Journal of Research in Pharmacy

Research Article

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Investigation of the pharmacological potential of myricetin on alcohol addiction in mice

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Received: 13 March 2022 / Revised: 18 May 2022 / Accepted: 19 May 2022

ABSTRACT: Alcohol addiction is one of the leading causes which is associated with morbidity and mortality with outcomes in high healthcare and economic costs. Myricetin is a flavonoid that demonstrates therapeutic actions in many central nervous system diseases. In the current study, the conditioned place preference (CPP) tests were performed to examine the effects of myricetin on ethanol reward. During conditioning, intraperitoneal (i.p) administration of ethanol (2 g/kg) and serum physiologic were given on alternate days for 8 days. In order to evaluate the effect of myricetin on the development of alcohol addiction, myricetin was injected into mice 30 minutes before ethanol administration. Subsequently, a daily myricetin injection was performed to evaluate the effect of myricetin on the extinction of alcohol addiction. Finally, ethanol was administered 900 seconds after different dose myricetin administration, and reinstatement was evaluated immediately thereafter. Systemic ethanol (2 g/kg, i.p) administration significantly produced CPP. Myricetin (5 and 10 mg/kg, i.p) attenuated the development of ethanol addiction ($p < 0.05$). Systemic myricetin injections immediately after each extinction period precipitated extinction and decreased reinstatement (10 mg/kg, i.p, $p < 0.05$, respectively). Ethanol alone and in combination with myricetin did not change locomotor activity and motor coordination. As a result, it can be suggested that myricetin is effective in attenuating the rewarding effect of alcohol in mice and can be used for the adjunctive therapy for alcohol addiction. In addition, it will be appropriate to conduct mechanistic experimental studies regarding these results in the future.

KEYWORDS: Alcohol addiction; myricetin; conditioned place preference (CPP); mice

1. INTRODUCTION

Compulsive alcohol use has a share of approximately 5% worldwide [1]. Alcohol addiction leads to 5.9% of all deaths worldwide and more than 25% of deaths in the age level 20-39 years [1, 2]. By many measures, the harm from alcohol far outweighs the harm from illicit drugs. Alcohol addiction is a chronic disease characterized by frequently repeated remission and recurrence/relapse. According to the DSM-5, alcohol addiction is described as a collection of brain function disability and uncontrollable behaviour, including absence, tolerance development, uncontrollable drinking, and seeking (craving) for alcohol [1-3]. Although there are various behavioural treatments for alcohol addiction, the success rate is low. Pharmacotherapy is not enough for alcohol addiction [1]. In addition, although behavioural treatments are applied together with pharmacotherapy, it is seen that the success of treatment is low [2]. It is claimed that drugs for alcohol addiction were not developed due to their low commercial potential [1, 2]. However, the

How to cite this article: Yunusoğlu O, Shahzadi A, Akünal Türel C, Demirkol MH, Berköz M, Akkan AG. Investigation of the pharmacological potential of myricetin on alcohol addiction in mice. J Res Pharm. 2022; 26(4): 722-733

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.170

data obtained show the opposite. Furthermore, uncontrolled alcohol consumption can cause a variety of diseases (e.g., liver diseases, mental diseases, cancers, neuropsychiatric disorders, diabetes, digestive diseases, cardiovascular diseases, etc.), alcohol-related accidents, economic losses, and violence [1-4].

Drugs with three different mechanisms (mu-preferring opioid antagonist naltrexone) are currently approved for the medicine of alcohol dependence by the Food and Drug Administration or the European Medicines Agency [1, 3]. The oldest, disulfiram (aldehyde dehydrogenase inhibitor), naltrexone (mu-preferring opioid antagonist), and acamprosate (homo-taurine analogue; glutamatergic mechanisms) remains considerable widely utilized [3]. Since these pharmacological agents are ineffective in the treatment and have many side effects, there is a need to develop new pharmacological molecules that are more effective and have fewer side effects. Although positive results are seen during or immediately after behavioural treatments, high recurrence is seen within the year after treatment (60% to 90%) [1, 2]. Although different combined treatment strategies were developed, similar recurrence/relapse rates were observed [3, 5].

