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NALOXONE-PRECIPITATED MITRAGYNINE WITHDRAWAL DID NOT ASSOCIATE WITH INCREASED ANXIETY-LIKE BEHAVIOUR IN RATS

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Abstract

Kratom (*Mitragyna speciosa* Korth) is a plant species currently used as an alternative self-treatment for pain and management of opioid dependence and withdrawal. However, information on the effect of its negative emotional state of withdrawal, particularly of the main psychoactive constituent, mitragynine (MG) is still lacking. The present study was designed to determine whether naloxone-precipitated MG withdrawal is associated with increased anxiety-like behaviour in both open-field test (OFT) and elevated-plus maze (EPM) test. Male Sprague Dawley rats were administered with MG and morphine twice daily for 14 consecutive days to develop substance dependence. The withdrawal was precipitated on day fifteen using naloxone (2 mg/kg, i.p.). This study has exhibited that challenge with opioid antagonist naloxone did not result in reliable expression of anxiety behaviour following chronic MG exposure in OFT and EPM tests. However, several somatic signs of withdrawal were observed in naloxone-precipitated MG withdrawn rats. The findings of this study suggest that rat behaviours during OFT and EPM tests might not be driven solely by anxiety levels following naloxone-precipitated withdrawal but rather by other withdrawal-induced behaviours. The presence of somatic signs in naloxone-precipitated MG withdrawn rats was the piece of evidence to prove that that discontinuation of MG or kratom is associated with physical symptoms of withdrawal. However, further studies are prompted to evaluate MG or kratom withdrawal severity to justify its use as opioid replacement therapy.

INTRODUCTION

Opioid misuse or abuse is the main cause of drug overdose in the USA, reflecting a rising opioid overdose addiction crisis globally [1]. A sharp hike in the number of people impacted by opioid use disorder (OUD) has caused less opioids being prescribed by USA health professionals [1,2]. As stricter restrictions on opioid-based prescribing are introduced, there is a strong likelihood for patients on opioid medication to seek an alternative treatment that could provide immediate pain relief [3].

Mitragyna speciosa (Korth, Rubiaceae) or kratom is a native medicinal plant of Southeast Asia [4]. Many extensive surveys have reported kratom's beneficial effects in reducing pain and managing opioid dependence and physical withdrawal [5,6,7]. As a new herbal alternative, kratom has attracted attention from researchers worldwide and is used widely by people or patients who are dependent on prescription and illicit opioid use [8,9,10]. However, kratom use may also cause substance dependence, where a number of users reported experiencing negative effects associated with physical and emotional symptoms of

withdrawal following kratom abstinence [10]. Previously, it was documented that anxiety is one of the negative emotional effects associated with withdrawal [10-12]. In fact, kratom users were reported to use benzodiazepine to treat kratom dependence and manage their withdrawal symptoms [8], which suggested the presence of anxiety following kratom abstinence. Earlier, most of the descriptive reports and surveys described the adverse and withdrawal effects of kratom as relatively mild compared with the classical opioids [5,8,12]. Nonetheless, scientific evidences on the assessment of withdrawal severity of the plant extracts or its active compounds are still very limited.

The pharmacological effects of kratom are largely mediated via opioid receptors and believed to be attributed to its constituent alkaloids. Mitragynine (MG), the main active alkaloid extracted from the leaves of *Mitragyna speciosa* Korth which accounts for 66% of the total alkaloid [4,13] was shown to modulate opioid receptors. It acts as an agonist at mu-opioid receptors [14,15] and antagonist at delta and kappa opioid receptors [16]. Previous studies reported various pharmacological effects of MG that include antinociceptive, anti-inflammatory, antidiarrhoeal, antitussive, antidepressant, and anxiolytic properties, highlighting the therapeutic potential of MG [17-19]. Although many kratom-related studies were conducted in the past decades, however the evaluation of MG dependency and withdrawal, especially its association with negative emotional states is still lacking.

