Ind. J. Pharm. Edu. Res., 2026; 60(1):247-257.
https://www.ijper.org

Original Article

Anti-nociceptive and Anti-inflammatory Effects of Withanone in Various Nociception and Inflammation-Induced Murine Model

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ABSTRACT

Background: Pain is an unpleasant sensation that may result from severe or harmful stimuli. It serves as an indication that there may be a problem within the body and it can aid in ensuring the protection. Pain is a ubiquitous experience, with a significant prevalence worldwide. Pain can arise from various sources, including injury, disease, or even emotional trauma and its manifestation can range from acute to chronic. **Objectives:** The current work has focused at exploring the anti-inflammatory and antinociceptive properties in various mice model. **Materials and Methods:** The current study employed Swiss mice, inducing nociception by several chemical and thermal stimulation methods. The experimental mice were treated with withanone at various concentrations including 10, 20 and 30 mg/kg before to the induction of stimuli. The anti-inflammatory activity of withanone were assessed utilizing a carrageenan-initiated air pouch technique. The concentrations of inflammatory cytokines were evaluated using commercial kits. **Results:** The results of the current work demonstrated that the withanone has significantly improved the reaction time on hot plate, increased the response time during tail immersion in hot water, reduced writhing frequency and diminished licking reflexes in the mice. Furthermore, the withanone successfully diminished the carrageenan-induced upsurge in inflammatory markers in the experimental mice, indicating the anti-inflammatory potentials of withanone. **Conclusion:** The present findings emphasize the anti-nociceptive and anti-inflammatory activities of withanone in various chemical- and heat-induced inflammation and pain models.

Keywords: Carrageenan, Cytokines, Formalin, Nociception, Tail flick test, Withanone.

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Received: 18-04-2025;
Revised: 05-06-2025;
Accepted: 26-08-2025.

INTRODUCTION

Pain is a multifaceted sensory phenomenon that has profound implications for human health and well-being. The International Association for the Study of Pain (IASP) characterizes pain as an aversive sensory and emotional experience linked to actual or potential tissue injury, or articulated in relation to such injury. This comprehensive description emphasizes the intricate interplay between the physiological and psychological aspects of pain, underscoring its subjective and multidimensional nature.[1] The types of pain can be broadly categorized into two major types: acute and chronic pain. Acute pain is characteristically a short-term response to a specific injury or illness, serving as a protective mechanism to alert the body to potential damage. In contrast, chronic pain continues beyond the predicted healing time and can become a devastating condition in itself, often significantly impairing an people's quality of life.[2] The causes of pain can be diverse, ranging from physical trauma and injuries to underlying medical conditions, such as neuropathic disorders, inflammatory diseases and cancer. Moreover, psychological causes, like stress, anxiety and past experiences, can profoundly influence the person's perception and experience of pain.[3] The global prevalence of pain is staggering, with studies indicating that chronic pain affects a major portion of the world's population. This widespread burden has significant implications for healthcare systems, economies and the overall well-being of individuals and communities.[4]

The fundamental pathophysiology of pain is a complicated interaction between the peripheral and central nervous systems. Nociceptors, specialized sensory receptors that detect various types of harmful stimuli, transmit pain signals to the brain, where the perception of pain is processed. Though, the specific mechanisms underlying the development and persistence of chronic pain are not fully known and research in this field continues to evolve.[5] Inflammation is a biological response to

DOI: 10.5530/ijper.20261166

several stimuli, including infections, physical injury and chemical irritants. Inflammation may be classified as acute, indicating a short-term process, or chronic, signifying a long-term condition. Acute inflammation is marked by the activation of innate immune cells, including neutrophils and macrophages, which penetrate from the bloodstream to the injured region. Chronic inflammation primarily entails the activation of monocytes into macrophages and is marked by concurrent destruction and repair of the affected area.[6] Inflammation and pain entail a complicated sequence of events involving several regulators, including prostaglandins and inflammatory markers, which significantly impact the physical and emotional well-being of patients.[7] Therefore, utilizing readily available anti-inflammatory drugs may be advantageous in the prevention and control of inflammation.

Anti-inflammatory drugs are commonly employed to manage various inflammatory conditions. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) may cause gastric ulcers, whereas opioids can lead to tolerance and dependence.[8] While these drugs provide substantial relief in alleviating pain and inflammation, some individuals may endure severe adverse effects. Consequently, researchers globally are seeking innovative NSAIDs and alternatives to opiates that lack those adverse effects. Consequently, it is essential to identify novel active compounds exhibiting anti-inflammatory properties with less detrimental effects, as plants represent a promising source of new anti-inflammatory compounds.[9] Withanone is a major bioactive steroidal lactone compound found extensively in the roots of *Withania somnifera*. Numerous previous studies has already disclosed the biological activities of the withanone including, neuroprotective,[10] anticancer,[11] anti-excitotoxicity,[12] and anti-influenza[13] properties. Apart from these biological properties, the antinociceptive and anti-inflammatory activities of the withanone was not studied yet. As a result, the current work has focused at exploring the anti-inflammatory and antinociceptive properties in various mice models.

MATERIALS AND METHODS

Chemicals

The major chemicals and reagents utilized in this work included: withanone, diclofenac sodium, carrageenan, capsaicin, acetic acid, morphine, formalin, dexamethasone and naloxone were purchased commercially from Sigma Aldrich, USA. The diagnostic kits to assess the inflammatory cytokines were acquired from Abcam, USA.