Myricetin, also known as arbutin, is an important flavanol commonly found in plants such as a natural product abundant in tea, fruits, vegetables, berries, and medicinal herbs, and they have various pharmacological actions [6]. Myricetin performs multiple important pharmacological processes, including potent anti-oxidant, epigenetic modulation, analgesic, hepatoprotective, anti-tumour, anti-diabetic, anti-allergic anti-microbial, anti-inflammatory, anti-amyloidogenic, anti-viral, anti-diabetic, anti-cancer, anti-ulcer, and cardioprotective properties [6-8]. In addition, the protective effects of myricetin on various central nervous system diseases have been shown in experimental and clinical studies [6]. In this sense, the anxiolytic, anti-Parkinson, anti-depressant, anti-neurodegeneration, anti-epileptic, anti-neuroinflammation and anti-Alzheimer effects of myricetin in central nervous system diseases can be given as examples [6-9].

Alcohol addiction, like different substance addictions, causes alcohol-seeking (craving) and maintenance of alcohol use, involving multiple neurotransmitter systems such as dopamine, glutamate, γ-aminobutyric acid (GABA), serotonin, and peroxisome proliferator-activated receptors (PPARs) system [10]. The publications review indicates that myricetin was demonstrated to affect the glutaminergic system, GABAergic system, peroxisome proliferator-activated receptors (PPARs) [11-16]. For example, an earlier investigation demonstrated that NMDA receptor antagonists reduced the development of ethanol-CPP [17-19]. The publications examination also indicated that GABA receptors and PPARs agonists reduced the development and reinstatement of ethanol-CPP [19, 20].

CPP is a method developed for the evaluation of addiction by experimental and clinical methods. However, it is widely used to study drug reward processes predominantly in rodents [21, 22]. CPP is a learned behaviour founded on Pavlovian conditioning. This method has been used to investigate the rewarding effects of abused drugs for many years [23]. CPP has face validity for drug addiction and dependence processes in humans [23]. Human substance abusers report robust relationships with environments in which they use drugs and substances; the procured stimulus characteristics of drug or substance-associated places can elicit craving and/or relapse in the abstinent addict [23]. Preliminary evidence defined in the subsequent section of this information demonstrates that the CPP process can be employed in a laboratory setting with humans [21, 23].

The purpose of this study was to determine the effects of myricetin on the acquisition/development and extinction and reinstatement of ethanol-induced CPP. Based on the results of the above scientific studies, we thought that myricetin would impact the rewarding effect of ethanol.

2. RESULTS

2.1. Ethanol Conditioned Place Preference Is Significantly Attenuated by Myricetin

The effects of myricetin on the development of ethanol-induced CPP are demonstrated in Figure 1. It can be stated that pre and post-conditioning ethanol administration had a significant impact on ethanol-induced CPP [$F(1,2\ 5) = 96.93$, $p < 0.01$, Two-way ANOVA]. A statistically significant effect was obtained for dosage groups [$F(4, 25) = 7.731$, $p < 0.01$]. There was also an interaction between pre-conditioning and post-conditioning tests (days), and myricetin doses [$F(4, 25) = 14.18$, $p < 0.01$]. 83 Ethanol administration at a dose of 2 g/kg produced statistically significant CPP in mice according to the post-hoc analysis ($p < 0.01$, Post-hoc Tukey's), which was similar to the combination administration, the myricetin 5 and 10 mg/kg plus ethanol 2 g/kg group significantly reduced the ethanol-induced CPP (Figure 1).

