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Ethanolic extract of *Moringa oleifera* alleviates the symptoms of migraine-like behavior in rats

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Abstract

OBJECTIVE: This study aimed to investigate the potential efficacy of *Moringa oleifera* ethanolic extract in alleviating migraine pain at different doses.

BACKGROUND: In this study, we employed an in vivo rat model in which systemic administration of nitroglycerin (NTG) was used to induce migraine-like and headache-like behaviors.

METHODS: Forty rats were randomly assigned to five groups (n = 8 per group): control (CT), NTG-only, sumatriptan (SMT), *M. oleifera* 50 mg/kg (MO 50 mg/kg), and MO 100 mg/kg. NTG (10 mg/kg, intraperitoneally) was administered every other day for nine days to induce migraine-like symptoms. Behavioral assessments–including climbing, face rubbing, body grooming, freezing, and head scratching–were conducted over a 60-minute period following NTG administration. Two hours post-treatment, the animals were sacrificed, and blood and hippocampal tissue samples were collected. Antioxidant activity, inflammatory biomarker expression (via enzyme-linked immunosorbent assay), and brain-derived neurotrophic factor (BDNF) levels were analyzed.

RESULTS: Our findings indicate that *M. oleifera* ethanolic extract at 50 mg/kg exhibited headache-relieving properties, enhanced BDNF levels, and mitigated depression-like behavior and anxiety indices. However, it did not significantly reduce inflammatory cytokines such as IL-6 and hs-CRP compared to the CT group. Notably, SOD activity was higher in the MO 50 and MO 100 mg/kg groups than in the SMT and NTG groups, although the CT group maintained the highest levels.

CONCLUSIONS: These findings suggest that *M. oleifera* demonstrates significant migraine-relieving effects compared to sumatriptan, with a more favorable safety profile, making it a promising candidate for migraine management.

Introduction

Migraine is a prevalent neurovascular disorder that affects individuals of all age groups (Wijeratne *et al.* 2019; Simmonds *et al.* 2023), manifesting as recurrent episodes of pain accompanied by associated symptoms (Demartini *et al.* 2022). It is one of the six most widespread illnesses globally, affecting both men and women (Avona *et al.* 2021). As a major headache disorder with a high prevalence, migraine significantly impacts patients' quality of life worldwide (Raoof *et al.* 2022). Approximately 2% of the global population suffers from chronic migraine, a complication of episodic migraines (Goschorska *et al.* 2020). Compared to other forms, chronic migraine is more strongly associated with conditions such as obesity, depression, and anxiety, imposing a substantial burden on individuals and healthcare systems (Ye *et al.* 2022). Migraine typically emerges in early childhood and peaks during adolescence, around 12 years of age in boys and 15 years in girls (Wijeratne *et al.* 2019), although it can develop at any stage of life (Haan *et al.* 2007). It is a debilitating condition characterized by intense, recurrent headaches often accompanied by photophobia, phonophobia, nausea, and vomiting. The associated pain significantly contributes to functional impairment, affecting nearly all aspects of daily life (Harriott *et al.* 2019). Additionally, migraine is classified as a primary episodic headache disorder with autonomic dysfunction. According to the International Classification of Headache Disorders, Third Edition (ICHD-3), migraines are categorized into two distinct types: migraine with aura and migraine without aura (Ye *et al.* 2022). Despite extensive research, the underlying mechanisms of migraine pathophysiology remain incompletely understood. Several factors, including genetic predisposition and neurovascular dysregulation, have been implicated in its etiopathogenesis (Goschorska *et al.* 2020; Ye *et al.* 2022). Moreover, recent studies suggest that environmental factors such as noise and air pollution may act as potential migraine triggers (Li *et al.* 2019). Given the limited understanding of this disorder and the scarcity of highly effective treatments, further investigation into novel therapeutic strategies is imperative (Tardiolo *et al.* 2019).

As a member of the family *Moringaceae*, *Moringa oleifera* – commonly known as the horseradish tree, drumstick tree, or sahajna (Mundkar *et al.* 2022) is widely recognized for its diverse medicinal properties. In developing countries, it serves as an edible plant for both humans and animals due to its well-documented antioxidant, anti-inflammatory, and apoptotic activities. These effects are attributed to its rich phytochemical profile, which includes α-carotene, quercetin, kaempferol, ascorbic acid, flavonoids, phenolic acids, rhamnose, glycosylates, glucomoringin, and isothiocyanates (Dhakad *et al.* 2019; Mundkar *et al.* 2022). Due to its extensive therapeutic potential, *M. oleifera* has been described by numerous researchers as a "wonder plant" with beneficial effects on nearly every physiological system (Igado & Olopade 2017). The neuroprotective and cognitive-enhancing properties of *M. oleifera* leaf extract are well-documented (Mundkar *et al.* 2022). This plant has been explored for its potential in managing neurological disorders, with preliminary findings suggesting that it may slow the progression of conditions such as Alzheimer's disease, Parkinson's disease, and epilepsy (Ghimire *et al.* 2021). Additionally, *M. oleifera* exhibits potent antioxidant activity, as its aqueous leaf extract has been shown to prevent lipid peroxidation by scavenging free radicals, including DPPH, superoxide, and nitric oxide (Sreelatha & Padma 2011). Notably, moringa contains nine times more iron than spinach and four times more fiber than oats, highlighting its nutritional value (Mushtaq *et al.* 2021). Previous studies have demonstrated that the ethanolic extract of *M. oleifera* leaves exhibits significant ($p < 0.001$) anxiolytic effects in albino mice, as observed in the elevated plus maze (EPM) and light-dark box (LDB) anxiety models at a dose of 200 mg/kg (i.p.) (Bhatia 2014). However, in the present study, lower doses of the ethanolic extract were used to evaluate its potential anti-migraine, anxiolytic, and antidepressant activities.

Regular administration of nitroglycerin (NTG) induces headaches in healthy individuals and migraines without aura in those predisposed to the condition (Moye & Pradhan 2017). In the present study, a migraine model was established in rats through intraperitoneal injection of NTG. As a nitric oxide (NO) donor, NTG has been widely utilized in migraine-inducing experiments, demonstrating reliable results in preclinical models (Latif *et al.* 2021; Li *et al.* 2016; Moye & Pradhan 2017). Oxidative stress has been implicated in migraine pathophysiology, with superoxide dismutase (SOD) playing a crucial role as a detoxifying enzyme and potent antioxidant that protects cells from reactive oxygen species (ROS) (Sannasimuthu *et al.* 2018). While previous research suggests that oxidative alterations contribute to migraine pathogenesis, clinical findings regarding oxidative stress markers in migraine patients remain inconclusive (Yigit *et al.* 2018). Some studies have reported a correlation between migraine and oxidative stress (Borkum 2016; Yilmaz *et al.* 2011), whereas others have found no significant association (Geyik *et al.* 2016; Neri *et al.* 2015). Notably, increased ROS levels in migraineurs have been linked to elevated nitric oxide (NO) metabolites and malondialdehyde (MDA), a key biomarker of lipid peroxidation, alongside reduced antioxidant defense, including lower SOD activity (Togha *et al.* 2019). Brain-derived neurotrophic factor (BDNF), both in its precursor form (proBDNF) and its mature form, is predominantly expressed in the central nervous system (CNS) and is secreted by neurons and microglia, playing a vital role in neuro-

inflammation (Zhou *et al.* 2022). Several studies have implicated BDNF in neurological disorders, including migraine (Lima Giacobbo *et al.* 2019). Additionally, high-sensitivity C-reactive protein (hs-CRP), a non-specific inflammatory marker, has been associated with migraine in cross-sectional studies (Hagen *et al.* 2020). However, further investigation is required to understand the role of emotional and affective components of pain in animal models of migraine. In behavioral migraine studies, the rat grimace scale (RGS) has been proposed as a reliable tool for assessing spontaneous pain responses (George *et al.* 2019). Previous research suggests that conditions inducing headaches in humans also result in widespread hypersensitivity in rats, supporting the translational relevance of preclinical migraine models (Avona *et al.* 2021). Therefore, this study aimed to provide an experimental framework for further research into migraine pathophysiology and potential therapeutic interventions. To achieve this, we assessed the anti-migraine effects of *M. oleifera* extract by measuring BDNF levels in hippocampal tissue, along with hs-CRP, SOD, and MDA levels in blood samples across different experimental groups.

