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Bhilawanol Reduces Morphine Conditioned Place Preference and Anxiety by Modulating Antioxidant, Anti-Inflammatory, and Nitric Oxide Pathways

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ABSTRACT: Opioid addiction is characterized by persistent drug seeking; the main objective of addiction treatment is to prevent relapses, which remains the primary limitation of pharmacological therapy. In the present work, the role of bhilawanol (BWL) on morphine conditioned place preference (CPP) and the anxiety-like behavior during the abstinence period in the mice model was investigated. Morphine CPP was induced with an increasing dose of morphine (MOR) from 5 mg/kg bid to 25 mg/kg bid for 5 days during the conditioning schedule. The effect of BWL (0.25, 0.5, and 1 mg/kg) was tested on the rewarding effects of MOR. BWL 1 mg/kg significantly reduced MOR acquisition and reinstatement and accelerated extinction of MOR CPP. BWL 1 mg/kg also markedly reduced anxiety-like behavior on the last extinction day, as measured by the open-field (OF) and elevated plus maze (EPM) tests. BWL significantly preserved the antioxidant enzymes in the brain and modulated the expression of tumor necrosis factor (TNF- α), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS) in both the cortex and hippocampus regions of the brain. Further, BWL also modulated the expression of TNF- α and brain-derived neurotrophic factor (BDNF) in MOR-treated mice, measured through ELISA. In addition, BWL displayed a significant reduction in total nitric oxide in the cortex and hippocampal region. These findings show the beneficial effects of BWL in MOR-induced CPP, extinction, and reinstatement, as well as reducing anxiety during abstinence from MOR through modulation of antioxidant, inflammatory, and NO pathways.

1. INTRODUCTION

Drug addiction is characterized by persistent, compulsive drug use despite adverse consequences and recurrent relapses that hinder the success of treatment [1,2]. Relapse is the hallmark of drug misuse and is seen as a significant issue in opioid use disorder (OUD) treatment [3,4]. Because of their exceptional analgesic qualities, opioids like morphine (MOR) are associated with the development of tolerance to their anti-nociceptive effects, physical and psychological dependence, and withdrawal symptoms after cessation. Often, these symptoms are so severe that they encourage a recovering individual to relapse to the use of the drug again and prevent the clinical use of medications for chronic pain [5].

In experimental work, the conditioned place preference (CPP) model, in which the animal chooses to remain longer in the area where it is exposed to addictive substances, supports relapse to drug use [6, 7]. CPP is a conditioning procedure wherein a drug's effects are paired with one distinct context, and another context is paired with the vehicle. The rewarding effects of the drug are measured by a higher preference towards the drug-paired context during subsequent drug-free tests [8]. Also, it is evident that abstinence from MOR, after prolonged use, is associated with increased anxiety [9]. The need to prevent unpleasant internal states contributes to the need to continuously seek out and acquire addictive drugs during periods of abstinence or recovery [9]. For this reason, one possible approach is to reduce one of the psychological aspects, which is anxiety during the abstinence phase, which may assist in preventing individuals from relapsing into the use of MOR in the future.

The administration of MOR not only increased the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) but also lowered the levels of antioxidants in target cells [10,11]. With the increase in oxidative stress and initiation of an inflammatory cascade, MOR addiction is associated with the generation of nitric oxide (NO), which contributes to disease progression [12]. It has also been observed that neuroinflammation results in elevated NO production, which may link neuroinflammation to mental disorders and illnesses, including anxiety and depression [13]. Nitric oxide (NO) is a highly diffusible gaseous molecule synthesized from L-arginine by the action of nitric oxide synthase (NOS) enzymes, namely inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS) [5, 12]. Moreover, addictive substances of different kinds have been shown to alter the levels of brain-derived neurotrophic factor (BDNF) protein and mRNA in various parts of the brain [14]. The elevated levels of free radicals and oxidative stress result in a decline in BDNF, thereby promoting depression and anxiety [15].

Currently, opioid dependence and withdrawal symptoms are treated with clonidine, methadone, and naloxone; however, these medications have side effects and increase the risk of relapse [16,17]. Hence, in the present study, we attempt to investigate the effect of bhilawanol (BWL) on morphine-induced conditioned place preference and anxiety during the abstinence period through behavioural approaches. Additionally, the role of protective antioxidant enzymes, inflammatory markers, and nitric oxide signalling pathways is also evaluated through various molecular and biochemical assays.

2. MATERIALS AND METHODS

2.1 Animals

Adult male albino mice (8–12 weeks old, 25–30 g) of the Swiss albino strain were employed in the investigation. Mice were obtained from the animal breeding facility at Riphah Institute of Pharmaceutical Sciences, Riphah International University, Pakistan. They were maintained under controlled conditions ($22 \pm 2^\circ\text{C}$, 12-hour light/dark cycle, optimal relative humidity of $65 \pm 5\%$) in polypropylene housings (4 mice per cage), with ad libitum access to food and water. Following a 7-day acclimatization period, an independent researcher randomly assigned mice to five experimental groups, each with 13 animals. All experimental procedures received approval from the Institutional Animal Care and Use Committee (IACUC), called the Research Ethics Committee (REC) of the Riphah Institute of Pharmaceutical Sciences (Ref. No: REC/125/Pharmacology/2025), and were conducted in strict accordance with institutional guidelines for animal care, reaffirming our dedication to ethical research standards. All animal experiments performed in the present investigation adhered to the ARRIVE guidelines.

Mice were grouped as follows, each consisting of 13 animals (**Figure 1**).