Experimental mice

At the institutional animal facility, the 8-10 weeks aged male Swiss mice (25-30 g) were housed under regulated laboratory environmental conditions at 23°C-25°C temperature with 12-hr light/dark sequence. They were given unrestricted access to both normal rodent meal pellets and drinking water. All animal experiments adhered to the recommendations of institutional animal ethical committee regarding the care and utilization of laboratory rodents.

Acetic acid-induced writhing test

The acetic acid-triggered writhing test was performed in this study to assess the antinociceptive efficacy of withanone. The test was conducted as per the procedure described earlier.[14] Briefly, the mice were treated with 10, 20 and 30 mg/kg of withanone, respectively along with diclofenac as a reference drug. After 30 min of post-final treatment of withanone and/or diclofenad, acetic acid (0.7%; 0.1 mL/b.wt) was administered (i.p.) into each mouse. The frequency of abdominal stretching and constrictions observed in each mouse over duration of 0-30 min was quantified and documented.

Capsaicin-induced licking assay

The capsaicin-induced licking assay was conducted in this study to study the antinociceptive effects of withanone. The test was performed using the protocols as previously indicated.[15] The mice received the withanone and morphine treatments as specified in the acetic acid assay 1-hr prior to the capsaicin treatment. Animals were individually positioned in transparent observation booths to assess nociceptive reactions and incidences of paw licking were documented and data generated.

Formalin-induced biphasic nociceptive assay

The biphasic nociceptive response generated by formalin was performed as previously described.[16] A 1% formalin solution in 0.9% saline was injected into the surface of the mice's hind paw, after which they were placed in transparent observation chambers. Mice received withanone (10, 20 and 30 mg/kg) and morphine as standard drug 30 min before formalin injection. The duration of time that mice exhibited licking in response to formalin injection in their paw was documented and the data were aggregated.

Analysis of inflammatory cytokine levels

The carrageenan-induced air pouch technique was utilized in this study to assess the anti-inflammatory activities of withanone. Before to the initiation of the assay, the mice were anesthetized and their dorsal surfaces were thoroughly shaved with sterile razors. The air pouches were formed by infusing sterile air into the dorsal area twice daily for 3 days. Then mice were administered carrageenan (0.5 mL) to initiate an inflammation, thereafter treated with withanone (10, 20 and 30 mg/kg) and dexamethasone (standard drug). After 1 hr, the mice were sacrificed and saline (2 mL) was given to the air pouches to collect the samples. The IL-6, TNF-α and IL-1β concentrations were studied using commercial diagnostic kits from Abcam, USA.

Behavioral analysis by Open-Field Test (OFT)

The OFT typically detects changes in exploratory behavior and emotionality of experimental animals under low stress situations.[17] The study was conducted in a square wooden box with 80×80×40 cm size, featuring red walls and a white floor delineated by black lines, which contained 16 identical squares, each measuring 4×4 cm. Animals were positioned individually at the box and meticulously observed for 3 min. The assessment of ambulation frequency was employed to assess the changes in behaviors.

Hot plate test

The hot plate technique was performed following an earlier established protocol.[18] The treatments were to the mice followed the protocols specified in the formalin test. The mice were positioned on a heated surface set at 50±1.0°C for maximum duration of 40 sec at 30, 60, 90 and 120 min post-treatment. The duration of forepaw licking or jumping was recorded as the latency time and an inhibitory percentage was assessed. Before commencing tests, animals were evaluated by locating them separately on a hot plate maintained at 50±1.0°C. Mice who did not exhibit nociceptive reactions, such as licking their hind paw or jumping, within 5 sec or beyond 30 sec were excepted.

Tail immersion test

The water bath was calibrated to the 55±0.5°C temperature. Mice were treated with withanone and/or morphine as specified in the formalin assay. Approximately 3 cm of the tail's distal segment was immersed in hot water. The pain response was noted through the quick withdrawal of the tail. The duration of tail dipping and removal was precisely documented at 30 min before and after the 30, 60, 90 and 120 min intervals. A limit immersion period of 15 sec was established to prevent tissue injury from prolonged exposure to hot water, with longer immersion times suggesting the analgesic effects of withanone.

Statistical analysis

Values are presented as mean±SD of three replicates. A one-way ANOVA and Duncan's Multiple Range Test (DMRT) was conducted for the statistical analysis of results using Graphpad Prism. A significant level of $p<0.05$ was fixed to compare the treatment groups.

RESULTS

Effect of withanone on acetic acid-induced writhing in the experimental mice

The results of withanone treatment on the acetic acid-induced writhing incidences in the experimental mice were presented in Figure 1. The results indicated that the acetic acid-treated mice exhibited more writhing numbers. However, the 10, 20 and 30 mg/kg of withanone treatment successfully diminished the incidences of writhing in acetic acid-triggered mice. In similar manner, the diclofenac treatment also reduced the incidence of writhing in acetic acid-induced mice (Figure 1). These findings clearly proved the antinociceptive properties of the withanone.

Effect of withanone on capsaicin-induced licking in the experimental mice

As indicated in Figure 2, the administration of capsaicin markedly elevated the licking numbers in the mice. Interestingly, the treatment of withanone at 10, 20 and 30 mg/kg dosages captivatingly diminished the capsaicin-triggered licking responses in the experimental mice, demonstrating its antinociceptive effects. Similarly, the standard drug morphine also significantly diminished the licking frequency in the experimental mice, which corroborates the activity of withanone (Figure 2).