Materials and methods

Animals

This study employed forty male Wistar rats (5–6 weeks old, 180–220 g), housed under standard laboratory conditions with a 12-hour light-dark cycle, ad libitum access to commercial rat chow and water, and a one-week acclimatization period prior to experimentation. All procedures were conducted in compliance with ethical guidelines set by the AJA University Medical Ethics Committee, the U.K. Animals (Scientific Procedures) Act of 1986, and the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978), with efforts to minimize animal use and suffering.

Experimental design

The animals were randomly assigned to five groups (n = 8 per group):

- Control (CT) group: Received no treatment.
- Migraine-induction (NTG) group: Received intraperitoneal (i.p.) injections of nitroglycerin (NTG) at a dose of 10 mg/kg every other day for nine days (a total of five injections) to induce a migraine-like condition.
- Sumatriptan-treated (SMT) group: Following migraine induction with NTG, rats received sumatriptan (1 mg/kg, i.p.).
- Moringa extract 50 mg/kg (MO-50) group: Received ethanolic extract of *Moringa oleifera* (50 mg/kg, i.p.) for 14 consecutive days.
- Moringa extract 100 mg/kg (MO-100) group: Received ethanolic extract of *Moringa oleifera* (100 mg/kg, i.p.) for 14 consecutive days.

Ethical approval

The experimental protocol was reviewed and approved by the Ethics Committee of AJA University of Medical Sciences (Approval ID: IR.AJAUMS.REC.1401.12). The study was conducted in compliance with animal welfare regulations, including the Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011).

Behavior testing on migraine

All behavior tests were conducted on Days 0 and 9 of the experimental period, between 08:00 and 13:00, corresponding to the rats' circadian daylight cycle. To ensure methodological consistency, the procedures were performed by two trained personnel.

Von Frey testing (mechanical threshold)

To assess mechanical sensitivity, the Von Frey filament test was used to measure facial and hindpaw withdrawal thresholds. Twenty-four hours before habituation in the behavioral chambers, rats were handled for a 5-minute session. Only rats that met the baseline withdrawal thresholds—≥ 8 g for facial withdrawal and ≥15 g for hindpaw withdrawal—were included in the study.

After baseline measurements, rats received the designated treatments. The mechanical sensitivity of the periorbital region (midline of the forehead at eye level) was assessed using Von Frey filaments, starting from the 1 g filament. Filaments were applied in an ascending order until the animal responded or the 8 g filament was reached. If a response was observed, lower-pressure filaments were tested until reaching the 0.4 g filament or until no further responses were detected. For facial sensitivity, the following filament forces were used: 1 g, 2 g, 4 g, 6 g, and 8 g. However, for hindpaw sensitivity, the filaments tested were: 2 g, 4 g, 6 g, 8 g, and 15 g (Avona *et al.* 2021).

Behavioral scoring

To assess migraine-related behaviors, rats in the NTG group and those treated with varying concentrations of *M. oleifera* ethanolic extract were placed in metal cages and allowed to acclimate for 15 minutes prior to testing. Each rat was videotaped for 60 minutes, with behavioral assessments conducted at 10-minute intervals. The following parameters were recorded as head scratching, head-flick frequency, grooming, cage climbing (rearing), and lying on the belly. The total time spent performing each behavior was summed to quantify migraine-associated behaviors.

RGS test

RGS was assessed according to established protocols (Benbow *et al.* 2022). Each rat was placed individually in a metal cage equipped with digital cameras positioned at both ends to capture their facial expressions. After a 15-minute acclimatization period, the rats were recorded for one hour. Still images were extracted every 5 minutes from the video to generate JPEG files

for analysis. A custom coding program was developed based on literature criteria, was used to evaluate the following facial action units:

- Orbital Tightening: Narrowing of the orbital area, partial closure of the eyelids, or squeezing of the eyes.
- Nose Flattening: The appearance of a circular bulge on the bridge of the nose that becomes flattened due to muscle contraction.
- Cheek Flattening: A transformation of the normally protruding cheek muscles (located between the eyes and the whisker pad) into a flatter appearance.
- Ear Position: Alterations in ear position, such as being pulled away from or forward relative to the baseline, with the ear tip potentially forming a vertical ridge with the face.
- Whisker Changes: Any deviation from the baseline whisker position, whether a backward shift, a forward extension, or a change from a gathered to a more erect posture.

Following a brief training session, three independent coders—blinded to the treatment conditions—evaluated randomized sets of digital photographs. For each action unit in every image, the coders assigned a score of 0 (absent), 1 (moderately visible), or 2 (severe). These scores were then used to determine the overall presence or absence of pain in each rat.

Behavioral testing on anxiety-like behavior

Elevated plus maze (EPM) test

The elevated plus maze is a well-established assay for assessing anxiety-like behavior in rodents and has been validated for evaluating anti-anxiety effects and identifying underlying neural mechanisms (Shemirani *et al.* 2022). In this test, each rat was placed at the center of the maze, facing an open arm. A video-tracking system (Borj Sanat, Tehran, Iran), along with an observer, recorded both the entries into and the duration spent in each arm over a 5-minute period. This study analyzed total open and closed-arms entries, open-arms entrance percentages, and closed-arms entry percentages. In addition, the percentage of crossings with open arms [open entrances/(open + closed entries) ×100] and the percentage of time spent in open arms [(open arms/300) ×100] were determined.

Prior to each trial, the maze was cleaned with an alcohol solution to eliminate olfactory cues. An anxiety index was calculated using methods described by Mazor *et al.* 2009 and Rao & Sadananda, 2016. The anxiety index incorporates both the time spent in the exposed open arms and the frequency of entries into these arms, and is computed using the following formula:

$$\text{Anxiety index} = 1 - \left(\frac{\text{Open} - \text{arm time}}{\text{Total time}} \right) + \left(\frac{\text{Open} - \text{arm entries}}{\text{Total entries}} \right)/2$$

This index quantitatively reflects anxiety-like behavior, with higher values indicating greater anxiety.

Open field test (OFT)

Locomotor activity and exploratory behavior were assessed using Plexiglas open-field boxes (90 × 90 cm with a height of 42 cm). The floor of each box was divided into 18 × 18 cm squares using black gridlines, with four 11 cm intervals from each wall defining the central and peripheral areas. Dependent variables included time spent in the center and distance traveled in the center relative to the total distance traveled. An observer, blinded to treatment groups, analyzed video recordings to document the number of squares crossed and missed. To eliminate potential olfactory cues, the apparatus was thoroughly cleaned between trials. After testing, each rat was returned to its home cage (Pak Aeen *et al.* 2022).

Light-dark test

Anxiety-related light aversion behavior was assessed using a modified light-dark test adapted from previous studies (Wu *et al.* 2021). The test was performed in a specialized chamber divided into two compartments: a brightly illuminated section and a dark, enclosed section. Baseline measurements were recorded prior to NTG injection, and rats were re-evaluated 10 minutes post-injection. Each rat was allowed to explore the light-dark box freely for 15 minutes, with the total time spent in the light compartment recorded. A decrease in time spent in the light chamber was interpreted as increased anxiety-like behavior.

Behavioral testing on depression-like behavior

Forced swimming test (FST)

The FST was conducted to evaluate depression-like behavior by assessing passive and active coping strategies in an inescapable stress scenario. The test was performed in a 2-liter glass beaker filled with water (21 ± 1°C) to a depth of 15 cm, preventing rats from touching the bottom or escaping. Each rat underwent a 5-minute trial, during which the following behaviors were recorded: immobility time (floating without struggling, indicative of behavioral despair), swimming time (active movement without climbing), and climbing time (vigorous attempts to escape). Following the trial, rats were immediately dried with towels to prevent hypothermia. The apparatus was thoroughly cleaned between trials to eliminate residual stress cues for subsequent subjects (Ibrahim *et al.* 2012; Modarresi Chahardehi *et al.* 2014).

Assessment of oxidative stress

Oxidative stress was assessed by measuring MDA levels, a marker of lipid peroxidation, and SOD activity, a key antioxidant enzyme, in blood samples (Wu *et al.* 2021). Blood was collected with 5 mmol/L butylated hydroxytoluene (BHT) to prevent lipid peroxidation. MDA levels and SOD activity were quantified using commercially available assay kits (SOD activity kit, Kiazist, Iran) in accordance with the manufacturer's instructions.

