Anxiolytic Effects of Thymoquinone in the Rats Exposed to MDMA
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Abstract—Ecstasy, 3, 4-Methylenedioxymethamphetamine (MDMA) is a psychostimulant drug capable of inducing psychiatric disorders in animals and humans. MDMA produces short-term pleasure effects but prompts to neuropsychiatric problems in the long-term, such as anxiety. The purpose of this study was to examine the anxiolytic potential of a natural compound known as thymoquinone (TQ) in rats that were stimulated to anxiety by MDMA. The administration of TQ into MDMA-induced 5-HT depletion rats was carried out in male Sprague Dawley via 1-week treatment dividing into four groups. The administration of TQ into MDMA-induced 5-HT depletion rats was carried out in male Sprague Dawley via a 1-week treatment dividing into four groups. The studied groups involved with the treatments comprise i) Control (1 mL/kg saline), ii) MDMA (20 mg/kg MDMA), iii) MDMA-TQ (20 mg/kg MDMA + 40 mg/kg TQ) and iv) TQ (40 mg/kg TQ). Anxiety test is subject to a light-dark test and social interaction test. Our results demonstrated an increase in anxiety behaviour in the MDMA group as compared to the control group. TQ prevented anxiety after undergoing a 1-week treatment in the MDMA+TQ group as compared to the untreated MDMA group. Hence, this study proposed that TQ could protect against anxiety-like behaviour upon MDMA exposure in rats.

Keywords—MDMA, ecstasy, anxiety, thymoquinone.

1. Introduction

Ecstasy, which is also known as 3, 4-Methylenedioxymethamphetamine (MDMA), is a synthetic drug that is included as a Class 1 drug in most countries despite it being legal and accepted in medical use in some countries. Over the last few years, the MDMA has suggested in the United States to use in a clinical trial as a tool to assist post-traumatic stress disorder (PTSD) treatment [1, 2, 3, and 4].

Unfortunately, the use of the MDMA in medicinal practice did not yet approve by the Food and Drug Administration (FDA). Despite many efforts attempted to legalize MDMA as an assisted psychotherapy in clinical settings, experts are worried about its harmful effects. The effects associated with MDMA are neurotoxic brain damage, memory impairment, psychological problems, behavioural disorders, and neurotransmitters alteration [5, 6, and 7].

In animal models, MDMA has been shown to confer animal behaviour and serotonergic neurotoxicity either in acute administration or repeated administrations. In MDMA exposure, the effects of MDMA vary depending on the frequency and duration of use and the dose [8]. In the clinical setting, MDMA administration showed a significant elevation in self-reported anxiety in adult volunteers who had never used the drug before [11]. A recent case report on MDMA described that a patient who ingested MDMA at a single dose had
developed anxiety for more than 2 months [12]. To date, there is no specific promising treatment available on the effects of MDMA. Several synthetic compounds that were introduced to treat the effects of MDMA include antidepressant drugs, dextromethorphan (DM), dextorphan (DX), ketoprofen, rilmenidine, and mephedrone. Most of the recent studies, suggestions from the previous researchers on the target and treatment for MDMA disorder, as well as ATS was focusing on attenuating neurotoxicity and neurotransmitters excitotoxicity in the brain by using synthetic drugs.

All of these synthetic drugs are accompanied by side effects. On the other hand, the uses of natural substances are widely believed as having no or less important side effects.

The active compound of *Nigella sativa* L. (Ranunculaceae), which is thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone) TQ, has traditional use as medicine and food. The active compounds of *Nigella sativa* modulate brain neurotransmitters, thus, producing its therapeutic effects [13]. These neurotransmitters such as 5-HT and dopamine (DA) in turn reduce anxiety, depression, and help in memory performance [14]. TQ has been extensively studied in the literature to have many therapeautic properties such as neuroprotective, gastroprotective, nephroprotective, anti-oxidative, anti-inflammatory, immunomodulatory and anti-microbial activities as well as anti-tumor effects [15]. Besides, anxiolytic effects of *Nigella sativa* and a decreased 5-HT turnover have been reported previously by Perveen et al. [16]. Hence, considering the potentials of TQ, our study aimed to evaluate the anxiolytic effects of thymoquinone (TQ) in the rats that were administered with MDMA.

