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Research Paper

Origanum vulgare Prevents Kaolin–induced Hydrocephalus Via Regulation of GFAP and Iba1 Proteins Expression in the Rat Brain

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ABSTRACT

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

Methods: A total of 30 male Wistar rats were injected with 25% kaolin directly into the cisterna magna. Twenty-one days after kaolin injection, 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. Then, the rats received different doses of OEO for 10 days. Additionally, to assess the expression levels of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba-1), immunohistochemical and Western blot analyses were performed 10 days after the completion of the treatment course.

Results: The size of the lateral ventricle in hydrocephalic rats treated with OEO was less than that 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, a significant difference was observed between the hydrocephalic control group and rats treated with OEO at a dose of 200 mg/kg/d.

Conclusion: Compared 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 by lowering the expression of GFAP and Iba-1. Therefore, OEO intervention might be effective as a novel treatment technique.

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Highlights

- Reactive astrocyte and microglial activation have been considered the cellular pathological hallmark in kaolin-induced hydrocephalus.

- *Origanum vulgare* extract displays anti-inflammatory and antioxidant activity in cell and animal models in kaolin-induced rat brains.

Plain Language Summary

When cerebrospinal fluid (CSF) accumulates excessively in the ventricles and cavities of the brain, it causes an increase in volume (hydrocephalus). This volume increase in the central nervous system can result from a disorder in the distribution, formation, flow, or absorption of cerebrospinal fluid. The increased pressure inside the skull and brain can cause 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, but the drugs may 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 magnetic resonance imaging (MRI) of the rats confirmed this volume increase within 3 weeks. In this study, it was demonstrated that the intraperitoneal injection of *Origanum vulgare* essential oil prevents lateral ventricle enlargement in a hydrocephalus model induced by intraventricular kaolin injection. Therefore, in related cases that occur pathologically, it may be effective in treatment.

Introduction

Hydrocephalus is a neuroanatomical disorder in which the secretion, absorption, and circulation of the cerebrospinal fluid (CSF) are disturbed. This condition results in an enlarged ventricle filled with CSF and an obstruction of its flow. Several congenital or environmental factors contribute to the pathogenesis of hydrocephalus, potentially causing damage to the surrounding brain tissues. Axonal damage in the periventricular white matter is one of the initial consequences of ventricular dilatation in both animals and humans. The accumulation of metabolic byproducts, 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 (Xu et al., 2012a). To experimentally explore brain injury pathogenesis, various approaches are used to induce hydrocephalus (Slobodian et al., 2007). The most typical procedure is the injection of kaolin (aluminum silicate) into the cisterna magna, as it is inexpensive, simple to perform, reliable, minimally invasive, and leaves no visible scar, thereby mimicking hydrocephalus following meningitis (Olopade et al., 2019; Slobodian et al., 2007). In addition, animal models of kaolin-induced hydrocephalus, linked to the activation of astrocytes and microglia, have been established to investigate 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 a high phenolic content, offering therapeutic benefits for various diseases and clinical symptoms, including digestive and respiratory disorders. In traditional medicine, this plant is used as a sedative, hepatoprotective, antioxidant, anticancer, anti-diabetic, anti-inflammatory, antiviral, and antifungal agent. It is effective in relieving pain associated with rheumatic arthritis (Bahmani et al., 2018; Chuang et al., 2018; Leyva-López et al., 2017). Ethnobotanical and ethnopharmacological studies have demonstrated the traditional medicinal use of this plant in treating various diseases (Bahmani et al., 2018). For example, Sajadi (2011) showed that the aerial parts of this herb contain compounds that improve neurological function. Zolfeghari et al. (2012) demonstrated that the therapeutic effects of this plant on flowering branches can enhance nervous system function and reduce migraine pain. Recently, *O. vulgare* extract has been shown to have a protective effect on the brain in an animal model of stroke, 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, specifically *O. vulgare* essential oil (OEO), on experimentally induced hydrocephalus. The primary goal of this research was to develop a novel pharmaceutical strategy for brain damage treatment before 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 magnetic resonance imaging (MRI) to assess lateral ventricular hypertrophy in the experimental groups. The effect of OEO on gliosis reduction was studied to suggest it as a novel treatment strategy. To this end, the expression levels of GFAP (glial fibrillary acidic protein) and Iba-1 (ionized calcium-binding adaptor molecule 1) were assessed, and immunohistochemical studies were performed for both proteins.

Materials and Methods

Study animals

Thirty adult male Wistar rats, weighing 250–300 g and 8–10 weeks old, were provided by the Iran University of Medical Sciences (Tehran, Iran). Animals were placed in cages with controlled conditions: A temperature of 21–22 ˚C, 34%-35% humidity, a 12/12-hour 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/d OEO for 21-days after the injections of 25% kaolin (n=5), 4) group receiving injections of 100 mg/kg/d OEO for 21-days after the injections of 25% kaolin (n=5), 5) group receiving injections of 200 mg/kg/d OEO for 21-days after the injections of 25% kaolin (n=5).

