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ORIGINAL ARTICLE

Mentha piperita Enhances the Antinociceptive and Ameliorates Pain through Antioxidant and Anti-Inflammation

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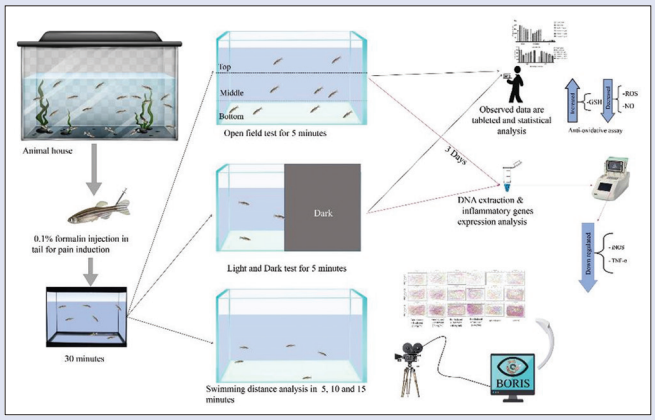
ABSTRACT

Background and Objectives: Pain or nociception is a devastating experience that alters both the physiological and psychological status of individuals. A variety of chemical and physiological tissue damage and physical parameters like temperature, pressure, etc. induce pain. Clinically, pain is managed through the administration of antinociceptive drugs. In the current work, the antinociceptive potential of *Mentha piperita* leaves against pain in the zebrafish model has been explored. **Materials and Methods:** Formalin was injected in the tail of the zebrafish to induce pain and was exposed to 5, 10, 15, and 20 mg of the extract. The behavioral pattern was analyzed using the Behavioral Observation Research Interactive Software (BORIS). Gene expression and enzymatic studies were performed to evaluate inflammation, oxidative stress and the antioxidant ability of *M. piperita*. **Results:** In the open field test, animals treated with the leaf extract restored their activity to normal in a dose-dependent manner. Pain-induced animals inclined toward darkness, whereas the treated animal preferred to stay in the light. Furthermore, behaviors such as freezing, inaction, and altered swimming patterns were all restored to normal conditions. The color change in the tail was prominent in pain-induced animals, whereas it was restored to normal in the treated group. The higher dose of the plant extract was effective in downregulating the expression of inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α), thereby impairing the neuroinflammation. The plant extracts reduced reactive oxygen species (ROS) and nitric oxide levels, whereas it increased reduced glutathione levels. **Conclusion:** The current work establishes that *Mentha piperita* is effective in ameliorating pain in a zebrafish model.

Key words: *Behavior, mentha piperita, nociception, pain, zebrafish*

SUMMARY

- Current treatment options for reducing nociception include the usage of modern drugs that cause side effects and are toxic to tissues. We evaluated *Mentha piperita* extract as an alternative source for antinociception in the formalin-induced zebrafish pain model. The induced groups showed abnormal behavior and on treatment with the plant extract, normal behavior was restored. Treating the pain models reduced oxidative stress and inflammation, thereby confirming antinociception activity. Further study needs to be carried out to understand the mechanism of action and clinical effectiveness involved in the antinociception activity.



Abbreviations used: BORIS: Behavioral Observation Research Interactive Software; PCR: Polymerase chain reaction; TNF-α: Tumor necrosis factor alpha; GSH: Glutathione; ROS: Reactive oxygen species; NO: Nitric oxide; *MP: Mentha piperita*; GABA: Gamma-aminobutyric acid, AChE -Acetylcholinesterase; NBT: Nitro blue tetrazolium, DMSO: Dimethyl sulfoxide; KOH: Potassium hydroxide.

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INTRODUCTION

Pain is a is an unpleasant, emotional, and cognitive experience that has its origin in the processing of noxious stimuli by neuronal cells. Pain is induced by a variety of factors including tissue damage, chronic inflammation, infections, cancer, etc. Nociceptive nerve endings are highly conserved among various animals including humans.[1–3] Several inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukins (IL-1β, IL-6, IL-8), etc. contribute to pain and hyperalgesia.[4,5] Pain is treated with agents such as glucocorticoids, non-steroidal anti-inflammatory drugs, opioids, etc. However, clinical side effects caused by such drugs discourage their use.[6,7]