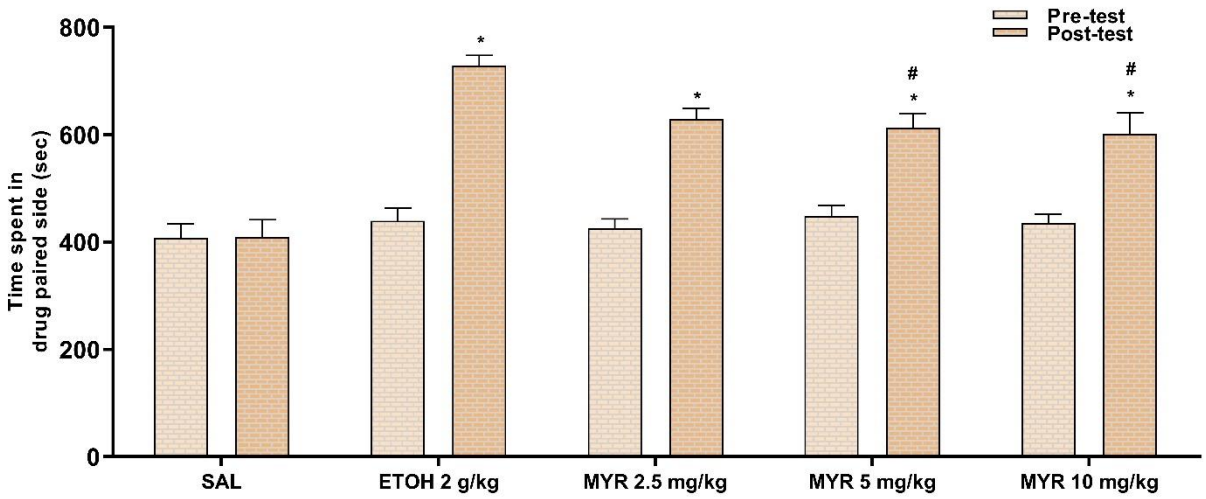

Figure 1. Effects of myricetin (2.5, 5, and 10 mg/kg) on the development of morphine-induced CPP in mice. Results are shown as mean (± SEM) values (n=6-8). *p < 0.01 versus the saline group; #p < 0.05 versus the ethanol group.

2.2. Impacts of Myricetin on the Extinction of Ethanol-Induced CPP

The impacts of myricetin on the duration for the extinction of CPP induced by ethanol in animals are shown in Figure 2. Comparison of extinction-1 [F (4, 25) = 25.90, p < 0.01; One-way ANOVA]; extinction-1 [F (4, 27) = 15, p < 0.01; One-way ANOVA]; extinction-2 [F (4, 27) = 4.804, p < 0.01; One-way ANOVA] and extinction-3 [F (4, 27) = 3.163, p < 0.05; One-way ANOVA] time spent in the drug-101 paired or ethanol-paired compartment between groups, there was a significant difference between the groups (Figure 2 A, B, C). Besides, one-way ANOVA indicated that the extinction-4 duration spent in the ethanol-paired chambers among groups was comparable [F (4,27) = 0.89, p > 0.05] (Figure 2 D). In Figure 2 demonstrated that myricetin at a dose of 10 mg/kg, i.p. was able to reduce ethanol-induced CPP on extinction-6 compared to the ethanol-paired compartment (p < 0.05; post hoc Tukey).

2.3. Impacts of Myricetin on Reinstatement of Ethanol-Induced CPP

The influence of myricetin on a single dose of ethanol triggering-induced CPP is shown in Figure 3, which demonstrated that the extinguished CPP was reinstated after the injection of a single low dose of ethanol (0.4 mg/kg) compared with the saline-paired chamber [F (4, 27) = 17.56, p < 0.01, one-way ANOVA] (Figure 3). This indicates that the CPP model was satisfactorily established. Pretreatment with myricetin (10 mg/kg, p < 0.05) reduced the effect of ethanol on reinstatement compared to the ethanol-paired chambers. The low doses of myricetin (2.5 and 5 mg/kg) reduced the impact of ethanol on reinstatement; nevertheless, the difference was statistically not significant (p > 0.05; Figure 3).

2.4. Influences of Myricetin on Locomotor Activity and Motor Coordination Ethanol-Induced CPP

There was no statistically significant change in locomotor activity after acquisition/development; post-conditioning, extinctions, and reinstatement tests caused by ethanol alone and co-administration with myricetin (p > 0.05) (Table 1). Ethanol alone and co-administration with myricetin did not change the remaining time of animals on the rota-rod apparatus (p > 0.05; One-way ANOVA) (Table 2).

