Oleuropein Against Gastric Cancer: A New Hope of Therapy

Kürşad Türkdoğan (kursad.turkdogan@izu.edu.tr)
Istanbul Sabahattin Zaim University: Istanbul Sabahattin Zaim Universitesi https://orcid.org/0000-0002-5594-782X
Abdurrahim Koçyiğit
Bezmialem Vakif University: Bezmialem Vakif Universitesi
Eray Metin Güler
Bezmialem Vakif University: Bezmialem Vakif Universitesi
Ömer Faruk Özer
Bezmialem Vakif University: Bezmialem Vakif Universitesi
Kübra Demir
Istanbul Sabahattin Zaim University: Istanbul Sabahattin Zaim Universitesi
Halime Uğur
Kütahya University of Health Sciences: Kutahya Saglik Bilimleri Universitesi

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Research Article

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Abstract

Oleuropein (OLE), the main phenolic compound of the olive fruit and leaves, has many healthful effects. Gastric cancer is the most fatal malignity in many parts of the world and it is generally related to harmful dietetic factors. The anticarcinogenic role of OLE in gastric cancer has not been studied sufficiently yet. In this study, we aimed to search the cytotoxic, genotoxic and apoptotic effects of OLE on gastric adeno cancer (G.CA) cells in vitro.

The performance of a standard cell line (G.CA) produced from G.CA cells was evaluated after a 24-hour exposure to OLE at varied dosages. The cytotoxicity, generation of reactive oxygen species (ROS), and genotoxicity were examined using the ATP analysis for viable cells, 2',7'-dichlorodihydrofluorescein-diacetate assay (H2DCF-DA), and alkaline single cell gel electrophoresis analysis (Comet Assay), respectively. Through the use of flow cytometry, the triggering of apoptosis was revealed.

OLE reduced G.CA cells viability (64%) at maximum concentration (500 μmol/L) and also resulted in approximately 100% DNA damage, 60% apoptosis and necrosis in G.CA cells depending on the increased doses. Malign cell viability was also significantly decreased in relation to growing amount intracellular reactive oxygen species (ROS) grades ($p < 0.05$-0.001).

In conclusion, Oleuropein has shown very significant anticarcinogen effects against G.CA cells in vitro. Nutrition rich in olive and extra virgin olive oil seems to be both protective and therapeutic against gastric cancer and Oleuropein may be a new, potential chemotherapeutic agent in the near future.

Introduction

The olive tree and fruits have been considered symbols of peace and hope for centuries and they are sacred in Holy Books. The classic and staple foods of the Mediterranean diet, olives and olive oil, are rich in nutrients and have numerous positive health effects. Oleuropein (OLE), the main phenolic chemical found in olive fruit and leaves of the Olea europaea L. (Olive tree), has a variety of medicinal properties, including those that are antibacterial, anti-inflammatory, anticarcinogenic, and anti-atherogenic. [18, 25]. Also, it has restorative efficacy in organ injuries induced by chemotherapy. Gastric cancer is the most frequent and fatal malignity in many parts of the world and it is generally related to harmful dietetic factors. The anticarcinogenic role of OLE in gastric cancer has not been studied sufficiently and the underlying mechanisms of its action remains yet unknown. In this study, we aimed to assess cytotoxic, apoptotic and genotoxic effects of OLE on gastric adenocarcinoma (G.CA) cells in vitro.

Materials And Methods

Chemical and reagents

OLE, FBS, 2,7-dichlorodihydrofluorescein-diacetate (H2DCF-DA), penicillin-streptomycin, ethidium bromide (EB), and F-12K medium were acquired from Sigma-Aldrich (Seelze, Germany). EBioscience provided the

Annexin V Human Apoptosis Detection Kit (300 tests/kit, Bendermed, San Diego, CA 92121 USA). Unless otherwise specified, all of the chemicals employed in the inquiry are of analytical quality.

Dimethyl sulfoxide (DMSO) was used to generate a 500 μM OLE stock solution, which was subsequently diluted with DMEM (which does not contain fetal bovine serum) to achieve the necessary concentrations before usage. All solutions had a final DMSO content of less than 0.1%. Before starting the studies, it was determined that the concentration of DMSO and the cells' DNA was not harmed by the serum-free medium. Before each test, the remaining chemicals were freshly made.

