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ORIGINAL ARTICLE

Oleuropein exhibits anticarcinogen effects against gastric cancer cell lines

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Abstract

Background Oleuropein (OLE), the main phenolic compound of the olive fruit and leaves, has many heathful effects. Gastric cancer is the most fatal malignancy 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 research the cytotoxic, genotoxic and apoptotic effects of OLE on gastric adenocancer (AGS) cells in vitro.

Methods and Results A standard cell line derived from gastric adeno cancer (AGS) cells was employed, and its performance following a 24-hour exposure to OLE at various doses was examined. The ATP cell viability assay, 2',7'-dichlorodihydrofluorescein-diacetate assay (H2DCF-DA) and alkaline single cell gel electrophoresis assay (Comet Assay) were used to study the cytotoxicity, production of reactive oxygen species (ROS) and genotoxicity respectively. The induction of apoptosis was discovered using flow cytometry. OLE reduced AGS cells viability about 60% at maximum concentration (500 µmol/L) and also resulted in approximately 100% DNA damage and about 40% apoptosis with necrosis in AGS cells depending on the increased doses. Cell viability was also significantly decreased in relation to increased intracellular reactive oxygen species (ROS) levels ($p < 0.05 - 0.001$).

Conclusions Oleuropein has shown significant anticarcinogen effects against gastric adenocancer (AGS) cells in vitro. Oleuropein, a nutrient rich in olive and olive oil, seems to be both protective and therapeutic against gastric cancer and may be a new chemotherapeutic agent in the future.

Keywords Oleuropein · Gastric cancer · Apoptosis · DNA damage

Introduction

The olive tree and its fruits have been considered symbols of peace and hope for centuries and are sacred in Holy Books. Olive and olive oil, the traditional and essential foods of the Mediterranean diet, possesses many nutritional and healthful properties.

The phytochemicals which are beneficial for human health in olives and olive oil are mainly oleuropein (OLE), hydroxytyrosol, luteolin, apigenin and verbascoside. Olive oil also contains high levels of triacylglycerols. The main fatty acids in their structure are oleic acid, linoleic acid, palmitic acid, palmitoleic acid, stearic acid and the triterpene maslinic acid. In addition, olive oil contains versatile useful compounds such as tocopherols, squalene, phytosterols and phenolic compounds. OLE, the predominant phenolic compound of the olive fruit and leaves of Olea europaea L. (Olive tree), has many therapeutic effects such as anticarcinogenic, antiatherogenic, anti-inflammatory, neuroprotective, hepatoprotective and antimicrobial etc. [1–3]. OLE also has hypotensive and hypoglycaemic specialities and it is a powerful antioxidant [4, 5].

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In addition, it has restorative efficacy in organ injuries induced by chemotherapy [6]. Gastric cancer is the most frequent and fatal malignancy in many parts of the world and it is generally related to harmful dietetic factors. Every year, hundreds of thousands of people die of gastric cancer in Eastern Turkey and all over the world [7, 8].

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 the cytotoxic, apoptotic and genotoxic effects of OLE on gastric adenocancer (AGS) cells in vitro.

Materials and methods

Chemical and reagents

OLE, FBS, H2DCF-DA, penicillin-streptomycin, ethidium bromide (EB), 2,7-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) and F-12 K Medium were purchased from Sigma-Aldrich (Seelze, Germany). EBioscience provided the Annexin V-FITC Human Apoptosis Detection Kit (300 tests/kit, Bendermed, San Diego, CA 92,121 USA). All of the reagents used in the investigation are analytical grade unless otherwise stated.

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 serum-free media did not cause DNA harm in the cells. The remaining reagents were freshly prepared before every experiment.

Cell culture and maintenance

American Type Cell Culture Collection provided AGS cells, a standard cell line derived from gastric adenocarcinoma cells (ATCC, CRL-1739, Germany). AGS cells were grown in F-12 K Medium at an equilibrium temperature of 37 °C with 5% CO_2. 10% FBS, 100 U/ml of penicillin, and 100 ng/ml of streptomycin were added to the medium as supplements. The quantity of living cells was calculated using the Trypan Blue Exclusion Test.

