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Original Article

Evaluation of the Effects of Aerial Parts of Hydro-Alcoholic Extract of *Tanacetum kotschyi* and Dextromethorphan on Morphine Withdrawal Symptoms

Leila Balaneshin Kordan[1], Bohloul Habibi Asl[1], Yadollah Azarmi[1], Meysam Balaneshin Kordan[2], Solmaz Asnaashari[3,*]

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, IRAN.
[2]Faculty of Naval Architecture and Ocean Engineering, Istanbul Technical University, Istanbul, TURKEY.
[3]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, IRAN.

ABSTRACT

Background: There are different mechanisms of dependence and tolerance to opioids. Dextromethorphan is a non-competitive N-Methyl-D-Aspartate (NMDA) glutamate receptor antagonist, whereas *Tanacetum kotschyi* (*T. kotschyi*) has antioxidant properties. **Objectives:** This study investigates inhibitory effect of *T. kotschyi* extract and dextromethorphan on the incidence of morphine dependence in male mice. **Materials and Methods:** 81 mice in 9 groups of 9 (weight: 20-30 g) were randomly selected and received drug regimens once a day for two weeks. Different doses of dextromethorphan (15, 30, 60 mg/kg, IP) and extract of *T. kotschyi* (25, 50, 100 mg/kg, IP) or both were injected half an hour before the daily injection of morphine (25 mg/kg, IP), then on the fourteenth day, two hours after morphine injection, naloxone (4 mg/kg, IP) was injected and withdrawal symptoms (number of jumps and standing on two legs) were measured within half an hour. After anesthetizing the animals, blood samples were collected from the heart to Measure Serum Malondialdehyde (MDA) and total Antioxidant capacity (TAC) levels. **Results:** Dextromethorphan and *T. kotschyi* doses decreased the symptoms as well as co-injection of dextromethorphan (15 mg/kg) and *T. kotschyi* (25 mg/kg). Moreover, dextromethorphan doses and *T. kotschyi* (50 and 100 mg/kg) diminished MDA level with $p<0.05$. Comparing to *T. kotschyi* (25 mg/kg), the co-injection increased standing on feet and decreased number of jumping and MDA level. **Conclusion:** *T. kotschyi* can be suggested as a new combination to reduce the symptoms of morphine withdrawal syndrome for further studies and to reduce the incidence of opioid addiction in communities.

Keywords: Dependence, Dextromethorphan, Morphine, Oxidative Stress, *Tanacetum kotschyi*.

Correspondence:
Dr. Solmaz Asnaashari
Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, IRAN.
Email: asnaasharisolmaz@gmail.com

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INTRODUCTION

Opioid withdrawal syndrome is a potentially fatal illness caused by opioid addiction. Opioids are a class of medications used to treat severe pain. They are also widely utilized as psychotropic drugs worldwide. Morphine, heroin, oxycontin, codeine, methadone, and hydromorphone hydrochloride are examples of opioids. They provide mental relaxation, pain alleviation, and euphoria.[1] Chronic opioid usage causes consumers to occur an incapacitating kind of dependency. Opioid addiction affects the drug user as well as society as a whole, rising health-care expenses, unemployment rates, absenteeism, and early death.[1] Repetitive use of opioid medications like morphine, either for pain relief or pleasure, engages motivation and reward-related pathways in the central nervous system. Long-term adaptive alterations in brain nuclei and neurons are the result of these systems. These adaptive alterations result in tolerance and dependency.[2] Although the molecular origins of morphine dependency and withdrawal symptoms are unclear, it has been postulated that repeated morphine use produces adaptation in many neurotransmitter systems across various brain areas, resulting in behavioral and neurochemical implications,[3] including dopaminergic,[4] glutamatergic,[5] nitric oxide,[6] orexinergic,[7] GABAergic,[8] and serotonergic systems.[9]

Dextromethorphan is a drug that does not affect opioid receptors. It is d-isomer of Levorphanol. It is used to treat coughs and may be used in a broad range of dosages without harming the human body.[10] According to preliminary research, this molecule may be able to inhibit the N-Methyl-D-Aspartate (NMDA) receptor by attaching to a specific region inside the channel and thereby limiting its activation.[11] Dextromethorphan has been shown in

