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Title: Origanum Vulgare Prevents Kaolin-Induced Hydrocephalus by Regulating the Expression Levels of GFAP and lba1 Proteins in the Brain of Rats

Running Title: Preventing the Progress of Kaolin–Induced Hydrocephalus in Rat

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Abstract

Introduction: Reactive gliosis contributes to damage and recovery patterns in hydrocephalus. The purpose of the research was to evaluate the impact of Origanum vulgare essential oil (OEO) on reactive astrogliosis and decreased lateral ventricle thickness in a rat model of kaolin-induced hydrocephalus.

Methods: 30 male Wistar rats were injected with 25% kaolin directly into the cisterna magna. After 21 days post-injection of kaolin, hydrocephalic rats were randomly divided into four groups, and the presence of hydrocephalus was confirmed by magnetic resonance imaging (MRI) to determine the lateral ventricle volume. Rats received different doses of OEO for 10 days. Also, to assess the expression levels of Iba-1 and GFAP proteins, immunohistochemical and western blot analyses were performed 10 days after the treatment course.

Results: The size of the lateral ventricle in hydrocephalic rats treated with OEO was less than in those treated with kaolin alone. Based on GFAP and Iba-1 immunostaining data, hydrocephalic rats showed more reactive astrocytes and activated microglia than OEO-treated animals. The findings of the immunohistochemistry analysis were also validated by western blot. In terms of GFAP and Iba-1 expression levels, there was a significant difference between the hydrocephalic control group and rats treated with OEO at a dose of 200 mg/kg/day.

Conclusion: In comparison to hydrocephalic rats, the OEO-treated groups exhibited stable ventricular dilatation. According to the results of this research, OEO may reduce hydrocephalus-related damage in kaolin-induced rat brains, potentially through lowering GFAP and Iba-1, and so OEO intervention might be effective as a novel treatment technique.

Keywords: *Origanum vulgare*; Hydrocephalus; GFAP; Iba-1; Reactive astrocytes; Microgliosis

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Highlights

- ✓ Reactive astrocyte and microglial activation have been considered as the cellular pathological hallmark in hydrocephalus (GFAP, Iba-1) by kaolin.
- ✓ *O. vulgare* extract displays anti-inflammatory and antioxidant activity in cell and animal models in kaolin-induced rat brains.

Plain Language Summary

When cerebrospinal fluid accumulates excessively in the ventricles and cavities of the brain, it leads to an increase in volume (hydrocephalus), and this increase in volume in the central nervous system can be caused by a disorder in the distribution, formation, flow or absorption of cerebrospinal fluid. The result is increased pressure inside the skull and brain damage and even death. Hydrocephalus may be a congenital or acquired abnormality that leads to abnormal skull enlargement. In mild cases, medicinal compounds are prescribed to reduce the cerebrospinal fluid, and the drugs themselves have side effects. In the past, it has been shown that the intra-cisternal injection of kaolin is a suitable model for creating hydrocephalus in the laboratory. In this study, kaolin injection caused hydrocephalus in more than 90% of the studied mice. Brain MRI of the rats showed this increase in volume within 3 weeks. In this study, it was shown that intraperitoneal injection of *Origanum vulgare* essential oil prevents lateral ventricle enlargement in hydrocephalus model caused by intraventricular injection of kaolin. Therefore, in related cases that occur pathologically, it may be effective in treatment.

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1. Introduction

Hydrocephalus is a neuroanatomical disorder in which the secretion, absorption, and circulation of the cerebrospinal fluid (CSF) are disturbed. This condition leads to an enlarged ventricle filled with cerebrospinal fluid and obstruction of cerebrospinal fluid flow. Several congenital or environmental factors are involved in the pathogenesis of hydrocephalus and can cause damage to the surrounding brain tissues. Axonal damage in the periventricular white matter is one of the initial ventricular dilatation consequences in both animals and humans. The accumulation of metabolic by products, compression, progressive physical stretching, and ischemia are pathophysiologic outcomes of hydrocephalus-induced brain injury.(Khan et al., 2003)

Hydrocephalus has been linked to gliosis and neuroinflammation. In a hydrocephalic brain, active astrocytes and microglia cause fibrosis and dysfunction.(Hao Xu et al., 2012) In order to explore brain injury pathogenesis experimentally, many approaches are used to induce hydrocephalus.(Slobodian et al., 2007) The most typical procedure for this purpose is kaolin (aluminum silicate) injection into the cisterna magna since it is inexpensive, simple to perform, reliable, minimally invasive, leaves no visible scar, and mimics hydrocephalus following meningitis.(Olopade et al., 2019; Slobodian et al., 2007) In addition, animal models of kaolin-induced hydrocephalus that are linked with activated astrocytes and microglia have been established with the goal of researching neuroinflammatory responses to ventricular enlargement in newborns, adolescents, and adults.(Olopade et al., 2019)

Origanum vulgare is an aromatic plant in the *Lamiaceae* family with high phenolic content that has therapeutic benefits in a variety of diseases and clinical symptoms such as digestive and respiratory disorders. In traditional medicine, this plant is used as a sedative, hepato-protective, antioxidant, anticancer, anti-diabetic, anti-inflammatory, antiviral, and antifungal agent and is effective in relieving pain in rheumatic arthritis.(Bahmani et al., 2018; Chuang et al., 2018; Leyva-López et al., 2017) Ethnobotanical and ethnopharmacological studies have shown the traditional effect of this plant as a medicine in the treatment of different diseases.(Bahmani et al., 2018) For example, a study conducted by Sajadi (2011) demonstrated that the aerial parts of this herb have compounds that improve the neurological functions. Another study performed by Zolfeghari et al. (2012) showed the therapeutic effects of this plant in flowering branches could enhance the nervous system function and reduce migraine pain. Recently, *O. vulgare* extract has been shown

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to have a protective effect on the brain in an animal model of stroke by reducing brain edema and neurological deficits.(Foroozandeh et al., 2014) Due to the scarcity of data on support for patients with hydrocephalus, the current study aimed to investigate the effect of the herbal essential oil from plant leaves, namely OEO, on experimentally-induced hydrocephalus. The primary goal of this research was to develop a novel pharmaceutical strategy for brain damage treatment prior to shunt therapy. This research hypothesized that OEO administration might enhance health status structurally and/or biochemically in a rat model of kaolin-induced hydrocephalus.

We established a murine model of kaolin-induced hydrocephalus and administered OEO to the animals. Then, we employed the MRI technique to assess lateral ventricular hypertrophy in experimental groups. The effect of OEO on gliosis reduction was studied to suggest it as a novel treatment strategy. To this aim, the expression levels of GFAP (glial fibrillary acidic protein) and Iba-1 (ionized calcium binding adaptor molecule 1) proteins were assessed, and immunohistochemical studies were performed for both of these proteins.

