Research Article

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Evaluation of Antioxidant Activity, Cytotoxicity and Genotoxicity of Eugenol in V79 cell line and Human Lymphocytes Respectively

V79 Hücre Hattında ve İnsan Lenfositlerinde Öjenolün Antioksidant Aktivitesinin, Sitotoksisitesinin ve Genotoksisitesinin Değerlendirmesi

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ABSTRACT

Eugenol (EUG) is a volatile phenolic constituent of clove essential oil obtained from Eugenia Caryophyllata buds and leaves. It has been used in pharmaceutical, cosmetic, agriculture and food industry. The derivatives have been used in medicine as a local antiseptic and anesthetic. Although EUG is considered safe as a food additive, due to the vast range of different applications and extensive use, there has been a great concern about its toxicity in recent years. However, studies about cytotoxicity and genotoxicity of EUG are very limited.

In the present study, we investigated the in vitro antioxidant activity and the cytotoxicity of EUG on V79 cell line by the TEAC Assay and Neutral Red Uptake Assay (NRU). Our results demonstrated that EUG has antioxidant activity and cytotoxic effect in V79 cell line in a dose dependent manner. The IC50 value of EUG in V79 cell line was found to be 341.5 µM.

The in vitro genotoxic effects of EUG are also studied on human peripheral lymphocytes by Single Cell Gel Electrophoresis (Comet) assay and micronucleus assay. Results show that EUG is potent to induce DNA damage at higher concentrations when compared with untreated control cells. Our study also indicated that EUG is potent to protect cells against H2O2-induced oxidative DNA damage.

Keywords: Eugenol (EUG), Cytotoxicity, Genotoxicity, Single Gel Electrophoresis (Comet) Assay, Cytokinesis-block Micronucleus (CBMN) Assay.

ÖZET

Öjenol, Eugenia Caryophyllata'nın yaprak ve tomurcuklarından elde edilen, karanfil yağının fenolik yapıdaki uçucu bir bileşenidir. İlaç, kozmetik, tarım ve gıda endüstrisinde kullanılmaktadır. Türevlerin lokal antiseptik ve anestezik olarak tıpta kullanımı bulunmaktadır. Öjenol genel olarak güvenli bir bileşik olarak değerlendirilir ancak çok farklı uygulamaları ve yaygın kullanım alanları nedeniyle, toksisitesi son yıllarda ilgi odağı olmuştur. Öjenol'ün sitotoksisitesi ve genotoksisitesi ile ilgili çalışmalar yetersiz ve çelişkilidir.

Bu çalışmada, öjenolün V79 hücre hattında in vitro antioksidan aktivitesi ve sitotoksisitesi TEAC test ve nötral kırmızı alım yöntemiyle incelenmiştir. Sonuçlara göre, öjenol'un doza bağımlı olarak V79 hücrelerinde antioksidan aktivite ve sitotoksik etkiye sahip olduğu gösterilmiştir.Öjenol'un IC50 dozunun 341.5 µM olduğu bulunmuştur.

Ayrıca, öjenol'un in vitro genotoksik etkileri, tek hücre jel elektroforez (Comet yöntemi) ve mikroçekirdek yöntemi ile insan periferal lenfositleri üzerinde incelennmiştir. Sonuçlarımıza göre, EUG negatif kontrol hücreleriyle karşılaştırıldığında, daha yüksek konsantrasyonlarda DNA hasarı yaratma potansiyeline sahiptir. Ayrıca bu çalışmada, a öjenol'un hücreleri H2O2 kaynaklı oksidatif DNA hasarına karşı koruduğunu göstermektedir.

Anahtar Kelimeler: Öjenol, Genotoksisite, Sitotoksisite, Tek hücre jel elektroforez (Comet) yöntemi, mikroçekirdek yöntemi.