Cell culture and maintenance

American Type Cell Culture Collection provided G.CA cells, a standard cell line derived from gastric adenocarcinoma cells (ATCC, Germany). G.CA cells were grown in F-12K Medium at equilibrium temperature of 37°C with 5% CO2. 10% FBS, 100 U/ml of penicillin, and 100 ng/ml of streptomycin were added to the medium as supplements. The Trypan Blue Exclusion Test was used to determine the number of live cells.

Cytotoxicity assay

A luminescence test was utilized to measure ATP levels in order to look into how cytotoxic OLE was to the cells (Cell-Titer-Glo Luminescent Cell Viability Assay, Promega). Cells were seeded onto 96-well plates at a density of 5×103 cells per well which were then incubated for the duration of the night at 37 oC with 5% CO2. The medium was then replaced with brand-new complete medium that included OLE in concentrations ranging from 25 to 500 μM. DMSO at 0.1% was used to treat the control cells. A humidified mixture of 5% CO2 and 95% O2 was used to incubate the cells for 24 hours at 37 oC. Following a culture medium wash, the cells were examined for the presence of ATP. Every sample received 100 L of the reagent (Cell Titer-Glo Luminescent Cell Viability Assay, Promega), which was then stirred for two min and allowed to stand at room temperature for 10 minutes. The results were evaluated with luminometry (Varioskan Flash Multimode Reader, Thermo, Waltham, MA). Relative light units (RLU) were used to measure the light that was produced when ATP was present. The amount of ATP in the test sample had a direct correlation with the intensity of light quants that were released. It was calculated how much of the cells were viable in comparison to the 100% negative control group. Non-linear regression analysis was used to derive the half maximum growth inhibitory concentration (IC50) values from the concentration-response curves. Three times each experiment was repeated to ensure that the standard deviation stayed within 5%.

Measurement of intracellular ROS generation

A cell-permeable fluorescent signal called CM-H2DCF-DA (2,7-dichlorodihydrofluorescein-diacetate) was used to measure ROS generation. As previously mentioned, the production of ROS causes H2DCF-DA to oxidize into a highly green fluorescent DCF (2,7-dichlorofluorescein). OLE was pretreated with a range of doses (25–500 μM) for 24 hours on G.CA cells. After the cells had been treated for 24 hours, they were washed with cold PBS before being incubated with 100 μM H2DCF-DA for an additional 30 minutes at

37°C. A fluorescent plate reader was used to quantify the DCF fluorescence density (Varioskan Flash Multimode Reader, Thermo, Waltham, MA) at Ex./Em = 488/525 nm. To ensure reproducibility, the forecasts were performed three times in triplicate, with the same amount of cells per treatment group being used each time. The percentages of relative fluorescence to the control were used to represent the values.

Apoptosis by flow cytometer

For the purpose of identifying apoptosis, the Annexin-V-FITC and PI staining kit (Roche Applied Science, Penzberg, Germany) was applied as directed by the manufacturer. Briefly, 1.5x105 G.CA cells per well were seeded into six-well plates, and they were then left to adhere over night before being exposed to a range of OLE concentrations (25–500 µM) for 24 hours. G.CA cells that had been trypsinized were centrifuged at 200 x g for five minutes. Inside an hour the cell pellet was re-suspended in 100 µL of Annexin-V-FITC labeling solution and it was then incubated at 15–20°C for 10–15 minutes. The next step was the analysis using flow cytometry (Becton Dickinson, FACS Canto II).

Genotoxic activity assay

With a small alteration from Singh et al. [22], the single-cell gel electrophoresis assay (Comet Assay) was carried out to assess the genotoxic impacts of OLE on AGC cell lines. AGC cells were plated into 6-well plates and exposed to various OLE dosages under the IC50 condition for 24 hours. The cells were extracted using trypsin-EDTA and centrifuged at 400xg after incubation. After being aspirated, the supernatant was washed with dPBS at 400xg for five minutes. A slide that had already been covered with 1% normal melting agarose received 15 microliters of cell solution and 85 microliters of 0.6% low melting agarose. The gel was first frozen at + 4°C, then incubated with lysis buffer at + 4°C, followed by 40 min of dark incubation in an alkaline solution to unwind DNA while cooling. Slides were electrophoresed for 25 minutes at + 4°C (condition: 26 V, 300 mA). After that, pictures were taken after the slides had been ethanol-dehydrated and stained with 2 µg/mL ethidium bromide. Using a fluorescent microscope and the Comet assay IV software, all DNA results were graded (Leica DM 1000, Solms, Germany).