Allodynia, hyperalgesia, and dysesthesia are some of the types of pain having various origins.[8] The global burden of neuropathic pain itself is estimated to range between 6.9% and 10% worldwide[9,10] and is likely to cause heavy economic loss to the patients.[10] Anti-depressants, anti-convulsants, and opioids are the choice of drugs for the management of pain.[11]

Mentha piperita, commonly known as peppermint, has been widely used in recipes across the world. It is cultivated across the globe. A variety of disorders and ailments have been treated using *M. piperita* in various

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folk medicines. The leaves possess a wide range of bio-active ingredients, such as palmitic acid, linolenic acid,[12] menthone, isomenthone, etc., *Mentha piperita* possesses strong antioxidant activity.[13] The plant extract has been explored for a range of biological activities, including anti-bacterial, anti-fungal, anti-viral, and anti-tumor activities. The plant has also been explored for its effects on the nervous system. In continuation of the above findings, we explored the antinociceptive potential of the plant using the zebrafish model system.

MATERIALS AND METHODS

Collection and maintenance

Zebrafish (*Danio rerio*) were purchased from the aquarium. The fish were acclimatized to laboratory conditions in a 55-L glass tank filled with deionized water, and continuous aeration was provided with an air compressor. The fish were maintained in a pH range of 7.3–7.5 with the temperature ranging between 26°C and 27°C, and the fishes were fed twice a day. The care and husbandry of the zebrafish used in this study were according to the guidelines that regulate the care and use of laboratory animals by humans for research purposes.

Preparation of *Mentha piperita* leaf extract

Mentha piperita plant sample was collected from the local market. The plant was identified by a taxonomist before proceeding further. *Mentha piperita* belongs to the Lamiaceae family and the Lamiales order. The collected leaves were washed three times using distilled water and air-dried for funder shade. Twenty grams of the leaf sample was ground using a pulverizer. Five grams of ground power was taken in a microwave-assisted extraction vessel (MAS-II Plus, Sineo Microwave, Shanghai, China), and 20 mL of distilled water was added to the same vessel. The vessel was kept in a microwave-assisted extractor and fixed parameter at 30°C for 20 min. Microwave irradiation was at 5 MHz in the extractor. After 20 min, the extract was recovered from the extractor and filtered to remove the debris. The filtrate was used for further experiments.

Toxicity assay and dose fixing

Fish with a body weight ranging between 0.4 and 0.5 g were selected for the experiments and grouped together. Each group consisted of five fish in a tank. We evaluated the toxicity of the extract between 1 and 100 mg/L concentrations. No toxicity was observed till 100 mg/L. Therefore, concentrations ranging from 5 to 20 mg/L were chosen for treatment in the pain-induced model. *Mentha piperita* extract at doses of 5 mg/L, 10 mg/L, 15 mg/L, and 20 mg/L were added to the respective tank. Control fish were maintained in a separate tank and the treatment for the fish was continued for one week. The following behaviors were observed for the assessment of pain: swimming distance, swimming pattern, zone test, dark and light test, aggression, active condition, and color change. Pain was induced by injecting 5 µl of formalin (0.1%) in the zebrafish tail. Behavior was evaluated 30 min after the pain induction by formalin/treatment doses of plant extract (5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L).

Group I = Control (without formalin)

Group II = (0.1%) formalin 5 µl

Group III = (0.1%) formalin 5 µl + 5 mg/L of *Mentha piperita* leaf extract

Group IV = (0.1%) formalin 5 µl + 10 mg/L of *Mentha piperita* leaf extract

Group V = (0.1%) formalin 5 µl + 15 mg/L of *Mentha piperita* leaf extract

Group VI = (0.1%) formalin 5 µl + 20 mg/L *Mentha piperita* leaf extract

Behavioral analysis

Software analysis

For analyzing the swimming distance of animals, the videos were subjected to scrutiny using the Behavioral Observation Research Interactive Software (BORIS) version 8.0.8.[14] Statistical analysis for open field test and aquatic light/dark test was done using GraphPad Prism 8.0 software.

Swim patterns

Swimming patterns of the fish were recorded to analyze the effect of the plant extract. To analyze the swimming patterns of the fish to identify if there is any prominent change in the swimming behavior of the treated fish compared to the untreated fish and formalin-treated fish [Figure 1a and 1b].

Aggression test

Aggression test was done by analyzing the attitude of each fish when it came across another fish, physical and behavioral changes such as the erection of dorsal, caudal, and pectoral regions, fast-swimming toward or against the fellow fish or a mirror image of itself.

