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RESEARCH ARTICLE

Antioxidant Effects of Oleuropein on Hydrogen Peroxide-Induced Neuronal Stress- An *In Vitro* Study

Altug Kucukgul[1,*], Mehmet M. Isgor[1], Vesile Duzguner[2], Meryem N. Atabay[1] and Azime Kucukgul[3]

[1]*Department of Biochemistry, Faculty of Veterinary, Mustafa Kemal University, Hatay, Turkey;*
[2]*Department of Nursery, High School of Health Sciences, Ardahan University, Ardahan, Turkey;*
[3]*Department of Aquaculture, Faculty of Fisheries, Munzur University, Tunceli, Turkey*

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Abstract: ***Background*:** Persistent oxidative stress can lead to chronic inflammation and mediate most chronic diseases including neurological disorders. Oleuropein has been shown to be a potent antioxidant molecule in olive oil leaf having antioxidative properties.

***Objective*:** The aim of this study was to investigate the protective effects of oleuropein against oxidative stress in human glioblastoma cells.

***Methods*:** Human glioblastoma cells (U87) were pretreated with oleuropein (OP) essential oil 10 µM. After 30 minutes, 100 µM H_2O_2 was added to the cells for three hours. Cell survival was quantified by colorimetric MTT assay. Glutathione level, total oxidant capacity, total antioxidant capacity and nitric oxide levels were determined by using specific spectrophotometric methods. The relative gene expression level of iNOS was performed by qRT-PCR method.

***Results*:** According to viability results, the effective concentration of H_2O_2 (100µM) significantly decreased cell viability and oleuropein pretreatment significantly prevented the cell losses. Oleuropein regenerated total antioxidant capacity and glutathione levels decreased by H_2O_2 exposure. In addition, nitric oxide and total oxidant capacity levels were also decreased after administration of oleuropein in treated cells.

***Conclusion*:** Oleuropein was found to have potent antioxidative properties in human glioblastoma cells. However, further studies and validations are needed in order to understand the exact neuroprotective mechanism of oleuropein.

Keywords: Anti-inflammatory, anti-oxidant, glioblastoma, *in vitro*, neuroprotection, oleuropein.

1. INTRODUCTION

Ageing is one of the most important factors in the pathologies of diseases such as Alzheimer's and Parkinson's that occur in advanced ages. Recent studies have supported that high intake of food rich in monounsaturated fatty acids has a protective effect against Alzheimer's or Parkinson's diseases [1]. This effect of monounsaturated fatty acids could be related to their role of maintaining the structural integrity of neuronal membranes [1, 2]. Oleuropein is the main phenolic compound in olive leaf and responsible for the characteristic bitterness of immature and unprocessed olives. It is heterosidic ester of elenolic acid and hydroxytyrosol and possesses beneficial effects on human health [3]. Oleuropein is known to have many biological activities like antimicrobial,

*Address correspondence to this author at the Department of Biochemistry, Faculty of Veterinary, Mustafa Kemal University, Hatay, Turkey; Tel: +90 326 2455845 - 1553; Fax: +90 326 2455867; E-mail: altugkucukgul@gmail.com

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antioxidant, anti-inflammatory, anti-carcinogenic and antiviral activity [4-10].

Many neurodegenerative diseases result in damage to neurons and disruptions in their structural functions. Oxidative stress is one of the most important key factors in aging and leads to the development and progression of neurodegenerative diseases [11]. Hydrogen peroxide is produced in neurons and it is one of the major contributors to oxidative damage. Olive, in its leaf and oil, contains some biologically active polyphenols like oleuropein, hydroxytyrosol, tyrosol and caffeic acid. These compounds have important antioxidant effects and protect brain cells against oxidative damage. Oleuropein may prevent oxidative stress by leading an enhancement of the antioxidant response and scavenging free radical species [12]. Therefore, in this study, it was aimed to investigate the effects of oleuropein on H_2O_2 induced neuronal toxicity in human glioblastoma cells.

2. MATERIALS AND METHODS

2.1. Cell Culture and Treatments

Human glioblastoma (U87) cells were obtained from American Tissue Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C with 5% CO_2 conditions. Then the cells were plated in 24-well plates (0.4×10^5cells/ml). In order to measure the effective concentration of exogenously applied H_2O_2 (5-250 µM), the maintenance medium was removed, medium containing H_2O_2 was added, and the cells were incubated for 24h. Similarly, the effective concentration of oleuropein (Sigma, EU) was identified. For this purpose, Oleuropein stock solution (1mM) was prepared by dissolving it in cell culture media. It was applied to the cells at different concentrations (5-100 µM) taking into account the required dilution factors. The cells were pretreated with oleuropein for 30 minutes before H_2O_2 application and then incubated for 24h.

