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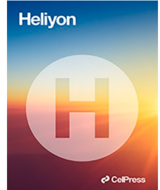

Research article

Carveol mitigates the development of the morphine anti-nociceptive tolerance, physical dependence, and conditioned place preference in mice

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ABSTRACT

Emergence of analgesic tolerance and dependence to morphine is frequently the limiting factor in the use of this agent in the management of pain. Hence, this study aimed to investigate the beneficial effects of the natural compound carveol (CV) against morphine antinociceptive tolerance, dependence and conditioned place preference (CPP) in mice. Behavioural paradigms included hot plate and tail-flick (for tolerance), observation of withdrawal signs (for dependence) while biochemical tests involved the assays for mRNA expression, nitrite levels, antioxidants, and immunohistochemistry studies. Behavioural tests indicated that treatment with CV significantly attenuated the morphine analgesic tolerance, physical dependence and CPP in mice. It was observed during biochemical analysis that CV-treated animals exhibited reduced mRNA expression of inducible nitric oxide synthase (iNOS) and NR2B (an NMDA subtype). In addition, decreased levels of nitrite were observed in mouse hippocampus following CV treatment than morphine administration only. Further, CV enhanced the neuronal innate antioxidants including Glutathione-S-Transferase (GST), glutathione (GSH) and catalase (CAT), while curtailed lipid peroxidase (LPO) levels in mice brain tissues. Moreover, CV exerted significant anti-inflammatory effects as evidenced by reduced expression of TNF-α and p–NF–κB in these animals than with morphine treatment only. Together, anti-inflammatory and antioxidant effects might confer needed neuro-protection following morphine administration. These observations warrant further investigations of the beneficial role of CV as a novel agent in overcoming the development of tolerance and physical dependence following morphine use.

1. Introduction

Pain, whether acute or chronic is an unpleasant sensory and emotional experience, frequently a debilitating disorder associated with disability and adversely affecting the quality of life [1]. The prevalence of pain varies considerably among various age groups.

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Recent estimates indicate that 1 in every 9 young adults might experience chronic pain, worldwide [2]. It is concerning to note that 50.2 million adults in the US (20.5%) reported experiencing pain most of the time or every day [1]. Opioid analgesics are some of the most commonly prescribed agents and 289 million prescriptions for opioid pain relievers were dispensed in US in 2012 [3]. Among them, morphine, for its rapid onset and prompt relief, is used for the treatment of pain associated with various diseases, such as cancer but its chronic use might lead to the development of tolerance [4,5]. Tolerance refers to a state where elevated doses of drug are required to maintain the desired analgesic effect [6]. Higher doses of morphine may lead to respiratory depression, withdrawal symptoms upon cessation and rewarding effects contributing to relapse of drug use [7].

Recreational use of abusive drugs develops compulsive behaviour towards its use, despite knowing its negative consequences [8,9]. The end result of addiction comprises of series of behavioural manifestations depending on that particular drug of abuse [10]. Long-term administration of morphine is associated with physical and psychological dependence [11]. Physical dependence involves formation of neuroadaptive alterations both at molecular and cellular level, reflecting the withdrawal signs after drug cessation [11]. Symptoms following the cessation of morphine use include anxiety, sneezing, anorexia, abdominal pain, diarrhoea among other effects in humans [11,12], while in animal models withdrawal effects include jumping, diarrhoea, wet dog shakes, paw tremors and teeth chattering [11]. Psychological dependence is compulsive drug use in order to improve the perception of well-being [11]. In humans, intensified drug-seeking behaviour, compromised decision making, drug use despite knowing its harmful effects, persistent and recurrent obsession after years of abstinence are included in psychological manifestations [11].

Another behavioural consequence of addiction is drug craving, which can be studied by assessing drug-seeking behaviours [10]. Drug of abuse leads to alterations in the brain reward circuits, resulting in craving, compulsive behaviour and relapse to substance use [13]. Relapse is major obstacle in the treatment of drug addiction and is triggered by re-exposure to the particular drug, drug associated cues and contexts, the positive and negative emotions, people or stress [14–18]. The ability of the particular drug to induce euphoria and reward is studied using self-administration and conditioned place-preference paradigms. In animals, conditioned place preference is based on their ability to pair rewarding drug with environmental cues in a two-chamber apparatus [10]. It is a well-established model to study the role of context associations in reward-related behaviours, including both natural rewards and drugs of abuse [19].

The intricate interplay between pain management and the potential risks associated with opioid analgesics necessitate exploration of alternative approaches. Despite the devastating impact of morphine tolerance and dependence, the current available pharmacological and psychosocial treatments remain inadequately effective for most people [8]. Natural moieties are an attractive source of new drugs, owing to their rich antioxidant potential [20]. Serafim et al. have reported that a natural monoterpene Carveol (CV) might exert cytoprotective, antioxidant and immunoregulatory effects in animal models of gastric ulcer [21]. Anti-oxidant, anti-inflammatory and