- Group-I Saline: Normal saline (10mL/kg) intraperitoneally.
- Group-II Morphine (MOR): Morphine (MOR) (CAS# 6211-15-0, Sigma-Aldrich) was injected at increasing doses of 5 mg/kg bid to 25 mg/kg bid intraperitoneally (i.p.) for 5 days during the conditioning schedule.
- Group-III to Group-V (Bhilawanol (BWL) + Morphine (MOR): BWL (CAT# SMB00116, Sigma-Aldrich) was administered at 0.25mg/kg, 0.5mg/kg and at 1mg/kg before MOR, respectively.

Figure-1

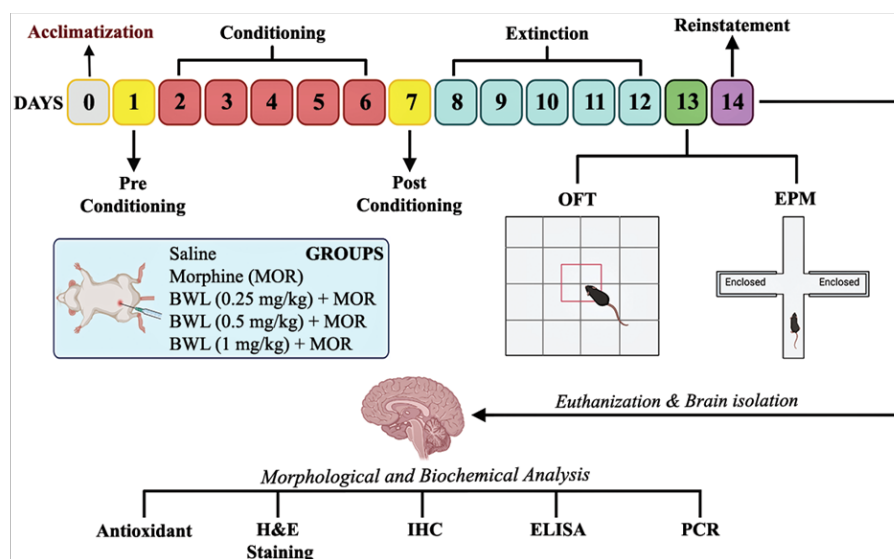


Figure 1. Experimental design of morphine (MOR) condition place preference in mice model. After pre-conditioning on day 1, conditioning was performed for 5 days with an increasing dose of intraperitoneal morphine from 5 mg/kg bid to 25 mg/kg. The effect of bhlawanol (BWL) on the rewarding effects of MOR was assessed on day 6 in post-conditioning.

Extinction to MOR was performed on the 8th day, which lasted for 5 days.

2.2 Conditioned place preference (CPP)

The induction of morphine (MOR) CPP was performed according to the previous work, with a few modifications [18]. MOR-induced CPP was conducted in a three-chamber apparatus, with two outer chambers having different cues for contextual conditioning and a central chamber that was kept neutral (Plain). One chamber was designed with a red mesh floor, while the other had black/white coloured striped walls and a mesh floor (MOR-paired chamber). The CPP assessments were categorized as follows: Preconditioning: During the initial phase, the animals were placed in the central chamber of the box and permitted to move freely within the entire chamber for 15 minutes to establish baseline preferences for the chamber. A conditioning session: During the following 5-day conditioning phase (Days 2–6), mice were administered intraperitoneal (i.p.) injections of morphine according to an escalating-dose regimen to establish conditioned place preference (CPP). By the fifth day of training, the morphine dosage had been raised from the initial 5 mg/kg twice daily (bid) to a final 25 mg/kg bid. Animals were conditioned to remain in a single chamber for 30 minutes, prohibiting movement to the alternate chambers, designated as the MOR-paired chamber. To assess its potential protective effects, bhlawanol (BWL) was administered at three distinct doses (0.25, 0.5, and 1 mg/kg, i.p.) to the corresponding treatment groups. BWL was administered as a pretreatment 30 minutes prior to each morphine injection during the conditioning period. The control group was administered normal saline (10 mL/kg, i.p.) according to the same schedule. On Day 7 following conditioning, all mice were permitted unrestricted access to the apparatus for 15 minutes to evaluate the rewarding effects of morphine by quantifying the duration spent in the drug-paired chamber.

2.3 Extinction

After the establishment of MOR-induced CPP, the mice underwent an extinction phase from Day 8 to Day 12. All the groups were placed in the CPP apparatus with free access to all the chambers for 15 minutes, and no drug injections were given until the preference for the drug-paired chamber was eliminated, and analyses were recorded. Behavioral tests, specifically the open field and elevated plus maze, were conducted on Day 13 to assess anxiety-like behavior during morphine abstinence.

2.4 Reinstatement

On Day 14, a priming dose of morphine (1 mg/kg, i.p.) elicited the reinstatement of drug-seeking behavior. The priming dose for the BWL-treated groups was administered for 30 minutes following the final pretreatment with their respective BWL doses. After the reinstatement test, all mice were euthanized, and brain tissues were collected for biochemical and molecular analysis.

2.5 Open-field maze (OF)

The OF test was performed according to the previously published work with slight modifications [13]. The mice were placed in the center of the OF apparatus (100 cm length × 100 cm width). The apparatus was located in a low-light room, and the experiment was recorded for 5 minutes. The time spent in the central and outer zones was recorded and analysed.

2.6 Elevated plus maze (EPM)

The EPM apparatus consisted of four arms, two open arms and two closed arms (50 cm length × 10 cm width × 100 cm height). Mice were placed in the center of the EPM, and their movements were recorded for 5 minutes. The parameters, such as the number of crossings in closed and open arms and time spent in open and closed arms, were evaluated [19].

