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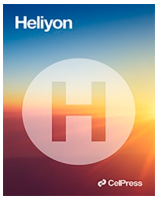

Research article

Neuroprotective effects of pink lotus oil in kainic acid-induced epilepsy

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ABSTRACT

Excitotoxicity-induced oxidative stress results in neuronal cell death. Pink lotus essential oil (PLO) is a concentrated volatile oil from lotus blossoms widely used in traditional medicine. This study aimed to explore the possible therapeutic effects of PLO and its underlying mechanisms on kainic acid (KA)-induced oxidative stress and hippocampal cell death in a mouse model of epilepsy. Mice were treated with 100 mg/kg or 200 mg/kg PLO to ameliorate neurodegeneration and seizure-induced behavior induced by KA injection. Pre- and post-treatment of PLO increased antioxidant activities, reduced the seizure score, prevented oxidative stress by increasing GSH and CAT levels, and reduced MDA (malondialdehyde) levels after KA-induced status epilepticus. KA injection created neuronal cell death in the pyramidal layers of CA1 and CA3 subfields of the hippocampus, and affected interneurons in the hilus of the dentate gyrus. PLO treatment notably diminished KA-induced neuronal cell death in these areas through activation of the Akt signaling pathway, increasing reactive astrogliosis, and up-regulation of GDNF expression. Moreover, caspase-3 expression, and microglia activation were significantly decreased in PLO treatments. Taken together, these results suggest that PLO possesses antiepileptic, anti-apoptosic, and neuroprotective effects on KA-induced epileptogenesis indicating that PLO may serve as a dietary supplement option in the treatment of epilepsy or of other neurodegenerative disorders.

1. Introduction

Epilepsy is a neurological disorder that affects about 50 million people of any age worldwide. Nearly 80 % of patients live in developing countries and do not get any treatment for epilepsy [1]. People with epilepsy suffer from recurrent and spontaneous seizures which are caused by an imbalance between excitatory and inhibitory neurotransmitters leading to over-excitation and abnormal discharges of neurons in the brain [2]. Kainic acid (KA), an analog of glutamic acid, has been widely used to study excitotoxicity-induced neuronal cell death, particularly in temporal lobe epilepsy [3–5]. Overstimulation by KA triggers a massive influx of Ca2+ into hippocampal neurons through kainate receptors with subsequent activation of Ca2+ dependent enzymes (kinases,

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proteases, phospholipases, nucleases) which induce mitochondrial dysfunction and massive generation of free radicals including oxygen (ROS) and nitrogen species (RNS). Moreover, high Ca2+ influx can also directly cause mitochondrial swelling followed by caspase-3 activation and DNA fragmentation. Therefore, the accumulation of free radicals has been suggested to be primarily responsible for the apoptotic neuronal death after epileptic seizures [6–8].

Nelumbo nucifera or pink lotus (Bualuang in Thai), has been widely used for a long time in the Asian traditional medicine. Several reports demonstrated beneficial effects of pink lotus [9,10], for example, it prevents HT22 cell, a mouse hippocampal neuronal cell line, from glutamate-induced neurotoxicity by reducing ROS and Ca2+ influx [11]. In oxidative stress conditions, treatment with lotus essential oil increases the expression of antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) and elevates glutathione (GSH) levels thereby protecting cells from oxidative stress induced injury [12]. Studies have shown that pink lotus essential oil (PLO) activates the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway leading to cell survival [13] and upregulation of B-cell lymphoma 2 (Bcl-2) which plays an important role in cellular survival [14]. Our previous finding had shown that the three main phytochemicals in PLO including palmitic acid ethyl ester, linoleic acid ethyl ester and methyl 8,11,14-heptadecatrienoate, which possess antioxidant activity, improve peripheral nerve regeneration by mitigating oxidative stress [15]. Based on the antioxidant activity and pro-regenerative effects of pink lotus, therefore, this study aimed to explore possible protective effects of PLO on neurodegeneration and epileptic behavior in KA-treated mice.

2. Materials and methods

2.1. Preparation of pink lotus oil (PLO)

PLO was prepared from whole lotus flowers using an absolute extraction method following the manufacturer's protocol of Tropicalife Co., Ltd. Then, the organic compounds of PLO were analyzed by gas chromatography-mass spectrometry (GC-MS) and the organic compounds identified with the data from the National Institute of Standards and Technology Mass Spectra database (NIST08) as described in our previous study [15].

2.2. Animals and intraperitoneal (i.p.) injection of kainic acid (KA)

Male C57BL/6NJcl mice (6–8 weeks of age, weighing 22–24 g) were purchased from Nomura Siam International Co., Ltd (Thailand). All mice were acclimatized for 1 week and kept under standard laboratory conditions: 12 h light-dark cycle, a consistent temperature (25 ± 2 °C), and free access to food and water. Experimental procedure was shown in Fig. 1A, briefly, mice were randomly divided into four groups of 10 mice each: Control group, KA group, KA+100 mg/kg PLO, and KA+200 mg/kg PLO. The mice in KA + PLO groups were given intraesophageal administration of 100 mg/kg PLO or 200 mg/kg PLO once daily for 7 days before and 14 days after KA injection. Kainic acid (cat. no. B6226, APExBIO, USA) was dissolved in sterile normal saline and used for a single i.p. injection at a dose of 30 mg/kg. The injection dose of KA was based on previous studies of KA-induced seizure [16,17], and the doses of PLO were chosen based on our previous study [15]. After KA injection, all mice were observed for seizure behavior for 90 min and scored as follows: stage 0) no behavioral change, score 1) facial muscle clonus, score 2) nodding head, score 3) forelimbs clonus, score 4) rearing, falling with forelimbs clonus, score 5) generalized tonic–clonic seizure, score 6) severe tonic-clonic seizures including death [18]. Only