Our previous work has successfully demonstrated a suppression of food-maintained operant responding in naloxone-precipitated MG withdrawn rats [20] that indicated the development of MG physiological withdrawal effects. In order to complement the aforementioned study with the emotional signs of withdrawal, simple models of the anxiogenic components following naloxone-precipitated MG withdrawal was proposed. Open field test (OFT) and elevated plus maze test (EPM) are exploration-driven paradigms that measure animal's propensity to remain in a relatively safe area (the OFT peripheral zone and the EPM closed arms) as opposed to a more aversive area (the centre of the OFT and the open arms of the EPM) [21]. These behavioural tests are often used together as a measure of anxiety in rodents [19]. In fact, EPM has been used extensively as an assessment tool of withdrawal from chronic opioid use where it showed sensitivity to both spontaneous and antagonist-precipitated withdrawal [22]. Therefore, the present study was conducted with the aim to assess anxiety-related behaviours resulting from antagonist-precipitated MG withdrawal using both OFT and EPM tests. In addition, the somatic signs of withdrawal were also assessed through behavioural scoring.

MATERIALS

Animals

Male Sprague Dawley rats weighing between 200-250 g at the start of the experiments were obtained from Animal Research Facility, Advanced Medical and Dental Institute, Universiti Sains Malaysia. Rats were socially housed in standard laboratory polycarbonate rat cages containing six individuals per cage and were acclimatised for 1 week prior to the study. All animals were maintained in a temperature-controlled room (21 to 22 °C) under a 12-h light/dark cycle. Food and water were available *ad libitum* throughout the experiment. Animal maintenance and experimental procedures were performed according to the local ethical requirements and guidelines recommended for the use of experimental animals that the Animal Ethics Committee approved of Universiti Sains Malaysia (AECUSM) [Reference number: USM/IACUC/2020/(125)(1095)].

Drugs

MG was extracted, isolated and verified from fresh leaves of *Mitragyna speciosa* at the Centre for Drug Research, Universiti Sains Malaysia, Penang, Malaysia as described in previous study [23]. The purity of MG extracted was approximately 98%, confirmed by HPLC and nuclear magnetic resonance (1H-NMR) analyses. Morphine hydrochloride was purchased from Pharmaniaga Berhad (Malaysia) and naloxone hydrochloride was obtained from Sigma Chemical Co. (USA). All drugs were dissolved in physiological saline (0.9% NaCl) with 20% (v/v) Tween-80 and diluted to the desired concentrations prior to experiments. Drug and vehicle injections (1.0 ml/kg per body weight) were administered via intraperitoneal route (i.p.).

Behavioural Apparatus

Open-field Test

The open-field (OF) apparatus (Panlab, Spain) consisted of a black painted arena (45 x 45 cm) with 50-cm high walls. The arena was virtually categorized by software (Acti Track, Panlab, Spain) into central and peripheral zones. Behavioural activities in the central zone, including the number of entries and the percentage of time spent in the central zone were automatically recorded by infrared beams and were used as a measure of anxiety. The overall motor activity during the open-field test was considered as the total distance travelled.

Elevated Plus Maze Test

The stainless steel EPM apparatus (Columbus Instruments, Ohio, USA) was consisted of four arms with two open (50 x 10 cm) and two closed (50 x 10 x 40 cm) arms which extending from a central platform (10 x 10 cm). The two enclosed arms had 50 cm high side walls and the entire maze was elevated to a height of 50 cm above the floor. The time spent on the open and closed arms and the number of entries made into each arm were recorded using a video camera (Sony). Arm entries were defined as entry of all four paws into an arm. The percentage of time spent on open arms (100 x time on open arms/total time spent in all arms) and the percentage of open arm entries (100 x number of open arm entries/total entries into all arms) were used as a measure of anxiety [19].

METHODS

Induction of MG and Morphine Dependence

Rats were randomly divided into three experimental groups (n=9 per group); a) MG-treated group, b) morphine-treated group and c) vehicle control group. MG-dependent group was given escalating doses of MG (15, 20, 25, 35, and 45 mg/kg, i.p.), twice daily for 14 consecutive days at 0900 and 1600h. The doses of MG injection (mg/kg) for each day were summarised in **Supplementary Table 1**.

For the morphine-dependent group, rats were also treated with escalating doses of morphine (5, 7.5, 10, 12.5, 15 mg/kg, i.p.), twice daily for 14 consecutive days. The injected doses of morphine (mg/kg) for each day were summarised in **Supplementary Table 1**. The selection of MG and morphine dosing regimens as well as the route of administration in this study were adapted from a previous study that had successfully developed MG and morphine dependence in rats [20]. These drugs dependence induction regimen were based on previous reports used to establish morphine dependence in rats [24, 25] but with a slight modification to comply with the 1:3 ratio of morphine to MG doses which was extrapolated from MG discrimination data [26].