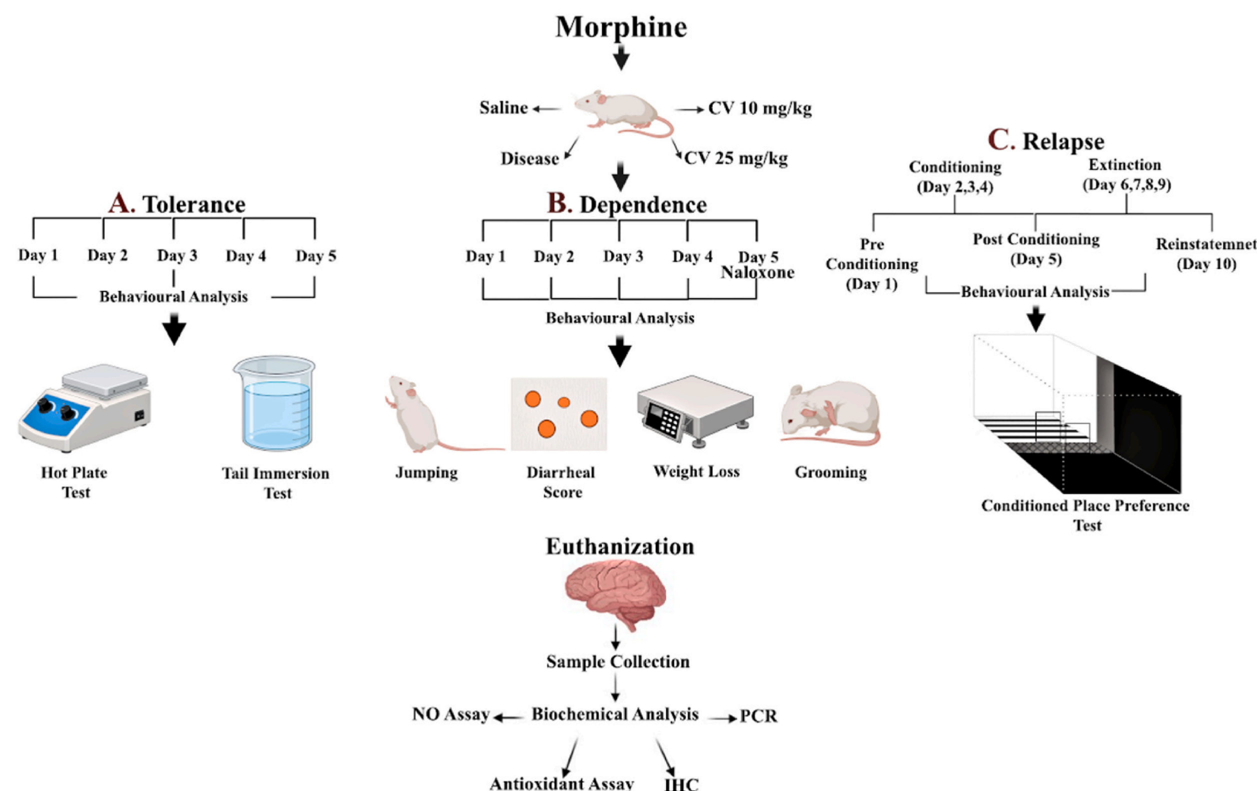

Fig. 1. Summary of the protocol used during the study.

neuroprotective effects of CV in PTZ-induced epileptic animals has also been reported [22]. Silva et al. have demonstrated that CV might exert relaxant effects on isolated human umbilical cord arteries [23]. Beneficial effects of CV against the memory impairment and oxidative stress induced by β-Amyloid-Peptide has also been reported [24]. Similar effects of CV on scopolamine induced memory impairments in rats have also been demonstrated by Latif et al. [25]. Alleviative effects of CV on the progression osteoarthritis have also been recently reported [26]. Interestingly, CV has also been shown to exhibit antibacterial effects against *Escherichia coli* and *Staphylococcus aureus* [27]. Devising successful interventions requires an understanding of the molecular mechanisms causing morphine tolerance, dependence and relapse. Hence, in present work, CV was studied for its effectiveness in reducing morphine tolerance and dependence using behavioural, *in-vitro* and molecular studies. Furthermore, the involvement of oxidative stress, NMDA and nitric oxide dependant mechanisms was also explored.

2. Materials and methods

2.1. Animals

Male albino mice (20–25g) used in this study were kept under standard laboratory conditions at 25 °C with 12 hourly alternating light and dark cycle with water and food provided *ad libitum*. All the experimental protocols were approved by Research Ethics Committee of Riphah Institute of Pharmaceutical Sciences (Ref. No. REC/RIPS/2023/17) and were in accordance with the guidelines set by “Principles of Laboratory Animal care”. At the end of the experimentation the animals were anesthetized using intraperitoneal injection of xylazine (9 mg/kg) and ketamine (90 mg/kg). Mice were then decapitated following AVMA guidelines. A summary of the protocol employed has been shown in Fig. 1.

2.2. Drugs and reagents

CV (#192384, PubChem ID: 24851543) was purchased from Sigma-Aldrich (USA). Other solvents and reagents such as Griess reagent, 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB, #D8130, PubChem ID: 24894189), trichloroacetic acid (TCA, #T6399, PubChem ID: 24900373), and N-(1-naphthyl) ethylenediamine dihydrochloride (#222488, PubChem ID: 24853334) were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.3. Induction of morphine tolerance

To induce the morphine tolerance to antinociceptive effect, morphine was injected at the dose of 50, 50 and 75 mg/kg at 9:00 a.m., 12:00 p.m. and 17:00 p.m., respectively. The dosing was performed for 4 consecutive days. On the last day (5th day) morphine at a dose of 50 mg/kg was injected. Behavioural tests, namely hot plate and tail immersion test were performed on each day, 60 min after 1st dose of morphine [28]. Hot plate test was performed by placing mice on a preheated hot surface having temperature of 50 ± 2 °C. The hot plate was covered with a transparent glass cylinder. After placing the mice on the hot plate, the latency time in seconds was recorded from zero till the mice jumped or licked its hind paws to avoid pain. To avoid any damage to the mice paw tissues, cut off time was set at 90 s [28]. Tail immersion test was performed by immerging mice tail in preheated water bath to 52 °C. The latency time was measured and to avoid any damage to the mice tail, the cut of time was set at 20 s [29].

2.4. Induction of morphine dependence and withdrawal

Morphine dependence was induced by injecting morphine at 8:00 a.m., 11:00 a.m., and 4:00 p.m. at doses of 50, 50 and 75 mg/kg, respectively for 4 consecutive days. On the 5th day, 100 mg/kg of morphine was injected and intraperitoneal injection of naloxone 4 mg/kg was injected 1 h later. Withdrawal signs (jumping, diarrhoea, weight loss and grooming) were observed by placing the animals in a separate plexiglass cylinder (40 cm × 25 cm × 45 cm dimension) for 1h. Weight loss was also determined before and after naloxone injection [4].

2.5. Induction of conditioned place preference (CPP)

The CPP apparatus consisted of two chambers, one with a mesh sheet floor coloured in red and the other had dotted floor and black walls [30]. The CPP tests was divided as follows; Preconditioning: On the first day, the preconditioning phase, the animals were kept in the middle chamber of the box and allowed freely to move in the whole chamber. Time spent in each chamber was recorded for 15 min. Conditioning: The conditioning was performed on 2nd, 3rd and 4th day for 30 min. Animals were conditioned to a single chamber and were not allowed to move to the other chambers. Animals were injected with morphine 60 mg/kg or saline 10 mg/kg. Post-conditioning: On the 5th day, the animals from the conditioning phase were allowed freely to move in all three chambers and test was recorded for 15 min. No drug was injected on this day. Extinction: The extinction was performed on 6th, 7th, 8th and 9th day. Animals received CV 30 min prior to extinction test on each day. Animals were allowed to freely move in all three chamber and test was recorded for 15 min. Reinstatement: On the last day (10th day), extinction of morphine-induced CPP was reinstated with a priming dose of morphine 1 mg/kg. Animals received saline or CV 30 min prior to each dose of morphine and immediately tested for reinstatement of CPP. Animals were allowed to move freely in all three chambers and the time spent in each chamber was recorded for 15 min.

