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ORIGINAL ARTICLE

Adhatoda vasica is Effective in Relieving Pain through Modulation of Inflammation

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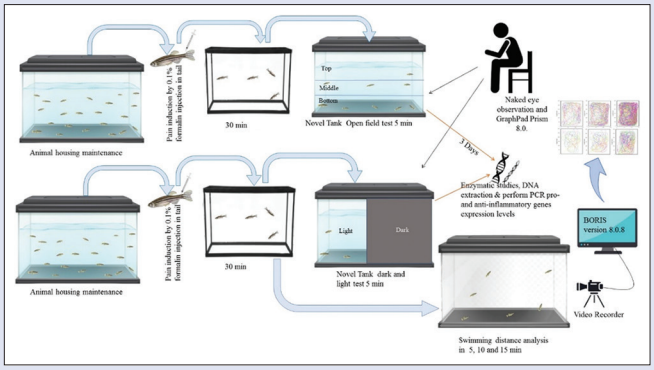
ABSTRACT

Background: Nociception is an unpleasant experience that has a negative effect on both the physiological and psychological status of individuals. Factors including temperature, and physical damage originating from mechanical, chemical, or even biological stimuli are likely to induce pain. Currently, a limited choice of antinociceptive medications is available for the management of pain. **Purpose:** Pharmacological activities of various plant extracts and derivatives are being actively explored for discovering novel plant-based antinociceptive agents. In the current work, we explored *Adhatoda vasica* for its antinociceptive activities. **Materials and Methods:** In the current experiment, we used zebrafish (*Danio rerio*) as a model organism, and to induce pain, formalin was administered. We used extracts from *A. vasica* at concentrations of 5, 10, 15, and 20 mg/ml for determination of the antinociceptive effect. To evaluate the antinociceptive activity of the A. *vasica* extract, animal behavior, reactive oxygen species (ROS) quantification, nitric oxide (NO) estimation, and reduced glutathione (GSH) estimation were performed. For behavioral analysis, the animal movement was recorded as a movie and analyzed using Behavioral Observation of Research Interactive Software (BORIS). **Results:** The behavior of fish was recorded by BORIS. Fishes treated with formalin preferred to stay in darkness. In addition, they also maintained their position at the bottom of the water tank. Upon treatment with the plant extract, the fish moved to light and the middle layer of the tank. The plant extract ameliorated the pain in fish, as evidenced by the swimming pattern. Further, the plant extract reduced the ROS and NO. **Conclusion:** The *A. vasica* plant extract reduced inflammation, as evidenced by a reduction in the inflammatory markers. The antinociceptive activity was further indicated by restoration of the color change induced by formalin. In conclusion, the current study emphasizes the significance of *A. vasica* plant extract in relieving pain.

Key words: *Adhatoda vasica*, behavior analysis, formalin, inflammatory markers, nociception, zebrafish

SUMMARY

- *A. vasica* extract has a strong antinociceptive potential.
- The plant extract improves the explorative nature impaired by pain in zebrafish, as evidenced by open field tests, implying the antinociceptive potential of the extract.
- Similarly, *A. vasica*-treated fishes moved to light, compared to pain-induced fishes.
- Inflammatory markers such as TNF-α and iNOS were reduced by the plant extract.
- Color change induced by formalin was restored by the plant extract.
- Overall, it is apparent that the plant extract is effective in ameliorating pain.



Abbreviations used: LC_{50}: lethal concentration 50; ROS: reactive oxygen species; DMSO: dimethyl sulfoxide; GSH: reduced glutathione; NO: nitric oxide; BORIS: Behavioral Observation of Research Interactive Software; PCR: polymerase chain reaction.

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INTRODUCTION

Nociception and inflammatory pathways together cause great implications for the health of an individual. The quality of life of the deceased individual is very much affected. Currently, a wide variety of small molecules are being used for the treatment of pain.[1,2] However, the clinical utility of these drugs is limited due to a variety of reasons including non-affordability, adverse side effects, poor response in certain individuals, etc., Pharmaceutical industry is in a great urge to discover effective drugs against nociception. Though new drugs are introduced into the market for the treatment of pain, they tend to be pretty expensive. Therefore, there is a pressing need for finding alternative drugs from inexpensive sources, preferably plant derivatives, for the treatment of pain.[3]

There are several analgesics used for pain management that cannot effectively relieve pain because of the complex processes involved with mediators and receptors related to nociceptive signals. The nociceptive