2. Materials and methods

Animals

Thirty adults male Wistar rats, 250–300 g weight and 8–10 weeks age, were provided by Iran University of Medical Sciences (Tehran, Iran). Animals were placed in cages with controlled conditions, 21–22°C temperature, 34%-35% humidity, a 12/12 hours light/dark cycle, and free access to water and food.(Nirogi et al., 2009)

The animals were randomly divided to five groups: 1- group receiving injections of normal saline (n=5), 2- group receiving injections of 25% kaolin (n=10), 3- group receiving injections of 50 mg/kg/day OEO for 21-days after the injections of 25% kaolin (n=5), 4- group receiving injections of 100 mg/kg/day OEO for 21-days after the injections of 25% kaolin (n=5), 5- group receiving injections of 200 mg/kg/day OEO for 21-days after the injections of 25% kaolin (n=5).

The experimental protocol was approved by Iran University of Medical Sciences' Committee on Ethics in Research (Research Code: 27517) (Ethical Code: IR.IUMS.REC 1395. 118.27517). The experiment was also designed in compliance with the ARRIVE Guildlines (Animal Research: Reporting of *In vivo* Experiments) guidelines and all technical and ethical considerations were taken into account.(Kilkenny et al., 2010; McGrath et al., 2010)

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Plant material

Oregano (*Origanum vulgare*), sometimes called wild marjoram, and its close relative, *O. majorana*, is known as sweet marjoram. The *Origanum vulgare* samples were obtained from a local green market in Tehran, and to confirm authenticity, samples were transferred to Dr Amin, herbarium of the School of Pharmacy, Tehran University of Medical Sciences. (Herbarium No. PMP384). The essential oil was obtained from dried plant several rounds (100 grams each time) and used for the study. The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus for 3 h from 50 to 100 g of air-dried leaves and shoots of the plant. Oil yields were then estimated on the basis of the dry weight of plant material. The yield in this procedure was 1.2%. Oils were recovered directly, from above the distillate, and stored in dark vials at 4°C.

Gas chromatography-mass spectrometry analysis (GC-MS)

The qualitative and quantitative analysis of the sample was carried out using GC/MS technique (Rtx-5MS, 30m× 0.25 mm× 0.25µm). The sample was injected using the split mode, helium, as the carrier gas passed with a flow rate of 0.5 mL/min. an Agilent 5973N mass detector was used for the separation and detection of chemical compounds. The chemical compounds were identified and the data are presented in Table 1. Identification of components for the sample was achieved by comparing their retention index and mass fragmentation patents with those available in the library, the National Institute of Standards and Technology (NIST).

Hydrocephalus induction

Hydrocephalus was induced by 25 µl sterile suspension of 25% kaolin (aluminum silicate; Sigma, 1332-58-7). In brief, 25 rats received kaolin injections and 5 received saline injection. After given a mixture of 75 mg/kg ketamine and 4mg/kg xylene, the animals were placed in a stereotaxic frame. The fur on the back of animal's neck was shaved and disinfected with chlorhexidine and 70% ethanol. The rat's head was positioned downward at a 45° angle. To collect CSF without incision, a sterile 23-gauge needle (shallow bevel tip) was horizontally and centrally inserted into the cisterna magna under aseptic condition; afterwards, a 25% sterile kaolin suspension was inoculated into the place at the rate of 6 µL/s and the controls received normal saline.(Nirogi et al., 2009) After recovering from anesthesia, the rats were then placed on a heating pad in order to

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monitor their neurological signs. To prevent further suffering, rats with weight loss or neurological disturbances were euthanized.(Hao Xu et al., 2012)

Magnetic resonance imaging and lateral ventricular volume measurement

After inducing anesthesia with 75 mg/kg ketamine, animals underwent brain MRI twice. T_2–weighted MR images were obtained at 3.0-Tesla on a horizontal-bore small animal MRI scanner (Center for Brain Mapping at Tehran University of Medical Sciences, Iran). The coronal MR images obtained from rats in both the hydrocephalous and saline control groups were used to measure the size of lateral ventricle. The first MRI was performed 21 days post kaolin injection to confirm the induction of hydrocephaly. After confirmation of the enlargement of the brain in hydrocephalus group compared to the saline group, the second MRI was obtained 10 days post kaolin injections with different experimental groups including, hydrocephalus group without treatment (number=10) and treatment groups at three OEO doses of 50, 100, and 200 mg/kg/day, ip, each treatment group contain 5 rats. For control group (0.9 % NaCl), MRI was also performed. Lateral ventricle was measured by Image J software (version 1.5). After measurement of the volume of lateral ventricle, the brains were detached and divided into two parts for further studies.

Drug preparation and administration

As mentioned earlier, animals received different doses of OEO and/or NaCl via injection into cisterna magna daily, after 21 days post-kaolin administration. OEO treated animals received OEO (prepared in laboratory), suspended in 0.9 % normal saline and were prepared at room temperature (RT). Animals received drug injections randomly based on the ratio of concentration to body weight.

Sacrifice and brain dissection

Rats were deeply anaesthetized with ketamine (75 mg/kg) and the vascular system was cleaned by transcardiac perfusion of 200 mL saline and 600 mL 4% paraformaldehyde solution (pH 7.4). To perform immunohistochemistry, following embedding in paraffin, the brain samples were processed after maintaining in 4% (PFA) for four days at 4°C. The samples were cut into 8-µm thick coronal sections.(Sajadian et al., 2015)

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Immunohistochemistry

To detect the expression level of GFAP and Iba-1, 8-µm thick paraffin-embedded brain sections were immunohistochemically stained. The sections were cleaned and rehydrated in xylol and ethanol, from 100% to 30%, and then rinsed in triplicate with phosphate buffered saline (PBS). The slides were incubated in the blocking solution (3% H_2O_2/methanol) for one hour. To facilitate antigen exposure for labeling, PBS was used to wash the slides, and then the antigen retriever solution (sodium citrate buffer 1 M at 95°C; pH 6.0) was added for 10 minutes.

After boiling, slides were kept in Room temperature for 20 minutes and then rinsed with distilled water and IHC washing solution each for five minutes. Then the slides were incubated for one hour following adding the blocking solution.

To label astrocytes and reactive astrocytes, the slides were incubated with rabbit polyclonal anti-GFAP (1:10,000 dilution), and for activated microglia labeling, with rabbit polyclonal Iba-1 (1:5000 dilution) (Abcom Biotechnology, USA) and left overnight in a moist chamber at 4°C.

Afterward, PBS was used to wash the sections in triplicate (each 10 minutes); then they were incubated with biotinylated secondary antibody. The sections reacted with streptavidin-peroxidase and then with 3-3′diaminobenzidine (Roche; 0.5 µL DAB and 1.5 µL peroxide buffer) for 5–10 minutes as chromogen substrates.(Sajadian et al., 2015)

Western blotting

Following the decapitation of anesthetized rats, the brains were dissected on ice after removal, placed in sterile tubes, immersed in liquid nitrogen, and kept at -80°C until use. Samples obtained from the control groups (n=4), hydrocephalic + OEO (n=5 for each concentration), and hydrocephalic (n=7) groups were separately kept in vials containing RIPA buffer [1% triton X-100, 1% sodium deoxycholate, 50 mM $NaCl_2$, 50 mM tris-HCl, 1 mM sodium vanadate, 2 mM phenylmethanesulfonyl fluoride] with a protease inhibitor tablet (Sigma) and then homogenized by ultrasound. Then, the vials were centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatants were collected for Bradford protein quantification. Proteins from the sham, hydrocephalic, and OEO + hydrocephalic groups were separated on 10% SDS-polyacrylamide gel in 1×Tris/Glycine/SDS buffer (90 minutes, 150 V, RT). Separated proteins were transferred to a PVDF membrane (30 minutes, 100 V, 4°C), soaked in Tris-buffered saline +Tween (TBS-t), and blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 for one hour