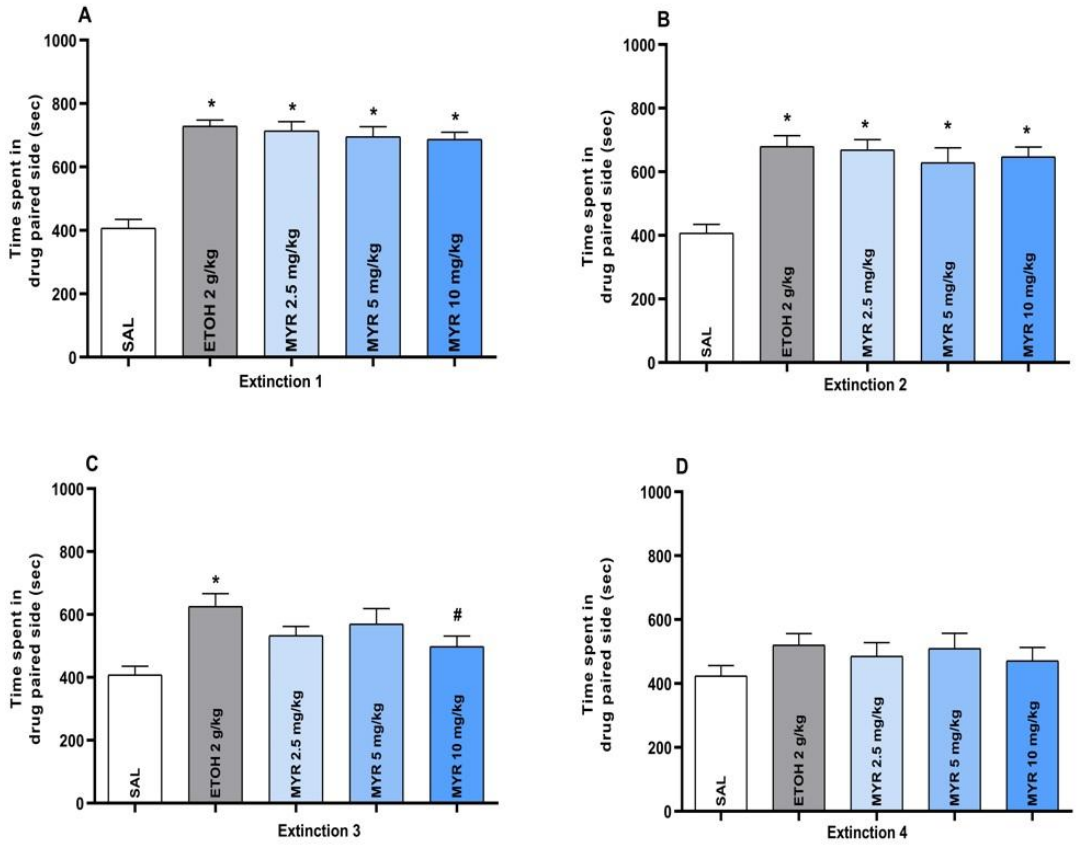

Figure 2. Effect of myricetin on the extinction of ethanol-induced CPP. Myricetin facilitated the extinction of CPP. Results are shown as mean (± SEM) values (n=6-8). *p < 0.05 versus the saline group; #p < 0.05 versus the ethanol group.

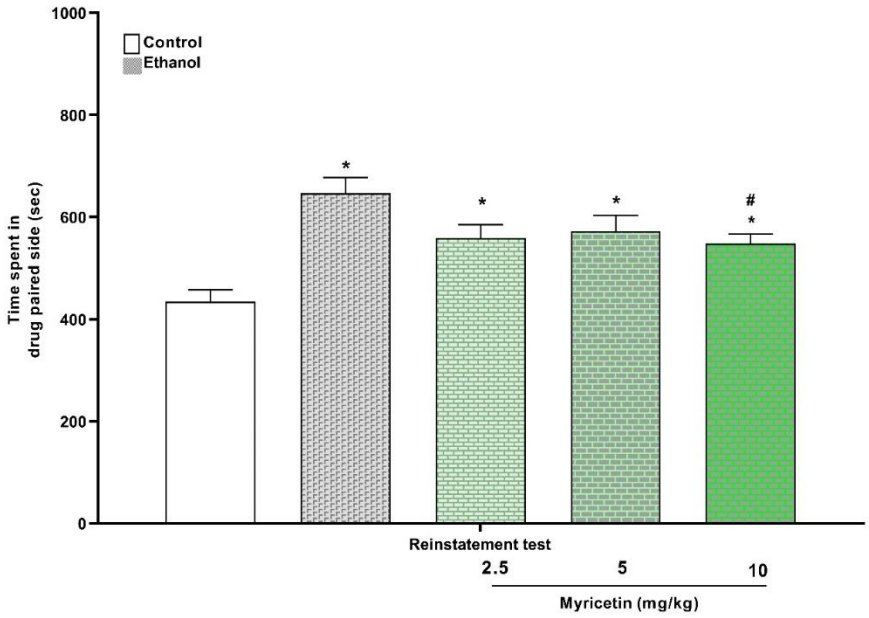

Figure 3. Effects of myricetin (2.5, 5 and 10 mg/kg) on the reinstatement of ethanol CPP caused by ethanol (0.4 g/kg). Results are shown as mean (± SEM) values (n=6-8). *p < 0.01 versus the saline group; #p < 0.05 versus the ethanol group.

