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Noni (*Morinda citrifolia* L.) fruit extract attenuates the rewarding effect of heroin in conditioned place preference but not withdrawal in rodents

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Abstract: The present study was designed to investigate the effect of a methanolic extract of *Morinda citrifolia* Linn. fruit (MMC) on the rewarding effect of heroin in the rat conditioned place preference (CPP) paradigm and naloxone-precipitated withdrawal in mice. In the first experiment, following a baseline preference test (preconditioning score), the rats were subjected to conditioning trials with five counterbalanced escalating doses of heroin versus saline followed by a preference test conducted under drug-free conditions (post-conditioning score) using the CPP test. Meanwhile, in the second experiment, withdrawal jumping was precipitated by naloxone administration after heroin dependence was induced by escalating doses for 6 days (3×/ day). The CPP test results revealed that acute administration of MMC (1, 3, and 5 g/kg body weight (bw), p.o.), 1 h prior to the CPP test on the 12th day significantly reversed the heroin-seeking behavior in a dose-dependent manner, which was similar to the results observed with a reference drug, methadone (3 mg/kg bw, p.o.). On the other hand, MMC (0.5, 1, and 3 g/kg bw, p.o.) did not attenuate the heroin withdrawal jumps precipitated by naloxone. These findings suggest that the mechanism by which MMC inhibits the rewarding effect of heroin is distinct from naloxone-precipitated heroin withdrawal.

Key words: conditioned place preference, heroin, naloxone, noni fruit, precipitated withdrawal

Introduction

Opioid abuse and dependence are chronic and enduring phenomena encountered throughout the world. Together, they are part of a compulsive pattern that is associated with drug seeking and taking and caused by positive reinforcement of the rewarding effect of the drug and negative reinforcement of the withdrawal syndrome that occurs after the cessation of substance abuse [8]. Heroin is known to be the most abused opioid drug, and studies have shown a steady increase in the number of addicts towards this substance over the years [36]. Although there are several FDA-approved pharmacotherapies, including methadone maintenance treatment (MMT), that have been used to date for the treatment of opioid dependence, their inadequacies have encouraged the search for new treatment options.

Numerous plants that have been used as traditional medicines, have recently turned out to be important sources of new drugs in health-care systems throughout the world. Several animal studies have determined the particular efficacies of plant-derived medicines for the treatment of morphine, alcohol, nicotine, and cocaine, as well as heroin dependence [20]. *Morinda citrifolia* L. (family: Rubiaceae) has been used as food and medicine by humankind for centuries. It is commonly known as noni, a small evergreen tropical tree that grows widely in many tropical regions of the world [22]. Traditionally, the roots, stems, bark, leaves, flowers, and fruits of the noni are used in various combinations in folk medicine for the treatment of many illnesses including arthritis, diabetes, high blood pressure, muscle aches and

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pains, menstrual difficulties, headaches, heart disease, cancers, gastric ulcers, sprains, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems, and drug addiction [40].

An aqueous and alcoholic extract of noni fruit and root showed an analgesic effect in mice, and this was suggested to be mediated through an opioidergic mechanism [34, 41]. Evidence in favor of an antidopaminergic effect of noni fruit have been provided by studies assessing its prokinetic effect in mice and antiemetic effect in humans [26]. Additionally, work from our laboratory demonstrated the antipsychotic-like effect of noni fruit juice and a methanolic extract of it on mouse models of apomorphine/methamphetamine-induced cage climbing/stereotypy and suggested that noni fruit has a neuromodulatory effect on the dopaminergic system [29]. Dopaminergic neurotransmission, especially the mesolimbic system, has been widely explored with respect to motivational (reinforcing) aspects of drug abuse [33]. It was therefore of interest to investigate whether the effect of a noni fruit extract resulting from the blockade of dopamine receptors and facilitation of opioid receptors, could be utilized to combat drug seeking and withdrawal syndrome.