2.6. Antioxidant activity

Assays of Glutathione (GSH) activity, Glutathione-S-Transferase (GST) activity, catalase and lipid peroxidise were performed in order to investigate the neuroprotective role of CV against morphine induced oxidative stress. GSH assay was performed by adding 0.2 mL of supernatant (brain tissue) in 0.2 mL of DNTB mixture. Final volume of 3 mL was made with 0.2 M phosphate buffer. Using spectrophotometer, absorbance was measured at 412 nm and the results were expressed as μmol/mg of protein. For evaluating GST activity, 1 mM CDNB and 5 mM GSH was added to 0.1 M phosphate buffer. About, 60 μL of tissue homogenate was added to the mixture. Absorbance was measured at 340 nm using ELISA plate reader (BioTek ELx808, Winooski, VT) by using 210 μL aliquots from the mixture. The GST activity was expressed as μmol of CDNB conjugate/min/mg of protein. Catalase activity was measured by adding 0.1 mL tissue homogenate to 0.1 mL of 1 M pyrogallol solution and 2.8 mL of potassium phosphate buffer (pH 7.4). Absorbance was measured at 312 nm. The catalase activity was expressed as U/mg of proteins. The extent of LPO was determined by detecting thiobarbituric acid reactive substances (TBARS). We added 200 μL of supernatant into 580 μL of 0.1 M phosphate buffer with pH 7.4, 200 μL of 100 mM ascorbic acid and 20 μL of ferric chloride. The assay mixture was incubated at 37 °C for 60 min in a water bath. The reaction was then stopped by adding 10 % trichloroacetic acid (TCA) and 1000 μL of thiobarbituric acid (0.66% TBA). The mixture was subjected to water bath for about 20 min in tubes. The tubes containing the mixture were cooled in an ice bath and centrifuged at 3000×*g* for 10 min afterwards. The absorbance was measured at 535 nm for both the supernatant and blank. There results were expressed as TBARS-nmol/mg protein [31].

2.7. Quantification of nitrite in hippocampus

In order to evaluate the levels of nitric oxide, nitrite concentration was measured using Griess reaction quantification method in the hippocampal part of the mice brain. Animals were decapitated and hippocampi were isolated from brain and kept on ice cold surface. Tissues were homogenized and placed at room temperature for 10 min and centrifuged for 15 min at 13,000 RCF. After that the supernatant was separated and analyzed for nitrite concentration [4].

2.8. NR2B and NOS gene expression analyses by quantitative real time reverse transcription-PCR (q RT-PCR)

From brain tissues, total RNA was extracted using TRIzol. NanoDrop (Skanit RE 4.1, Thermo Scientific) was applied to evaluate RNA quality and quantity. Conversion of RNA to cDNA was done with Viva cDNA synthesis kit (Vivantis cDSK01-050). Galaxy XP Thermal Cycler (BIOER, PRC) and 2X Amplifyme Universal qPCR mix (Blirt, Germany) were used for performing PCR [32]. Following sequences for the forward and reverse primers were used; NR2B gene: (Forward): 5′-GCCATGAACGAGACTGACCC-3′, (Reverse): 5′-GCTTCCTGGTCCGTGTCATC-3'; iNOS gene: (Forward): CAGAAGCAGAATGTGACCATC, (Reverse): CTTCTGGTCGATGTCATGA.

2.9. Histological preparation

For molecular studies, extracted brains were preserved in 4% paraformaldehyde solution, washed and cut (thickness of 3 mm coronal sections) with a sharp blade and fixed in paraffin blocks. After, they were sliced (4 μm thin coronal sections) using a microtome.

2.10. Immunohistochemistry analysis

Immuno-staining as performed by hydrating tissues with xylene, serial dilutions of graded alcohol and with distilled water. Tissues were then washed using PBS for about 5 min. In the next step, antigen recovery was performed using Proteinase K. Tissues were again washed with 3% H_2O_2 solution for 5 min in order to avoid endogenous peroxidase activity. Tissues were left for an hour at room temperature and then anti-mouse p–NF–κB antibody and anti-mouse TNF-α antibody (dilution 1: 100, Santa Cruz Biotechnology, Dallas, TX, USA) were applied and left overnight at 4 °C. Slides were treated with secondary antibody and left for 2 h. Slides were again washed with PBS and then treated with ABC staining kit and left for an hour at room temperature. Further, slides were stained using DAB solution and left for about 5 min and washed with distilled water. In the final step, slides were dipped in xylene and 100% ethanol and covered using mounting media. Slides were left to air dry for a day. Using Olympus microscope at 10x and 40x magnification, images were obtained and evaluated using ImageJ software [33].

2.11. Statistical analysis

Graph Pad prism-8 software was used for performing Statistical analysis. One-way and two-way ANOVA (*post-hoc* Tukey's test) were applied for analyzing data. Statistical significance was set at $P < 0.05$. Symbol * represents $P < 0.05$; ** represents $P < 0.01$, whereas, *** or ### represents $P < 0.001$. Data was expressed as mean ± standard deviation (SD).

3. Results

3.1. Carveol delays the development of morphine antinociceptive tolerance

Animals treated with morphine developed tolerance to its antinociceptive effect in both hot plate and tail immersion tests exhibited by progressively decreased latency time (Fig. 2A and B). In contrast, CV prolonged the development of tolerance at doses of 10 mg/kg ($P < 0.01$) and 25 mg/kg ($P < 0.001$) in hot plate test (Fig. 2A). Similar prolongation was observed in tail immersion test at CV doses of 10 mg/kg ($P < 0.05$) and 25 mg/kg ($P < 0.01$) (Fig. 2B).

3.2. Carveol ameliorates morphine physical dependence following withdrawal

Administration of morphine followed by naloxone was associated with significantly increased jumps and diarrhoea score with reduced grooming behaviour in addition to weight loss compared to control animals (Fig. 3A–D). Interestingly, CV treatment resulted in significant amelioration of these withdrawal symptoms at doses of 10 mg/kg (jumping: $P < 0.05$, grooming: $P < 0.05$, diarrhoea: $P < 0.01$ and weight loss: $P < 0.01$) and 25 mg/kg (jumping: $P < 0.001$, grooming: $P < 0.01$, diarrhoea: $P < 0.001$ and weight loss: $P < 0.001$) as compared to animals given morphine only (Fig. 3A–D).

