

The potential neuroprotective roles of olive leaf extract in an epilepsy rat model induced by kainic acid

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Abstract

Background and purpose: Epilepsy is recognized as a chronic neurologic disease. Increasing evidence has addressed the antioxidant and anti-inflammatory roles of olive leaf extract (OLE) in neurodegenerative diseases. So, the current study aimed to investigate the neuroprotective roles of OLE in epilepsy.

Experimental approach: Forty rats were divided into 4 groups including a control group, sham group, kainic acid (KA) group, and KA + OLE group. KA (4 µg/rat) was injected intrahippocampal, and OLE (300 mg/kg) was orally administered for 4 weeks. Animals were sacrificed, and their hippocampi were isolated. KA-induced seizure activity was recorded. Oxidative stress index was assessed by measuring its indicators including malondialdehyde (MDA), nitrite, nitrate, and glutathione (GSH) as well as the catalase (CAT) activity. The supernatant concentration of tumor necrosis factor-α (TNF-α) and the apoptosis rate in neurons were measured.

Findings/Results: Treatment with OLE significantly reduced the seizure score. OLE decreased oxidative stress index by reducing the concentration of MDA, nitrite, and nitrate as well as increasing the level of GSH. OLE had a significant anti-apoptotic effect on neurons. However, CAT activity and the level of TNF-α were not affected.

Conclusion and implications: Our findings indicated neuroprotective properties of OLE, which is mainly mediated by its antioxidant and anti-apoptotic effects, therefore, could be considered as a valuable therapeutic supplement for epilepsy.

Keywords: Epilepsy; Inflammation; Kainic acid; Olive leaf extract; Oxidative stress.

INTRODUCTION

Epilepsy is recognized as one of the most widespread and serious neurologic disease, which is characterized by recurrent epileptic seizures and emotional dysfunction (1). This disorder affects 0.5-1% of the world population (~50 million people worldwide) (2). Epilepsy causes several momentous physical, psychological, and economic consequences

(1,3). In spite of the availability of various antiepileptic drugs (such as acetazolamide, carbamazepine, clobazam, rivotril, and so on), epilepsy is not completely curable and about one-third of patients with this disorder suffer from enduring seizures that do not respond to antiepileptic drugs (4).

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Temporal lobe epilepsy, as one of the most persistent types of epilepsy, is associated with a structural alteration in the hippocampus which is known as hippocampal sclerosis, the formation of neurodegeneration, and extensive reorganization of hippocampal circuits. This situation is distinguished by a specific pattern of neuronal death. The most sensitive neurons are in the CA1 and CA3 regions and the hilus of the dentate gyrus (5,6).

Increasing research evidence has addressed the crucial role of inflammation in the pathophysiology of several neurodegenerative and autoimmune brain diseases such as Parkinson's disease, multiple sclerosis, and, Alzheimer's disease (7-9). Moreover, data from several clinical studies indicated the clinical efficacy of anti-inflammatory drugs for the treatment of epilepsy by suppressing inflammation, which could be considered as one of the potential mechanisms in the pathophysiology of epilepsy (8,10). Additional evidence emerged from experimental studies and demonstrated that brain inflammation is the key aspect of epilepsy (8). During chronic inflammation in the brain, several pro-inflammatory mediators are produced by peripheral immune cells and brain resident cells including endothelial cells of the blood-brain barrier, astrocytes, and microglia (11,12). Tumor necrosis factor- α (TNF- α) is one of the most important pro-inflammatory mediators and is a major cause of hyperexcitability and seizures (7,12). On the other hand, oxidative stress, which is closely related to inflammation, is another key mechanism involved in neuronal death and seizures (13,14). It has been demonstrated that increased production of free radicals causes cellular damage, neuronal irritability, and excitability, which might contribute to the pathophysiology of several neurodegenerative disorders (15). With regard to this mounting evidence, inflammation and oxidative stress can be considered as two main targets for the treatment of epilepsy.