Freezing time

The objective of this test was to identify the role of the sample and formalin-treated, control fish in inducing stress in the fish by analyzing the maximum time spent by the fish without any movement.

Open field test

In this study, to analyze fish how many seconds were spent in the top, middle, and bottom of the test tank for 5 min observation.

Aquatic dark and light test

One half of the test tank was kept dark and the other half of the tank with a light transparent. The control, formalin-treated, and drug (*Mentha piperita* leaf extract)-treated fish spent how many seconds in the dark and light side of the test tank.

Active condition

This study was used to identify fish active conditions (less, active, less active) in treated fish as compared to control fish.

Colour change

The color of the formalin-treated fishtail changed to light brown, and after drug-treated fish was changed to normal tail colour [Figure 2].

Estimation of reactive oxygen species (ROS)

Estimation of reactive oxygen species (ROS) was performed following the NBT reduction assay. The protocol was adapted from Hackel *et al.*[15] with slight modifications. Tissues were isolated from zebrafish of different groups. The tissues were homogenized and centrifuged for 5 min at 5000 rpm. To the homogenized tissues, 0.1 mL of NBT solution (0.1%) was added and the tissues were incubated for 10 min. After incubation, the samples were subjected to centrifugation again for 5 min at 5000 rpm. The supernatant was discarded and the resulting pellet was washed with phosphate-buffered solution (PBS). One hundred twenty microliters of dimethyl sulfoxide (DMSO) and 120 µl of 2M KOH were added. Following this, the absorbance was read at 630 nm.

Estimation of reduced glutathione (GSH)

Estimation of reduced glutathione (GSH) was performed according to the guidelines previously published by Ellman *et al.* and Wang *et al.*[16,17] Zebrafish tissues were homogenized using ice-cold 0.5 M

potassium phosphate buffer, and 4% sulfosalicylic acid was employed to deproteinate the tissues. The principle behind this assay is that dithiobis 2-nitrobenzoic acid reacts with reduced GSH at 25°C and produces yellow colour that can be measured at 412 nm. GSH was measured in mMol/g wet weight.

mMol/g wet weight

$$= \frac{Absorbance}{(Path\ length\ \text{X}\ 14.15)} \times 20 \times Dilution\ factor$$

- path length in centimeters (cm).

- 20 is the dilution factor of 50µl sample to 1 mL assay volume

Estimation of nitric oxide (NO)

Estimation of nitric oxide (NO) in the zebrafish tissues was performed using the method detailed by Giustarini *et al.*[18] NO is determined calorimetrically by measuring the breakdown of NO_3^- (nitrite) to NO_2^- (nitrate). Griess reagent was employed to determine NO_2^-. Sulphanilamide (2%) and N-(1-naphthyl) Ethylenediamine (0.2%, w/v) were mixed with phosphoric acid (5%, *v/v*), separately. Nitrate gives a purple colour when it reacts to the Griess reagents in the dark. The absorption maxima of the azo dye product was 549 nm. The nitric oxide concentration was expressed in terms of µM/mL.

RESULTS

Open field test

Exploring and surveying is a normal phenomenon exhibited by animals, especially zebrafish and rodents.[6,19,20] We evaluated the spatial-temporal positioning of zebrafish after treatment with *Mentha piperita*. The pain-induced fish preferred to stay at the bottom, which was a kind of thigmotaxis, whereas treatment with the extract improved their positioning and restored their activity to near normal [Figure 3 and Table 1]. The healthy fish preferred staying at an intermediate level while the pain-induced fish were relatively lesser compared to the bottom, and a shift in the positioning of animals to the intermediated level in a dose-dependent manner was observed [Figure 3] ($P < 0.0001$). Overall, there was an equal distribution of fish at the bottom and intermediate levels. However, on the top, the number of fish was significantly lesser compared to the other two levels. In conclusion, a higher dose of 20 µg/mL of *Mentha piperita* extract restored the fish's positioning to normal.