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animal studies to attenuate ischemia-hypoxia induced neuronal damage and seizures, as well as other neuropathologies caused by NMDA receptor overactivation.[12-14] Different researches has been demonstrated that dextromethorphan may successfully reduce morphine tolerance and withdrawal symptoms in adult animals with no obvious negative effects.[15,16] Dextromethorphan also potentiates the analgesic effects of the μ-opioid receptor agonist morphine under certain conditions. Cases of aggressive psychosis have been reported in the setting of regular dextromethorphan abuse.[17] Using dextromethorphan for an extended period of time during pregnancy may result in fetal dextromethorphan toxicity, hence it is not recommended.[18]

The NMDA excitatory amino acid receptor, glutamate, which is the primary stimulatory messenger in most central nervous system receptors, is one of the biochemical mechanisms accepted as an important principle in the development of morphine dependence. Glutamate and glutamate receptors activate and depolarize all nerve cells,[19] so NMDA receptor antagonists have been shown in several studies to prevent morphine tolerance and dependence. Another effect of excitatory amino acid stimulation of NMDA receptors is an increase in Nitric Oxide (NO) production.[20] In general, long-term opioid usage is associated with a number of behaviors such as seeking, craving, restlessness, sleeplessness, and elevated blood pressure, which are recognized in the individual over time owing to neurological adaptations and modifications. They are a component of a person's mood. Many of these addictive behaviors have been drastically reduced by the use of NMDA receptor antagonists.[21] On the other hand, recent studies have shown that dependence on morphine is strongly related to oxidative stress, and morphine causes oxidative stress in different tissues of the body by factors such as increasing the formation of various types of free radicals and suppressing antioxidant enzymes. Some studies showed that single dose morphine administration decreaseed the level of revived Glutathione (GSH) in brain cells and evacuation of GSH under frequent chronic injections caused central nervous system highly vulnerable to oxidative stress. An increase in the level of Glutathione Disulfide (GSSG), a decrease in the level of intracellular GSH in the brain, and a decrease in the activity of Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), as well as an increase in the production of malondialdehyde and NO in chronic use morphine has been reported. Chronic use of morphine could stimulate glial cells and increase the production or activity of inflammatory cytokines and neurotropic factors such as interleukins, TNFα and NO, which could increase the activity of inflammatory mediators to facilitate the transmission of pain messages and thus it could reduce the analgesic effect of morphine.[22-24]

The use of herbal medicine in the treatment of opioid addiction and withdrawal symptoms is seen as a viable approach.[25,26] *Tanacetum* is one of the biggest and most extensively spread genera in the *Asteraceae* family, tribe Anthemideae. In Iran, 36 species belong to this genus.[27] *Tanacetum* species include a high concentration of essential oils and sesquiterpene lactones. *Tanacetum* species are used to treat arthritis, fever, migraine, menstrual difficulties, stomachache, toothache, and bug bites.[28-30] Acetylenes and sesquiterpene lactones have been discovered in this genus, and lactones are listed as important ingredients in *Tanacetum* species.[31,32]

T. kotschyi is an endemic species found in Iran and Turkey.[33,34] It is a perennial plant with the highest levels of camphor, esters, terpenes, and flavonoids which have been reported to have antioxidant characteristics.[33] Considering the effects of dextromethorphan and the effects of T. *kotschyi* extract in reducing oxidative stress,[34] it seems that dextromethorphan and *T. kotschyi* extract can inhibit morphine dependence in chronic conditions in mice. The results of previous studies indicated the beneficial effects of dextromethorphan on morphine tolerance and dependence in animal and human phases.[35,36] Also, the antioxidant effects of *T. kotschyi* species extract have been mentioned in previous researches.[37] In this study, A comparative study of the injection alone and the combination of these substances was evaluated.

MATERIALS AND METHODS

Materials

Dextromethorphan and morphine sulfate were supplied by Dana Pharmaceutical Co. (Tabriz, Iran) and Darou Pakhsh Pharmaceutica Co. (Tehran, Iran), respectively. Naloxone was purchased from Tolid Darou Company (Tehran, Iran). Thiobarbituric acid, hydrogen peroxide, n-Butanol, and phosphoric acid, were obtained by Sigma-Aldrich (Sigma Aldrich Inc., Missouri, USA). Ketamine and xylazine were also purchased by Alfasan Diergeneesmiddelen B.V. (Utrecht, Netherlands). The aerial parts of *Tanacetum* species was prepared from Arasbaran forests (Tabriz, Iran).