Effect of withanone on formalin-induced biphasic nociceptive reaction in the experimental mice

The results of withanone on a formalin-induced nociception in the experimental mice was presented in Figure 3. The mice administered with formalin displayed an increased frequency of licking in both phases, signifying the occurrence of nociception. Captivatingly, the 10, 20 and 30 mg/kg of withanone effectively decreased the occurrences of licking responses produced by formalin (Figure 3). Likewise, the administration of morphine also diminished the frequency of formalin-induced licking responses in the mice.

Effect of withanone on the inflammatory cytokine levels

As indicated in Figure 4, a significant elevation in the concentrations of IL-6, TNF-α and IL-1β was noted in the mice with carrageenan-induced air pouch. Interestingly, withanone treatment at several dosages (10, 20 and 30 mg/kg) appreciably reduced the IL-6, TNF-α and IL-1β concentrations in the carrageenan-induced mice. Likewise, the results of standard drug dexamethasone treatment also demonstrated the successful diminution in these cytokine levels, which highlighting the anti-inflammatory activity of the withanone.

Effect of withanone on the behaviors of the experimental mice assessed by OFT

Figure 5 indicates the results of withanone treatment on the behavioral changes in experimental mice, which is assessed by OFT. The experimental mice treated with 10, 20 and 30 mg/kg of withanone revealed a lower number of walked squares than the control, suggesting the sedative effects of withanone (Figure 5). In similar manner, the standard drug morphine also significantly reduced the number of squares walked due to its sedative properties, hence corroborating the effects of withanone.

Effect of withanone on hot plate-induced nociceptive response in the experimental mice

The antinociceptive activity of withanone on hot plate-induced nociceptive response was assessed and the results are given in Table 1. The results of this study demonstrated that the response time of control mice was reduced than the withanone-treated animals. The withanone (10, 20 and 30 mg/kg)-treated mice exhibited prolonged reaction times on the hot plate, suggesting its antinociceptive properties. Standard drug morphine also elevated the reaction time of mice. Moreover, the withanone also increased the response time when administered simultaneously with naloxone, which supports its antinociceptive effects.

Effect of withanone on tail immersion-induced nociceptive response in the experimental mice

The findings indicated in Table 2 demonstrated the response times of mice treated with withanone (10, 20 and 30 mg/kg) were successfully improved in the hot water. The control mice exhibited a reduced response time compared to the withanone-treated mice. The standard drug morphine also markedly increased the tail immersion duration, comparable to the results of withanone. Notwithstanding the difficulty posed by naloxone, treatment with withanone and/or morphine prolonged the response time in hot water, demonstrating the antinociceptive efficacy of withanone (Table 2).

DISCUSSION

Pain is a ubiquitous experience, with a significant prevalence worldwide. It is estimated that up to 80% of people with chronic or terminal conditions in hospital and hospice conditions experience significant pain, which can cause physical and psychological distress, interfere with daily activities and impair the quality of life.[19,20] Pain can arise from various sources, including injury, disease, or even emotional trauma and its manifestation can range from acute to chronic. Chronic pain, in particular, is a pivotal public health issue, with studies indicating that it affects a substantial proportion of the population globally.[21] The incidence of chronic pain differs widely, with estimates ranging from as low as 2% to over 55% worldwide. This wide range can be attributed to differences in the definitions of chronic pain, the populations studied and the methodologies employed in the research.[22]

The acetic acid-induced writhing test in mice is a broadly utilized method for evaluating the analgesic or pain-relieving properties of various compounds. This test involves the administration of acetic acid, which induces a characteristic writhing response in the mice, characterized by abdominal contractions, stretching and extension of the hind limbs.[23] The writhing test is considered a reliable and sensitive technique for assessing the antinociceptive (pain-relieving) activity of drugs and natural products. It provides a simple and cost-effective way to screen for potential analgesic compounds, making it a valuable tool in drug discovery. One of the key applications of this test is in the assessment of

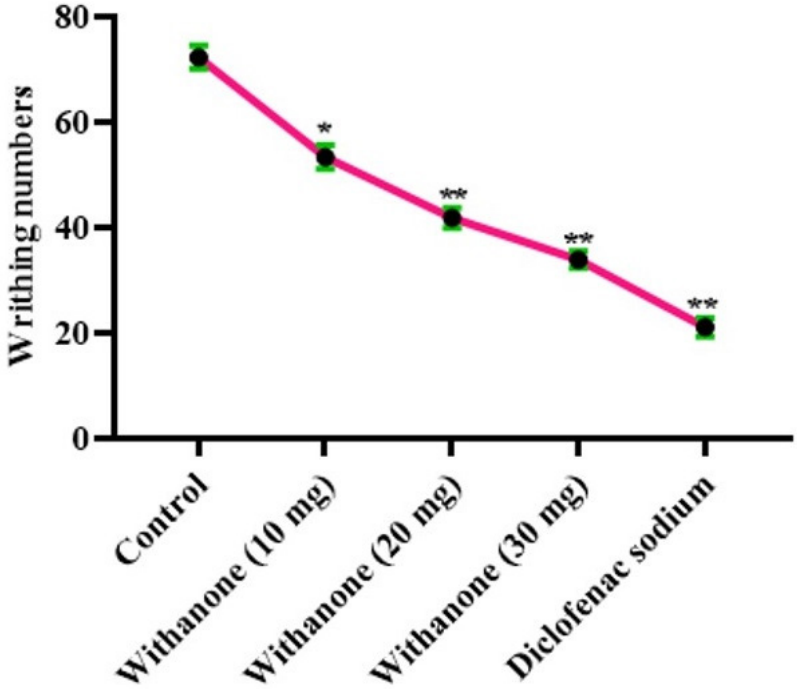

Figure 1: Effect of withanone on acetic acid-induced writhing in the experimental mice.