From this perspective, the present study was primarily undertaken to determine the effect of a standardized methanolic extract of *Morinda citrifolia* Linn. fruit (MMC) on a rat model of heroin conditioned place preference (CPP), a model that investigates the reinforcing effect of drugs with dependence liability. In addition, an attempt was undertaken to examine the possible effect of MMC on naloxone-precipitated withdrawal jumping behavior in mice.

Materials and Methods

Animals

Male Sprague–Dawley rats weighing 250–300 g upon arrival (7–8 weeks old) and ICR mice weighing 20–26 g upon arrival (7–8 weeks old) were obtained from the Laboratory Animal Centre, University of Malaya. Animals were housed four per cage in polycarbonate cages in a temperature-and humidity-controlled environment, given *ad libitum* access to food and water, and maintained at 22 ± 1°C with a 12 h light: 12 h dark cycle. All experiments were carried out according to an experimental protocol (ACUC Ethics No. FAR/27/01/2012/PV (R)) approved by the Animal Care and Use Committee, Faculty of Medicine, University of Malaya, Kuala Lumpur.

Standardized extract of M. citrifolia fruit

The standardized MMC was prepared using cold extraction with sonication as mentioned in our recent publication [30]. The dried solvent-free standardized MMC was stored at 4°C in a container until further use.

Drugs and chemicals

Naloxone hydrochloride from Sigma-Aldrich, St. Louis, MO, USA and methadone hydrochloride in the form of syrup (5 mg/ml) (Aseptone®) from Duopharma (M) SDN BHD, Malaysia, were used. Heroin hydrochloride (diacetylmorphine hydrochloride) was generously provided by the Chemistry Department, Ministry of Health, Malaysia. The drug solution was prepared fresh in normal saline prior to the start of experimentation and administered intraperitoneally (i.p.) in a constant volume of 1 ml/kg body weight of rats and 1 ml/100 g body weight of mice. The standardized MMC was suspended in 1% w/v sodium carboxymethylcellulose (CMC) solution and administered orally (p.o.). CMC solution served as the vehicle control (VEH).

Apparatus

The conditioned place preference (CPP) procedure was carried out as described previously [37] with the following specifications. In brief, place preference conditioning was performed in plexiglass boxes measuring (90 × 22 × 30 cm) (L×W×H) that were divided into two chambers of equal size (40 × 22 × 30 cm) by insertion of a removable plexiglass wall and separated by a small middle grey zone (10 × 22 × 30 cm) in the middle of the chamber. Both chambers possessed visual cues along with tactile cues: one side of the chamber had black walls with white horizontal stripes attached white wire mesh on the floor, while the other side had white walls with vertical black lines and a smooth black plexiglass floor. Detachable dividers, complementing the chamber walls, were used to close-off each chamber. Transparent plexiglass lids allowed observation of an animal's behavior on a computer connected to a Logitech HD Webcam placed above the apparatus. Rat behavior was recorded and later scored by an experimenter who was blind to the treatment condition.

Conditioned place preference (CPP) procedure

The CPP test was comprised of three specific phases, the preconditioning, conditioning, and post-conditioning phases and was performed over the course of 12 con-