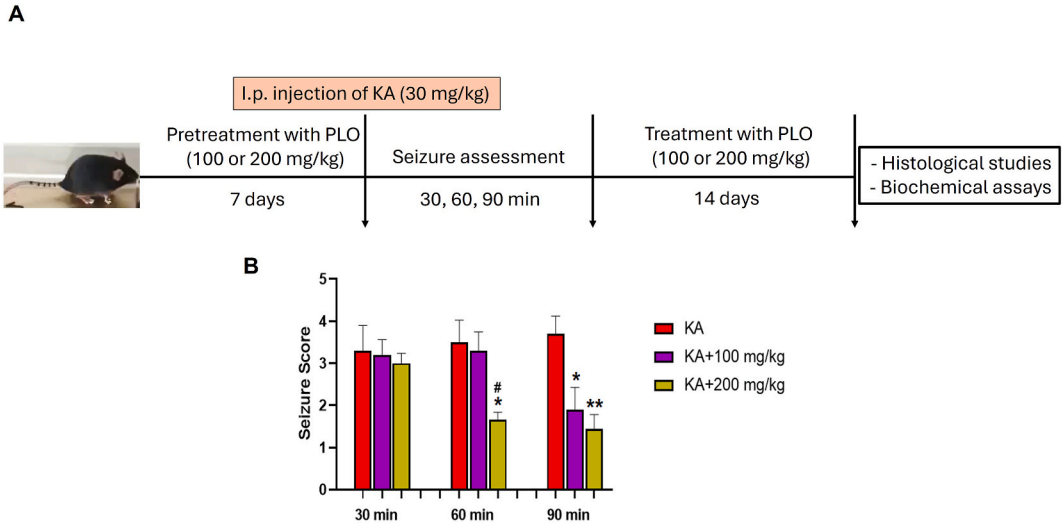

Fig. 1. Experimental arrangement for KA-induced seizure induction in PLO treated mice (A). PLO treatment ameliorates KA-induced seizure activities at different time points (B). Data are represented as mean ± SEM (n = 10). *p < 0.05, **p < 0.01 versus KA alone. #p < 0.05 versus 100 mg/kg.

mice with score 3–5 were included in this study. All animal experiments were approved by the Animal Ethics Committee of the University of Phayao, Thailand (approval no. 1-026-65).

2.3. Histology and immunohistochemistry

Fourteen days after KA injection, all mice were anesthetized with an i.p. injection of 70 mg/kg thiopental sodium, then perfused intracardially with sterile normal saline. Brains from 5 mice of each group were extracted and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.4 at 4 °C for 48 h, then cryoprotected with 30 % sucrose in PBS at 4 °C for 48 h. Serial 20-μm thick coronal sections were cut by a cryostat (Leica Microsystems, Inc., CM1950). Nissl staining of brain sections at 1.8–2.0 mm posterior to the bregma according to the mouse brain atlas [19] was performed using a cresyl violet dye (cat. no. 1.05235, Sigma-Aldrich, USA) to determine the neuronal density in CA1, CA3 and dentate gyrus. The nuclei of pyramidal neurons were counted by a blinded observer in regions of 125 μm in length (and 125 μm width) in Nissl-stained sections covering the whole layer width of the CA1 and CA3 sub-fields, and the interneurons were counted in the entire hilar area.

For immunohistochemical staining, the sections were incubated free-floating in 10 % normal goat serum in PBS for 90 min at 25 °C followed by mouse anti-Iba1 antibody (1:500, cat. no. MABN92, Sigma-Aldrich, USA) or mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:500, cat. no. MABN92, Sigma-Aldrich, USA) overnight at 4 °C. The sections were then stained at 25 °C for 2 h with donkey biotinylated anti-mouse secondary antibody (1:500, cat. no. 715-065-150, Jackson ImmunoResearch, USA) followed by an incubation for 1 h in extravidin peroxidase (1:1,000, cat. no. E2886, Sigma-Aldrich, USA), then marked with 3,3′-diaminobenzidine (DAB) (cat. no. D12384, Sigma-Aldrich, USA). All images were captured at 40× magnification using an upright microscope (Eclipse Ni-U, Nikon, Japan) and analyzed by the NIS Elements imaging software version 5 (Nikon, Japan). All histology and immunohistochemical data were shown as the percentage of control and all experiments were repeated three times for each animal.

2.4. Tissue preparation for biochemical assay

The hippocampi were collected from 5 mice and washed with PBS. After that, the tissues were homogenized in 500 μl of RIPA buffer (cat. no. RB4475, BioBasic, Canada) supplemented with 1 % protease inhibitor cocktail set I (cat. no. 539131, Sigma-Aldrich, USA), then kept on ice for 30 min. The lysates were centrifuged at 10,000 g for 15 min at 4 °C. Protein concentrations of the supernatants were determined by the Lowry protein assay using bovine serum albumin (BSA) (cat. no. 9048-46-8, Sigma-Aldrich, USA) as standard [20].

