

THE EFFECTS OF RUTIN HYDRATE ON THE RESPONSE-REINSTATEMENT OF MICE RUNNING AN ALLEY FOR ETHANOL REWARD

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ABSTRACT: Pure compounds belonging to flavonoid family were studied for their biological potential such as rutin hydrate (3, 3', 5, 7-pentahydroxyflavone-3-rhamnoglucoside). These molecules were chosen based on their neuroactive properties. The evaluation of the in vitro de-addiction activity were exploited for the first time for such purposes. The aim of the present study was to investigate the effects of rutin hydrate (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside), a flavonoid that is an important dietary constituent of foods and plant-based beverages, on response-reinstatement of mice using running alley method of ethanol reward. Rutin (0.1, 1, and 10 mg/kg) was administered orally (p.o) one hour before post conditioning test, during extinction trials, and on re-instatement day. It was noted that rutin significantly ($P < 0.001$) increased runtimes on reinstatement day. No changes in body weight, food and water intake when compared with saline control group was observed. These results indicate that flavonoid rutin hydrate has a potential role in decreasing ethanol reinforcement. This can be used as a powerful food to delineate ethanol re-inforced running. Several mechanisms may contribute to the potential role of rutin in ethanol re-inforced running. Therefore, further studies are warranted to understand the exact mechanism of rutin hydrate.

Key words: Alcohol, Rutin hydrate, Response reinstatement, runway alley, craving

(Received 19.08.2023

Accepted 15.09.2023)

INTRODUCTION

Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonol type of flavonoid that is an important dietary constituent of foods, and plant-based beverages including apples and tea. It is also found in various medicinal plants, such as *Melia azedarach* buckwheat, passion flower (Kuntic *et al.*, 2007). Several reports have provided evidence that rutin possesses pharmacological properties, including antioxidant, anticarcinogenic, cytoprotective, antiplatelet, antithrombic, vasoprotective, and cardioprotective activities (La Casa *et al.*, 2000; Janbaz *et al.*, 2002; Schwedhelm *et al.*, 2003; Sheu *et al.*, 2004; Mellou *et al.*, 2006; Trumbeckaite *et al.*, 2006). Moreover, rutin was found to be a neuroprotective agent (Pu *et al.*, 2007). It has shown anticonvulsant activities in pentylenetetrazol model of rats and mice (Nassiri-Asl *et al.*, 2008), and has also ameliorated ischaemic reperfusion injury in the brain (Gupta *et al.*, 2003). The prolonged supplementation with rutin exhibited a significant reversal of trimethyltin (TMT)-induced impairments in spatial memory, and pyramidal neurons in the hippocampal CA3b region which are linked to its antioxidative (Koda *et al.*, 2008), and anti-inflammatory effects including suppression of pro-inflammatory cytokines and microglial activation (Koda *et al.*, 2009). There are several lines of evidence

which show fruit- and vegetable derived phytochemicals, especially flavonoids, have beneficial effects on memory and learning (Spencer, 2007). The beneficial effects of flavonoid-rich foods such as ginkgo biloba, green tea, blueberry, pomegranate juice, and pure flavonoids on neuro-cognitive ability have been shown in rodents (Joseph *et al.*, 1999; Hartman *et al.*, 2006; Kim *et al.*, 2004; Yamamoto *et al.*, 2007).

Rodent models have been used as models of human declarative memory to predict the potential effects of flavonoids on human cognitive performance (Rendeiro *et al.*, 2009). Previous reports have also established a role for flavonoids in preventing dementia in humans (Commenges *et al.*, 2000). It has been shown that flavonoids can influence peripheral blood flow in human (Schroeter *et al.*, 2006). These vascular effects are potentially significant because increased cerebrovascular function is known to facilitate adult neurogenesis in the hippocampus, based on in vitro study (Gage, 2000). In the present study, we utilized a modified runway paradigm that serves to lengthen the time required for the animal to reach the goal box, thereby limiting concerns about possible "floor effects" that can obscure group differences in animals (such as mice) that traverse the apparatus quickly. This is important since the primary dependent measure in this paradigm is the time required for the animal to travel across the length of the alley (i.e.,

Run Time) from the start box to the goal box where incentive drugs are given. Faster run times in runway models provide evidence of the animal's motivation to seek the stimulus (such as a drug of abuse) that is made available upon goal box entry (Khan and Pandey, 2016).