2.7 Antioxidant activities

Protein quantification was performed using a BCA kit. For antioxidant assays, tissue samples were lysed in phosphate buffer, except for GSH which used 5% metaphosphoric acid. GSH was measured by combining supernatant with DTNB and reading absorbance at 412 nm. Catalase activity was assessed by mixing supernatant with H₂O₂ and monitoring decreased absorbance at 240 nm over 60 seconds. GST levels were determined using a reaction mixture of GSH and CDNB, with absorbance measured at 340 nm for 5 minutes. Lipid peroxidation was evaluated by incubating supernatant with ferric chloride and ascorbic acid, terminating with TCA/TBA, and reading absorbance at 535 nm, with results expressed as TBARS nmol/mg protein.

2.8 Nitric Oxide Assay

For nitric oxide (NO) levels, the concentration of nitrite in the cortex and hippocampus regions of the mouse brain tissue was determined with the Griess reaction quantification method [20]. First, the cortex and hippocampus tissues were homogenized and centrifuged at 13,000 RCF for about 15 min. From the resultant homogenate, the supernatant was separated. An equal volume of supernatant and Griess reagent was mixed and incubated at room temperature in a dark room. Further, the absorbance was measured at 540 nm, and the nitrite levels were expressed as μmol/milligram of protein.

2.9 Hematoxylin and Eosin (H&E) staining

Tissue slides were immersed in hematoxylin solution (Sigma) and an Eosin solution. Slides were dehydrated with graded ethanol and xylene, and a coverslip was applied and examined via a light microscope (Olympus BX53, Japan) to assess the surviving neurons and their morphology. Each group produced three slides, from which two images were captured using a light microscope (Olympus BX53, Japan). Two regions of interest (ROIs) were once more identified: the hippocampus and specific areas of the parietal cortex (PC).

2.10 Immunohistochemistry

For immunostaining, the primary antibodies, such as inducible nitric oxide synthase (iNOS) (SC-7271), tumour necrosis factor alpha (TNF-α) (SC-52B83), and nuclear factor kappa-light-chain-enhancer of activated B cells (p-NFκB) (SC-271908), were purchased from Santa Cruz Biotechnology, Dallas, TX, USA, and neuronal nitric oxide synthase (nNOS) (18984-1-AP) from ProteinTech, UK. Following antigen retrieval and blocking, primary antibodies were applied to the slides and incubated in a damp container overnight at 4°C. The next morning, the secondary antibody was applied, followed by ABC treatment. The slides were stained with DAB solution and cover-slipped. The Images (10x and 40x magnification) of the cortex and hippocampus regions were captured using an Olympus microscope and processed using ImageJ software.

2.11 Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of brain-derived neurotrophic factor (BDNF) (Cat#PRS-20038Mo) and tumor necrosis factor-α (TNF-α) (Cat#PRS-20038Mo) ELISA kits from Elabscience were used following the manufacturer's instructions. The cortex and hippocampus regions of the brain were homogenized in PBS with a protease inhibitor (PMSF) with a Heidolph crusher. The samples were centrifuged at 4000 × g for about 10 min. Further, the supernatant was separated and processed for total protein concentration using the BCA method. An equal quantity of protein was processed using the ELISA microplate reader (BioTek ELx808).

2.12 Statistical Analysis

The Shapiro-Wilk test was employed to evaluate the normality of the numerical data across the four groups. The ImageJ software was employed to quantify histology data. GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was utilized for graph preparation and statistical analysis. The values obtained from the results were expressed as mean $\pm$ standard deviation (S.D.), and statistical disparities between groups were assessed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A statistically significant result was established as $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Effect of bhilawanol (BWL) on the acquisition and extinction of morphine (MOR) conditioned place preference (CPP)

In the post-conditioning phase, all the mice showed an equal preference for both outer chambers (**Figure 2A**). After the establishment of MOR-induced CPP, the mice showed a significant preference towards the MOR-paired chamber compared to the saline-treated mice on the post-conditioning day (**Figure 2A**, $###p < 0.001$ vs. Saline). BWL pretreatment decreased the acquisition of MOR-induced CPP in a dose-dependent manner. The preference for the MOR-paired chamber was considerably reduced by BWL at 1 mg/kg (**Figure 2A**, $***p < 0.001$ vs. MOR), but BWL at 0.25 and 0.5 mg/kg had a modest impact.

In the extinction phase, BWL attenuated MOR-induced CPP in a dose-dependent manner throughout the course of the 5-day extinction period. When compared to the MOR group, mice treated with BWL 1 mg/kg exhibited a sharp decrease in preference for the MOR-paired chamber during the extinction experiment for five days (**Figure 2B**, $***p < 0.001$ vs. MOR). While BWL 0.25 mg/kg did not exhibit any significant effect, BWL 0.5 mg/kg also facilitated extinction, but to a lesser extent, specifically on day 1 (**Figure 2B**, $*p < 0.05$ vs. MOR).

After the extinction of MOR CPP, the mice were treated with a MOR priming dose of 1 mg/kg for reinstatement. The MOR group showed a significant preference towards the MOR-paired chamber ($###p < 0.001$ vs. Saline), while BWL 0.5 mg/kg ($*p < 0.05$) and 1 mg/kg ($***p < 0.001$) significantly reduced the time spent in the MOR-paired chamber (**Figure 2C**). BWL 0.25 mg/kg did not prevent reinstatement.