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neuron sensation is regulated through neuromodulators, which in turn activate signaling cascades that control pain perception.[4] There is a high demand for potent analgesics for pain management.[5] Screening of plant extracts and scrutinizing ingredients from plants for antinociceptive activity is a promising strategy in the search for new drugs against pain.[6,7]

Adhatoda vasica Nees (Acanthaceae) is generally called "Adosa." It is a small shrub broadly distributed in various parts of the world. There are a variety of phytochemicals found in the plant, which include vasicinone, vasicinol, adhatodine, and pegamine. Further, certain steroids and flavonoids like astragalin, kaempferol, apigenin, and quercetin are also associated with the plant. Traditionally, it has been used as an antispasmodic and expectorates cough, and is used to treat respiratory illnesses like asthma, chronic bronchitis, etc. Combined with powdered herbs and boiled with sesame oil,[8-10] it is also used to treat ear infections and bleeding. The analgesic activity of *A. vasica* has recently been shown. Rheumatic pain and the pain of urinary tract infections are treated with the boiled leaves of *A. vasica*. Therefore, in the current study, we attempted to evaluate the effect of a *A. vasica* in ameliorating pain.

MATERIALS AND METHODS

Preparation of extract

The plants were collected during the month of July from Jinsha botanical garden and authenticated by a botanist. The leaves from the plant were shade dried before processing them. The aqueous extract was prepared from the collected powder. The extraction process involved boiling 5 g of powder in 100 ml of distilled water in a water bath at 50°C for 1 h followed by filtration with Whatman filter paper. The filtrate was concentrated in a rota evaporator (Buchi Rotavapor® R-215) under reduced pressure and temperature. The final extract was collected and stored in a screw cap vial at 4°C for further studies. For treating the fish, 5, 10, 15, and 20 mg concentrations of plant extract were used.

Zebra fish model studies

Collection and maintenance

Zebrafish (*Danio rerio*) was purchased from the aquarium. The fish were maintained in laboratory condition under 50 L of deionized water in a glass tank with continuous aeration from an air compressor. Then, the fishes were maintained at a pH range between 7.2 and 7.5; the temperature was maintained between 26°C and 27°C. The fishes were fed twice per day. The laboratory animal, zebrafish, used in this study was maintained in compliance with the guidelines for animal care and safe use.

Pain induction and plant extract treatment

Formalin test is one of the most common methods used for the induction of pain.[11,12] The respective treatment group was induced with formalin. A known volume of formalin (5 µl of 0.1%) was injected into the tail of the selected fishes using an insulin syringe Intraperitoneal injection (IP). After 30 min of formalin injection, different concentrations of plant extracts like 5, 10, 15, and 20 mg were added separately.

Treatment groups

Adult zebra fishes of both sexes weighing 0.4–0.5 g were selected for this study. Selected fishes were maintained in a treatment tank separately with varying concentrations of plant extracts (5, 10, 15, and 20 mg/l). The control group was maintained in a separate tank without plant extracts. The study group was divided into six treatment groups: group I – control (without formalin); group II – (0.1%) formalin 5 µl; group III – (0.1%) formalin 5 µl + 5 mg/l of *A. vasica* plant extract; group IV – (0.1%) formalin 5 µl + 10 mg/l of *A. vasica* plant extract; group V – (0.1%) formalin 5 µl + 15 mg/l of *A. vasica* plant extract; and group VI – (0.1%) formalin 5 µl + 20 mg/l *A. vasica* plant extract. Respective treatment groups were maintained separately for 1 week. Lethal concentration 50 (LC_{50}) was determined from the respective treatment.

Estimation of reactive oxygen species

For the quantification of reactive oxygen species (ROS), nitro blue tetrazolium (NBT) reduction assay was performed with slight modification from previous methods.[13] Cell pellets isolated from the muscle tissue treated with *A. vasica* plant extract at concentrations 5, 10, 15, and 20 µg/ml were centrifuged at 5000 rpm for 5 min. One hundred microliters of 0.1% NBT solution was added to cells, incubated for 10 min, and centrifuged again at 5000 rpm for 5 min. Cell pellets were washed twice with phosphate-buffered saline (PBS), and to this mixture, 120 µl of 2 M potassium hydroxide (KOH) and 120 µl of dimethyl sulfoxide (DMSO) were added. Following centrifugation, the supernatant was separated and absorbance was recorded at 630 nm with KOH/DMSO as blank.