The values are scrutinized using one-way ANOVA and DMRT were conducted using Graphpad Prism to study the results. Results are expressed as Mean±SD of three replicates. '*' and '**' reveal the statistically significant levels at $p<0.05$ and $p<0.01$, respectively, when comparing treatment groups.

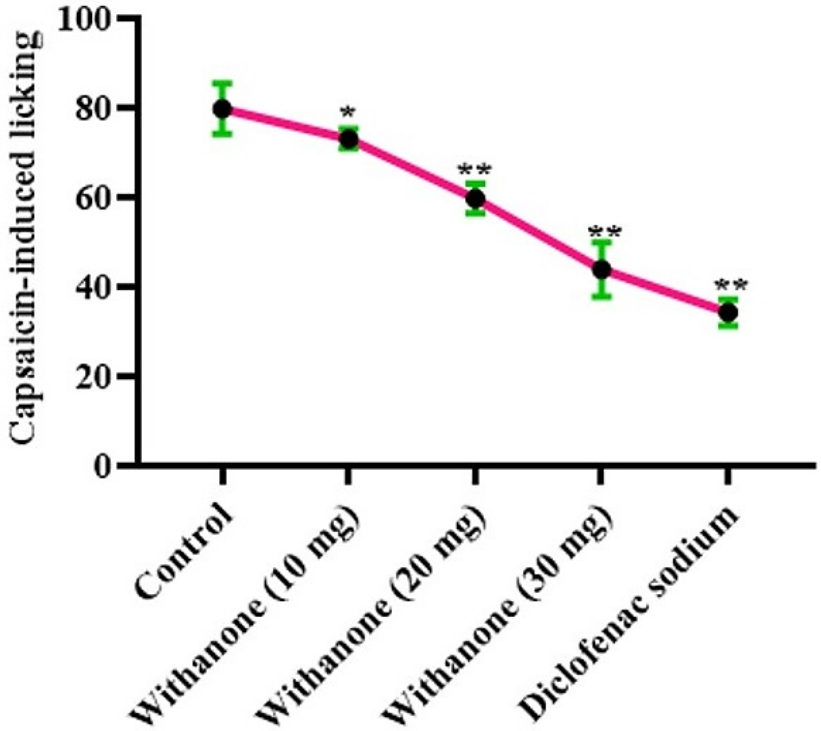

Figure 2: Effect of withanone on capsaicin-induced licking in the experimental mice.

The values are scrutinized using one-way ANOVA and DMRT were conducted using Graphpad Prism to study the results. Results are expressed as Mean±SD of three replicates. '*' and '**' reveal the statistically significant levels at $p<0.05$ and $p<0.01$, respectively, when comparing treatment groups.

the analgesic effects of various natural products with potential pain-relieving properties.[24] The writhing test is also useful for studying the mechanisms of pain and inflammation. The test can help researchers understand the pathways involved in the perception and transmission of pain signals and the modulation of these processes by pharmacological interventions. Furthermore, the writhing test can be used to evaluate the anti-inflammatory properties of compounds, as the acetic acid-triggered writhing response is associated with the release of inflammatory markers.[25] The present results indicated the increased writhing in the acetic acid-treated mice. Interestingly, the treatment with withanone successfully decreased the incidences of writhing in acetic acid-induced mice, which proves its antinociceptive properties.

Capsaicin, the pungent compound found in chili peppers, has long been a subject of interest in the area of pain research due to its capacity to induce nociception, the perception of pain. The capsaicin-induced nociception test in mice has become a widely used experimental model to study the mechanisms and therapeutic potential of sample bioactive compounds.[26] The nociceptive response triggered by capsaicin is mediated through the activation of the transient receptor potential vanilloid 1 channel, which is expressed on a subset of primary afferent nociceptive neurons. This activation leads to the opening of the gate for pain transmission, as the receptor's stimulation by capsaicin results in the influx of cations and depolarization of the neurons. The capsaicin-induced nociception test in mice has been instrumental in elucidating the mechanisms underlying vanilloid-mediated pain perception and exploring potential therapeutic interventions.[27] One of the primary applications of the capsaicin-induced nociception test in mice is the study of analgesic and anti-inflammatory compounds. The ability of a drug to attenuate the nociceptive response to capsaicin can provide insights into its potential to alleviate pain and inflammation.[28] In this work, the findings revealed that the administration of capsaicin markedly elevated the licking responses in the mice. Whereas, the withanone treatment significantly reduced the capsaicin-induced licking incidences in the experimental mice, demonstrating its antinociceptive effects.