2.2. Viability Assay

Cell viability was determined by MTT (4-5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay [13]. Briefly, the viable cells produced a dark blue formazan product, whereas no such staining was formed in the dead cells. The cell viability was calculated by the normalization of optical densities (OD 570 nm) to the untreated control cells.

2.3. Homogenate Preparation

Cells were homogenized in ice-cold homogenization buffer (10 mM Tris, 1 mM EDTA, 25 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.25 M sucrose, pH 7.4) containing complete protease inhibitor cocktail (aprotinin, phenylmethylsulfonyl fluoride, leupeptin, sodium fluoride) (Sigma, Germany). Homogenates were centrifuged at 4ºC, 15000×g for ten minutes and the soluble fraction was retained. The protein concentrations of cell extracts were measured by the Bradford reagent using bovine serum albumin as a standard.

2.4. Spectrophotometric Analyses

Nitric oxide concentration in cultured cell medium was determined indirectly by measuring the nitrite levels based on Griess reaction [14]. Samples were deproteinized with 75 mM zinc sulphate. Total nitrite was determined by spectrophotometrically at 546 nm after conversion of nitrate to nitrite by copperized cadmium granules. Non-enzymatic antioxidant (GSH) content of U87 cell homogenates was determined according to the method of Sedlak and Lindsay [15].

2.5. Total Antioxidant Capacity (TAC) Analyses

TAC levels in homogenates were analyzed by using commercial kits (Rel Assay Diagnostics, Turkey). Absorbance measurements were performed on the spectrophotometer at the wavelength of 660 nm. TAC values of the samples were recorded as µmol Trolox Eqiv./L.

2.6. Total Oxidant Capacity (TOC) Analyses

TOC levels in homogenates were analyzed using commercial kits (Rel Assay Diagnostics, Turkey) by following the procedures given. The absorbance values of the standard and the samples were read at the wavelength of 530 nm in the spectrophotometer. The TOC values of the samples were recorded in µmol H_2O_2 Eqiv/L.

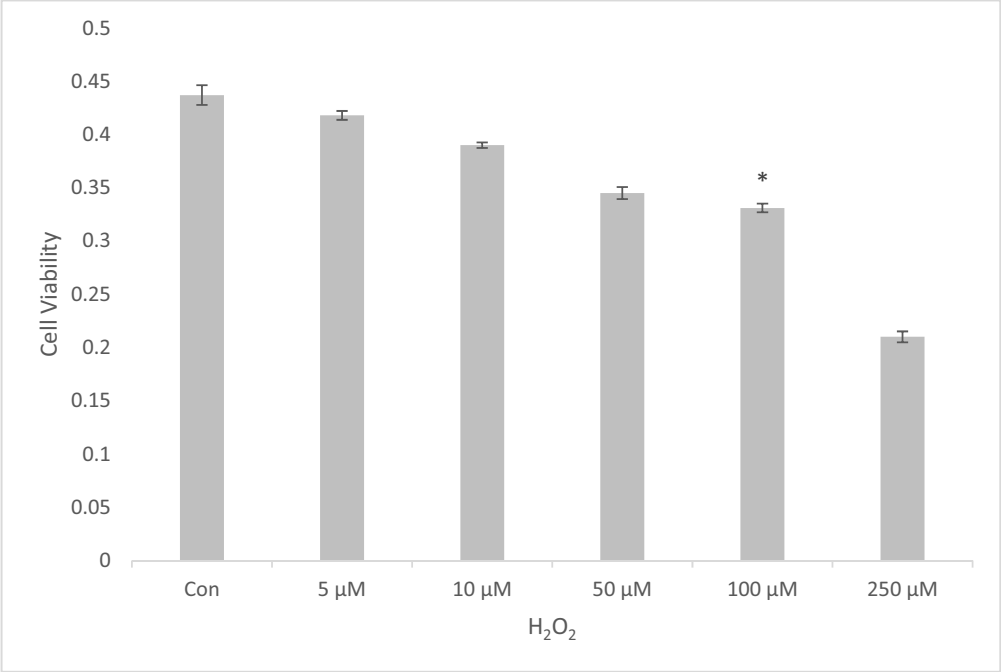

Fig. (1). Dose dependent effect of H_2O_2 on U87 cell viability. The data is represented as Mean ± SEM of five independent experiments (*p<0.05 compared to control group).