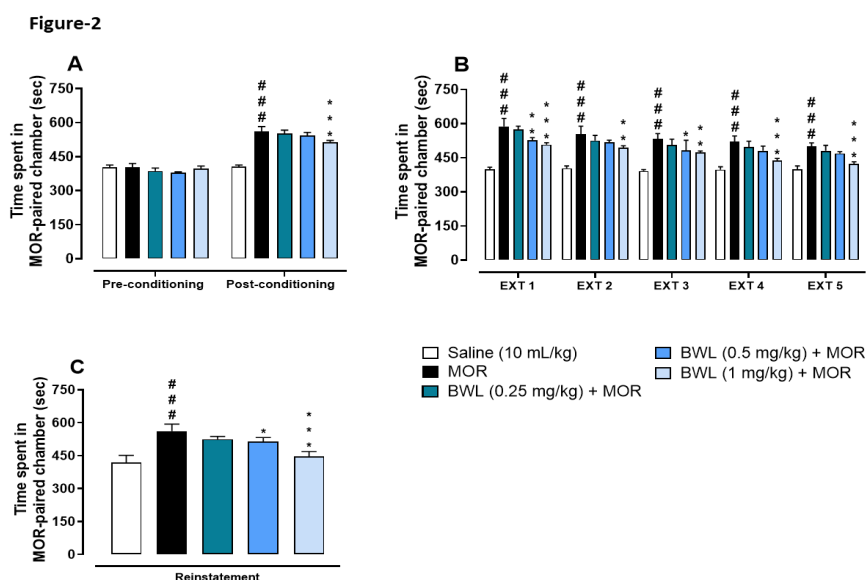


Figure 2. Effects of bhilawanol (BWL) on the **(A)** Acquisition, **(B)** Extinction, and **(C)** reinstatement to morphine (MOR) conditioned place preference (CPP). For statistical analysis, two-way repeated measures ANOVA (*post-hoc* Tukey's test) was applied. On the 14th day of reinstatement, mice received BWL 30 minutes before a MOR 1 mg/kg priming dose. For statistical analysis, one-way ANOVA (*post-hoc* Tukey's test) was applied. The results were evaluated as time spent by the mice in the MOR-paired chamber. Data are expressed as means $\pm$ S.D. ($n=13$ /group). The $***$ represents $p < 0.001$ vs saline group, whereas $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs MOR group.

3.2 Effect of bhlawanol on anxiety-like behavior during abstinence from morphine (MOR)

Mice treated with morphine exhibited a marked increase in anxiety-like behavior during the MOR abstinence phase, as assessed by the open field (OF) and elevated plus maze (EPM) tests (### $p < 0.001$ vs. Saline). In the open field test (**Figure 3A, 3B**), MOR-treated mice exhibited a marked reduction in time spent in the central zone and an increase in time spent in the outer zone, suggesting the presence of anxiety-like behavior. Pretreatment with BWL reduced this effect in a dose-dependent manner. BWL 1 mg/kg markedly enhanced the duration in the inner zone (**Figure 3A**, *** $p < 0.001$ vs. MOR) and reduced the time in the outer zone (**Figure 3B**, *** $p < 0.001$ vs. MOR). BWL 0.5 mg/kg demonstrated a significant effect (* $p < 0.05$ compared to MOR), whereas BWL 0.25 mg/kg did not exhibit any effect.

In the elevated plus maze test (**Figure 3C, 3D**), MOR-treated mice spent less time in the open arms and more time in the closed arms, indicating anxiety-like behavior (### $p < 0.001$ vs. Saline). Treatment with BWL 1 mg/kg substantially increased time spent in open arms (**Figure 3C**, *** $p < 0.001$ vs. MOR) and reduced time in closed arms (**Figure 3D**, *** $p < 0.001$ vs. MOR). BWL 0.5 mg/kg showed stronger anxiolytic effects in the EPM test (* $p < 0.001$ vs. MOR) than in the OF test. There was no significant impact of BWL 0.25 mg/kg in any behavioural test.

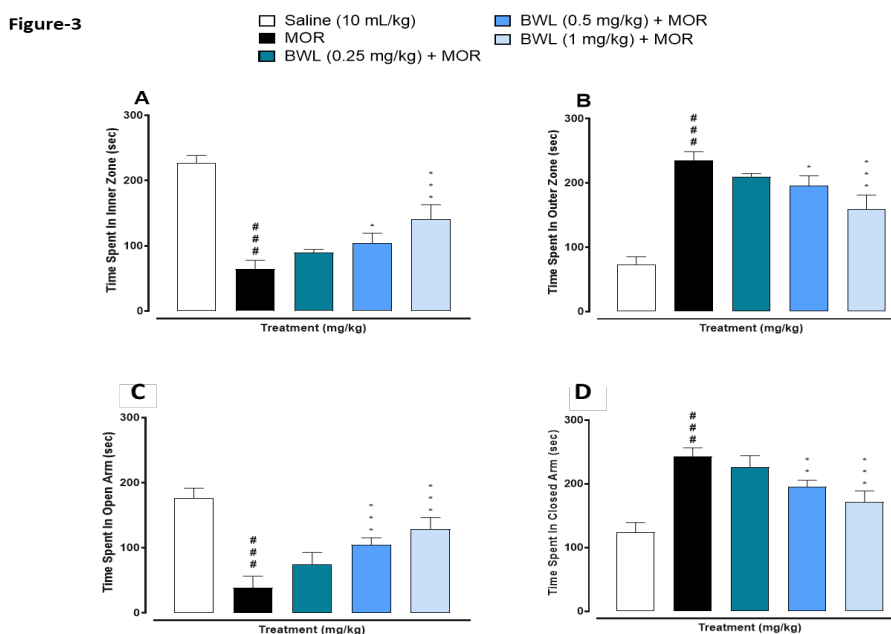


Figure 3. Effect of bhlawanol (BWL) on anxiety-like behavior during the abstinence period to morphine (MOR) in the extinction phase, as analyzed through the open-field (OF) test and elevated plus maze (EPM). (A) Presenting time spent in the inner zone and (B) outer zone of the open-field arena; (C) Time spent in the open arm and (D) Time spent in the closed arm of the EPM apparatus. Values were expressed as mean $\pm$ S.D. (n=13/group). For statistical analysis, one-way ANOVA (post-hoc Tukey's test) was applied. The p-values, ### $p < 0.001$, denote significance against the saline, whereas * $p < 0.05$ and *** $p < 0.001$ denote significance against the MOR group.