In earlier studies, our research group established the anti-dopaminergic effect of MMC (Methanolic *Morinda Citrifolia*) and its bioactive principles, such as scopoletin and rutin. We proposed that these bioactive principles could be responsible for the antipsychotic-like activity of *Morinda citrifolia* fruit (Pandey and Vijeeppallam, 2017). Therefore, the present study was designed to evaluate the effects of rutin on response re-instatement of ethanol reward using modified runway paradigm. In this study, we focused on the possible effects of rutin on memory retrieval in mice. However, so far, rutin have not been studied for effective treatment of relapse which is an important parameter for the novel drug development.

MATERIALS AND METHODS

Animals: Animal Male ICR mice weighing from 20–30 g were used for this study. The animals were randomly transferred from the shipping container they were sent in into appropriate cages (n=4 in each cage) with free access to food and water, and maintained in a controlled vivarium environment with a temperature of $22 \pm 1^\circ\text{C}$, relative humidity of 45–60%, and a 12 h light: 12 h dark normal cycle (lights on at 7 AM) in a quarantine room. The quarantined animals were then randomly selected, marked to permit individual identification, and kept in their corresponding cages for at least two weeks prior to the start of the experiment to enable acclimatization to the laboratory conditions. Mice with abnormal conditions (excessive aggression/excessive licking/self-injury/any physical abnormalities) were excluded from the study. Selected mice were further randomized by body weight. The selected mice were assigned into different treatment groups (control and treatment) by randomized weight distribution so that the mean body weight of each group would not be statistically different from those of the other groups. All experimental protocols were approved by the University Animal Care and Use Committee, University of Karachi. All experimental protocols and animal care adhered to the guidelines of the National Research Council of the National Academies of Sciences, Engineering, and Medicine, USA. The behavioral experiments were performed during the light cycle between 10.00 AM and 6.00 PM. All efforts were made to minimize suffering of the mice.

Drugs: Rutin hydrate (quercetin-3-rutinoside hydrate) at a dose of (0.1, 1 and 10 mg/kg) procured from Sigma Aldrich were prepared as suspensions in a 1% w/v aqueous solution of sodium carboxymethyl cellulose

(CMC), and administered orally (p.o.). Ethanol (10% v/v) was obtained by dilution of 99.8% v/v ethanol (Scharlau S.L. Spain) in sterile water for injection. In this study, both ethanol and saline were administered intraperitoneally. All drug solutions were prepared fresh prior to start of the experiment and administered in a constant volume of 1ml/100g body weight of the animal. Acamprosate (Sigma-Aldrich, USA) (333 mg/kg, p.o.) was suspended in 0.5% w/v sodium carboxy methyl cellulose (CMC) solution.

Modified Straight Alley Runway Paradigm of Ethanol Self-Administration in mice

Apparatus: The Composite Aluminium was used to make this apparatus as described in our earlier publication (Khan and Pandey, 2016b). The runway apparatus was modified from earlier straight alley runway designs and incorporated a zig zag path to the goal box as a means of increasing the run times of mice traversing the apparatus (i.e., to prevent floor effects resulting from the relatively fast runtimes of the subjects). The apparatus was arranged in a Z-shaped configuration consisted of a square-shaped start box and a goal connected with three straight runway joined together in a zig-zag manner. The apparatus was situated on a tabletop at a height of 1200 mm from the floor (to minimize the mouse's visual contact with the experimenter). Each segment of the runway included 2 hurdles at a height of 30 mm, to again reduce the speed with which the animals reached the goal box. The start box had black walls with white horizontal stripes and a black polished floor surface. A guillotine door separated the start box from the alley. In contrast, the goal box had white walls with black vertical stripes and a white wire-mesh floor. The mice tracement using this apparatus was noted in real time with the help of a Logitech webcam (C270) mounted above the apparatus and also attached with a computer. Using a digital stopwatch, the run time (seconds) was assessed manually.