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at RT. Then, the membranes were incubated with primary rabbit anti-GFAP antibody (Abcom, ab7260, Cambridge US.), primary rabbit anti-Iba1 antibody (Abcom, ab178846, Cambridge US.), and the reference protein B-actin (Abcom, ab8226 Cambridge US.) with 1:10000 dilutions at 4°C overnight. Afterwards, the samples were washed with TBS-t in triplicate. Secondary antibody, peroxidase-conjugate anti-rabbit IgG (Abcom, ab6721, Cambridge US.), was incubated for one hour at a dilution of 1:1000 at RT. Finally, chemiluminescence reagent (ECL, Amersham Bioscience, USA) was added to the membrane and signals were detected. To quantify Western blots, densitometric readings from the appropriate bands (a 48-kDa GFAP and 17-kDa Iba-1) were obtained. The GFAP and Iba-1 relative intensities were divided by the loading control B-actin (42 kDa) relative intensity in order to quantify the bands. Image J version 1.5 was employed to measure the density of bands.

Statistics

Data were expressed as mean±standard error. To compare data obtained from groups, one-way ANOVA with Dunnett post hoc and two-tailed *t* tests were used. The level of significance was $P \leq 0.05$. Data were analyzed in Prism version 7.

3. Results

GC-MS analysis data

According to the GC-MS analysis, OEO was made up of 35 compounds (representing 99.75%). The main component was Thymol (28.32%), followed by γ-Terpinene (11.22%), Terpinene-4-ol (7.91%), O-Cymene (6.55%), Cis-β-Terpineol (5.58%), α-Terpinene (5.44%), Carvacrol (3.50%), α-Thujene (3.44%), α-Thujone (2.99%), Caryophyllene (2.22%), Sabinene (2.09%), trans-Sabinene hydrate (2.07%), as shown in Table 1. These components (12) accounted for 81.33% of the yield, while the other detected components represented (Fig. 1 and Table 1).

Mortality

From 25 rats receiving kaolin, four died shortly after injection due to tonic-clonic seizure. Gradual reversion of anesthesia averted seizure. Totally, five rats (four kaolin-injected, one saline-injected)

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died. The three-week intervention was completed with the remaining animals, ultimately sacrificed for analysis.

Effects of OEO on lateral ventricular volume of rat hydrocephalus model

A moderate enlargement in lateral ventricle was observed in all rats receiving kaolin, based on MR images obtained three weeks after the injection. A progressive enlargement of lateral ventricle was observed in MR images obtained 31 days after the injection in the hydrocephalus treatment groups (Fig. 2A and B). However, lateral ventricle size for OEO treatment groups were significantly smaller (0.039±0.001, 0.037±0.001, 0.033 ±0.002 for 50, 100 and 200 respectively) compared with hydrocephalus group (0.065±0.005) at 31 days after kaolin injection. Although there were no significant differences between three concentrations of OEO, 200 mg/kg/day OEO treatment group had a smaller ventricular volume compared to other OEO concentrations (0.033 ±0.002).

Immunohistochemistry

Reactive astrocyte is the pathological hallmark of hydrocephalus. The immunohistochemistry for GFAP expression was performed in the study to evaluate OEO effects on astrocyte reactivity (Fig. 3A and 3B).

Some reactive astrocytes in lateral ventricle were found by immunohistochemical staining for GFAP (Fig. 3A). Significant differences were observed between the treatment groups in the semi-quantitative grading of GFAP immunolabeling in the white matter of the lateral ventricle. After induction of hydrocephalus, the mean percentage of positive reacted cells in lateral ventricle was significantly higher in the kaolin groups than the sham control (47.32±4.5% vs 2.66±0.6%). The GFAP immunostaining level in the kaolin + OEO 200, 100, and 50 mg/kg groups (11.54±0.7%, 22.46±0.9%, and 34.33±2.3% respectively) were considerably lower than that of the kaolin group in a dose-dependent manner. However, 200 mg/kg OEO group was comparable to sham control group (Fig. 3A and 3B).

All the hydrocephalic rats showed reactive microglia in immunostaining for Iba-1 (Fig. 4A and B). Significant differences among the treatment groups were observed by semi-quantitative grading. After induction of hydrocephalus, the mean percentage of positive reacted cells was

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significantly higher in the lateral ventricle of the hydrocephalus group than the sham control (43.66± 0.7% vs 2.43± 0.5%). In the OEO groups, expression of Iba-1 was decreased compared with hydrocephalus group in a dose-dependent manner and 200 mg/kg/day OEO treatment group had the smallest expression than two other doses.

The Western blot

The results of Western blot analysis on day 31 showed that the GFAP and Iba-1 expression levels were significantly higher in hydrocephalic rats than the sham control. The GFAP expression measured by Western blot, 31 days after kaolin injection demonstrated a decrease in 50, 100, and 200 mg/kg/day OEO groups by 1.21 ± 0.16%, 1.09 ± 0.09% and 0.85 ± 0.05%, respectively, relative to hydrocephalus group, however this effect did not differ significantly between these doses. In hydrocephalic groups treated with 50, 100 and 200 mg/kg/day of OEO, Iba-1 expressions decreased by 1.25 ± 0.04%, 1,11± 0.02 and 0.86 ± 0.01, respectively compared with hydrocephalic group alone (Fig. 5).

4. Discussion

Several approaches for developing an animal model of the disease currently exist to investigate the etiology, pathophysiology, and neurological abnormalities of hydrocephalus, as well as analyze different surgical and/or non-surgical treatments for its treatment.(Suryaningtyas et al., 2019)

When the CSF circulation is blocked, the cerebral ventricles become enlarged. Successful and established models have been used for more than 50 years to induce acute hydrocephalus, inflammatory responses, and gliosis in the posterior fossa of rats and larger animals by injecting stimuli, such as kaolin, into the cisterna magna. However, the mode of action of these factors, as well as the deficiency produced by them, remain unknown. There is a lack of information on the involvement of gliosis—for example, reactive astrocytosis and microgliosis—and neuroinflammation in the etiology of hydrocephalus. Further research into gliosis and gene expression may aid in the diagnosis and treatment of the hydrocephalus brain.(Bloch et al., 2006; Deren et al., 2010; Suryaningtyas et al., 2019)

Mechanical and biochemical cellular changes are among the factors that play a role in the pathophysiology of brain damage caused by hydrocephalus. Ependymal cells are the first structures to be affected by mechanical damage and appear through cell loss, denudation, and

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detachment. According to evidence, ependymal cells in the adult brain contribute to the proliferation and migration of progenitor cells in the subventricular zone (SVZ) using a branching fractal structure known as a fracton. Fractone, as an extracellular matrix, promotes neural stem cell production by allowing cytokine penetration into the SVZ. Fractones in hydrocephalus enable inflammatory cytokines to penetrate the SVZ and limit production. Detachment of the ependymal layer of the lateral ventricles causes SVZ disruption, loss of germinal zone, and aberrant migration of neuroblasts to the ventricle in hydrocephalic embryos.(Suryaningtyas et al., 2019)