2. MATERIALS AND METHODS

*The materials chemicals*

TQ was purchased from Sigma Chemical Co. (Beta Element Sdn. Bhd., Malaysia). d,1-MDMA HCl purchased from Labchem Sdn. Bhd., Malaysia. Both MDMA and thymoquinone dissolved in saline (0.9%). Anesthetize reagents (ketamine, zoletil, and xylazine) supplied from the Animal House, Universiti Kebangsaan Malaysia, UKM.

*Animals*

Sprague Dawley rats (230–250 g), was obtained from the Animal House, Universiti Sains Malaysia (USM) and housed under a controlled environment (22 ± 2°C temperature, 12 hours light and dark cycle, and 50% relative humidity) with food and water provided ad libitum. The animals acclimatized to the environment seven days before starting the experiments. The protocol of this study approved by the Animal Experimentation Ethics Committee of Universiti Sultan Zainal Abidin (ID: UAPREAC/04/18/001) In Rats were given with a single dose of the MDMA (20mg/kg) through oral gavage to induce anxiety. The dose 20-
mg/kg used as previously shown to produce 5-HT depletion and neurotoxic effects in the laboratory rats [17]. The oral route was selected because MDMA is usually taken orally by humans [18].

**TQ treatment**

The method of TQ treatment and dosage stated in a study by Ullah et al.[19] The rats receiving TQ treatment were administered with 40mg/kg, TQ once a day starting from the first day of the experiment until day seven.

**Experimental Design**

The rats were randomly divided into 4 groups (n=6). The details of the groups involved were as follows:

Group 1: a negative control group. The rats received 1 ml/kg of normal saline every day until seven days.

Group 2: MDMA group. The rats were given with MDMA (20 mg/kg) through oral gavage, followed by 1 ml/kg normal saline every day until seven days. Group 3: MDMA+TQ group. The rats received 40 mg/kg TQ in the first day before the administration MDMA (20 mg/kg), followed by 40 mg/kg TQ on the next day until day seven. The rats received 40 mg/kg TQ in the first day before the administration MDMA (20 mg/kg), followed by 40 mg/kg TQ on the next day until day seven. Group 4: TQ control group. The rats were given 40 mg/kg TQ during the first day until the last day of the experiment. The MDMA injection procedure was conducted at an environmental condition of 25°C as it could produce hyperthermia and exacerbate MDMA neurotoxicity [20]. The weight of each animal recorded every day during the experiment. In the end experiment, a subsequent anxiety test conducted.

**a) Light Dark Exploration test (LDT)**

Light/dark Exploration test (LDT) is widely used to measure anxiety behaviour in rodents, including the rats. The test was developed by Crawley and colleagues to study the anxiolytic effects of benzodiazepine in mice, where he observed that the anxiolytic drug increased the number of crossings between light and dark compartments [21]. The use of the LD test was expanded later apply to rats, and these rats were used by researchers to perform this test. Researchers observed that the distance travelled in the light area and the time inside the compartment, reflecting an anxious behaviour [22].

In the current study, LDT conducted based on the natural behaviour of the rat to have an aversion to the brightly illuminated areas and on their spontaneous exploratory behaviour in response to mild stressors, such as brightness and new place or environment [23]. In this study, the LD test conducted as described by Takao & Miyakawa with slight modification [24]. The apparatus used for the light/dark test consisted of a box (21 × 42 × 25 cm) divided into two sections of equal size by a partition with a door. The apparatus used for the light/dark test consisted of a box (21 × 42 × 25 cm) divided into two sections of equal size by a partition with a door. One box brightly illuminated by white diodes and the other box dark. Rats placed on the light side, and the door opened after 3 seconds. The rat was allowable to explore the box for 5 minutes. The recording
carried on time of rats stays in the light area and the latency of the head emergence of the dark area.