Table 1. Effect of ethanol alone and in combination with myrcetin on locomotor activity in CPP stages in mice. Locomotor activity was assessed by dividing the floor of the CPP into 8 equal parts for 15 minutes. No statistically significant difference was found between all groups ($p > 0.05$). Results are expressed as means ± SED (n=6-8). Saline-SAL, Ethanol-ETOH, Myricetin-MYR.

Groups	SAL 12.5 ml/kg	ETOH 2 g/kg	ETOH 2 g/kg+ MYR 2.5 mg/kg	ETOH 2 g/kg+ MYR 5 mg/kg	ETOH 2 g/kg+ MYR 10 mg/kg
Postconditioning; Means/errors	117.3±9.55	136±12.79	124.5±12.23	128.7±12.05	115.±11.08
Extinction1; Means/errors	95.83±6.670	119.0±12.28	115.3±16.79	112.5±15.76	118.2±13.33
Extinction2; Means/errors	102.0±8.85	124.3±9.89	117.5±11.41	106.0±10.91	103.5±9.84
Extinction3; Means/errors	113.0±14.14	121.5±9.82	114.5±11.53	113.5±11.61	102.0±10.29
Extinction4; Means/errors	106.3±8.24	129.5±13.80	127.2±12.37	132.0±15.21	114.2±12.4
Groups	**SAL 12.5 ml/kg**	**ETOH 0.4 g/kg**	**ETOH 0.4 g/kg+ MYR 2.5 mg/kg**	**ETOH 0.4 g/kg+ MYR 5 mg/kg**	**ETOH 0.4 g/kg+ MYR 10 mg/kg**
Reinstatement Means/errors	110.8±11.61	134.0±16.37	127.2±9.31	121.3±18.05	118.8±17.20

Table 2. Effect of ethanol alone and in combination with myrcetin on rota-rod test in mice. No statistically significant difference was found between all groups ($p > 0.05$). Results are expressed as means ± SED (n=6-8). Saline-SAL, Ethanol-

Groups	SAL 12.5 ml/kg	ETOH 2 g/kg	ETOH 2 g/kg+ MYR 2.5 mg/kg	ETOH 2 g/kg+ MYR 5 mg/kg	ETOH 2 g/kg+ MYR 10 mg/kg
Postconditioning; Means/errors	134.2±16.31	131.3±15.98	112.0±4.52	143.5±14.22	140.7±16.31
Groups	**SAL 12.5 ml/kg**	**ETOH 0.4 g/kg**	**ETOH 0.4 g/kg+ MYR 2.5 mg/kg**	**ETOH 0.4 g/kg+ MYR 5 mg/kg**	**ETOH 0.4 g/kg+ MYR 10 mg/kg**
Reinstatement; Means/errors	131.2±15.95	124.6±17.17	117.2±16.18	120.5±19.50	128.0±20.46

ETOH, Myricetin-MYR.

3. DISCUSSION

Alcohol addiction is one of the widely prevalent mental health conditions related to important morbidity and mortality [1, 17]. While therapy options exist, the currently available pharmacotherapies for use disorder are not consistently effective, and less than twenty percent of individuals with a lifetime prevalence of use disorder have ever sought treatment [1, 17]. Hence, research on novel potential treatment approaches remains a priority.

In our research, systemic ethanol administration significantly induced CPP. These outcomes are consistent with other issued findings [24, 25]. Myricetin treatment significantly reduced the development of ethanol dependence. In addition, myricetin administration accelerated the extinction (disappearance) of ethanol-related addiction. Finally, myricetin slowed the reinstatement of low-dose ethanol-induced addiction. Systemic ethanol administration alone and in combination with myricetin did not alter locomotor activity and motor coordination. Drug doses were selected based on similar prior examinations [25-28]. These results are consistent with other published conclusions [25, 29].

It is not possible to say for certain by which mechanism myricetin affects. Myricetin has a broad pharmacology profile [6, 8]. In this sense, myricetin affects various mechanisms of alcohol addiction [8]. For instance, myricetin influences GABAergic, glutamatergic, nitrergic, and PPAR systems, which has been proven by various studies in alcohol addiction [6-8].

The GABAergic system plays a critical role in a broad scope of alcohol impacts, from initial intoxication to alcohol craving and relapse [30]. The γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter of the brain. Experimental models and clinical examinations have supplied proof to demonstrate the involvement of the GABA system in alcohol other substance addictions [30]. It was documented in different studies that GABA agonists decrease ethanol addiction [31, 32]. Myricetin has GABAergic activation that can contribute to the decrease of ethanol-CPP via this mechanism [7, 9, 13, 33].