Procedure: After 3 days of each 15 min habituation to the runway device (except the target box), the experimental data collection was initiated. On a given trial, every mouse was kept for testing in the initiation (start) box, and then permitted to traverse the alley and enter the goal box, the required to do so (run time) was recorded on every trial and constituted the primary dependent measure. Following 3-day habituation, a baseline runtime was recorded which was served as preconditioning runtime. Conditioning/acquisition trials were then initiated for the next 5 days (Day 1-Day 5) during which each animal received ethanol (2 g/kg, i.p.) or saline upon entry into the goal box and conditioned for half an hour in the target (goal) box from Day 1 to Day 5 as described previously (Khan and Pandey, 2016). The Post-conditioning test was performed 24 h after the last conditioning (on Day 6) during which no saline or

ethanol doses were given upon target box entry. The test group received Rutin (0.1, 1 and 10 mg/kg) 1 hour prior to the post-conditioning trial. The saline and ethanol control groups received CMC (1% w/v; 1 ml/100 g, p.o). Next in extinction trial from Day 7 to Day 11, animals were subjected to extinction trials during which animals did not receive ethanol or saline upon goal box entry. However, Rutin at different doses (0.1, 1 and 10 mg/kg) or CMC (1% w/v) 1 h prior to extinction trials (Ext 1 to Ext 5) was administered.

In order to test the effect of Rutin at different doses on ethanol reinforced reinstatement along the alley, new set of saline treated and ethanol treated animals were selected and they all went through post-conditioning and extinction testing. On Day 12 pre-reinstatement test was performed in the treatment groups. Then on Day 13, the animals underwent reinstatement testing in which animals got a small dose of ethanol 0.8 g/kg, i.p. (1/5th of the maximum dose of ethanol used in conditioning) to reinstate the subjects' motivation to receive ethanol in the goal box. The priming dose was injected 15 mins prior to behavioral task. The test/vehicle groups received different oral doses of Rutin (0.1, 1 and 10 mg/kg) or CMC (1% w/v) 1 h prior to post reinstatement testing. See figure 1.

Statistical analysis: Values are expressed as mean $\pm$ S.E.M. The results were analysed using repeated measures analysis of variance (ANOVA) with one "between-subjects" variable "Group" and a "within-subjects" variable "Trial" using Bonferroni test and one-way analysis of variance (one-way ANOVA) followed by *post hoc* Newman-Keuls multiple comparison tests. Statistical significance was set as $p < 0.05$

Effect of Rutin (0.1, 1 and 10 mg/kg, p.o.) on ethanol reinforced reinstatement in mice running on alley:

Mice were randomly selected and divided into six groups as: Group 1- Saline control group; Group 2- Ethanol control group; Group 3, 4, 5- Rutin (0.1, 1, and 10 mg/kg, p.o.) treated groups, respectively; Group 6- a reference drug, acamprosate (ACAM) (333 mg/kg, p.o.) treated group. During the conditioning stage, the saline-control group (Group 1) was administered with saline whereas Groups (2, 3, 4, 5, and 6) received ethanol upon goal box entry as depicted in Figure 1. The test groups served with Rutin (0.1, 1, and 10mg/kg, p.o.) and/or ACAM (333 mg/kg, p.o.), 1 h prior to post conditioning, extinction trials, and reinstatement testing. Data are expressed as mean $\pm$ S.E.M. The results were analysed using repeated measures analysis of variance (ANOVA) using one —between-subject's variable —Group and a —within-subject's variable —Trial and one-way analysis of

variance (one-way ANOVA) with subsequent post hoc Bonferroni's multiple comparison tests. Statistical significance was set as $p < 0.05$.

RESULTS

Effect of Rutin (0.1, 1 and 10 mg/kg, p.o.) on ethanol reinforced reinstatement in mice running on alley:

Figure 2 depicts the effect of RUTIN and ACAM on the preconditioning and post conditioning runtimes (Day 6). ANOVA results revealed significant effects of the interaction (Group $\times$ Trial) [$F(5, 48) = 7.880$; $P < 0.0001$]. Bonferroni test revealed that the runtimes of ethanol treated group on post-conditioning day was significantly ($p = 0.0009$) decreased when compared with the preconditioning day. However, the runtimes on the post-conditioning day was not altered in ACAM treated group. Mice treated with Rutin at a dose of 0.1 mg/kg displayed less fast runtimes ($P = 0.0407$) as compared with 1.0 mg/kg ($P = 0.0059$), and rutin 10 mg/kg ($p = 0.0042$) on post-conditioning day which suggest that mice treated with rutin were still seeking ethanol on post-conditioning day.