The experimental protocol was approved by the Ethics Committee in Research at Iran University of Medical Sciences. The experiment was also designed in compliance with the animal research: reporting of in vivo experiments (ARRIVE) guidelines), and all technical and ethical considerations were taken into account (Kilkenny et al., 2010; McGrath et al., 2010).

Plant material

O. vulgare, sometimes referred to as wild marjoram, and its close relative, *Origanum majorana*, are known as sweet marjoram. The *O. vulgare* samples were obtained from a local green market in Tehran. To confirm authenticity, the samples were transferred to Dr Amin's herbarium at the School of Pharmacy, Tehran University of Medical Sciences (Herbarium No. PMP384). The essential oil was obtained from the dried plant in 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 hours from 50 to 100 g of air-dried leaves and shoots of the plant. Oil yields were then estimated based on the dry weight of the 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

The qualitative and quantitative analysis of the sample was carried out using a gas chromatography-mass spectrometry (GC-MS) technique (Rtx-5MS, 30 m × 0.25 mm × 0.25 µm). The sample was injected using the split mode, with helium as the carrier gas, at 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 in the sample was achieved by comparing their retention indices and mass fragmentation patterns with those available in the National Institute Of Standards And Technology Library.

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 injections. After being given a mixture of 75 mg/kg ketamine and 4 mg/kg xylene, the animals were placed in a stereotaxic frame. The fur on the back of the 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 conditions; 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 placed on a heating pad to monitor their neurological signs. To prevent further suffering, rats with weight loss or neurological disturbances were euthanized (Xu et al., 2012a).

MRI and lateral ventricular volume measurement

After inducing anesthesia with 75 mg/kg ketamine, the animals underwent brain MRI twice. T2-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 hydrocephalus and saline control groups were used to measure the lateral ventricle size. 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 the hydrocephalus group compared to the saline group, the second MRI was obtained 10 days post-kaolin injections with different experimental groups, including the hydrocephalus group without treatment (number=10) and treatment groups at three OEO doses of 50, 100, and 200 mg/kg/d, IP. Each treatment group contains 5 rats. For the control group (0.9% NaCl), MRI was also performed. The lateral ventricle was measured by ImageJ software, version 1.5. After calculating the volume of the lateral ventricle, the brains were detached and divided into two parts for further study.

Drug preparation and administration

As mentioned earlier, animals received different doses of OEO and or NaCl via injection into the cisterna magna daily, 21 days after kaolin administration. OEO-treated animals received OEO (prepared in the laboratory) suspended in 0.9% normal saline and were administered 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 anesthetized with ketamine (75 mg/kg), and the vascular system was flushed with 200 mL of saline and 600 mL of 4% paraformaldehyde solution via transcardiac perfusion (pH 7.4). To perform immunohistochemistry, following embedding in paraffin, the brain samples were processed after being maintained in 4% paraformaldehyde (PFA) for four days at 4 ˚C. The samples were cut into 8-µm thick coronal sections (Sajadian et al., 2015).

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 in methanol) for one hour. To facilitate antigen exposure for labeling, the slides were washed with PBS, and then the antigen retrieval solution (1 M sodium citrate buffer at 95 ˚C, pH 6.0) was added for 10 minutes.

After boiling, the slides were kept at room temperature for 20 minutes and then rinsed with distilled water and immunohistochemistry (IHC) washing solution for 5 minutes each. The slides were then incubated for one hour after the addition of the blocking solution.

To label astrocytes and reactive astrocytes, the slides were incubated with rabbit polyclonal anti-GFAP (1:10000 dilution). For activated microglia labeling, the slides were incubated with rabbit polyclonal Iba-1 (1:5000 dilution) (Abcam Biotechnology, USA) and left overnight in a moist chamber at 4 ˚C.

Afterward, PBS was used to wash the sections in triplicate (each for 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 13000 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 a 10% SDS-polyacrylamide gel in 1×Tris/Glycine/SDS buffer (90 minutes, 150 V, RT). Separated proteins were transferred to a PVDF (Polyvinylidene fluoride or polyvinylidene difluoride) 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 at RT. Then, the membranes were incubated with primary rabbit anti-

GFAP antibody (Abcam, ab7260, Cambridge, US), primary rabbit anti-Iba1 antibody (Abcam, ab178846, Cambridge, US), and the reference protein B-actin (Abcam, ab8226, Cambridge, US) at 1:10000 dilutions overnight at 4 ˚C. Afterwards, the samples were washed with TBS-t in triplicate. Secondary antibody, peroxidase-conjugated anti-rabbit IgG (Abcam, ab6721, Cambridge, US), was incubated for 1 hour at a dilution of 1:1000 at room temperature. 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 relative intensities of GFAP and Iba-1 were divided by the loading control B-actin (42 kDa) relative intensity to quantify the bands. Image J version 1.5 was employed to measure the density of bands.

