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Research Article

HESPERIDIN INHIBITS THE ACQUISITION AND EXPRESSION OF SENSITIZATION TO THE LOCOMOTOR STIMULANT EFFECT OF ETHANOL IN SWISS ALBINO MICE

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ABSTRACT

Objectives: The objective of the present study was to investigate the influence of hesperidin on both the acquisition (development) and expression (manifestation) of sensitization to ethanol's locomotor stimulant effects in Swiss albino mice. By examining these parameters, this study aims to elucidate the possible therapeutic role of hesperidin as a natural neuroprotective agent for preventing or attenuating ethanol-induced neurobehavioral adaptations and addiction.

Methods: The presenter search set out to examine the effect of hesperidin on ethanol-induced behavioral sensitization in Swiss albino mice.

Results: The results revealed that treatment with hesperidin across all doses of 4 mg/kg, 8 mg/kg, and 16 mg/kg notably decreased the ethanol-induced count ($p<0.05$) of locomotion, suggesting a potent inhibitory action of hesperidin on ethanol-induced locomotor sensitization. Hesperidin (8 and 16 mg/kg, i.p.) during development (before each ethanol injection on days 1, 4, 7, and 10) decreased acquisition as well as expression (day 15) of sensitization to locomotor stimulant effect of ethanol. Moreover, chronic administration of hesperidin 30 min before the challenge dose of ethanol (2.0 g/kg, i.p.) produced a dose-dependent attenuation of expression of sensitization to locomotor stimulant effect of ethanol (ethanol-induced locomotor sensitization). Hesperidin *per se* did not affect locomotor activity. These effects highlight its potential therapeutic role in preventing ethanol addiction or ethanol-triggered behavioral sensitization.

Conclusion: Overall findings suggest that hesperidin may have therapeutic potential to attenuate the ethanol-induced locomotor sensitization which could be due to its neuro-modulatory action, which also suggests its use, as adjuvant for therapy of ethanol addiction. However, future investigations are needed to demonstrate the detailed underlying mechanisms of hesperidin on ethanol addiction.

Keywords: Hesperidin, Drug relapse, Ethanol addiction, Locomotor activity, Stimulant effect of ethanol, Behavioral sensitization.

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INTRODUCTION

Frequent use of psychostimulants, including cocaine and amphetamine, opioids like morphine, or sedative-hypnotics like ethanol typically leads to a progressive enhancement of their initial locomotor stimulant effects. The phrase behavioral sensitization refers to this phenomenon. When long-term medication delivery is stopped, behavioral sensitization usually lasts for a while. For instance, it has been demonstrated that sensitization to cocaine can last up to 3 months, amphetamine up to a year, and morphine up to 8 months. These findings imply that long-term alterations in the brain processes that underlie sensitization may take place. These brain systems are thought to become and stay more sensitive to the drug with repeated drug administration, and a link between this phenomenon and drug recurrence following intervals of abstinence has been suggested [1].

Regarding sensitization, psychostimulant medications have been the subject of a lot of research. Increasing sensitivity to the stimulatory effects of ethanol (EtOH) has received little investigation. If the complicated neurobiology of ethanol is to be characterized, animal models of its effects on the central nervous system (CNS) must be continuously developed and improved. Regarding this, it has been demonstrated that ethanol causes biphasic shifts in laboratory mice' locomotor activity. This study has garnered much interest as a prospective animal model of ethanol impact on central stimulant and depressing effects on CNS function [2]. It is well known that relatively high ethanol dosages reduce mice' locomotor activity [3]. whereas it has been noted that lower dosages enhance locomotor activity [4].

Hesperidin is a flavonoid that belongs to the flavanone class and is the aglycone form of hesperidin. Citrus fruits, specifically their epicarp, mesocarp, endocarp, and juice, are the primary source of hesperidin, the flavanone that is most prevalent in oranges. Hesperidin is present in trace amounts in citrus fruits, while glycosides make up the majority of the flavonoids [5-8]. Due to their antioxidant qualities, polyphenols have garnered a lot of attention in the past 20 years. Numerous positive effects, including those on degenerative diseases, cardiovascular diseases, cancer, and osteoporosis, have also been documented, as has their impact on the immune system [9,10].

Hesperidin has been shown to have a number of pharmacological actions to date. Previous experiments have shown that hesperidin has a range of pharmacological effects, including enhancing memory, boosting neurogenesis, and reducing inflammation [11]. Hesperidin's capacity to interact with important neurotransmitter systems in the CNS allows it to produce a wide range of neuropharmacological effects. Numerous studies have shown that hesperidin can change the activity of three neurotransmitters that are crucial for mood, motivation, arousal, and reward-related behaviors: serotonin, gamma-aminobutyric acid, dopamine, and norepinephrine [8]. Recent evidence suggests that hesperidin's neuroprotective effects are mediated by its ability to attenuate oxidative stress, reduce neuroinflammation, and regulate neurotransmitter balance – mechanisms that overlap with those involved in behavioral sensitization and addiction. However, its potential role in modulating ethanol-induced behavioral sensitization remains unexplored.