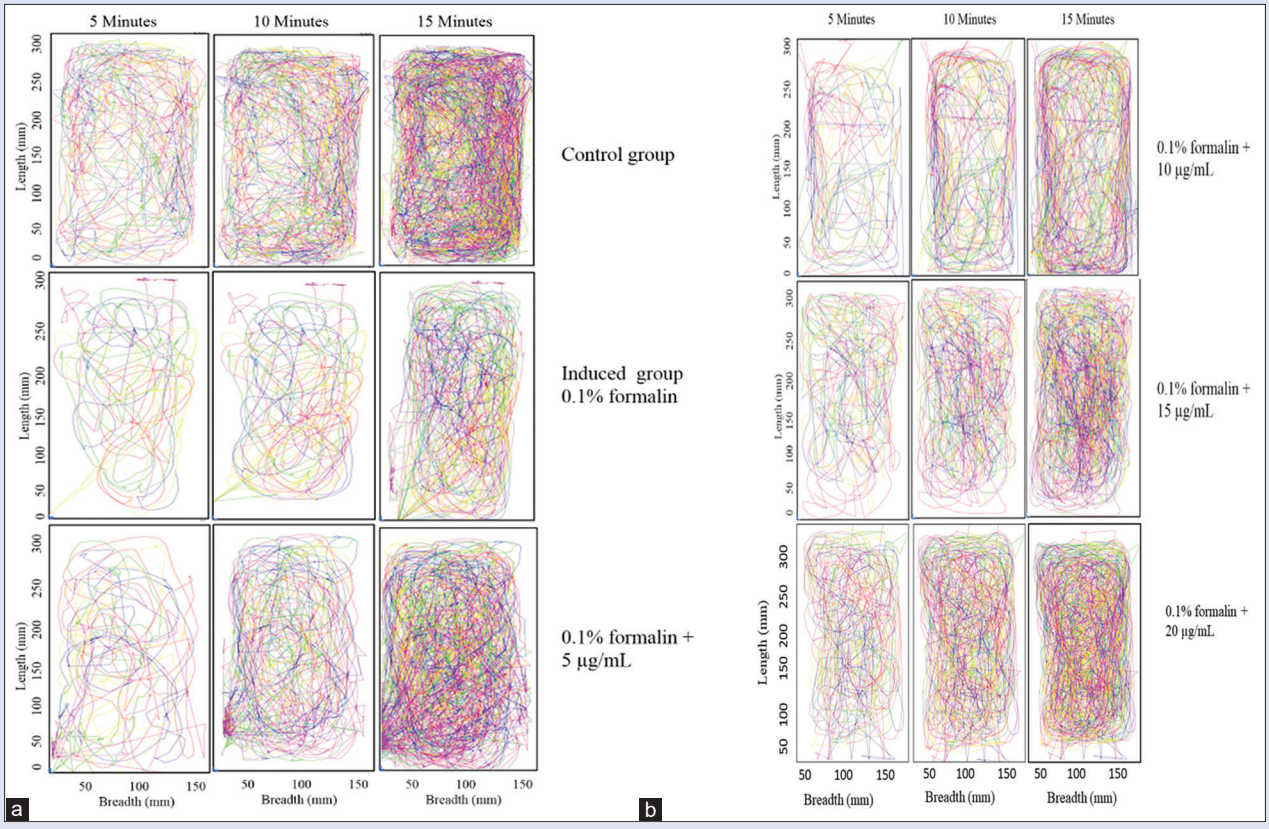

Figure 1: (a) Swimming Distance 2 D Plot (b) Swimming Distance 2 D Plot

Table 1: Open Field Test

S. No.	Top (Times in Sec's)	Intermediate (Times in Sec's)	Bottom (Times in Sec's)
Control group	132±3.55903	147±3.74166	21±1.63299
Formalin	197±1.41421	5.3333333±1.24722	97.666667±2.49444
Formalin+5 µg/mL	188.66667±3.77124	101.66667±4.10961	9.6666667±2.49444
Formalin+10 µg/mL	164.33333±4.49691	120.66667±3.29983	15±1.63299
Formalin+15 µg/mL	148±5.71548	134.66667±5.79272	7.333333±1.24722
Formalin+20 µg/mL	137.33333±2.62467	144.33333±3.85861	18.333333±1.69967