2.7. RNA Isolation and qRT-PCR Analyses

Real-time PCR was performed in a qPCR system (Bio-RAD CFX96, Touch-South Korea). Total RNA from U87 cells were extracted using TRI-reagent (Sigma, USA) according to the manufacturer's instructions. One µg of total RNA was reverse-transcribed with reverse transcriptase kit (Thermo, Germany) in 20µl reaction volume. One µl of each cDNA was used as a template for amplification by using SYBER Green PCR amplification reagent and gene-specific primers. The human primer sets used were obtained from Thermo Electron Corporation (Germany): iNOS forward: 5'-GGC CTC GCT CTG GAA AGA A-3', reverse: 5'-TCC ATG CAG ACA ACC TT-3'. The amount of RNA was normalized to β-actin amplification in a separate reaction. β-actin forward: 5'-CAT CGT CAC CAA CTG GGA CGA C-3', reverse: 5'-CGT GGC CAT CTC TTG CTC GAA G-3'. The cycling method was performed shortly as an initial 5m denaturing step at 95°C, followed by 40 cycles of 95°C for 10s, 60°C for 30s, and 72°C for 15s. Relative quantification of iNOS expression levels was elevated by the 2^{-DDCt} method.

2.8. Statistical Analyses

The one-way analysis of variance (ANOVA) and post hoc Duncan tests was performed on the data to examine the differences among the groups using the SPSS statistical software package. The results are presented as Mean ± SEM. A value of $p<0.05$ was considered significant.

3. RESULTS

The viability of U87 cells was decreased steadily in a concentration-dependent manner over the range of 5 to 250µM following H_2O_2 treatment. The data showed that 100µM H_2O_2 killed about 24% (0.331 ± 0.009) of the cells at the end of the incubation when compared to the control (0.437 ± 0.021) (*p<0.05) (Fig. **1**).

Our viability results indicated that 10µM OP (0.480 ± 0.016) increased the number of viable cells by 7.6% when compared to the control (0.446 ± 0.011). This concentration was used as a cell protective concentration for further experiments. Treatments utilizing 50µM and higher concentrations of OP decreased cell viability (Fig. **2**) (*p<0.05). Dose-response studies demonstrated that 10µM OP pretreatment (0.428 ± 0.014) decreased cell losses by 18% caused by H_2O_2 (0.347 ± 0.006) (**p<0.05) (Fig. **3**).

In the present study, H_2O_2 exposure of the cells in culture medium elevated nitrite levels by 4.4

