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ANTI-GENOTOXIC EFFECT OF OLEUROPEIN AGAINST AFLATOXIN B_1

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Abstract

Aflatoxin B_1 when binds to DNA bases, cause DNA damage, thus, affects the functioning, growth, division, and control of cells. Oleuropein is a fundamental polyphenol founded in olive leaf, fruit, oil and has many benefits for human health. In this study, the possible effect of oleuropein was assessed against toxicity of aflatoxin B_1. For this purpose, the single cell gel electrophoresis and micronuclei assays were performed using the cells of human peripheral blood. As a result of this study, it was determined that oleuropein has not genotoxic effect and exhibited anti-genotoxic properties caused by aflatoxin B_1 (with especially 625 µM application) ($p<0.05$). On this basis, the observed anti-genotoxic effect of oleuropein against aflatoxin B_1 may occur as oleuropein has sufficient capability to reduce the lipid peroxidation and inhibit the loss of membrane integrity.

Key words: aflatoxin B_1, carbonic anhydrase, comet assay, micronuclei assay, oleuropein

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1. Introduction

Most of the synthetic drugs used in the treatment of different diseases are known to have undesirable side effects (Kısa et al., 2018). Therefore, the scientific community has turned to medicinal plants and other organisms to produce alternative solutions. The family *Oleaceae* is reported to have 16 genera in the world flora (Kısa et al., 2018). Olea *europaea L.,* also known as olive, is a member of this family grown in Mediterranean countries as Turkey, Spain, Portugal, Italy and Greece (Kısa et al., 2018). For many years, the leaf of this plant (*O. europaea* L) has been consumed in conventional medicine (Lee and Lee, 2010). In former times, people have consumed the leaf of this plant as a medication against malaria and fever associated disorders (Benavente-Garcıa et al., 2000).

Oleuropein is the most effective active ingredient in olive leaves and the most abundant phenolic compound (Albertos et al., 2018; Soler-Rivas et al., 2000). Although oleuropein was first discovered by Bourquelot and Vintilesco in 1908, structure of oleuropein was defined in 1960 (Benavente-Garcıa et al., 2000). Oleuropein is a glycosylated secoiridoid which consists of several structural subunits including a glucose molecule, a secoiridoid (elenolic acid) and a polyphenol, as shown in Fig. 1.

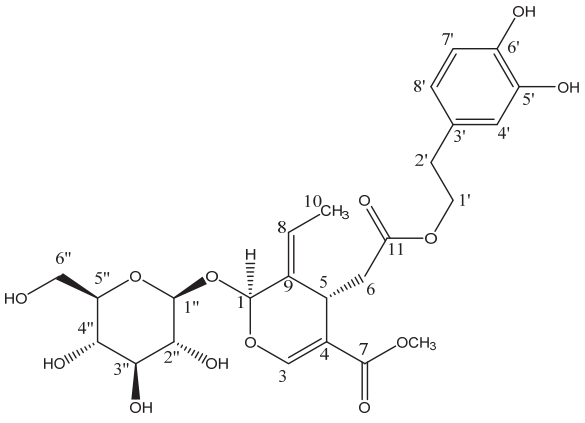


Fig. 1. Chemical structure of oleuropein (Aggul et al., 2020)

Some studies have determined the beneficial physiological and pharmacological properties of oleuropein (Ahamad et al., 2019; Hassen et al., 2015). As shown in many reports, oleuropein and its derivatives have many biological activities including anti-inflammatory, anti-diabetic, anti-tumour, anti-aging, cardio protective, neuroprotective, antioxidant and hepatoprotective (Bakır et al., 2018; Carrera-González et al., 2013; Fuentes and Palomo, 2014; Rubió et al., 2014; Sepporta et al., 2014; Qabaha et al., 2018; Žukovec Topalović et al., 2015). On the other hand, it was reported that oleuropein exhibited prooxidant activity and increased DNA damage [DNA oxidation of guanines (8-oxo-dG) in human breast cancer MDA-MB-231 cells] (Odiatou et al., 2013).

Aflatoxins are toxic compounds produced by a fungi genus (*Aspergillus*) when they grow in human and animal foods. These toxic compounds are known to cause many diseases in both human and animals (Wu and Khlangwiset, 2010). Aflatoxins are substances with the greatest potential to cause liver cancer in humans and are evaluated among group I carcinogens by the World Cancer Research Center (Claeys et al., 2020). Aflatoxin B_1 (AFB_1) is the most potent carcinogen/hepatogenic agent among aflatoxins (Orhan et al., 2016). However, it is known that AFB_1 can be found as a common contaminant in various crops such as rice, sorghum, nuts, and dried fruits, causing people to be easily exposed to AFB_1 in everyday life (Singto et al., 2020).