Alcohol craving and seeking have been mainly related to glutamatergic changes in the brain reward system [30]. In various studies, the various pharmacological molecular inhibiting glutamatergic system reduced alcohol addiction [26, 30]. It has been previously demonstrated in different studies that Myricetin inhibits the glutamatergic system [8, 30, 34]. Through this mechanism, myricetin can decrease ethanol-CPP.

Nitric oxide is a potent messenger in the central nervous system and various kinds of evidence indicate that it mediates numerous ethanol impacts [35]. The different investigation has demonstrated that NO agents produce a marked decrease in ethanol withdrawal symptoms and ethanol-induced CPP in rodents [35, 36]. General, the publications examination demonstrates that myricetin decreases NO levels [8, 37-39]. Myricetin can lessen ethanol-CPP by utilizing this system.

Peroxisome proliferator-activated receptors (PPARs) are a subfamily of nuclear receptors [20, 40]. Three isoforms of PPARs have been recognized: α, γ, and β/δ [20, 40]. PPAR agonists have recently received attention for their potential to treat substance abuse [20]. PPAR agonists have recently attracted attention to their potential to treat alcohol and similar substance addiction [20]. It has been suggested by scientific studies that exogenous PPAR agonists attenuate alcohol, nicotine, and heroin addiction [20, 40]. In different studies, myricetin has been demonstrated to have an agonist effect on PPAR receptors [14, 41].

Despite the shown study delivering interesting findings and casting novel light on the vulnerability factors contributory to ethanol addiction, we identified some limitations that should be taken into consideration and developed in future investigations [26, 42, 43]. It should be highlighted that these experiments were performed on male mice. Nevertheless, sex differences are present for all the stages of drug and substance addiction in animals and humans [42]. In age groups, men have more heightened rates of use or addiction to alcohol and substances/drugs than do women [42, 44]. Nevertheless, women are just as possible as men to develop a drugs and substance use disease [42, 43]. Moreover, women may be further sensitive to craving and relapse, which are critical stages of the dependence process.

The combination of pharmacological techniques and exposure medicines accelerated extinction of craving and attenuation of the recurrence/relapse of substance addiction have been demonstrated by contemporary proofs [3]. A strategy to combine the approaches of precipitating the extinction development and reducing the motivational influence of substance-associated triggers on behavior might be the most beneficial and clinically useful for achieving a robust and ongoing suppression of relapse behaviors [45]. Based on the principles of classical conditioned extinction and learning, cue exposure therapy involves replicated exposure to drugs-associated or substance-associated cues to extinguish conditioned seeking or cravings and decrease the likelihood of relapse [26]. In common, the therapeutic agents evaluated in animal (e.g., mice, rats, monkeys, etc.,) models for relapse, target brain mechanisms that keenly control drug-craving and drug-craving seeking induced by relapse-recurrent effects [26, 46, 47]. The primary recovery strategy for any substance abuse contains management of acute withdrawal (detoxification), abstinence initiation, and relapse prevention [48]. Whereas the majority of the medical lookout is spent on the management of acute abstinence and short-term detoxification schedules, there is very little proof to offer these short-term interventions to improve long-term withdrawal rates [26]. The principal challenge of addiction treatment is to prevent relapse, which appears in more than >60% of newly withdrawn patients with alcohol disorders within three months. In this sense, myricetin also significantly reduced addiction reinstatement.

4. CONCLUSION

As a consequence, this study demonstrates significant effects of myricetin on development, extinction, and reinstatement in ethanol-induced CPP. Therefore, it can be proposed as a proof of the reducing of effect of myricetin on the development of ethanol dependence, accelerated extinction, and reduced reinstatement. Based on these results, myricetin may be considered as a promising agent for the development of pharmacological approaches for treatment of alcohol-addiction.

5. MATERIALS AND METHODS

5.1. Drugs and Chemicals

Ethanol and myricetin were obtained from Sigma-Aldrich. Ethanol (95%) was prepared 20% in a diluted serum physiologic and injected intraperitoneally (i.p) with 12.5 mL volume at a dose of 2 g/kg. Control groups were injected with serum physiologic solutions (0.9% NaCl) via the same route at the same volume. Drug solutions were prepared freshly on each experimental day. All drugs were injected at room temperature, drug treatment and behavior tests were conducted during the light period.