Determination of serum inflammatory factor levels

The concentrations of interleukin-6 (IL-6) and hs-CRP in blood plasma were measured using enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturers' protocols to ensure accurate detection and quantification.

Determination of hippocampal BDNF levels

At the conclusion of the experiment, rats were anesthetized with diazepam hydrochloride (4 mg/kg) and ketamine hydrochloride (60 mg/kg) (Husseini *et al.* 2021). Transcardiac perfusion was performed using cold 0.9% saline (pH 7.4) to remove blood from the tissues (Hiromasa *et al.* 2020). Following decapitation, the hippocampus was carefully dissected. For BDNF level determination, 20 mg of hippocampal tissue was placed in a 1.8-mL microtube, to which 500 µL of 100 mM PBS buffer (pH 7.4) and 20 µL of antiprotease containing 0.16 mg/mL heparin and cooled Tris-HCl buffer (pH 7.5, 5 mM EDTA) were added. Samples were stored at 5–7°C for 30 minutes prior to homogenization. Homogenization was performed using a Heidolph homogenizer (Germany) at 12,000 rpm for 3 minutes, followed by centrifugation at 6,000 rpm for 10 minutes (Hitech). The supernatant was collected and stored at −20°C for further analysis. BDNF levels were quantified using a ZellBio GmbH ELISA kit (Cat. No.: ZB-0476-R9648) in accordance with the manufacturer's instructions.

Statistical analysis

The sample size of eight rats per group was determined based on the statistically significant effects of NTG treatment observed on behavioral outcomes. All results are presented as the mean ± standard deviation (SD). All statistical analysis was performed using GraphPad Prism® 8.1 software for Windows (GraphPad Software, San Diego, CA, USA). The data were analyzed using a one-way analysis of variance (ANOVA) with Dunnett's test to compare the groups with the control. Bonferroni post hoc and Student t-tests were used when necessary. The tracking paths were analyzed using

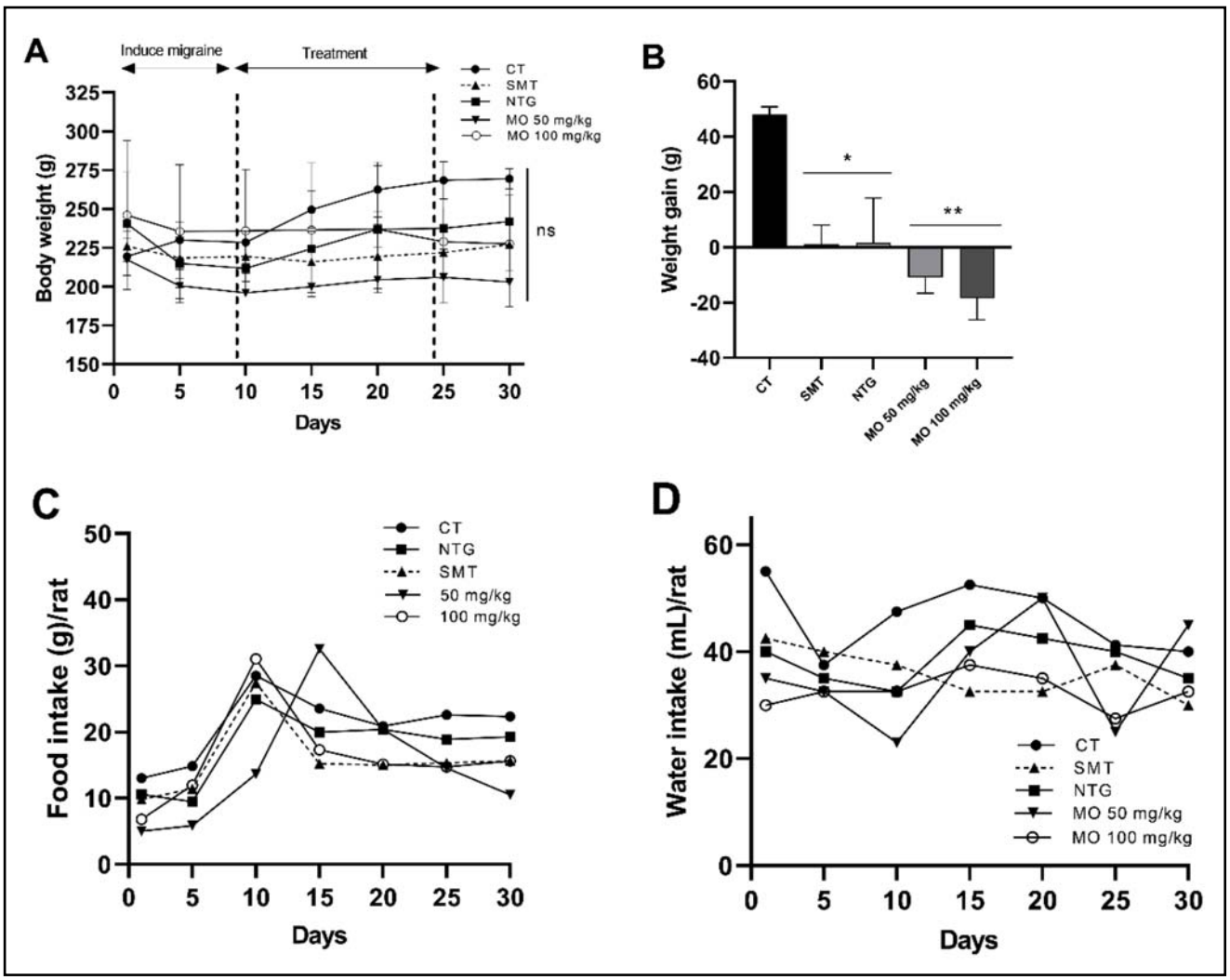

Fig. 1. Changes in body weight in male rats for various tested groups **(A)**, weight gain **(B)**, food intake **(C)**, and water intake **(D)**. All data are presented as mean ± SD and analyzed using repeated-measures ANOVA (comparing all groups) using Dunnett's test. * $p < 0.05$ and ** $p < 0.01$; ns = not significant.

a computerized video-tracking system by Borj Sanat, Tehran, Iran. Statistical significance was set at $p < 0.05$, with additional thresholds of $p < 0.01$, $p < 0.001$, and $p < 0.0001$ for highly significant results.

Results

Body weight, food and water intake

As illustrated in Fig. 1, no significant difference in body weight was observed between the control and treatment groups at the end of the four-week study (Fig. 1A). However, during NTG-induced migraine, the MO 50 mg/kg group showed a lower body weight, while the MO 100 mg/kg group exhibited the highest body weight until day 10. From day 11 onward, the CT group achieved the highest body weight (269.5 g), followed by the NTG group (242 g), while the MO 50 mg/kg group maintained the lowest weight ($p > 0.05$). With the exception of the CT group, all other groups exhibited relatively stable body weight trends with minor fluctuations but no statistically significant differences over time. A significant difference in weight reduction was observed between the CT and treatment groups. The SMT and NTG groups showed slight weight reductions of 1 g and 1.5 g ($p < 0.01$), respectively. In contrast, the MO 50 mg/kg and MO 100 mg/kg groups demonstrated substantial weight reductions of 11 g and 18.5 g ($p = 0.0039$ and $p = 0.0023$, respectively).

As shown in Fig. 1C, food intake followed a similar trend across all groups. Initially, all groups displayed a gradual increase in food intake until day 5, followed by a more pronounced rise until day 10 (the end of migraine induction). From day 10 to day 30, food intake remained relatively stable in all groups except for the MO 50 mg/kg group. The CT group exhibited the highest food intake, while the MO 50 mg/kg group had the lowest. Moreover, there were noticeable differences in water consumption across the groups (Fig. 1D).