3.3 Effect of bhlawanol on morphine-induced increases in the nitric oxide pathway

Mice administered morphine exhibited a substantial elevation in nitrite concentrations in both the cortex and hippocampus compared to the saline group (**Figure 4A**, ### $p < 0.001$ vs. Saline). Immunohistochemistry demonstrated a substantial increase in the protein expression of iNOS (**Figure 4B**) and nNOS (**Figure 4C**) in the cortex and hippocampus of MOR-treated mice (### $p < 0.001$ vs. Saline).

Treatment with bhlawanol (BWL) reduced these effects in a dose-dependent manner. BWL at all administered doses (0.25, 0.5, and 1 mg/kg) significantly decreased the increased nitrite levels in both cerebral regions (**Figure 4A**, *** $p < 0.001$ vs. MOR). BWL 1 mg/kg markedly reduced the protein expression of both iNOS and nNOS in the brain and hippocampus, as demonstrated in the IHC investigations (**Figure 4B, 4C**, *** $p < 0.001$ vs. MOR). The low doses of BWL exhibited region-

specific effects; BWL 0.25 and 0.5 mg/kg markedly decreased iNOS expression in the cortex, but not in the hippocampus (Figure 4B, $^{***}p<0.001$ and $^{*}p<0.05$ vs. MOR). These findings indicate that BWL regulates the NO pathway by inhibiting both the expression and the enzymatic production of NO.

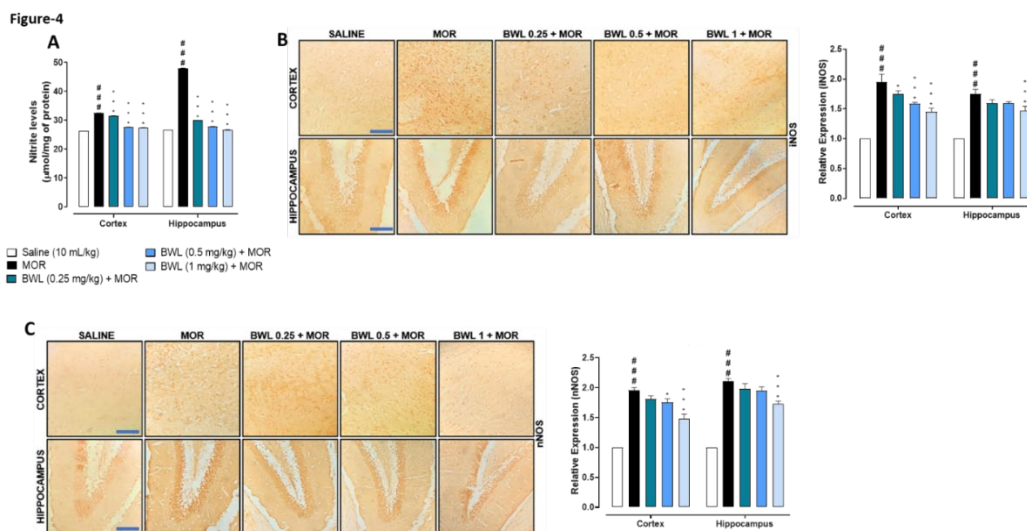


Figure 4. Effect of bhilawanol (BWL) on the nitric oxide pathway: (A) nitrite levels in cortex and hippocampus regions of the brain in morphine (MOR)-treated mice; (B) Immunostaining of inducible nitric oxide synthase (iNOS) expression; (C) Immunostaining of neuronal nitric oxide synthase (nNOS) expression. Values are presented as mean $\pm$ S.D. (n=13/group), and replicates per group were 3; scale bar 40 μ m, magnification 40X. For statistical analysis, one-way ANOVA (post-hoc Tukey's test) was applied. $^{###}p<0.001$ denotes significance against the saline group, whereas $^{***}p<0.001$ denotes significance against the MOR group

3.4 Effect of bhilawanol on the morphine-induced morphological alteration in cortex and hippocampus parts of the brain

Mice administered morphine displayed notable morphological changes, such as neuronal atrophy, densely marked pyknotic nuclei, cytoplasmic vacuolization, and compromised cellular integrity in both the cortex and the hippocampus DG region (Figure 5, $^{###}p<0.001$ vs. Saline). BWL 1 mg/kg markedly maintained neuronal morphology, demonstrated by diminished pyknosis, reestablished cellular architecture, and reduced vacuolization in comparison to the MOR-treated group (Figure 5, $^{***}p<0.001$ vs. MOR). BWL 0.5 mg/kg demonstrated moderate neuroprotective effects; however, BWL 0.25 mg/kg did not mitigate morphine-induced neuronal damage.

Figure-5

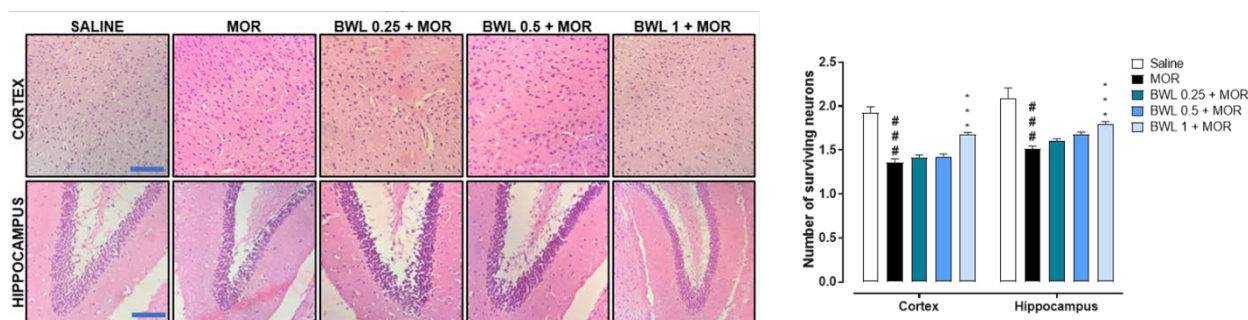


Figure 5. Photomicrograph of morphine (MOR)-induced morphological changes in mice cortex and hippocampus. Values are presented as mean $\pm$ S.D. (n=13/group), and replicates per group were 3, scale bar 40 μ m; magnification 40X. For statistical analysis, one-way ANOVA (post-hoc Tukey's test) was applied. $^{###}p<0.001$ denotes significance against the saline group, whereas $^{***}p<0.001$ denotes significance against the MOR group.