O. vulgare is an herbaceous shrub native to the Mediterranean region and temperate regions of western and southwestern Eurasia.(Chuang et al., 2018) *O. vulgare* has been widely consumed for a long time as a foodstuff and a medical herb worldwide to treat different conditions and control symptoms. Some reports it exerts antimicrobial activity and could be used for relieving rheumatic pain, decreasing the serum levels of cholesterol and glucose, and suppressing the development of some types of cancer.(Chuang et al., 2018; Leyva-López et al., 2017)

Recent in-vitro and in-vivo studies have shown that *O. vulgare* extract has antioxidant, anti-inflammatory, anti-apoptotic, and anti-cancer properties.(Chuang et al., 2018; Leyva-López et al., 2017; Vujicic et al., 2015)

O. vulgare may inhibit several inflammatory indicators and tissue regeneration in a human skin disease model study.(Han & Parker, 2017) Therefore, the objective of the current research was to determine how various dosages of OEO (50, 100, and 200 mg/kg/day) affected the anti-inflammatory properties and volume of the lateral ventricle in the brain of rats with hydrocephalus brought on by kaolin. Following kaolin injection, tonic-clonic convulsions might be fatal in rare situations. This was shown in rats that had received kaolin injections; however, in our test model, a few seizures occurred when the kaolin injection was combined with OEO. According to MRI data obtained three weeks following kaolin treatment, the lateral ventricles of the intervention rats were significantly larger than those of the control group. The lateral ventricle increment lasted until day 31 after injection. The size of the lateral ventricle was then lowered in the intervention groups receiving various dosages of OEO. Similarly, when compared to the hydrocephalus group, the saline control group did not develop hydrocephalus. Although 200 mg/kg OEO was not significantly different compared to 50 and 100 mg/kg, all three doses were significantly different compared to the kaolin group, and 200 mg/kg was comparable to the saline control group. Based on the results of earlier research, the injection of kaolin into mice may increase and produce

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significant ventricular dilatation, as measured by the MRI technique.(Di Curzio et al., 2016; Hao Xu et al., 2012)

In this context, Hao Xu et al. (2012) demonstrated that after 14 days of kaolin injection, hydrocephalic adult male Sprague–Dawley rats had a greater degree of ventricular dilatation compared with the control group. Another study found that injecting kaolin into the basal cistern of 21-day-old rats might produce hydrocephalus. The MRI technique was carried out after two weeks to assess ventricular enlargement in the animals. They showed that kaolin could cause hydrocephalus and ventricular enlargement in juvenile rats.(Botfield et al., 2013) Olopade et al. (2012) also indicated that in three-week-old rats with hydrocephalus produced by intracisternal injection of kaolin, ventricle size was larger than in the control group. According to the research stated above, the steady rise in neuron density and long-term expansion of the ventricles may be used as biomarkers of persisting injury in the brain, such as an inflammatory response.

GFAP is an astrocyte-specific intermediate filament protein. In reactive astrogliosis and the glial scar generated by astrocytes, GFAP plays a regulatory function in CNS inflammation.(Sofroniew, 2009; Hao Xu et al., 2012)

Reactive astrogliosis in the brain is caused by GFAP upregulation and astrocytic process hypertrophy. Activated astrocytes may be useful in the acute phase of CNS damage, but they may be detrimental in the chronic phase.(Botfield et al., 2013) The brain is less compliant in hydrocephalic individuals with chronic reactive astrogliosis, which might compromise the effectiveness of shunt treatment and inhibit axon regeneration and remyelination.(Botfield et al., 2013) The immunohistochemical data obtained in this research showed that OEO therapy reduced the expression levels of GFAP in hydrocephalic rats compared to those treated with kaolin alone. The levels of GFAP immunohistochemistry in the 200 mg/kg OEO+kaolin group, on the other hand, were comparable to the saline-treated control group. A study performed by Lin et al. (2015) discovered that lithospermic acid (LSA) extracts found in plants such as *Salvia miltiorrhiza Bge* (*Labiatae*) and *Origanum vulgare*, might be employed for the treatment of Parkinson's disease. They demonstrated that LSA effectively reduced MPP+-induced neurotoxicity by lowering GFAP and Iba-1 expression levels as well as abnormal neurogenesis. Correspondingly, Jeong et al. (2020) examined the effects of carvacrol, an essential oil extracted from *Origanum vulgare*, on lithium-pilocarpine-induced status epilepticus (SE) as a model of temporal lobe epilepsy by the reduction

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of intracellular free zinc accumulation. However, carvacrol treatment significantly reduced glial and astroglial activation (Iba-1 and GFAP) in the hippocampus following SE.

GFAP-labeled reactive astrocytes have been detected in the CSF of a variety of hydrocephalic animals, including newborn rats, mice and rats of different ages, ferrets, and human patients with hydrocephalus.(Del Bigio et al., 1994; Olopade et al., 2019; Hao Xu et al., 2012) A previous study found GFAP-labeled reactive astrocytes in a feline model of kaolin-induced hydrocephalus.(Del Bigio et al., 1994) Clinical trials indicated a significant increase in GFAP levels of CSF, and histopathological studies demonstrated elevated reactive astrogliosis in hydrocephalic patients compared with healthy subjects.(Beems et al., 2003) In another clinical study, cerebrospinal fluid GFAP levels were found to be an important tool for estimating astrogliosis and astrocyte activation in patients with neurological disorders.(Petzold et al., 2004) Therefore, GFAP protein level can be used as an indicator for current diagnostic purposes.

Microglial cells may have a dual function (protective or neuroprotective roles) in brain injury, depending on conditions such as ischemia and hemorrhagic brain injury.(Hao Xu et al., 2012) Astrocytes and microglia may lower glutamate in the extracellular space in the early stages of injury, as well as store and provide cells with high-energy substances.(Suryaningtyas et al., 2019) The activation of microglia is polarized towards a neurotoxic phenotype one week after the protective effects are activated, and this causes inflammatory reactions such as the production of chemokines and cytokines as well as the production of an extracellular matrix that is destructive and causes the formation of glial scars.(Suryaningtyas et al., 2019)

In this research, microglia have reached the chronic phase three weeks after kaolin injection, and the effects of increased expression of Iba-1-immunoreactive microglia may be investigated under these conditions. Compared with the untreated hydrocephalic group, Iba-1-immunoreactive microglia were significantly decreased in the three OEO + kaolin treatment groups. However, a significant difference was observed between the three OEO + kaolin groups, which indicated that 200 mg/kg/day was the most effective dose to inhibit Iba-1 protein production. These results strongly support the anti-inflammatory activity of OEO in a rat model of kaolin-induced hydrocephalus. Javadian et al. (2016) evaluated the anti-inflammatory effects of *Origanum species*. They found that *Origanum vulgare* extracts had very strong anti-inflammatory effects on the activated mixed and microglial cells through the inhibition of iNOS and TNF-α expression. Another study discovered a volatile substance, beta-caryophyllene (BCP), which is abundant in

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the essential oils of several popular spices and edible plants, including *Origanum vulgare L*. and *Cinnamomum spp*. In animal models, it may attenuate inflammatory pain responses (end-stage) in the formalin test in a cannabinoid 2 (CB2) receptor-dependent manner. They demonstrated that oral treatment of BCP reduced GFAP expression, Iba1 spine level, and thermal hyperalgesia. They proposed that BCP belongs to a class of common herbal natural compounds with potential health effects.(Klauke et al., 2014) Also, Ojha et al. (2016) demonstrated that daily administration of 50 mg/kg (i.p.) of Beta (β)caryophyllene (BCAR) for four weeks could protect dopaminergic neurons and reduce microglia and astrocyte activation in Wistar rat models of Parkinson's disease, as evidenced by decreased Iba1 and GFAP expression.