Statistical analysis

Data were expressed as Mean±SE. To compare data obtained from groups, one-way ANOVA with the Dunnett post hoc test and a two-tailed t-test were used. The level of significance was $P \leq 0.05$. Data were analyzed in GraphPad prism software, version 7.

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 (Figure 1 and Table 1).

Mortality

Of 25 rats receiving kaolin, 4 died shortly after injection due to tonic-clonic seizure. Gradual reversion of anesthesia averted seizure. Totally, 5 rats (4 kaolin-injected, 1 saline-injected) died. The 3-week intervention was completed with the remaining animals, ultimately sacrificed for analysis.

Effects of OEO on the lateral ventricular volume of the rat hydrocephalus model

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

Immunohistochemistry

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

Some reactive astrocytes in the lateral ventricle were found by immunohistochemical staining for GFAP (Figure 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 the 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) was considerably lower than that of the kaolin group in a dose-dependent manner. However, the 200 mg/kg OEO group was comparable to the sham control group (Figures 3A and 3B).

All the hydrocephalic rats exhibited reactive microglia as indicated by immunostaining for Iba-1 (Figures 4A and 4B). Significant differences were observed among the treatment groups using semi-quantitative grading. After induction of hydrocephalus, the mean percentage of positive-reacted cells was 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 the hydrocephalus group in a dose-dependent manner, and the 200 mg/kg/day OEO treatment group had the smallest expression compared with the two other doses.

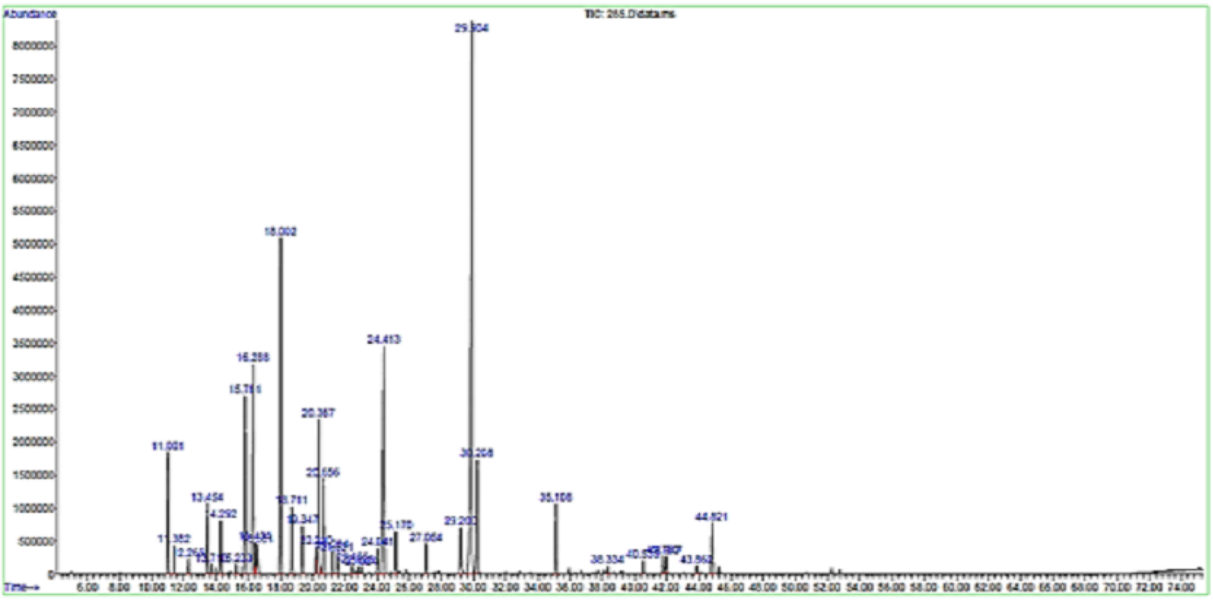

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Figure 1. GC-MS chromatogram of origanum vulgare essential oil

The Western blot

The results of Western blot analysis on day 31 showed that the expression levels of GFAP and Iba-1 were significantly higher in hydrocephalic rats than in 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/d OEO groups by 1.21±0.16%, 1.09±0.09% and 0.85±0.05%, respectively, relative to the hydrocephalus group; however, this effect did not differ significantly between these doses. In hydrocephalic groups treated with 50, 100, and 200 mg/kg/d of OEO, Iba-1 expressions decreased by 1.25±0.04%, 1.11±0.02%, and 0.86±0.01%, respectively, compared with the hydrocephalic group alone (Figure 5).

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 to analyze different surgical and non-surgical treatments for its management (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 they produce, remains unknown. There is a lack of information on the involvement of gliosis—for example, reactive astrocytosis and microgliosis—as well as neuroinflammation in the etiology of hydrocephalus. Further research into gliosis and gene expression may aid in the diagnosis and treatment of hydrocephalus (Bloch et al., 2006; Deren et al., 2010; Suryaningtyas et al., 2019).

