keratinocytes, Eug was observed to induce a marginal reduction in cell viability, which was concentration-dependent. At the lowest concentration tested (0.1 mM), the viability of cells did not demonstrate a significant variation relative to control cells, with a viability value of approximately 93%. Conversely, a concentration of 1 mM resulted in a noteworthy decrease in cell viability of approximately 70%, as illustrated in Figure 1.

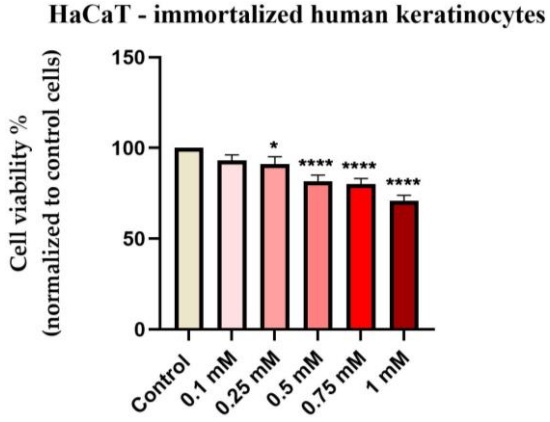

Figure 1.

In vitro assessment of the cytotoxic effects of Eug (0.1, 0.25, 0.5, 0.75 and 1 mM) at the level of human keratinocytes - HaCaT after 72 hours of treatment

Results are presented as a percentage (%) normalized to control cells, and as the mean ± standard deviation of three independent experiments conducted in triplicate

Statistically significant differences between the test and control groups were determined through one-way ANOVA analysis and Dunnett's multiple comparison post-test (* $p < 0.1$; **** $p < 0.0001$)

On the other hand, in tumour cells, Eug decreased the percentage of viable cells according to the concentration tested. Therefore, at the lowest concentration tested (0.1 mM), a significant decrease in cell viability of approximately 88% was observed. The most significant effect was observed at a concentration of 1 mM, when the percentage of viable cells decreased to approximately 34% (Figure 2).

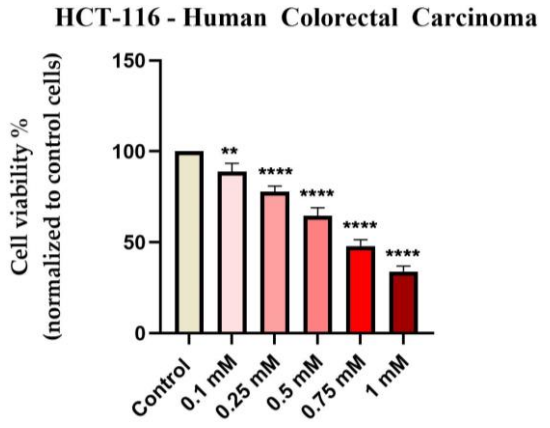

Figure 2.

In vitro assessment of the cytotoxic effects of Eug (0.1, 0.25, 0.5, 0.75 and 1 mM) at the level of human colorectal carcinoma - HCT-116 after 72 hours of treatment

Results are presented as a percentage (%) normalized to control cells, and as the mean ± standard deviation of three independent experiments conducted in triplicate Statistically significant differences between the test and control groups were determined through one-way ANOVA analysis and Dunnett's multiple comparison post-test (** $p < 0.01$; **** $p < 0.0001$)