3.3. Carveol attenuates the acquisition while accelerates the extinction of morphine conditional place preference

No significant difference was observed between the groups in the pre-conditioning phase (Fig. 4A). In the post conditioning, animals spent significantly greater time in the morphine paired side than control group ($P < 0.001$). However, this time was significantly reduced after the administration of CV in animals injected with morphine (CV 10 mg/kg: $P < 0.01$ and CV 25 mg/kg: $P < 0.001$) (Fig. 4A). During the extinction phase, CV treated groups exhibited rapid reduction in conditional place preference (CV 10 mg/kg: $P < 0.01$ and CV 25 mg/kg: $P < 0.001$) demonstrated by time spent in morphine paired site than animals' injected with morphine only on 6th, 7th, 8th and 9th days, respectively (Fig. 4B). As for the reinstatement, CV caused statistically significant reductions (CV 10 mg/kg: $P < 0.01$ and CV 25 mg/kg: $P < 0.001$) in the CPP as compared with mice treated with morphine only and thus reduced the reinstatement to morphine (Fig. 4C).

3.4. Carveol exhibits significant antioxidant effects in morphine treated mice

Morphine induced oxidative stress, and the neuroprotective effect of CV was measured by performing antioxidant activities of enzymatic and non-enzymatic antioxidants in hippocampus including GST, GSH, CAT and LPO. Mice treated with morphine showed significantly reduced activities of GST, GSH and CAT (Fig. 5A, B, 5C) than control animals ($P < 0.001$). In contrast, CV (25 mg/kg) restored the levels of GST, GSH and CAT (Fig. 5A, B, 5C) ($P < 0.001$). LPO activity was markedly elevated in hippocampi of morphine treated groups as compared to the saline treated groups (Fig. 5D) ($P < 0.001$). However, CV (25 mg/kg) treated animals exhibited reduced LPO in hippocampus as compared to group treated with morphine only ($P < 0.001$).

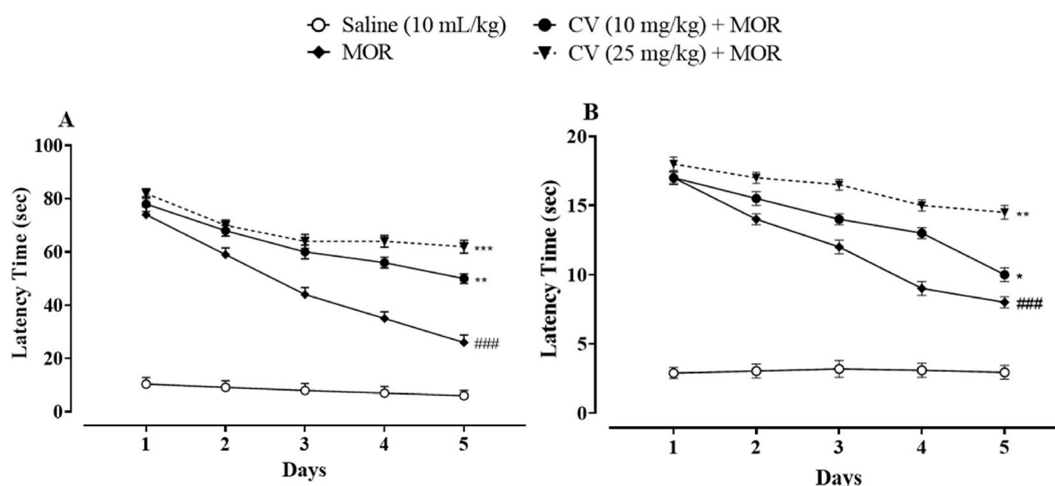

Fig. 2. The effect of Carveol (CV) on morphine induced antinociceptive tolerance. The animals received morphine (50, 50 and 75 mg/kg/day for 5 consecutive days) for induction of morphine tolerance and 30 min prior to each dose of morphine received **(A)** Hot plate test and **(B)** Tail immersion test were carried out on the 1st,2nd, 3rd, 4th and 5th day. Each group comprised 6–7 mice. Two-way ANOVA with *post-hoc* Tukey's test. ###$P < 0.001$ *vs* saline group and *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* morphine group on the last day.