2.5. Estimation of malondialdehyde (MDA) level

The MDA assay was used to determine the lipid peroxidation activity. MDA concentration was measured based on the MDA-thiobarbituric acid (TBA) reaction according to the previously described protocol [21]. Briefly, 150 μl of lysates was mixed with 125 μl of 5 mM EDTA, 125 μl of 8 % sodium dodecyl sulfate, 125 μl of 10 % trichloroacetic acid, and 10 μl of 0.5 μg/ml butylated hydroxytoluene and kept for 10 min at room temperature. After that, 500 μl of 0.6 % TBA was added and boiled for 30 min followed by centrifugation at 2,800 g for 20 min. The supernatant was read at 532 nm using a microplate reader (BioTek Cytation5, USA). The MDA concentration was compared with the standard curve using 1,1,3,3-tetraethoxypropane and presented as nmol/mg protein.

2.6. Measurement of reduced glutathione (GSH) level

GSH was used to indicate antioxidant levels in the hippocampus. The reduced GSH concentration was measured based on the reaction between the sulfhydryl group of GSH and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). According to described protocols [22], reduced GSH (cat. no. 70-18-8, Sigma-Aldrich, USA) was dissolved in 0.1 N HCl and used for a standard curve. Then, 20 μl of the standard GSH or tissue lysates was mixed with 250 μl of 0.1 M phosphate buffer pH 7.6, and 50 μl of DTNB (cat. no. 69-78-3 Sigma-Aldrich, USA). The solution was kept in the dark for 5 min, then read at 412 nm using a microplate reader (BioTek Cytation5, USA). The concentration of reduced GSH in the lysates is shown as nmol/mg protein.

2.7. Estimation of catalase (CAT) activity

The activity of CAT was measured based on the enzymatic degradation of H2O2 following published protocols with some modifications [23]. In brief, the enzymatic reaction was initiated by adding 20 μl of lysates to 100 μl of 6 mM H2O2, then incubated at 37 °C for 1 min. Thereafter, the reaction was stopped by adding 100 μl of 32.4 mM ammonium molybdate, then the absorbance was read at 405 nm by a microplate reader (BioTek Cytation5, USA). CAT activity is expressed in units/mg protein.

2.8. Western blotting

The supernatants containing 20 μg of protein were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated in 5 % skim milk at 25 °C for 1 h followed by the incubation with anti-β-actin antibody (1:10,000, cat. no. T0022, Affinity Biosciences, USA), anti-caspase 3 antibody (1:1,000, cat. no. AF6311, Affinity Biosciences, USA), anti-pan-Akt1/2/3 antibody (1:1,000, cat. no. AF6261,

Affinity Biosciences, USA), anti-phospho-Akt1/2/3 antibody (1:1,000, cat. no. AF0016, Affinity Biosciences, USA) or anti-GDNF antibody (1:1,000, cat. no. ab244211, Abcam, USA) at 4 °C overnight. The membranes were then incubated with the appropriate horseradish peroxidase conjugated secondary antibodies goat anti-rabbit (1:5,000, cat. no. AP307P, Sigma-Aldrich, USA) or goat anti-mouse (1:5,000, cat. no. 62–6520, Invitrogen, USA) at 25 °C for 2 h. The protein bands were developed by enhanced chemiluminescence and detected using a chemiPRO XS Western Blot Imaging System (Cleaver Scientific, UK). The protein band density was analyzed using ImageJ software (Pierce, Rockford, IL, USA), and data were normalized to β-actin.

2.9. Statistical analysis

All data analyses were performed using GraphPad Prism software version 9.0. Statistically significant differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data are presented as means ± SEM or means ± SD. Statistical difference was considered when p value is less than 0.05 ($p < 0.05$).

3. Results

3.1. Alteration of seizure score by PLO treatment

Seizure activity was observed at different time points for 90 min after i.p. injection of KA to determine the anti-epileptic effect of PLO. The KA injected mice displayed seizure behavior including myoclonus, rearing, and falling within 20 min after injection. The seizure scores were 3.3 ± 0.6 in KA alone, 3.2 ± 0.4 in KA+100 mg/kg PLO, and 3.0 ± 0.2 in KA+200 mg/kg PLO at 30 min of KA injection. At 60 min, seizure scores significantly decreased in the mice receiving 200 mg/kg PLO (1.7 ± 0.2, $p < 0.05$) when compared to mice receiving KA alone (3.5 ± 0.5). There was no statistical difference in KA+100 mg/kg PLO (3.3 ± 04) when compared to KA alone. The mice treated with PLO continually exhibited decreased seizure scores, whereas the seizure score in KA alone was still high after 90 min of KA injection. Seizure scores were significantly reduced to 1.9 ± 0.5 ($p < 0.05$) and 1.4 ± 0.3 ($p < 0.01$) in KA+100 mg/kg PLO and KA+200 mg/kg PLO, respectively, when compared to KA alone (3.7 ± 0.4) (Fig. 1B).