3.5 Effect of bhlawanol on inflammatory markers and BDNF levels in the cortex and hippocampus

Immunohistochemistry demonstrated that morphine-treated mice displayed markedly increased expression of TNF- α (**Figure 6A**) and phosphorylated NF- κ B (p-NF- κ B) (**Figure 6B**) in both the cortex and hippocampus compared to the saline-treated group (### p <0.001 vs. Saline). Pretreatment with BWL reduced this inflammatory response in a dose-dependent manner. BWL 1 mg/kg markedly reduced the expression of TNF- α and p-NF- κ B in the hippocampus (** p <0.001 vs. MOR). BWL 0.5 mg/kg exclusively influenced the expression of inflammatory markers solely in the hippocampus area (** p <0.01 vs. MOR), with no significant impact noted in the cortex. BWL 0.25 mg/kg did not demonstrate any meaningful reduction in either brain region.

These results were additionally validated by ELISA quantification (**Figure 6C, 6D**). Morphine administration markedly diminished BDNF levels in both the cortex and hippocampus (Fig. 6C, ### p <0.001 vs. Saline), signifying a reduction in neurotrophic support. Simultaneously, TNF- α levels were markedly increased in the identical brain areas of MOR-treated mice (Fig. 6D, ### p <0.001 vs. Saline), corroborating the IHC results. The use of BWL rectified these imbalances in a dose-dependent manner. BWL 1 mg/kg markedly elevated BDNF levels (** p <0.001 vs. MOR) and reduced TNF- α levels (** p <0.001 vs. MOR) in both cerebral regions. BWL 0.5 mg/kg demonstrated considerable efficacy, however BWL 0.25 mg/kg proved ineffective.

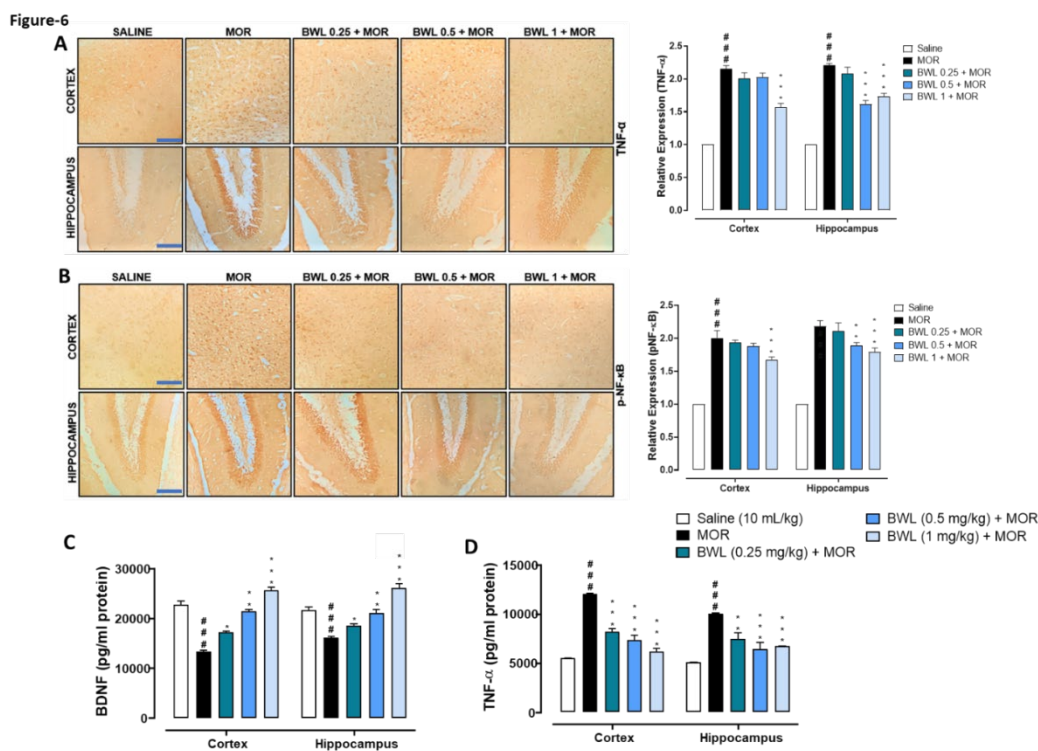


Figure 6. Effect of bhlawanol (BWL) on inflammatory and growth factors: **(A)** Immunostaining of tumor necrosis factor- α (TNF- α); **(B)** Immunostaining of phosphorylated nuclear factor- κ B (p-NF- κ B) expression. Values are presented as mean $\pm$ S.D. (n=13/group), and replicates per group were 3; scale bar 40 μ m, magnification 40X. **(C)** ELISA analysis of brain-derived neurotrophic factor (BDNF); **(D)** ELISA analysis of TNF- α . For statistical analysis, one-way ANOVA (*post-hoc* Tukey's test) was applied. the p-values, ### p <0.001 denotes significance against the saline group, while ** p <0.001 denotes significance against the MOR group