Previous research has shown that the effects of the chronic phase in glial cells are comparable to brain damage caused by hydrocephalus.(Olopade et al., 2019; Suryaningtyas et al., 2019; Hao Xu et al., 2012; H Xu et al., 2012) A study demonstrated a link between microglial process retraction and increased Iba-1 antibody in the white matter, as well as kaolin induction of hydrocephalus in rats.(Olopade et al., 2012) A study on adult male Sprague-Dawley rats found that reactive microglial cells with dense packing have a positive function in the development of hydrocephalus in the cortex and hippocampus, whereas glial cells play a negative influence.(H Xu et al., 2012) Suryaningtyas et al. (2019) reported that inoculating 20% kaolin into the cistern of ten-week-old Sprague-Dawley rats increased microglial activation as observed by increased Iba-1 protein expression. The expression of Iba-1 and GFAP proteins in the lateral ventricle was then examined using the western blot analysis. The current research found increasing Iba-1 and GFAP elevation in the kaolin-treated group, indicating neuroinflammatory profile alterations in chronic hydrocephalus. The western blot analysis revealed that reactive astrocytes and microglia dramatically elevated GFAP and Iba-1 protein expression in hydrocephalous rats, validating the immunohistochemistry findings of the present investigation. GFAP and Iba-1 expression levels were significantly lower in the 200 mg/kg/day OEO group than in the hydrocephalus group, although there was no significant difference between the 50 and 100 mg/kg/day OEO and hydrocephalus groups.

According to the findings of the study, GFAP and Iba-1 expression levels were considerably greater in hydrocephalic rats than in controls.(Deren et al., 2010; Hao Xu et al., 2012). Hao Xu et al. (2012) revealed that kaolin-induced hydrocephaly increased the expression of GFAP and Iba-

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1 in rats. Their analysis indicates a link between GFAP and Iba-1 protein levels and the degree of ventricular dilatation.Deren et al. (2010) showed that reactive astrocytes and reactive microglia increased 21 days following intra-cisternal injection of kaolin to rats to induce hydrocephalus, based on immunohistochemical and Western blot analyses. According to research, in adult rats with hydrocephalus caused by intraventricular injection of 3% kaolin, GFAP and Iba-1 expression levels dramatically increased in the experimental groups compared to saline controls, and this had a direct association with disease severity.(H Xu et al., 2012)

The findings show that the increased expression of GFAP and Iba-1 in astrogliosis and microgliosis, respectively, is associated with the development of hydrocephalus, and OEO may reverse these effects. In addition, studies have shown that OEO significantly reduces gliosis and inflammation in severe hydrocephalus, especially at high doses, and may be considered an effective agent for the treatment of hydrocephalus.

Conclusion

In conclusion, intraventricular administration of OEO at varying concentrations for 10 days after kaolin administration prevents the development of kaolin-induced hydrocephalus in rats by inhibiting GFAP and Iba-1 protein-mediated lateral ventricular enlargement and protecting against the progression of brain damage caused by hydrocephalus. Our findings demonstrated that OEO might be an effective substance for reducing hydrocephalus and subsequent mortality. Animal models of hydrocephalus and human subjects are needed to determine the therapeutic effectiveness of OEO in humans.

Ethical Considerations

Acknowledgments

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Ethical statement

All animal experiments were approved by the Ethics Committee of Iran University of Medical Sciences' Committee on Ethics in Research (Research Code: 27517) (Ethical Code: IR.IUMS.REC 1395. 118.27517).

Conflict of interest

The authors declare no conflict of interest.

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Authors' Contributions

Manijeh Motevalian conceived and designed the analysis and also critical revision.

Gelareh Vahabzadeh wrote the manuscript and performed statistical analyses.

Ashrafsadat Moazam collected the data of Fig 1 and Fig 4.

Mandana Rahimi provided data for Fig 2 and Fig 3.

Fatemeh Khajehasani helped in collecting MRI data.

All authors reviewed the final manuscript.

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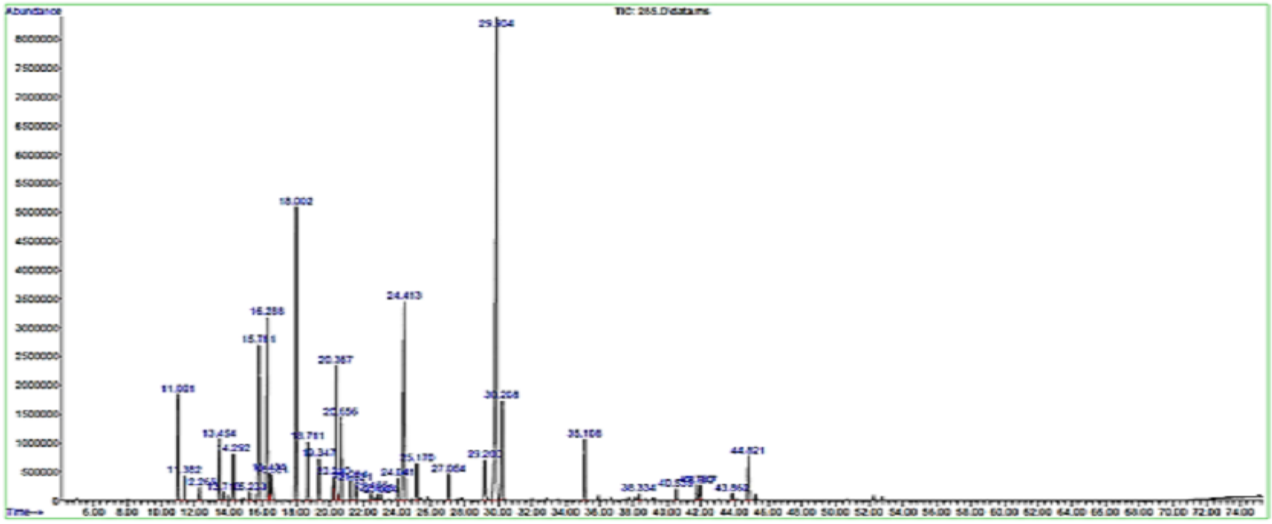

Fig. 1. GC-MS chromatogram of *Origanum vulgare* essential oil.