Mechanical and biochemical cellular changes are among the factors that contribute to the pathophysiology of brain damage resulting from hydrocephalus. Ependymal cells are the first structures to be affected by mechanical damage, typically characterized by cell loss, denudation, and detachment from the surrounding tissue. 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 fractone. 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 the germinal zone, and aberrant migration of neuroblasts to the ventricle in hydrocephalic embryos (Suryaningtyas et al., 2019).

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

No.	RT	%	Components	KI	Type
1	11	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	0.3	β- pinene	982	MH
6	14.3	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	11.22	γ-Terpinene	1065	MH
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	Carvacrol, methyl ester	1248	MO
27	29.20	1.87	Trans-bornyl acetate	1294	MO
28	29.90	28.32	Thymol	1309	MO
29	30.21	3.5	Carvacrol	1316	MO
30	35.11	2.22	Caryophyllene	1428	SH
31	40.53	0.39	Elemol	1561	SO
32	41.76	0.53	Spathulenol	1592	SO
33	41.95	0.54	Caryophyllene oxide	1597	SO
34	43.86	0.35	γ-eudesmol	1648	SO
35	44.82	1.66	α-eudesmol	1673	SO

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Abbreviations: RT: Retention time; KI: Kovats indices; MH: Monoterpene hydrocarbons; MO: Oxygenated monoterpenes; SH: Sesquiterpene hydrocarbons; SO: Oxygenated sesquiterpenes.

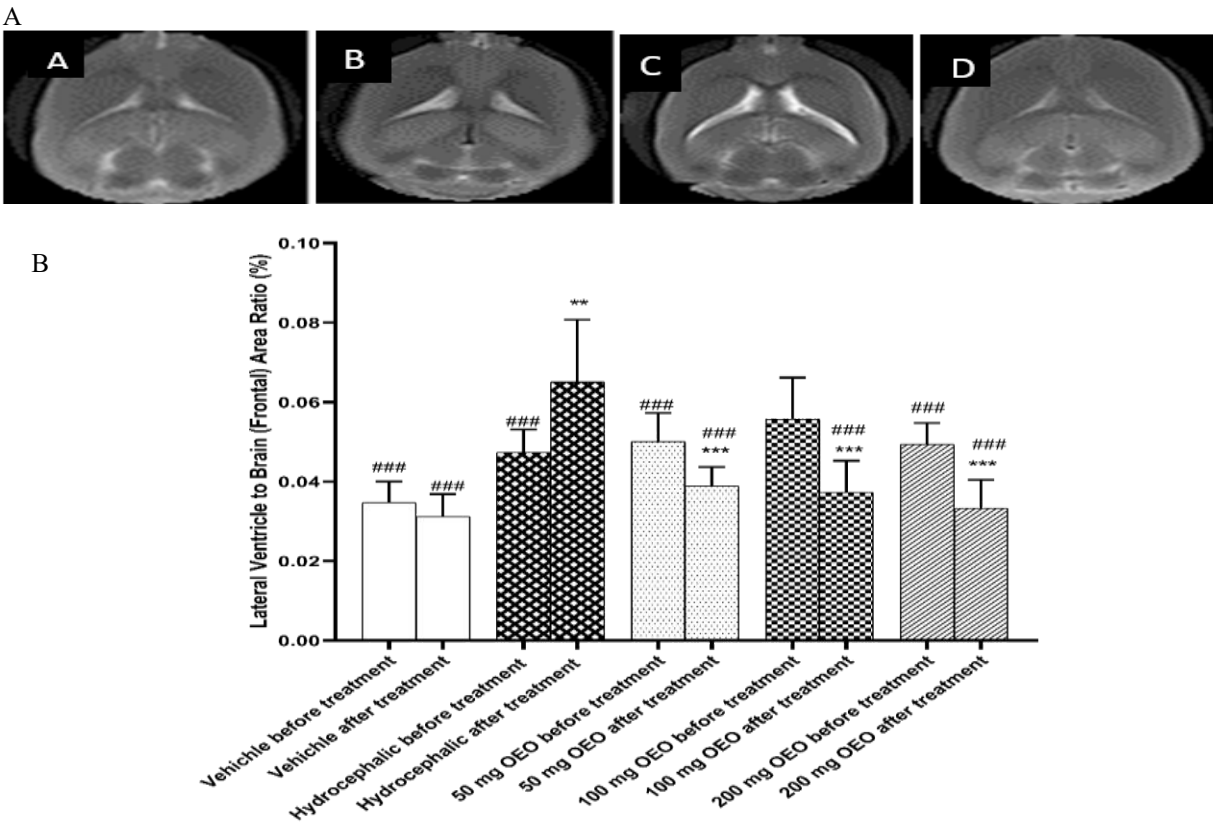

Figure 2. MRI changes of kaolin-induced hydrocephalus with and without *O. vulgare*

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A) T2-weighted magnetic resonance (MR) images of brain coronal slices showing lateral ventricle size in a hydrocephalic rat brain; A) Thirty-one days after normal saline injection in the control group; B) Twenty-one days after the injection of kaolin in the hydrocephalus group; C) Thirty-one days after kaolin injection in the hydrocephalus group; D) Thirty-one days after kaolin injection in the group receiving 200 mg/kg/d OEO therapy. CSF in the lateral ventricle appears white.