A review of the specialized literature guided the selection of the concentrations of Eug tested in the present study [29-31]. In accordance with Aburel *et al*., Eug was assessed at 24, 48 and 72-hour intervals to investigate its impact on human keratinocytes. The investigation revealed that at a concentration of 50 μM, Eug did not exhibit a significant cytotoxic effect on HaCaT cells [32]. Furthermore, in a previous study conducted by Surducan *et al*., Eug was tested at concentrations identical to those in the present study at the level of human gingival fibroblasts and tongue squamous carcinoma cells. Based on the findings of the study, Eug is associated with a relative reduction of human gingival fibroblasts viability of approximately 76% [33]. In accordance with ISO Standard 10993-5:2009, a compound is considered cytotoxic if it causes a reduction in cell viability of more than 30% [34]. The present study found that Eug had no cytotoxic effect on human keratinocytes, with cell viability remaining above 88%. Additionally, Elham Ghodousi-Dehnavi *et al*. investigated the possible cytotoxic effects of Eug on colorectal adenocarcinoma cells - HT-29. After 72 hours of treatment with doses similar to those used in the present study, the researchers observed a pronounced cytotoxic effect [29]. Eug exhibited an IC_{50} between 130 and 750 μM in similar studies conducted on colorectal adenocarcinoma cells [30,35]. Moreover, Eug was also tested on other tumour cell lines, with the IC_{50} varying according to the type of cell. Accordingly, Eug's IC_{50} value for human melanoma cells was 0.5 μM, while its IC_{50} value for metastatic ovarian cells was 1600 μM [36, 37]. Additionally, it has been suggested that Eug induces apoptosis of HCT-116 cells at concentrations of 0.3 mM and 0.5 mM by dissipating MMP, activating caspase-3 and PARP, and up-regulating the tumour suppressor gene p53 [30].

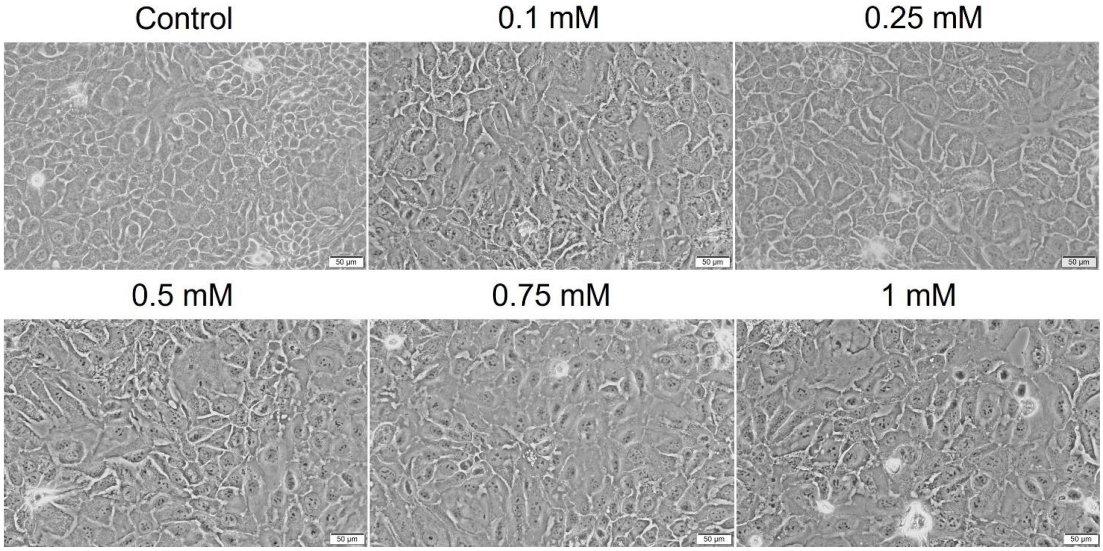

Figure 3.

Morphological appearance and confluence of human keratinocytes - HaCaT after 72 hours of treatment with Eug (0.1, 0.25, 0.5, 0.75 and 1 mM). Scale bars indicate 50 μm

Assessment of cell morphology has been carried out to further comprehend the cytotoxic implications of Eug. No significant variations were observed with regards to the impact of Eug on HaCaT cells. However, at a concentration of 1 mM, a reduction in cell count was observed alongside slight cell rounding, as demonstrated in Figure 3.

On the other hand, tumour cells were observed to undergo changes starting from the lowest concentration tested. Accordingly, a reduction in the number of cells attached to the plate as well as a rounding of the cells were observed at a concentration of 0.1 mM. The most significant changes in morphology were observed at a concentration of 1 mM, at which cells showed signs of rounding and shrinking, reduced confluence and loss of connections between neighbouring cells (Figure 4).