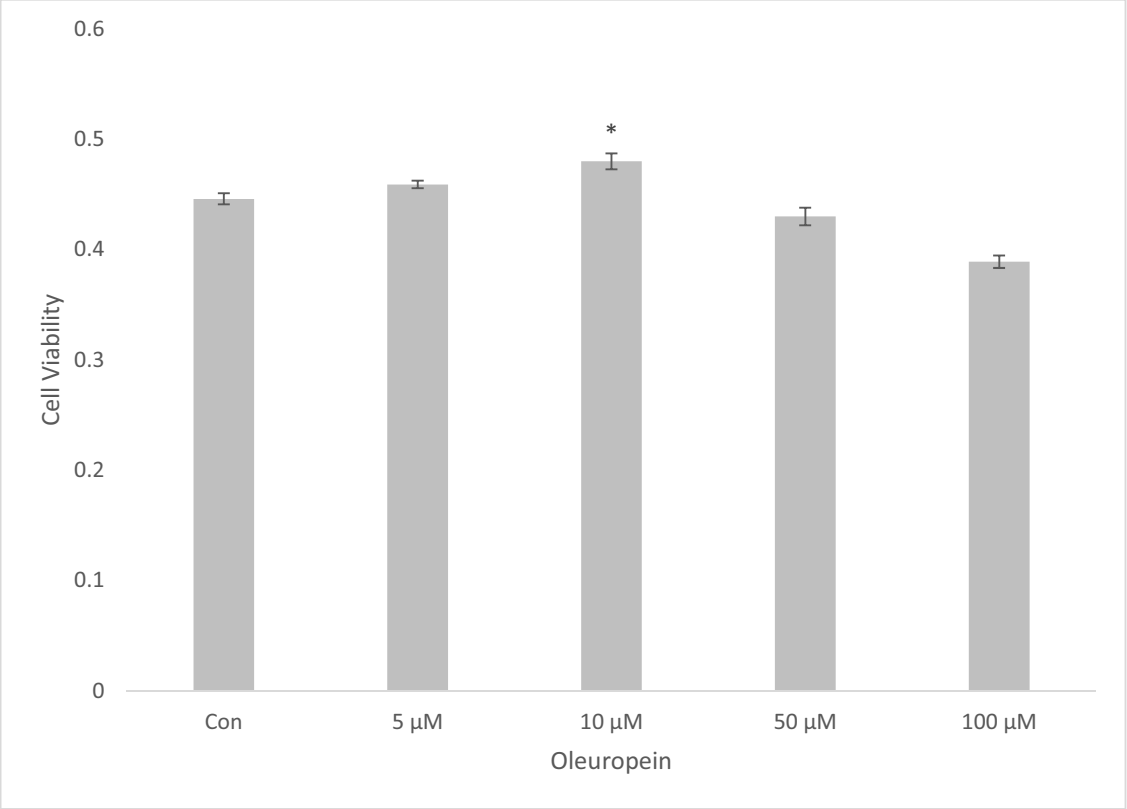

Fig. (2). Dose dependent effect of OP in viability. The data is represented as Mean ± SEM of five independent experiments. *P<0.05 compared to control group.

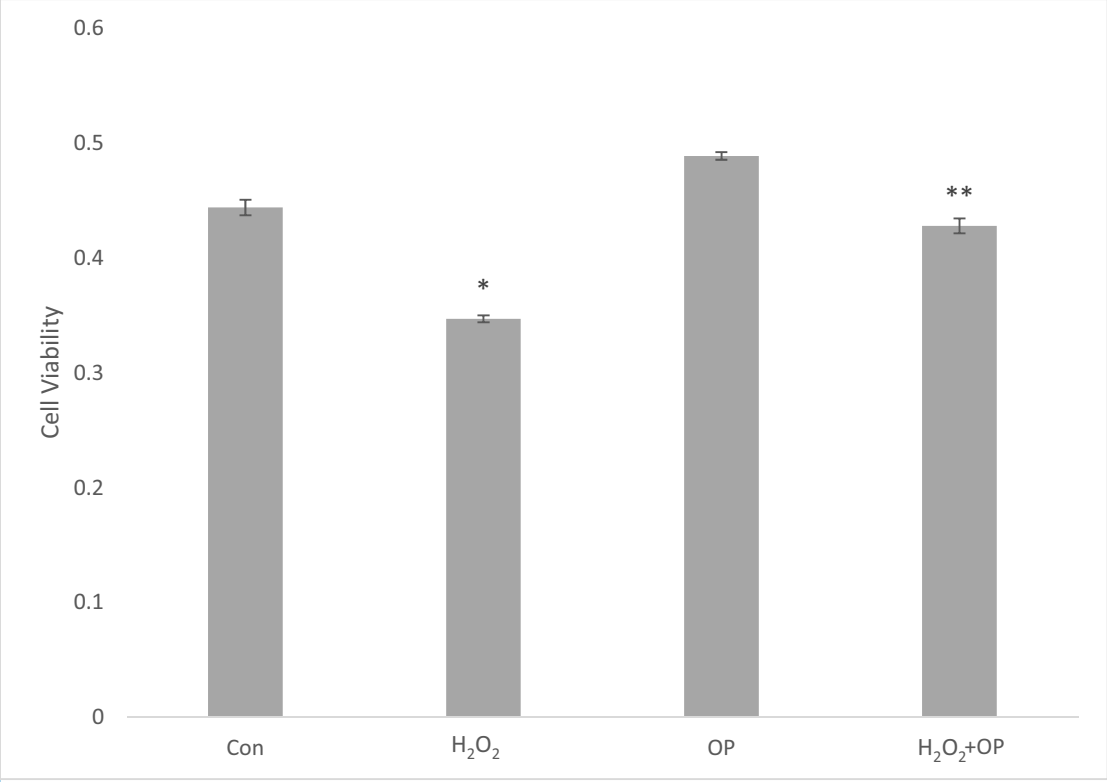

Fig. (3). Dose dependent protective effect of OP on H_2O_2-induced cytotoxicity in cells. The data is represented as Mean ± SEM of five independent experiments. *P<0.05 compared to control group. **P<0,05 *versus* H_2O_2 group.