B) Bar graph (Mean±SE) showing the size of the lateral ventricle measured based on MR images obtained 3 weeks after the injection of kaolin (before treatment), 10 days after kaolin injection (31 days), and drug therapy (after treatment); enlargement of the lateral ventricle was obvious in all the hydrocephalic groups compared to non-hydrocephalic rats.

Note: $^{**}P<0.01$ and $^{***}P<0.001$ indicate differences between pre- and post-treatment results in all the groups. $^{###}P<0.001$ indicated a difference between the hydrocephalic treatment group after 31 days and other groups.

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 suggest that it exhibits antimicrobial activity and may be used to alleviate rheumatic pain, lower serum levels of cholesterol and glucose, and inhibit the development of certain types of cancer (Chuang et al., 2018; Leyva-López et al., 2017).

Recent in vitro and in vivo studies have demonstrated that *O. vulgare* extract exhibits antioxidant, anti-inflammatory, anti-apoptotic, and anticancer 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/d) affected the anti-inflammatory properties and the volume of the lateral ventricle in rats with hydrocephalus induced by kaolin. Following kaolin injection, tonic-clonic convulsions might be fatal in rare situations. This condition was seen 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

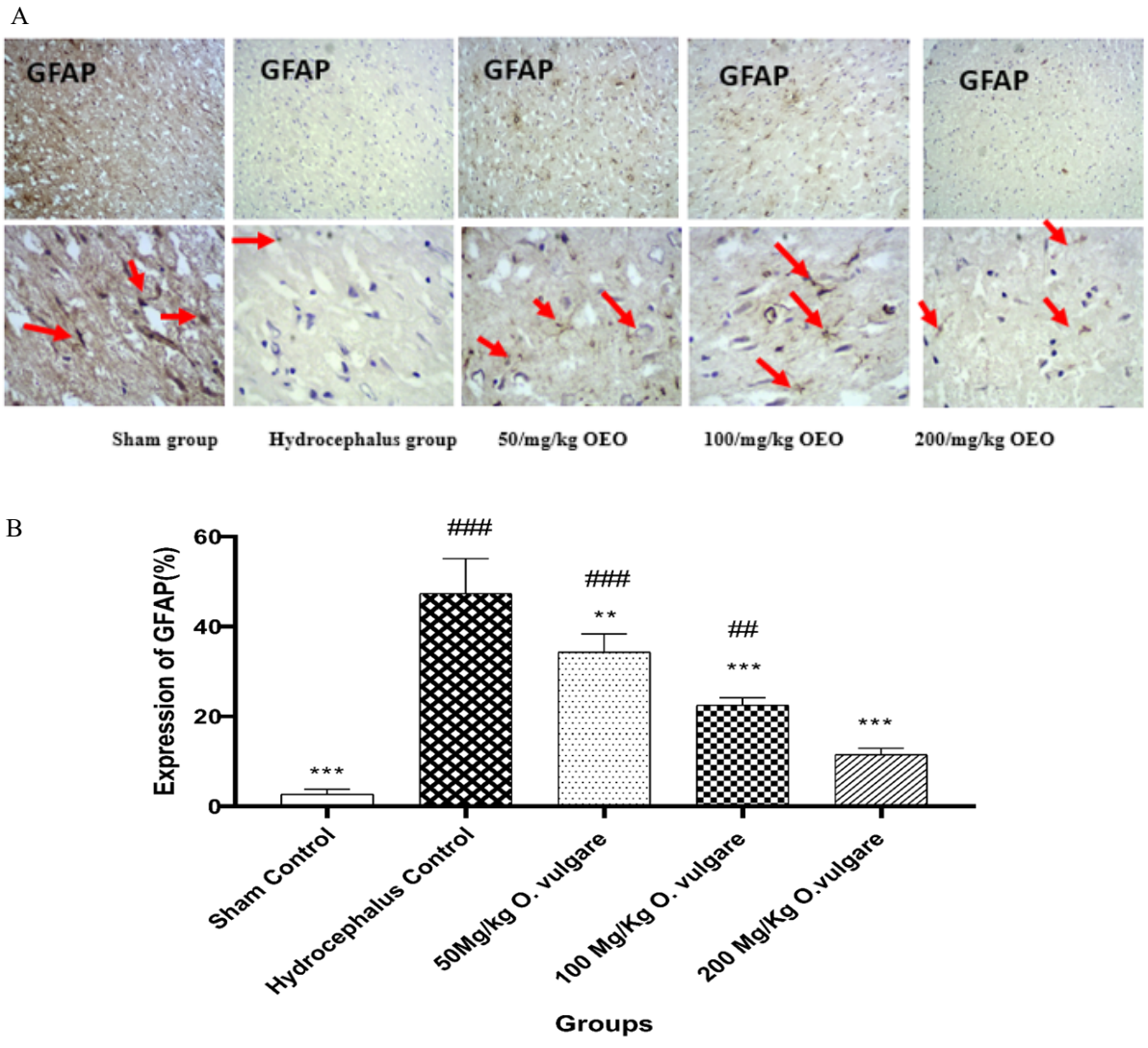

Figure 3. Mean ranking score for GFAP expression in brain tissue for hydrocephalic rats and the *O. vulgare* group