Effect of M. oleifera extract on NTG-induced rats and their migraine-like behavior

Fig. 2A displays representative images from JPEG files of the RGS test, which evaluated nocifensive behavior. However, no significant differences in RGS scores were observed among the treatment groups, including the NTG group, during the one-hour monitoring period (Fig. 2B). A two-way ANOVA indicated a significant difference between groups ($F_{4,60} = 2.81$, $p = 0.0333$), but no significant differences were found in interaction effects ($F_{20,60} = 0.823$, $p = 0.6771$) or time-point effects ($F_{5,60} = 0.112$, $p = 0.9894$). As expected, orbital tightening was observed in the NTG group.

Facial mechanical threshold assessments were performed before NTG injection and after extract administration. A significant increase in RGS scores was observed in the NTG and MO 100 mg/kg groups at the end of the one-hour experiment ($p < 0.0001$ and $p < 0.05$, respectively; Fig. 2D). Treatment with MO 50 and 100 mg/kg resulted in a substantial reduction in the frequency of head flicks (Fig. 2C), head scratches (Fig. 2E), facial grooming (Fig. 2F), rearing (Fig. 2G), and lying on the belly (Fig. 3H). A significant effect of time ($F_{4,25} = 3.071$, $p = 0.0346$), treatment group ($F_{4,25} = 7.357$, $p = 0.0005$), and time/treatment interaction ($F_{16,25} = 4.589$, $p = 0.0004$) was observed on head-flick frequency. As illustrated in Fig. 2C, neither the MO 50 mg/kg nor the MO 100 mg/kg groups exhibited any head flicks throughout the observation period. Grooming behavior was significantly reduced in the MO 50 mg/kg group compared to the NTG and CT groups (Fig. 2F).

Fig. 2I(a) illustrates the motor coordination and balance assessment using a rotarod device. The NTG and MO 100 mg/kg groups showed the highest latency, though no significant differences were observed among the groups compared to the CT group ($p > 0.05$). A similar trend was noted for the distance traveled on the rotarod (Fig. 2I(b)). The NTG and SMT groups exhibited significantly higher rates of resting behaviors, such as motionless postures or sleep-like behavior, compared to the CT group ($p < 0.01$; Fig. 2H). The LOB response induced by NTG, known to reflect nausea, was significantly suppressed in the MO 50 mg/kg and MO 100 mg/kg groups compared to the CT group from P1 to P6 (Fig. 2H). Dunnett's post-hoc analysis revealed a significant increase in rearing behavior in the MO 50 mg/kg and MO 100 mg/kg groups at P3 and P4 ($p < 0.05$). Additionally, rats in the MO 50 mg/kg and MO 100 mg/kg groups exhibited significantly shorter LOB durations from P3 to P6 compared to the CT and NTG groups ($p < 0.05$). A significant effect of treatment group ($F_{4,30} = 1255$, $p < 0.0001$), time ($F_{5,30} = 704.2$, $p < 0.0001$), and time/treatment interaction ($F_{20,30} = 262.6$, $p < 0.0001$) was observed.

Elevated plus maze (EPM) test

Fig. 3A illustrates the tracking paths in the EPM, including the percentage of time spent in the open, center, and closed arms, the percentage of entries into each arm, and the anxiety index. Male NTG-induced rats treated with 50 and 100 mg/kg of ethanolic *M. oleifera* extract and the CT group had their time spent in the open and closed arms recorded. Notably, the MO 50 mg/kg group spent significantly more time in the open arms compared to the CT group ($p = 0.0078$; Fig. 3B). The CT group exhibited the highest percentage of time spent in the center of the EPM (21.79%), followed by the MO 50 mg/kg (12.98%, $p = 0.0039$) and MO 100 mg/kg groups (8.56%, $p < 0.0001$). No significant differences were observed in open- and closed-arm entries between the groups ($p > 0.05$; Fig. 3E and 3F).

These findings suggest that the MO 50 mg/kg group exhibited lower anxiety-like behavior than other groups. Conversely, the SMT, NTG, and MO 100 mg/kg groups showed a significant increase in time spent in the closed arms ($F_{4,35} = 19.7$, $p < 0.0001$, $p = 0.0001$, $p < 0.0001$;

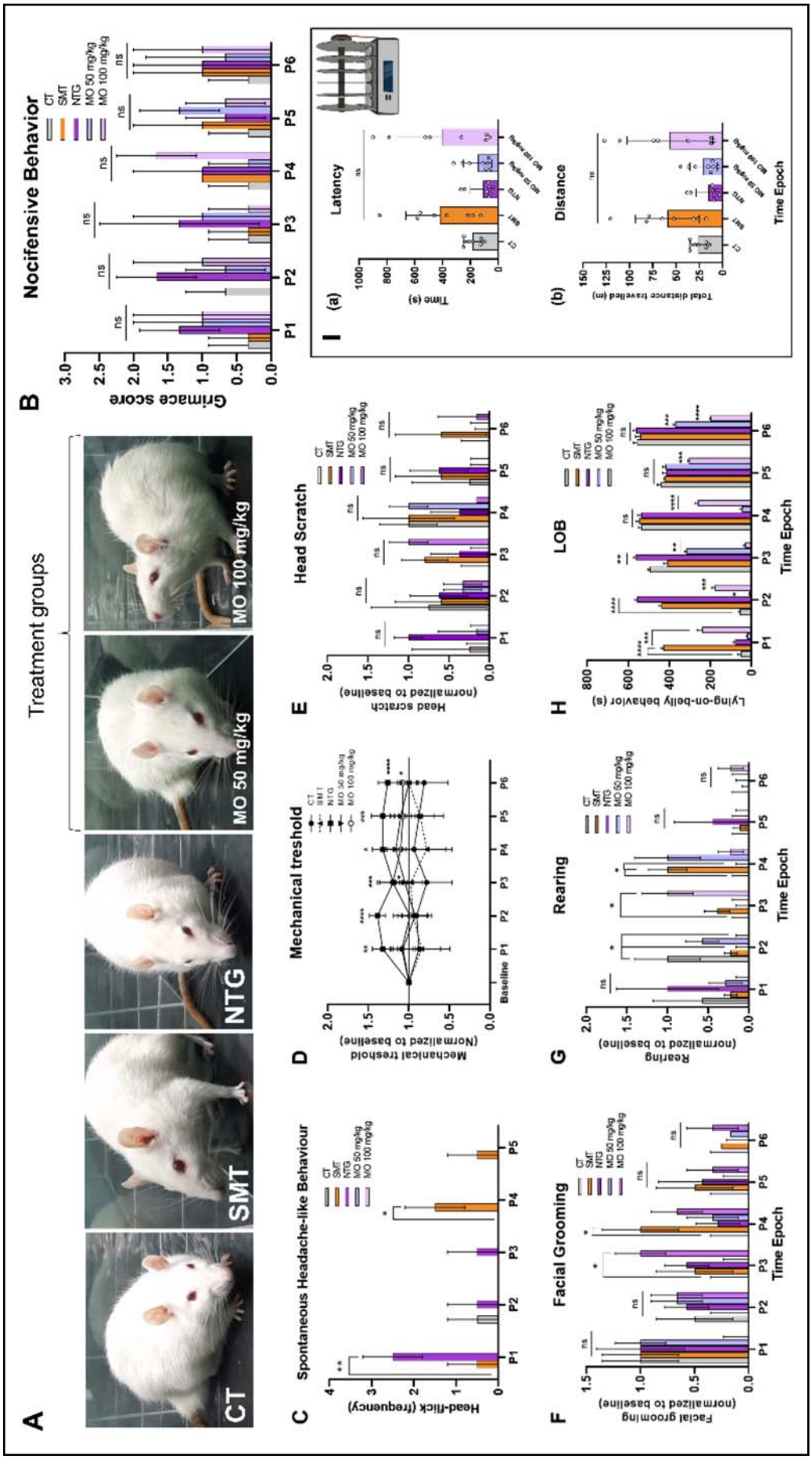

Fig. 2. Various headache-like behaviors are related to pain and migraine in rats. **A)** samples of different images obtained for nocifensive behavior (grimace score); **B)** results from nocifensive behavior were taken on the 14th day of treatment (the last day of treatment); **C)** head-flick frequency; **D)** temporalis muscle mechanical threshold; **E)** head scratching; **F)** facial grooming; **G)** rearing behavior; **H)** lying on the belly (LOB) behavior; **I)** motor function ability using the rotarod test: **a)** latency; **b)** total distance traveled. The data are mean ± SD (n = 30). Using Dunnett's test, a one-way ANOVA was used to compare the control and treatment groups. Time Epoch = P1 (0–10 min), P2 (10–20 min), P3 (20–30 min), P4 (30–40 min), P5 (40–50 min), and P6 (50–60 min) post-injection. A statistical difference in the data obtained from the tested groups was determined using a one-way ANOVA using Dunnett's test (*post hoc*) compared to the CT group. * $p < 0.05$ and ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = no significant.