2.7 Statistical analysis

The average and standard deviation of the tree replicates were used to represent the results. The Kolmogorov-Smirnov test for normality was used in the statistical analysis. In all trials, the statistical significance of nonparametric data was assessed using the Kruskal-Wallis test. A statistically significant result was one with a p value less than 0.05. Nonlinear regression analysis was used to get the IC50 values of OLE for the cell lines. By using Pearson correlation analysis, associations between ROS production and cell viability metrics were examined. The SPSS package application for Windows was used to conduct all statistical analyses (Version 20, Chicago, IL, USA).

Results

Effects of OLE on the cell viability of G.CA cells

Test solutions without OLE (negative control) or with OLE (25–500 µmol/l) were applied to human G.CA cells for 24 hours. After incubation, the ATP cell viability assay was used to assess OLE's effects. Before any trials, the cell viability in G.CA cell cultures was more than 95%. Cell viability was dramatically decreased when OLE was added (p 0.001; Fig. 1). In comparison to the untreated control cells (0 µmol/l), the concentration-response curve revealed a loss of cell viability of 20.22% at 25 µmol/l and 63.95% at 500 µmol/l OLE. In a dose-dependent manner (200–500 µmol/l), significant differences were seen between the control cells and OLE exposed cells (p0.05-0.001). The viability of gastric cancer cells decreased to 40% as the OLE dose increased from 25 µmol/l to 500 µmol/l. 42 µmol/l was discovered to be the IC50 value.

Effects of OLE on reactive oxygen species (ROS) generation in G.CA cells

The fluorescent probe H2DCF-DA was used to identify ROS production. In the human G.CA cells, OLE was observed to stimulate the production of ROS. In percentages, the measured relative fluorescence was displayed (Fig. 2). The findings demonstrated that, in comparison to the control, ROS production in cells increased considerably following a 24-hour exposure to OLE. Following exposure to 200–500 µmol/l OLE, cells showed significant alterations in ROS levels ($p = 0.05–0.001$).

Apoptotic effect of OLE on G.CA cells detected by flow cytometry

We also used a flow cytometry technique to look at the impact of OLE on apoptosis. Induction of early (within 1 hour) and late (at 24 hours) apoptosis and necrosis (dead cells) were found considerably in G.CA cells in a concentration-time dependent manner (OLE 200–500 µmol/l), as shown in Fig. 3, following treatment with increasing doses of OLE (25–500 µmol/l) for 24 h. At 500 µmol/l OLE, the combined percentage of apoptotic and necrotic (dead) cells was about 60%.

Genotoxic effect of OLE on G.CA cells detected by the comet assay

Using the comet assay, a specialized test for genotoxicity, DNA damage caused by OLE was identified. DNA deterioration was noticed as the production of comets [13]. Between the control cells and the cells exposed to OLE, there were discernible differences in the DNA's tail. OLE-exposed cells (150–500 µmol/l) dramatically increased DNA damage (tail) compared to control cells. DNA damage and elevated OLE levels were shown to be positively correlated ($p = 0.05–0.001$) (Fig. 4).

Discussion

In a meta-analysis of 13800 cancer patients and 23340 controls, consumption of olive oil (OLO) was found to be negatively related to cancer prevalence, and those who consumed the most OLO had a lower chance of developing any sort of cancer than those who consumed the least [20]. Similarly, two