1. Introduction

EUG ($C_{10}H_{12}O_2$ or $CH_3C_6H_3$) is a weakly acidic, clear to pale yellow oil. It is a major volatile phenylpropanoid compound extracted from clove essential oil with a spicy clove-like aroma, extracted from Eugenia Caryophyllata buds and leaves [1]. It has been used as herbal medicine, spice, fragrance and as natural analgesic and antiseptic in dentistry[2]. Further, EUG used for treatment of digestive diseases and skin infections. Also it has been discovered in insect repellents and UV absorbers [3]. The US Food and Drug Administration approved use of clove oil as a flavoring substance in food industry, as a fragrance in cosmetics industry and in dentistry as a natural analgesic and antiseptic [4].

In recent years, there is an increasing tendency for replacement of synthetic antioxidants with natural substances and phenolic compounds such as EUG shown to have antioxidant capacity. EUG at low concentrations has shown to exert an antioxidant activity; however, at high concentrations it is suggested to act as a pro-oxidant, which may lead to enhancement of free radical generation and finally results to tissue damage [2, 5]. However, there are limited and contradictory studies about cytotoxicity and genotoxicity effects of EUG.

It was demonstrated that EUG had the ability to inhibit both the liver microsomal monooxygenase and lipid peroxidation induced by carbon tetrachloride (CCl_4). A protective effect of EUG at doses of 5 and 24 mg/kg against CCl_4 induced hepatotoxicity was also shown both *in vitro* and *in vivo* [6].

EUG has also found to have a preventive effect on dopamine depression and lipid peroxidation, which can protect depression induced by 6-hydroxyl dopamine (OHDA). EUG has prevented depression by decreasing lipid peroxidation and stimulating reduced glutathione (GSH) may lead to a protecting effect [7].

The aim of this study was to investigate the antioxidant activity of EUG by the trolox equivalent antioxidant capacity (TEAC) assay. The cytotoxicity effects of EUG were determined by the Neutral Red Uptake Assay (NRU) method in Chinese hamster lung fibroblast cell line (V79). Also, the genotoxic and antigenotoxic effects of EUG against H_2O_2 were investigated by the Single Cell Gel Electrophoresis (Comet) and cytokinesis blocked micronucleus (CBMN) assay in human peripheral lymphocytes. The study has approved by Local Ethics Committee of Hacettepe University.

2. Material and Methods

2.1. Chemicals

EUG (99% purity), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), L-glutamine, phytohaemagglutinin M (PHA-M), Trypsin-EDTA were purchased from Sigma-Aldrich Chemicals, Germany); Giemsa, hydrogen peroxide (35%) (H2O2), NaCl, NaOH, glacial acetic purchased from Merck Chemicals (Darmstadt, Germany); penicillin–streptomycin were obtained from PAA The Cell Culture Company (Cansera,Canada); neutral red (NR) , (ABTS), RPMI 1640, dimethyl sulfoxide (DMSO),Triton X-100, ethidium bromide (EtBr), phosphate buffered saline (PBS) tablets, KCl, potassium peroxodisulfat ,(±)6-hydoxy-2,5,7,8-tetramethylchromon-2-carboxylic acid (trolox) (purity >97%) and cytochalasin B (Cyt-B) were prepared from Sigma (St. Louis, USA), normal melting point agarose (NMA) and low melting point agarose (LMA) were obtained from Boehringer Mannheim (Mannheim, Germany), ethylene diamine tetra acetic acid disodium salt dihydrate (EDTA -Na2) and Tris purchased from ICN Biomedicals Inc.(Aurora,Ohio,U).

2.2. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC is a spectrophotometrically measurement of antioxidant strength. A cell-free system was used to detect the antioxidant capacity of EUG by TEAC assay as previously described [8]. The decolorization

of stable radical cation; ABTS, in several concentrations of EUG and synthetic antioxidant trolox was analyzed at 734nm. The experiment performed three times.