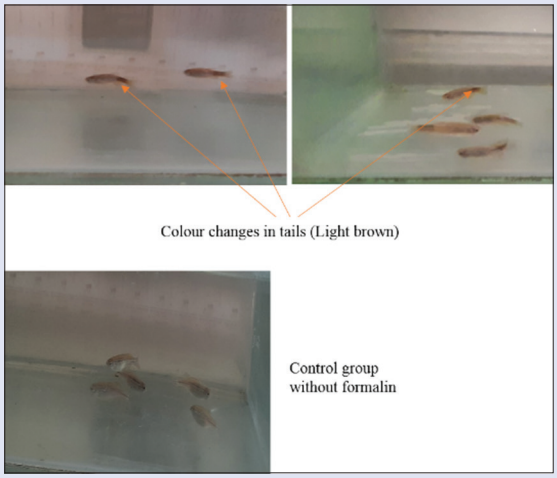

Figure 2: Tail Color Appearance

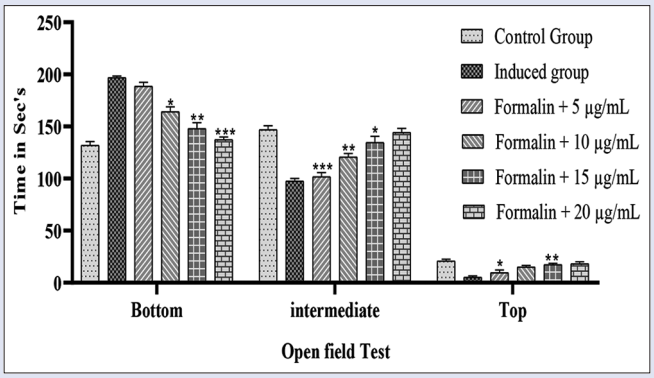

Figure 3: Open field test. Swimming behavior was observed at the bottom, intermediate, and top levels for 5 min. The data are expressed as mean and SD analyzed by two-way analysis of variance (ANOVA) using GraphPad Prism version 8.0 software. The values are highly significant ($P < 0.0001$) in the 20 µg/mL treated group relatively equated to the control group

Table 2: Aquatic Light/Dark Test

S. No	Dark (Times in Sec's)	Light (Times in Sec's)
Control group	97±12.55211359	202±12.55211359
Induced	184±8.219218671	115.7±8.219219
Formalin+5 µg/mL	166±7.874007874	137.3±8.259674
Formalin+10 µg/mL	149±9.533566431	150.7±9.533566
Formalin+15 µg/mL	127±5.906681716	172.7±5.906682
Formalin+20 µg/mL	106±9.273618495	194±9.273618

Aquatic light/dark test

Animals with pain and anxiety are evaluated by an aquatic light/dark test. Certain fishes prefer to stay in the dark whereas the others stay in the light; it can be interpreted that the ones that prefer the dark are the ones with fear/anxiety, whereas the bolder fishes stay in the light areas. In the present experiment, pain-induced animals preferred to be in the darkness as opposed to the control group [Figure 4 and Table 2]. Upon treatment with *Mentha piperita* extract, their exposure to light increased in a dose-dependent manner. A higher dose of the extract restored the behavior of the fish near to that of control ($P < 0.0001$) The behavior of animals returned to normal in a dose-dependent manner.

Behavioral activity

Pain significantly alters the behavior of animals. In the current experiment, we evaluated the behavior of the zebrafish in terms of the freezing duration, active condition, swimming pattern, aggression, and color change. The behavior of the fish was recorded and analyzed. Aggression was not observed in any group including the pain-induced groups. Freezing was a behavior observed in the zebrafish, marked by complete immobility for a period not less than 2s. The pain-induced fish exhibited a prolonged freezing behavior. However, freezing behavior was completely absent when the fish were treated with higher doses of *Mentha piperita* [Table 3; row 1]. The lowest dose (5 mg) had little effect on the freezing pattern.

The activity of the fish of each group was monitored. In the pain-induced groups, the activity was very poor, whereas treatment with increasing doses of *Mentha piperita* restored the activity of the fish to normal, which happened in a dose-dependent manner. The highest dose of the extract completely restored the activity of zebrafish to normal.

The swimming pattern of fish is an indication of their health status. In general, a healthy fish swims in all possible patterns, such as circular, zig-zag, and straight, as observed in the control group. On contrary, in the pain-induced group, the fish moved only in circular patterns and lacked zig-zag patterns and straight movement; however, they had an erratic movement. With a 5 mg dose of the extract, the erratic movement was absent and only circular motion was observed. In the group treated with 10 mg of the extract, the zig-zag movement was observed along with circular movement. In the 15-mg group, straight and circular movements were observed and zig-zag movement was absent. In the 20-mg group, zig-zag, straight, and circular movements indicated complete recovery from pain.

Color change was observed in the tails of the fish. In the control group, the color did not change at all. After inducing pain, the tail color changed to light brown. Only in the highest dose of the extract did the color restore to normal, whereas in all treated groups, the tail color remained light brown [Figure 2].