A) The representative images of GFAP in the lateral ventricular white matter; B) Mean percentage of GFAP expression in the brain tissue of the sham control, hydrocephalic + OEO, and hydrocephalic rats

***P<0.001 and **P<0.01 compared with the hydrocephalus control groups, ###P<0.001 compared with 200 mg/kg/d OEO treatment groups.

group. The lateral ventricle increment lasted until day 31 after injection. The size of the lateral ventricle was then reduced in the intervention groups that received 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 from 50 and 100 mg/kg, all three doses were significantly different from the kaolin group, and the 200 mg/kg OEO group was comparable to the saline control group. Based on the results of earlier research, the injection of kaolin into mice may lead to significant ventricular dilatation, as measured by the MRI technique (Di Curzio et al., 2016; Xu et al., 2012a).

In this context, Xu et al. (2012a) 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 demonstrated that kaolin can induce hydrocephalus and ventricular

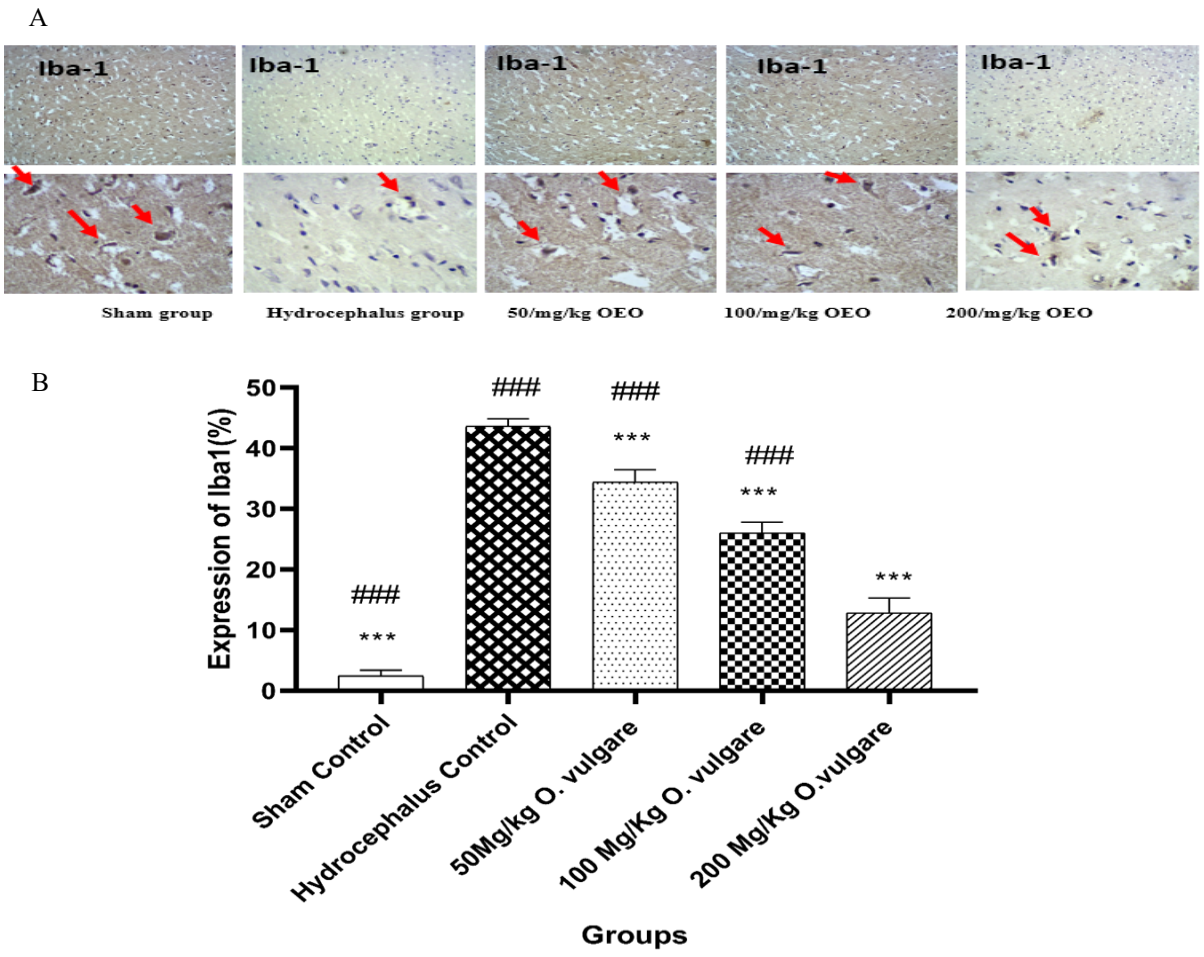

Figure 4. Mean ranking score for Iba-1 expression in brain tissue for hydrocephalic rats and the *O. vulgare* group