Following the 14-day protocol, a single dose of MG or morphine was injected in the morning of day fifteen. Rats from the control group were injected with vehicle solution twice daily for the first 14 days, then with a single vehicle injection on day fifteen. Naloxone (2 mg/kg, i.p.) was administered after 2 hours following the last injection of MG, morphine or vehicle on day 15 to precipitate withdrawal. All testing took place between 0900 and 1400, with varying injection time on the test day to produce appropriate interval between the injection and test sessions.

Open-Field, Measurement of Naloxone-Precipitated Withdrawal Signs and Elevated Plus Maze Tests

Prior to testing session, rats were habituated to the testing room for a duration of at least 1 hr. Following naloxone administration, each rat was immediately placed at the centre of the open-field arena and allowed to freely explore the apparatus for 30 mins.

During the open-field testing period, measurement of somatic signs induced by naloxone precipitated MG and morphine withdrawal was performed. The somatic signs of naloxone-precipitated withdrawal syndrome were assessed as described in previous studies [27, 28]. The occurrence of wet dog shakes, rearing, stretching, teeth chattering, jumping and grooming behaviour were counted and the frequency of diarrhoea was checked over a period of 30 mins. The counted and checked signs were multiplied with the respective weighing factors for evaluation of the withdrawal signs severity using scoring methods by Rahman et al. [27] and Hassan et al. [28]. **(Supplementary Table 2).**

Following the OFT and withdrawal behaviour scoring, the rat was then transferred to the EPM apparatus, placed in the maze center to face an open arm and allowed to explore the maze for 5 mins freely. An entry was counted when the rat's superior portion, including the head and neck, the shoulders, the forelimbs and forepaws and thoracic region, moved into an arm. Anxiety-like behaviour was determined by calculating the amount of time and number of entries each rat made in the open and closed arms and denoted by the percentage of the total time or number of entries. Between each trial, all apparatus was thoroughly cleaned with 70% ethanol to diminish the residual odours and traces of the previously tested animal.

Data Analysis

All graphical output data were expressed as mean ± SEM. Data were analysed by one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. Counts of each naloxone precipitated withdrawal signs were presented as means ± SEM, and the data were analysed by two-way ANOVA followed by Bonferrroni's post hoc test. A significance level of $p<0.05$ is used to test for statistical significance. Prism statistical software was used to perform the statistical analysis (version 5.01; GraphPad Software, Inc., San Diego, California USA).

RESULTS

There was no significant decrease in the number of entries and percentage time spent in the central zone by the rats following naloxone-precipitated MG withdrawal when compared to the vehicle control group, as shown in Fig. 1. The naloxone-precipitated MG withdrawn rats also showed no significant changes in the locomotor activity (total distance travelled, $p>0.05$). However, increased number of centre entries ($p<0.05$) and total distance travelled ($p<0.05$) were observed in morphine-dependent rats undergoing naloxone-precipitated withdrawal when compared with the vehicle control group.