2.3. Determination of cytotoxicity of EUG by Neutral Red Uptake (NRU) assay

The cytotoxicity assay of EUG was determined in V79 cells by NRU assay according to the protocol explained previously [9]. NR assay is one of the most widely used tests for cytotoxicity assays. Basically, the viable cells are potent to bind to dye neutral red dye in the lysosomes. The V79 cells are widely sensitive to chemical compounds and also have good characteristics in cell culture such as excellent cloning proficiency [10].

Cells were cultured in 96 well culture plates (microtitration plates) at a density of 1×10^5 cells per well and a medium containing Neutral Red dye. Then after the plates were allowed to incubate at 37°C and 5% CO_2. Following 24 h of incubation, the different dilutions of EUG (25, 50, 100, 125, 150, 200, 250, 500, 1500, 2000 µM/ml) were added to the medium. Cells were incubated for 18 h in the humidified incubator at 37°C and 5% CO_2. During the incubation time, the weakly cationic dye penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes. Then the medium was discarded and the absorbance of each well was read at 540 nm in a microplate spectrophotometer and compared with the absorbance of wells of untreated cells. Final results were presented as the mean percentage of cell growth obtained from three separate experiments.

2.4. Determination of genotoxicity of EUG by the alkaline comet assay

The basic alkaline technique of Singh et al. 1988, [11] and N Basaran [12] was followed. Collected whole blood samples were centrifuged and lymphocytes were separated by Ficoll-Hypaque [13]. 1×10^4/ ml lymphocytes in 50 µl were treated with various concentrations of EUG (50, 100, 150, 200 and 250 µg/ml) for 30 min, then oxidative damage was induced by a solution containing 50 µM H_2O_2. The lysing, electrophoresis and neutralization procedures were described in the previous studies. The 0.4 M Tris–HCl solution was used for washing slides. The ethidium bromide (EtBr 20 µg/ml in distilled water, 35 µl/ slide) used to staining slides to recognize the DNA damage. To detect the DNA damage, captured pictures were analyzed by a Leica fluorescence microscope under green light. A device camera was connected to the microscope and analysis was performed by the Comet image analysis software, version 3.0, Kinetic Imaging Ltd., UK. The experiment was repeated three times and for each experiment, one hundred cells from two replicate slides were evaluated. Results of DNA damage were represented as tail intensity and tail moment.

2.5. Determination of the genotoxicity of EUG by the CBMN Assay

The presence of MN of binucleated cells was detected by using the protocol described before[14] . Briefly, the blood samples were collected in sterile blood culture tubes containing 0.5 ml of heparin. Then samples were incubated for 72h at 37 ℃ and 5% CO_2 in a humidified incubator. After the first 24h, blood samples separated into two groups; one group were treated with EUG alone (50, 100, 150, 200, and 250 µg/ml) and the other group treated with a combination of same concentrations of EUG and 50 µM H_2O_2.Then samples were incubated at the same conditions for another 48 h. The untreated cells were chosen as negative control and the cells treated with 50 µM H_2O_2 alone were chosen as positive control. At the 44h of incubation, Cytochalasin B (Cyt-B) at a final concentration of 6 µg/ml was added to the same tubes. During incubation of blood samples, the slides were stained in 5% Giemsa and were stored in 70% ethanol at -20 ℃. Then 1000 binucleated cells were scored to assay the presence of MN. To determine the percentage of the cells with 1-4 nuclei, 500lymphocytes from per donor were scored. The total experiment was repeated for three times.

2.6. Data Analysis

The obtained data of the TEAC assay and alkaline comet assay were indicated as the mean ± standard deviation. SPSS for windows 22.0 package program was used for the final statistical analysis. Differences between the means of data were compared by the one-way ANOVA test and post hoc analysis test. The accepted statistically significance level of *P* value was <0.05. For statistical analysis of obtained results of the CBMN assay, the z-test was used. The final results were shown as the mean ± SEM.

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3. Results

3.1. Antioxidant activity of EUG

The antioxidant activity of EUG was measured by the TEAC assay and results are expressed in figure 1. Due to the results at the studied concentrations of 2-200 µM and compared to the trolox, EUG was discovered to have considerably more antioxidant activity.