The formalin-induced paw licking assay is a widely used technique for evaluating nociception and pain-related behaviors in mice. This test involves the administration of a dilute formalin into the hind paw of the animal, which elicits a characteristic biphasic behavioral response. The 1st phase (acute phase), is characterized by immediate and intense paw licking, biting and lifting, reflecting the direct activation of nociceptive afferents. The second, more prolonged phase, known as the tonic phase, is thought to involve both peripheral and central sensitization and is often utilized to assess the efficacy of analgesic drugs.[29] The formalin test has several advantages over other pain models, like hot-plate and tail-flick tests. Unlike these tests, which measure

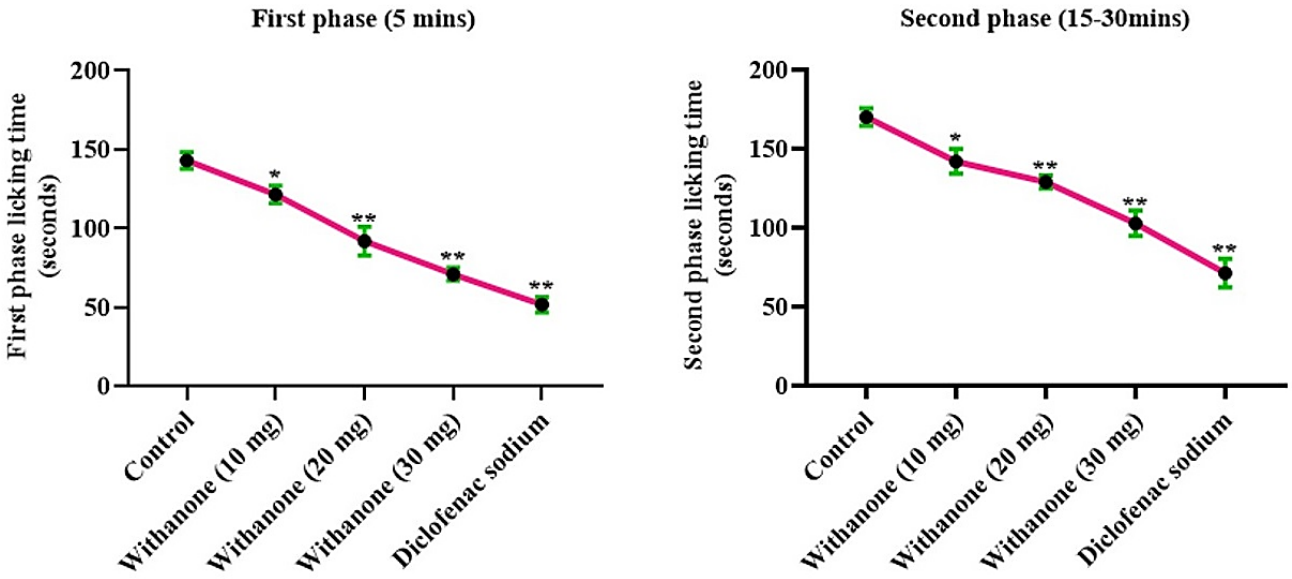

Figure 3: Effect of withanone on formalin-induced biphasic nociceptive response in the experimental mice.

The values are scrutinized using one-way ANOVA and DMRT were conducted using Graphpad Prism to study the results. Results are expressed as Mean±SD of three replicates. '*' and '**' reveal the statistically significant levels at $p<0.05$ and $p<0.01$, respectively, when comparing treatment groups.