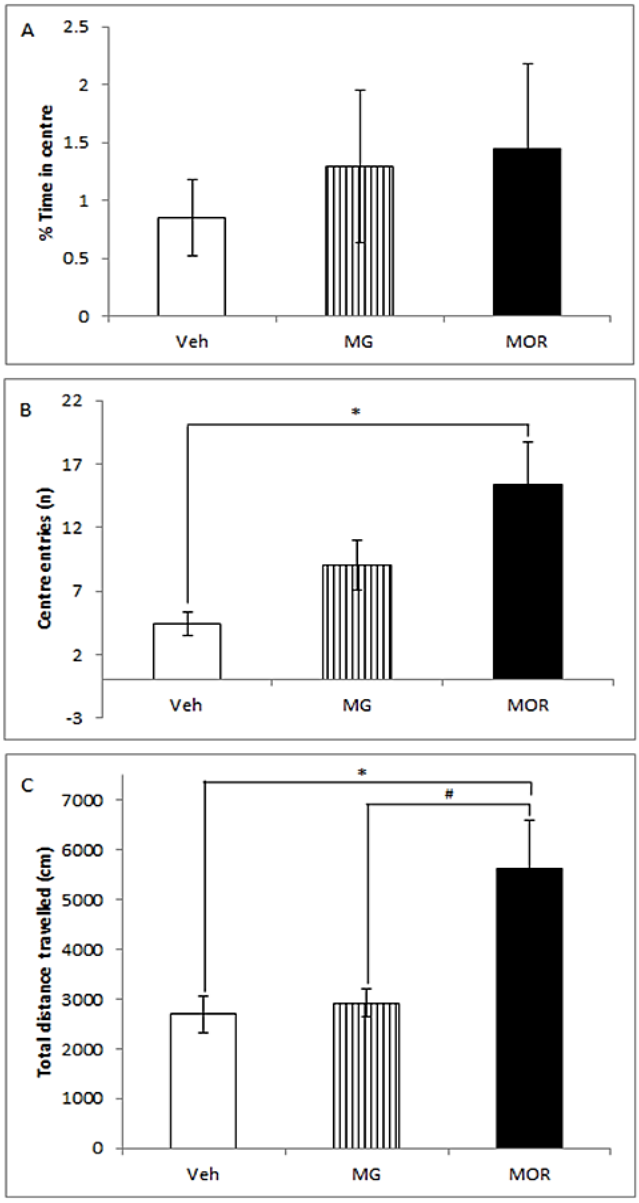

Figure 1: Effects of naloxone precipitated mitragynine (MG) and morphine withdrawal on the number of entries into the central zone (A), the percentage of time spent in centre (B) and the total distance travelled (C) in the open-field. Each bar represents mean ± SEM, n=9. *p< 0.05 when compared with the vehicle control group. #p<0.05 when compared with the MG group.

Fig. 2 demonstrated that naloxone-precipitated MG withdrawal did not reduce the number of entries ($p>0.05$) and percentage of time spent in the open arms ($p>0.05$) compared with the vehicle. Additionally, no significant difference in the number of total arms entries ($p>0.05$) was observed in the MG-treated rats when compared to the vehicle-treated rats. Similarly, naloxone-precipitated morphine withdrawn rats also did not exhibit significant effects on the percentage of open arm entries ($p>0.05$), time spent in open arms ($p>0.05$) and total arms entries ($p>0.05$) when compared with the vehicle.

Nevertheless, the data significantly showed that the rats entered the closed arms more frequently and spent longer time in the closed arms than in the open arms in all treatment groups [vehicle ($p<0.01$), naloxone-precipitated MG withdrawal ($p<0.001$) and naloxone-precipitated morphine withdrawal ($p<0.01$)].