Blank: (Ethanol + ABTS), TR: (Trolox +ABTS), EUG :(Eugenol +ABTS)

3.2. Cytotoxicity of EUG in V79 cell line by Neutral Red Uptake Ass

The cytotoxic effect of EUG in V79 cell line was determined by NRU assay and results are presented in figure 2. The concentrations of EUG up to 250 µM had no effect on survival of V79 cells when compared with untreated control cells but at the concentrations higher than 500 µM, the viability of V79 cells decreased below 50 %. The IC_{50} value of EUG in V79 cell line was found to be 341.5 µM.

Results were shown as the mean percentage of cell growth inhibition. Cell viability was given as the percent of control. The IC_{50} value was 341.5 µM.

3.3. Genotoxicity and antigenotoxicity of EUG by the alkaline Comet assay

The results obtained by alkaline comet assay in lymphocytes are shown in figure 3. The DNA damage is presented as DNA tail intensity (percent of DNA in tail) and tail moment (fraction of total DNA in tail). According to data obtained from three individual experiments, no significant increase in DNA damage, was observed at different dilutions of EUG (50,100,150,200 µM) when compared with untreated control cells. But at the highest concentration (250 µM) of EUG, a considerable increase in DNA damage was detected in compare with untreated cells. At the concentration of 150µM, EUG seemed to decrease the H_2O_2 induced DNA damage ($p < 0.05$). Although at the higher concentration (250 µM) of EUG increase in the tail intensity was detected, such effect was not confirmed by the evaluation of tail moment data.

3.4. Genotoxicity of EUG by CBMN Assay

The observations of the CBMN experiments are given in Table 1. According to the obtained data from studied concentrations (50-200 µM), EUG had no genotoxic effect in treated lymphocytes as compared with the negative control group. But as the data obtained with CBMN test, at the highest concentration

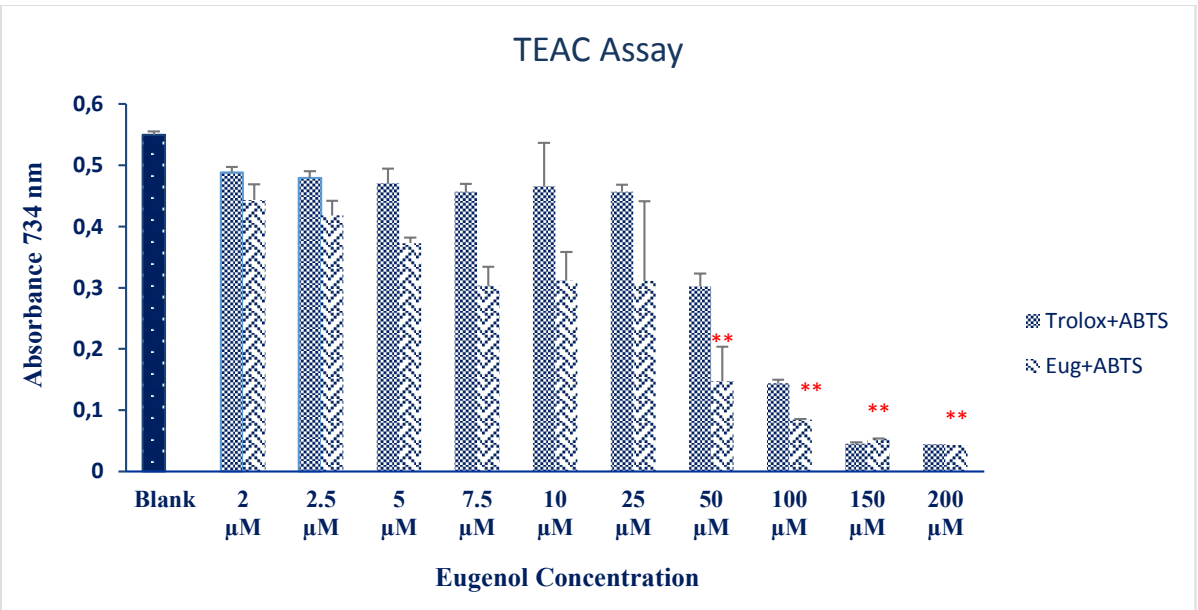

Figure 1. The antioxidant capacity of EUG in comparison with trolox. Values are given as the mean ± SEM.
**$p < 0.05$ has been reported as significantly different from trolox.