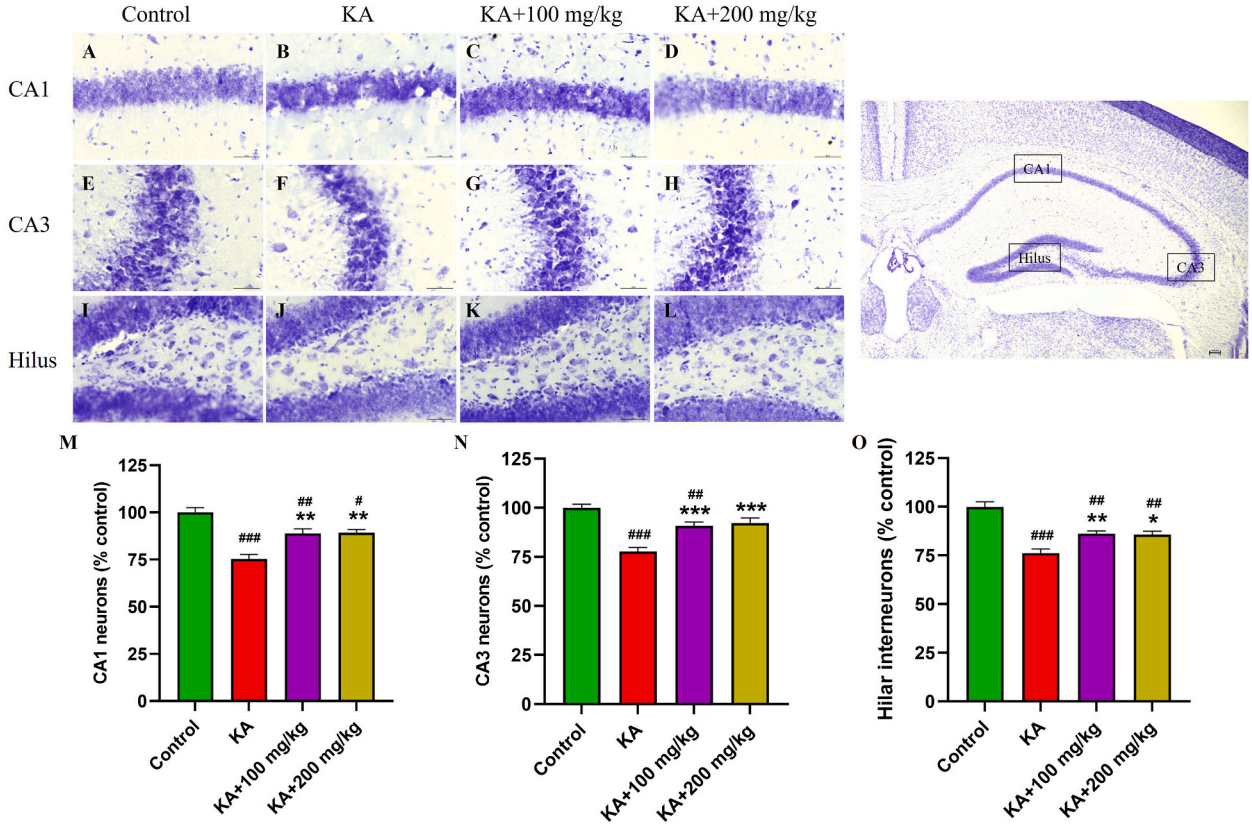

Fig. 2. Effect of PLO on KA-induced neuronal cell death in the hippocampus. Nissl staining of the dorsal hippocampus shows the CA1 region (A–D), CA3 region (E–H) and the dentate hilus (I–L) of the hippocampus. Measurements depict neuroprotective effects of PLO in CA1 (M), CA3 (N), and hilus (O). Scale bar = 50 μm. Data are expressed as mean ± SEM (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus KA alone. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$versus control.

3.2. Histological changes in the hippocampus after KA injection

KA injection produced typical histological changes in CA1 and CA3 subfields of the hippocampus [24]. In this study, KA injection at a dose of 30 mg/kg resulted in the loss of pyramidal neurons in CA1 and CA3, and of interneurons in the hilus that were counted in defined areas of interest using Nissl staining (Fig. 2A–L). In the KA alone group, the percentage of CA1 pyramidal neurons significant decreased to 75.37 ± 2.41 % ($p < 0.001$) when compared to the control group (100 ± 2.58 %). Pre- and post-treatment of PLO at doses of 100 mg/kg and 200 mg/kg significantly increased the percentage of CA1 pyramidal neurons compared to the KA alone group (KA+100 mg/kg PLO: 88.95 ± 2.35 %; $p < 0.01$, KA+200 mg/kg PLO: 89.19 ± 1.80 %; $p < 0.01$) (Fig. 2M). The loss of pyramidal neurons was also confirmed in the CA3 area. KA injection alone led to a significant decrease in CA3 pyramidal neurons (77.79 ± 2.09 %, ($p < 0.001$) when compared to the control group (100 ± 1.80 %). Noteworthy, 100 mg/kg and 200 mg/kg treatment with PLO significantly increased neuron numbers in the CA3 region (KA+100 mg/kg PLO: 90.75 ± 1.99 %; $p < 0.001$, KA+200 mg/kg PLO: 92.24 ± 2.55 %; $p < 0.001$) (Fig. 2N).

Hilar, GABAergic interneurons play an important role in maintaining the balance between excitatory and inhibitory neurons and prevent KA-induced overexcitation resulting in hippocampal cell death [25,26]. Degeneration of hilar interneurons was observed in KA injected mice after 14 days of administration (KA: 76.25 ± 2.03 % versus 100 ± 2.56 % in control, $p < 0.001$). Degeneration of hilar interneurons was decreased in both PLO-treated groups. Pre- and post-treatment of PLO at doses of 100 and 200 mg/kg exhibited a greater number of hilar interneurons as compared to the KA alone group (KA+100 mg/kg PLO: 86.14 ± 1.44 %; $p < 0.01$, KA+200 mg/kg PLO: 85.72 ± 1.72 %; $p < 0.05$) (Fig. 2O).