Estimation of reduced glutathione

Quantification of reduced glutathione (GSH) was performed following the method described by Jollow *et al.*[13] Tissue homogenization was done using 0.5 M potassium phosphate buffer and deproteinated using 4% sulfosalicylic acid. The principle of the assay is based on the reaction between GSH and dithiobis 2-nitrobenzoic acid, which produces a yellow color with absorption maxima of 412 nm. The concentration of GSH is expressed in terms of µM/ml.

Estimation of nitric oxide

Tissue-specific nitric oxide in the fish tissues was quantified using the method of Giustarini e*t al.*[14] with slight modifications. Nitric oxide concentration was determined by quantifying stable decomposition products of nitric oxide, such as NO_3^- and NO_2^-, colorimetrically. NO_3^- is reduced to NO_2^- and can be colorimetrically estimated using Griess reaction. The Griess reagent is composed of 0.2% (w/v) naphthyl ethylenediamine and 2% sulfonamide in 5% (v/v) phosphoric acid. NO_2^-, upon reacting with Griess reagent in the dark, gives a purple/magenta color with absorption maxima ranging from 520 to 550 nm. The absorbance is compared with the standard values, and the nitric oxide concentration is expressed in terms of mg/l.

Analysis of behavioral parameters

After 30 min of treatment, alterations in various behavioral parameters such as swimming pattern (any prominent change in the swimming behavior in the respective treatment compared to the control and formalin treatment), aggression (analyzing the attitude of the respective treatment group when it comes in contact with another treatment group – physical and behavioral changes such as the erection of dorsal, caudal, and pectoral regions, fast swimming), and freezing time (analyzing the maximum time spent by the respective treatment groups without any movement). To analyses active condition parameters like identification of active, moderate and less active fishes from the respective treatment groups and dark- light cycle parameter. In this test, half of the tank was covered with black background, whereas the remaining half was exposed to the light side. Duration of the respective treatment group in the light and dark sides of the tank was recorded. Determination of changes in color also reveals behavioral changes (color changes in the respective treatment group). Behavioral

Observation of Research Interactive Software (BORIS) was used to record live observations of the respective treatment groups.

Analysis of gene expression pattern

The effect of the plant extract on the major inflammatory marker genes was studied by analyzing tumor necrosis factor (TNF)-α and Inducible nitric oxide synthase (iNOS) expression using gradient polymerase chain reaction (PCR). The primers were designed using National center for biotechnology information (NCBI) Primer-BLAST tool. The sequences are listed in Table 1. β-Actin was used as a housekeeping gene. DNA samples were isolated from the treated group after 24 h by standard isolation protocol. Gradient PCR was carried out with the respective primer and isolated DNA, with initial denaturation (95°C for 10 min) followed by amplification consisting of denaturation (95°C for 15 s), annealing (60°C for 15 s), and extension (72°C for 30 s). Amplified products were resolved in agarose gel electrophoresis under standard conditions. Amplification pattern of the PCR products reveals changes in the expression pattern.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance of differences between values (mean ±SD [SD] with $n = 3$) and was followed by Duncan's comparison tests, with a P value of 0.05 being considered the minimum value acceptable for a statistically significant difference.

RESULTS

Open field test

Fishes have the tendency to survey and explore their surroundings.[15-17] The movement pattern of fishes was analyzed by BORIS [Figure 1]. Behavioral evaluation of animals that underwent pain induction with formalin revealed that the fishes stayed at the bottom of the tank more often throughout the time frame recorded, compared to the control [Figure 1: induced group]. In the control group, the distribution of fish was interspersed throughout the tank. [Figure 1: control group] The extent of spread of fishes throughout the tank increased with increasing concentrations of *A. vasica* plant extract [Figure 2: 5, 10, 15, and 20 mg/l]. The healthy animals preferred to explore the entire tank, whereas the pain-induced animals preferred to stay at the bottom of the tank ($P < 0.0001$).

Aquatic light/dark test

Fishes with pain/anxiety can be distinguished clearly using the dark–light test. Fishes that undergo pain or anxiety have the tendency to stay in darkness, whereas healthy animals prefer to stay in the light. In our experiment, we observed that pain-induced fishes were in dark for a relatively higher period of time, whereas normal fishes were distributed in the light [Figure 3]. On the other hand, fishes treated with *A. vasica* plant extract moved into lighted areas from dark areas in a dose-dependent manner ($P < 0.0001$). Higher dose (20 mg/l) of *A. vasica* completely restored the behavior of animals in lighted areas, indicating the antinociceptive potential of *A. vasica*.