Expression of pro- and anti-inflammatory genes

The expression pattern of genes was evaluated via polymerase chain reaction. TNF-α is a cytokine that acts both as a pro-inflammatory as well as an anti-inflammatory cytokine. TNF-α level in the fish increased upon treatment with formalin and its expression remained constant throughout all doses of the plant extract, whereas 20 mg/mL significantly decreased the expression of TNF-α. A similar pattern of expression was observed with iNOS expression as well. A relatively higher expression was observed after the treatment with formalin. The highest dose (20 mg/mL) of the plant extract significantly downregulated the expression of iNOS [Figure 5].

Reactive oxygen species levels

ROS levels were elevated in the tissues of zebrafish in which nociception was induced by injecting formalin. Treating the formalin-induced group with the extracts of *Mentha piperita* gradually reduced the ROS levels. A dose-dependent suppression of ROS levels was observed. At a dose of 20 µg/mL of *M. piperita,* ROS reduced significantly. However, at a dose of 5 µg/mL, minor effect was seen ($P < 0.0001$) [Figure 6].

Nitric oxide levels

Nitric oxide (NO) acts as a retrograde neurotransmitter in synapses. In this regard, we evaluated the nitric oxide in tissues. Compared to the control group, the NO levels in the formalin-induced group were elevated. Treating the induced group with *M. piperita* significantly reduced the NO levels in a dose-dependent manner. At the highest dose (20 µg/mL), NO levels were greatly reduced ($P < 0.0001$) [Figure 7].

Table 3: Behavioral Activity

Behavioral Parameters	Control Group	Induced Group	Formalin+5 mg (MP)	Formalin+10 mg (MP)	Formalin+15 mg (MP)	Formalin+20 mg (MP)
Freezing	None	3 s	2 s	None	None	None
Active condition	Normal	Less	Less active	Below active	Active	Active equal normal
Swimming pattern	Circular, zig-zag, straight	Circular and erratic	Circular	Zig-zag, circular	Straight, circular	Circular, zig-zag, straight
Aggression	None	None	None	None	None	None
Color Change	Normal	Tail (light brown) color change	Tail (light brown) color change	Tail (light brown) color change	Tail (light brown) color change	Tail color change to normal

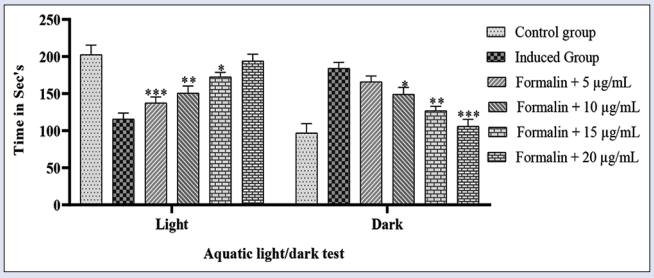

Figure 4: Aquatic light/dark test**.** The dark and light swimming duration was observed between control, induced, and treated groups for 5 min. The values are expressed as mean and SD analyzed by two-way analysis of variance using GraphPad Prism version 8.0. The values are highly significant ($P < 0.0001$) in the 20 µg/mL treated group relatively equated to the control group