**b) Social Interaction test (SIT)**

The social interaction test was the first model of anxiety-like behaviour that was developed by File & Hyde [25]. In this study, the test performed by placing two rats of different partners in a cage filled with sawdust. Partners selected roughly equal to the bodyweight and same treatment condition, like MDMA, or vehicle, which is from different home cage. [10] Each of the social interaction sessions lasted for 5 minutes. Each of the social interaction sessions lasted for 5 minutes, the number of these interactions recorded. Behaviours recorded as social interaction included sniffing, following, crawling over/under, adjacent lying, and mutual grooming [10, 26].

**Data Analysis**

Statistical analysis was performed using GraphPad Prism version 6. The data for anxiety-like behaviour were analysed by nonparametric test Kruskal-Wallis test followed by Dunn's multiple comparisons test. The data are expressed as mean ± SEM. Treatment differences are considered statistically significant at $P \leq 0.05$.

**2. FINDINGS**

The effect of anxiety behaviours in rats was determined by two tests, which include the light/dark exploration test and social interaction test.

**Light/Dark Exploration Test**

The results for the anxiety-like behaviour, as measured by the light/dark, exploration test is shown in Figure 1 (a, b, and c). The following behavioural measures recorded include; the latency of first passage to the different compartment, time spent in the light area, and the emerging to light area.

The result showed that the single administration of MDMA (20 mg/kg) in the MDMA group increases the latency of first passage into the dark compartment as compared to the control group, although it was not significant. Moreover, it found out that the TQ treatment for a week had shown a decreased interval for the first passage in the MDMA+TQ group. The TQ control group showed no significant difference in the latency of the first passage with the control group.

The second parameter of the light/dark exploration test, i.e., the time spent in the light area, demonstrated no significant difference between the groups. Further analysis carried out to evaluate rat's tendency to develop anxiety by investigating the number of the emergence of rat's half body or head enter the light area. Our results showed that the MDMA group had a significant increase in the number of emergences in the light area as compared to the control group ($P \leq 0.05$). Meanwhile, TQ treatment showed an insignificant decrease
in the number of emergences into the light area in the MDMA+TQ group as compared to the MDMA group. The TQ control group showed an insignificant increase in the entering number to the light area as compared to the control group.

a.

![Graph showing latency (in seconds) for different groups.](image)

b.

![Graph showing time spent in the light area (in seconds) for different groups.](image)

c.

![Graph showing number of emergences into the light area for different groups.](image)
Figure 1 The effects of MDMA and TQ on the anxiety parameters in rats after the administration of a single dose (20 mg/kg orally) MDMA and TQ (40 mg/kg, orally) for 1 week, i.e.; (a) the latency of first passage entering dark area; (b) the time spent in the light area; (c) the number of emergence into the light area. The data are expressed as the mean ± SEM, (n= 6) ^aP≤0.05 vs control. Statistical analysis was done using nonparametric test Kruskal-Wallis followed by Dunn’s Multiple Comparison Test. TQ= Thymoquinone). The result for the anxiety-like behaviour, as measured by the social interaction test is shown in Figure 2. A single administration of MDMA (20 mg/kg) resulted in decrease time spent on social interaction than that of the control group (P≤0.05). TQ treatment had significantly shown an increased social interaction time in the MDMA+TQ group as compared to the MDMA group (P≤0.05).

DISCUSSION

By contrast, MDMA treated rats demonstrated less preference towards the novel object as compared to the saline-treated rats, indicating that the MDMA treatment attenuates the recognition memory of the rats. These group differences were very significant (P≤0.05). TQ treatment before MDMA administration led to a significant increase in the recognition index compared with the MDMA group (P≤0.05). The difference between the saline group and MDMA + TQ group was significant (P≤0.05). The group treated with TQ alone showed an insignificant difference in the recognition index as compared to the saline-treated group.
Two different anxiety tests on the animals performed in this study, the first test the light/dark exploration test, and the second test is the social interaction test. The results of the light-dark test demonstrated an insignificant increase of anxiety-like behaviour in the MDMA group as compared to the control group when measured by the latency for the first movement of the rats into the next compartment. Apart from that, it finds that the rats in the MDMA group had higher anxiety-like behaviour than that of the control group; when assessing the number of the emergence of half of the body/head entered the light area. Unfortunately, the time spent in the light area showed in homogeneously.