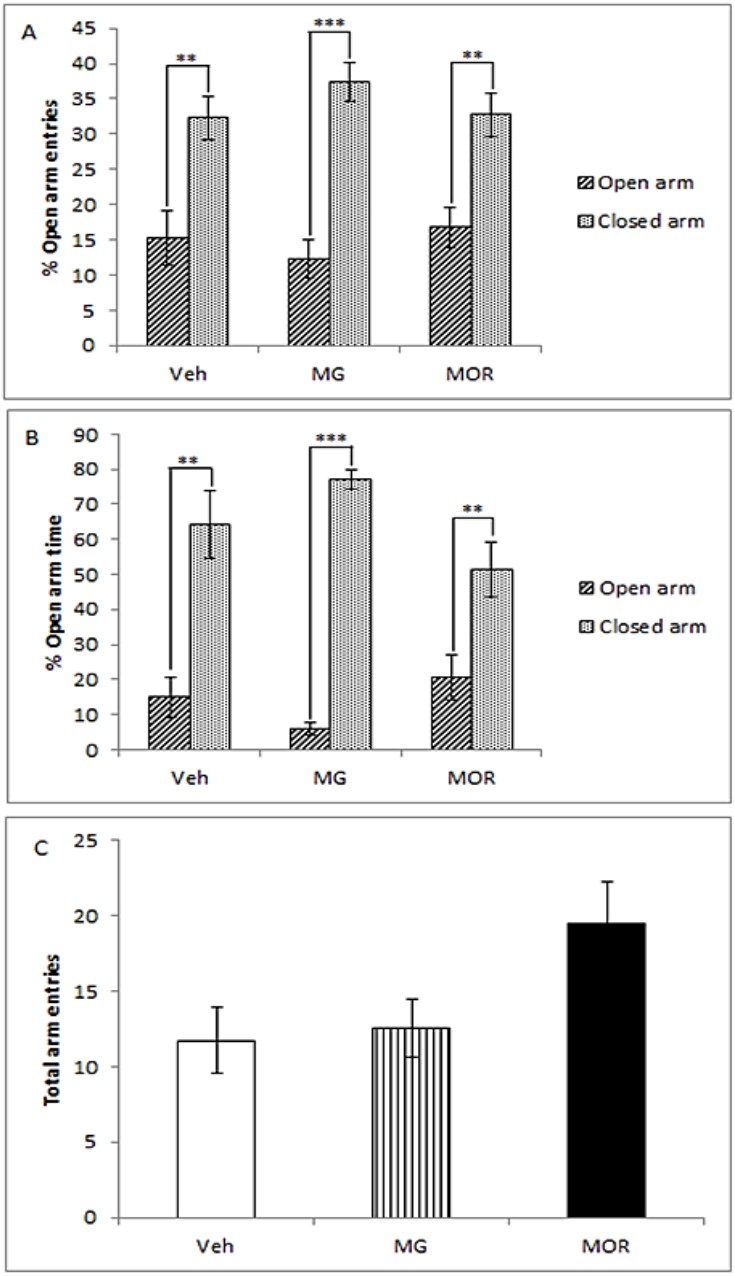

Figure 2: Effects of naloxone precipitated mitragynine (MG) and morphine withdrawal on the number of entries into open and closed arms (A), the percentage of time spent on open and closed arms (B) and the total arm entries (C) in the elevated plus maze (EPM) test. Each bar represents mean ± SEM, n=9. **$p<0.01$, p***<0.001 when compared between open and closed arms.

The global withdrawal signs occurrence for all groups are summarised in Table 1. In naloxone-precipitated MG withdrawn rats, the frequency of rearing ($p<0.001$) and chewing behaviours ($p<0.001$) were significantly elevated when compared to the vehicle control group. Other signs including wet-dog shaking and stretching were also observed in some, but not in all rats. The wet-dog shaking behaviour or sometimes called "whole body shake" involves the quivering shudder of the body up to the rat's shoulders. However, jumping and teeth-chattering behaviours were not observed. In contrast, naloxone-precipitated morphine withdrawn rats showed significant increase in the number of rearing ($p<0.001$), chewing ($p<0.001$) and grooming behaviours ($p<0.001$). The frequency of other signs including wet-dog shaking, stretching, jumping, teeth-chattering, and diarrhoea were also increased, but were not significant.

Table 1. Global withdrawal signs occurrence are presented as the means ± SEM of total number for 30 mins. Results for diarrhoea are expressed as the number of rats showing diarrhoea as a proportion of the total number of rats tested. ***$p<0.001$ compared with the vehicle control group.

Withdrawal signs	Vehicle-treated group	Mitragynine-treated group	Morphine-treated group
Wet-dog shaking	0 ± 0	0.22 ± 0.27	2.44 ± 0.54
Rearing	9.33 ± 0.97	18.67 ± 1.45***	63.78 ± 3.28***
Stretching	0.22 ± 0.27	2.22 ± 0.57	3.11 ± 0.52
Jumping	0 ± 0	0 ± 0	0.22 ± 0.27
Teeth chattering	0 ± 0	0 ± 0	1.11 ± 0.50
Chewing	7.11 ± 0.75	26 ± 1.15***	18.89 ± 1.25***
Grooming	24.89 ± 1.26	25.33 ± 1.52	37.11 ± 1.43***
Diarrhoea	0.22 ± 0.22	0.11 ± 0.19	0.44 ± 0.24

The analysis of the results using two way ANOVA revealed significant effect of drug treatment ($F_{2, 192} = 227.6$, $p<0.001$), withdrawal signs ($F_{7, 192} = 534.3$, $p<0.001$) and effect of the interaction ($F_{14, 192} = 105.6$, $p<0.001$) (Fig. 3). Bonferroni's multiple comparison test also confirmed the significant difference ($p<0.05$) of withdrawal scores between naloxone-precipitated MG withdrawal and vehicle control group. The result also showed a significant difference of withdrawal scores between naloxone-precipitated MG and morphine withdrawn groups ($p<0.001$) (Fig. 3).