Table. 1. Chemical composition of volatiles in the Origanum vulgare essential oil

No	RT	%	Components	KI	Type
1	11.00	3.44	α-Thujene	928	MH
2	11.38	0.82	α-Pinene	935	MH
3	12.27	0.46	Camphene	953	MH
4	13.45	2.09	Sabinene	976	MH
5	13.71	0.30	β- Pinene	982	MH
6	14.30	1.56	Myrcene	993	MH
7	15.23	0.32	α- Phellandrene	1011	MH
8	15.78	5.44	α-Terpinene	1022	MH
9	16.29	6.55	O-Cymene	1032	MH
10	16.43	0.93	Limonene	1035	MH
11	16.55	1.04	β - Phellandrene	1037	MH
12	18.00	11.22	γ-Terpinene	1065	MH

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13	18.71	2.07	trans-Sabinene hydrate	1079	MO
14	19.35	1.48	α-Terpinolene	1091	MH
15	20.22	0.75	Linalool	1108	MO
16	20.36	5.58	Cis-β-Terpineol	1111	MO
17	20.66	2.99	α-Thujone	1117	MO
18	21.22	0.72	β-Thujone	1128	MO
19	21.52	0.61	Cis-p-Menth-2-en-1-ol	1134	MO
20	22.47	0.43	Trans- Menth-2-en-1-ol	1154	MO
21	22.81	0.25	Camphor	1161	MO
22	23.02	0.25	Borneol	1165	MO
23	24.04	0.93	L-Borneol	1186	MO
24	24.41	7.91	Terpinene-4-ol	1193	MO
25	25.17	1.34	α-Terpineol	1209	MO
26	27.05	0.94	Carvacol,methyl esther	1248	MO
27	29.20	1.87	Trans-Bornyl acetate	1294	MO
28	29.90	28.32	Thymol	1309	MO
29	30.21	3.50	Carvacrol	1316	MO
30	35.11	2.22	Caryophyllene	1428	SH
31	40.53	0.39	Elemol	1561	SO
32	41.76	0.53	Sapathulenol	1592	SO
33	41.95	0.54	Caryophyllene oxide	1597	SO
34	43.86	0.35	γ-Eudesmole	1648	SO
35	44.82	1.66	α-Eudesmole	1673	SO
		99.75	Total Identified		

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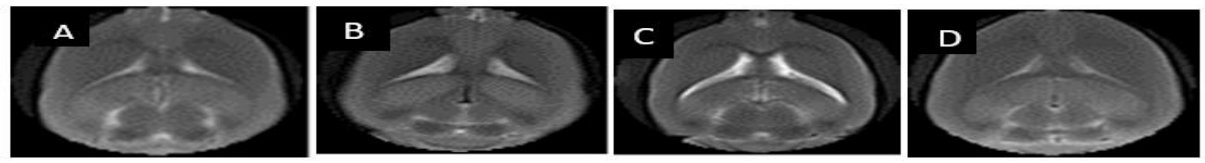

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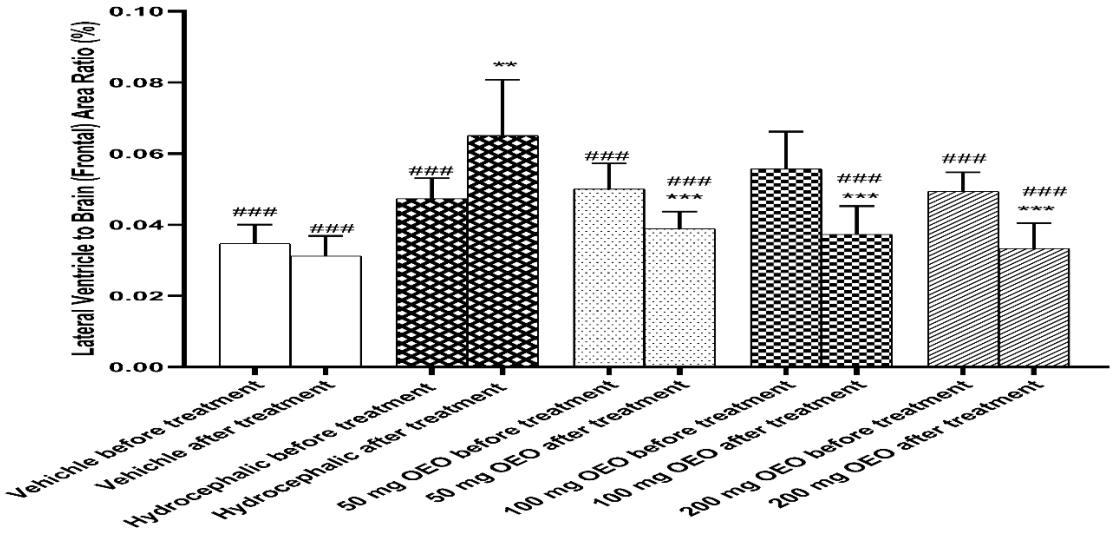

Fig. 2. A,T_2-weighted MR images of brain coronal slices showing lateral ventricle size in hydrocephalic rat brain, a 31 days after normal saline injection in the control group. b 21 days after the injection of kaolin in the hydrocephalus group, (c) 31 days after kaolin injection in hydrocephalus group and (d) 31 days after kaolin injection in group receiving 200 mg/kg/day OEO therapy. CSF in the lateral ventricle appears white. (B), Bar graph (mean ± SE) shows the size of lateral ventricle measured based on MR images obtained three weeks after the injection of kaolin (before-treatment), and 10 days after kaolin injection (31 days) drug therapy (after-treatment). Enlargement of lateral ventricle was obvious in all the hydrocephalic groups compared to non-hydrocephalic rats. ** *P<0.01* and ****P <0.001*indicate differences between pre- and post-treatment results in all the groups. $^{###}p<0.001$ indicated difference between hydrocephalic treatment group after 31 day and others groups.

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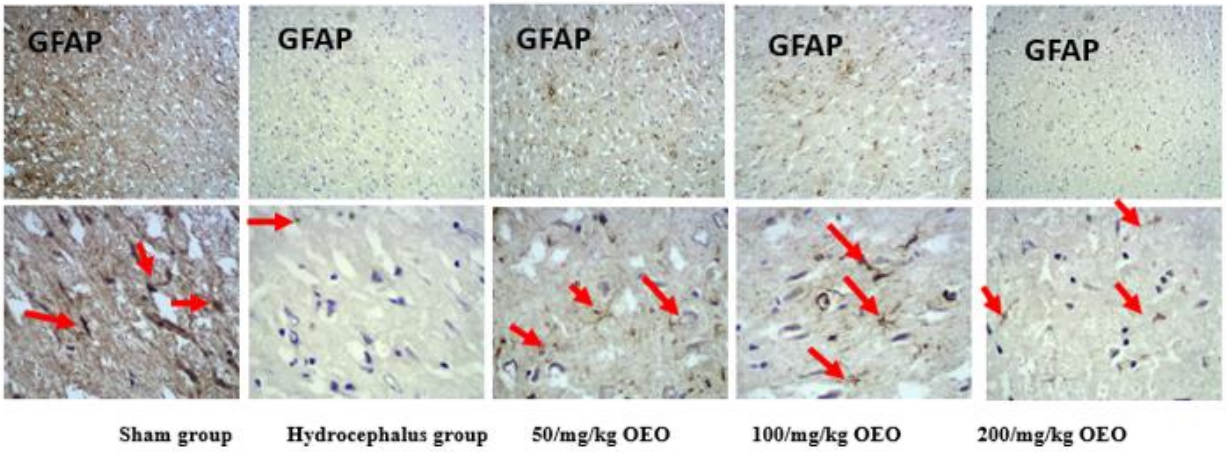