Cytotoxicity assay

ATP levels obtained using a luminescence assay were used to investigate the cytotoxic effects of OLE on the cells (Cell-Titer-Glo Luminescent Cell Viability Assay, Promega). Cells were seeded onto 96-well plates at a density of 5×10^3 cells per well, which were then incubated for the duration of the night at 37 °C with 5% CO_2. The medium was then replaced with the 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. Cells were incubated for 24 h at 37 °C with humidified 5% CO_2 and 95% O_2. The cells were then washed with the culture media and tested for the presence of ATP. 100 µL of the reagent (Cell Titer-Glo Luminescent Cell Viability Assay, Promega) was added to each sample before being mixed for 2 min and left to sit for 10 min at room temperature. The outcomes were assessed using 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 is directly correlated with the intensity of light quants that were released. It was calculated how much of the cells were viable compared to the 100% negative control group. Non-linear regression analysis derived the half maximum growth inhibitory concentration (IC_{50}) values from the concentration-response curves. Each experiment was repeated three times 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 pre-treated with a range of doses (25–500 µM) for 24 h on AGS cells. After the cells had been treated for 24 h, they were washed with cold 1xdPBS (1xDulbecco's Phosphate Buffered Saline, Gibco) before being incubated with 100 µM H2DCF-DA for an additional 30 min at 37 °C. DCF fluorescence density was measured using a fluorescent plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, MA) at Ex./Em = 488/525 nm. To ensure reproducibility, the estimations were performed three times in triplicate, with the same number of cells per treatment group being used each time. The values were presented as percentages of relative fluorescence to the control.

Apoptotic activity

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, 1.5×10^5 AGS cells per well were seeded into six-well plates, and they were then left to adhere overnight before being exposed to a range of OLE concentrations

(25–500 µM) for 24 h. Trypsinized AGS cells were centrifuged at 200 x g for 5 min. Within an hour, the cell pellet was re-suspended in 100 µL of Annexin-V-FITC labeling solution and incubated at 15–20 °C for 10–15 min. The flow cytometry (Becton Dickinson, FACS Canto II) analysis was then performed.

Genotoxic activity assay

With a small alteration from Singh et al. [9], the single-cell gel electrophoresis assay (Comet Assay) was carried out to assess the genotoxic impacts of OLE on AGS cell lines. AGS cells were plated into 6-well plates and exposed to various OLE dosages under the IC50 condition for 24 h. The cells were extracted using trypsin-EDTA and centrifuged at 400 x g after incubation. After being aspirated, the supernatant was washed with dPBS at 400 x g for 5 min. A slide that had already been covered with 1% normal melting agarose received 15 µl of cell solution and 85 µl 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 min at + 4 °C (condition: 26 V, 300 mA). Subsequently, pictures were taken after the slides were ethanol-dehydrated and stained with 2 µg/mL ethidium bromide. Using a fluorescent microscope and the Comet assay IV software, all DNA damage results were recorded as tail intensity (Leica DM 1000, Solms, Germany).

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 IC_{50} 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 25, Chicago, IL, USA).

Results

Effects of OLE on the cell viability of AGS cells

Test solutions without OLE (negative control) or with OLE (25–500 µmol/l) were applied to human AGS cells for 24 h. After incubation, the ATP cell viability assay was used to assess OLE's effects. Before any trials, the cell viability in AGS cell cultures was more than 95%. The cell viability dramatically decreased when OLE was added ($p < 0.001$, Fig. 1). Compared to the untreated control cells (0 µmol/l), the concentration-response curve revealed a loss of cell viability of 18.31% at 100 µmol/l and 57.96% 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 ($p < 0.05 - 0.001$). The viability of gastric cancer cells decreased up to about 40% as the OLE dose increased from 100 µmol/l to 500 µmol/l. and the IC_{50} value was discovered to be 42 µmol/l.

Effects of OLE on reactive oxygen species (ROS) generation in AGS cells

The fluorescent probe H2DCF-DA was used to identify ROS production. In the human AGS 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).