Although different studies have shown that olive phenolics have a strong antioxidant activity and prevent ROS mediated cell damage, there are few studies on the effect of oleuropein on DNA. Therefore, the goal of this work was to find out the anti-genotoxic potential of oleuropein, against AFB_1 induced DNA damage on human peripheral blood lymphocytes, by using the micronuclei (MN) and the single cell gel electrophoresis (Comet) assays. In addition, the effect of oleuropein on human carbonic anhydrase I, II isoenzyme activities were also investigated.

2. Material and methods

This study was conducted after the approval of Agri Ibrahim Cecen University Ethics Committee (14.11.2018-66).

2.1. Plant material, extraction, and isolation

The leaves of *O. europaea* L. collected from Artvin region in Turkey in September 2018 were cleaned and dried at room temperature. Dry leaves were ground into powder by grinding with liquid nitrogen. Then, extraction and isolation of oleuropein from the dried leaves were carried out as indicated by Kısa et al. (2018). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy data were used to characterize the chemical structure of the obtained material. It was determined that the substance was oleuropein and the ^1H and ^{13}C NMR data consonant with the literature (Zhang et al., 2014). The NMR data of the oleuropein isolated from the ethyl alcohol extract of the *O. europaea* L. leaf are given in Table 1.

Table 1. ^1H and ^{13}C-NMR data of oleuropein

DMSO-d6			
	C/H	***δC ppm***	***δH ppm, J (Hz)***
1	CH	93.5	5.82 (s, 1H)
3	CH	154.0	7.47 (s, 1H)
4	C	108.1	-
5	CH	30.6	3.79 (dd, J1= 4,5 Hz, J2= 13,2 Hz, 1H)
6	CH_2	40.1	2.36 (d, 1H, J = 9.20 Hz, H6a), 2.33 (d, 1H, J = 9.10 Hz, H6b)
7	C	171.5	-
8	CH	123.7	5.92 (q, 1H)
9	C	129.3	-
10	CH_3	13.3	1.66 (d, 3H, J = 6.24 Hz)
11	C	167.0	-
-	OCH_3	51.8	3.65 (s, 3H)
1'	CH_2	65.6	4.13 (t, 2H)
2'	CH_2	33.9	2.68 (t, 2H)
3'	C	129.3	-
4'	CH	116.5	6.59 (s, 1H)
5'	C	145.2	-
6'	C	143.9	-
7'	CH	115.9	6.63 (d, J = 7.96 Hz, 1H)
8'	CH	120.2	6.46 (d, J = 8.00 Hz, 1H)
glc-1"	CH	99.3	4.65 (d, J = 7.76 Hz, 1H)
glc-2"	CH	73.5	nd
glc-3"	CH	76.6	nd
glc-4"	CH	70.2	nd
glc-5"	CH	77.3	nd
glc-6"	CH_2	61.4	nd

** nd: not determined, ^1H: 400 MHz, ^{13}C: 100 MHz*

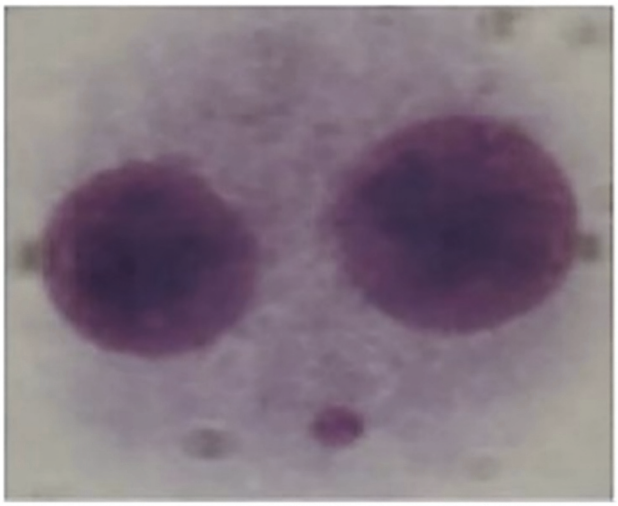
2.2. In vitro MN and Comet tests

Peripheral blood to be used in both MN and Comet tests was obtained from young volunteers who were not exposed to factors that may increase the frequency of micronucleus (Ceker, 2019; Ceker and Agar, 2021). Enough peripheral blood samples (0.5 mL) were added to sterile tubes containing chromosome medium B (Biological Industries, Beit Haemek, Israel) that allows cells to undergo mitosis, under sterile conditions (Nartop et al., 2020).