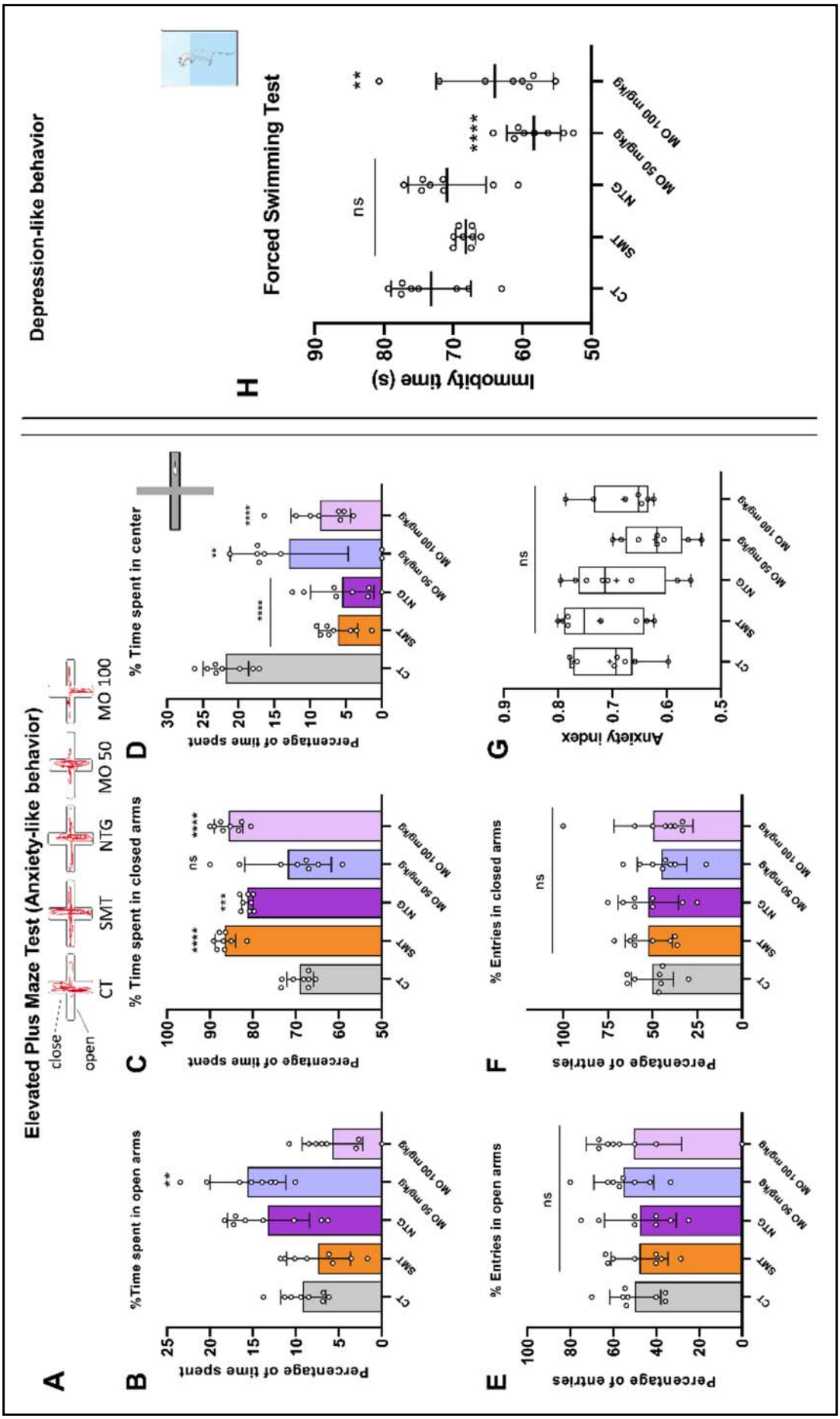

Fig. 3. Effects of various doses of ethanolic extract of *M. oleifera* on anxiety and depression-like behavior in the elevated plus maze test in open and closed arms. **A)** A representative of a tracking path obtained from the EPM; **B)** the percentage of time spent in the open arms; **C)** the percentage of time spent in the closed arms; **D)** the percentage of time spent in the center area in the EPM; **E)** percentage of entries in the open arms; **F)** percentage of entries in the closed arms; **G)** anxiety index; and **H)** FST test. Data represent the mean ± SD (n = 8 per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against the control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ against the sham group; one-way ANOVA followed by Dunnett's multiple comparison test. ns = The experiment and control groups showed no statistically significant differences.

Fig. 3C). The anxiety index, which ranges from 0 to 1 and is based on open- and closed-arm entries and cumulative time spent in the EPM test, ranged from 0.5 to 0.8 in this study. However, the MO 50 mg/kg group demonstrated no significant difference in anxiety levels compared to the CT group ($p = 0.0716$; Fig. 3G).

Effect of ethanolic M. oleifera extract in various doses on forced swimming test

The FST was used to evaluate the impact of ethanolic *M. oleifera* extract on depression-like behavior (Fig. 3H). The results demonstrated a significant reduction in immobility time in the MO 50 mg/kg and MO 100 mg/kg groups compared to all other groups, including the CT group ($p < 0.0001$ and $p = 0.0077$, respectively; Fig. 3H).

Effect of ethanolic M. oleifera extract in various doses on open-field and light-dark box tests

Fig. 4A presents the tracking path and heatmap for each experimental group. As illustrated in Fig. 4B, the NTG and MO 50 mg/kg groups exhibited significant alterations in exploratory behavior, with a marked decrease in time spent in the peripheral area and an increase in time spent in the central area of the OFT. One-way ANOVA analysis revealed a significant difference in the percentage of time spent in the center area between the NTG and MO 50 mg/kg groups compared to the CT group ($p < 0.0001$). In terms of the number of center entries in the OFT, the SMT, NTG, and MO 50 mg/kg groups demonstrated a significant increase compared to the CT group ($p < 0.0001$, $p = 0.0088$, and $p = 0.0002$, respectively). A similar trend was observed in the total distance traveled within the center zone. The MO 50 mg/kg and SMT groups exhibited the greatest distance traveled in the center area, measuring 3.51 and 2.85 meters, respectively ($p < 0.0001$; Fig. 4D). This was followed by the NTG and MO 100 mg/kg groups, with distances of 2.06 and 1.78 meters ($p = 0.0054$ and $p = 0.00456$, respectively).

In the LDB test, the MO 50 mg/kg group showed a significant increase in the percentage of time spent in the light chamber compared to the CT group ($p = 0.0031$; Fig. 4E). As expected, the NTG group exhibited a significant increase in time spent in the dark chamber ($p = 0.0009$; Fig. 4F). Additionally, the MO 50 mg/kg group displayed a significantly higher frequency of head pokes into the light chamber, with an average of 22 occurrences ($p < 0.0001$; Fig. 4G). However, there were no statistically significant differences in the number of 10-minute intervals across groups ($p > 0.05$), except for the MO 50 mg/kg group (data not shown).

Effect of ethanolic M. oleifera extract in various doses on spatial learning in Barnes maze test

The impact of various concentrations of *M. oleifera* extract on learning and spatial memory was assessed using the Barnes maze test. Several parameters were evaluated, including time spent in the Q1 zone, strategy, escape latency, number of errors, velocity, and total distance traveled (Fig. 5).

Latency to escape

In the probe trial, latency to reach the target hole exhibited a noticeable reduction in the CT group during the learning phase, whereas other groups showed slight variations. The MO 50 mg/kg and MO 100 mg/kg groups demonstrated significantly prolonged latency to reach the escape hole ($p = 0.0007$ for both groups), whereas the NTG group exhibited a significantly shorter latency ($p = 0.0202$). Despite these differences, latency to escape consistently decreased over the training phases across all groups, presenting similar learning curves ($F_{3,140} = 2.17$, $p = 0.0940$, Bonferroni post hoc; Fig. 5C). Further statistical analysis using two-way ANOVA revealed no significant differences between groups ($F_{4,140} = 18.1$, $p > 0.05$) or interactions ($F_{12,140} = 0.718$, $p = 0.7324$) during the learning phase.