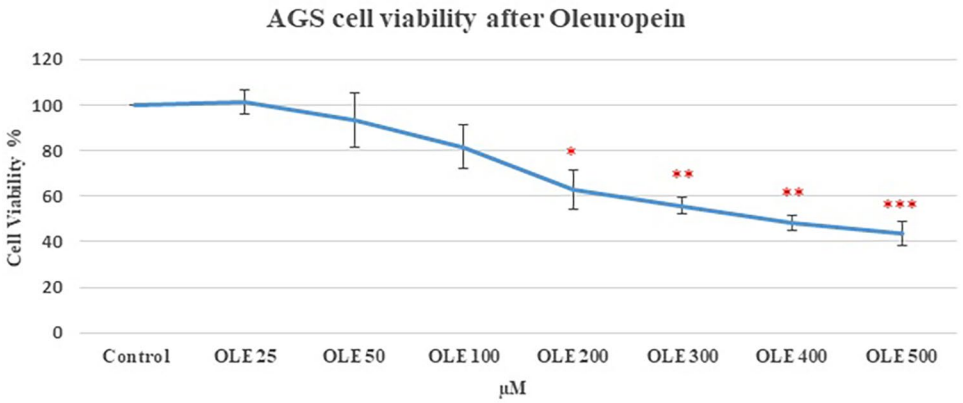

Fig. 1 Cytotoxicity analysis of OLE on AGS cell line. In a dose-dependent manner (200–500 µmol/l), significant differences were seen between the control cells and OLE-exposed cells. Results for survival, compared with control samples are presented as mean ± SD. OLE doses are in µM concentration. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

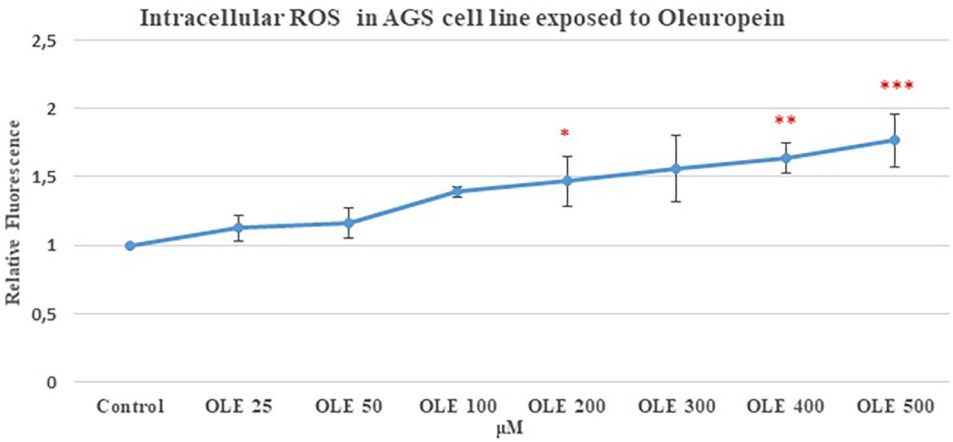

Fig. 2 Reactive oxygen species (ROS) content in AGS cells exposed to OLE in comparison to the control. ROS production in cells increased considerably following a 24-hour exposure to OLE. Following exposure to 200–500 µmol/l OLE, cells showed significant alterations in ROS levels. The results are presented as the means from three independent experiments ± SD. *p < 0.05, ** p < 0.01, *** p < 0.001

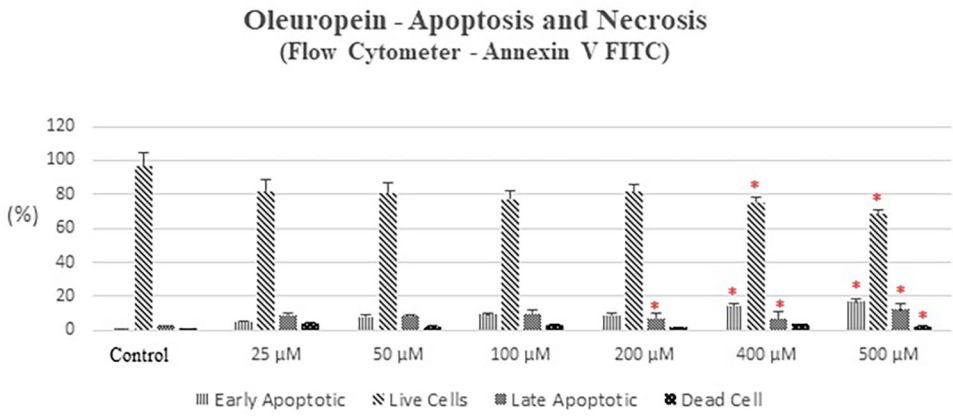

Fig. 3 Apoptosis and necrosis of AGS cells after treatment with OLE (Flow cytometry). Apoptosis and necrosis were considerably found in AGS cells in a concentration-time dependent manner following treatment with increasing doses of OLE (25–500 µmol/l) for 24 h. The combined percentage of apoptotic and necrotic (dead) cells at 500 µmol/l OLE was about 40%. *p < 0.05 indicate significant differences compared to the control group

Apoptotic effect of OLE on AGS cells detected by flow cytometry

Flow cytometry technique was used to observe the impact of OLE on apoptosis. Induction of early (within 1 h) and late (at 24 h) apoptosis and necrosis (dead cells) were found in treated AGS cells in a concentration and time dependent manner (OLE 200–500 µmol/l), following treatment with increasing doses of OLE (25–500 µmol/l) for 24 h (Fig. 3). At 500 µmol/l of OLE, the combined percentage of apoptotic and necrotic (dead) cells was about 40%.