Table 1: Primers sequence

Primer sequence	Nucleotide sequences
β-Actin forward	5'TCAAGGTGGGTGTCTTTCCTG3'
β-Actin reverse	5'ATTTGCGGTGGACGATGGAG 3'
TNF-α forward	5'ACACAGAAGACACTCAGGGA3'
TNF-α reverse	5'CCGGTACTAACCCTACCCCC 3'
iNOS forward	5'ACACTTCGAAAAGCAAGATGG 3'
iNOS reverse	5'ACGGGCATCGAAAAGCTGTA 3'

TNF = tumor necrosis factor

ROS levels

The tissues of animals induced with pain by injecting formalin displayed an elevated level of ROS. However, with increasing concentration of *A. vasica* plant extract, there was a gradual reduction in the ROS levels [Figure 4]. A significant reduction in ROS was observed with 20 μg/ml of *A. vasica* plant extract, whereas there was little effect on the ROS levels in 5 μg/ml-treated animals ($P < 0.0004$).

Nitric oxide assay

Since nitric oxide also acts as a neurotransmitter, we estimated the tissue level of nitric oxide. There was an increase in nitric oxide levels in pain-induced animals compared to the controls. On the other hand, treatment with *A. vasica* plant extract significantly reduced the level of nitric oxide in a dose-dependent manner [Figure 5]. Especially, 20 μg/ml of the plant extract potentially decreased the level of nitric oxide ($P < 0.0004$).

GSH levels

GSH is an effective antioxidant. To evaluate the involvement of oxidative stress in the antinociceptive activity of *A. vasica* plant extract, GSH levels were measured. There was a significant reduction in the level of GSH in the tissues of animals induced by pain. In contrast, a dose-dependent increase in the GSH level was evident upon treatment with *A. vasica* plant extract [Figure 6]. At the highest dose of (20 μg/ml) of *A. vasica* plant extract, there was a significant increase in the GSH level ($P < 0.0004$).

Physiological changes

Zebrafishes have a tendency to change their color in response to adverse conditions such as pain. A well-pronounced change in the color of fishes was observed in formalin-treated group [Figure 7]. Upon treatment with varying doses of *A. vasica* extract, the color of fishes was restored in a dose-dependent manner. A higher dose (20 mg/l) of *A. vasica* completely recovered the color of fishes to normal.

Gene expression analysis

Gene expression analysis was performed for TNF-α and iNOS. TNF-α is both a pro- and anti-inflammatory cytokine. The expression of TNF-α significantly increased upon treatment with formalin [Figure 8]. On the other hand, the expression of the cytokine decreased in a dose-dependent manner in response to increasing concentrations of *A. vasica* extract. A remarkable reduction in the expression of TNF-α could be observed in the group which was treated with 20 mg/l of *A. vasica* extract. A strikingly similar pattern of expression was observed with iNOS. An elevated level of iNOS was evident in the group which was treated with formalin, whereas a higher dose of *A. vasica* extract reduced the effect of formalin.

DISCUSSION

Pain is a physiological as well as a psychological complication. Chronic pain leads to a variety of factors contributing to nociception. The antinociceptive efficacy of *A. vasica* was evaluated in the current study. Formalin was used to induce pain in fishes, and subsequently, varying concentrations of *A. vasica* extract were supplemented. We observed a striking difference in the swimming pattern among various groups. Following exposure to the pain inducer, most fishes preferred staying at the bottom and relatively fewer animals reached the intermediate level of the fish tank. The effect was well pronounced in the pain-induced fishes