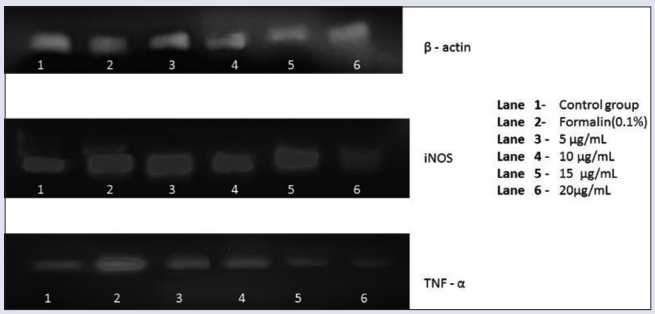

Figure 5: Expression of β-Actin, iNOS and TNF-α

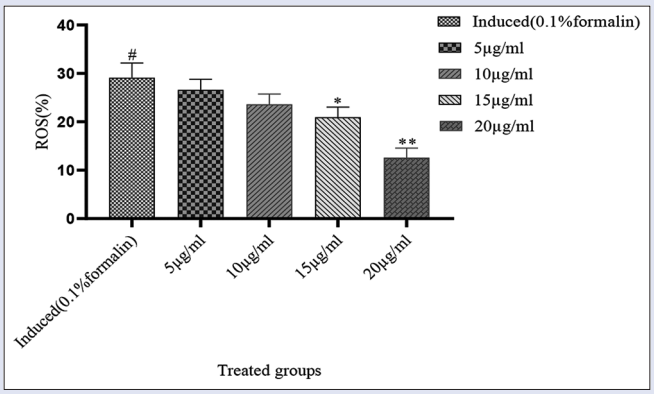

Figure 6: Reactive Oxygen Species Assay

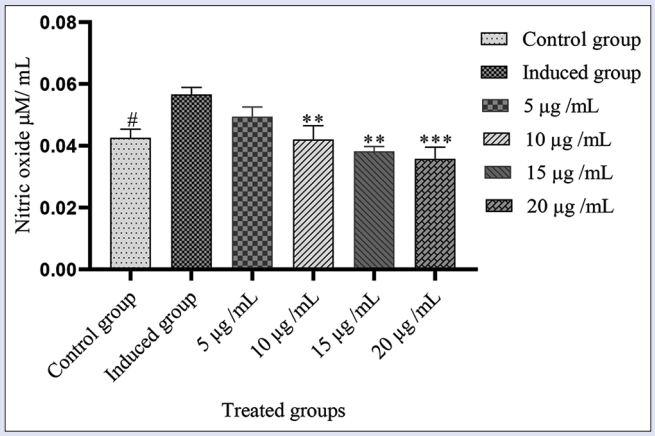

Figure 7: Nitric Oxide Assay

Reduced glutathione levels

GSH levels were evaluated to assess the oxidative stress in the antinociceptive activity of *M. piperita*. The GSH levels were significantly reduced in the pain-induced group compared to the control group. On administering the *M. piperita* extract, a dose-dependent increase in the level of reduced GSH was seen. At maximum dosage (20 µg/mL), GSH significantly increased ($P < 0.0001$) [Figure 8].

DISCUSSION

The present study explores the possibility of exploiting *Mentha piperita* leaf extract for the treatment of pain using the zebrafish model. We assessed the effectiveness of the extract in controlling pain through various behavioral analyses. Open field test of the zebrafish was one of the effective tests to evaluate the physiological and psychological health of the animal. Exploration is an inherent nature of rodents as well as zebrafish.[21–23] The fish that were treated with formalin to induce pain exhibited a preference to stay at the bottom of the tank, and a relatively lesser number of fish moved to the intermediate level in the tank as opposed to the control group [Figure 3]. This kind of thigmotaxis—preferential adherence to the bottom or edges—could be due to the pain. This kind of behavior can be explained by classic exploratory behavioral therapy.[20,24,25] On the other hand, a higher dose of the extract restored the level of the fish's position. This indicated that *Mentha piperita* extract was effective in ameliorating pain.

Pain has been associated with anxiety and depression.[26] Scototaxis is a behavior of animals wherein pain and/or anxiety induce animals to have a preferential adherence to darkness. In the present study, zebrafish induced with pain preferred to stay in the darkness [Figure 4; Table 2]. From this behavior, it can be interpreted that the pain-induced fish were experiencing anxiety and depression, which might have caused alterations in the behavior of home-based phenotype. Consequently, animals in pain

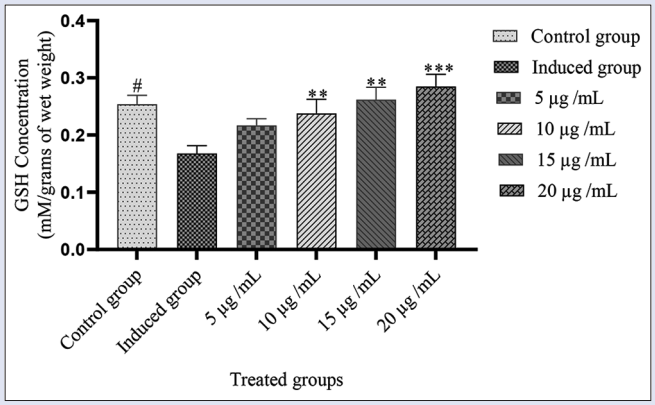

Figure 8: Reduced Glutathione Assay

tend to change their home base to a darker place. However, the fish treated with *Mentha piperita* extract were restored to normalcy, which indicates the effectiveness of the extract in reducing pain.