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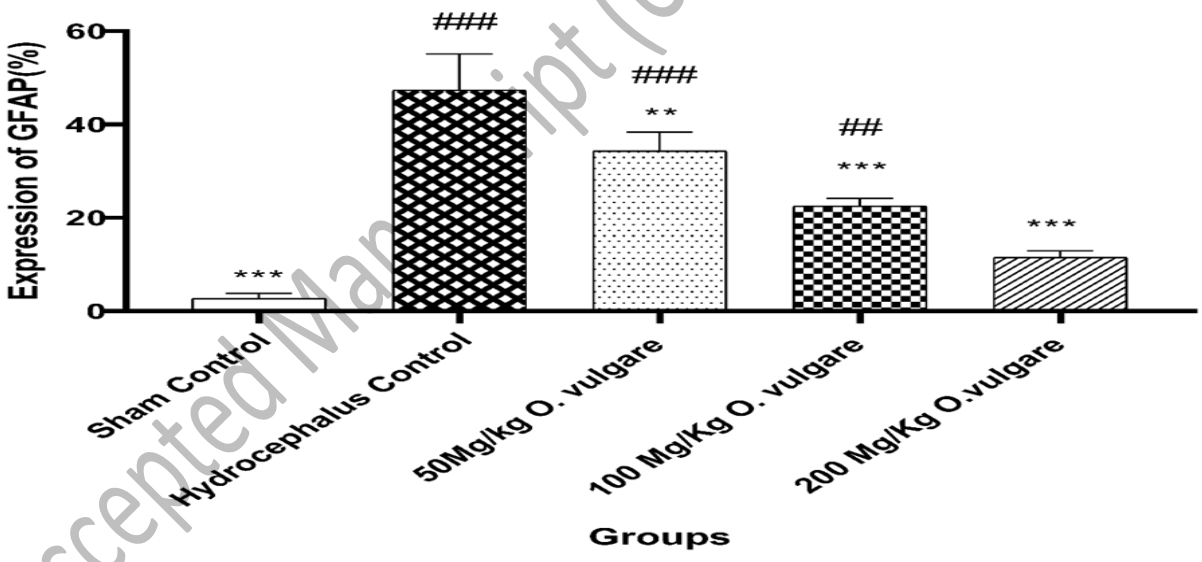

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Fig. 3. A The representative images of GFAP in lateral ventricular white matter. B Mean percentage of GFAP expression in the brain tissue of the sham control, hydrocephalic + OEO, and hydrocephalic rates. $^{***}P < 0.001$ and $^{**}P < 0.01$ in comparison with hydrocephalus control groups. $^{###}P < 0.001$ in comparison with 200 mg/kg/day OEO treatment groups.

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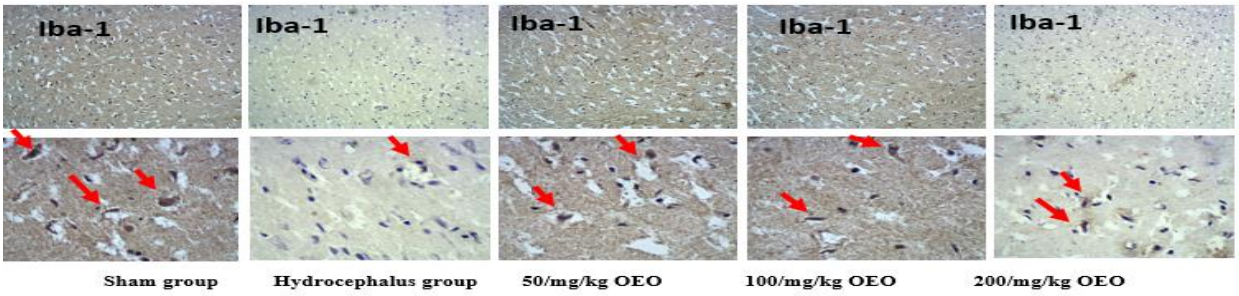

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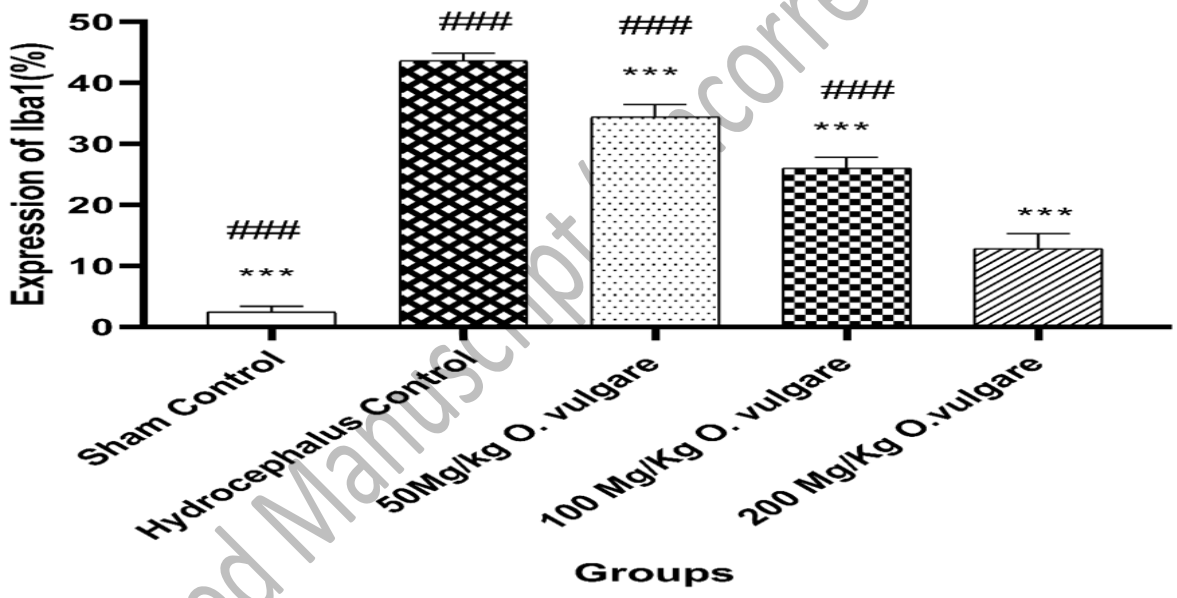

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Fig. 4. A The representative images of Iba-1 protein in lateral ventricular white matter. B The mean percentage of protein expression in positive reacted cells of Iba-1 in the brain tissue of the sham control, hydrocephalic + OEO and hydrocephalic groups. $^{***}P < 0.001$ in comparison with the hydrocephalus control group. $^{\#\#\#}P < 0.001$ in comparison with the 200 mg/kg/day OEO treatment group, F (4, 10) = 259.8.