3.3. Immunohistochemistry findings

Hypertrophic or reactive astrocytes were increased after intrahippocampal or i.p injection of KA in response to inflammatory processes [24,27]. Astrocytes play a crucial role in protecting neurons from hyperexcitation and restoring homeostasis of the brain leading to recovery [28]. Glial fibrillary acidic protein (GFAP) as a marker for reactive astrocytes was detected using immunohistochemical staining on day 14 after KA injection. GFAP positive astrocytes significantly increased in size and number when compared to the control brain (Fig. 3A–D). The quantification of reactive astrocytes in the hilus of PLO treated mice revealed significantly increased number as compared to the KA alone group (control: 100 ± 3.87 %, KA: 128.5 ± 3.22 %, KA+100 mg/kg PLO: 144.0 ± 2.08 %; $p < 0.05$, KA+200 mg/kg PLO: 150.0 ± 5.93 %; $p < 0.01$) (Fig. 3I).

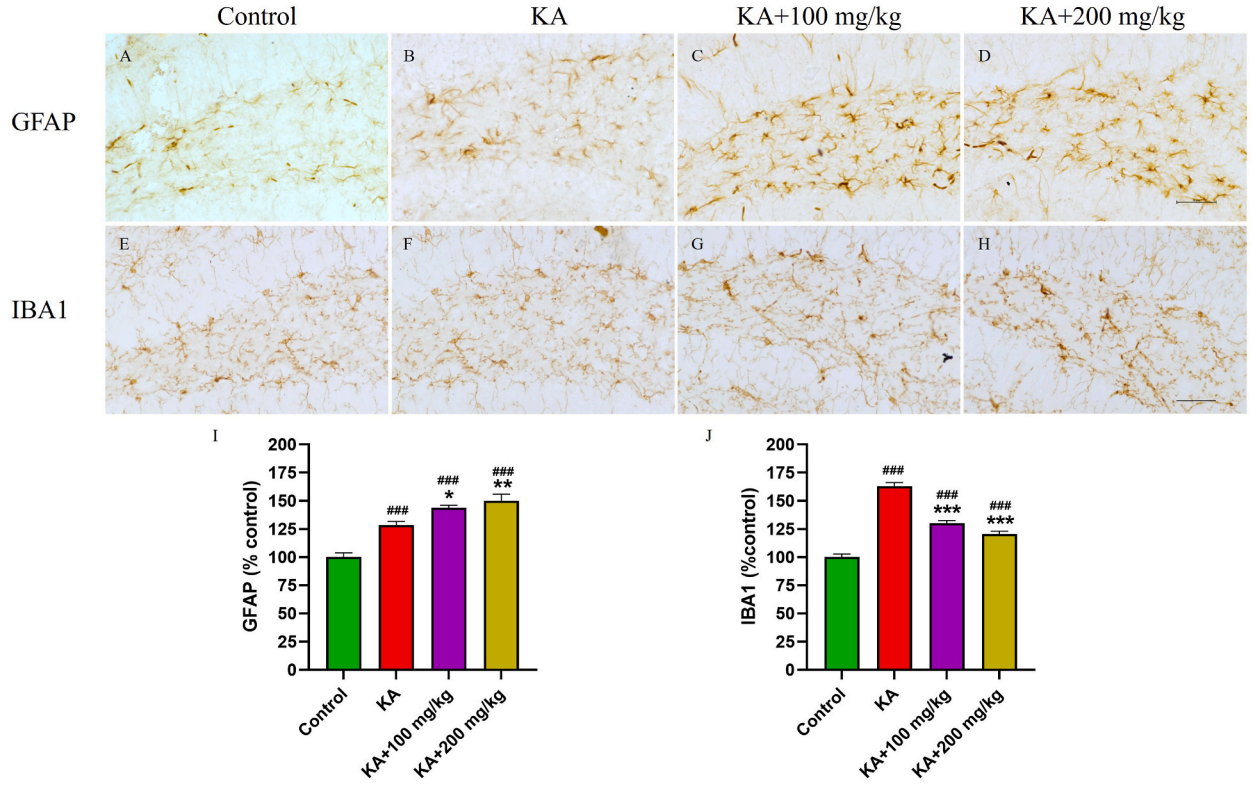

Fig. 3. Alterations in GFAP and IBA1 immunoreactive cells after i.p. injection of KA. GFAP positive astrocytes in the hilus (A–D), and quantification of GFAP expression (I). IBA1 positive microglia in the hilus (E–H), and quantification of IBA1 expression (J). Scale bar = 50 μm. Data are expressed as mean ± SEM (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus KA alone. ###$p < 0.001$versus control.

Activated microglia has been reported to be involved in inflammatory processes in the lesioned brain, and KA leads to an over-activation of microglia causing a massive secretion of inflammatory cytokines, ROS and RNS resulting in neuronal cell death [29, 30]. Two weeks after KA administration, activated microglia was stained by ionized calcium binding adaptor molecule 1 (IBA1) antibodies. In this study, KA injection showed an increase in number of IBA1 positive cells in the hilus (Fig. 3E–H), however, mice treated with PLO exhibited significantly decreased percentages of IBA 1 positive cells in the hilus when compared to the KA alone group (control: 100 ± 2.92 %, KA: 163.2 ± 3.14 %, KA+100 mg/kg PLO: 130.3 ± 2.34 %; $p < 0.001$, KA+200 mg/kg PLO: 120.5 ± 2.63 %; $p < 0.001$) (Fig. 3J).