Mediterranean studies approved the chemo-preventive role of OLO against gastric CA [5, 19]. Also, another epidemiological study concerning 28 countries from four continents has showed the protective role of OLO on colorectal cancer (CRC) development [23]. Oleuropein (OLE) is a phenolic secoiridoid chemical that is mostly found in the leaves, fruits, and flowers of the olive tree. It is also found in the form of oleuropein aglycon in OLO. Many experimental studies revealed that OLE and its metabolite hydroxytyrosol (HT) inhibited the development of both the digestive system (colorectal, hepatic etc.) and other systemic (breast, lung, blood, brain, urogenital, skin and soft tissue) malignancies. Both OLO phenols (OLE and HT) are effective at inhibiting the growth, migration, invasion, and angiogenesis of cancerous cells. By altering a number of oncogenic signaling pathways, they also function as anticancer agents [2, 14, 15, 18]. Numerous studies examined the chemoprotective and curative properties of olive oil against colon cancer. They found that OLE and HT suppressed COX-2 and BCL-2 protein expression moreover avoided DNA damage. Thus, the start, development, and metastasis of colorectal cancer cells were all prevented by OLE and HT [7, 9, 17]. Oleuropein has been shown to have chemo-preventive effects against colon cancer in C57bl/6 mice with colitis. OLE treatment inhibited inflammatory responses, cancer initiation and tumor development via its antioxidant activity [10].

Several studies have reported the induction of apoptosis of cancer cell lines and the inhibition of malign cell proliferation by OLE [6, 24]. Another research about the effectiveness of OLE and HT on pancreatic cancer cells revealed their cytotoxicity and apoptosis in these malign cells. OLE also displayed a selective protective effect on non-tumorigenic pancreas cells [11]. Similarly, in another study on prostate cancer, OLE has shown an anti-oxidant effect in normal cells, whereas it induced pro-oxidant and anti-proliferative actions in cancer cells [1]. Moreover, OLE analogs stimulated immune (natural killer and lymphokine-activated killer lymphocytes) attacks against several malign cell lines [21]. Also, in a study related to Albino mices affected by soft tissue sarcoma, 1% OLE in drinking water provided complete tumor regression [12]. Oleuropein has also been shown to potentiate the effects of conventional chemotherapy in a number of trials [18].

Nowadays, the effectiveness and anticarcinogenic role of OLE and HT on gastric cancer cells has not been studied satisfactorily and the underlying mechanisms of its action remains yet unknown. OLE significantly reduced ROS levels, increased total antioxidant status levels, and repaired cisplatin-induced stomach cell damage in rats in an experimental investigation about the antioxidant and restorative effects of the substance [8]. Recently, synthesis of Nano-Paramagnetic Oleuropein has been reported as an inducer of KRAS Over-Expression and inhibition of G.CA Cancer Cells. Also, it could trigger apoptosis in the G.CA cell line [4].

Our study is the first research about cytotoxic, genotoxic, apoptotic and ROS generating effects of OLE on G.CA cells simultaneously. We have detected that OLE decreased G.CA cells viability nearly 70% by increasing doses to the maximum concentration (500 μmol/L). Apoptosis is a wellknown mechanism induced by OLE on cancer cells. In our study, we observed that OLE induced apoptosis and necrosis nearly 60%, associated with reduced G.CA cells viability by two methods. In parallel with the induction of apoptosis and the reduction of G.CA cell viability, intracellular ROS levels were increased 1.5 times at the maximum concentration of OLE. Several studies reported that OLE caused ROS production and ROS accumulation is involved in the apoptosis of cancer cell lines by inhibition of some mitochondrial pathways [3, 6, 16, 24]. In our study increased ROS levels in parallel to OLE doses suggest their apoptotic roles in G.CA cells. Moreover, we established that increasing doses of OLE ensured almost complete (100%) DNA damage in G.CA cells, suggesting its very successful genotoxic effect in malign cells.

In conclusion, we detected significant cytotoxic, apoptotic and genotoxic effects of oleuropein on G.CA cells. Nutrition rich olive and olive oil seems to be both protective and therapeutic against gastric cancer and oleuropein may be a new, potential chemotherapeutic agent in the next future.

Declarations

Statements & Declarations

Funding

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Competing Interests

“The authors have no relevant financial or non-financial interests to disclose.”