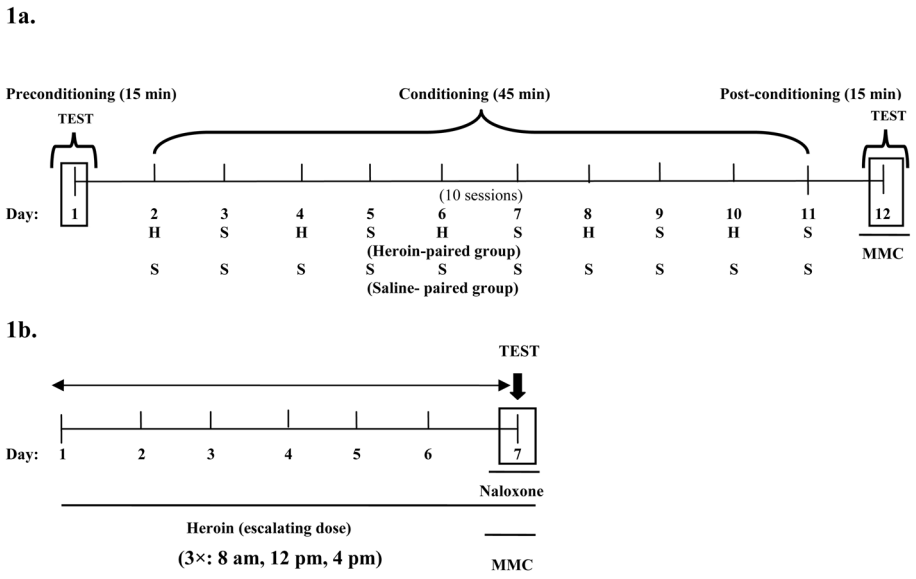

Fig. 1. Experimental design for (a) the conditioned place preference paradigm and (b) naloxone-precipitated withdrawal jumping.

secutive days. A schematic diagram of the study plan is depicted in Fig. 1a. Preliminary data from our laboratory indicated that, naïve rats spent more time in the black compartment in comparison with the white compartment when given free choice regarding access to the whole apparatus for 15 min. As a result, to assess conditioning, we paired heroin with the initial non-preferred white compartment. On the first day, each rat was individually preexposed to the test apparatus for 15 min.

Initially, each animal was placed in the center grey compartment for one min; following that, the guillotine doors were raised, and each animal was allowed to move freely between the two compartments. The times spent in each of the compartments were recorded on the preconditioning day. The rats that showed innate place preference for the white compartment in preconditioning phase were excluded from further study. The day after preconditioning session, the conditioning (days 2–11) phase began. During this phase, the guillotine doors were set in place, and the animals were restricted to either the white or black compartment. Rats were moved from the animal holding room into the testing room, weighed, and allowed to habituate them to the testing room for at least 30 min. After habituation, the rats were placed in their respective compartments for 10 min for adaptation and later injected with either escalating doses (1.25, 2.5, 5, 10, and 10 mg/kg bw, i.p.) of heroin HCl on days 2, 4, 6, 8, and 10, respectively, or saline on days 3, 5, 7, 9, and 11 (heroin-conditioned group). Control rats were treated with saline prior to all conditioning sessions from day 2 to day 11 (saline-conditioned [SAL] group). Immediately after heroin/saline injections, rats were confined to the appropriate side of the test apparatus for 45 min and then were returned to their home cages.

On day 12, neither saline nor heroin was administered to either the heroin-conditioned group or the saline-conditioned group. As in the preconditioning phase, the guillotine doors were raised, and the time spent by the drug-free rats in the two compartments was recorded for 15 min. CPP is defined by an increase in the time spent in the drug-paired chamber during the preference test. The data are expressed as the differences between the time spent in the compartment associated with heroin (white) and the time spent in that associated with saline (black) [37].

The effect of MMC (1, 3, and 5 g/kg bw, p.o.) and methadone (3 mg/kg bw, p.o.) on heroin-seeking behaviour in rats was investigated. The MMC-treated groups received different doses of MMC (1, 3, and 5 g/kg bw, p.o.) by oral gavage, 60 min prior to the test during the post-conditioning phase (12th day). The methadone-treated group (MTD group), which was the positive control group, received an oral dose of methadone (3 mg/kg bw), 60 min before the test. The vehicle control group was given 1%w/v CMC (1 ml/kg bw, p.o.) according to the same time schedule.