Repeated or prolonged exposure to chemicals such as ethanol can cause behavioral sensitization, a condition in which the intensity

of a drug's effect increases. The neuroadaptive alterations linked to drug addiction, including altered dopaminergic signaling, oxidative stress, and neuroinflammation, are thought to be mirrored by this phenomenon. Addiction and behavioral dependence may be less likely if these pathways are altered. Nevertheless, little is known about hesperidin's precise effects on ethanol-induced behavioral sensitization, especially in animal models, despite its intriguing pharmacological profile. The majority of research to date has been on hesperidin's antidepressant, anxiolytic, and memory-boosting properties; however, little is known about its possible function in regulating drug-induced locomotor alterations and addiction-like behaviors [12].

The purpose of present research work is to fulfil the gap by examining how hesperidin affects the development (growth) and expression (acquisition) of sensitization to the stimulatory effects of ethanol in Swiss albino mice. This could help find natural, plant-based ways to treat substance use disorders and associated neurobehavioral problems. Therefore, the present study was designed to investigate the influence of hesperidin on both the acquisition (development) and expression (acquisition) of sensitization to ethanol's locomotor stimulant effects in Swiss albino mice. By examining these parameters, this study aims to elucidate the possible therapeutic role of hesperidin as a natural neuroprotective agent for preventing or attenuating ethanol-induced neurobehavioral adaptations and addiction-like behaviors.

MATERIALS AND METHODS

Materials

EtOH (100% v/v) was purchased from Merck Ltd., India, and Absolute ethanol was diluted in 0.9% saline to a 20% w/v solution for intraperitoneal injection. Ondansetron injection (Emeset; Cipla Ltd.) was purchased from a local pharmacy store. Hesperidin was isolated from dried peels of *Citrus sinensis*.

Methods

Extraction of hesperidin from C. sinensis peels

Dried and powdered orange peel were placed in the extraction sleeve of a Soxhlet extractor and covered with glass wool. The petroleum ether was placed in round bottom flask. A reflux condenser was put on the Soxhlet extraction unit, and extraction was done for 4 h with constant temperature between 40 and 60°C. The petroleum ether extract was discarded. The adherent petroleum ether was removed by placing the content of the extraction sleeve in a dish. The petroleum ether extracted sample was again kept in extraction sleeve and reextracted with methanol for 3 h, unless the solvent leaving the extraction sleeve was color less. The extract was filtered and washed with hot methanol. The filtrate was concentrated and dried. The dried residue was mixed with 6% acetic acid to precipitate crude hesperidin. Crude hesperidin was filtered using a Buchner funnel, washed with 6% acetic acid, and dried to constant weight.

Animals

Every procedure used in the research was authorized by the Institutional Animal Ethics Committee (IAEC) (Protocol no. SCOP/IAEC/2011–12/13), which was formed by the Ministry of Environment and Forests, GOI, New Delhi, India, to control and supervision of experimental animals. The current research was performed in a silent room with a 12:12 cycle of light and dark and temperature and humidity controls (22±3°C and 60±5%, respectively). Inbred adult male Swiss albino mice weighing between 22 and 26 grams were utilized. They were raised at the Animal House of the Sinhgad College of Pharmacy in Vadgaon (Bk), Pune, India. Mice were housed together (six per cage) in 28×21×14 cm opaque polypropylene cages that have open access to drinking water and standard rodent food. Each group in the experiment had six mice. A behavioral study was carried out between 09.00 and 14.00 h to lessen the effects of the clock. Swiss albino mice were employed in the current investigations because ethanol (2.0 g/kg) causes a locomotor-stimulating effect in these animals that gets stronger with repeated exposure.

Locomotor activity

Locomotor activity was performed using actophotometer (Dolphin, India). It is made up of a 40×40×10 cm enclosed square arena with four infrared beam cells mounted on the wall, 2 cm above the ground. A digital counter records the breaks in the light beam caused by the movement of the animals. The experiments were performed in a room with indirect, low-level incandescent lighting (40 lx) and sound attenuation. Locomotor activity was measured as the overall count of interruptions to the light beam throughout a 30-min period. To acclimatize to the testing environment, mice were relocated from their residence cages to the experimental laboratory 60 min before the drug administration on test day [13].