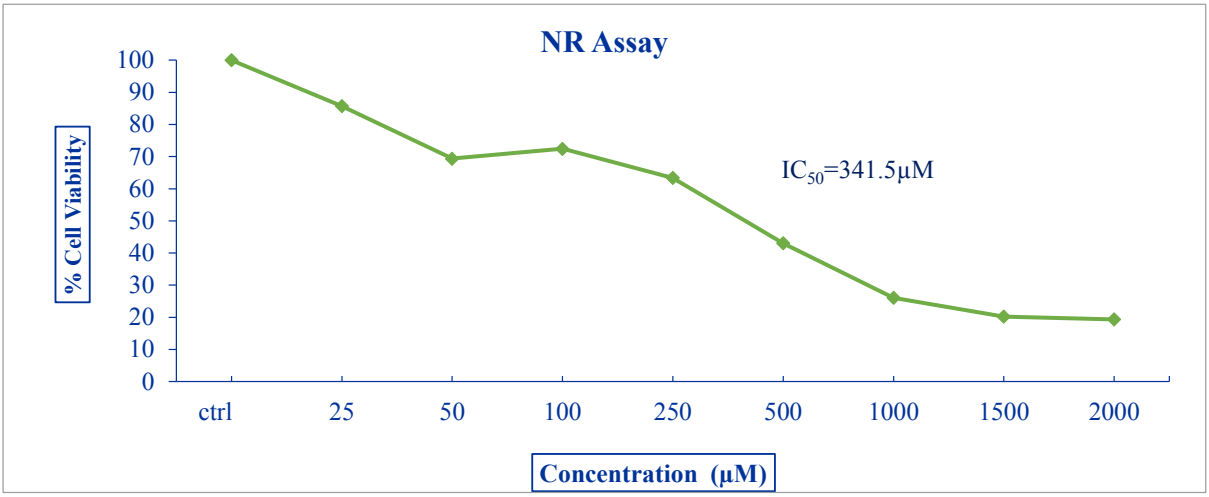

Figure 2. The cytotoxic effect of EUG in V79 cell line. Cell viability was detected by NRU assay.

of EUG (250 µM), genotoxic effect was observed. The treatment of EUG with H_2O_2 (50-250 µM) revealed a decrease in the micronuclei frequency at the studied concentrations when compared with the samples treated with only 50 µM H_2O_2.

4. Discussion

EUG, the major volatile compound of clove essential oil, is a phenylpropane derived from the Eugenia Caryophyllat1a [15]. Its pharmacological properties which include [16] anti-inflammatory, analgesic and antioxidant activities have been the subjects of many studies [17, 18]. In recent years, there is a great concern about the activity and toxicity of EUG due to the wide range of usage. The antioxidant activity of EUG is related to the presence of the phenolic groups in molecular structure. EUG has been recognized as a safe food additive in the GRAS substance classification under the sections of Federal Food, Drug and Cosmetics Administration. Nevertheless, there are very limited and contradictory studies about the cytotoxicity and genotoxicity of EUG [4].

EUG is able to form complexes with reduced metal which may lead to its antioxidant effects. The iso-EUG shows a potent inhibitory effect on lipid peroxidation. EUG inhibits the oxidation of the LDL by suppression the formation of free radicals [19]. Studies showed that EUG has higher inhibitory effect on hydrogen peroxide in comparison with other reactive oxygen species. EUG also is potent to block oxidation of DNA and lipid peroxidation induced by hydroxyl radical. In some cases of metastasis, EUG acts as a potent preventive agent due to its antioxidant activities [3] . In this study, by using TEAC assay, we found that at our studied concentrations, EUG shows greater antioxidant activity than trolox.