Freezing was a behavioral response in the zebrafish groups that were treated with formalin for inducing pain. This can be correlated to a decline in acetylcholinesterase (AChE) activity.[27] have established the link between altered locomotion in zebrafish and decreased AChE activity. Accumulation of acetylcholine at the synapses might have induced disrupted muscular movements in zebrafish due to abnormalities in neuronal functions.[28-30] Therefore, it is likely that *Mentha piperita* is effective in restoring the AChE activity impaired by formalin, as evidenced by the lack of freezing activity in groups administered with higher doses of the extract.

In continuation with freezing behavior, alterations in swimming patterns were also observed in the pain-induced groups, which could also be due to impaired AChE activity induced by formalin. However, treatment with the *Mentha piperita* extract restored the swimming pattern to normal with circular, zig-zag, and straight patterns. Pain thus hurts neuromuscular junctions through AChE.

A variety of stimuli, such as light activation, stress, pain, panic response, etc., can cause a change in coloration in the body, which might be a camouflage activity.[15,31] In the present work, color change was observed in the fish tail. However, treatment with *M piperita* extract returned the tail color to normal, indicating that the pain, panic, and stress response were aborted.

ROS is a key player in the development and maintenance of both acute and chronic pain.[15] Elevated ROS levels in the spine can change the nociception, which results in hyperexcitability of the nervous system.[21] This sensitization leads to hyperalgesia. Another mechanism is that ROS levels elevate pain by impeding inhibitory transmission by suppressing the release of gamma-aminobutyric acid (GABA) in the synapses.[32] In the acute nociceptive phase, oxidative process and inflammation play a vital role, and it is the production of ROS that causes the pain in the chronic phase.[33] Overall, a reduction in ROS levels can reduce the progression and maintenance of pain. From our results, it is evident that *M. piperita* can alleviate high ROS levels and therefore reduce pain. Furthermore, a dose-dependent increase in GSH levels substantiates the reduction in ROS levels.

NO acts as a neurotransmitter and plays a dual role in pain regulation. NO plays a role in the establishment of central sensitization and mediates nociception. However, NO is also known to induce analgesia.[34] In our study, the NO levels were elevated in the formalin-induced group where the fishes experienced pain; however, treatment with *M. piperita* extract regulated the NO levels, indicating a reduction in oxidative stress and pain.

There was no effect of the plant extract (up to a dose of 15 mg/mL) on the expression levels of iNOS and TNF-α. However, a significant level of reduction could be observed with the highest dose of 20 mg/mL. Reduction in the level of iNOS has been positively correlated with pain relief. TNF-α has been established as a promoter of neuroinflammatory signalling through the p38 MAPK signalling pathway.[35] Therefore, it is highly likely that a higher dose (20 mg/mL) of *Mentha piperita* extract could have been effective in abolishing the neuroinflammatory signalling and aided in relieving pain.

Pain is a response that drastically affects the normal behavior of individuals. The present study highlights the potential of *Mentha piperita* in alleviating pain using the zebrafish model. The animals exhibited spatiotemporal preference in response to pain stimuli. The pain-induced fish preferred to stay at the bottom of the tank. Furthermore, the pain-induced fish were shown to stay in the dark. Both responses were abolished by the plant extract. Additionally, the animals displayed a freezing response due to the exposure to formalin. The animals exposed to pain-inducing agents displayed a latency in their activity. In addition, the swimming pattern was also altered by the pain-inducing agent. Furthermore, there was a color change in the tail in response to pain stimuli. The plant extract likely impaired the inflammatory signalling, as evidenced by a reduction in the expression of TNF-α and iNOS. All of these responses to formalin were restored by exposure to the plant extract. And a response to the plant extract was in a dose-dependent manner. Overall, the present indicates the treatment potential of the extract.

CONCLUSION

Mentha piperita extract displays antinociceptive activity, which alleviates oxidative stress and inflammation, as evidenced by behavioral studies, antioxidant enzymes, and inflammation markers in the formalin-induced zebrafish pain model. There was a significant decrease in ROS and NO levels with an increase in GSH in a dose-dependent manner. Color change induced by formalin was restored by the plant extract. The regulation of inflammatory markers was confirmed by a decrease in TNF-α and iNOS gene expression. Thus, *Mentha piperita* can be an alternative source of treatment for pain. In future, the study may be extended using neuronal cells *in vitro* to understand the mechanism of pain reduction. Furthermore, using the mammalian model system, the study would be extended.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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