Figure 3 depicts the effects of RUTIN and ACAM on the rewarding properties of ethanol self-administration during extinction days (Day 7- Day 11). ANOVA results revealed significant effects of Group [$F(5, 47) = 7.887$; $P < 0.001$]. A separate one-way ANOVA on the data from each extinction trial revealed that there was no significant difference in runtime on extinction days. The vehicle control group (VEH) showed a marked preference for the ethanol-paired compartment from Day 7 to Day 9, after that it returned to the same level of conditioning score as the respective saline control group (SAL). The post hoc analyses revealed that Rutin did not significantly enhance the running times during days 7 to 11. However, the acamprosate (333 mg/kg, p.o.) treated animals showed a significant enhancement in running time during extinction days (Day 7 to Day 8).

Figure 4 depicts the effect of Rutin and ACAM on the rewarding properties of ethanol on the reinstatement (Day 13) of ethanol self-administration in mice. ANOVA results revealed a significant effect between Groups [$F(5, 48) = 6.171$; $P = 0.0002$]. Bonferroni's multiple comparison test revealed that the runtime of vehicle-treated group on reinstatement (day 13) was significantly ($p = 0.0481$) decreased when compared with the pre-reinstatement (day 12). However, the runtimes on the reinstatement day was not altered in RUTIN, and ACAM treated group.