The last two decades have seen a growing interest in the use of medicinal plant-derived extract or compounds for the treatment or control of several diseases (16,17). In this regard, olive leaf extract (OLE) has been studied by many researchers (18,19). The

therapeutic potential of OLE is mainly associated with the olive phenolic compounds (such as oleuropein), which have powerful anti-inflammatory and antioxidant properties (19-21). Moreover, the anti-apoptotic effects of OLE have been shown in several experimental studies (22-24). Recent work by Sarbishegi *et al.* has indicated that hydro-alcoholic extract of olive leaf effectively reduced both oxidative stress index and neuronal apoptosis in an experimental model of Parkinson's disease (25). Up to now, however, no previous study has addressed the protective roles of OLE in epilepsy. So, this study set out to investigate the potential anti-inflammatory, antioxidant, and anti-apoptotic roles of OLE in hippocampus neurons under conditions of kainic acid (KA)-induced temporal lobe epilepsy in rats.

MATERIALS AND METHODS

Reagents

KA, cresyl violet, and ketamine hydrochloride/xylazine hydrochloride solution purchased from Sigma-Aldrich (Germany). Diethyl ether was purchased from Merck (Germany). Lipid peroxidation (malondialdehyde, MDA) assay kit, nitrite/nitrate assay kit, and glutathione (GSH) assay kit were procured from Sigma-Aldrich (Germany). Catalase (CAT) activity assay kit was supplied by Abcam (UK).

Preparation of OLE

The extract was provided in the Herbarium of the School of Pharmacy (Shahid Beheshti University of Medical Sciences, Tehran, Iran). The olive leaf powder extract was purchased from the local herbal medicines market (Tehran, Iran) in 2018.

The preparation of the olive extract was performed with ethanol as an extracting solvent by the maceration method. All extracted samples were then condensed using a rotary evaporator. Finally, 15 g of dried extracts were obtained. In order to prepare OLE gel, 6% polymer Carbopol[®] (base gel) was added to the obtained extract. Total phenolic content of OLE assayed using Folin's phenol reagent (Folin-Ciocalteu colorimetric method; Sigma-Aldrich, Germany), and the absorbance read at 760 nm

and compared with gallic acid as the calibrator, according to the previous study (26). Total flavonoid content of OLE measured and calculated per mg equivalents of quercetin/g of the extract, as previously reported (27).

Animals

Forty male Wistar rats (weighing 200-250 g) were purchased from Pasteur Institute (Tehran, Iran). A 12/12-h light/dark cycle was maintained and the rats kept three per cage with free access to water and pellet chow. The Ethics Committee of Tehran University of Medical Science (Tehran, IRAN) approved all procedures of the current animal study (Ethic code: IR. TUMS. VCR. REC. 1395. 1380). All rats received human care in conformity with the National Institutes of Health (NIH) guidelines.

Experimental procedure

Four groups of rats (n = 10) determined as follows: (1) control group, didn't receive any interference; (2) sham group, received the intrahippocampal injection of normal saline and gavaged with water; (3) KA group, injected intrahippocampal with 4 µg of KA dissolved in 5 µL of normal saline at a rate of 1 µL/min using a Hamilton microsyringe; and (4) KA + OLE group, injected with 4 µg of KA dissolved in 5 µL of normal saline and orally treated with OLE at a dose of 300 mg/kg/daily for 4 weeks before surgery (25).

For intrahippocampal injections, rats anesthetized with xylazine (5 mg/kg, ip) and ketamine (60 mg/kg, ip). The dorsal surface of the skull exposed and a burr hole was drilled, according to the atlas of Paxinos Thebregma point used as the reference: anteroposterior: 4.1 mm, Lateral: 4.1 mm, and ventral to the dura: 4 mm (28).

Behavioral assessment of seizures

Rats rated for KA-induced seizure activity during a 4-h period after the surgery, according to Racine's classification, as previously described (14,29). (0), No reaction; (1) stereotypic mounting, eye blinking, and/or mild facial clonus; (2) head nodding and/or multiple facial clonus; (3) myoclonic jerks in the forelimbs; (4) clonic convulsions in the forelimbs with rearing; and (5) generalized clonic convulsions and loss of balance.

Hippocampi isolation and preparation

After 24 h, rats decapitated under diethyl ether anesthesia. The brains removed and the hippocampi were isolated and prepared, as previously described (30). Briefly, brains were removed and hippocampi isolated and prepared as a 10% tissue homogenate in ice-cold 0.9% saline solution containing protease inhibitor cocktail. After centrifugation (1,000 g, 4 °C, 10 min), the supernatant was aliquoted and stored at -70 °C until assayed.