Animals

Healthy male mice (20-30 g) were purchased from the Tabriz University of Medical Sciences animal center, housed in a standard polypropylene cage at 25±2°C temperature, and provided 12-hr light/12-hour dark intervals with ad libitum feeding.

Ethics

Approval for this study was granted by the Research Ethics Committee of "Tabriz University of Medical Sciences guidelines" (Approval ID: IR.TBZMED.VCR.REC.1400.177).

Experimental Protocols

The randomly selected mice (81 mice in 9 groups of 9 in the weight range of 20-30 g) received drug regimens once a day for two weeks. The groups received different doses of dextromethorphan (groups

3-5), extract of *T. kotschyi* (groups 6-8), or both (group 9) half an hour before the daily injection of morphine (Table 1). Then, on the 14th day, two hours after morphine injection in animals, naloxone (4 mg/kg, ip) was injected and withdrawal symptoms (number of jumping and number of Standing on feet) were measured within half an hour. The animals were then anesthetized and blood samples were taken from the heart to Measure Serum Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC) levels (tests for oxidative stress biomarkers) in the groups. The Thiobarbituric Acid Reactive Substances (TBARS) test was used to detect and quantify MDA.[38] TAC is a common analyte used to examine the antioxidant state of biological materials and may quantify the antioxidant response to free radicals generated in a specific illness. In this study, the TAC Assay Kit was used. Antioxidants of the whole body increase in situations where oxidative stress is reduced or receiving antioxidant substances increases, thereby increasing the antioxidant capacity Table 1.[39]

Preparation of plant powder and extraction

The extraction of *T. kotschyi* was done by the maceration method. The aerial part of the plant was dried in the dark and away from the sunlight and the temperature of the laboratory, and then it was ground with an electric mill, then it was mixed with 50% ethanol and then staying overnight in hydro-ethanol solvent. Finally, the supernatant solution was filtered and again added 50% ethanol solvent to the powder left in the container and this process was repeated three to four times. The obtained solution was evaporated and the extract was collected. The amount of total phenol present in 1 g of plant extract was obtained based on folin ciocalteu method.[40]

Statistical analysis

GraphPad InStat software was used in the statistical analysis of the results with 95% confidence interval. The results in the present study were adjusted as Mean ± SEM. Unpaired student *t*-test method was used to compare two groups, and One Way ANOVA and Tukey's post-test were used to compare the results of more than two groups, $p < 0.05$ results were considered significant.

RESULTS

The results of morphine withdrawal symptoms and the changes of TAC and MDA serum levels in different animal groups were presented in Figures 1 and 2, respectively. Moreover, total phenol content of hydroalcoholic extract of *T. kotschyi* was evaluated and the result showed the presence of 29.98 mg GAE/g of phenolic compounds in the extract.

Figure 1, Comparing morphine withdrawal symptoms of (A) group 2 and group 1, (B) group 3-5 and group 2, (C) group 6-8 and group 2, and (D) group 9 and group 2, 3, and 6; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ (S=Saline, M=Morphine, TK=*Tanacetum Kotschyi*, D=Dextromethorphan). The numbers in parenthesis are injection doses according to Table 1.

Figure 2, Comparing changes in TAC and MDA serum levels of (A) group 2 with group 1, (B) group 3-5 with group 2, (C) group 6-8 with group 2, and (D) group 9 with group 2, 3, and 6; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ (S=Saline, M=Morphine, TK=*Tanacetum Kotschyi*, D=Dextromethorphan). The numbers in parenthesis are injection doses according to Table 1.

DISCUSSION

Drug withdrawal syndrome is a group of physiological responses of the body that occur with the sudden cessation or reduction of the use of medicinal or recreational drugs. These symptoms are common to many abused drugs and may often complicate the care of critically ill patients.[41] Morphine has been introduced as the first purified active substance from plant sources. This structure is a strong alkaloid compound and has significant medical uses. Because of this alkaloid structure directly affects the central nervous system, it is commonly used to relieve moderate to severe pain. However, prolonged abuse leads to opiate addiction, which commonly associated with increased tolerance and physical dependence.[42] Morphine withdrawal syndrome symptoms are observed after long-term exposure to morphine and most of the brain regions show less activity due to long-term abstinence and acute morphine withdrawal.[42] The withdrawal

Table 1: Grouping of animals with their daily injections (Intraperitoneal) doses; saline dosage is in mL/kg and doses of morphine, Dextromethorphan, and *T. kotschyi* are in mg/kg.