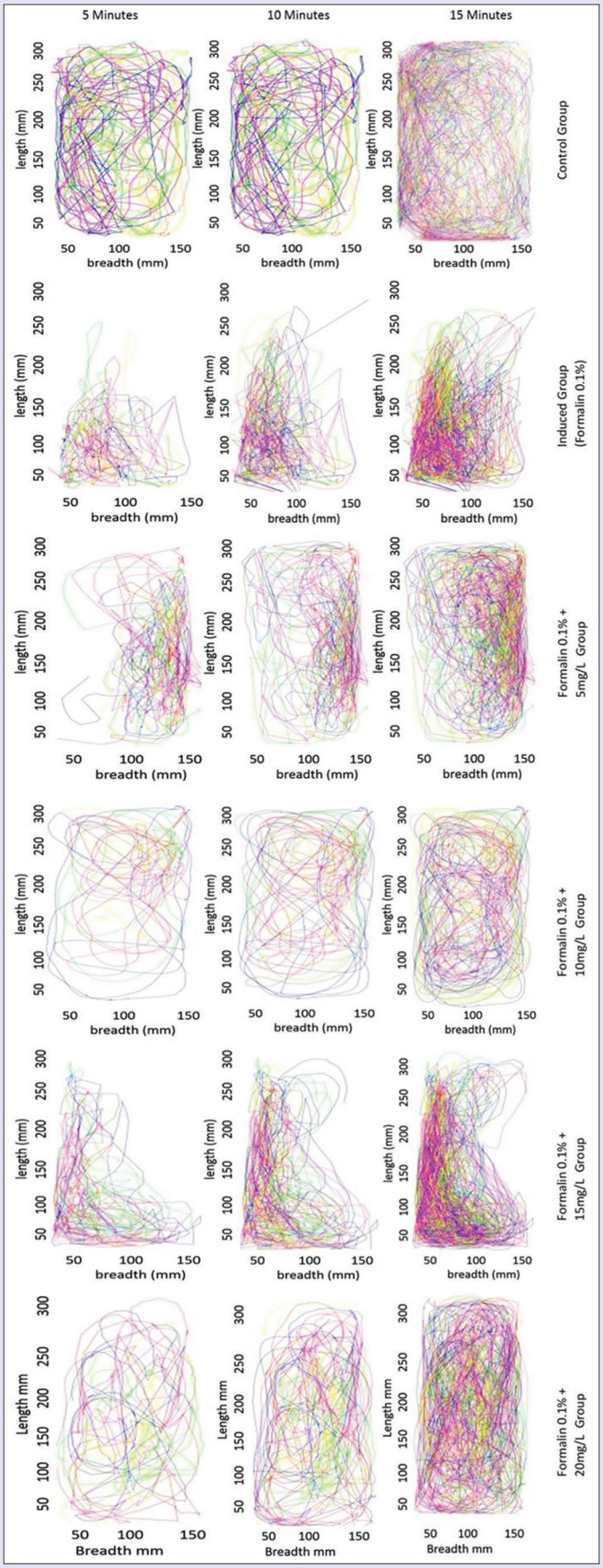

Figure 1: Open field test

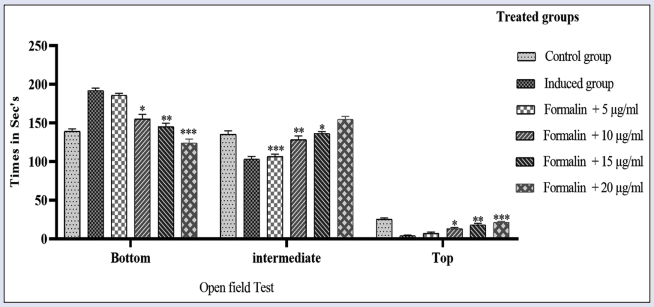

Figure 2: Positioning of fishes in tank

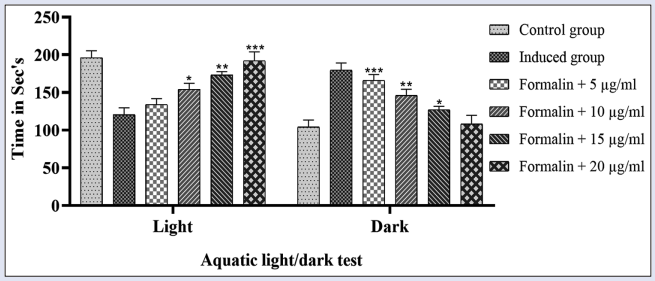

Figure 3: Light/dark preference

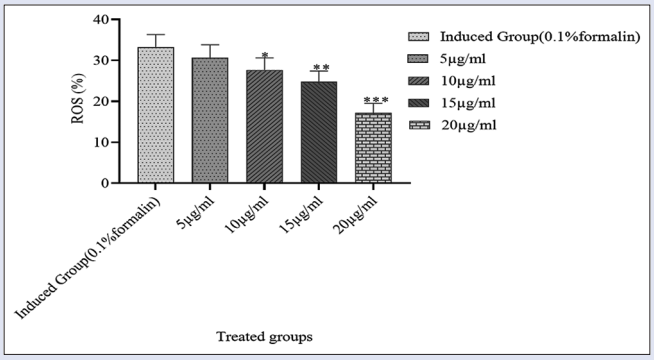

Figure 4: Reactive oxygen species assay

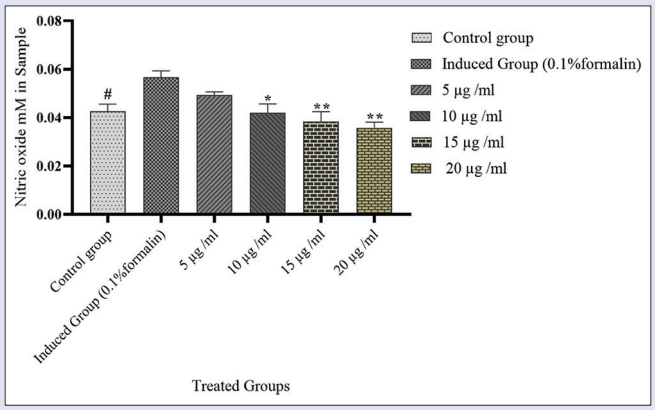

Figure 5: Nitric oxide assay

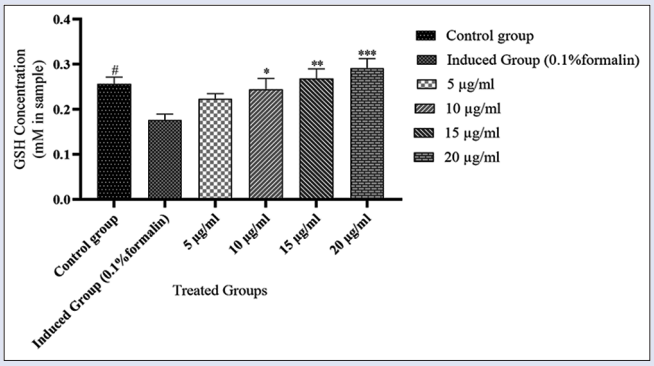

Figure 6: Reduced Glutathione assay

Figure 7: Color change in zebrafish

with respect to the top level of the fish tank [Figures 1 and 2]. The results emphasize that pain-induced animals show anxiety-like behavior, which is evidenced by the reluctancy of the fishes to reach the middle layer or top of the tank. The relative amount of time spent at the bottom of the tank is directly proportional to the extent of anxiety.[15]

Analysis of the fishes' movement in the tank also revealed another striking observation that the fishes predominantly swam near the wall of the tank [Figure 1]. 2D plots derived by BORIS indicated thigmotaxis, which is the preferential movement of fishes near the walls of the tank rather than at the center of the tank.[18] On the other hand, upon treatment with the extracts of *A. vasica*, the fish movement was restored near to normal [Figure 2]. This indicates that *A. vasica* extract was effective in reducing pain, as shown by the reduction of anxiety-like behavior in fishes. Restoration of locomotor behavior occurred in a dose-dependent manner. With a higher dose of the extract, a near to normal activity could be observed. Inflammation is one of the factors that could lead to anxiety-like behavior.[19,20] Therefore, it is highly likely that the alterations in behavior could have originated due to the inflammation caused by the pain inducer.[21]

The role of oxidative stress in aggravating pain is well established.Click or tap here to enter text.[22,23] On the other hand, *A. vasica* has been shown to possess strong antioxidants. Therefore, it is very much clear that the plant extract exerts antinociceptive activity by reducing oxidative stress. There is also evidence that during pain, there is an elevation in oxidative stress.[24] Therefore, it appears that oxidative stress is both a cause and one of the outcomes of pain. In the present work, since the oxidative stress is decreased by the plant extract, it is apparent that the pain is reduced. Further, supporting the above observation, there was a dose-dependent increase in GSH in response to treatment with plant extract at varying doses [Figure 6]. An increase in GSH levels indicates active glutathione reductase. A dose-dependent increase in the activity of glutathione reductase by the plant extract could be a plausible explanation for the increased levels of GSH.

Neuroinflammation is commonly observed in animals exposed to pain stimuli. Therefore, we evaluated some of the inflammatory markers. The inflammatory markers such as TNF-α and iNOS were analyzed by PCR.