Naloxone-induced withdrawal jumping

To develop heroin dependence, mice were injected subcutaneously with escalating doses of heroin three times daily for 6 days, with injections starting at 08.00 h and being given 4 h apart. A schematic diagram of the study plan is shown in Fig. 1b. The different doses of heroin from day 1 to day 6 were 1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 mg/kg bw, respectively. The straub tail reaction and hyperactivity were seen in the animals after heroin injections. At 08.00 h on the test day (day 7), a final dose of heroin (20 mg/kg bw, i.p.) was administered, and 2 h later, heroin withdrawal was precipitated with a single injection of naloxone (25 mg/kg bw, i.p.), an opioid receptor antagonist. In a previous study, the maximum jumping response was observed at a dose of 30 mg/kg of naloxone in morphine-dependent mice [5]. Hence, in the present study, we used a relatively high dose of naloxone (25 mg/kg bw) to ascertain the maximum withdrawal effect in mice. Thirty min before naloxone injection, mice were placed in a clear 5 l beaker (15 cm in diameter, 30 cm in height) to allow them to habituate to the new environment. Immediately after naloxone injection, each mouse was placed gently again in the beaker, which was and then closed with a plexiglass plate. Then the mice were observed for naloxone-precipitated withdrawal jumping behavior for 30 min. Withdrawal jumping was defined as simultaneous removal of all four paws from the bottom of the beaker [19]. Withdrawal jumping was manually evaluated by a researcher who was blind to the treatment protocol. Control animals were given saline according to the same schedule and tested after naloxone administration. All mice received only one dose of naloxone injection during the study protocol.

To observe the influence of MMC on naloxone-precipitated withdrawal jumping in heroin-dependent mice, MMC at different doses (0.5, 1, and 3 g/kg bw, p.o.), was administered 60 min after the final heroin injection (day 7). Vehicle control mice received 1% w/v CMC (1 ml/100 g, p.o.).

Statistical analysis

The data are expressed as mean ± SEM. Effects of MMC on CPP scores were analyzed using two-way analysis of variance (ANOVA) and one-way ANOVA followed by Dunnett's Multiple Comparison Test. For naloxone-precipitated withdrawal, one-way ANOVA was performed, followed by Dunnett's *post hoc* test. All data analyses were conducted using the GraphPad Prism 5 statistical software. Values of $P<0.05$ were regarded as statistically significant.

Results

Effect of MMC on expression of heroin-induced CPP

The effect of MMC on the expression of heroin-induced CPP is represented in Figs. 2a and 2b. ANOVA results revealed a significant effect of treatment [$F(5, 74)=3.931$; $P<0.01$], time [$F(1, 74)=16.83$; $P<0.001$], and the interaction of treatment by time [$F(5, 74)=3.068$; $P<0.05$]. Separate one-way ANOVA results revealed that the preconditioning scores of the different groups (SAL, VEH, MMC, and MTD) were statistically insignificant [$F(5, 37)=0.05250$; $P>0.05$], as shown in Fig. 2a. The escalating doses of heroin (1.25, 2.5, 5, 10, and 10 mg/kg bw, i.p.) injected on alternate days produced significant CPP ($P<0.001$) when compared with the saline-treated rats. However, acute oral administration of MMC (1, 3, and 5 g/kg bw) and MTD (3 mg/kg bw) 60 min prior to testing on day 12 (post-conditioning) resulted in a significant dose-dependent decrease in the post-conditioning scores [$F(5, 37)=7.924$; $P<0.0001$], as shown in Fig. 2b. The *post hoc* analysis revealed that MMC (3 and 5 g/kg bw) significantly ($P<0.001$) reversed drug seeking in the white (heroin-paired) compartment, which was similar to the effect observed in the MTD-treated group.

Effect of MMC on naloxone-precipitated withdrawal jumping in heroin-dependent mice

One-way ANOVA results revealed that there was a significant difference between the treatment groups [$F(4, 35)=4.315$; $P<0.01$] as shown in Fig. 3. Heroin-dependent, mice when treated with naloxone, exhibited significant ($P<0.01$) precipitated-withdrawal jumping behavior. Pretreatment with MMC (0.5, 1, and 3 g/kg bw, p.o.) 60 min prior to naloxone injection did not significantly alleviate the naloxone-precipitated withdrawal jumping behavior in mice (Fig. 3).