Groups	Injections and amount of injections	Sample size (*n*)
Group 1	Saline (10)+Saline (10)	9
Group 2	Saline (10)+Morphine (25)	9
Group 3	Dextromethorphan (15)+Morphine (25)	8
Group 4	Dextromethorphan (30)+Morphine (25)	7
Group 5	Dextromethorphan (60)+Morphine (25)	7
Group 6	*T. kotschyi* (25)+Morphine (25)	6
Group 7	*T. kotschyi* (50)+Morphine (25)	6
Group 8	*T. kotschyi* (100)+Morphine (25)	9
Group 9	Dextromethorphan (15)+*T. kotschyi* (25)+Morphine (25)	7

symptoms of morphine addiction usually occur at the short time before the next scheduled dose, usually between 6 to 12 hr of taking the drug. Several symptoms such as severe depression, nausea, vomiting, increased systolic and diastolic blood pressure, and heart rate during the period of acute withdrawal, muscle spasms, bones and muscles severe pain are a variety of morphine withdrawal signs.[42-44]

Previous studies demonstrated that medicinal plants could be effective in treatment of different stages of addiction and decreasing withdrawal symptoms.[25] According to the outcomes of this study, comparing withdrawal symptoms of the group 2 (morphine and saline injection) with those of the group 1 (saline and saline injection) can be seen in Figure 1A. There was a significant increase in withdrawal symptoms of the group 2 with $p<0.001$ for number of both jumping and standing on feet. According to Figure 1B, withdrawal symptoms were decreased by IP injection of dextromethorphan (15, 30, and 60 mg/kg) in comparison with group 2 which received morphine and saline. In the case of 15 mg/kg of dextromethorphan injection, jumping number had $p<0.01$, while number of standing on feet had $p<0.001$. For 30, and 60 mg/kg of dextromethorphan injection, both of jumping and standing on feet (as withdrawal symptoms) have $p<0.001$.

Based on the results set out Figure 1C, IP injection of different doses of *T. kotschyi* hydroalcoholic extract (25, 50, 100 mg/kg) could significantly reduce withdrawal symptoms (number of jumps and number of times standing on feet) compared to the group 2 receiving morphine and saline. For injection of 25 mg/kg of *T. kotschyi* extract, number of both jumping and standing on feet have $p<0.01$. However, in the case of injecting 50, and 100 mg/kg of *T. kotschyi* doses, number of jumping has $p<0.01$ and number of standing on feet has $p<0.001$. As can be seen in Figure 1D, co-injection of *T. kotschyi* (25 mg/kg) and dextromethorphan (15 mg/kg) in comparison with the group 2 caused significant reduction in the symptoms of withdrawal of morphine ($p<0.05$ for number jumping and $p<0.001$ for number of standing on feet). Furthermore, the combined injection of *T. kots*chyi and dextromethorphan compared to *T. kotschyi* alone injection (25 mg/kg) showed significant decline of morphine withdrawal symptoms ($p<0.01$ for number of jumping and $p<0.05$ for