Number of errors

The total number of errors was analyzed using repeated-measures ANOVA (Fig. 5D). Unlike latency to escape, the number of errors exhibited a downward trend throughout the learning phases, with no significant differences observed across days ($F_{3,140} = 1.67$, $p = 0.1752$), between groups ($F_{4,140} = 2.04$, $p = 0.0924$), or interactions ($F_{12,140} = 1.11$, $p = 0.3542$). These findings suggest that the rats struggled to utilize spatial cues effectively. Additionally, one-way ANOVA comparison in the probe trial did not reveal any significant differences between experimental groups and the CT group ($F_{4,15} = 0.763$, $p = 0.5652$).

Velocity

Throughout the learning session, velocity remained stable across all groups, except for the CT group, which showed variations during days 1 to 3. During the probe trial, the SMT and MO 100 mg/kg groups exhibited significantly reduced velocity ($p = 0.0095$ and $p = 0.0079$, respectively) compared to the CT group, followed by the MO 50 mg/kg group ($p = 0.0362$). The CT group displayed the highest velocity, indicating possible differences in exploratory behavior or motivation.

Total distance

The total distance traveled by each group during the learning phase is illustrated in Fig. 5F. Distance traveled followed a similar trend to the number of errors, progressively decreasing over training days ($F_{3,140} = 6.54$, $p = 0.0004$). Interaction effects were statistically significant ($F_{12,140} = 1.95$, $p = 0.0330$), while intergroup differences did not reach statistical significance ($F_{4,140} = 2.36$, $p = 0.0564$). In the probe trial, no significant difference was found between the treated and CT groups ($F_{4,15} = 2.56$, $p = 0.0814$).

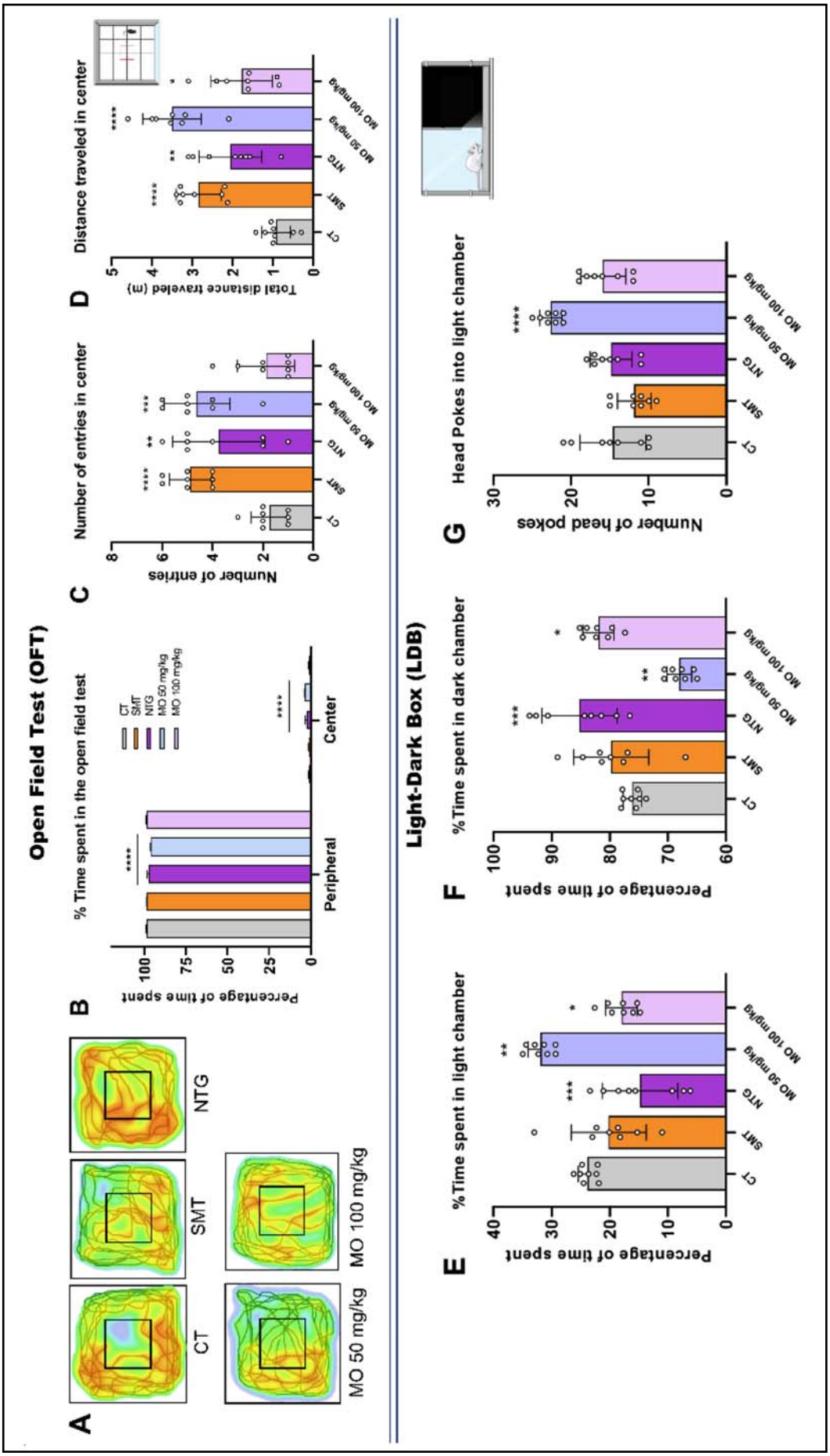

Fig. 4. Comparison of three behavioral parameters in the open-field test on different groups. **A)** representing the heat map and tracking the pace of rats in different treatment groups (CT and NTG groups); **B)** the percentage of time spent in the peripheral and central areas in the OFT; **C)** the number of entries to the center of the OFT; and **D)** the total distance traveled in the center area. The data represent the mean ± S.D. (n = 8 per group). A statistical difference in the data obtained from the tested groups was determined using a one-way ANOVA using Dunnett's test (post hoc) compared to the CT group. * $p < 0.05$ and ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns = not significant.

Based on one-way ANOVA with Dunnett's test, only the SMT group ($p = 0.04$) showed a significant difference compared to the CT group regarding time spent in the Q1 zone during the probe trial. The NTG group exhibited the highest percentage of time spent in the Q1 zone (yellow zone), though this difference was not statistically significant ($p > 0.05$; Fig. 5B). The tracking paths obtained for all groups (Fig. 5G) indicate that only the CT group employed a direct strategy, achieving the shortest latency to escape.

Determination of BDNF, hs-CRP, and IL-6 levels

To assess learning and memory processes, BDNF expression levels in the hippocampus were measured. Post-hoc analysis indicated that BDNF levels were significantly increased in the MO 50 mg/kg group compared to the CT group ($p = 0.0002$). However, the NTG group exhibited significantly lower BDNF levels compared to the CT group ($p < 0.0001$; Fig. 6A). Notably, no significant differences were observed between the SMT and MO 100 mg/kg groups relative to the CT group ($p > 0.05$), suggesting a possible non-linear dose-response effect of *M. oleifera* on BDNF expression.

Serum hs-CRP levels were significantly elevated in all experimental groups, including NTG, MO 50 mg/kg, MO 100 mg/kg, and SMT, compared to the CT group ($p < 0.0001$; Fig. 6B). Similarly, IL-6 levels were significantly increased in all experimental groups ($p < 0.0001$), with the NTG group displaying the highest concentration at 9.88 ng/mL ($p < 0.0001$; Fig. 6C). These findings suggest a systemic inflammatory response across treatment groups.