Assessment of oxidative stress markers

Measurement of hippocampal MDA

The concentration of MDA, as an *in vitro* marker of lipid peroxidation, was determined by measuring thiobarbituric acid-reactive substances in the supernatant, as previously described (31). Briefly, the reaction was carried out at pH 2-3 and 90 °C for 90 min. After cooling samples on ice, centrifuging was carried out at 1000 g for 10 min, and then, the absorbance of the supernatant read at 532 nm. Thiobarbituric acid-reactive substances were determined using tetramethoxypropan as standard and MDA concentration presented as nanomoles/mg of protein.

Measurement of hippocampal nitrite and nitrate concentration

Supernatant nitrite and nitrate concentration measured using Griess assay (14). Briefly, 1 mL of the homogeneous tissue and 1 mL of Griess solution kept at room temperature for 10 min. Then, the absorbance determined at 540 nm, using a spectrophotometer (752N, Jiangsu, China).

Hippocampal GSH measurement

GSH was assayed, using Ellman's method (32). Briefly, to 0.1 mL of the homogenate supernatant, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 5'5 dithiobis (2-nitrobenzoic acid) (DTNB or Ellman's reagent), and 0.4 mL of double distilled water were added. The absorbance measured at 412 nm within 15 min, using a spectrophotometer (752N, Jiangsu, China). The final concentration of these optical absorbencies calculated using the standard curve.

Antioxidant activity of CAT

The antioxidant activity of CAT was measured, according to the procedure of Claiborne (30,31). Briefly, the rates of H₂O₂ decomposition in the supernatant, containing 50 mM potassium phosphate buffer (pH 7.0), measured by monitoring the optical absorbance changes at 240 nm for 2 min, using a spectrophotometer (752N, Jiangsu, China). The antioxidant activity of CAT is expressed as unit/mg protein. One unit of catalase activity is defined as 1 μmol of H₂O₂ that is decomposed in 1 min.

Protein assay

The protein concentration of the hippocampus tissue supernatant assayed, according to the procedure used by Bradford (14). Briefly, 100 mg of Coomassie Brilliant Blue (G-250) was dissolved in 50 mL of 95% ethanol and added to 100 mL of phosphoric acid 85%. When the color was completely dissolved, the volume adjusted to 1 L and passed the solution to the paper before use. Then, 5 mL of reagent and 100 μL of the sample were added and after 5 min, the absorbance read at 595 nm, using a spectrophotometer (752N, Jiangsu, China).

Cytokine assay

The supernatant concentration of TNF-α measured using a TNF-α ELISA kit (Blue Gene Biotech Co., Shanghai, China), according to the manufacturer's protocol. Samples measured in duplicate.

Neuronal death detection

Cell death detection ELISA plus kit (Sigma-Aldrich, USA; #11287100) was used to assess DNA fragmentation as an index of apoptosis using a plate reader (BioTek, USA).

Statistical analysis

All analyses were carried out, using the GraphPad Prism software program (Version 6.07, San Diego, California). Descriptive data

were generated for all variables and presented as mean ± SEM. The significance level was set at $P < 0.05$.

RESULTS

The phenol and flavonoid content of OLE

The total content of phenol and flavonoid in the extract has been shown in Table 1.

The effect of OLE on seizure activity and behavior

As can be seen from Fig. 1, no seizure symptoms were observed in the control and sham groups. Besides, treatment with OLE markedly reduced the mean score of seizure strength compared with the KA group ($P < 0.01$).

Oxidative stress index

The effect of OLE on membrane lipid peroxidation

In Fig. 2, the concentration of MDA, as a marker of membrane lipid peroxidation, was shown in the hippocampal supernatant. Treatment with OLE markedly reduced MDA concentration in the treatment group in comparison with the KA group ($P < 0.01$).