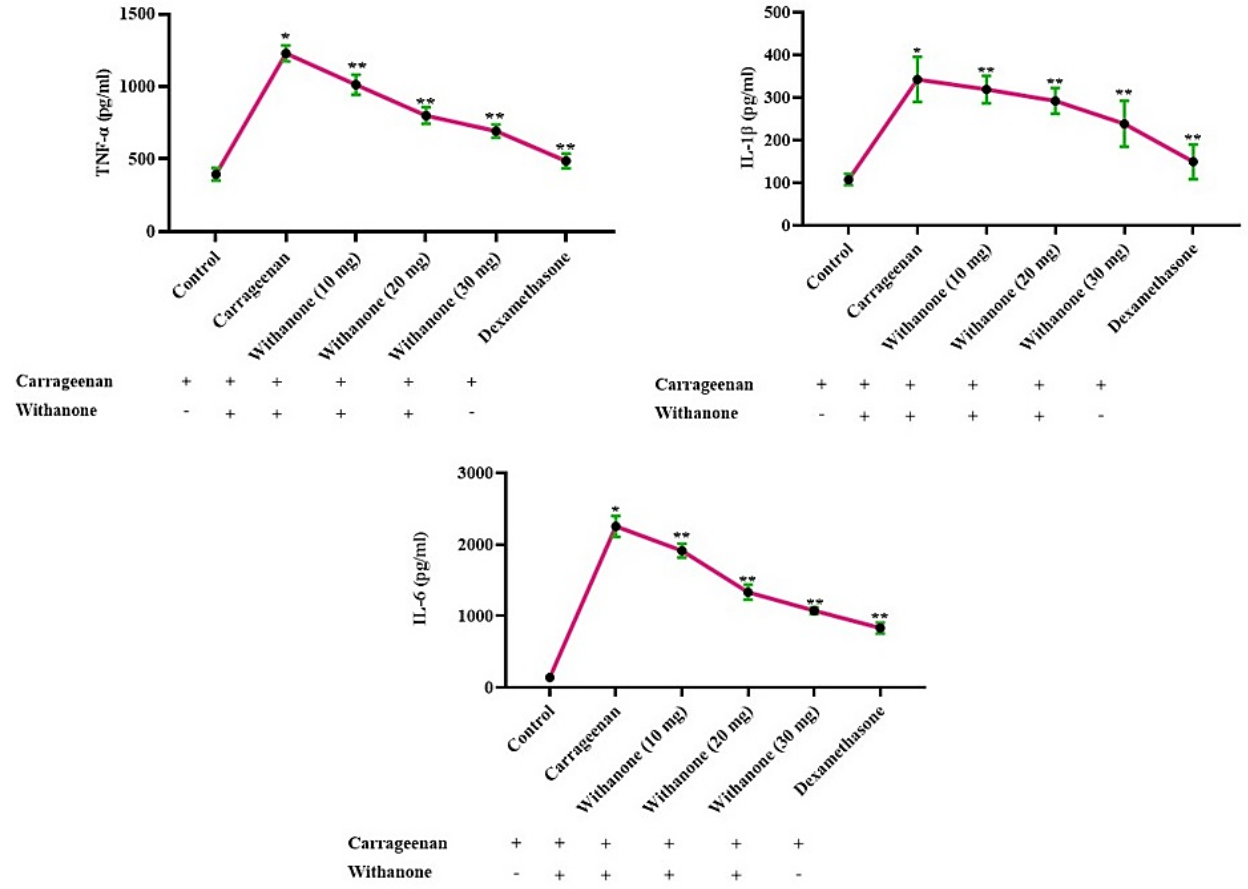

Figure 4: Effect of withanone on the level of inflammatory cytokines.

The values are scrutinized using one-way ANOVA and DMRT were conducted using Graphpad Prism to study the results. Results are expressed as Mean±SD of three replicates. '*' and '**' reveal the statistically significant levels at $p<0.05$ and $p<0.01$, respectively, when comparing treatment groups.

acute nociceptive responses to thermal stimuli, the formalin test provides a more sustained and complex pain experience, which is more representative of clinical pain conditions. Additionally, the formalin-induced test is relatively easy to perform, requires minimal equipment and provides a quantifiable, dose-dependent behavioral response.[30] The formalin-induced test has been widely employed to study the natural products. By measuring the duration and intensity of paw licking during the different phases of the test, researchers can assess the efficacy of various pharmacological interventions in modulating pain-related behaviors. Moreover, the formalin test has been used to study the underlying mechanisms of pain, including the roles of neurotransmitters, ion channels and inflammatory mediators.[31] The findings of this work has demonstrated that the mice administered with formalin indicated an increased licking numbers in both phases, signifying the onset of nociceptive response. Interestingly, the treatment with withanone significantly reduced the occurrences of licking responses produced by formalin in both phases.

The hot plate-induced nociceptive test is an extensively utilized technique for evaluating pain sensitivity and the analgesic effects of various compounds in mice. The hot plate technique is considered a reliable and sensitive model for assessing nociceptive thresholds in rodents and it has been employed extensively in the study of pain and the discovery of new analgesic drugs.[32] The hot plate-induced nociceptive test has several important applications in the field of pain research. One of the primary applications is the evaluation of the analgesic properties of natural products with potential analgesic activity. By observing the alterations in the animal's response latency to the hot plate stimulus, researchers can assess the ability of a compound to alleviate pain and determine its potency and duration of action.[33] In addition to drug testing, the hot plate technique has also been employed to study the mechanisms underlying nociceptive processing and the modulation of pain perception. By comparing the responses of normal and genetically modified mice, or by using pharmacological interventions, researchers can investigate the role of specific neural pathways and neurotransmitter systems in the regulation of pain sensitivity.[34] Another important application of the hot plate method is its use in the study of pain-related disorders, such as neuropathic pain and inflammatory pain. These conditions are often accompanied by altered nociceptive thresholds, which can be studied using hot plate method. By comparing the responses of animals with pain conditions to those of healthy controls, researchers can gain valuable insights into the underlying mechanisms and discovery of new therapies.[35] The results of this work demonstrated that the response time of control mice was lower than that of the withanone-treated mice. Captivatingly, the withanone-treated mice exhibited prolonged

Table 1: Effect of withanone on hot plate-induced nociceptive response in the experimental mice.

Treatment (mg/kg)	Pre-treatment	Response time (s) (% MPE)			
		30 min	60 min	90 min	120 min
Control	8.24±0.20	9.74±0.40	8.43±0.35	7.71±0.47	11.30±0.14
Withanone (10 mg)	9.17±0.06	11.22±0.14 (13.26)*	13.10±0.68 (15.22) *	11.52±0.41 (19.45) *	13.31±0.40 (32.33) *
Withanone (20 mg)	8.28±0.28	11.24±0.31 (15.12)**	13.83±0.33 (26.32) **	14.07±0.64 (35.66) **	12.42±0.62 (15.99) **
Withanone (30 mg)	8.84±0.03	12.36±0.17 (19.56)**	13.23±0.61 (21.39) **	15.52±0.27 (22.25) **	14.71±0.61 (35.26) **
Morphine (5 mg)	8.63±0.05	14.66±0.56 (16.56)**	15.75±1.06 (32.25) **	16.57±0.36 (39.48) **	18.60±0.47 (41.56)**
NLX (2 mg)+ Control	8.38±0.16	9.62±0.25 (12.32)**	10.31±0.41 (12.33)**	8.89±0.13 (9.32)**	9.22±0.56 (10.36)**
NLX (2 mg)+ Withanone (10 mg)	8.0±0.47	10.46±0.32 (22.32)**	10.21±0.14 (23.45) **	10.73±0.41 (0.41) **	12.14±0.12 (23.62) **
NLX(2 mg)+ Withanone (20 mg)	7.73±0.30	9.51±0.005 (11.32)**	9.17±0.34 (12.98) **	11.42±0.31 (25.45) **	12.78±0.20 (21.52) **
NLX(2 mg)+ Withanone (30 mg)	9.36±0.07	9.10±0.004 (12.41)**	9.27±0.12 (16.45) **	12.75±0.86 (28.52) **	12.76±0.87 (55.46) **
NLX(2 mg)+ Morphine (5 mg)	9.76±0.01	9.42±0.04 (10.23)**	10.83±0.85 (19.99) **	11.74±0.52 (31.25) **	16.39±0.17 (22.95) **

The values are scrutinized using one-way ANOVA and DMRT were conducted using GraphPad Prism to study the results. Results are expressed as Mean±SD of three replicates. '*' and '**' reveal the statistically significant levels at $p<0.05$ and $p<0.01$, respectively, when comparing treatment groups.