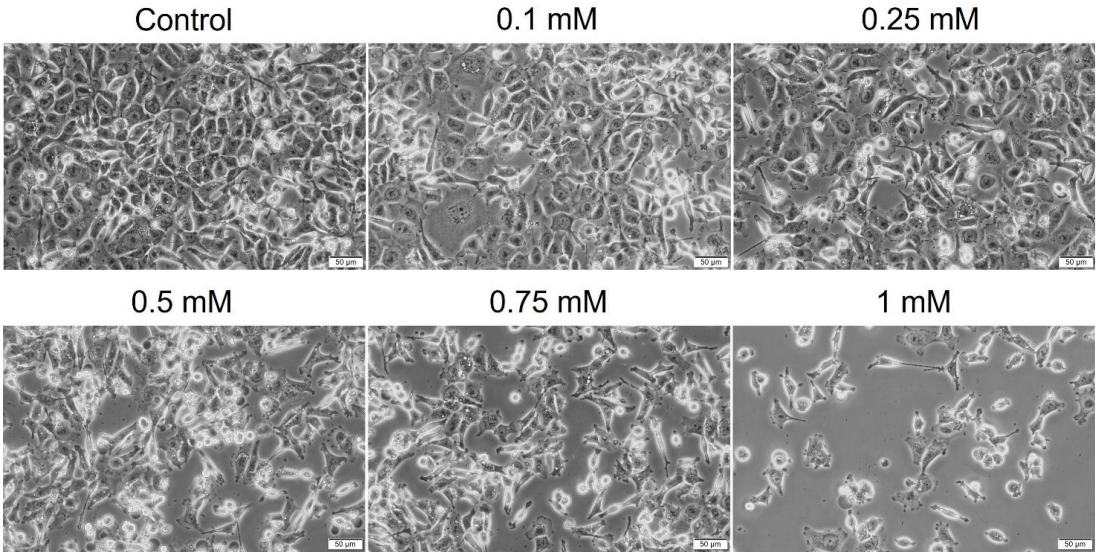

Figure 4.
Morphological appearance and confluence of colorectal carcinoma cells - HCT-116 after 72 hours of treatment with Eug (0.1, 0.25, 0.5, 0.75 and 1 mM). Scale bars indicate 50 µm

Cell apoptosis is characterized by a series of morphological changes such as cell contraction, the appearance of apoptotic bodies, and cell rounding [38]. All these changes were also observed in the present study, indicating that Eug can induce an apoptotic-like effect. Additionally, Eug has been shown to induce apoptosis in other types of cell lines. Accordingly, Fathy and colleagues have demonstrated that eugenol induces the apoptosis of cervical cancer cells - HeLa by increasing the expression of caspase-3, caspase-9, and decreasing the expression of Bcl-2 [39]. Moreover, Eug reduced cell proliferation and induced apoptosis in breast cancer cells, MDA-MB-231 and SK-BR-3, by increasing the activity of caspases-3 and -9 [40].

The effect of Eug 0.5 mM on the structure of the nuclei and actin filaments of HaCaT and HCT-116 cells was examined in order to obtain a more detailed understanding of potential targets and biological mechanisms.

Based on the results obtained at the level of human keratinocytes, Eug 0.5 mM did not significantly alter the number or shape of nuclei. Analysis revealed the occurrence of certain chromatin condensation, albeit in a negligible percentage of the samples. Moreover, Eug did not have a major impact on the actin filament distribution in HaCaT cells; their distribution was similar to that in control cells (Figure 5).

In contrast, 0.5 mM of eugenol induced chromatin condensation and a reduction in nuclei size within colorectal carcinoma cells. Additionally, apoptotic bodies were observed. Furthermore, Eug induced a sequence of alterations at the level of actin filaments, including condensation and restructuring into peripheral rings, as illustrated in Figure 6.

The process of apoptosis is a type of programmed cell death that is marked by a series of changes depending on the type of cell and the apoptotic inducer. On the nuclear level, apoptosis involves a series of extremely conservative changes, such as the condensation of chromatin, nuclear contraction, nuclear fragmentation and the formation of apoptotic bodies [41]. In addition, apoptosis is characterized by a series of other changes that can affect the cytoskeleton, causing a decrease in cell volume and contraction of all the cells. Actin filaments undergo reorganization during apoptosis, becoming condensed and arranged mainly at the edges of cells during the process [42].

Based on a previous study conducted on gingival fibroblasts and tongue cancer cells, Eug 0.5 mM did not significantly affect the structure and organization of the nuclei of gingival fibroblasts. Conversely, in

tumour cells, intense chromatin condensation and fragmented nuclei were observed. Furthermore, the actin filaments were strongly modified, forming a peripheral ring of condensed filaments [33]. In the current study, all of these changes were also observed.