Genotoxic effect of OLE on AGS 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 there was a production of comets [10]. 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 intensity) compared to the control cells. Elevated OLE levels and DNA damage were shown to be positively correlated (p < 0.05–0.001), (Fig. 4). We observed that DNA damage and tail formation were started after 25 µmol/l OLE exposure compared to control and DNA damage was a distinct comet appearance after exposure to 500 µmol/l OLE. (Fig. 5a and 5b).

Discussion

In a meta-analysis of 13,800 cancer patients and 23,340 controls, consumption of olive oil was found to be negatively related to cancer prevalence and those who consumed the most olive oil had a lower chance of developing any sort of cancer than those who consumed the least [11]. Similarly, two Mediterranean studies described the chemo-preventive role of olive oil against gastric CA [12, 13]. Also, another epidemiological study concerning 28 countries from four continents has shown the protective role of olive oil on colorectal cancer (CRC) development [14]. OLE is a phenolic secoiridoid chemical that is primarily found in leaves, fruits, and flowers of the olive tree. It is also found in the form of OLE aglycon in olive oil. 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,

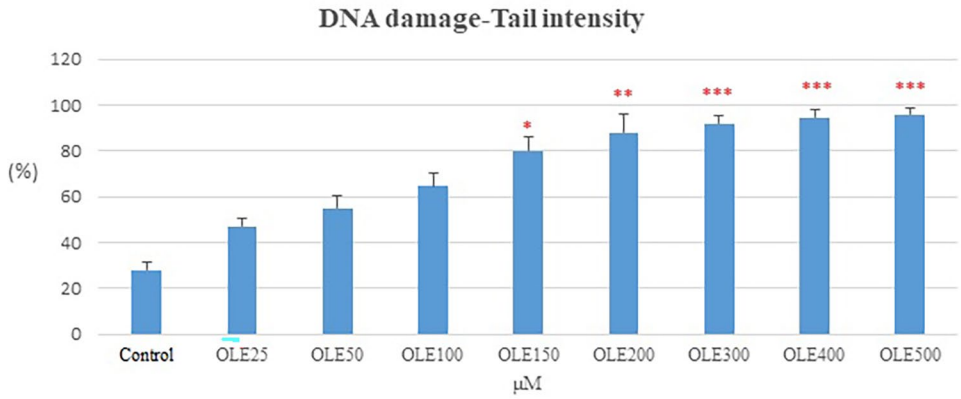

Fig. 4 DNA damage-Tail intensity (% Tail) of AGS cells exposed to OLE (25–500 µmol/l) by comet assay. There were noticeable differences in the DNA's tail between the control cells and the cells exposed to OLE. OLE-exposed cells (150–500 µmol/l) dramatically increased DNA damage (Tail intensity) compared to the control cells. Elevated OLE levels and DNA damage were shown to be positively correlated. The results are presented as the means ± SD. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

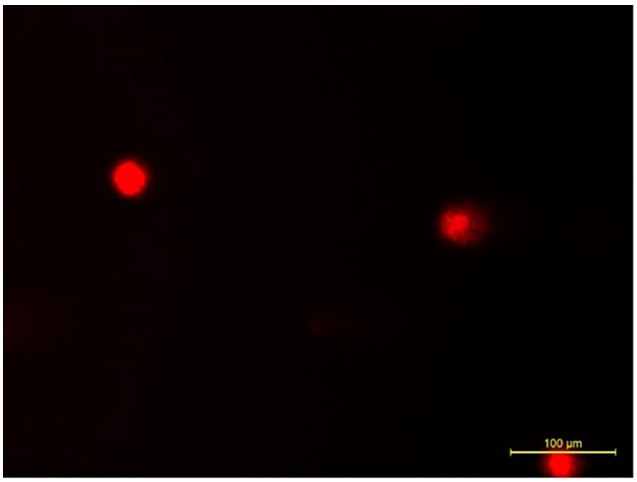

Fig. 5a DNA damage and "Tail" formation during the comet assay. DNA damage and Tail formation started after 25 µmol/l OLE exposure compared to control