Table 2: Effect of withanone on tail immersion-induced nociceptive response in the experimental mice.

Treatment (mg/kg)	Pre-treatment	Response time (s) (% MPE)			
		30 min	60 min	90 min	120 min
Control	4.17±0.09	5.04±0.02	5.46±0.12	5.45±0.23	4.51±0.34
Withanone (10 mg)	3.48±0.44	5.43±0.18 (6.66) *	5.63±0.21 (7.33)*	5.63±0.27 (7.45)*	6.47±0.14 (7.77)*
Withanone (20 mg)	4.00±0.93	5.51±0.25 (7.15)**	6.30±0.07 (9.96)**	6.48±0.33 (8.85) **	6.56±0.22 (13.62)**
Withanone (30 mg)	2.23±0.18	3.77±0.28 (7.32) **	6.11±0.46 (8.25) **	7.74±0.08 (9.09)**	7.56±0.23 (11.15)**
Morphine (5 mg)	3.86±0.56	6.15±0.13 (9.25)**	6.35±0.48 (12.36) **	7.29±0.09 (65.22) **	4.08±0.44 (22.12) **
NLX (2 mg)+ Control	4.46±0.15	4.39±0.30 (5.32)**	4.34±0.22 (6.33)**	5.42±0.54 (6.32)**	5.51±0.24 (6.33)**
NLX (2 mg)+ Withanone (10 mg)	4.26±0.03	5.31±0.27 (7.56) **	5.43±0.25 (6.19) **	5.41±0.23 (8.55) **	5.47±0.21 (6.32) **
NLX (2 mg)+ Withanone (20 mg)	4.02±0.03	7.48±0.29 (9.33) **	7.51±0.21 (9.99) **	7.55±0.26 (13.23) **	8.37±0.13 (9.33) **
NLX (2 mg)+ Withanone (30 mg)	3.83±0.56	5.42±0.16 (11.23) **	5.54±0.18 (6.99) **	5.36±0.16 (8.59) **	6.50±0.21 (8.85) **
NLX (2 mg)+ Morphine (5 mg)	2.36±0.25	4.44±0.21 (5.25) **	4.39±0.21 (5.12) **	4.28±0.14 (7.32) **	5.43±0.13 (11.96) **

The values are scrutinized using one-way ANOVA and DMRT were conducted using GraphPad Prism to study the results. Results are expressed as Mean±SD of three replicates. '*' and '**' reveal the statistically significant levels at $p<0.05$ and $p<0.01$, respectively, when comparing treatment groups.

reaction times on the hot plate when compared with control group, suggesting its antinociceptive properties.

The tail immersion test is a widely used experimental paradigm in the area of pain research, particularly in the context of studying the analgesic (pain-relieving) properties of various pharmacological agents in mice. This test, which involves immersing the distal portion of a mouse's tail in warm or hot water and measuring the latency to withdrawal, is a valuable tool for assessing nociceptive responses and evaluating the efficacy of pain-relieving drugs.[36] One of the primary advantages of the tail immersion test is its capacity to model acute pain, which is often a primary concern in clinical settings. By exposing the mouse's tail to a thermal stimulus, the test can elicit a reliable and quantifiable nociceptive response, allowing researchers to measure the degree of analgesia induced by different treatments. This information is crucial for the development and evaluation of new analgesic drugs, as it provides a means to assess their potency and efficacy in reducing pain-related behaviors.[37] The tail immersion test has been broadly employed in studies exploring the mechanisms underlying pain perception and the modulation of pain responses. Additionally, the test has been utilized to assess the analgesic properties of nonsteroidal anti-inflammatory drugs, as well as novel pain-relieving compounds.[38] Furthermore, the tail immersion test has been instrumental in understanding the neural pathways involved in pain processing. By examining the effects of lesions or pharmacological interventions on the latency to tail withdrawal, researchers can elucidate the specific brain regions and neural circuits that are responsible for the transmission and modulation of nociceptive information.[39] The present findings indicated that the response times of withanone-treated mice were successfully improved in the hot water. Notwithstanding the difficulty posed by naloxone, withanone treatment increased the response time in hot water, demonstrating its antinociceptive efficacy.