Author Contributions

M.Kürşad Türkdoğan, Abdürrahim Koçyiğit contributed to the study conception and design. Material preparation, data collection were performed by Abdürrahim Koçyiğit, Eray Metin Güler. Data analysis were performed by M.Kürşad Türkdoğan, Abdürrahim Koçyiğit. The first draft of the manuscript was written by M. Kürşad Türkdoğan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. No ethical approval is required since this is a cell culture study.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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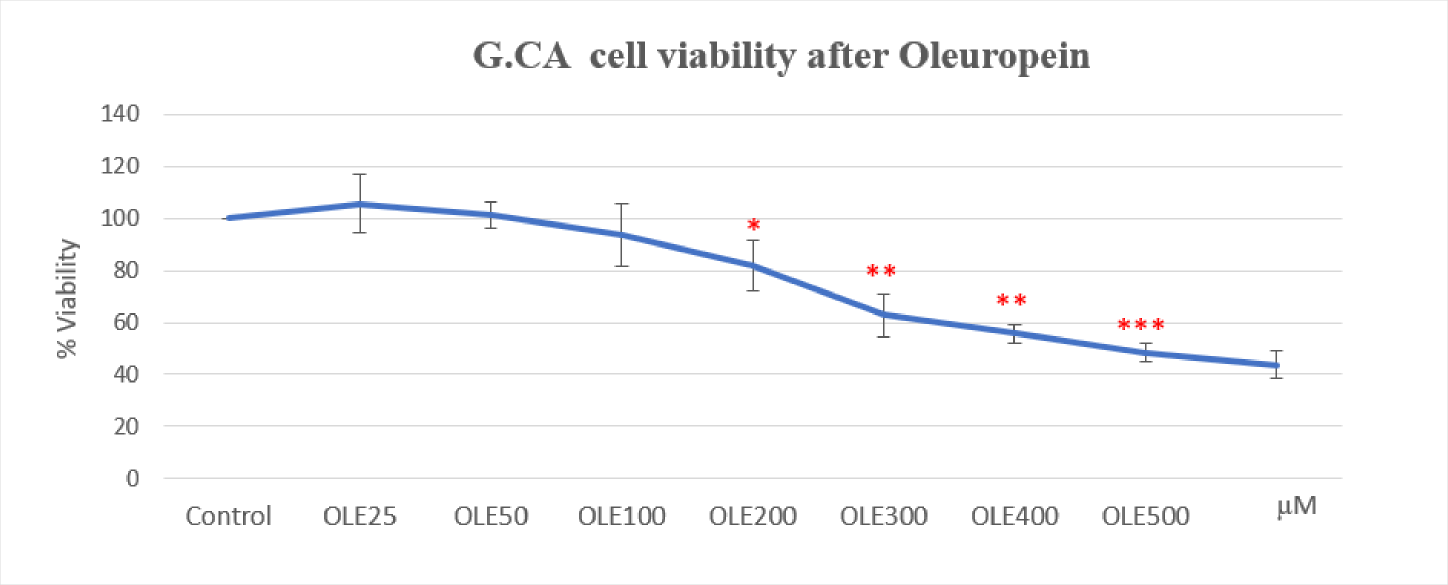
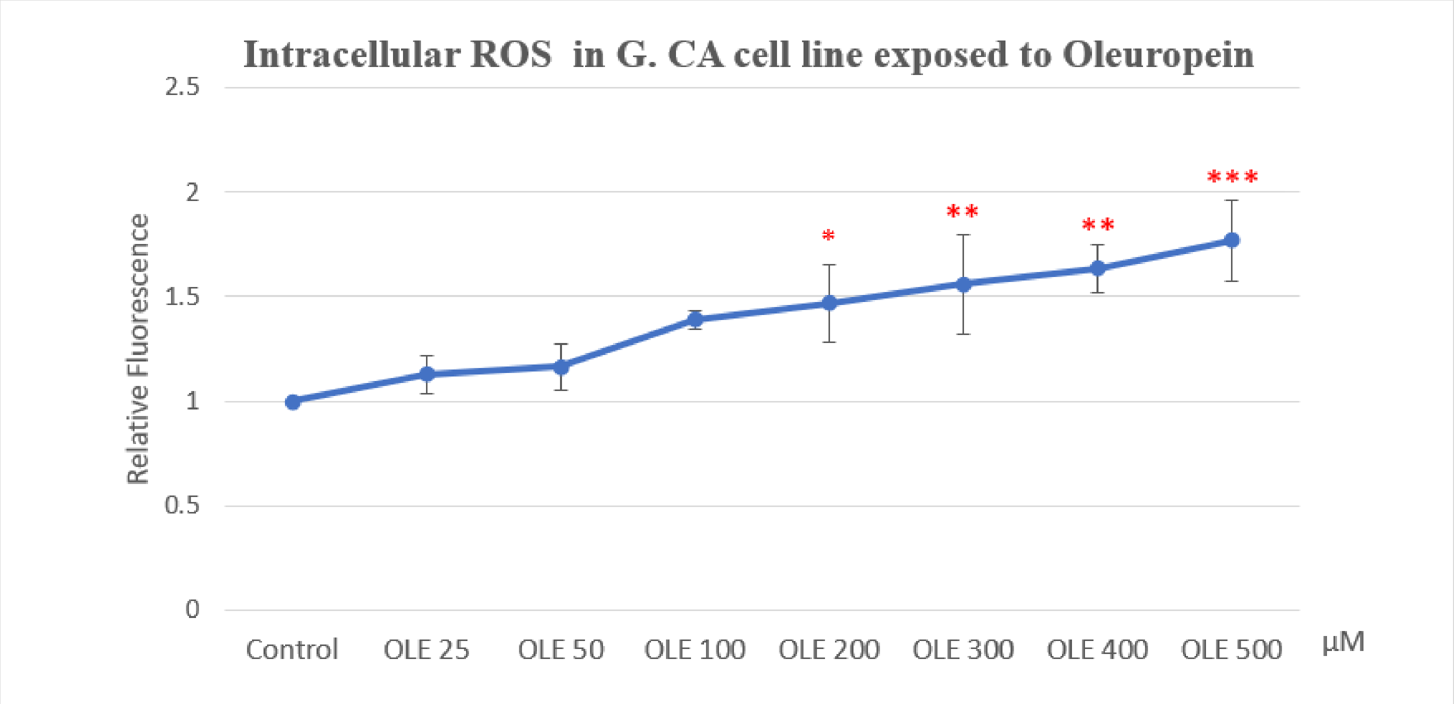
Figures