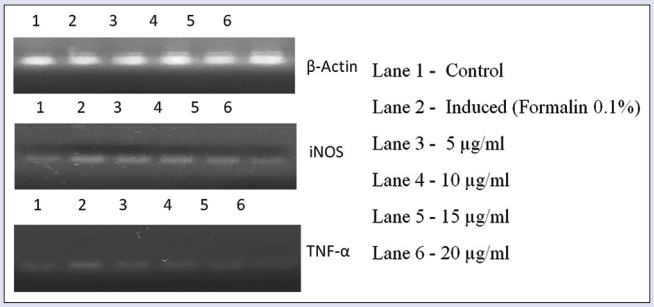

Figure 8: Gene expression

There was a dose-dependent decrease in the expression of both TNF-α and iNOS [Figure 8], which indicates the anti-inflammatory potential of *A. vasica*. The role of TNF-α in neuropathic pain is well established.[25,26] Inhibition of iNOS reduces pain.[27] Nitric oxide is one of the agents causing oxidative stress. Oxidative stress facilitates anxiety-like behavior in the zebrafish model.[28] Therefore, it is reasonable to speculate that *A. vasica* has strong anti-inflammatory activity and subsequently reduces the pain induced by formalin.

The role of nitric oxide in nociception remains controversial. It is both pro- and anti-nociceptive.[29] The antinociceptive activity of nitric oxide has been shown to be mediated through adenosine triphosphate (ATP)-sensitive K + channels in NO–cGMP pathway.[30-32] In our experiment, the NO level was significantly increased in the pain-induced group, whereas treatment with the plant extract reduced the NO level in a dose-dependent manner [Figure 5]. Therefore, it is reasonable to conclude that the plant extract normalized the NO level and subsequently ameliorated the pain. The results are in agreement with the reduced expression of iNOS [Figure 7]. The plant extract drastically reduced NO level through the impaired expression of iNOS, resulting in a reduction of pain.

Further, to analyze the protective effect of *A. vasica* on pain-induced fishes, we evaluated the light–dark preference of all the groups. The control group preferred to stay in the light, whereas pain-induced fishes stayed in darkness [Figure 3]. In parallel, *A. vasica*-treated fishes preferred light in a dose-dependent manner compared to pain-induced fishes without any treatment. Scototaxis is a behavior in which animals display a bias to stay in darkness, Click or tap here to enter text.[33,34] which is well correlated with anxiety. In the present case, anxiety-like behavior can be correlated to pain.

Tail color change is one of the signs of pain and anxiety in zebrafish models. Camouflaging is a response by zebrafish in response to stress.[35] The fishes exposed to pain stimuli displayed a color change, whereas treatment with the *A. vasica* extract restored the color to normal in a dose-dependent manner [Figure 7], indicating the protective effect of the extracts on pain induction.

Overall, the present study is in line with the hypothesis that *A. vasica* would reduce pain. Primarily, the behavioral outcomes strongly emphasize the pain-relieving potency of *A. vasica* plant extract. Secondarily, the gene expression analysis implies the involvement of inflammation and ROS as contributing factors to the development of the pain, and the plant extract reverses the condition caused by pain induced by administration of formalin. However, our study is limited by the animal model. Though a number of review articles suggest zebrafish as a model to study nociception ("Current methods to investigate nociception and pain in zebrafish," "The zebrafish as a model for nociception studies," "A novel zebrafish-based a model of nociception"), further studies have to be performed using rodent model and primate model. In addition, a detailed mechanism-based analysis needs to be done for understanding the ameliorative potential of the plant extract.

CONCLUSION

The present study highlights the possibility of exploiting *A. vasica* in ameliorating pain. The antinociceptive activity of *A. vasica* was evaluated by analyzing the behavior of zebrafish. Pain-induced animals displayed a preference for the bottom of the tank and also had an inclination toward staying in darkness (thigmotaxis and scototaxis). Upon treatment with the plant extract, animals restored their activities to normal in a dose-dependent manner. Further, inflammatory markers such as TNF-α and iNOS were elevated in pain-induced animals, whereas the expression levels were found to be normal in *A. vasica*-treated fishes. The color of fishes changed in response to pain, and the colors were restored back to normal by the plant extract. In short, the present study highlights the antinociceptive potential of *A. vasica*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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