3.4. Effect of PLO on antioxidative activities after KA injection

Administration of KA triggers a high production of free radicals, a cause of oxidative stress that leads to neuronal cell damage and apoptosis [8]. After treatment with increasing concentrations of PLO, the endogenous antioxidants including GSH and CAT were evaluated to confirm the antioxidative activities of PLO. Treatments of PLO at doses of 100 and 200 mg/kg significantly increased GSH levels when compared to the KA alone group (control: 1.88 ± 0.22 mM/mg protein; $p < 0.01$, KA: 0.74.2 ± 0.12 mM/mg protein, KA+100 mg/kg PLO: 1.88 ± 0.26 mM/mg protein; $p < 0.01$, KA+200 mg/kg PLO: 2.16 ± 0.08 mM/mg protein; $p < 0.01$) (Fig. 4A). Moreover, PLO treatments showed a significant increase in CAT activities. After KA injection, mice in the KA alone group revealed a significant reduction in CAT level (8.64 ± 1.43 U/mg protein) when compared to control mice (18.41 ± 1.43 U/mg protein, $p < 0.01$). However, PLO treatments significantly increased in CAT levels when compared to the KA alone group (KA+100 mg/kg PLO: 15.00 ± 1.73 U/mg protein; $p < 0.05$, KA+200 mg/kg PLO: 14.93 ± 1.15 U/mg protein; $p < 0.05$) (Fig. 4B). Oxidative stress induced by KA injection was also evaluated using the MDA assay. After KA injection, the MDA content as an indicator of lipid peroxidation significantly increased in the KA alone group (16.71 ± 0.49 mM/mg protein) as compared to the control group (12.52 ± 0.64 mM/mg protein, $p < 0.05$). However, treatment with PLO resulted in a significant reduction of MDA content (KA+100 mg/kg PLO: 12.55 ± 0.37 mM/mg protein; $p < 0.05$, KA+200 mg/kg PLO: 9.98 ± 1.27 mM/mg protein; $p < 0.001$) (Fig. 4C). These results suggest that neuroprotective effects of PLO against KA-induced neuronal cell death might be due to its ability to mitigate oxidative stress in neurons.

3.5. Alterations in protein expression after KA injection

Western blotting was used to detect the changes in expression of Akt, GDNF and caspase-3 (Fig. 5). The protein kinase B (Akt) signaling pathway plays a crucial role in various cellular processes including proliferation, migration, and survival [31–33]. According to our results, PLO treatment promoted neuronal survival as indicated by significant increases in relative p-Akt/Akt expression when compared to the KA alone group (Fig. 5A). The relative expression of p-Akt/Akt was dramatically increased in mice receiving a PLO dose of 200 mg/kg (1.01 ± 0.23 in the 200 mg/kg versus 0.53 ± 0.13 in the KA alone group; $p < 0.01$). A significant difference of relative p-Akt/Akt expression was also found in the 100 mg/kg PLO group as compared to the KA alone group (0.88 ± 0.10; $p < 0.05$ versus KA alone group). The p-Akt/Akt levels were not different between mice in the PLO-treated groups and those in the untreated control group (0.70 ± 0.17 in normal control group) (Fig. 5B).

Cysteine-aspartic acid protease or caspase-3 is an enzyme involved in cellular apoptotic processes. Activated or cleaved caspase-3 has been shown to play a major role in neuronal apoptosis induced by KA [34]. After KA injection, the relative expression of caspase-3

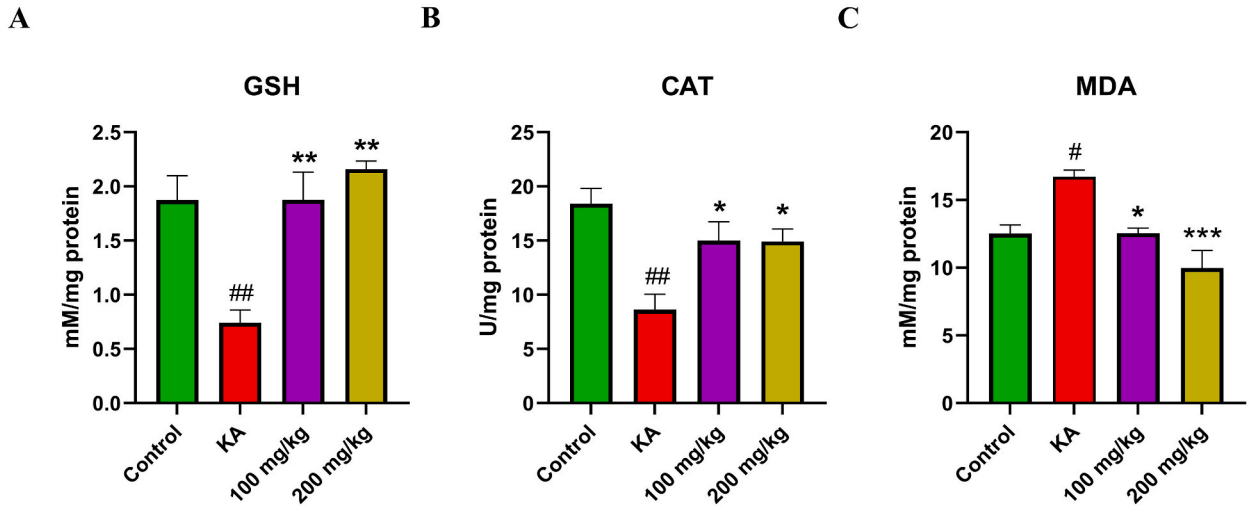

Fig. 4. Alterations in endogenous antioxidants and MDA after i.p. injection of KA. Treatment with PLO caused an upregulation of GSH (A) and CAT (B) as antioxidative enzyme, whereas MDA, a marker for oxidative stress, is decreased in the PLO-treated groups (C). Data are shown as mean ± SEM ($n = 5$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus KA alone. #$p < 0.05$, ##$p < 0.01$ versus control.