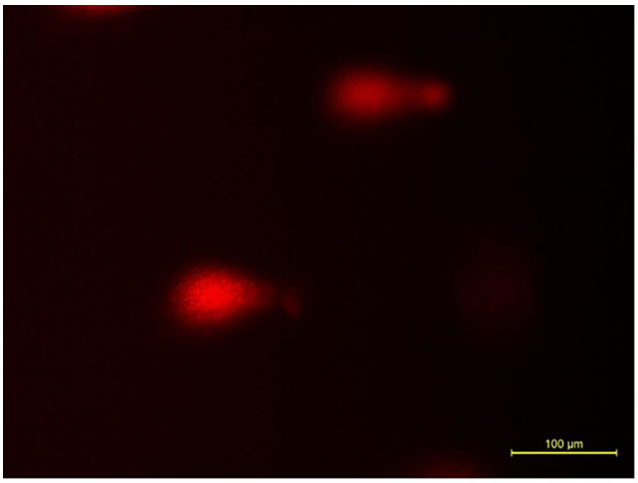

Fig. 5b DNA damage and "Tail" formation during the comet assay. DNA damage is a distinct comet appearance after exposure to 500 µmol/l OLE

blood, brain, urogenital, skin and soft tissue) malignancies. Both olive oil phenols (OLE and HT) effectively inhibit the growth, migration, invasion, and angiogenesis of cancerous cells. By altering a number of oncogenic signalling pathways, they also function as anticancer agents [1, 15–17]. 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, they avoided DNA damage. Thus, the beginning, development and metastasis of colorectal cancer cells were all prevented by OLE and HT [18–20]. OLE has been shown to have chemo-preventive effects against colon cancer in C57bl/6 mice with colitis. OLE treatment prevented inflammatory responses, cancer initiation and tumor development via its antioxidant activity [21].

Several studies have reported the induction of apoptosis of cancer cell lines and the inhibition of malignant cell proliferation by OLE [22, 23]. Further research about the effectiveness of OLE and HT on pancreatic cancer cells revealed their cytotoxicity and apoptosis in these malignant cells. OLE also displayed a selective protective effect on non-tumorigenic pancreas cells [24]. 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 [25]. Moreover, OLE analogs stimulated immune (natural killer and lymphokine-activated killer lymphocytes) activation against several malignant cell lines [26]. Furthermore, in a study performed in Albino mice affected by soft tissue sarcoma, 1% OLE in drinking water provided complete tumor regression [27].

OLE has also been shown to potentiate the effects of conventional chemotherapy in a number of trials [1].

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 remain yet unknown. OLE has 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 therapeutic effects of the substance [6]. Recently, the synthesis of Nano-Paramagnetic Oleuropein has been reported as an inducer of KRAS Over-Expression and inhibition of AGS Cancer Cells. Also, it could trigger apoptosis in the AGS cell line [28].

Our study is the first research on the cytotoxic, genotoxic, apoptotic, and ROS-generating effects of OLE on AGS cells simultaneously. We have detected that OLE decreased AGS cell viability nearly 60% by increasing doses to the maximum concentration (500 µmol/L). Apoptosis is a well known mechanism induced by OLE on cancer cells. In our study, we observed that OLE induced apoptosis and necrosis for about 40%, associated with reduced AGS cell viability by two methods. In parallel with the induction of apoptosis and the reduction of AGS 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 are involved in the apoptosis of cancer cell lines by inhibition of some mitochondrial pathways [22, 23, 29, 30]. In our study, increased ROS levels in parallel to OLE doses suggest their apoptotic roles in AGS cells. Moreover, we established that increasing doses of OLE ensured almost complete (100%) DNA damage in AGS cells, suggesting its very successful genotoxic effect on malignant cells.

Conclusion

In conclusion, we detected significant cytotoxic, apoptotic and genotoxic effects of Oleuropein on AGS cells. Nutrition rich in 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 future.

Author contributions M.Kürşad Türkdoğan and Abdürrahim Koçyiğit contributed to the study conception and design. Material preparation and 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.

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

Declarations

Competing interests "The authors have no relevant financial or non-financial interests to disclose."

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.

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