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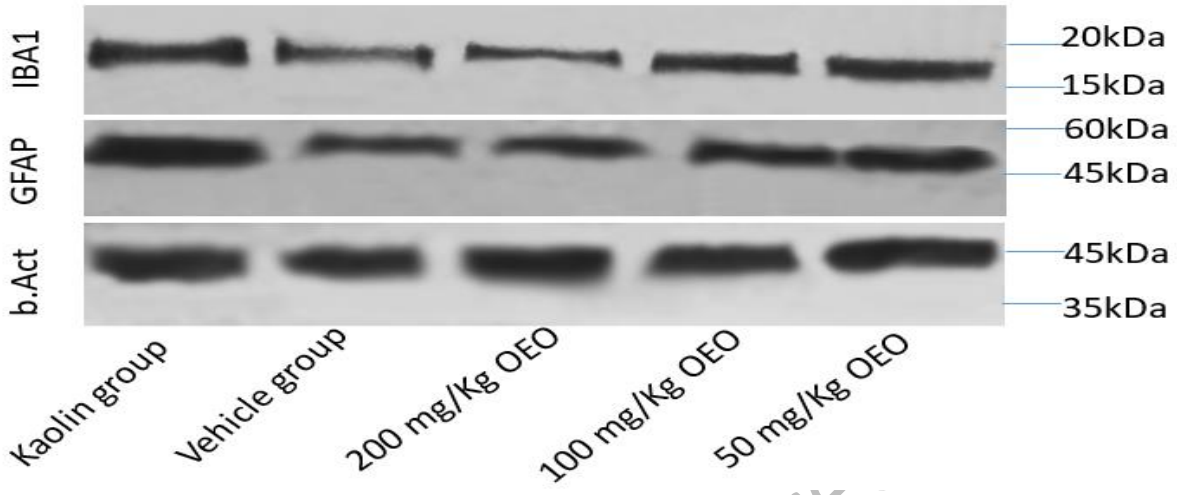

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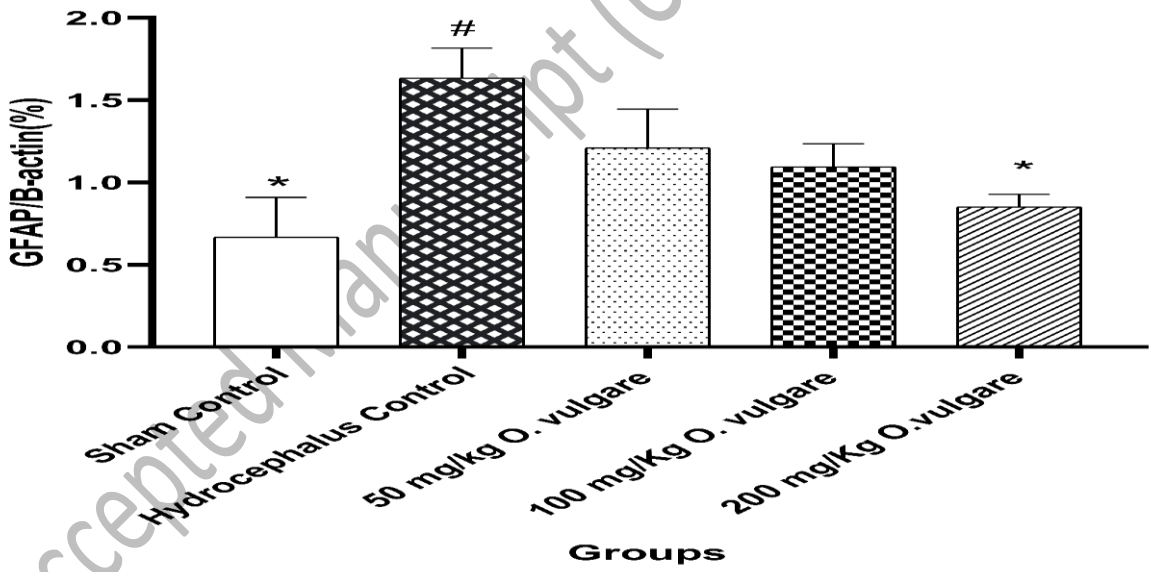

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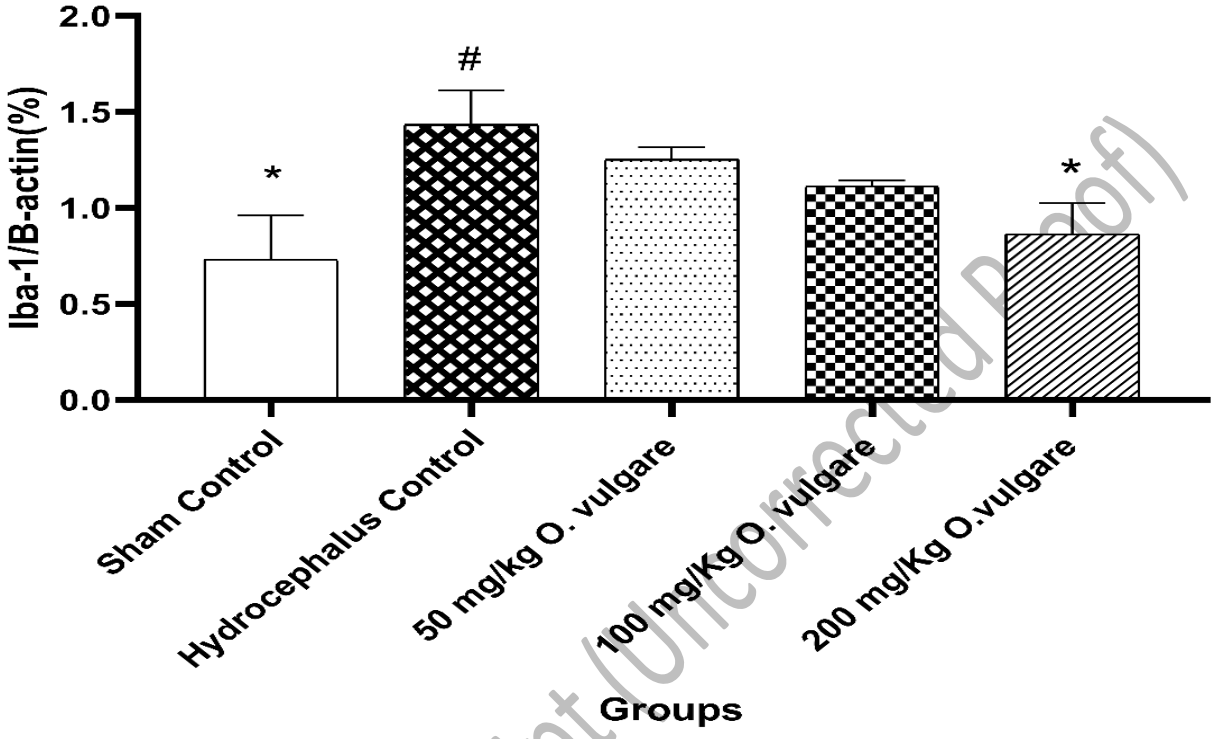

Fig. 5. (A) Proteins were processed for Western blot to assess levels of GFAP and Iba-1. Mean ranking score of GFAP (B) and Iba-1(C) in brain tissue for Sham Control group. $^{*}P < 0.05$ in comparison with the hydrocephalus control group; $^{\#}P < 0.05$ in comparison with the 200 mg/kg/day OEO treatment group.

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