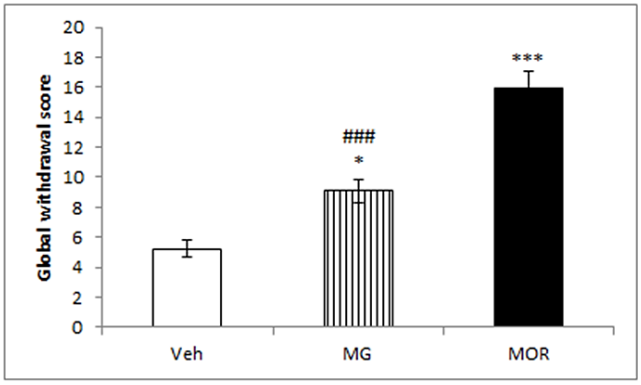

Figure 3: Effects of naloxone-precipitated mitragynine (MG) and morphine withdrawal on the global withdrawal score (means ± SEM, n=9). *$p<0.05$, *** $p<0.001$ when compared to the vehicle control group. ###$p<0.001$ when compared to the morphine group.

DISCUSSION

Studies have demonstrated that withdrawal from dependence-producing drugs would cause anxiety behaviour. While drug withdrawal is known to be anxiogenic in rats and both OFT and EPM were used to measure potential changes in anxiety-like behaviour induced by drugs withdrawal [29], the present study has characterize the OFT and EPM behaviours of rats during naloxone-precipitated MG withdrawal.

In the OFT, naloxone-precipitated MG withdrawn rats showed no significant effect on the percentage of centre time and number of centre entries. The similar insignificant effect was also observed in the total distance traveled, suggesting no motor function changes. In contrast, precipitation by naloxone to morphine-dependent rats showed a significant increase in both centre entries and total distance traveled, suggesting that morphine dependent rats had developed anxiolytic effects, which coincides with previous reports [30]. The increment in total distance observed from morphine-treated group could suggest that the rats had developed hyperlocomotion due to sensitisation similar to previous findings [14, 31]. Besides, the exposure to novel environment immediately before testing in the exploration-driven paradigm also increase motor activity [32] which could also associate with the greater likelihood of entering the central zones in morphine-treated rats.

However, in EPM, no significant decrease was observed in the open-arm time for both MG and morphine groups

after precipitated with naloxone. Decreased in time spending on open arm during withdrawal was anticipated because opioid withdrawal is usually associated with anxiety behaviour [22]. Hence, the rats will spend more time in the closed arm due to anxiety behaviour. However, similar finding was also reported in mice underwent naloxone-precipitated morphine withdrawal [33] which suggested that the variabilities (i.e. level of illumination, duration of testing, sex difference) could also influence the exploration-driven behaviours using the EPM [34]. In addition, this finding could also be due to the pre-exposure to OFT environment prior to EPM test. It was reported that pre-exposure to other testing environment prior to EPM could alter the subsequent behaviour of rats during the EPM test [32]. Besides, the utilization of single test session in EPM test could also influence the EPM behaviour where previous studies reported that second exposure to the EPM task relative to the first exposure had reduced the activity on the open arms [35-37]. Therefore, this possibility is worth considering, and careful considerations should be taken when planning a future behavioural experiment using EPM.

Drug abstinence also involves physical signs, aside from psychological symptoms that lead to anxiety [27,28]. Together with affective changes, physical symptoms also provide a predictive value for the severity of drug withdrawal. The finding from this study demonstrated a significant increase in somatic signs, including the number of rearing and chewing behaviour in naloxone-precipitated MG withdrawn rats. It is worth noting that naloxone-precipitated morphine withdrawn rats exhibited more withdrawal signs which coincides with previous reports that the physical signs of withdrawal appeared to be more severe and intense in naloxone-precipitated morphine withdrawal than those of MG [20]. The presence of somatic signs following naloxone precipitated MG withdrawal has confirmed that the dependence induction regimen adopted from Harun et al. [20] was effective for the occurrence of withdrawal arising from naloxone precipitation. This finding also coincides with previous reports, which had established the association between naloxone-precipitated MG withdrawal and physical signs of withdrawal [20, 38, 39].

In conclusion, this study suggests that the rats behaviour during the tests might not be driven solely by the levels of anxiety following naloxone-precipitated withdrawal but rather by other withdrawal-induced behaviours. Although absence of withdrawal-induced anxiety observed in rats during OFT and EPM tests, however, future studies involving other behavioural models (i.e. Vogel conflict test, forced swimming test, defensive-probe burying paradigm, marble burying test, social interaction test) should be conducted to measure other affective symptoms of withdrawal and corroborate the present findings. Nonetheless, the presence of global withdrawal score has provided strong support that discontinuation of MG or kratom is associated with physical symptoms of withdrawal. However, further studies are required to evaluate the severity of MG or kratom withdrawal to justify its use as opioid replacement therapy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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