Discussion

The present study results provide evidence that MMC significantly attenuated the positive reinforcing properties (drug seeking) of heroin in the rat CPP test. CPP is widely used as a behavioral animal model to study the rewarding properties of various abused substances and is compatible with the classical Pavlovian conditioning

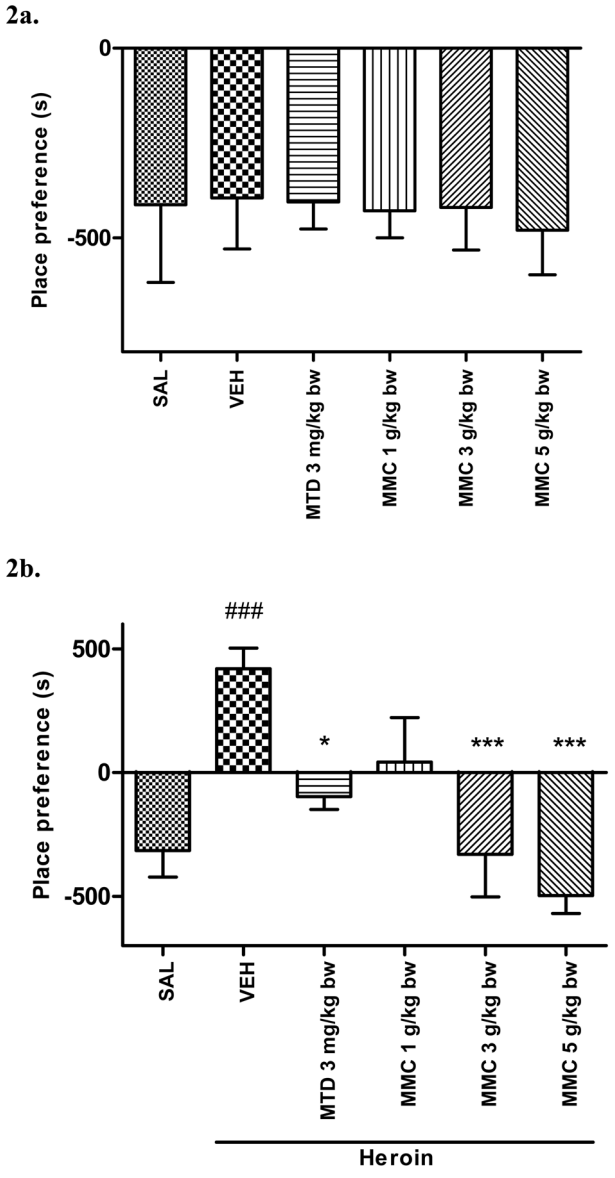

Fig. 2. Effect of different doses of MMC (1, 3, and 5 g/kg bw, p.o.) and methadone (MTD; 3 mg/kg bw, p.o.) on the expression of heroin-induced CPP in the (a) preconditioning and (b) post-conditioning phases. Data represent the differences between the times spent in the compartment associated with heroin and saline. The negative values represent a preference for the black compartment and vice versa. Each bar represents the mean ± SEM (n=6–8). Statistical significance: $^{###}P<0.001$ compared with the saline control group (SAL); $*P<0.05$ compared with the vehicle control group (VEH); $***P<0.001$ compared with the vehicle control group (VEH); when not indicated, the differences were not statistically significant.

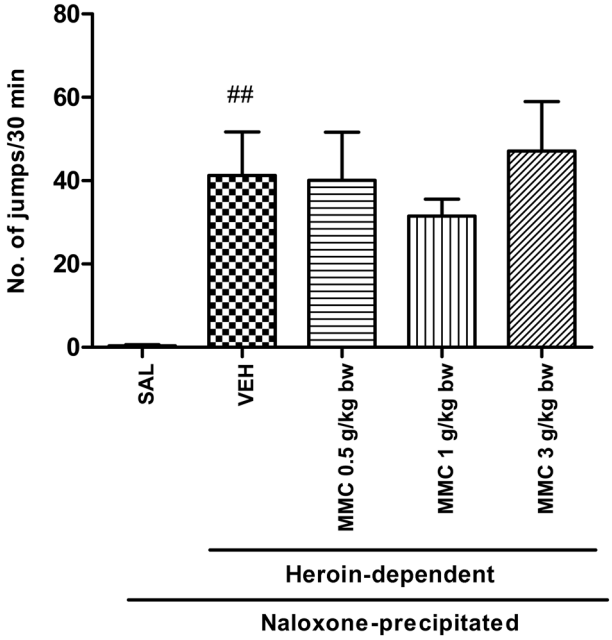

Fig. 3. Effect of MMC (0.5, 1, and 3 g/kg bw, p.o.) on naloxone-precipitated withdrawal jumping behavior in mice. Each bar represents the mean ± SEM (n=8). Statistical significance: $^{##}P<0.01$ compared with the saline control group (SAL); when not indicated, the differences were not statistically significant.