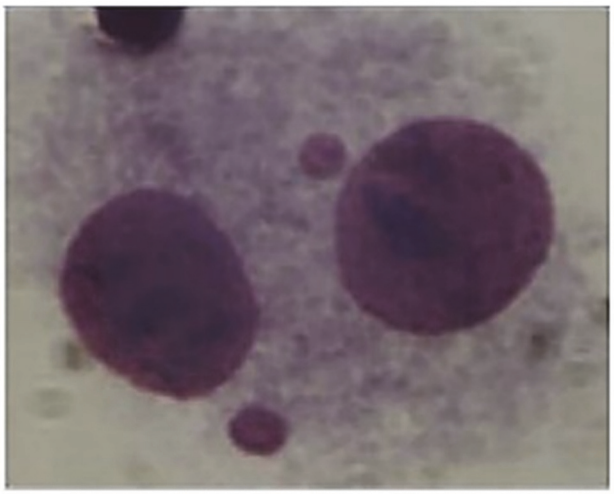
AFB_1 (Sigma, MO, USA) (5 μM) alone, oleuropein alone (500μM) and cotreatment of AFB_1 (5 μM) and different concentrations (125, 250, 500 and 625 μM) of oleuropein were added into this solution and cell culture was incubated (at 37 °C for 72 h). AFB_1 was used as positive control and pure water was used as negative control (Ceker, 2019; Ceker and Agar, 2021; Nartop et al., 2020).

The concentrations of oleuropein investigated in the study were determined in a wide range based on the concentrations studied in previous studies (Anter et al., 2011; Čabarkapa et al., 2014; Liman et al., 2017; Türkez and Toğar, 2011; Žukovec Topalović et al., 2015). For the determination of micronuclei, the procedure previously described by Fenech (2000) was used. 44 h later the initiation of incubation at 37°C, cytochalasin-B (Sigma, MO, USA) was added to each

tube at a final concentration of 3 µg / mL to prevent cytoplasmic division. After 72 h, the cells were removed from the incubator. It was centrifuged at 1000 rpm for 10 min. After removing the tops of the tubes (supernatant), a hypotonic solution (6 mL 0.075 M KCl) was added and put back into the incubator (7 min.) The cells were then immediately centrifuged and fixed three times with cold methanol / glacial acetic acid (3:1). The fixed cells were dropped onto slides and allowed to dry at room temperature (72 h). The preparations were stained with 6% giemsa (Merck, Darmstadt, Germany) for 10 min. For MN analysis, bi-nucleated cells were evaluated under light microscope (magnification 1000x) and scored (Fig. 2) (Nartop et al., 2020).



(a)



(b)

Fig. 2. Cells containing micronuclei:
(a) One MN; (b) Two MN

The comet test was first established by Ostling and Johansson (1984), then modified by various researchers and presented with new techniques (Azqueta et al., 2020; Ceker and Agar, 2021; Langie et al., 2015; Singh et al., 1988). In this study, the Comet test was performed specifically as described by Ceker and Agar (2021).

In the comet test, firstly, lymphocytes were isolated from peripheral blood. Slides were prepared by embedding cells into agarose gels. In the next step, cells were treated with lysis solutions to remove membranes and other cellular materials. In the fourth step, after the loosening of the supercoil structure of DNA in alkaline medium and the emergence of fractures, the sample was subjected to electrophoresis. In the neutralization step, removal of solutions which contained high salt concentrations used during electrophoresis was performed. In the final step, staining with ethidium bromide and imaging of the slide under fluorescence microscope were performed (Fig. 3). Afterwards, DNA damage was determined with the obtained data (Ceker and Agar, 2021).

2.3. Measurement of CA activity

The effects of oleuropein on the activity of carbonic anhydrase isoenzyme (CA I and II) purified from human erythrocytes were investigated *in vitro* by the method indicated by Kuzu et al. (2018). Enzyme activity was measured at five different concentrations (between 18.5 - 185 µM) of oleuropein. The activity without oleuropein was accepted as control (100% activity).

2.4. Statistical analysis

In this study, three replicates of all experiment groups were accomplished for the reliability of the data. The data of each experiment groups were analyzed with SPSS 18.0 version using one-way analysis of variance. Significance was determined by Duncan's test. The level of significance was regarded $p<0.05$ and 0.01 for all statistical analysis.

3. Results and discussion

In the study, it was observed that AFB_1 increased MN frequency and DNA damage. In addition, different concentrations of oleuropein treatment were observed to reduce the frequency of AFB_1 induced MN and DNA damage. In both test systems, it was determined that the most effective result was obtained especially from the 625 µM concentration of oleuropein (Table 2) ($p < 0.05$).