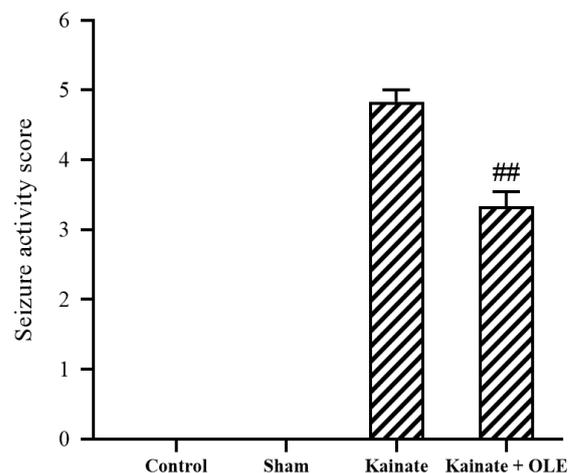


Fig. 1. Seizure strength score in different groups. Data are presented as mean ± SEM. ^{##} $P < 0.01$ Indicates significant difference compared with kainate group. OLE, olive leaf extract.

Table 1. Total phenols and flavonoids in the olive leaf extract

Total phenols; mg equivalent of gallic acid /g of the extract	6.72 mg/g
Total flavonoids; mg equivalent of AlCl ₃ /g of the extract	0.36 mg/g

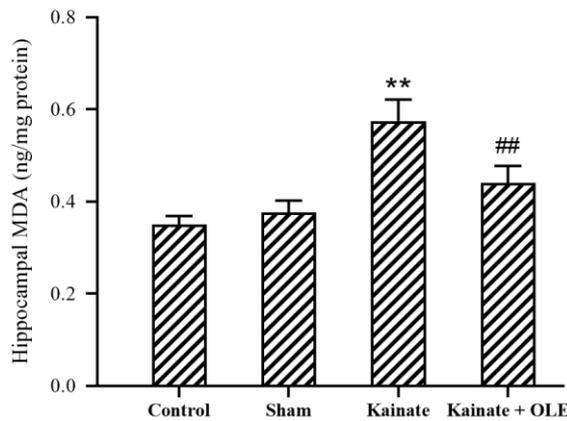


Fig. 2. Alterations of hippocampus MDA in different groups. Data are presented as mean \pm SEM. ** $P < 0.01$ Indicates significant difference compared with sham group and ## $P < 0.01$ vs kainate group. MDA, Malondialdehyde; OLE, olive leaf extract.

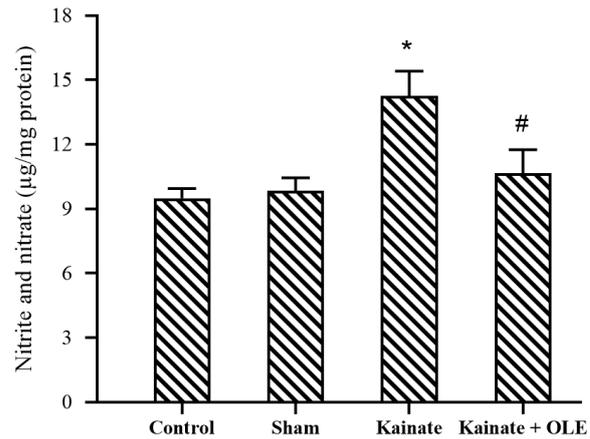


Fig. 3. Alterations of hippocampus nitrite and nitrate in different groups. Data are presented as mean \pm SEM. * $P < 0.05$ Indicates significant difference compared with sham group and # $P < 0.05$ vs kainate group. OLE, Olive leaf extract.

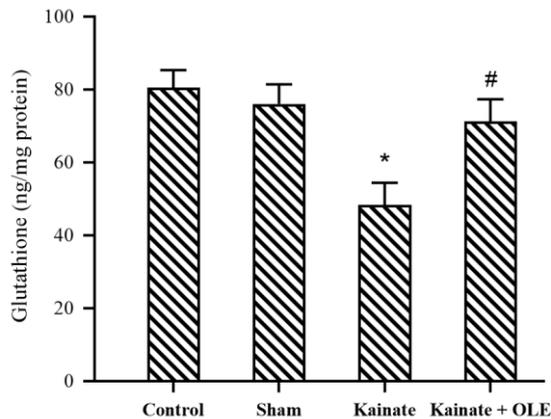


Fig. 4. Alterations of hippocampus glutathione in different groups. Data are presented as mean \pm SEM. * $P < 0.05$ Indicates significant difference compared with sham group and # $P < 0.05$ vs kainate group. OLE, Olive leaf extract.