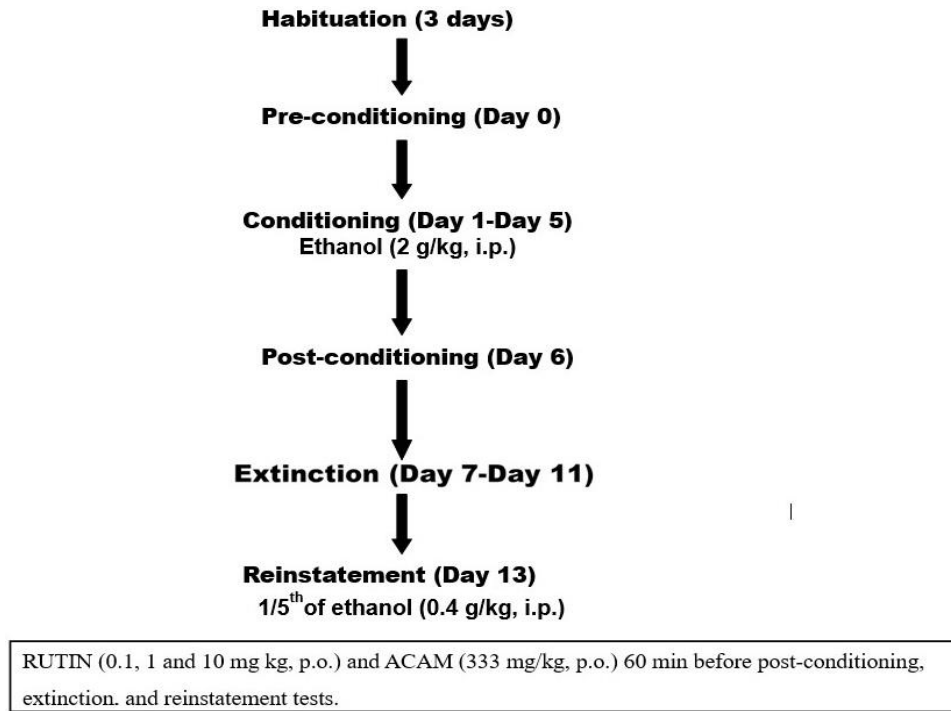


Figure 1: A schematic diagram of the Experimental Design

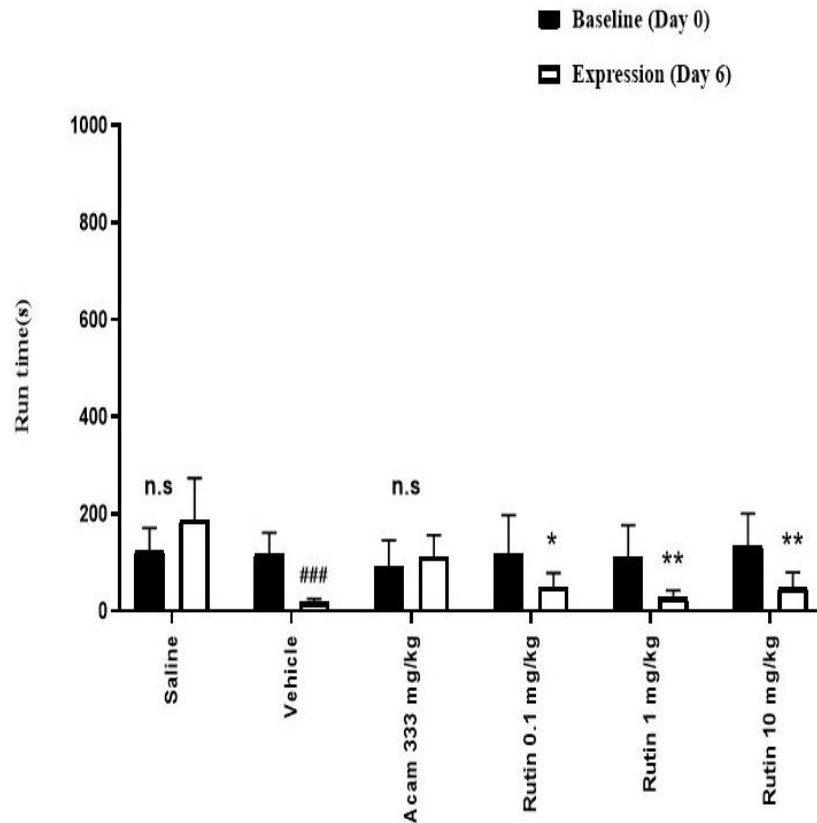


Figure 2: Effect of Rutin (0.1, 1.0, 10 mg/kg, p.o.) and ACAM (333 mg/kg, p.o.) on post conditioning day (Day 6) of ethanol reinforcement. All data are presented as means $\pm$ SEM of the runtimes obtained from eight to ten animals. Statistical significance at * $p < 0.05$, ** $p < 0.001$ (Post conditioning versus preconditioning runtimes in seconds).