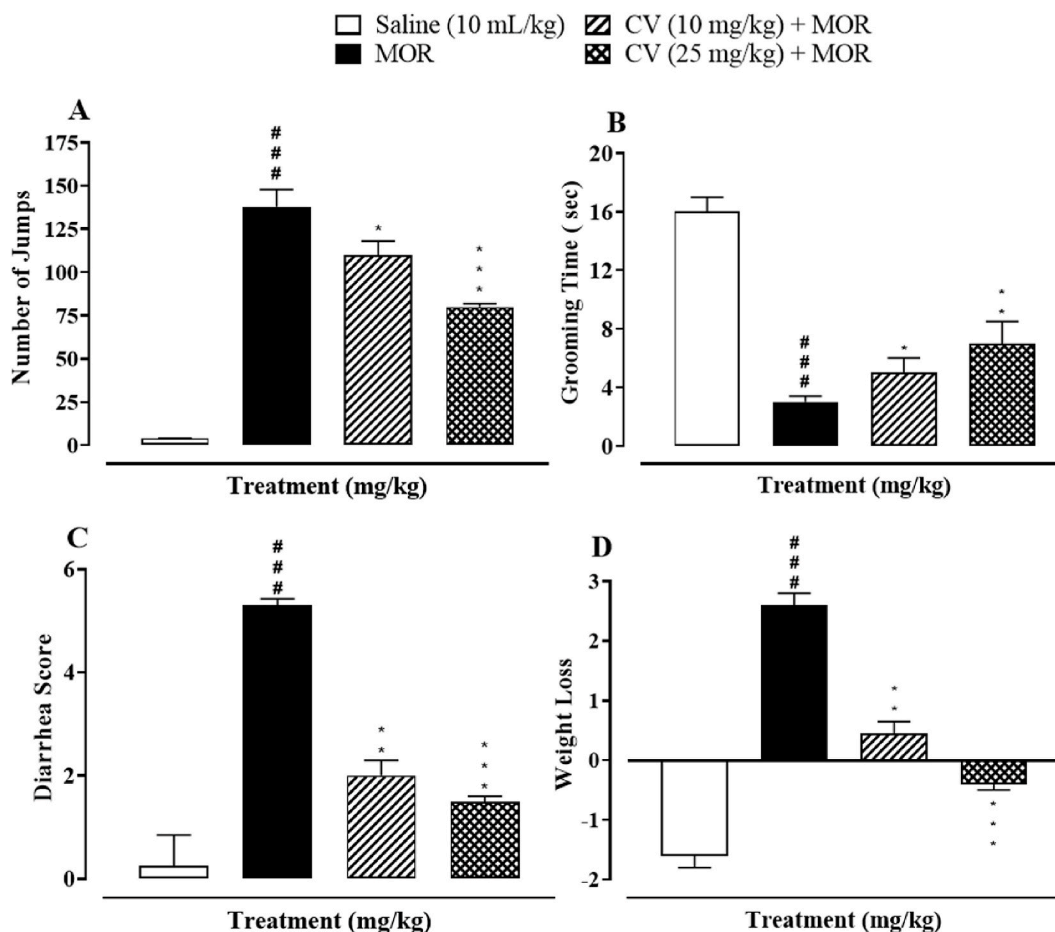

Fig. 3. Effect of Carveol (CV) on morphine (MOR) induced dependence and withdrawal. Carveol was injected 45 min prior to each dose of morphine for 5 consecutive days. Data is expressed as mean ± S.E.M for withdrawal signs of 6–7 mice. **A**: Number of jumping after naloxone injection. **B**: Number of grooming. **C**: Diarrheal score and **D**: Weight loss after naloxone injection. Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. ###P < 0.001 *vs* saline group and *P < 0.05, **P < 0.01, ***P < 0.001 *vs* morphine group.

3.5. Carveol exhibits significant anti-inflammatory effects in morphine treated mice

We recall that administration of morphine is associated with the activation of inflammatory cascade. IHC results indicated hyper expression of TNF-α and downstream target p–NF–κB in morphine treated animals in cortex and hippocampi (CA1, CA2 and DG regions; Fig. 6 and Fig. 7). Statistically reduced expressions of TNF-α and p–NF–κB were observed after pre-treatment with CV 25 mg/kg in both cortex (P < 0.01) and hippocampus (CA1: P < 0.01, CA2: P < 0.01, and DG: P < 0.001) than in the morphine only treated group (Figs. 6 and 7).

3.6. Hippocampal mRNA expression of NR2B and iNOS are curtailed by carveol after morphine treatment

Further, the involvement of iNOS (Fig. 8A) and NR2B (Fig. 8B) was estimated through RT-PCR. Overexpression of iNOS and NR2B was observed in morphine treated group (Fig. 8A and B) while the respective expressions of iNOS (Fig. 8A) and NR2B (Fig. 8B) were significantly curtailed following treatment with CV (CV 10 mg/kg: P < 0.01 and CV 25 mg/kg: P < 0.001).

3.7. Carveol curtails hippocampal expression of nitrite in in morphine treated mice

Assay for the hippocampal expression of nitrite was conducted in order to estimate the nitric oxide production. Levels of nitrite in hippocampus was significantly elevated in morphine treated mice (P < 0.001 *vs* saline), while, nitrite levels were curtailed in the CV treated (P < 0.05 *vs* MOR) animals (Table 1).