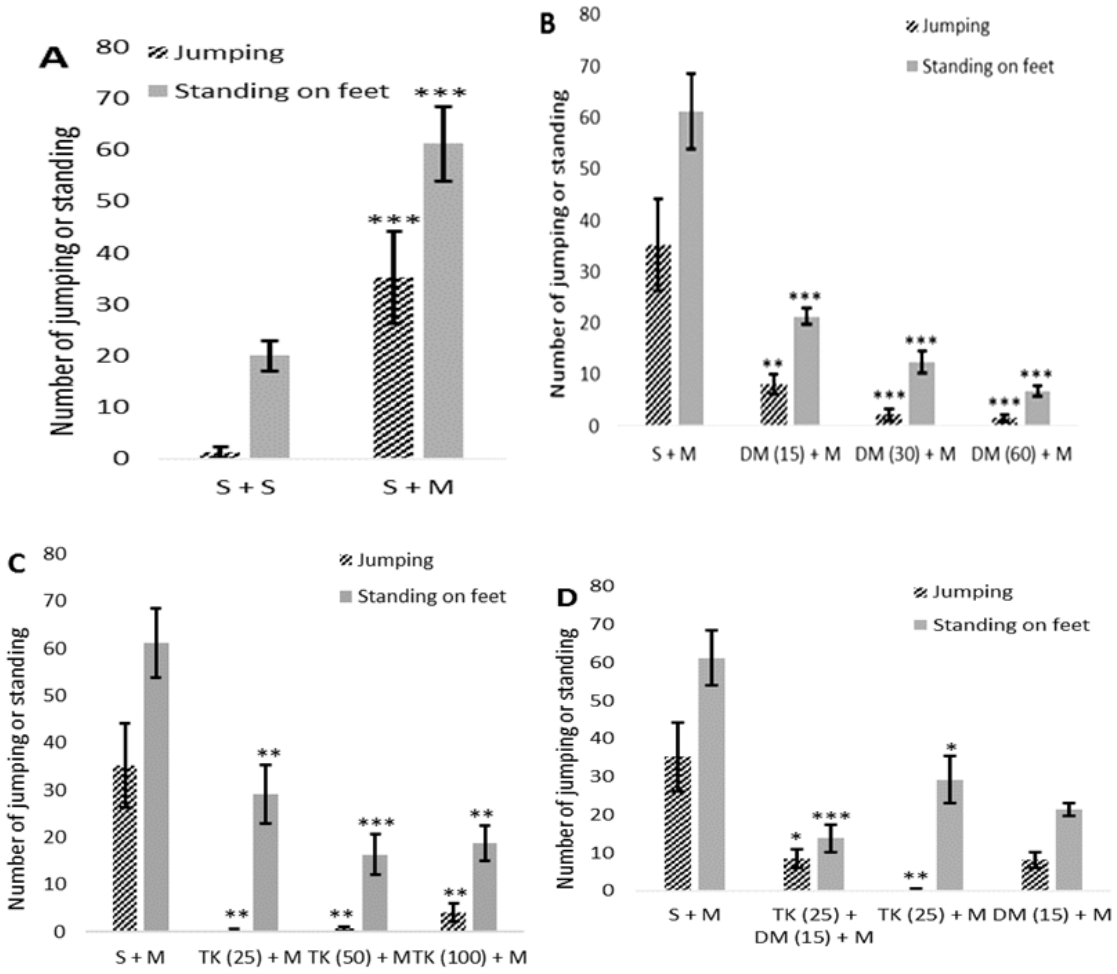

Figure 1: Comparing morphine withdrawal symptoms of (A) group 2 and group 1, (B) group 3-5 and group 2, (C) group 6-8 and group 2, and (D) group 9 and group 2, 3, and 6; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ (S=Saline, M=Morphine, T =*Tanacetum Kotschyi*, D=Dextromethorphan). The numbers in parenthesis are injection doses according to Table 1. image2

number of standing on feet). However, the co-injection of *T. kotschyi* and dextromethorphan compared to dextromethorphan alone injection (15 mg/kg) did not significantly affect morphine withdrawal symptoms. Therefore, in the simultaneous use of the 25 mg/kg of extract and 15 mg/kg dextromethorphan, synergistic effects were not evident compared to dextromethorphan (15 mg/kg) alone. A previous *in vivo* study, Yeh *et al.*, showed that dextromethorphan reduced the morphine withdrawal syndrome in neonatal rats, which passively exposed to morphine.[45] In the other study they reported dextromethorphan and morphine co-administration during pregnancy in animal model significantly reduced naloxone-induced morphine withdrawal behavior in their offspring.[46] A recently published study demonstrated that herbal medicine could enhanced bioavailability of dextromethorphan with inhibiting several metabolic enzymes and decreasing dextromethorphan metabolism.[47] Moreover, several previous studies reported different *Tanacetum* species antinociceptive and anti-inflammatory properties. For example, *Tanacetum fisherae* essential oil had anti-inflammatory properties and reduced the pain responses in dose dependently manner during late phases of formalin test.[48] Furthermore, chronic orally used of *Tanacetum parthenium* extract alleviated CCI (Chronic Constriction Injury)-induced neuropathic pain in animal model.[49]

Therefore, it seems that the evaluation of other doses of *T. kotschyi* extract might be useful in the observation of these two drugs synergistic effects in current study.