A) The representative images of Iba-1 protein in the lateral ventricular white matter; B) The mean percentage of protein expression in positively reacted cells of Iba-1 in the brain tissue of the sham control, hydrocephalic + OEO, and hydrocephalic groups

***P<0.001 compared with the hydrocephalus control group, ###P<0.001 in contrast with the 200 mg/kg/d OEO treatment group, $F_{4, 10}$=259.8.

enlargement in juvenile rats (Botfield et al., 2013). Olopade et al. (2012) also reported that in 3-week-old rats with hydrocephalus induced by intracisternal injection of kaolin, the 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 serve as biomarkers of persistent brain injury, 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; Xu etal., 2012a).

Reactive astrogliosis in the brain is caused by the upregulation of GFAP and the hypertrophy of astrocytic processes. 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 individuals with hydrocephalus and chronic reactive astrogliosis, which may 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. Lin et al. (2015) discovered that lithospermic acid (LSA) extracts found in plants such as *Salvia miltiorrhiza* Bge (Labiatae) and *O. vulgare* might be employed for the treatment

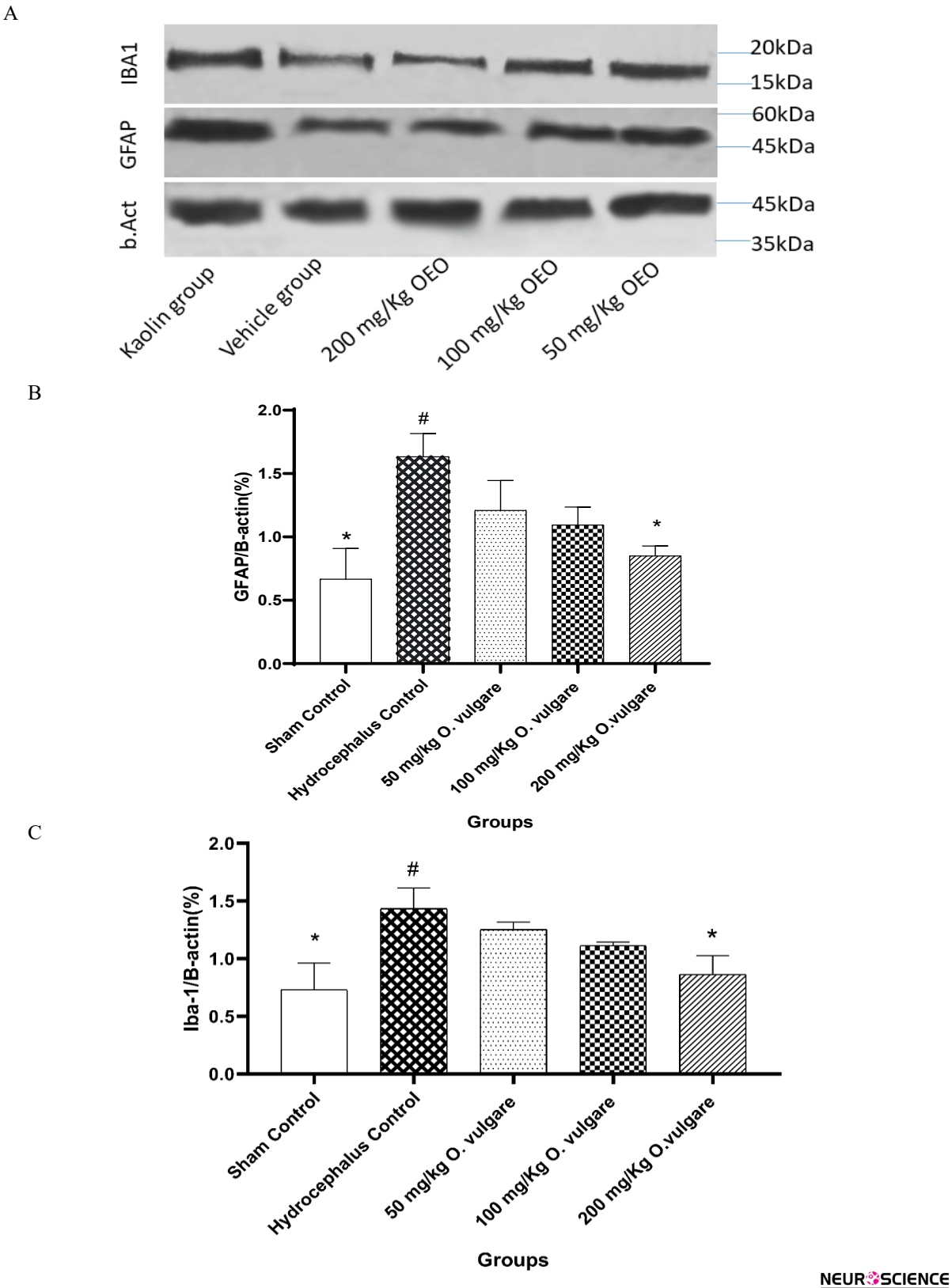

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Figure 5. A) Western blot image showing proteins GFAP and Iba-1 in the lateral ventricular volume of rat hydrocephalus model; B) Mean ranking of GFAP in the brain tissue of the study groups; C) Mean ranking of Iba-1 protein in the brain tissue of the study groups