According to observations, the cytotoxic effect of EUG is not mediated by ROS-dependent mechanisms. Such effect is possibly in relation with involvement of phenoxyl radicals and/or EUG quinone methide [20]. The cytotoxicity of EUG was studied in three different malignant and nonmalignant human derived cells. The malignant Hep G2 hepatoma cells, the malignant Caco-2 colon cells and the nonmalignant human VH10 fibroblasts were chosen to evaluate the cytotoxicity of EUG and results showed the cytotoxic effects in all mentioned cell lines. EUG acted as a genotoxicant in human VH10 fibroblasts and Caco-2 colon cells however, but not in Hep G2 hepatoma cells. EUG at the concentrations under 600µM significantly caused an increase in DNA breaks in human VH10 fibroblast cells. However, the degree of such damage in Caco-2 colon cells was lower. The DNA damaging effect was not observed in Hep G2 cells [21].

In a dose dependent manner, EUG had a cytotoxic effect in human osteoblastic cells and the IC_{50} of EUG 0.75 mmol/L. EUG inhibited cell proliferation in a 4-days culture period. At the concentrations higher than 0.01 mmol/L, EUG seemed to have significant toxicity potential [22] . In another study, researchers examined the cytotoxicity of EUG by using MTT assay in HL-60 cancer cells. EUG showed different

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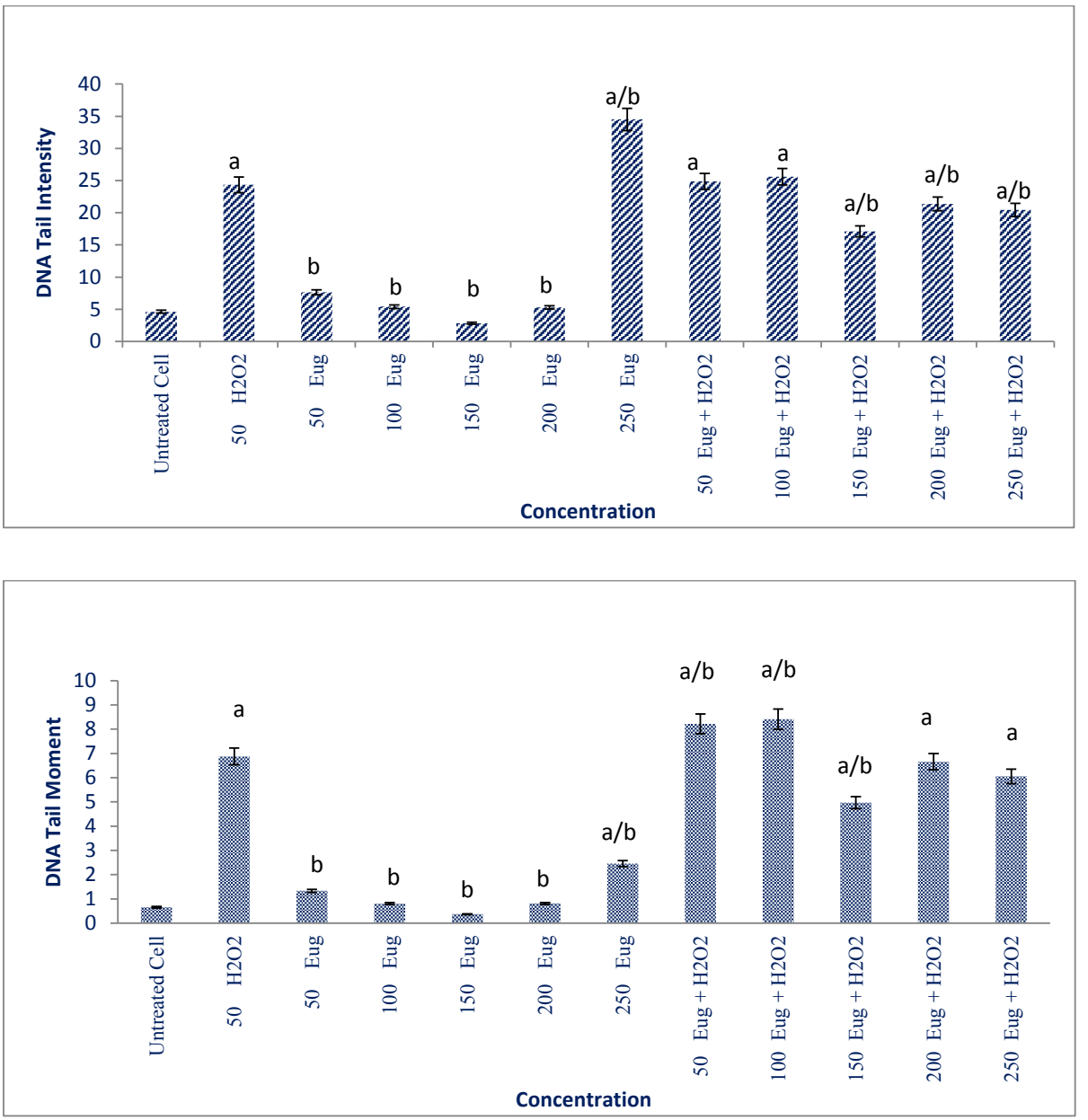