Effects of M. oleifera extracts on the blood level of MDA level and SOD activity

To evaluate oxidative stress, serum MDA levels were measured. The NTG group exhibited significantly higher MDA levels compared to the CT group ($p < 0.0001$), followed by the MO 50 mg/kg, MO 100 mg/kg, and SMT groups ($p = 0.0005$, $p = 0.0007$, and $p = 0.0015$, respectively). Conversely, SOD activity was markedly reduced in the NTG group (9.608 U/mL) compared to the CT group (26.285 U/mL). The MO 50 mg/kg and MO 100 mg/kg groups displayed significantly elevated SOD activity (21.035 ± 1.496 and 20.475 ± 2.052, respectively) compared to the NTG and SMT groups ($p = 0.044$ and $p = 0.025$, respectively), though still lower than the CT group.

The MDA/SOD ratio, an indicator of oxidative stress (Chen *et al.* 2016), was significantly higher in the NTG group ($p < 0.0001$), followed by the SMT group ($p = 0.001$). However, the MO 50 mg/kg and MO 100 mg/kg groups showed no significant difference from the CT group ($p > 0.05$), indicating a potential antioxidative effect of *M. oleifera* at these doses. The complete results are summarized in Table 1.

Discussion

Migraine is a complex neurological condition that necessitates a multifaceted treatment approach due to its intricate pathophysiology and pharmacological management. The need for more effective treatments remains critical (Tardiolo *et al.* 2019). Characterized by a pulsating pain of varying severity, migraines are often accompanied by debilitating symptoms that worsen with physical activity (Demartini *et al.* 2023). Currently, over 276 million people worldwide suffer from this severe neurological disorder, which significantly impacts quality of life and is a leading cause of disability (Mundkar *et al.* 2022). The etiology of migraine involves a complex interplay between genetic and environmental factors (Foudah *et al.* 2022).

A well-established model for studying migraines involves the chemical activation of the trigeminovascular system (TS) using NTG, a NO donor (Farajdokht *et al.* 2018; Sadeghi *et al.* 2022). NTG induces mild-to-moderate headaches in non-migraine individuals but can trigger migraine-like attacks in migraineurs. This model has been extensively used to assess behavioral and neural consequences of treatments in rodents (Sureda-Gibert *et al.* 2022; Zhang *et al.* 2017). In our study, we employed NTG to induce migraine using the method described by Latif *et al.* (2021). The hyperalgesia observed in NTG-treated rats effectively mimics the sensory hypersensitivity associated with migraines (Markovics *et al.* 2012; Moye & Pradhan 2017).

Ethanolic extracts of *M. oleifera* at 50 and 100 mg/kg resulted in reduced weight gain compared to the control group ($p < 0.01$). However, no significant changes in body weight were observed across all treatment groups ($p > 0.05$). NTG has been reported to induce weight loss in Wistar rats, as demonstrated by Farajdokht *et al.* (2018), though we observed fluctuating water consumption patterns in most groups. Activation of the trigeminovascular system by dural infusion of inflammatory mediators has been associated with appetite loss, a symptom commonly linked with pain (Vuralli *et al.* 2019).

Regarding headache-like behaviors, neither the MO 50 mg/kg nor the MO 100 mg/kg groups exhibited head flicks after one hour of observation. Grooming frequency was lower in the MO 50 mg/kg group than in the NTG and CT groups. A persistent nausea-related behavior known as LOB was previously found to be more prevalent in male rats, potentially as a means of coping with a sensation akin to gastric distension (Benbow *et al.* 2022). In our study, MO 50 mg/kg and 100 mg/kg significantly suppressed LOB behavior compared to the CT group.

The behavioral effects of NTG persisted for 2–3 hours and were assessed through visual observation. NTG-treated rats exhibited increased resting, grooming, and freezing behaviors, along with reduced

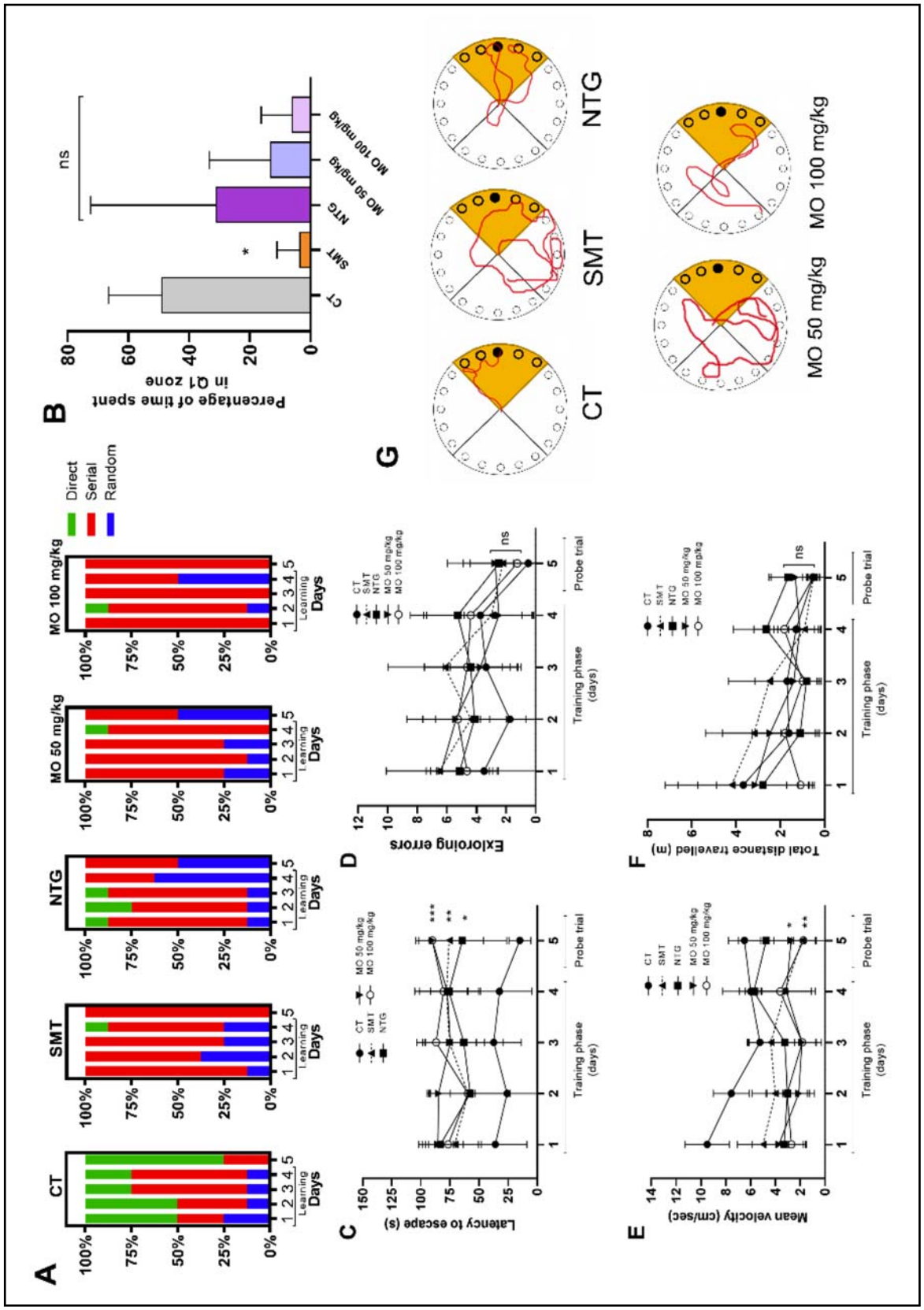

Fig. 5. Results of the Barnes maze test. **A)** Percentage of search strategy; **B)** total time spent in the quadrant target zone (Q1); **C)** latency to escape in the learning phase and probe trial; **D)** number of errors in the learning phase and probe trial; **E)** mean velocity (cm/sec); F) total distance until escape in the learning phase and until the target hole in the probe trial; and **G)** tracking paths of different groups in the probe trial.