3.6 Effect of bhlawanol on antioxidant enzymes and lipid peroxidation

Morphine (MOR)-treated mice displayed a significant decrease in antioxidant enzymes such as GST, GSH, and CAT, while showing elevated expression of LPO levels as compared to the saline-treated group (**Figure 7**, ### p <0.001). BWL 1 mg/kg preserved the GST levels in MOR-treated mice brain tissues both in the cortex (* p <0.05, **Figure 7A**) and hippocampus

(*** $p < 0.001$, **Figure 7A**), while BWL at the doses of 0.25 and 0.5 mg/kg did not affect GST levels. On the other hand, the GSH levels were also significantly preserved with BWL 0.5 and 1 mg/kg, with no effect at the dose of BWL 0.25 mg/kg (**Figure 7B**). The CAT levels were also protected with BWL 0.5 and 1 mg/kg only in the cortex region of the brain (**Figure 7C**, *** $p < 0.01$), with no effect observed in the hippocampus region. BWL at all mentioned doses showed a significant decline in the MOR-induced increase in LPO levels in both the cortex and hippocampus regions of the brain (**Figure 7D**, *** $p < 0.001$).

Figure-7

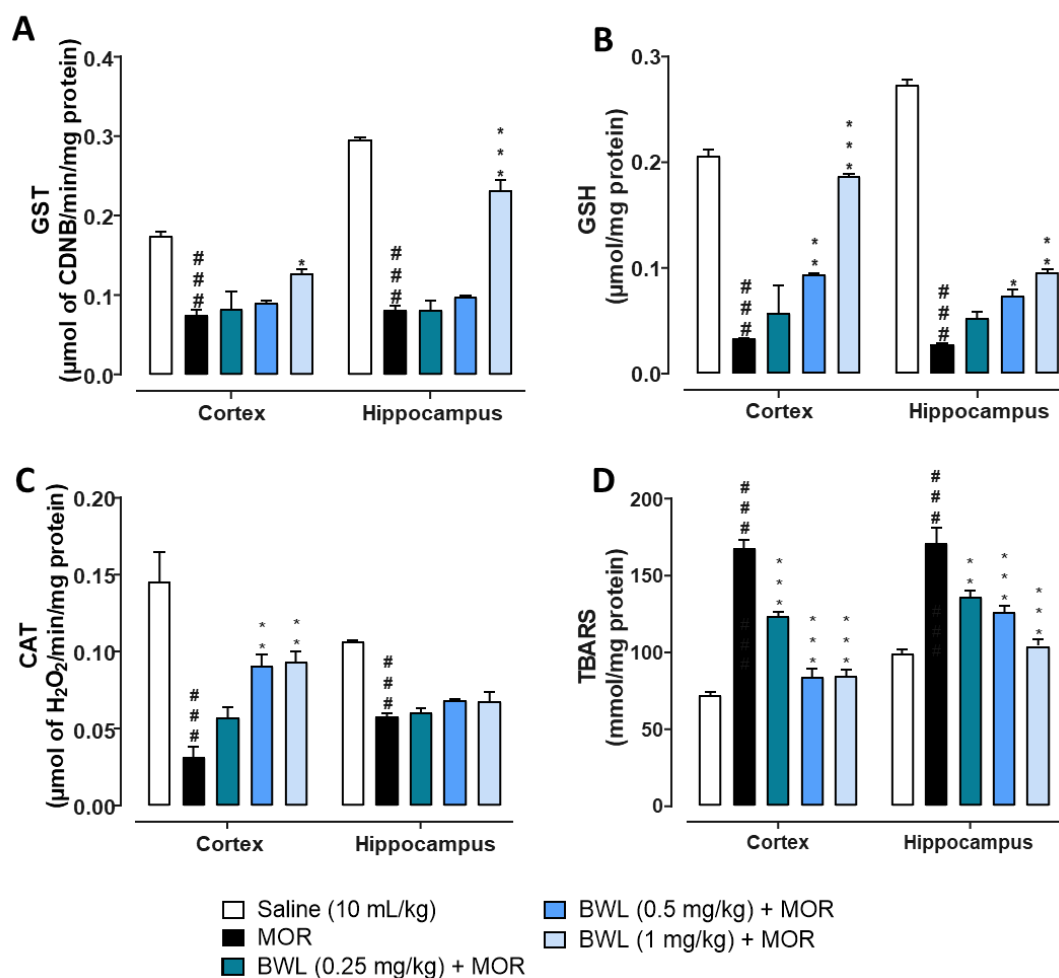


Figure 7. Effect of bhilawanol (BWL) against morphine (MOR)-induced oxidative stress. (A) GST, (B) GSH, (C) CAT, and (D) LPO in the cortex and hippocampus regions of mice brains. Values are presented as mean $\pm$ S.D. ($n=13$ /group), 3 replicates per sample. For statistical analysis, one-way ANOVA (post hoc Tukey's test) was applied. ### $p < 0.001$ denotes significance against the saline group, whereas * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ denote significance against the MOR group.

3.7 DISCUSSION

Opioid addiction is a chronic relapsing brain disorder characterized by dysregulation within the brain reward pathway and presents a significant obstacle to the treatment of drug addiction [21]. Approximately 50% of individuals with opioid use disorder (OUD) relapse within 24 weeks, despite the availability of various treatment options [22]. In the current work, we investigated the potential of bhilawanol (BWL), a naturally occurring compound, to attenuate morphine relapse, using conditioned place preference, extinction, and reinstatement behavioural tests. Our findings indicate that BWL contributes to these positive outcomes by modulating antioxidant defenses, inflammatory mediators, the nitric oxide (NO) pathway, and neurotrophic support.