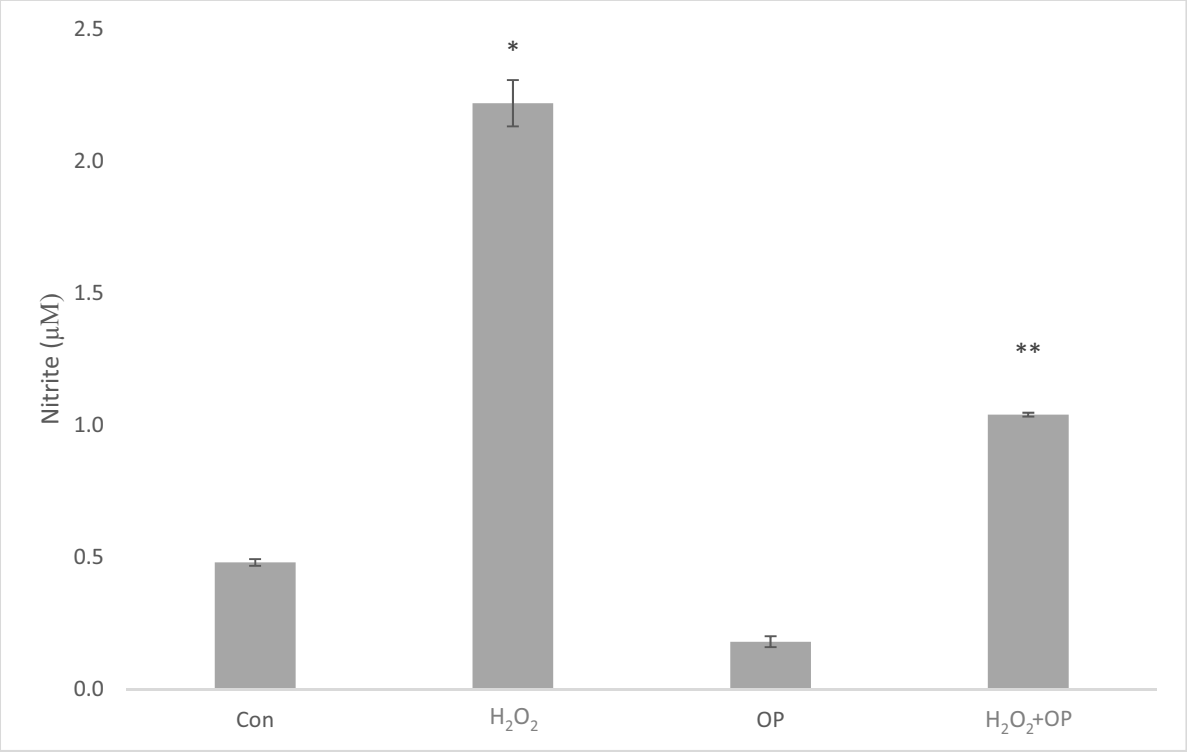

Fig. (4). Effects of OP, H_2O_2 and H_2O_2+OP treatments on nitrite levels in neuronal cells The data is represented as Mean ± SEM. *P < 0.05 compared to control group; **P < 0.05 compared to H_2O_2-treated group (n = 5).

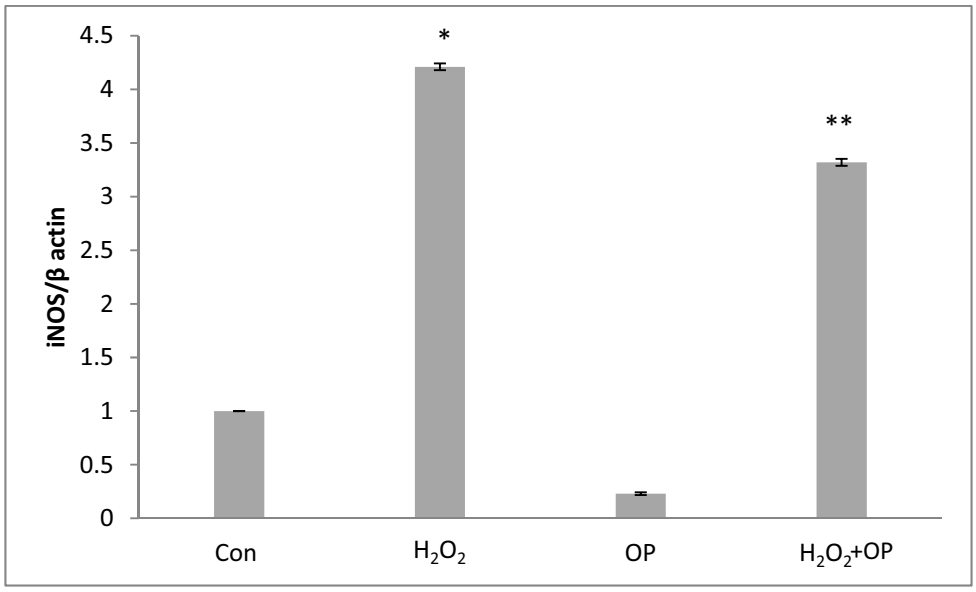

Fig. (5). Analysis of iNOS expression in U87 cells. The results were normalized with housekeeping gene β actin. The data is represented as Mean ± SEM. *P < 0.05 compared to control group; **P < 0.05 compared to H_2O_2-treated group (n = 6).

fold (2.2 ± 0.196) in comparison to control group (0.5 ± 0.029)(*P<0.05). A significant decrease (54%) in nitrite production was observed in cells pretreated with 10µM OP (1.01 ± 0.016) (**p<0.05) according to H_2O_2 treatment (Fig. **4**). In addition, H_2O_2 exposure significantly induced iNOS (4.2 fold) mRNA expressions in comparison with control cells (Fig. **5**). As shown in the same figure, OP pretreatment decreased iNOS mRNAs by 21%, when compared to the H_2O_2 group.