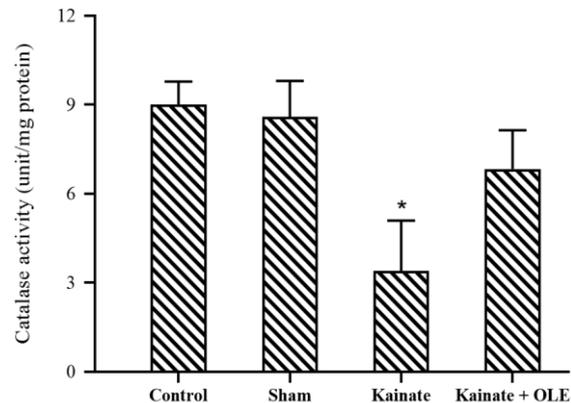


Fig. 5. Alterations of hippocampus catalase activity in different groups. Data are presented as mean \pm SEM. * $P < 0.05$ Indicates significant difference compared with the sham group and. OLE, Olive leaf extract.

Oxidative stress index

The effect of OLE on membrane lipid peroxidation

In Fig. 2, the concentration of MDA, as a marker of membrane lipid peroxidation, was shown in the hippocampal supernatant. Treatment with OLE markedly reduced MDA concentration in the treatment group in comparison with the KA group ($P < 0.01$).

The effects of OLE on nitrite and nitrate concentration

As shown in Fig. 3, an increase in the hippocampal concentration of nitrite and nitrate was observed in the KA group which significantly lowered under oral treatment with OLE ($P < 0.05$).

The effect of OLE on GSH level

In Fig. 4, the level of GSH was significantly decreased in the KA group ($P < 0.05$) compared to the sham group; however, there is a clear increase in the level of GSH in hippocampal supernatant of OLE-treated rats ($P < 0.05$).

Antioxidant enzyme activity

As shown in Fig. 5, despite the elevated enzyme activity of CAT following the treatment with OLE, however, this increased activity of CAT was not statistically significant in comparison with the KA group ($P > 0.05$).

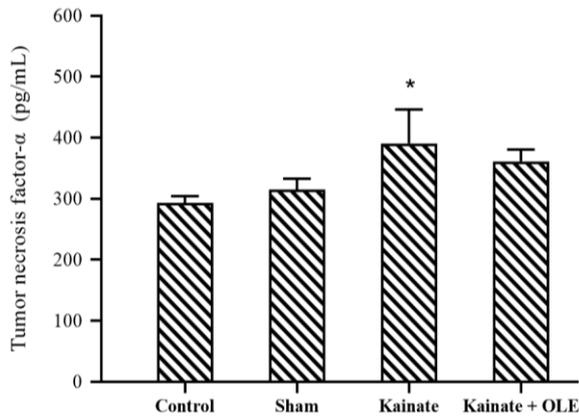


Fig. 6. Alterations of hippocampus tumor necrosis factor- α in different groups. Data are presented as mean \pm SEM. $P < 0.05$ Indicates significant difference compared with the sham group and. OLE, Olive leaf extract.

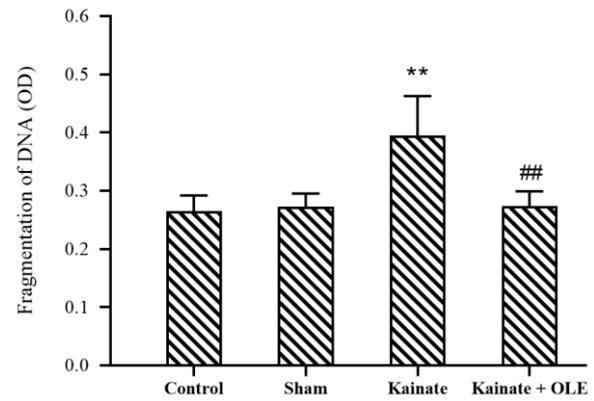


Fig. 7. Alteration of hippocampus fragmentation of DNA in different groups. Data are presented as mean \pm SEM. ** $P < 0.01$ Indicates significant difference compared with sham group and ## $P < 0.01$ vs kainate group. OLE, Olive leaf extract.