*P<0.05 compared to the hydrocephalus control group, #P<0.05 compared to the 200 mg/kg/d OEO treatment group.

of Parkinson disease. They demonstrated that LSA effectively reduced MPP + -induced neurotoxicity by lowering the expression levels of GFAP and Iba-1, as well as abnormal neurogenesis. Correspondingly, Jeong et al. (2020) examined the effects of carvacrol, an essential oil extracted from *O. vulgare* , on lithium-pilocarpine-induced status epilepticus, a model of temporal lobe epilepsy, by reducing intracellular free zinc accumulation. However, carvacrol treatment significantly reduced glial and astroglial activation (as indicated by Iba-1 and GFAP) in the hippocampus following status epilepticus.

GFAP-labeled reactive astrocytes have been detected in the CSF of various 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; Xu et al., 2012a). 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, CSF 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, the 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 (Xu et al., 2012a). Astrocytes and microglia may lower glutamate levels in the extracellular space during the early stages of injury, while also storing and providing 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 initiated, and this leads to inflammatory reactions, including the production of chemokines and cytokines, as well as the formation of a destructive extracellular matrix that causes the development 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, the number of Iba-1-immunoreactive microglia was significantly decreased in the three OEO + kaolin treatment groups. However, a significant difference was observed between the three OEO + kaolin groups, indicating that 200 mg/kg/day was the most effective dose in inhibiting 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 *O. vulgare* extracts had a very strong anti-inflammatory effect on activated mixed and microglial cells, inhibiting the expression of inducible nitric oxide synthase and tumor-necrosis factor-alpha. Another study discovered a volatile substance, beta-caryophyllene (BCP), which is abundant in the essential oils of several popular spices and edible plants, including *O. 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 intraperitoneal (IP) of β-caryophyllene for 4 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 those caused by brain damage resulting from hydrocephalus (Olopade et al., 2019; Suryaningtyas et al., 2019; Xu et al., 2012a; Xu et al., 2012b). A study demonstrated a link between microglial process retraction and increased Iba-1 antibody expression in the white matter, as well as kaolin-induced hydrocephalus in rats (Olopade et al., 2012). Another 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. In contrast, glial cells have a negative influence (Xu et al., 2012b). Suryaningtyas et al. (2019) reported that inoculating 20% kaolin into the cistern of 10-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 research found an increase in Iba-1 and GFAP elevation in the kaolin-treated group, indicating alterations in the neuroinflammatory profile in chronic hydrocephalus. The Western blot analysis revealed that reactive astrocytes and microglia exhibited dramatically elevated GFAP and Iba-1 protein expression in hydrocephalus rats, vali-

dating the immunohistochemistry findings of the present investigation. GFAP and Iba-1 expression levels were significantly lower in the 200 mg/kg/d OEO group than in the hydrocephalus group, although there was no significant difference between the 50 and 100 mg/kg/d OEO and hydrocephalus groups.

According to the study's findings, GFAP and Iba-1 expression levels were significantly higher in hydrocephalic rats than in controls (Deren et al., 2010; Xu et al., 2012a). Xu et al. (2012b) revealed that kaolin-induced hydrocephaly increased the expression of GFAP and Iba-1 in rats. Their analysis suggests a correlation between GFAP and Iba-1 protein levels and the extent of ventricular dilatation. Deren et al. (2010) demonstrated that reactive astrocytes and reactive microglia increased 21 days after the intracisternal injection of kaolin in rats to induce hydrocephalus, as determined by immunohistochemical and Western blot analyses. According to research, in adult rats with hydrocephalus caused by the intraventricular injection of 3% kaolin, the expression levels of GFAP and Iba-1 dramatically increased in the experimental groups compared to saline controls, and this had a direct association with disease severity (Xu et al., 2012b).

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 treatment agent for 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

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran (Code: IR.IUMS.REC 1395. 118.27517).

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Authors' contributions

Conceptualization: Manijeh Motevalian and Ashraf'osadat Moazam; Methodology: Ashraf'osadat Moazam and Fatemeh Khajehasani; Investigation: Ashraf'osadat Moazam; Data collection: Mandana Rahimi and Ashraf'osadat Moazam; Data analysis: Gelareh Vahabzadeh; Writing: Gelareh Vahabzadeh; Supervision and funding administration: Manijeh Motevalian.

Conflict of interest

The authors declared no conflict of interest.

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