OFT is a widely used behavioral assessment technique in neuroscience and pharmacology research to evaluate the locomotor activity and anxiety-like behaviors in rodents, particularly in mice. This method allows researchers to observe the spontaneous exploratory behavior of mice in a new environment and assess the effects of various pharmacological interventions, including sedatives, on their motor and emotional responses.[40] The OFT typically involves placing a mouse in a brightly lit, enclosed arena and recording its movement and exploratory behavior over a specified time period.[41] Several studies have employed the OFT to investigate the sedative properties of natural and synthetic compounds in mouse models.[42,43] By analyzing the alterations in locomotor activity, researchers can gain insights into the

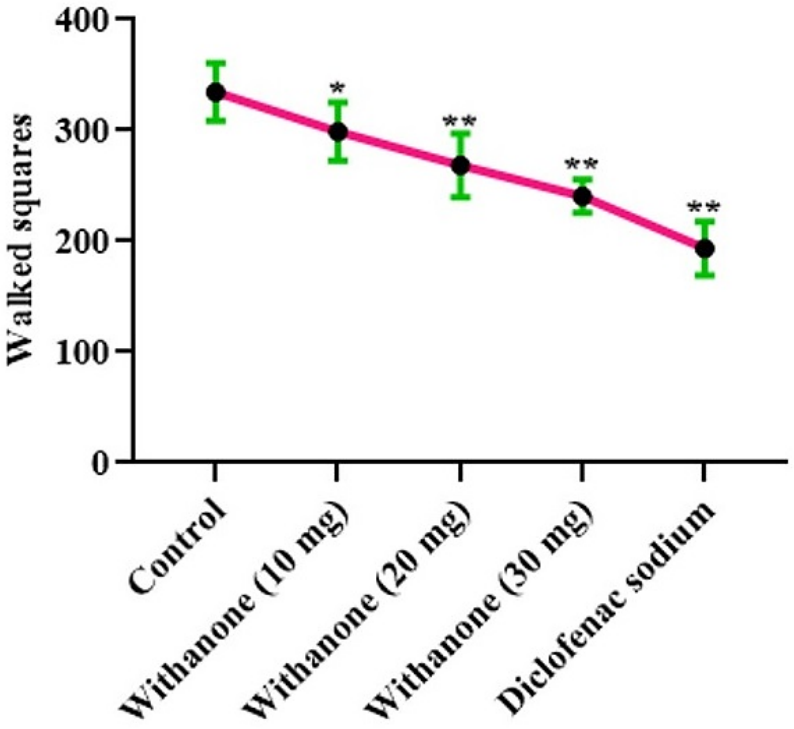

Figure 5: Effect of withanone on the behaviors of the experimental mice assessed by OFT.

The values are scrutinized using one-way ANOVA and DMRT were conducted using Graphpad Prism to study the results. Results are expressed as Mean±SD of three replicates. '*' and '**' reveal the statistically significant levels at $p<0.05$ and $p<0.01$, respectively, when comparing treatment groups.

potential sedative or anxiolytic effects of the tested samples.[44] The current findings has demonstrated that the experimental mice treated with withanone exhibited the substantial diminution in the number of walked squares, suggesting the sedative effects of withanone.

The carrageenan-induced air pouch method is a widely utilized experimental approach in the field of inflammation and immunology research, providing invaluable knowledge into the underlying processes of various pathological conditions. This model involves the subcutaneous injection of carrageenan, which induces a localized inflammatory response within the air pouch.[45] Furthermore, this model is also a well-established system for studying the inflammatory response and the involvement of various cytokines. Inflammatory cytokines, including IL-6, TNF-α and IL-1β play a crucial role in the onset of inflammatory response.[46] These pro-inflammatory molecules can enhance the generation of other mediators, including NO and prostaglandins, further enhancing the inflammatory cascade. The carrageenan-triggered model allows for the assessment of the temporal dynamics of these cytokines and their potential therapeutic targeting.[47] One of the primary applications of this model is the study of anti-inflammatory properties of the sample's drugs.[48] The present findings has demonstrated the significant elevation in the concentrations of IL-6, TNF-α and IL-1β in the mice with carrageenan-induced air pouch. Fascinatingly, withanone treatment at various concentrations substantially reduced the IL-6, TNF-α and IL-1β concentrations in the carrageenan-induced mice, which highlights its anti-inflammatory activities.

CONCLUSION

The present findings emphasize the anti-nociceptive and anti-inflammatory activities of withanone in various chemical- and heat inflammation and pain models in mice. The treatment with withanone markedly suppressed the inflammatory response induced by carrageenan and mitigated the nociceptive responses elicited by several thermal and chemical-induced models. Furthermore, additional studies are still necessitated in the future to extend the understanding of the therapeutic benefits of withanone.

ACKNOWLEDGEMENT

Report all funding sources includ.ing any grant numbers. This work is supported by ZhongkeInno(Beijing)International Medical Research Institute.

FUNDING

2022 Nantong Social and People's Livelihood Science and Technology Plan Project Number: MSZ2022028.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

IASP: International Association for the Study of Pain; **NSAIDs:** Non-steroidal Anti-inflammatory Drugs; **IL-6:** Interleukin-6; **TNF-α:** Tumor Necrosis Factor-alpha; **IL-1β:** Interleukin-1beta; **OFT:** Open-Field Test.

ETHICAL APPROVAL

This work has approved by the institutional animal ethical committee by Nantong Hospital of Traditional Chinese Medicine, Nantong, Jiangsu Province, 226001, China.

SUMMARY

Pain is an unpleasant sensation that may result from severe or harmful stimuli. Pain can arise from various sources, including injury, disease, or even emotional trauma and its manifestation can range from acute to chronic. Withanone is a major bioactive steroidal lactone compound found extensively in the roots of *Withania somnifera*. The present findings emphasize the anti-nociceptive and anti-inflammatory activities of withanone in various chemical- and heat inflammation and pain models in murine.

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Cite this article: Wang F, Zhu Y. Anti-nociceptive and Anti-inflammatory Effects of Withanone in Various Nociception and Inflammation-Induced Murine Model. Indian J of Pharmaceutical Education and Research. 2026;60(1):247-57.