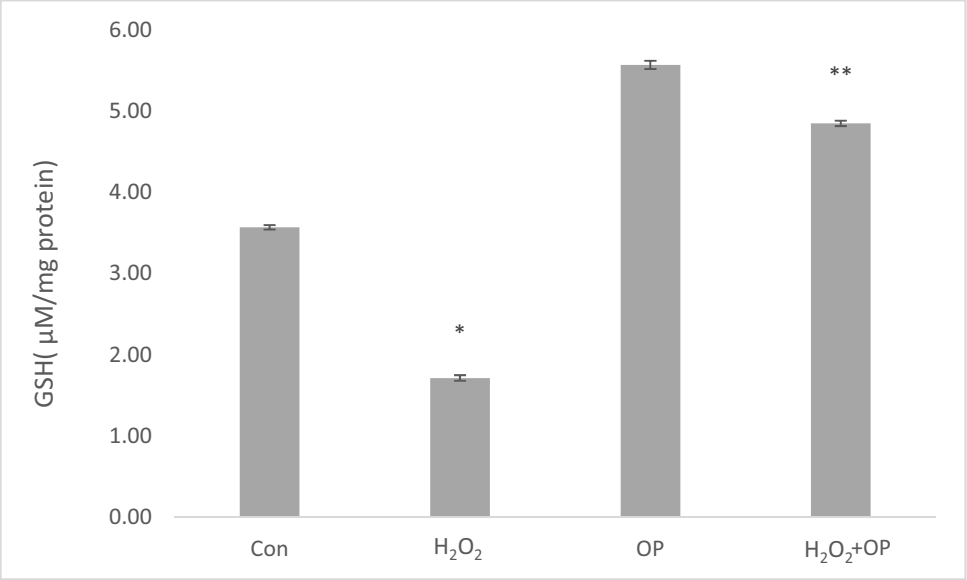

Fig. (6). Measured GSH levels of U87 cells with H_2O_2, OP and H_2O_2+OP. The data is represented as Mean ± SEM. *P < 0.05 compared to control group; **P < 0.05 compared to H_2O_2-treated group (n = 6).

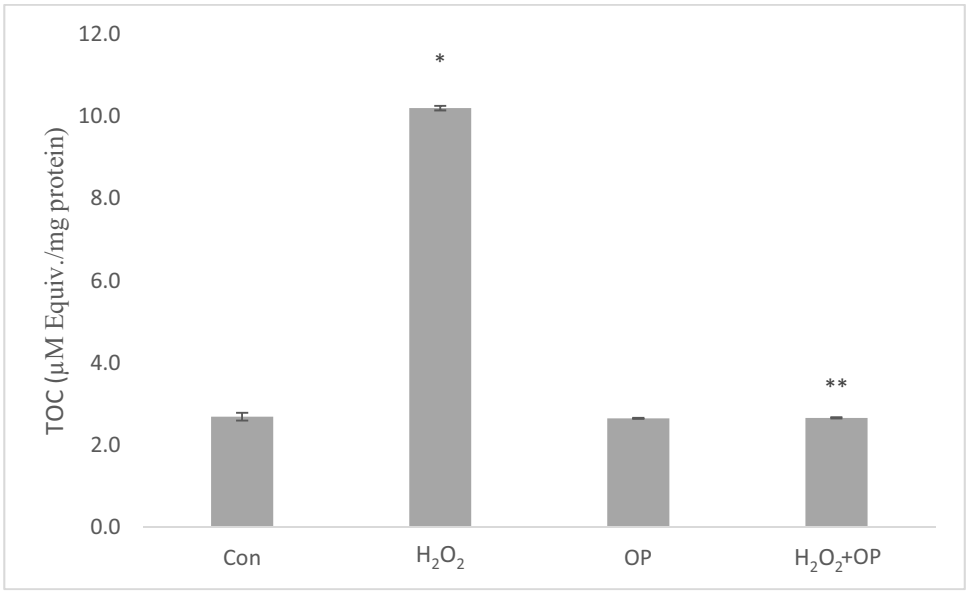

Fig. (7). Total oxidant status of the cells in the groups after the incubation periods. The data is represented as Mean ± SEM. *P < 0.05 compared to control group; **P < 0.05 compared to H_2O_2-treated group (n = 6).