5.2. Animals and laboratory

Male adult Swiss albino mice (eight weeks old) were used in all experiments. The mice were preserved under normal laboratory situation/conditions (22-24 °C, 12 h light/dark cycle, lights on at 8 am, approximate humidity was about 50-60%) with *ad libitum* to tap water and rodent food. All experimental techniques were done in agreement with the Van Yüzüncü Yıl University and Behavior Guidelines for the Care and Use of Laboratory Animals and were approved by the local ethics committee under a protocol (permission number:1/2021).

5.3. Place Conditioning Paradigm

5.3.1. Conditioned Place Preference Apparatus

Place preference conditioning was performed as described elsewhere. Four individual devices made of Plexiglass were used to assess CPP. A two-chambers CPP apparatus, with different tactile cues in the chambers, was employed with open-able/shut-able gates between the compartments (20×20 ×20). The unbiased CPP procedure was based on our prior experiences and the publications. After each test, the CPP device was cleansed with 70% ethanol to mask the olfactory cues as much as possible.

5.3.2. Experimental Procedure

5.3.3. Behavior Testing

The experiment consisted of handling/habituation, pre-conditioning (day 1), conditioning (days 2-9), post-conditioning (day 10), extinction (days 10-22) and reinstatement (day 23) (Figure 4).

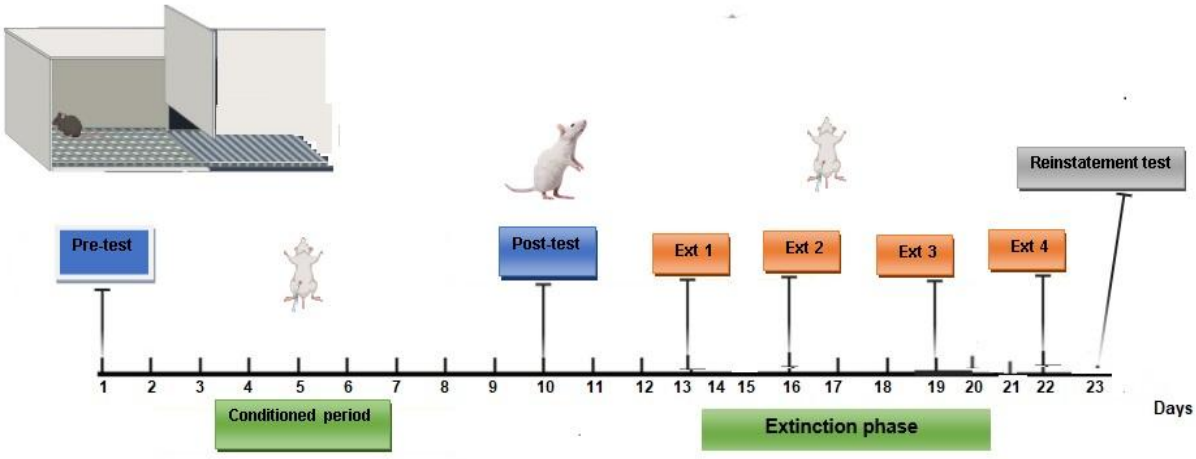

Figure 4. Schematic diagram of the CPP experimental design.

5.3.4. Habituation

In an effort to eradicate the possibility of any confounds and to reduce the stress levels of the mice, specific procedures were followed to provide habituated testing environments, handling. Mice did not receive any medication during this period.

5.3.5. Pre-conditioned

On day 1 (pre-conditioning), no injection, was placed in the central chamber with both gates open, and initial/spontaneous place preference was determined during the 15 min. At the end of the pre-conditioning test, mice were immediately removed from the CPP device and returned to their home cages. A preference standard was also employed, whereby mice that spent more than 66% [540 s] of the time exploring the preferred side were eliminated from the study.

5.3.6. Conditioning

One day after the preconditioning test, mice were started on an 8-day conditioning period. The mice were administered ethanol on days (2-4-6-8) and were administered serum physiologic on days (1-3-5-9) of the conditioning tests.

5.3.7. Evaluation of the Effects of Myricetin on the Development of Ethanol-Induced CPP

The mice were divided into five groups: the control group (saline + saline); the alcohol group (saline + alcohol) and the myricetin treatment groups (myricetin 2.5, 5, and 10 mg/kg + alcohol, respectively) (Table 3). During the conditioning phase 2.5, 5, and 10 mg/kg myricetin doses were administered i.p 30 min before alcohol injections [26, 27].

Table 3. Schematic diagram of the conditioned place preference (CPP) experimental design (development acquisition).