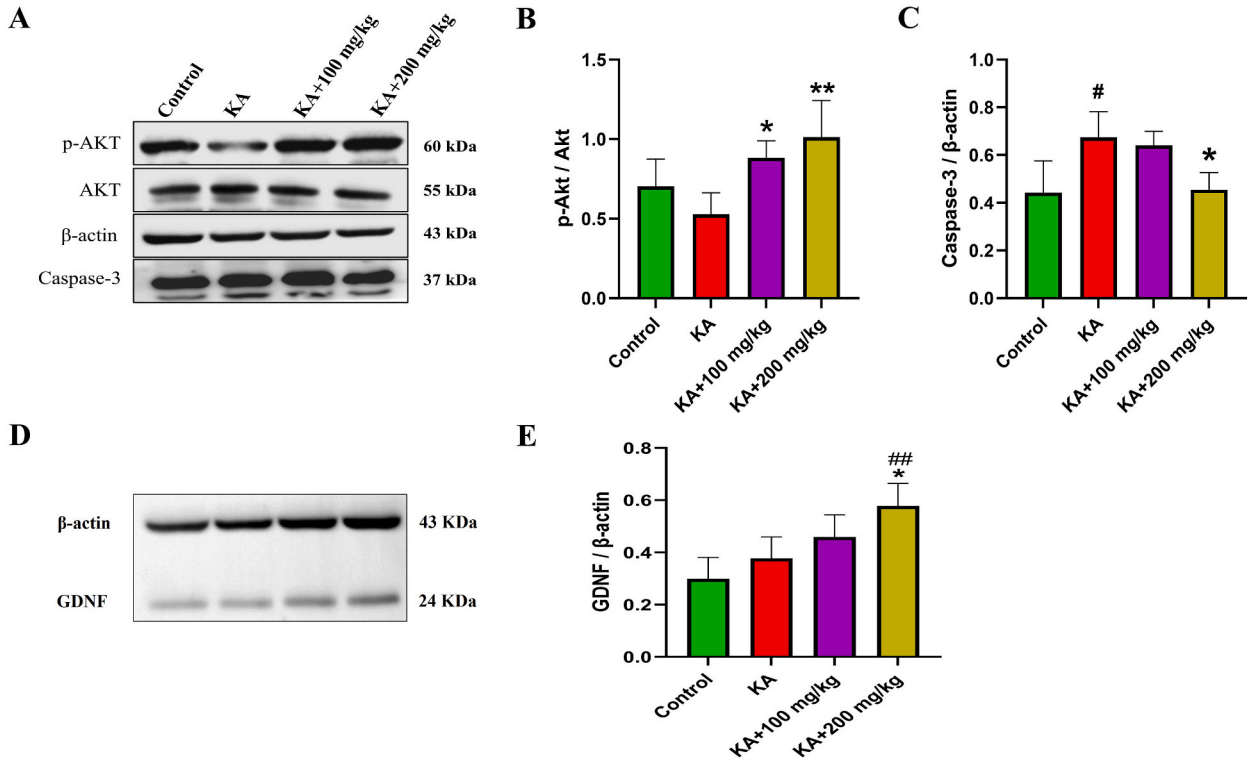

Fig. 5. Effect of PLO treatment on Akt, p-Akt, and caspase-3 expression after KA injection (A). Quantification of p-Akt/Akt and caspase-3 levels (B–C). GDNF protein expression after KA injection (D) with quantification (E). Data are shown as mean ± SD (n = 5). *p < 0.05, **p < 0.01 versus KA alone. #p < 0.05, ##p < 0.01 versus control. The original images for all relevant western blots have been provided as supplementary data.

was increased in the KA-injected mice when compared to the control group (Fig. 5A). PLO treatment at a dose of 200 mg/kg revealed a significant reduction of caspase-3 expression when compared to the KA alone group (0.44 ± 0.13 versus 0.67 ± 0.11, p < 0.05, 0.64 ± 0.06 in the 100 mg/kg PLO group) (Fig. 5C).

Glial cell line-derived neurotrophic factor or GDNF is considered as survival factor for neurons and has been shown to protect hippocampal neurons from death induced by KA [35,36]. In this study, pre- and post-treatment of PLO at a dose of 200 mg/kg showed a clear increase in GDNF expression as compared to the control group and the KA alone group (0.29 ± 0.08 in the control group, 0.37 ± 0.08 in the KA alone group, 0.58 ± 0.09 in the 200 mg/kg PLO group; p < 0.01 versus control and p < 0.05 versus KA alone). However, the expression of GDNF protein was not significantly different between the control group, the KA alone group and the KA+100 mg/kg PLO group (0.046 ± 0.08 in 100 mg/kg) (Fig. 5D and E).

4. Discussion and conclusion

Excitotoxicity-induced oxidative stress leads to neuronal cell death and is considered as one of the etiologies of neurodegeneration in epilepsy [6,8]. It has been reported that the overexcitability of neurons causes spontaneous seizures and ongoing epileptic activities [2]. Previous research has shown that ethanol extraction of pink lotus fruit displays antiepileptic activities as delaying the onset of convulsions [37]. So far, many studies reported that antioxidative substants can inhibit the mechanisms involved in seizures production such as the inhibition of opioid receptors resulting in anticonvulsant effects [38] and the antioxidative effect of flavonoids can significantly increase in seizure threshold [39]. In the present study, PLO which contains antioxidative activity revealed the antiepileptic activity resulted in significant decrease in seizure score within 90 min after KA injection.