This study also investigated the effect of five different concentrations (between 18.5 - 185 µM) of oleuropein on the human erythrocyte carbonic anhydrase activity (CA I and II). All the tested concentrations neither activated nor inhibited the carbonic anhydrase. Based on this result, it was concluded that oleuropein has no effect on the activity of carbonic anhydrase. Having no effect of oleuropein on CA I and II enzymes can be explained as not being negatively affected by many cell-important biological processes (acid-base regulation, transport of ions and carbon dioxide, lipogenesis, respiration, osteolysis and tumor development in different tissues by facilitating the conversion of carbon dioxide to HCO_3) affecting carbonic anhydrase isomers (Kuzu et al., 2018; Şentürk et al., 2011).

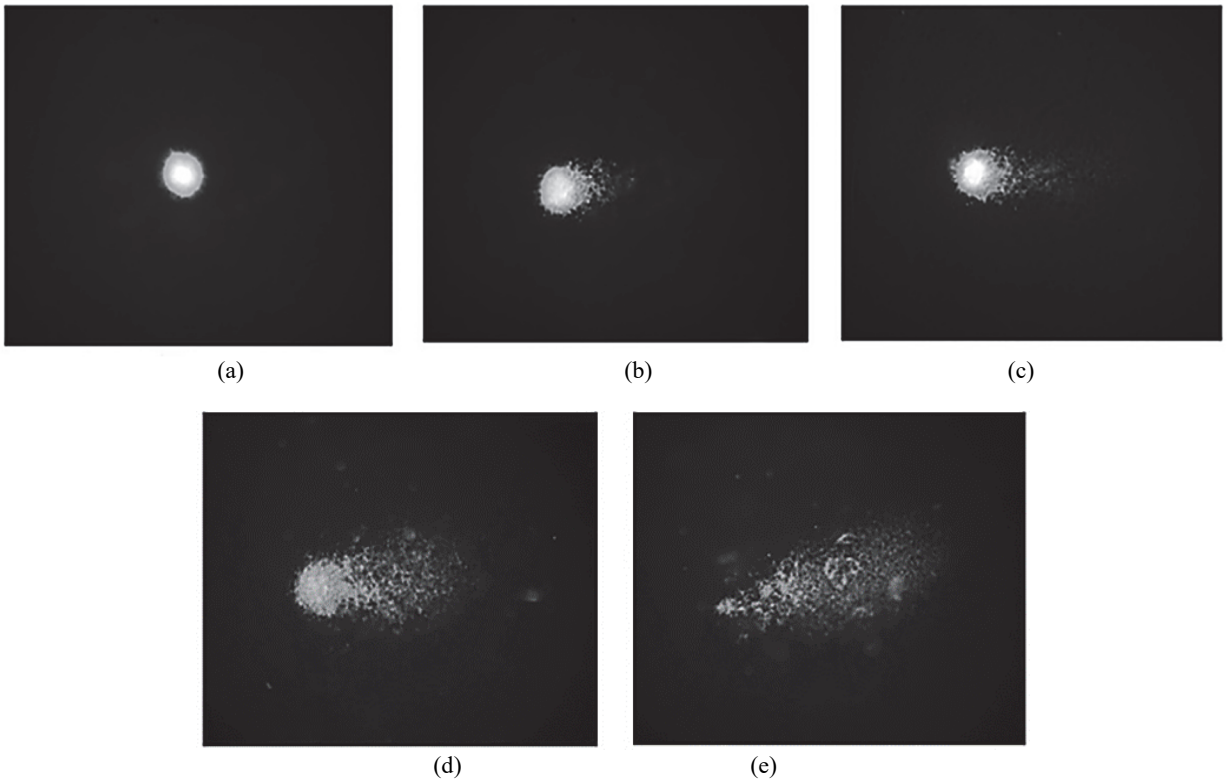


Fig. 3. Comet assay images of DNA at different degree of damages: (a) No damaged, (b) Low level damaged, (c) Medium level damaged, (d) High level damaged, (e) Total damaged

Table 2. The effects of AFB_1 and oleuropein on Comet and MN in human peripheral lymphocytes