Malondialdehyde (MDA) is known as the final product obtained from the peroxidation of poly-unsaturated fatty acids and is used as a biomarker to assess oxidative stress in biological samples in a wide range of diseases.[50] Oxidative stress also appears as an abnormality and imbalance between antioxidants and pro-oxidants as a key mechanism in molecular signaling pathways that disrupts enzyme activity and leads to tissue damage.[50,51] There are many evidences show that morphine, in addition to activating the relevant receptors and their classical signaling pathways, can cause oxidative stress under certain conditions by increasing the formation of free radicals. Imbalance of antioxidant levels and

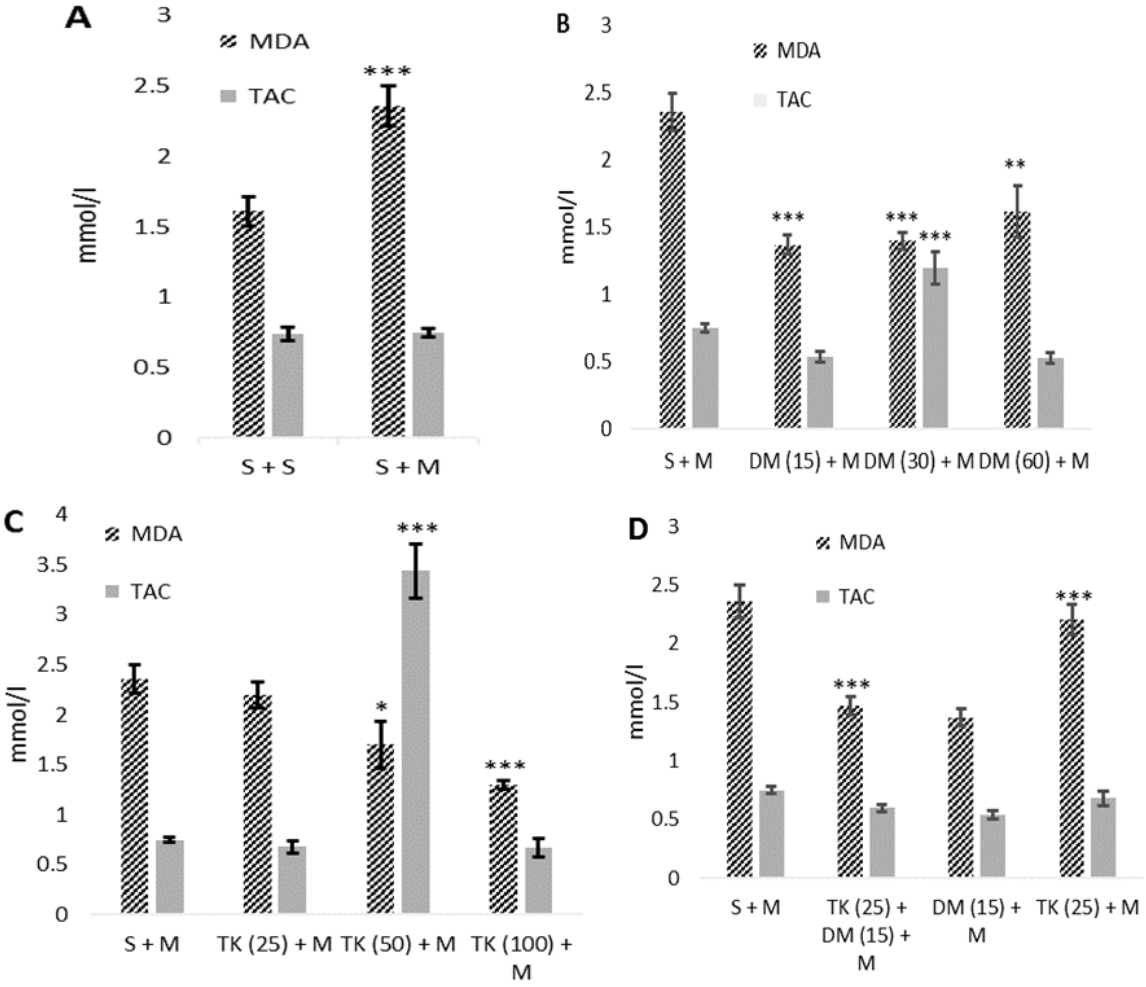

Figure 2: Comparing changes in TAC and MDA serum levels of (A) group 2 with group 1, (B) group 3-5 with group 2, (C) group 6-8 with group 2, and (D) group 9 with group 2, 3, and 6; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ (S=Saline, M=Morphine, TK=*Tanacetum Kotschyi*, D=Dextromethorphan). The numbers in parenthesis are injection doses according to Table 1.