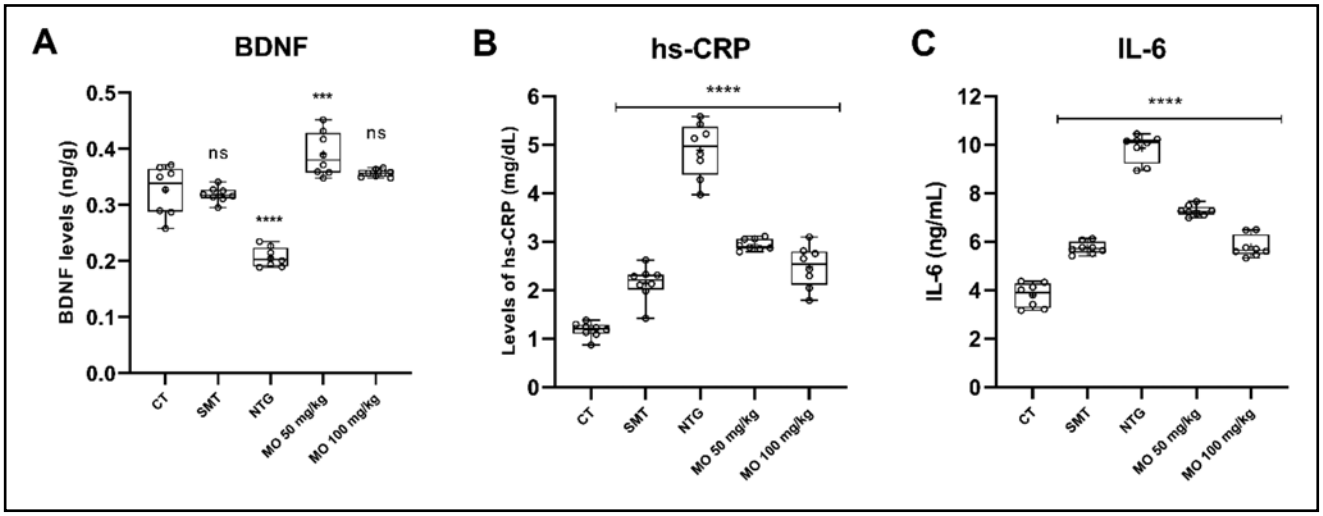

Fig. 6. Effects of various concentrations of ethanolic extracts of *M. oleifera* on BDNF, serum IL-6, and hs-CRP levels in rats. **A)** BDNF levels, **B)** levels of high-sensitivity CRP serum, and **C)** IL-6 levels in the blood. A statistical difference in the data obtained from the tested groups was performed using one-way ANOVA using Dunnett's test (*post hoc*) compared to the CT group. * $p < 0.05$ and ** $p < 0.01$, **** $p < 0.0001$; ns = not significant.

exploratory and locomotor activities, consistent with previous findings (Foudah *et al.* 2022). A strong association between migraines and mental health disorders has been reported (Zhang *et al.* 2017). In the EPM assay, the MO 50 mg/kg group spent significantly more time in open arms than the CT group, indicating reduced anxiety. Anxiety levels, as measured by an index of 0.5 to 0.8, were not significantly different between the MO 50 mg/kg and CT groups ($p = 0.0716$). The OFT revealed that the MO 50 mg/kg and NTG groups spent significantly more time in the central area compared to the CT group. In contrast, the FST test showed that the MO 50 mg/kg and 100 mg/kg groups exhibited significantly less immobility time than the CT group, suggesting reduced depression-like behavior. This is consistent with findings by Demartini *et al.* (2022), who reported increased anxiety and pain-related behaviors, along with decreased locomotor and exploratory activity, in NTG-treated animals (Demartini *et al.* 2022). Additionally, the MO 50 mg/kg group spent significantly more time in the light chamber in the LDB test. In the Barnes maze test, only the CT group selected the direct escape route and exhibited the shortest escape time.

Inflammatory pathways and elevated cytokines, such as hs-CRP, play a pivotal role in migraine pathophysiology by inducing repeated, sterile neurogenic inflammation through the production of pro-inflammatory cytokines (Demartini *et al.* 2023; Tanik *et al.* 2015). Our findings revealed that all treatment groups exhibited significantly higher hs-CRP levels compared to the control group, consistent with previous studies linking elevated hs-CRP to migraines (Lippi *et al.* 2014). However, administration of *M. oleifera* extract did not reduce hs-CRP levels in NTG-induced rats. Similarly, IL-6, a pro-inflammatory cytokine known to increase during migraine attacks (Avona *et al.* 2021), showed no significant alteration following *M. oleifera* treatment ($p > 0.05$). These results contrast with the findings of Cuellar-Núñez *et al.* (2021), who reported a reduction in serum levels of MCP-1, IL-6, and TNF-α in mice supplemented with *M. oleifera* extract in a colorectal cancer model (Cuellar-Nunez *et al.* 2021).

To assess oxidative stress, we measured serum MDA as a biomarker of lipid peroxidation and oxidative damage, along with SOD activity as an antioxidant defense marker. MDA, an end product of lipid peroxidation (Haddadi *et al.* 2014), has been linked

Tab. 1. The level of SOD activity and MDA concentration after administration of various concentrations of *M. oleifera* extracts

Study parameters	Mean ± SD				
	CT	SMT	NTG	MO 50 mg/kg	MO 100 mg/kg
MDA (nmol/mL)	1.142±0.686	1.773±0.103**	2.540±0.393****	1.862±0.0848***	1.834±0.140***
SOD (U/mL)	26.285±3.881	12.608±3.619****	9.608±0.776****	21.035±1.496*	20.475±2.052*
MDA/SOD	0.044±0.009	0.148±0.039**	0.267±0.055****	0.089±0.010	0.090±0.012

A statistical difference in the data obtained from the tested groups was determined using a one-way ANOVA using Dunnett's test (*post hoc*) compared to the CT group. * $p < 0.05$ and ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns = not significant. CT: control; SMT: sumatriptan; NTG: nitroglycerin.

to cortical spreading depression, a key migraine pathophysiological process (Togha *et al.* 2019). Our results showed that SOD activity was highest in the CT group but was also increased in the MO 50 and 100 mg/kg groups compared to the NTG and SMT groups. NTG-treated rats exhibited a substantial increase in midbrain lipid peroxidation, as indicated by elevated MDA levels. However, the SMT group exhibited lower MDA levels than the CT group ($p < 0.001$), suggesting potential oxidative damage modulation.

Mitochondrial oxidative metabolism dysfunction has been implicated in migraine pathogenesis (Tuncel *et al.* 2008). Although several studies have explored the role of oxidative stress and neuroinflammation in migraine, findings remain inconclusive. Elevated ROS have been associated with increased MDA levels in migraine sufferers (Togha *et al.* 2019). While some studies have reported no significant changes in antioxidant enzyme activities, including catalase (CAT), SOD, and glutathione peroxidase (GPx) (Shukla *et al.* 2004), others, such as Shimomura *et al.* (1994), suggested that reduced platelet SOD levels may contribute to migraine pathogenesis. Additionally, oxidative stress markers may correlate with headache frequency, with increased ROS and decreased antioxidant capacity observed in chronic migraine patients (Togha *et al.* 2019). However, Tuncel *et al.* (2008) found no association between SOD and MDA levels and headache frequency. Our findings align with their observations, reinforcing the complexity of oxidative stress in migraine pathophysiology.

In conclusion, understanding the underlying mechanisms of migraine and its associated inflammatory and oxidative stress pathways is critical for developing novel therapeutic strategies. While *M. oleifera* extract demonstrated potential behavioral benefits, its effects on inflammatory cytokines and oxidative stress markers remain inconclusive, warranting further investigation.

Conclusion

Our study demonstrated that treatment with *Moringa oleifera* could improve locomotor, depression-like behavior, anxiety-like behavior, grooming, and rearing in the NTG-induced migraine rat model. All these findings recommended that *M. oleifera* at a dose of 50 mg/kg (low dose) reduced anxiety and depression-like behavior, the development of migraine headaches, and improved memory processing and antioxidant defense levels. However, for inflammatory functions, both concentrations failed to control and alleviate inflammatory cells.

Compliance with ethical standards

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Author's contribution
AMC and YH wrote and organized the first draft; AMC and YN collected data; AMC analyzed the data; YH and MC checked the latest version of manuscript. All authors read and approved the final manuscript.

Conflict of interest
The authors declare no conflict of interest.

Data availability statement
The data for the current study are available upon request.

Ethical approval
This study was approved by the Ethics Committee of AJA University of Medical Sciences (Approval ID: IR.AJAUMS.REC.1401.12). All procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Eighth Edition 2011) and complied with relevant institutional and national guidelines for the welfare of animals.

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