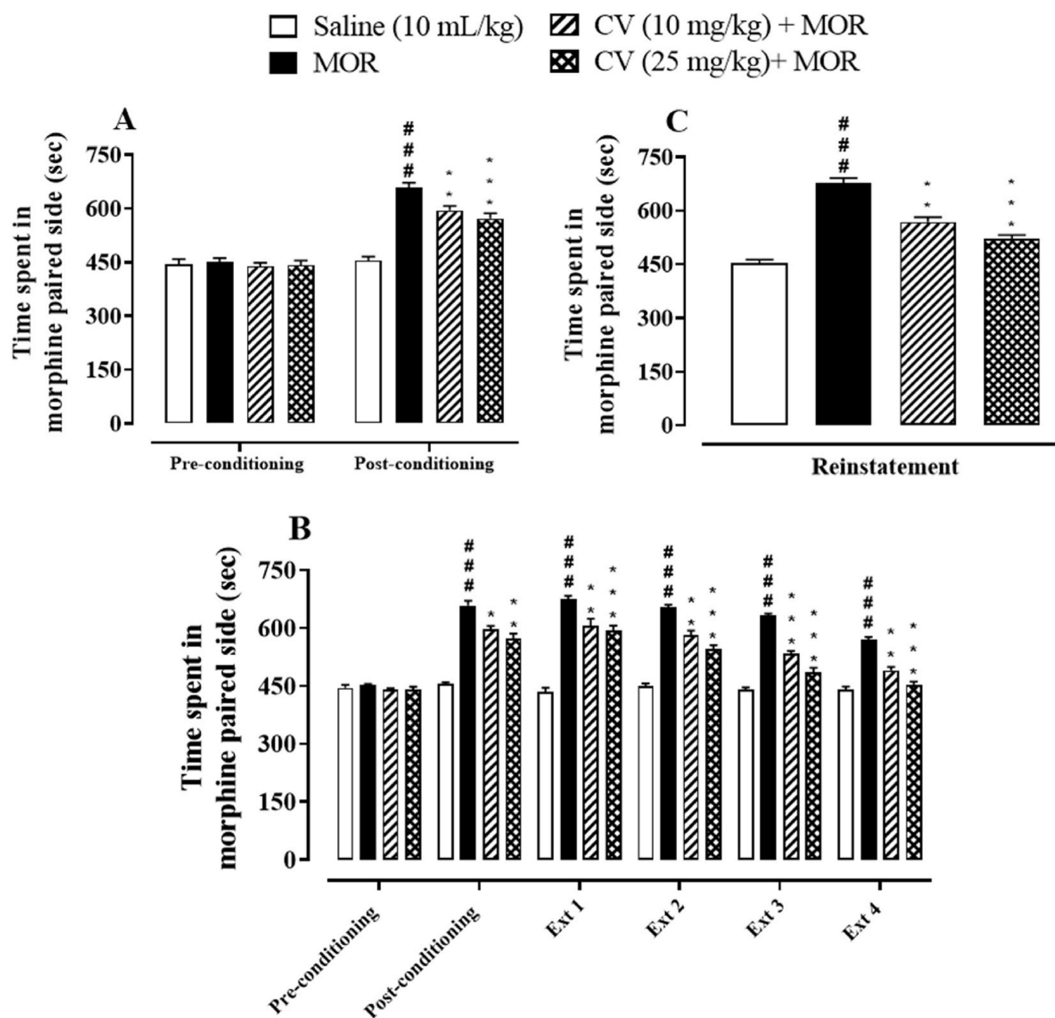

Fig. 4. Effects of carveol (CV) on the **(A)** acquisition, **(B)** extinction and **(C)** drug-priming reinstatement of morphine-induced CPP. Mice displayed an apparent CPP after morphine (MOR) treatment. **(A)** CV pre-treatment significantly attenuated the acquisition of MOR-induced CPP. Values are expressed as means ± S.E.M (n = 6) of time spent in the morphine paired chamber. Statistical analysis was performed with two-way repeated measures ANOVA and post-hoc Tukey's test. **(B)** CV significantly accelerated the extinction of MOR-induced CPP. Values are means ± S.E.M. of time spent in the morphine-paired chamber. Two-way repeated measures ANOVA followed by a post-hoc Tukey's test was applied to determine if there were any significant differences between the groups on each day in the extinction (Ext day 1–4) phase. **(C)** One day after the last extinction trial, mice that received CV or saline 30 min before a priming injection of MOR, were immediately tested for reinstatement of CPP. Values are expressed as means ± S.E.M (n = 6) of time spent in the morphine-paired chamber. Statistical analysis was performed with one-way ANOVA and post-hoc Tukey's test. ###P < 0.001 *vs* saline group and *P < 0.05, **P < 0.01, ***P < 0.001 *vs* MOR group.

4. Discussion

Repeated administration of morphine is frequently associated with the emergence of morphine tolerance and dependence [7]. During the present study, CV administration significantly prolonged the development of antinociceptive tolerance and dependence in mice. This effect involved the inhibitory action of this agent on nitric oxide as well as NMDA dependant pathways in addition to the amelioration of oxidative and inflammatory stress secondary to the administration of morphine.

Involvement of nitric oxide in the development of morphine tolerance and dependence has been reported in various studies. Motahari et al. argued that the role of nitric oxide in morphine addiction may involve changes in the pattern of dopamine release in different reward-related brain regions [34]. According to Majeed et al., two inhibitors of nitric oxide synthase L-NG-nitroarginine methyl ester and NG-nitro-L-arginine inhibited the development of tolerance while prolonging the analgesic effects of morphine in albino Swiss mice [35]. Studies by Ozdemir et al. have demonstrated that the nitric oxide-cGMP signalling pathway is crucial for the development of tolerance to morphine's analgesic effects [36]. In another study by Abdel-Zaher et al., *Nigella sativa* oil prevented mice from developing excess nitric oxide, which improved morphine-induced tolerance and dependency [37]. In the current study, CV delayed the development of anti-nociceptive tolerance in mice during 5 days of treatment. This effect involved, in-part, the nitric oxide

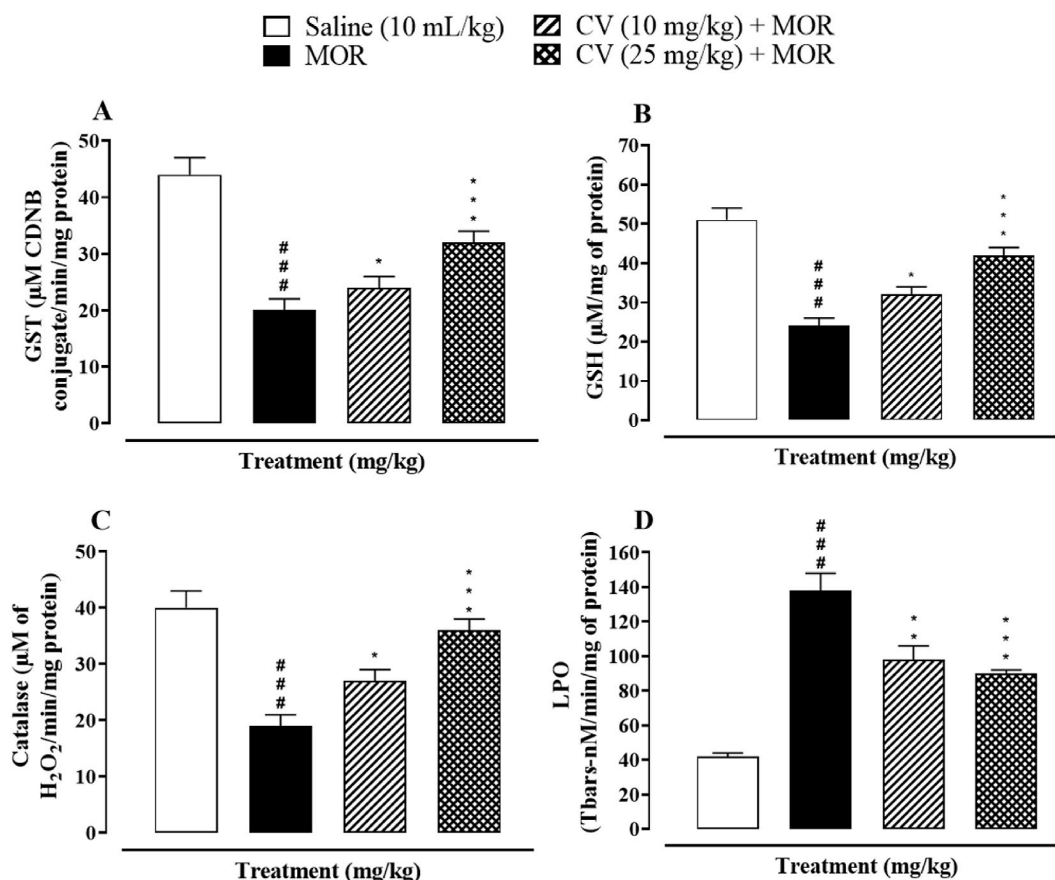

Fig. 5. Effect of carveol against **(A)** Glutathione-S-Transferase (GST), **(B)** Reduced Glutathione (GSH), **(C)** Catalase and **(D)** Lipid Peroxidation (LPO) in brain tissues of morphine treated mice. Values are expressed as mean ± SEM (n = 6). One-way ANOVA with post-hoc Tukey's test. ###P < 0.001 *vs* saline group and *P < 0.05, **P < 0.01, ***P < 0.001 *vs* morphine group.

dependant pathway as the administration of this agent significantly reduced mRNA expression of iNOS. This was also associated with significantly reduced nitrite levels in these animals in hippocampus. The accumulation of nitrite in supernatant has been related with the production of nitric oxide that can be measured indirectly through the use of Griess reagent, as performed in the current study [38].