Our results showed that BWL dose-dependently reduced the MOR CPP, extinction, and reinstatement. Our injection regimen of an escalating dose of MOR for 5 days showed an increase in preference towards the MOR-paired chamber in the post-conditioning phase, due to the positive reinforcement associated with MOR use, consistent with the positive reinforcing properties of morphine mediated through dopaminergic activation in the mesolimbic pathway [5]. During the extinction phase, the MOR-conditioned mice preferred the MOR-paired chamber, which may be due to the negative reinforcement to avoid withdrawal symptoms. Additionally, re-exposure to the priming dose of MOR after 5 days of abstinence showed an increase in time spent in the MOR-paired chamber, effectively modeling relapse, as previously described [8]. BWL pretreatment (1 mg/kg) significantly reduced all three phases of CPP, indicating that BWL disrupts both the formation and recall of morphine-associated memories. These findings correspond with recent studies emphasizing the capacity of natural compounds to influence addiction-related behaviors [16, 17].

Moreover, the literature studies show an increase in anxiety symptoms and behaviour during opioid abstinence [9]. Patients with opioid use disorder go through psychological and physical withdrawal symptoms when they stop using opioids. The high recurrence rates can be attributed to prolonged negative affect, which persists even after physical withdrawal symptoms diminish and includes anxiety and increased stress reactivity [22]. Based on our results, MOR abstinence facilitates an anxiety state in mice, which augments the expression of MOR CPP. We find that BWL significantly ameliorated anxiety-like behaviour during MOR abstinence in both OF and EPM behavioural tests. BWL administration at doses of 0.5 and 1 mg/kg demonstrated a dose-dependent reversal of these behavioral deficits, suggesting notable anxiolytic effects. Alleviating abstinence-induced anxiety is clinically significant, as it may diminish the motivation to relapse to escape this negative affective state.

Nitric oxide (NO) plays a major role in the progression and development of MOR tolerance, dependence, and relapse [23]. Based on our findings, BWL dose-dependently decreased the levels of NO in both the cortex and hippocampus regions of the mice brain in the MOR group. Oliva, I., et al., provided behavioural and neurochemical credence to the theory that morphine reward and relapse-like behaviour are reduced when PSD95-nNOS protein-protein interactions are inhibited [24]. Also, according to recent findings, glial activation triggers the release of pro-inflammatory cytokines like TNF- α and IL-1 β as well as iNOS throughout the inflammatory process [13]. Interestingly, BWL at the higher doses decreased the expression of both iNOS and nNOS in mice brain tissues.

Chronic opioid exposure dysregulates BDNF signaling in a region-specific manner, which contributes to the maladaptive neuroplasticity associated with addiction [14]. Decreased BDNF levels have been associated with depression and anxiety [15], suggesting an additional mechanism for the anxiety-like behaviors noted during abstinence. BWL treatment restored BDNF levels in a dose-dependent manner, exhibiting the most significant effect at 1 mg/kg. Our findings collectively indicate a multifaceted mechanism of action for BWL. BWL disrupts the cycle of oxidative damage, inflammation, and excitotoxicity associated with opioid-induced neurotoxicity by scavenging oxidative stress, suppressing NF- κ B-driven neuroinflammation, inhibiting pathological NO production through iNOS and nNOS, and restoring BDNF-mediated neurotrophic support. This integrated mechanism elucidates its effectiveness across various behavioral domains: diminishing drug reward (CPP), expediting extinction, inhibiting reinstatement, and mitigating abstinence-induced anxiety. The dose-dependent characteristics of these effects, particularly the consistent efficacy observed at 1 mg/kg, underscore the necessity for future studies focused on dose optimization.

3.8 Limitation:

The application of a pretreatment paradigm, although valuable for mechanistic investigations, fails to accurately reflect the clinical context in which treatment usually commences post-addiction establishment. The effects on NF- κ B, NOS enzymes, and BDNF have been demonstrated; however, it remains uncertain whether BWL directly interacts with these proteins or operates upstream through receptors or signaling kinases. Considering the sex differences in opioid addiction and anxiety, it is recommended that future research incorporate female subjects. Further investigation is necessary in other brain regions essential for addiction, including the nucleus accumbens, ventral tegmental area, and amygdala.

4 CONCLUSIONS

The outcomes of the current work may have several implications in exploring potential therapies to overcome negative effects like anxiety during morphine (MOR) abstinence. The involvement of the nitric oxide (NO) pathway in the progression of the disease merits additional targeted therapy for the treatment of psychological aspects during MOR abstinence and withdrawal.

The effect of natural compounds such as bhlawanol in ameliorating disease progression by its antioxidant, anti-inflammatory, and morphine-preserving morphological alteration provides a novel treatment strategy. Although further research work is needed to test bhlawanol in addiction models, which will help in clarifying its effectiveness and could be a possible candidate for clinical investigations in treating morphine withdrawal and relapse.

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Conflicts of Interest

The author declares no conflict of interest.

AUTHOR CONTRIBUTIONS

Aleena Muskan, Imran Malik, and Ismail Badshah: managed the experimental work and data curation.

Sagheer Ahmed: helped with the methodology and lab facilities.

Najeeb Ur Rehman, Mohd Faiyaz Khan, and Mohd Nazam Ansari: helped in the analysis and data interpretation.

Fawad Ali Shah: supervised, provided materials, antibodies, and kits.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

All experimental procedures received approval from the Institutional Animal Care and Use Committee (IACUC), called the Research Ethics Committee (REC) of the Riphah Institute of Pharmaceutical Sciences (Ref. No: REC/125/Pharmacology/2025), and were conducted in strict accordance with institutional guidelines for animal care, reaffirming our dedication to ethical research standards. All animal experiments performed in the present investigation adhered to the ARRIVE guidelines.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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