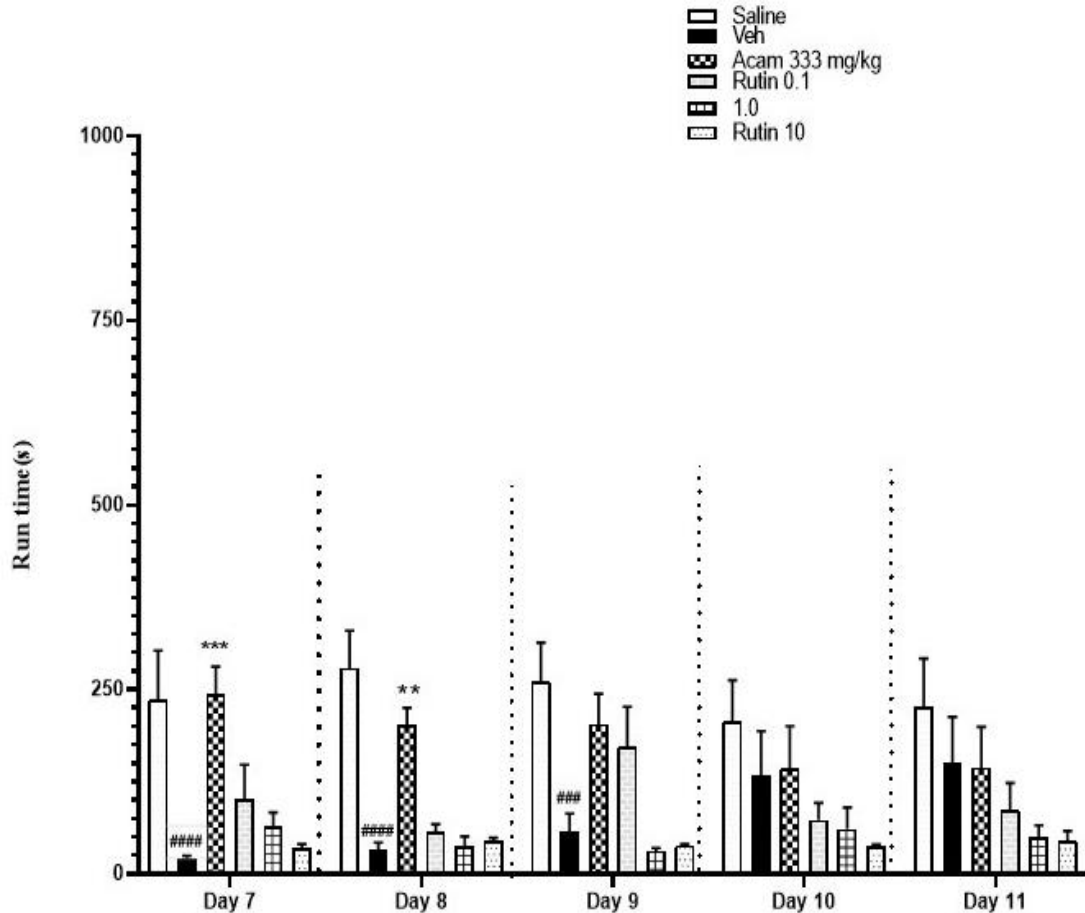


Figure 3: Effect of Rutin (0.1, 1, 10 mg/kg, p.o.) on ethanol runtime during extinction (day 7 to day 11). All data are presented as mean $\pm$ P, 0.05, ##: P<0.01. when compared with the saline control group, *p<0.05, **P, 0.01 when compared with vehicle-ethanol control.

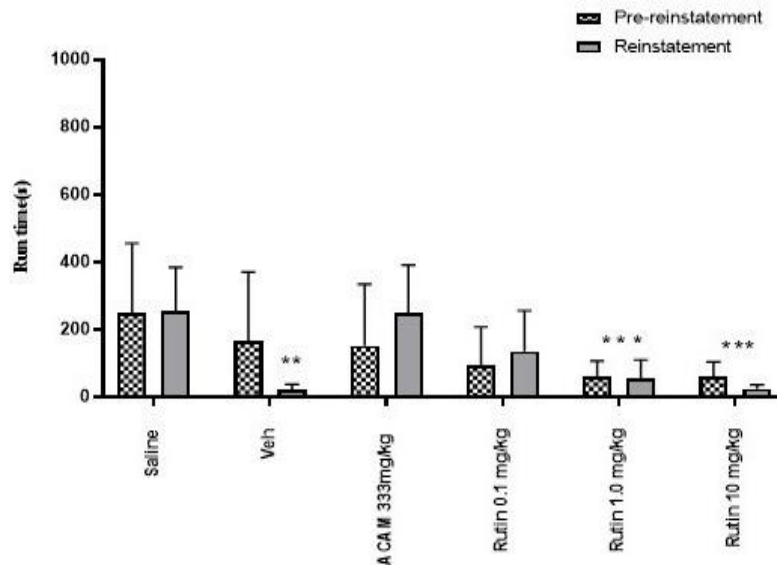


Figure 4: Effect of Rutin (0.1, 1, 10 mg/kg, p.o.) on reinstatement of ethanol reinforcement runtime on day 13 in mice. All data are presented as mean $\pm$ SEM of the runtimes obtained from eight to ten animals. Significant differences were noted as #: P<0.05; ##: P<0.01 when compared with the pre-reinstatement runtime in seconds.

DISCUSSION

The present study result confirms the possible role of rutin from *Melia azedarach* and ACAM on the relapse properties of ethanol as measured after different phases i.e acquisition, extension and reinstatement in mouse model of runway paradigm. Each phase in runway paradigm imitates a real clinical situation like expression for craving, extinction for abstinence, and reinstatement for relapse. The results of the present study provide evidence that: (1) intraperitoneal administration of ethanol (2 g/kg) can produce reward in mice as previously reported (2) the reward can be weakened by repeated exposure to the conditioning environment in an ethanol-free state; (3) a priming intraperitoneal administration of a low dose of ethanol (0.4 g/kg) successfully reinstated the animals as previously reported (Khan and Pandey, 2016); (4) Rutin (0.1 mg/kg, p.o.) and ACAM (300 mg/kg, p.o.) significantly weakened the reinstatement of ethanol-induced reward in mice in the mouse modified runway paradigm was demonstrated and postulated the involvement of antidopaminergic property and its bioactive principles rutin.

Rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonoid found in many plants and fruits have been reported to have many beneficial effects on the central nervous system, especially improvement of cognitive function mainly mediated by suppressing astrocytes and microglia activation, and facilitating synaptic plasticity (Du *et al*, 2014). They have also been reported to have antidepressant activity mediated by their interaction with monoaminergic systems (Du *et al*, 2014). In the previous study, rutin (0.1 mg/kg, p.o.) significantly reduced the intensity of climbing and stereotyped behaviors in mice induced by apomorphine and methamphetamine, respectively, which indicates the antipsychotic-like activity of rutin (Pandey and Vijayapallam, 2017). There is another *ex vivo* study reported on rutin using rat vas deferens in which rutin (156 and 312 µg/ml) significantly inhibited the dopamine-induced contractile response indicated the antipsychotic-like effect of rutin could be mediated by interaction with dopamine D2 receptors (Pandey *et al*, 2014). In present study, we showed anti-motivational property of rutin (0.1, 1.0 and 10 mg/kg, p.o.) against ethanol reinforcement in mice and it is postulated the involvement of antidopaminergic property of rutin for its anti-motivational property against ethanol reinforcement.

This present study results do not facilitate any underlying neuronal or neurochemical mechanisms responsible for the efficacy of rutin in the relapse reinstatement of mice running an alley for ethanol. Studies have confirmed the positive reinforcing effects of ethanol in animals primarily through the dopaminergic systems (reward pathways) that terminate in certain areas of the brain, such as the nucleus accumbens (Davis and

Myers, 2002; Goodman, 2008; Aguilar *et al*, 2009). Administering any substances of abuse, eating (especially sweets), and sexual behavior or even gambling can increase intra synaptic levels of dopamine (DA) in the nucleus accumbens (Khan and Pandey, 2016b). In a recent report, demonstrated antipsychotic-like effect of rutin (0.1 mg/kg p.o.) in mice (Pandey and Vijayapallam, 2017) and suggested antidopaminergic activity of rutin might be mediated by its interaction with dopamine D2 receptors. However, rutin at higher doses (0.5 and 1 mg/kg, p.o.) failed to demonstrate any antipsychotic-like effect in mice (Pandey and Vijayapallam, 2017). Moreover, Pandey *et al* demonstrated the biphasic effect of the ethyl acetate fraction of a methanolic extract of unripe noni (*Morinda citrifolia* Linn.) fruit (EAMMC) that is antidopaminergic effect at lower dose and dopaminergic facilitatory effect at higher dose and indicated the involvement of its bioactive principles scopoletin and rutin for antidopaminergic mechanisms (Pandey *et al*, 2017). *In silico* molecular docking analysis of phytoconstituents from another plant named *Morinda citrifolia* fruit extract showed that rutin possessed the maximum hydrogen bond interaction with dopamine D2 receptors which was comparable to the standard antipsychotic drugs paliperidone and haloperidol (Jeyabalan *et al*, 2017). In this study, rutin (0.1 mg/kg) prevented the establishment of ethanol reinforced reinstatement in mice. Thus, we propose that the effects of rutin on the relapse reinstatement of ethanol-induced reinforcement may stem from its antidopaminergic activity.

Furthermore, Pachauri *et al*. (2013) demonstrated that there was no significant difference in spontaneous locomotor activity when mice were treated with a noni fruit extract containing rutin. Similarly, we performed locomotor activity test on rutin and we found no significant change in animal's locomotion. This report indicated that the current results cannot easily be accounted for by impairment in the subjects' ability to move. Finally, we note that, an acute oral toxicity study of rutin showed no toxicity in cytotoxic assay (Muzafar *et al*, in preparation). In earlier study, rutin (10 mg/kg) significantly increased the step-through latency of the passive avoidance response compared to the control in the three retention tests of the passive avoidance paradigm which demonstrated memory enhancement property of rutin (Nassiri-Asl *et al*, 2009). Prolonged supplementation with rutin significantly reversed trimethyltin (TMT)-induced spatial memory impairment and damage to pyramidal neurons in the hippocampal CA3b region. These effects were correlated with antioxidative effects of rutin (Koda *et al*, 2008). It also suppressed microglial activation and pro-inflammatory cytokines (Koda *et al*, 2009). The results from present study revealed a decrease in establishment of ethanol reinstatement in rutin-treated mice (0.1 mg/kg p.o.) might not be mediated through simple memory impairment.

This result suggests that the anti-relapse activity of rutin is not accounted by its alteration of locomotion in mice.

Acknowledgement: This study was supported by animal resource facility ICCBS University of Karachi.

Conflict of interests: The author declares no conflict of interests

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