For example, instead of having increased time spent for the control group and TQ group, which indicates no anxiety or reduce anxiety behaviours, the results obtained show a contradicting finding, in which, the time spent in the light area reduced in two groups as compared to MDMA group This finding opposite to the initial hypothesis, which is expecting the MDMA group would have a decreased time spent in the light area when compared to the control group, indicating an anxiety sign. Therefore, a contradicting result observed that interestingly discussed the anxiety behaviour in the rats.

Based on these findings, the evidence of anxiety caused by MDMA by the light/dark test was not strong enough to conclude that the MDMA exposure had cause anxiety to the rats. In this case, there should be several factors that influence the results of anxiety behaviours using the light/dark test. Firstly, the age of the animals might affect the sensitivity of the light/dark test; either they were adults or adolescences, thus influencing the result of the light/dark test. Adolescent male rats, which are around postnatal day 28, would have either more or less anxiety-like behaviour than adults, and adolescent male mice found with greater anxious like behaviour than adults [27, 28, 22], [29]. However, the adolescents also reported exhibiting greater anxious to stress, but are less sensitive to the anxiogenic effects of drugs targeting the endocannabinoid and serotonergic systems.

The result of this study that used adolescence rats, thus the anxiety behaviour might be difficult to be observed due to their less sensitivity to the effect of MDMA that was known could alter the serotonergic system. This study analyses that the changes in the anxious behaviour regarding the time spent in the light area after the MDMA treatment not significantly different from the control group. As mentioned earlier that the rats in the control group might experience stress conditions rather than anxiety, thus resulting in a decrease in the time spent in the light area.

To further support the result and explanation, there is the second reason behind this present finding. The time spent in the light or dark compartments was not significantly affected by MDMA because the results could be a consequence of the anti-exploratory properties of MDMA [30]. Hence, this could answer the contradicting result between the increased times spent in the light area, and increase the latency of the first passage in the MDMA group. Hence, this could answer the contradicting result between the increased times spent in the light area, and increase the latency of the first passage in the MDMA group. The TQ control
group had a higher time spent in the light area than the control group.

As explained previously, the result of time spent in the light area obtained in this study was not very clear whether the administration of MDMA could cause anxiety or not. Therefore, it was unclear whether the effect of TQ was due to the anti-anxiety effects of TQ or anti-depressant. In these cases, there would be more inhibitory effect of the TQ against stress on rats regardless of the anti-anxiety effect, as mentioned earlier that the control group might experience stress rather than anxiety. Overall, without ignoring the results of the other two parameters (latency of first passage and emergence into the light area, we conclude that the rats administered with MDMA could result in anxiety-like behaviour when tested by light/dark exploration test. Here, we agreed with the study by Maldonado and Navarro that reported an anxiogenic-like activity of rats administered with MDMA in the light/dark test even though the result was not significantly affected by the drug [30]. We also concluded that TQ treatment was able to reduce the anxiety-like behaviour in the rats even though it was not significant.

To further support the available result, another anxiety-like behaviour test was carried out on the rats, i.e., the social interaction test. The result showed a significant decrease in social interaction in the MDMA group as compared to the control group. On the other hand, the MDMA+TQ group demonstrated a significant increase in social interaction when comparing it with the MDMA group. This second test supports the result of the light/dark test on the anxiety behaviours of the rats. This study postulates that the animal models of anxiety behaviours by using social interaction were more sensitive to the effects of anxiogenic compounds like MDMA [30, 31]. Hence, this study concludes that MDMA develop anxiety-like behaviour in the rats by considering all of the factors and parameters tested. Moreover, a clinical study demonstrates psychological changes of patients, in which the excitement feeling in the volunteers of MDMA users was reduced significantly four days after MDMA ingestion [32].