Alcohol dehydrogenase (ADH) assay

Using the ADH assay, the blood ethanol content was measured by Bhutada *et al*. [25]. After locomotor activity tests, which were conducted 35 min after the administration of ethanol, 40 μL of blood were extracted from the retro-orbital sinus. Then, in a cooling centrifuge, 160 μL of after adding 3% perchloric acid and vortexing, the mixture was centrifuged at ×10,000 g. Before being analyzed, the supernatant was kept at 4°C. To conduct the experiment, 60 μL of the supernatant was incubated for 40 min at room temperature in 3 mL of 0.5 M Tris–Cl buffer (pH 8.8) that included 1.5 mM β-nicotinamide adenine dinucleotide (β-NAD) and 5.5 μg/mL of ADH. After then, absorbance at 340 nm was used to quantify the buildup of β-NADH. A standard calibration curve was used to estimate the ethanol content of the samples.

Acquisition and expression of sensitization to locomotor stimulant effect of ethanol

The following procedure was used to generate ethanol sensitization to its locomotor-stimulating effect by Kotlinska *et al*., and Bhutada *et al*. [14,15]. Ethanol or saline was administered intraperitoneally at a dose of 2.0 g/kg, i.p., in volume 12.5 mL/kg body weight and 12.5 mL/kg body weight, respectively, to mice on days 1, 4, 7, and 10. As previously stated, the movement of each mouse was assessed for 30 min after each ethanol or saline injection. To assess the expression of sensitization to locomotor activity, the ethanol and saline pre-treated groups were given ethanol at a dose of 2.0 g/kg and intraperitonially were challenged on day 15. As stated earlier, a 30-min measurement of locomotor activity was conducted. The group that was chronically receiving saline therapy was given ethanol on the day of the challenge, which was day 15.

Influence of hesperidin acute treatment on the acquisition of sensitization to the locomotor stimulant effect of ethanol

Hesperidin (4, 8, and 16 mg/kg, i.p.) [16-18] or vehicle (0.9% saline, 10 mL/kg body weight, i.p.) was administered to six different mouse groups 30 min before each ethanol (2.0 g/kg, i.p.) or saline injection on days 1, 4, 7, and 10 (acquisition). All mice on day 15 were given only an ethanol challenge dosage. Five minutes after the challenge, locomotor activity was observed for 30 min as previously stated (expression), and the blood ethanol levels were measured on day 15 following the conclusion of the locomotor session.

Table 1: Observation table of HPLC analysis of standard and isolated hesperidin

Standard hesperidin			Isolated hesperidin		
Rt	**Area**	**Percentage area**	**Rt**	**Area**	**Percentage area**
9.94	48300	0.97	9.938	133142	3.82
10.262	4886769	97.96	10.254	3065354	87.93
11.625	30779	0.62	11.626	138879	3.98
14.006	22888	0.46	14.007	24386	0.70

HPLC: High-performance liquid chromatography, Rt: Retention times

Influence of hesperidin chronic treatment on the expression of sensitization to locomotor stimulant effect of ethanol

The impact of hesperidin on the expression of sensitization to the locomotor-stimulating effect of ethanol was investigated by sensitizing six mice in separate groups to ethanol as previously described. Hesperidin at a dose of 4, 8, and 16 mg/kg administered intraperitonially was given to mice on day 15, 30 min before the ethanol challenge dosage. As previously noted, locomotor activity was observed for 30 min after the ethanol injection, and when the locomotor activity test session was over, blood ethanol levels were measured using the same protocol.

Influence of hesperidin and ethanol treatment on motor coordination in mice

An accelerated rota rod test was used to verify the specificity of hesperidin's inhibitory effect on ethanol-induced locomotor sensitization. A separate group of mice was assessed using an accelerated rota rod technique, which consists of a training phase and a testing phase. Five consecutive rota rod trials were provided to each mouse during training (INCO, Ambala, India) with a 30-s rest interval between each trial and a constant acceleration rate of 20 rpm. Each mouse had to fall or reach 180 s to end the experiment. The experiment was terminated for animals who failed at least three out of five trials (each lasting at least 180 s). Training and testing were separated by a 24-h period. The test involved accelerating the rota rod from 20 to 30 rpm. Thirty minutes after administering hesperidin (4, 8, and 16 mg/kg intraperitonially to the animals were given an injection of ethanol (0, 1.25 g/kg) at 20 rpm, and the amount of time to fall was noted [19].

Data analysis

Horizontal, vertical, and ambulatory activities were added up to determine total locomotor activities. The findings were presented as mean±standard error of the mean for recordings that lasted 30 min. Either the two-way analysis of variance (ANOVA)/repeat measure ANOVA or the one-way ANOVA followed by Tukey's multiple comparison test was used to examine the data. A significance criterion of $p<0.05$ was established, and every instance was considered statistically significant.