[37]. Most substances of abuse are generally known to induce CPP, including heroin [6]. Typically, the drugs used in the treatment of substance addiction are able to attenuate acquisition and/or expression of substance-induced CPP. Nevertheless, several drugs act without having an impact on drug reward pathways and instead impair learning as well as memory (e.g., NMDA receptor antagonists) [1]. Interestingly, Muralidharan *et al.* (2010) claimed that an ethyl acetate extract of noni fruit prevented amyloid beta-induced memory dysfunction in mice [24]. Similarly, the nootropic effect of the *Morinda citrifolia* fruit was established in a scopolamine-induced memory impairment animal model [28]. The attenuation of heroin-induced CPP by MMC is therefore unlikely a result of interference with the memory impairment mechanism.

The different brain areas, including the nucleus accumbens, ventral tegmental area, locus coeruleus, and amygdala and, their connections within the midbrain are well-known to be involved in positive reinforcement, persistent compulsive drug craving, and vulnerability to relapse to morphine and other drugs of abuse [10]. Most importantly, the mesolimbic dopaminergic system plays an essential role in the mechanisms related to memory, which is involved in the establishment of morphine-induced CPP [13]. Use of substances of abuse, gambling,

eating (especially sweets), and sexual behavior are usually linked to elevated intra-synaptic levels of dopamine (DA) in the nucleus accumbens (NAc). Reports of positron emission tomography (PET) as well as single-photon emission computed tomography (SPECT) in addictive users of cocaine, ethanol, methamphetamine, and heroin demonstrated reductions of D_2 receptor density within the ventral striatum that persisted after prolonged detoxification [18]. It has been reported that dopamine D_2 receptor antagonists significantly reduced ethanol self-administration in rats [14] and hindered reinstatement associated with cue-induced cocaine-seeking behavior in rats [16]. Drugs having a neuromodulatory effect on dopaminergic system could possibly be useful in the treatment of drug addiction. In our recent publications, we reported the antidopaminergic activity of MMC and its major bioactive compounds, scopoletin and rutin, using an *ex vivo* study of the vas deferens isolated from rats and *in vivo* behavioral studies in mice [29, 30]. Thus, it could be predicted that the antidopaminergic property of MMC could be involved in the attenuation of heroin-induced CPP in rats. Furthermore, no significant difference in spontaneous locomotor activity was reported for group treated with a noni fruit extract [27]. This report eliminates the possibility that an alteration in locomotor activity contributed toward the observed behavioral changes in rats following noni administration.

Physical dependence is often associated with chronic consumption of a substance of abuse. Acute withdrawal from a substance of abuse has been certainly linked to somatic signs and symptoms. Administration of a single dose naloxone, an opioid receptor antagonist, in opioid-dependent mice resulted in distinct jumping behavior in addition to other somatic signs in mice [35]. The naloxone-precipitated withdrawal jumping behavioral mouse model is a reliable and commonly used animal model for physical dependence on opioids. Drugs that are effective in treating physical dependence in human beings are expected to alleviate naloxone-precipitated withdrawal jumping in mice. The present results revealed that MMC could not attenuate naloxone-precipitated withdrawal jumping behavior in mice.