Another aspect of our study involved the investigation of the effect of CV on the acquisition and extinction of CPP. Interestingly, CV treatment attenuated the acquisition while accelerated the extinction of morphine CPP in mice. In addition, CV blunted the reinstatement following a morphine priming dose. Karami et al. have previously demonstrated that NO in the CA1 hippocampal area in rats might be involved in the morphine CPP [39]. Gholami et al. have shown that the nitric oxide in the nucleus accumbens might mediate the morphine CPP in rats [40]. These findings are further corroborated by Peng et al. who have demonstrated that insular nitric oxide signalling pathway was involved in the expression of morphine CPP [41]. In our study, reduced mRNA expression of iNOS as well as nitrite in hippocampus was observed which is in agreement with the previous reports.

NMDA receptor has been demonstrated as an interesting target in overcoming the morphine associated anti-nociceptive tolerance. Trujillo et al. have reported that MK-801, an NMDA antagonist inhibited the development of tolerance without affecting the acute analgesia caused by this agent [42]. Previous studies have suggested that inhibition of NMDA subtype NR2B could be a target in overcoming analgesic tolerance associated with morphine use [43]. Jin et al. have demonstrated that NR2B but not NR2A receptors submit of spinal cord were augmented in acute opioid tolerant rodents [44]. In the current study, CV treatment resulted in the reduced expression of NR2B in morphine treated animals. This observation suggests that the beneficial effects of CV might also involve the modulation of NMDA dependent mechanisms.

Based on available data, morphine appears to enhance the production of free radicals, such as reactive oxygen species (ROS), and reduce antioxidant activity within target cells [7]. One of the main contributors to many biological processes, including inflammation, is oxidative stress [45]. In current the study, morphine increased the oxidative stress markers in the hippocampus of mice brain. However, after CV administration, the mice' GST, GSH, and CAT levels returned to levels that were similar to those of the normal control group. This was further augmented by the decrease in the LPO activity levels in these mice. When using morphine excessively or for extended periods of time, CV's strong antioxidant properties may protect against oxidative damage linked to morphine consumption. In addition to antioxidant effect, CV also exerted significant anti-inflammatory effects in mouse brain, as evidenced by the

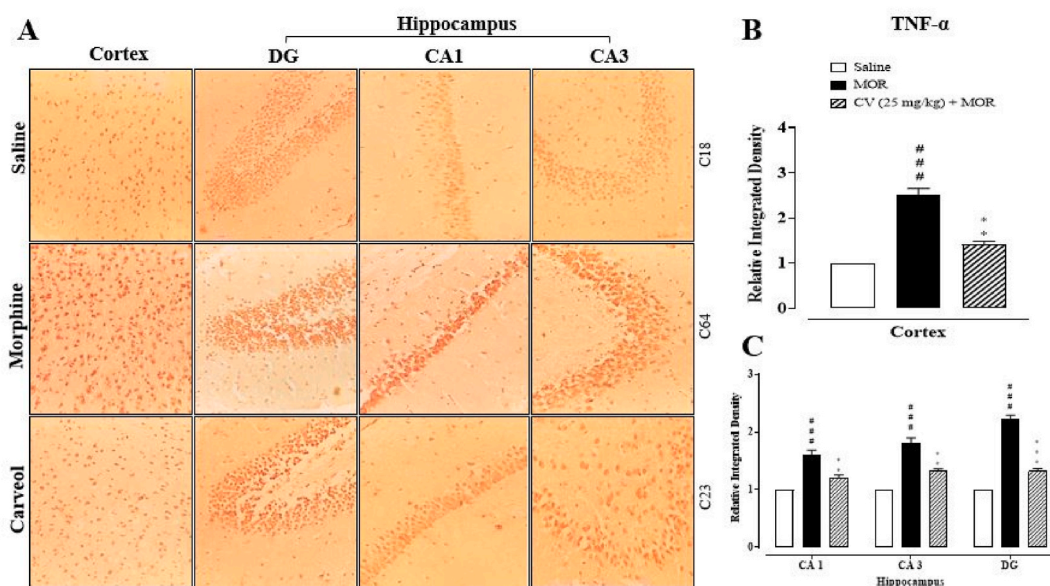

Fig. 6. Effect of Carveol (CV) on outcomes of morphine-induced inflammatory mediators. Immunohistochemistry results for TNF-α. Immunohistochemistry results for TNF-α in the cortex and hippocampal tissues of the brain. Data were expressed as the mean ± SEM (n = 6/group). One-way ANOVA with post-hoc Tukey's test. Where, ###P < 0.001 *vs* saline, while **P < 0.01 or ***P < 0.05 *vs* Morphine (MOR).