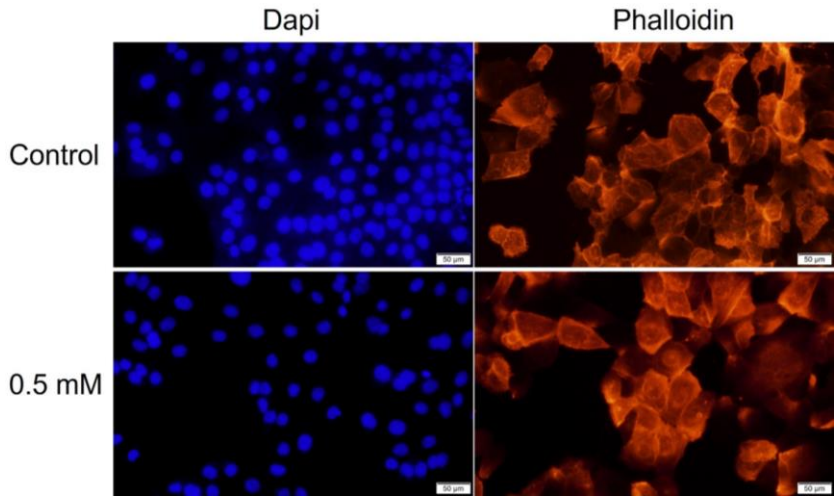

Figure 5.

. The impact of Eug 0.5 mM in HaCaT cells on: nuclei - DAPI staining (blue) and F-actin fibers - Phalloidin (red). The pictures were taken using 20× objective with a scale bar of 50 µm

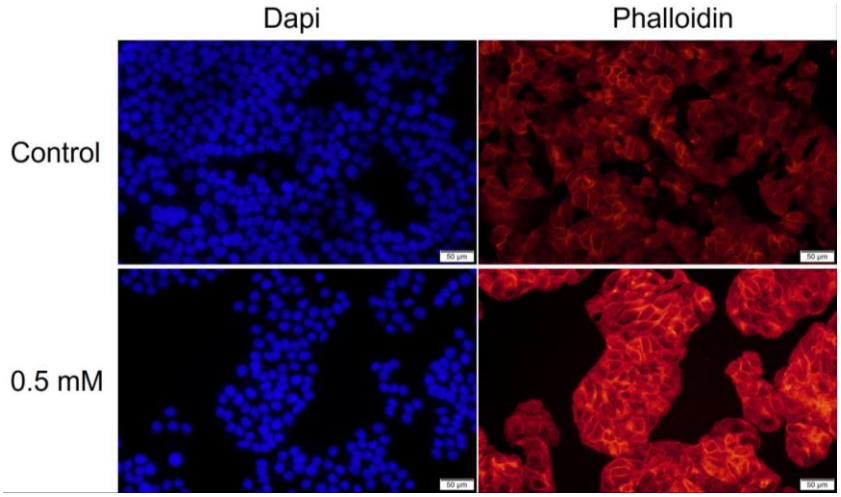

Figure 6.

The impact of Eug 0.5 mM in HCT-116 cells on: nuclei - DAPI staining (blue) and F-actin fibers - Phalloidin (red). The pictures were taken using 20× objective at a scale bar of 50 µm

Conclusions

The current study aimed to assess the cytotoxic properties of eugenol in relation to both colorectal carcinoma cells and human keratinocytes, the latter of which was utilized as a representative healthy cell line. The findings of our research indicate that eugenol has the ability to impede the growth of colorectal carcinoma cells in a manner that is dependent on the dosage. A series of morphological changes were also observed as a result of the cytotoxic effect - rounding of cells, condensation of nuclei, and reorganization of actin filaments. On the other hand, eugenol did not show an intense effect in inhibiting cell proliferation and altering cell morphology at the level of healthy cells. The findings of these studies suggest that eugenol exhibits promise as a possible antineoplastic agent. It is, however, necessary to perform additional studies in order to gain a complete understanding of the pharmacotoxicological profile and biological mechanism of action of this substance.

Conflict of interest

The authors declare no conflict of interest.

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