Administration of KA results in mitochondrial dysfunction through an over-stimulation of kainate receptors causing high Ca2+ influx into neurons. Mitochondrial failure results in massive production of ROS and activation of caspase-3 which leads to progressive neuronal apoptosis [6,40]. Pink lotus has antioxidative, anti-inflammatory, and neuroprotective effects probably because of its rich content in cyanidin, quercetin and naringenin [41,42]. Our previous study reported that PLO contains fatty acids such as linoleic acid and palmitic acid [15], which have been shown to increase antioxidants via the AMPK signaling pathway [43]. In this study, systemic injection of KA displayed an increase in oxidative stress as indicated by high expression of MDA levels, whereas antioxidative GSH and CAT levels were decreased. Noteworthy, PLO at doses of 100 mg/kg and 200 mg/kg could restore the antioxidative activities by increasing GSH and CAT and decreasing the MDA level.

Neuronal cell death occurs in all regions of the hippocampus but mostly in the CA3 area which leads to epileptic seizure after administration of KA [44–46]. In this study, KA-induced neuronal loss occurred in CA1, CA3 and in the hilus region. However,

treatment with 100 mg/kg PLO or 200 mg/kg PLO showed neuroprotective effects as revealed by an increase in number of CA1 and CA3 neurons as well as of hilar interneurons. Moreover, treatment with PLO decreased the seizure score which correlated with the higher number of hippocampal neurons and interneurons.

Microglial activation and reactive astrogliosis are known to play an important role in response to inflammation following status epilepticus induced by KA [47]. Activated microglia was detected in CA1, CA3 and in the hilar subfields of the hippocampus with their morphology changing to enlarged and elongated cell bodies as well as dense and large processes after prolonged status epilepticus [48]. Microglia activation releases pro-inflammatory cytokines, i.e. tumor necrosis factor alpha (TNF-α), interleukin-1β and interleukin-6 leading to progressive neurodegeneration [49]. Attenuation of microglial activation clearly reduces inflammation and neurodegeneration in the hippocampus following KA-induced seizure [50]. In the present study, we observed a marked reduction of activated microglia after 100 mg/kg PLO and 200 mg/kg PLO treatment. This clearly suggested that neuroprotective effects of PLO can be at least partially ascribed to its anti-inflammatory effect. In recent years, studies show that palmitic acid, a long-chain saturated fatty acid and linoleic acid, a polyunsaturated fatty acid in PLO can induce microglial apoptosis and inhibit microglia-induced neuroinflammation [51–53]. Therefore, the anti-inflammatory effect of PLO may involve fatty acids inhibiting microglial activation.

Status epilepticus induces reactive astrogliosis by an increase in astrocyte population as well as inducing morphological and functional changes [47]. Reactive astrocytes protect neurons from hyperexcitability-induced cell death by uptake of glutamate [28], and by stabilizing the ion balance for reducing the seizure threshold [54], and increasing glutathione production [55,56]. Our findings indicate that PLO enhances reactive astrocyte formation leading to neuroprotection in a mouse model of KA-induced epilepsy.

Furthermore, we investigated the effect of PLO on the activation of the Akt signaling pathway and on caspase-3 and GDNF protein expression after KA-induced status epilepticus. Treatment with 100 mg/kg PLO and 200 mg/kg PLO clearly increased p-Akt and GDNF levels. Phosphorylated Akt plays an important role in various cellular responses such as proliferation, growth and survival following stimulation of PI3K [57]. Previous studies have shown that enhanced Akt phosphorylation in astrocytes through the eNOS/sGC/PKG/PI3K pathway results in the up-regulation of neuroprotective and pro-survival transcription factors [58]. Moreover, several studies have indicated that stimulation of extracellular signal-regulated kinase 1/2 (ERK1/2) also contributes to improved cell survival [24,58]. Other reports demonstrated that increased p-Akt and GDNF levels in astrocytes protect neurons from KA-induced neuronal death [59]. Several findings have demonstrated that GDNF significantly decreases hippocampal cell loss and epileptic seizure following KA injection [36,60]. Additionally, our previous study showed that increases in GDNF expression results in protection of hippocampal neurons in a model of neurotoxicity-induced Parkinson's disease [61]. Furthermore, treatment with 200 mg/kg PLO showed a significant decrease in caspase-3 expression. Since activation of caspase-3 has been shown to cause apoptotic neurodegeneration induced by KA in the hippocampus [62], PLO may ameliorate KA-induced apoptotic neurodegeneration through inhibition of caspase-3 [63,64].

In summary, the present study demonstrated that administration of PLO increases antioxidant activities, Akt phosphorylation, and GDNF expression while reducing oxidative stress, inflammation, and apoptosis resulting in reduced hippocampal cell loss and decreased seizure behavior in a mouse model of KA-induced epilepsy. These findings suggest that PLO at high dose may provide a novel pharmacological treatment for the therapy of neurodegenerative diseases including seizure-induced neuronal loss.

Ethics statement

All animal procedures were performed in accordance with the institutional guidelines and approved by the Animal Ethics Committee of the University of Phayao, Thailand (approval no. 1-026-65).

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Additional information

No additional information is available for this paper.

Data availability statement

All data analyzed during this study are provided in this article, there is no necessary to deposit data into a publicly available repository. However, the data are available upon request.

CRediT authorship contribution statement

Ratchaniporn Kongsui: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Teera Chanmanee:** Validation, Methodology, Investigation, Data curation. **Tichanon Promsrisuk:** Methodology, Formal analysis, Data curation. **Lars Klimaschewski:** Writing – original draft, Data curation. **Napatr Sriraksa:** Methodology, Data curation. **Jinatta Jittiwat:** Methodology, Data curation. **Sitthisak Thongrong:** Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38070.

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