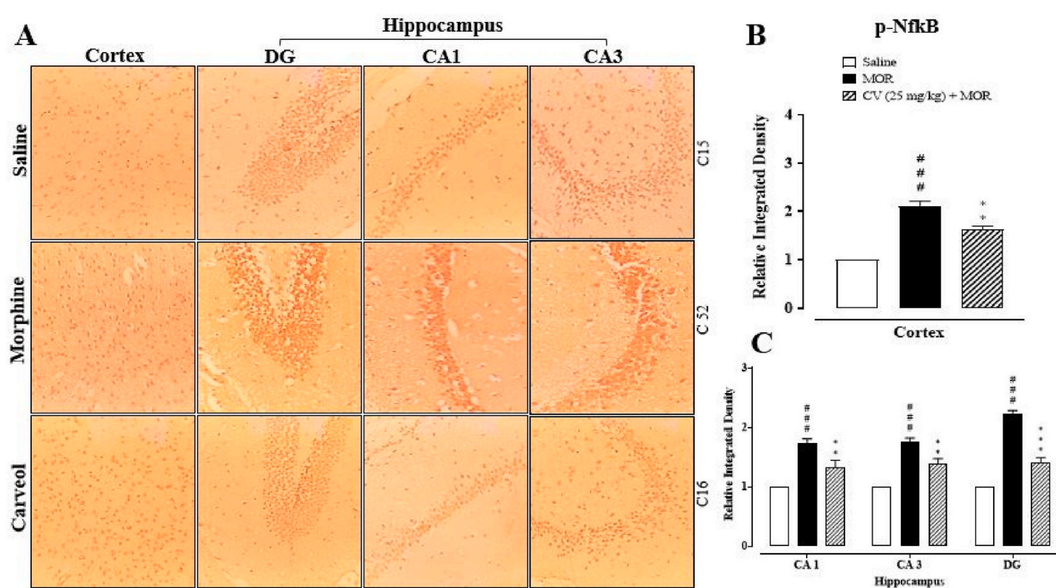

Fig. 7. Effect of carveol (CV) on outcomes of morphine-induced inflammatory mediators. Immunohistochemistry results for p-Nf-κB in the cortex and hippocampal tissues of the brain. One-way ANOVA with post-hoc Tukey's test. Data were expressed as the mean ± SEM (n = 6/group). Where, ###P < 0.001 *vs* Saline, while **P < 0.01 or ***P < 0.05 *vs* Morphine (MOR).

reduced expressions of TNF-α and p–NF–κB which are well known inflammatory mediators, when compared with treatment with morphine only in dentate gyrus (DG) region of hippocampus [46]. This implies that CV administration might also result in significant neuroprotective effects that along with anti-oxidant effects might be crucial in overcoming the morphine associated neurotoxicity [47, 48]. DG has been shown to be crucial for cognition and susceptible to opiate-induced alterations [49]. Indeed, DG is widely known for its role in hippocampal memory formation [50]. DG is critical for several processes in rodents, including memory, forgetting, mood regulation, stress coping, attention, and others that affect and support cognition generally and are necessary for successful drug-free abstinence in the future. It is noteworthy that DG neurogenesis and various DG activities are causally related [49].

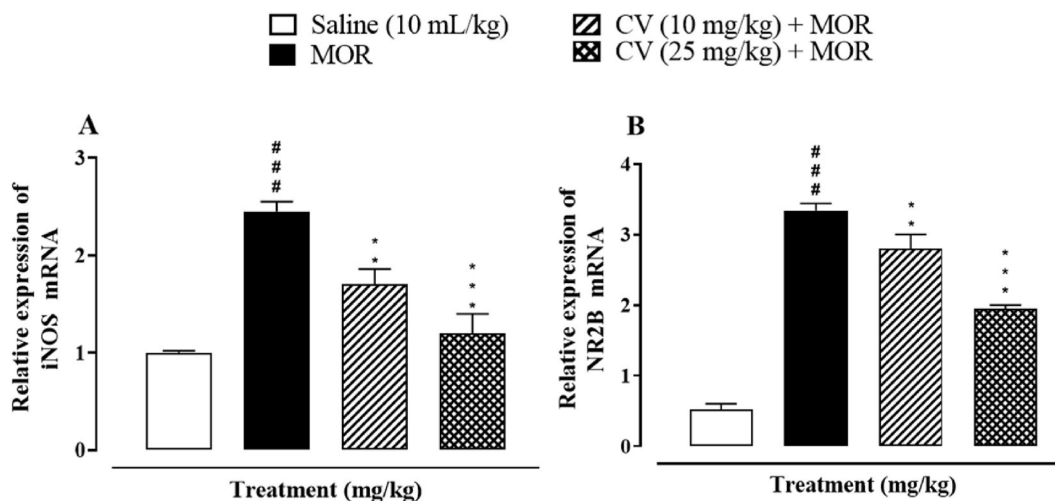

Fig. 8. Inhibitory effect of carveol against mRNA of **(A)** iNOS and **(B)** NR2B expression in brain tissues of morphine treated mice, using Real Time-Polymerase Chain Reaction (RT-PCR) technique. Values were expressed as mean ± SEM (n = 3). One-way ANOVA with post-hoc Tukey's test. ###P < 0.001 vs. saline group, **P < 0.01, ***P < 0.001 vs. morphine group.

Table 1
Effect of carveol on nitrite expression in hippocampi of treated animals.

Groups	Nitrite (μmol/mg of Protein)
Saline (10 mL/kg)	46.35 ± 1.78
Morphine	124.43 ± 2.1###
CV (25 mg/kg) + Morphine	101.52 ± 1.43*

The data were expressed as the mean ± SEM, (n = 3). One way ANOVA followed by *post-hoc* Tukey test. ###$P < 0.001$ vs. Saline *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. Morphine.

5. Conclusion

Taken together, our study suggests that CV attenuates the morphine associated anti-nociceptive tolerance, physical dependence and CPP through the modulation of Nitric oxide and NMDA dependent pathways. In addition, this agent exerts significant anti-inflammatory and anti-oxidant effects that may be crucial in conferring neuro-protection against morphine use. In the realm of pain management, where finding effective and sustainable solutions is paramount, the exploration of natural compounds like CV opens up exciting possibilities.

6. Limitations

Several limitations have emerged during the current study. The report focused on mice, and the results may not be directly applicable to human beings due to differences in physiology, metabolism, and drug response among several other reasons. Mice models may not capture the complexities of human pain pathways and addiction mechanisms, thus limiting the data' applicability to clinical application. While the current piece of work focused on NMDA and iNOS pathways, other molecular mechanisms and neurotransmitter systems may also play significant roles in the development of morphine tolerance and dependence, warranting further investigation. Additional behavioural approaches could also be useful in strengthening the results of behavioural paradigms. Biochemical analysis especially PCR could be extended to include a diverse set of biomarkers that could not be done due to financial limitations. Further, in order to assess the effectiveness of innovative therapies and role of natural compounds that can target DG neurogenesis, future research is also required to deepen our understanding of how DG neurogenesis may prevent opiate relapse.

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Data availability statement

Data will be available on request from corresponding authors.

Ethics approval

The animal study was reviewed and approved by Research and Ethics Committee of Riphah Institute of Pharmaceutical Sciences (Ref. no. REC/RIPS/2023/17) along with the guidelines of "Principles of Laboratory Animal care".

CRediT authorship contribution statement

Ismail Badshah: Writing – original draft, Investigation, Formal analysis, Data curation. **Neelum Gul Qazi:** Resources, Methodology, Investigation, Formal analysis, Data curation. **Maira Anwar:** Methodology, Investigation, Data curation. **Bushra Shaukat:** Writing – original draft, Formal analysis, Data curation. **Muhammad Imran Khan:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Babar Murtaza:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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