Study/period	Pre-test	Conditioning phase		Post-test
	Day 1	Day 2, 4, 6, and 8	Day 3, 5, 7, and 9	Day 10
Acquisition/ development	NA	Saline + Saline	Saline + Saline	NA
	NA	Saline + ethanol	Saline + Saline	NA
	NA	Myricetin+ ethanol	Saline + Saline	NA

CPP; conditioned place preference test; NA, not applicable

5.3.8. Post-conditioning Test

The pre-test period started one day after the conditioning period. After the door was opened on the tenth day, the mice were placed in the CPP apparatus and allowed to voluntarily choose for 15 minutes. The time the mice spent in each compartment for 900 seconds was recorded. On day 10 (test day), mice did not receive any drug.

5.3.9. Evaluation of the Effects of Myricetin on the Extinction of Ethanol-Induced CPP

To assess whether myricetin affected the extinction time of ethanol-induced CPP, mice were subjected to the daily extinction test for 12 days. During this period, mice were equally divided into 5 groups by administration of myricetin (2.5, 5, and 10 mg/kg, i.p) or saline 30 minutes prior to each extinction test. Immediately after myricetin (2.5, 5, and 10 mg/kg, i.p) or saline administration, mice were placed in the CPP and tested for 900 seconds. The preferences of the mice were recorded 900 seconds and evaluated (Table 4).

Table 4. Schematic graph of the conditioned place preference (CPP) experimental design (extinction and reinstatement).

Study/phases	Pre-test	Conditioning phase		Post-test	Extinction	Reinstatement
	Day 1	Day 2, 4, 6, and 8	Day 3, 5, 7, and 9	Day 10	Day 11 to 22 CPP-test	Day 23 CPP-test
Extinction and Reinstatement	NA	Saline + Saline	Saline + saline	NA	Saline + saline	Saline + saline
	NA	Saline + ethanol	Saline + saline	NA	Saline + saline	Saline + ethanol
	NA	Saline + ethanol	Saline + saline	NA	Myricetin + saline	Myricetin + ethanol

CPP; conditioned place preference; NA, not applicable

5.3.10. Evaluation of the Effects of Myricetin on the Reinstatement of Ethanol-Induced CPP

On day 22, the time spent in the rooms of the experimental groups was similar to the time in the preconditioning phase. On the 23rd day, the myricetin groups were injected with ethanol (0.4 g/kg) 30 minutes after the myricetin (2.5, 5, and 10 mg/kg) treatment and immediately tested. The saline groups were injected with saline in both injections (Table 4). The ethanol group was injected with ethanol (0.4 g/kg) 30 minutes after the physiological saline injection, and it was tested. Immediately after the second's injections, the animals were placed in the CPP set up and allowed to freely choose sections for 900 seconds and a camera recording was made (Table 4).

5.3.11. Locomotor Activity, Motor Coordination, and Conditioned Place Preference Determination

To assess locomotor activity, the floor of the CPP device was divided into 8 equal squares. The total square passes of the mice within 900 seconds were counted and evaluated. Mice were not injected in locomotor activity measurements. The rota-rod test was performed to evaluate the sensorimotor coordination and grip strengths of mice. The test was conducted 900 seconds after post-conditioning and reinstatement tests. The rotor was divided into two compartments which permitted two mice to be tested together. In the rotarod apparatus, a total of five tests were performed: the first 2 tests were "training," and the other 3 tests. The results were evaluated by considering the average of 3 tests. The maximum time allowed for the tests was determined as 5 minutes. The results were evaluated by considering the average of 3 tests.

5.4. Statistical Analysis

For data analyzed using GraphPad Prism software, version 8.1.3, a one and two-way analysis of variance (ANOVA) were utilized to determine the general interaction of drug treatment. Values of $p < 0.05$ were assumed to be statistically significant. Significant results were also analyzed using the Tukey's post hoc test.

Author contributions: Concept – O.Y; Design– O.Y., A.S., M.H.D., M.B; Supervision–O.Y., A.S., C.A.T.; Recources – O.Y., A.S., M.B., A.G.A., M.H.D; Materials – O.Y., A.S., M.B., A.G.A; Data Collection and Processing – O.Y., A.S., M.B., A.G.A., C.A.T., M.H.D; Analysis and /or Interpretation – O.Y., A.S., M.B., C.A.T., A.G.A; Literature Research – O.Y., A.S, M.B, A.G.A. M.H.D; Writing – O.Y., A.S; Critical Reviews – O.Y., A.S, M.B, A.G.A.

Conflict of interest statement: The authors declared no conflict of interest.

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