suppression of antioxidant enzymes can lead to oxidative damage in various tissues. Lipid peroxidation, DNA damage, protein oxidation, and induction of apoptosis are normal deleterious events associated with this deleterious process.[52]

According to the presented results in Figure 2A, it can be seen that the use of morphine (25 mg/kg) for two weeks, induced a significant increase in the level of MDA in the group 2 (morphine and saline injection) compared to the group 1 (saline and saline injection) with $p<0.001$, where there is no significant change in the level of TAC. Moreover, IP injection of dextromethorphan different doses could induced a significant decrease in MDA serum levels in different groups ($p<0.001$ for 15 and 30 mg/kg and $p<0.01$ for 60 mg/kg) (Figure 2B). TAC serum level showed a significant increase ($p<0.01$) only in the case of 30 mg/kg of dextromethorphan injection. Injection of hydroalcoholic extract of *T. kotschyi* could reduce MDA level in doses of 50 and 100 mg/kg with $p<0.05$ and $p<0.001$, respectively; while it improved TAC level considerably ($p<0.001$) only in 50 mg/kg dosage compared to the group 2 (saline and morphine) (Figure 2C). According to Figure 2D, co-injection of *T. kotschyi* (25 mg/kg) and dextromethorphan (15 mg/kg) in comparison with the group 2 led to a significant reduction only in MDA level with $p<0.001$. The combined injection resulted to decrease of MDA level significantly ($p<0.001$) when compared with *T. kotschyi* alone injection (the group 6).

Various studies have investigated the possible benefits of antioxidants in modifying, reversing or preventing the negative effects of oxidants. According to Esmaeili *et al*., treatment of K-562 cells with ethanol extract of various species of *Tanacetum* increased the intracellular GSH (Glutathione) level, decreased ROS (Reactive Oxygen Species), and main enzyme defense system. Therefore, ROS scavenging and influence on antioxidant defence systems was the main possible mechanism that inhibited the oxidative stress in H2O2-treated K-562 cells. They were reported that the highest antioxidant activities were found for ethanol extract of *T. hololeucum* and *T. kotschyi*. Furthermore, the maximum phenolic and flavonoid contents were belonged to these two species extracts (34). Antioxidant activities were also reported from the methanol extracts of five *Tanacetum* taxa collected from Antalya and the obtained results demonstrated that *T. prae*teritum ssp. *Massicyticu*m extract was the most potent antioxidant between tested extracts. It had also the highest total phenolic and flavonoid contents.[53]

CONCLUSION

T. kotschyi extract inhibited oxidative stress and reduced morphine dependence due to its antioxidant properties. Therefore, after more extensive studies, *T. kotschyi* can be proposed as a new agent to reduce the symptoms of morphine withdrawal syndrome and reduce the incidence of opioid addiction in communities. Moreover, this species extract in combination with dextromethorphan could have the beneficial role on oxidative stress. As well as the antagonistic effect of dextromethorphan on NMDA receptors, it can be used to reduce the symptoms of morphine withdrawal syndrome along with intensifying the *T. kotschyi* effect. According to the results of this study, it can be said that the administration of dextromethorphan and *T. kotschyi* can reduce the experience of morphine dependence in mice.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

MDA: Malondialdehyde; **NMDA:** N-Methyl-D-Aspartate; **TAC:** Total Antioxidant Capacity.

SUMMARY

This study explored the effects of *T. kotschyi* extract and dextromethorphan on morphine dependence in male mice. Dextromethorphan, an NMDA receptor antagonist, and *T. kotschyi*, known for its antioxidant properties, were administered at various doses-alone and in combination-prior to morphine injections over two weeks. On the final day, withdrawal symptoms were triggered using naloxone and assessed through behavioral observations and biochemical markers (MDA and TAC). Results showed that both substances, individually and in combination, significantly reduced withdrawal symptoms and oxidative stress markers. The combination of low-dose dextromethorphan (15 mg/kg) and *T. kotschyi* (25 mg/kg) proved particularly effective. The study concludes that *T. kotschyi* may be a promising adjunct therapy for mitigating opioid withdrawal symptoms and warrants further investigation.

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