It has been previously revealed that the dopamine D_2 receptor plays a significant role in controlling opioid withdrawal. Acute withdrawal from addictive substances such as ethanol, morphine, cannabinoid, nicotine and cocaine decreased the spontaneous activity of the ventral tegmental area dopaminergic (VTA DA) neurons in rodents *in vivo* and *in vitro* [23]. This dampened VTA DA neuronal function was also observed when somatic signs of withdrawal were not detectable. Moreover, when pharmacologically precipitated withdrawal was induced with the specific cannabinoid antagonist SR 141716A, the somatic signs of withdrawal were accompanied by diminished VTA DA neuronal activity [23]. Interestingly, short-term treatment with D_2 receptor agonists restored the hypodopaminergic neuronal function and suggested a potential treatment for addictive substance withdrawal [23]. Similarly, systemic treatment with dopamine agonists or activation of dopamine D_2 receptors in the nucleus accumbens of the rat brain prevented the somatic symptoms of withdrawal caused through blockade of opioid receptors [3, 39]. Therefore, these findings provide some support for the ineffectiveness of MMC on naloxone-precipitated jumping behavior in heroin-dependent mice, which could be due to noni's antidopaminergic activity, which was established in our earlier studies [29, 30].

The hyperactivity of noradrenergic neurons within the locus coeruleus continues to be suggested to be a key player that underlies the cause of opioid withdrawal [21, 38]. The animals with naloxone-precipitated opioid withdrawal showed a significant increase in the noradrenaline concentration of dialysates obtained from locus coeruleus, hippocampus, and ventral bed nucleus of the stria terminalis [15, 25]. It has been well-documented that the inhibition of monoamine oxidase enzyme-A (MAO-A) can drastically increase the synaptic availability of noradrenaline [31]. Noni fruit has been reported to have the ability to inhibit both MAO-A and MAO–B [11], thereby facilitating noradrenergic transmission. Hence, another possible explanation may be related to the fact that the noradrenergic facilitatory property of noni fruit may not be able to alleviate naloxone-precipitated withdrawal jumping behavior in mice.

From our point of view, the difference in the results of the rat CPP study and the mouse naloxone-precipitated withdrawal study is not likely to be due to the species difference but is likely due to the actual pharmacological effects of MMC. In general, the pharmacological effects observed in rats and mice for similar kinds of experiments are alike. For example, apomorphine showed a dopaminergic agonistic effect in rats (0.2–0.8 mg/kg bw, subcutaneous [s.c.]) and in mice (0.1–1.0 mg/kg bw, i.p.). Similarly, haloperidol showed an antidopaminergic effect in both species (rat and mouse) (10 mg/kg bw, s.c.)

[17]. Likewise, in drug addiction preclinical studies, heroin showed robust place conditioning (CPP) in rats [4] as well as in mice [9]. Moreover, naloxone-precipitated withdrawal signs and symptoms, particularly jumping behavior, were reported in both morphine-dependent rats [2] and mice [32].

It has been claimed that *M.citrifolia* fruit has multifaceted therapeutic benefits due to its multiple nutritional and functional properties. These valuable outcomes may derive from some key compounds, especially, scopoletin, nitric oxide, alkaloids, and flavonoids, along with sterols [7]. A number of biologically active substances have been determined in the extracts of *M.citrifolia* [42]. The high-performance liquid chromatography (HPLC) fingerprint profile of the methanolic extracts of noni fruit revealed three major peaks representing scopoletin, rutin, and quercetin, together with several minor peaks [28]. In our recent report, the levels of scopoletin and rutin (retention times: 14.51 and 15.86 min, respectively) were quantified in MMC and found to be 18.95 μg/mg and 1.66 μg/mg, respectively. These major bioactive constituents of noni have been reported to have various biological and pharmacological activities *in vitro* and *in vivo* [12]. Therefore, although the present work could not delineate the active compound that is responsible for the effects observed, it is predicted that the anti-reward effect of noni fruit against opioid dependence is attributable to these phytoconstituents. Further, similar studies are warranted using the bioactive phytoconstituents scopoletin and rutin in novel drug discovery for the treatment of opioid addiction.

In conclusion, our findings suggest that MMC attenuates the rewarding effects of heroin in the rat CPP paradigm but that it fails to alleviate the naloxone-precipitated withdrawal jumping behavior in mice. These results limit its therapeutic potential for treatment of opioid seeking but not its therapeutic potential for the treatment of opioid withdrawal.

Conflict of Interest

The authors declare that they have no competing interests.

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