In order to determine the effect of H_2O_2 exposure on oxidant and antioxidant homeostasis, we measured reduced glutathione (GSH) levels in U87 cells. There was a significant decrease (52%) in GSH levels in H_2O_2 treated cells (1.71 ± 0.24) when compared to the control (3.57 ± 0.61) (*p≤0.05). A significant increase (2.83 fold) in GSH production was observed in cells pretreated with 10 µM OP (4.86 ± 0.34) according to H_2O_2 treated group (**P≤0.05) (Fig. **6**).

After incubation periods, H_2O_2 increased the total oxidant capacity about 4 times (10.2 ± 0.21) with respect to control group and it was found that this increase was suppressed by oleuropein in OP pretreated group (2.7 ± 0.25) by 3.7 fold (Fig. **7**). On the other hand, H_2O_2 increased total antioxidant capacity by 12% (1.33 ± 0.14) and in the oleuropein pretreated group, this increase was observed as 35% (1.6 ± 0.08) according to control group (Fig. **8**).

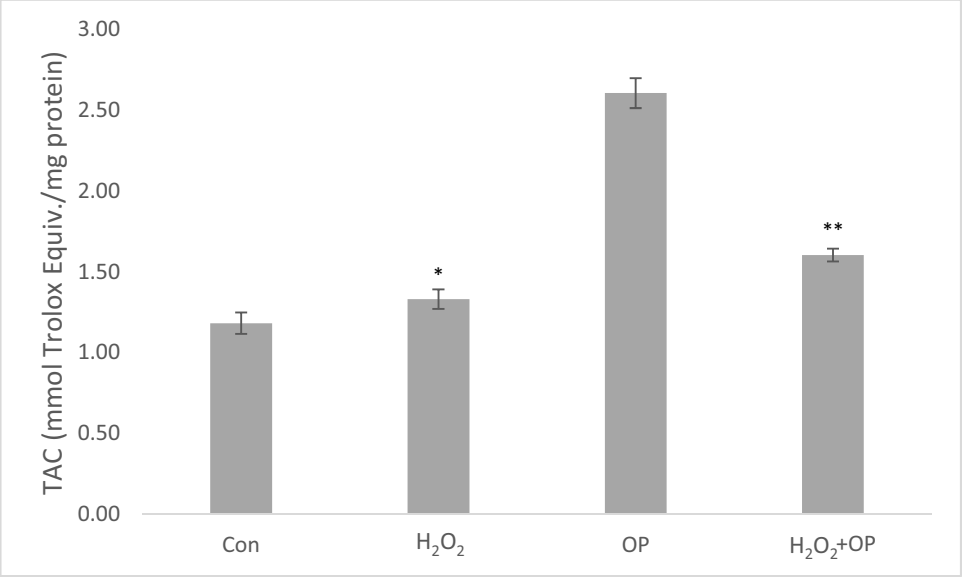

Fig. (8). Total antioxidant status in the groups after incubation periods. The data is represented as Mean ± SEM. *P < 0.05 compared to control group; **P < 0.05 compared to H_2O_2-treated group (n = 6).

4. DISCUSSION

Oxidative stress is one of the most important intracellular stimuli caused by reactive oxygen species such as H_2O_2 and superoxide anion [16]. The process of neuron injury is complicated and associated with oxygen free radical injury, inflammatory factor damage [17]. Many researches have demonstrated that H_2O_2 is used extensively in cell culture studies to explore the molecular mechanism of oxidative stress to glial cells, because the production of H_2O_2 is one of the main causes of oxidative damage [18, 19]. Sun *et al*., [20] documented that the application of H_2O_2 (100μM) caused significant cell loss in *in vitro* neuroblastoma culture. This data is in agreement with those of Malhotra *et al*., [21] who reported that 1h incubation of H_2O_2 caused significant cell losses on viability. In previous researches [22-24], it has been reported that H_2O_2 caused cell losses by increasing ROS production, pro-apoptotic Bax protein levels and DNA fragmentation. Here, we used an oxidative stress model of neuronal injury in U87 cells by addition of exogenous H_2O_2. H_2O_2 (100 μM) decreased cell proliferation in a dose-dependent manner and caused significant cell loss in neuronal cells.