* p <0.05, ** p <0.01, *** p <0.001.

Figure 1

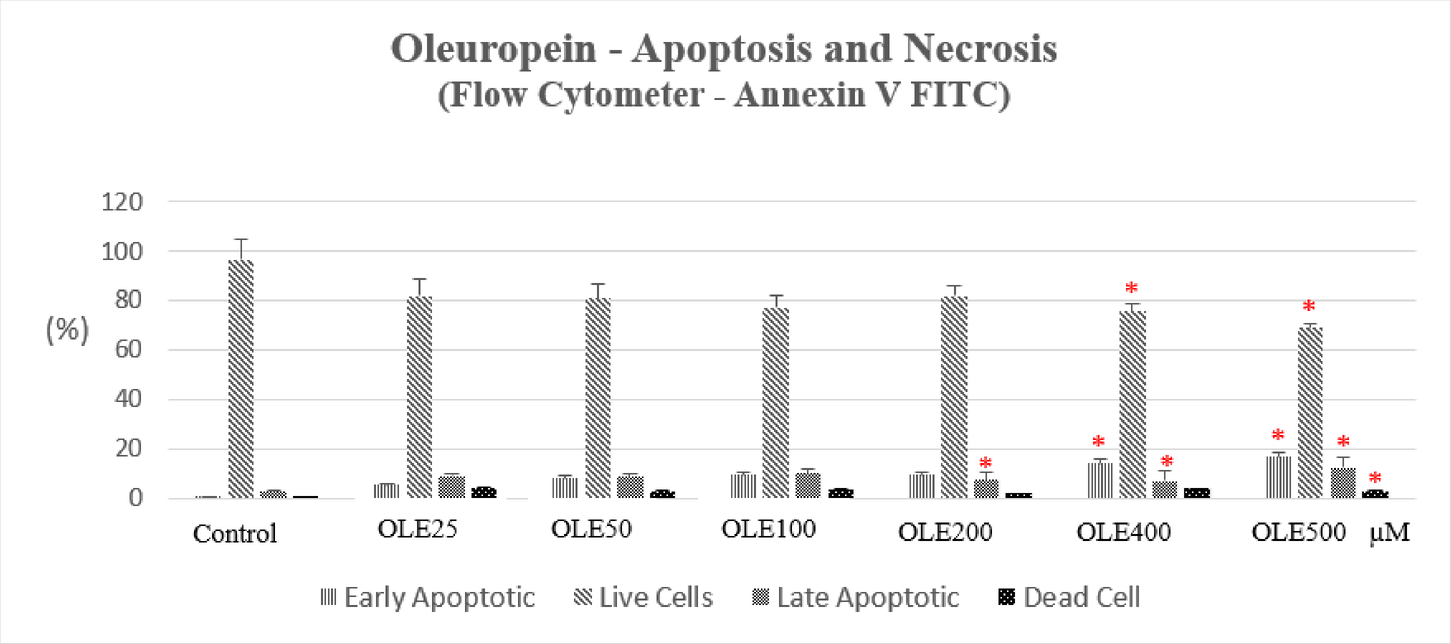
Decrease of G.CA cells viability due to increased doses of OLE