Figure 3. The DNA damage detected in treated lymphocytes compared to control group. DNA damage expressed as DNA tail intensity, and DNA tail moment. Values are given as mean ± SD.

a: p <0.05 reported as statistically different from negative control (1% DMSO)

b: p <0.05 reported as statistically different from positive control (50µM H_2O_2)

degrees of cytotoxicity in these cells and inhibited the cell growth by 50% at the concentration of 23.7 µM [15].

It has been shown that EUG inhibited the growth of the colon cancer cells (HT-29 cells) in a dose and time dependent manner. After 24h exposure, the growth of cells was reduced below 50% at the concentration of 250 µM [23]. Elia Martins et al, investigated the genotoxic and apoptotic activities of EUG in AA8 and EM9 cells. The cell viability decreased below 50% after 24h exposure to higher concentrations of

Table 1. Genotoxicity Findings of EUG by Micronucleus Assay*

Treatment group	BN cells scored	Number of BN cells according to donors			MN /10^3 cells	MN% ± SE
		1st	2nd	3rd		
Untreated control	3000	1	2	1	1.33	0.13± 0.07
50 µM H_2O_2	3000	10	11	10	10.33	1.03± 0.18 *
50 µM EUG	3000	3	4	1	2.67	0.27 ± 0.09
100 µM EUG	3000	5	2	4	3.67	0.37± 0.11
150 µM EUG	3000	2	4	2	2.67	0.27± 0.09
200 µM EUG	3000	1	3	1	1.66	0.17± 0.08
250 µM EUG	3000	7	1	3	3.67	0.37± 0.11 #
50 µM EUG+ H_2O_2	3000	4	3	4	3.67	0.37± 0.11 #
100 µM EUG+H_2O_2	3000	7	2	3	4	0.4 ± 0.12 #
150 µM EUG + H_2O_2	3000	2	2	2	2.33	0.20 ± 0.08 #
200 µM EUG + H_2O_2	3000	2	3	2	2.33	0.23 ± 0.09 #
250 µM EUG + H_2O_2	3000	2	5	4	3.66	0.37± 0.11 #

*Micronucleus frequencies in human lymphocytes for the genotoxicity and antigenotoxicity of EUG. BN=binucleated; MN=micronucleus; SE=standard error

Negative control=untreated cells; Positive control= 50 µM H_2O_2 treated cells.