Olive oil phenols are known to have strong antioxidant activity [25]. Recent literature indicates that oleuropein has also neuroprotective activity against Alzheimer's and Parkinson's diseases [1]. It was reported that OP significantly inhibited cell losses on PC-12 neuron cells [22] and also prevented H_2O_2-induced β cell losses [26]. In our study, we observed increased cell proliferation at increasing concentrations of oleuropein and 10 μM oleuropein was found to be the most effective concentration. In addition, it was observed that oleuropein pretreatment showed good protection against neuronal cell losses induced by H_2O_2.

Oxidative stress in the brain of elderly people is mainly caused by the excessive production of ROS and impaired function of the antioxidant system [27]. Glutathione, a tripeptide present in almost all cells, has important roles like taking part in oxidation-reduction reactions, acting as cofactors in enzymatic reactions, scavenging free radical species and toxic xenobiotics. Porres-Martínez *et al*., [28] reported that administration of 100 μM H_2O_2 for 30 m significantly reduced intracellular GSH levels in cells in the hydrogen peroxide-induced neuronal oxidative damage model. Moreover, GSH levels were shown to be reduced significantly with 100 μM H_2O_2 treatment in H_2O_2-induced neuronal oxidative damage [29]. In a recent study, the amount of GSH increased significantly in a dose-dependent manner by the application of oleuropein to the rats in Alzheimer model of hippocampal area neurons. In addition, oleuropein significantly

increased antioxidant capacity and GSH in the carbon tetrachloride-stimulated hepatotoxicity [30]. Similarly, our results indicated that 100 μM H_2O_2 reduced intracellular GSH contents of the cells by half. On the other hand, oleuropein pretreatment prevented the reduction in GSH levels. Oleuropein treatment alone increased the intracellular GSH levels and oleuropein administration before H_2O_2 regenerated the levels of GSH reduced by hydrogen peroxide. Moreover, oleuropein administration decreased the total oxidant capacity that increased by H_2O_2 and enhanced total antioxidant capacity in a similar manner. Moreover, similar to the data we obtained in our study, it was reported that GSH injection decreased NO levels at 3 and 6 hours in an *in vivo* study [31]. Based on this information, it can be said that one of the causes of cell damage of hydrogen peroxide is its reducing effect in the amount of GSH levels in the cells. Also, it can be proposed that one of the most important reasons for the antioxidant properties of oleuropein is that it regulates the intracellular GSH molecule responsible for the scavenging of reactive oxygen and nitrogen species.

NO is an important molecule in the development, maintenance and regulation of brain circuits. However, under oxidative stress, nitric oxide (NO) and its metabolites give rise to the formation of neurodegeneration by oxidizing biomolecular targets such as proteins, lipids and nucleic acids [32, 33]. Hu *et al*., [34] reported that H_2O_2 application increased both iNOS and nitrite levels in neuronal damage. In addition, it was shown that iNOS expression was significantly increased in hydrogen peroxide toxicity model of spiral ganglion cells [35]. Our study indicated that H_2O_2 significantly increased NO levels and upregulated iNOS gene expression in the cells. However, oleuropein pretreatment attenuated the increase in a considerable amount in NO and iNOS gene expression levels. Oxidative stress-induced cell damage is particularly associated with prostaglandins, interleukins and nitric oxide-induced inflammation. Cabrerizo *et al*., [36] used hydroxytyrosol, a metabolite of oleuropein, in their *in vitro* and *in vivo* neurodegeneration studies. They reported that hydroxytyrosol has an anti-oxidant and anti-inflammatory effect by suppressing the increase of hypoxia-induced nitric oxide levels *via* increasing intracellular glutathione capacity. Besides, hydroxytyrosol was found to reduce both the activity and expression levels of inducible enzymes of COX-2 and iNOS [37]. Cell death due to neuroinflammation is mostly due to the long-term effects of reactive oxygen and nitrogen species (RONS) that play an important role in the emergence of apoptotic cell death due to irreversible oxidative or nitrosative damage of neuronal cells. Therefore, oleuropein in our study can be said to have the antioxidant and anti-inflammatory potential to neurodegeneration by increasing glutathione level, ameliorating increased NO amounts and downregulating iNOS expression.

CONCLUSION

Many researches focus on the neuroprotective role of oleuropein which constitutes a possible pharmacological agent against oxidative stress-related neurodegeneration. Taken together, above results indicated that oleuropein, as the most active polyhydroxyl component of olive leaf and olive oil, can be said to be an effective natural compound for decreasing oxidative and nitrosative stress in hydrogen peroxide-induced neuronal toxicity. However, recent researches on the neuroprotective role of oleuropein are still very few and further analysis is a need in this area.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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