*p<0.05, ** p<0.01, *** p<0.001.

Figure 2

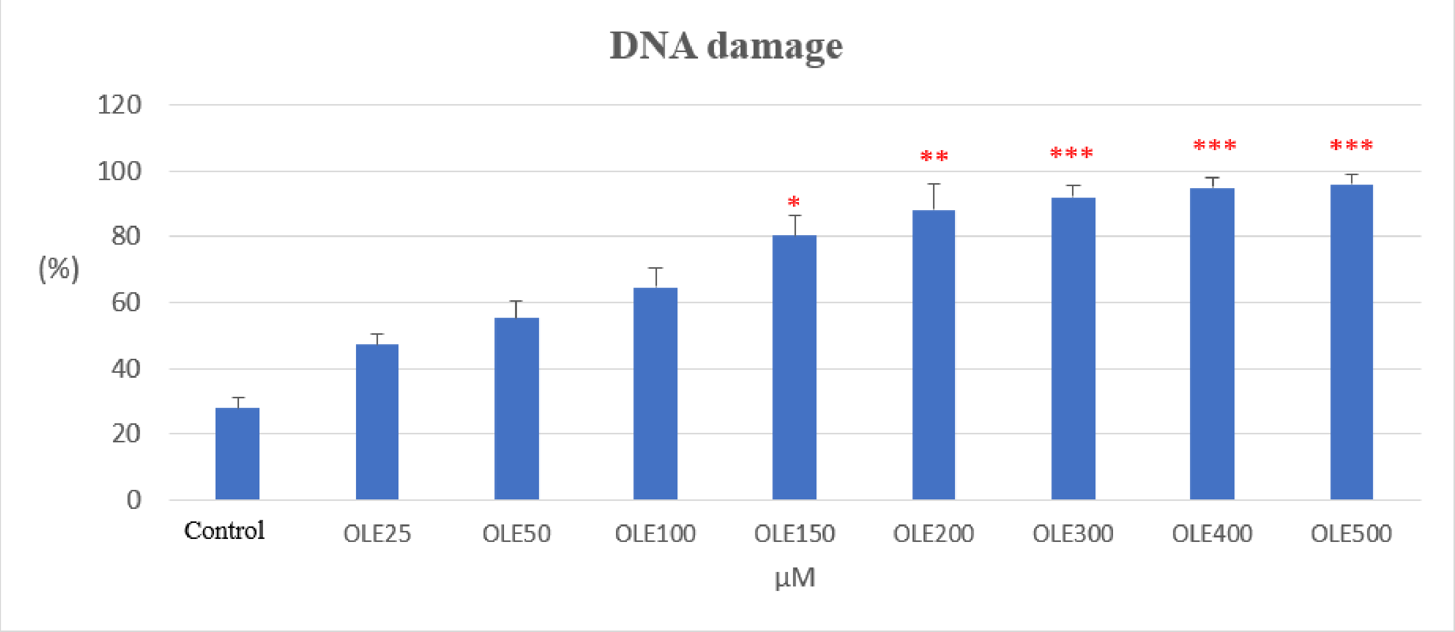
Reactive oxygen species (ROS) content in G.CA cells exposed to OLE



$^*p<0.05$

Figure 3

Apoptosis and necrosis of G.CA cells after treatment with OLE (Flow cytometry)



$^*p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4

DNA damage of G.CA cells exposed to OLE (25 - 500 µmol/l)