Test Items	*Concentrations*	*Visual Score (AU) ± SE*	*MN numbers ± SE*
Negative control		7.25 ± 0.16^{a}	2.42 ± 0.92^{a}
Positive control	5 µM	146.08 ± 0.08^{d}	4.65 ± 0.13^{e}
Oleuropein	500 µM	7.26 ± 0.12^{a}	2.61 ± 0.24^{a}
AFB_1 (5 µM) + Oleuropein (125-1000 µM)	5 µM + 125 µM	142.30 ± 0.06^{d}	3.85 ± 0.65^{d}
	5 µM + 250 µM	138.93 ± 0.22^{d}	3.38 ± 0.14^{c}
	5 µM + 500 µM	92.42 ± 0.25^{c}	2.84 ± 0.17^{ab}
	5 µM + 625 µM	56.80 ± 0.18^{ab}	2.58 ± 0.16^{a}
	5 µM + 750 µM	78.50 ± 0.07^{b}	2.98 ± 0.26^{b}
	5 µM + 1000 µM	108.14 ± 0.16^{cd}	3.16 ± 0.12^{bc}

$^{a-e}$Means ± SE; values within each column not sharing a common superscript are significantly different ($p<0.05$ and 0.01*) as determined by Duncan test*

In previous studies, the important roles of carbonic anhydrase isomers in various physiological processes have been demonstrated and the relations of abnormal levels or activities of these enzymes with human diseases have been revealed (Supuran, 2008, 2011). The result obtained from this study can be interpreted that oleuropein has no side effects, since oleuropein has both anti-genotoxic character and does not affect CA I and II, which have important functions in cell, thus these enzymes can perform their functions

The mutagenic and carcinogenic effects of aflatoxins are due to the toxic products formed because of their biotransformation (Gross-Steinmeyer and Eaton, 2012). In the liver, AFB_1 metabolization occurs via activation of cytochrome P-450 monooxygenase (Orhan et al., 2016). Cytochrome P-450 monooxygenase converts the nontoxic AFB_1 into the electrophilic, reactive, and toxic AFB_1-8, 9-epoxide. The reactive AFB_1-8, 9-epoxide can easily interact with cellular constituents including DNA, RNA, and proteins (Kim et al., 2011). Impaired membrane integrity and lipid peroxidation increase have been shown to be caused by an increase in the level of AFB_1-8, 9-epoxide (Verma, 2004). Similarly, the oxidative damage and lipid peroxidation have been mainly linked with the increased level of AFB1 Kim et al. (2011).

Giamarellos-Bourboulis et al. (2006) reported that oleuropein is used as an immune-regulating component and plays an active role in strengthening the immune system by maintaining total antioxidant capacity reducing bacterial growth in blood and organs. In a study investigating the effects of oleuropein enriched olive leaf extracts on Wistar mice, oleuropein has been shown to slow down the lipid peroxidation process and increase antioxidant enzyme

activities as well as cholesterol lowering effect (Jemai et al., 2008). In another study, it was determined that olive leaf extract and oleuropein reduced activities of GSH-Px and CAT, increased the cell viability, improved necrotic and apoptotic deaths and inhibited ROS production of cells treated with H_2O_2 (Cumaoğlu et al., 2011). Other studies have also reported that oleuropein (Kyriakopoulou et al., 2012) and O. *europaea* L., leaf extract (Čabarkapa et al., 2014) reduce DNA damage caused by H_2O_2.

Anter et al. (2011) in their study performed with *Drosophila melanogaster*, reported 73.7% reduction of H_2O_2 oxidative stress by oleuropein (555 µM). Oleuropein was found to decrease permethrin induced genotoxicity and oxidative damage in human lymphocyte cells (Türkez and Toğar, 2011). Also, Žukovec-Topalovic et al. (2015) and Čabarkapa et al., (2014) reported that *O. europaea* L., extract of leaf has been shown to alleviate oxidative DNA damage caused by thyroxine and adrenaline in human leukocytes under two different experimental conditions (pre and post treatment). On the other hand, Odiatou et al. (2013) determined that oleuropein causes cells to produce H_2O_2 due to sodium bicarbonate in standard culture medium and thus exhibits prooxidant properties. Furthermore, in another study, oleuropein was reported to increase DNA damage for 48 h at IC_{50} and $2xIC_{50}$ and 72 h at all tested concentrations (Liman et al., 2017).

4. Conclusions

In this study, the protective properties on DNA and anti-genotoxic effect of oleuropein, which is thought to be used as a preservative in foods, against AFB_1, which is known to be a good mutagenic substance and frequently encountered in daily diet, were investigated.

As a result of this study, it is determined that oleuropein has not genotoxic property but reduced the mutagenic/genotoxic effect caused by AFB_1 (625 µM). As previously stated, that AFB_1 increase lipid peroxide level and destroy the integrity of cell membrane integrity. The effect of oleuropein against AFB_1 toxicity determined in this study could be due to its ability to minimize both membrane integrity damage and the lipid peroxidation.

Taking these effects of oleuropein into account more studies should be performed in order understand the precise mechanisms underlying its antigenotoxicity.

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