Proinflammatory cytokine concentration

According to Fig. 6, the high concentration of TNF- α was observed in the hippocampal supernatants of rats treated with KA ($P < 0.05$), however, a clear anti-inflammatory effect of OLE in the prevention of TNF- α could not be identified in the treatment group ($P > 0.05$).

Neuronal death

The apoptosis rate of the CA3 neurons was measured using the *in situ* assay and results are presented in Fig. 7. Generally, the fragmentation of DNA, as a marker of apoptosis, in the neurons was significantly increased under intervention with the KA ($P < 0.01$). Interestingly, after treatment with OLE, a lower apoptosis rate in CA3 neurons was found compared with the KA group ($P < 0.01$).

DISCUSSION

Understanding the mechanisms of epilepsy has long been the subject of human research. Despite extensive research in this field, the mechanism of this disorder is still unknown, so it is important to achieve animal models that are most similar to humans. Currently, one of the most similar animal models of epilepsy is temporal lobe epilepsy induced by KA (33).

Hence, KA can be the best substance to create an animal model of temporal lobe epilepsy. The CA3 region in the hippocampus has a high density of glutamate receptors. The

activation of the glutamate receptor increases the intake of sodium ions and calcium into the cell, where calcium ions can be the initiator of apoptosis and neuronal death (34).

Prior research studies have addressed the anti-inflammatory, antioxidant, and anti-apoptotic roles of OLE in several neurodegenerative disorders (19-23). So, the current study was designed to assess the potential neuroprotective roles of OLE in a rat model of epilepsy. In this study, OLE was found to have neuroprotective effects against the temporal lobe epilepsy model in rats.

Increasing evidence has shown that oxidative stress is a common feature of several neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, stroke, and dementia, which causes neural dysfunction or death, and neuronal excitability (15). Interestingly, several research-reports indicate that neurodegenerative disorders may get some aspects of epilepsy with time (35,36). Therefore, oxidative stress is considered as a possible mechanism in epileptogenesis (37). High levels of polyunsaturated fatty acids in the brain are potent substrates for oxidation (38). Moreover, defect in the elimination of free radicals by antioxidant mechanisms is a major cause that promotes the increased generation of the reactive oxygen species (ROS) in the brain. As a consequence of these events, free radicals damage susceptible targets including phospholipids, proteins, and mitochondrial

DNA of neuronal cells (38,39). On the other hand, several reports have shown that mitochondrial ROS is increased after durable epilepsy, which causes lipid peroxidation, DNA damage, gliosis, impairment of intracellular calcium homeostasis, and neuronal overexcitability in hippocampus neurons (39-42). Increased ROS production has been related to the pathology of diseases as stroke, amyotrophic lateral sclerosis, diabetes, cancer, atherosclerosis, and neurodegenerative illnesses such as Alzheimer's and Parkinson's diseases and epilepsy. Extracellular levels of ROS cause injury to lipids, nucleic acids, proteins, and membranes which can begin cell death signaling pathways, for example, apoptosis (43). In the current study, epilepsy was induced by KA injection. This model emulates many pathophysiological features of epilepsy such as increased-ROS production, impaired-mitochondrial function, impairment of intracellular calcium homeostasis, and neuronal apoptosis in different areas of the brain, especially in the hippocampus (44,45).

However, treatment with OLE for 4 weeks significantly decreased KA-induced oxidative stress by reducing the concentration of MDA, nitrate, nitrite, and increasing the level of GSH in the hippocampus. These findings accord with prior research reports, in which OLE effectively reduced the production of free radicals and lipid peroxidation (23-25). As we mentioned above, the potential antioxidant properties of OLE arise from its two important bioactive components including polyphenols and flavonoids (19,20). Oleuropein as a main phenolic constituent of OLE effectively decreases the level of MDA in the hippocampus of an animal model of Alzheimer's disease (46). Concerning this precise evidence, OLE has antioxidant effects in epilepsy.