In contrast, the treatment with TQ successfully reversed the effects of MDMA. The results of the study found that both of the control groups and the TQ control group. However, when comparing the anxiety behaviour of those two groups, there was no significant difference. Reduced anxiety-like behaviour in the rats could be due to the anti-anxiety property of TQ. Unfortunately, in the current study, the detailed mechanism on the TQ reduction of anxiety is not explored, which did not identify the genes associated with anxiety such as tryptophan hydroxylase-2 [33], SERT [34], CMYA5, MCTP1, RXRG, and TNR [35].

Besides that, biochemical regulation, such as the 5-HT, plays an important role; in the modulation of animal behaviours [36, 37]. Our previous report indicated that the treatment with TQ enhanced the level of 5-HT in MDMA+TQ treated rat compared; to depletion in the 5-HT level in the MDMA group [38]. Hence, the enhancement of 5-HT level by TQ possibly one of the factors that contribute to the decreased anxiety levels in the rats. Further studies on the correlation between these two components (5-HT level and anxiety level) were needed to support the theory. A study on animal models of depression demonstrated that TQ (20 mg/kg)
was able to elevate the 5-HT levels and reduce depression in mice [39]. Besides that, Perveen and his colleagues indicated in their study that a repeated administration of Nigella sativa to the rats had increased brain level of 5-HT, eventually produced an anti-anxiety effect in rats when tested in an open field test and elevated plus maze [6].

Apart from the overall normal function of the serotonergic system to determine anxiety development not solely through the neurotransmission; based on the result that we obtained, TQ probably prevents the severe effects of MDMA on the serotonergic system via its protection on the 5-HT receptors. Several studies provided evidence on the relationship between anxiety and dysfunction of the serotonergic system, especially 5-HT receptors. For example, Daniels et al., (2000) reported in their study that the number of serotonin, 5HT-1a receptors in the rats decreased significantly, in anxious rats as compared to controls [40]. They also suggested that the serotonergic system plays a role in the manifestation of anxiety behaviour in the rats. The role of 5HT-1a receptors in anxiety proposed by Garcia-Garcia et al. (2014), later hundreds of publications have taken base on this research [41]. As for TQ effects on the modulation of 5HT-1a receptors, in our knowledge, lack of evidence has been shown, but conceptualized, that Nigella sativa oil was reported to induce the cardiovascular depressant effects mediated via 5-HT mechanisms through the activation of the 5HT-1a receptor.

In addition, other proposed pathways related to the anxiolytic effects of TQ evidenced in the literature studies, which explain the mechanism in this study.

In the current study, the TQ could protect the animal from anxiety via its property as an anti-oxidative agent. As a study report, the rats induced by arsenic and supplemented the TQ for three days, at 2.5 and 5 mg/kg/day to reduce anxious behaviour, in an elevated plus-maze and open field test, which attributed to its anti-oxidant and anxiolytic properties [43]. Besides that, another study indicates, the TQ at 20 mg/kg administered to the mice produced antianxiety-like effects through the modulation of GABA and NO levels [44]. Hence, researchers of this study hypothesize the effects of the TQ in reducing anxiety in the MDMA induced-neurotoxic rats are contributing through different mechanisms that are worth exploring.

5. Conclusion

The current study suggests TQ would produce anti-anxiety effects in the rats that exposed to MDMA. This study also suggests that the social interaction test more sensitive than the light/dark test to observe the anxiety effects in the rats, especially the rats that administered with MDMA. It was analysed that TQ treatment attenuated the sign of anxiety in rats, which is tested by the social interaction test, which indicates an increase in social interaction time. In addition, it was found that TQ treatment attenuated the sign of anxiety of rats, particularly tested by the social interaction test, which was indicated by the increase of social interaction time. Hence, it would be worthwhile in the future to discover more about the potential of TQ regarding its mechanism of actions in attenuating the anxiety effects of MDMA.
6. Acknowledgement

The authors wish to thank the UniSZA Fundamental Research Grant Scheme FRGS/1/2018/SKK10/UNISZA/01/1, grant from Malaysia Ministry of Higher Education for funding.

7. References


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