*p< 0.05 reported as significantly different from negative control

# p<0.05 reported as significantly different from positive control

Values are given as the mean ± standard error.

EUG. The IC_{50} for AA8 cells was about 500µM and for EM9 cells was about 1000µM[16].

Studies demonstrated that all the zinc-oxide EUG based root canal sealers have moderate to severe cytotoxic effects in V79 cultured cells, but the degree of cytotoxicity is different due to the dose and duration of exposure. However, the results did not indicate the genotoxic effects of these dental products [24].

In this study, we also investigated the *in vitro* cytotoxicity effects of EUG by the NRU assay in V79 cell line which is highly used in many *in vitro* assays. The IC_{50} values of EUG have been found to be different according to the cell-line, duration of incubation and the method used in different studies. Generally, cancer cell-lines were used in these studies however; we used V79 cells because they are suitable and wide used cells in cytotoxicity assays in the biomaterials. The doubling time of this cell is about 16-18 hr. The plating efficiency is about 80%. These cells are also widely used in DNA damage induced by oxidizing agents. As V79 cells are very fast growing and also based on literature review in phenolic compounds there is no significant difference between 18 and 24 h of exposure time [25].

Our findings indicated that the concentrations of EUG up to 250 µM had no effect on cell viability during 18 hr exposure, however, at concentrations higher than 500 µM the cell viability reduced below 50%. The IC_{50} value of EUG in this study has been found at the concentration of 341.5 µM.

The data of our study are consistent with the data of Martins et al, who indicated cytotoxicity of EUG at high concentrations, although the IC_{50} value determined in that study is lower than our finding [16]. Evaluation the genotoxicity of EUG was studied in V79 cell line *in vitro*. Results demonstrated that EUG leads to a significant increase in chromosomal aberrations in V79 cells (3.5% aberrant cells) at 2500 µM, indicating genotoxicity at higher concentrations. The DNA damaging effect of EUG was assessed in both AA8 and EM9 cells (Chinese hamster cell line) with the alkaline comet assay[26]. The DNA damage was induced by EUG in AA8 cells, but EUG did not induce DNA damage in EM9 cells [16].

A dose and time dependent study in rats investigated the genotoxicity of methyl-eugenol (MEG) by using comet assay. Results demonstrated no significant dif-

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ferences in DNA damage after 24 hr exposure with doses that produce tumors in rodents [27].

In the present study, the genotoxic effect of EUG in the range of 50-250 μM concentrations (non-cytotoxic concentrations), was investigated by two important genotoxicity assays, cytokinesis-blocked micronucleus (CBMN) and Comet test, to evaluate the DNA damage. Furthermore, it was also evaluated whether EUG provided protection against H_2O_2 induced DNA damage in human peripheral lymphocytes. Based on the obtained results from comet test, at the concentration of 250 μM, EUG indicated genotoxic effect due to DNA tail intensity and tail moment parameters. At lower concentrations of 50 and 100 μM EUG no decrease in the H_2O_2 -induced DNA damage was observed. When EUG used in combination with H_2O_2, it appeared to prevent H_2O_2-induced DNA damage only at 150 μM concentrations. EUG alone induced increase in MN just at the 250 μM concentration. On the other hand, EUG, in all concentrations, decreased H_2O_2 -induced DNA damage.

5. Conclusion

In conclusion, our results suggest that EUG has antioxidant properties and cytotoxic effects in a dose dependent manner in V79 cell line. EUG in the concentrations below the IC_{50} value showed no significant genotoxic effects. Our results of CBMN and comet assay on human lymphocytes, also showed that EUG is potent to protect against H_2O_2-induced oxidative DNA damage in all studied concentrations. It is suggested that EUG has the potential ability to induce DNA damage at higher doses. However, our results obtained from only *in vitro* data assays, further detailed *in vivo* animal research are warranted to discover the genotoxic or antigenotoxic potential of EUG.

Competing interest: The authors declare that they have no competing interest.

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