Apoptosis of neurons or other susceptible cells in the brain is one of the major causes in the pathogenesis of neurodegenerative diseases (47). Elevated levels of oxidative stress by damaging to mitochondrial and nucleus DNA are the most important stimulator of apoptosis in neurons (38,39). It has been shown that KA injections (systemic or intracerebral) by increasing generation of free radicals contribute to the neuronal death *via* apoptosis in the CA3

region of the hippocampus, which causes epilepsy in the experimental temporal lobe model (48). Moreover, inhibition of the NO formation in the brain prevents KA-induced neuronal apoptosis in the CA3 area (49). In the current study, oral treatment with OLE effectively protected neurons against apoptosis induced by KA. In line with our findings, Sarbishegi *et al.* indicated that administration of OLE inhibits rotenone-induced apoptosis of dopaminergic cells in Parkinson's disease rat model (25). Our finding also accords with earlier *in vitro* and *in vivo* observations, which showed that OLE has an anti-apoptotic role against neuronal death induced by neurotoxin and aging (50-52). Molecular mechanisms by which OLE plays its anti-apoptotic role has been described. The first mechanism of OLE's anti-apoptotic action is tightly associated with its antioxidant effects. OLE by suppressing the production of the free radicals (such as hydroxyl ($\cdot\text{OH}$), superoxide (O_2^-), and NO), and increasing the level of GSH indirectly inhibits the neurons' death *via* apoptosis. The second mechanism is related to the function of hydroxytyrosol, which is one of the important phenolic constituents of OLE. Hydroxytyrosol by increasing the mRNA levels of NAD(P)H quinone oxidoreductase 1 (NQO1) effectively prevents quinone cytotoxicity to protect neurons against cell death (53,54). Moreover, it has been reported that oleuropein by up-regulating the expression of BCL-2, a main anti-apoptotic member of BCL-2 family, and downregulating the expression of BAX, a main pro-apoptotic member of BCL-2 family, play crucial roles to protect neurons against apoptosis induced by ischemia (22).

Existing researches recognize the critical roles played by inflammatory mediators in the epileptogenic process (7). Pro-inflammatory mediators including cytokines and chemokines are produced by brain resident cells such as microglial cells, astrocytes, and endothelial cells of the blood-brain barrier, leading to vascular inflammation and increased infiltration of peripheral leukocytes into sites of inflammation in the brain (11,12). Precise evidence has been supported that neuro-inflammation might be both a consequence and cause of seizures and epilepsy (11). TNF- α is

one of the most important inflammatory mediators involved in the pathogenesis of seizures by increasing the excitability of the neurons (7). In the current study, intrahippocampal injection of KA significantly increased the level of TNF- α in the rat model of epilepsy. However, the finding of the current study does not support the previous research reports, in which administration of OLE, significantly reduced the mRNA or protein levels of TNF- α (24,55). There are several possible explanations for this result including the period time and the dose of treatment, and also different animal models of diseases. Finally, parallel to the reduced-oxidative stress index and apoptosis, treatment with OLE also significantly attenuated the seizure activity score in the rat model of epilepsy, therefore represents the clinical benefit of OLE for the treatment of temporal lobe epilepsy.

CONCLUSION

Collectively, the findings of the current study provide important insights into the neuroprotective properties of OLE, which is mainly mediated by its antioxidant and anti-apoptotic effects, therefore, could be considered as a valuable supplement for the treatment of epilepsy. However, more research on this topic needs to be undertaken to confirm the potential neuroprotective effects of OLE in human temporal lobe epilepsy.

Acknowledgments

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Author's contribution

S. Khamse was responsible for the selection and implementation of the design subject, responsible for statistical analysis, and helped

with the performance of the oxidative stress parameters, assistance in reviewing the manuscript and final edition of the manuscript. S. Mohammadian Haftcheshmeh contributed to writing this article, responsible for reviewing the manuscript and the final edition of the manuscript. SS. Sadr contributed to the monitoring and design of the study. M. Roghani was responsible for the performance of the oxidative stress and ELISA test. M. Kamalinejad was responsible for preparation of the aqueous extract. R. Golchoobian helped with the performance of the ELISA test. P. Mohseni Moghaddam was responsible for the animal's works. F. Ebrahimi helped to animal's works and record the behavior of the animal. All authors monitored the article.

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