RESULTS AND DISCUSSION

Extraction of hesperidin from *C. sinensis* peels

The chromatograms (Fig. 1) and Table 1 display distinct peaks corresponding to the retention times (Rt) of the components detected in both the standard and isolated samples. In the standard hesperidin chromatogram, a major peak was observed at Rt=10.262 min, with a % area of 97.96%, indicating high purity of the standard compound. Minor peaks at Rts 9.94, 11.625, and 14.006 min correspond to negligible impurities or secondary components with % areas below 1%.

In contrast, the isolated hesperidin sample exhibited a major peak at Rt=10.254 min with % area of 87.93%, closely matching the Rt of the standard hesperidin peak. This strong correlation confirms that the isolated compound corresponds to hesperidin. Minor peaks at Rt=9.938, 11.626, and 14.007 min with % areas of 3.82%, 3.98%, and 0.70%, respectively, may represent traces of co-eluting phytoconstituents or residual impurities that persisted during extraction or purification.

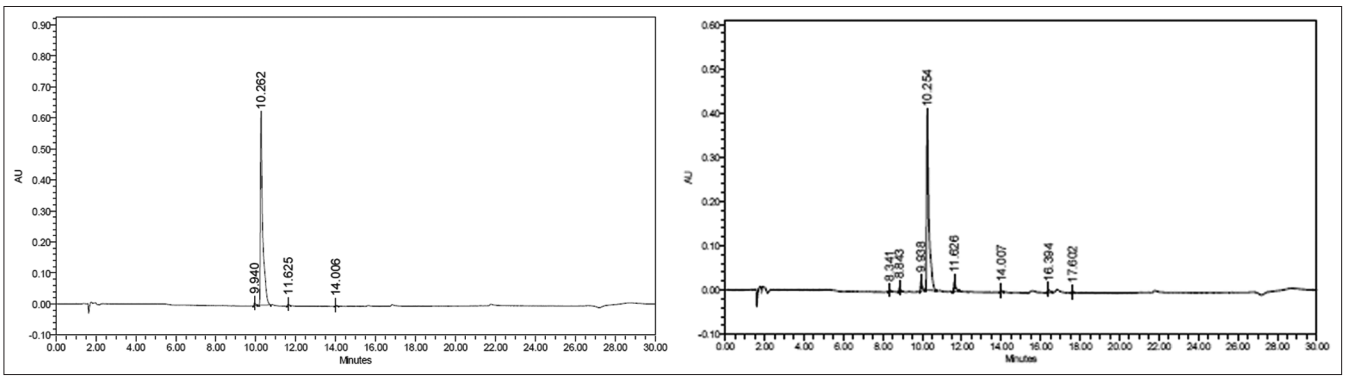

Fig. 1: High-performance liquid chromatography chromatogram of standard hesperidin and isolated hesperidin

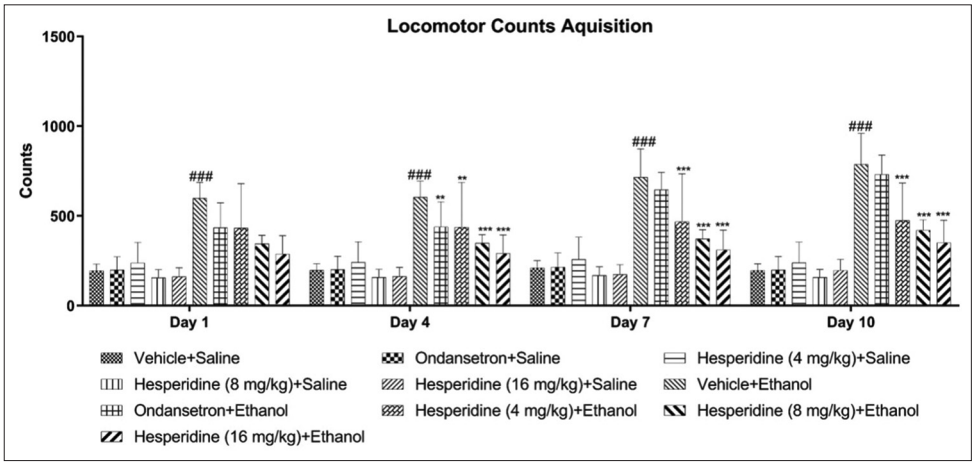

Fig. 2: Influence of hesperidin acute treatment on the acquisition of sensitization to the locomotor stimulant effect of ethanol. The data presented is mean±standard deviation (n=6). Two-way analysis of variance followed by Tukey's multiple comparison test was used for the analysis. Statistical significance was considered at *p<0.05, **p<0.01, ***p<0.001 as compared to Vehicle+Ethanol and #p<0.05, ##p<0.01, ###p<0.001 as compared to Vehicle+Saline in all cases

Influence of hesperidin acute treatment on the acquisition of sensitization to the locomotor stimulant effect of ethanol

The impact of hesperidin on the development of ethanol-induced locomotor sensitization (EILS) during a 10-day period is depicted in the bar graph (Fig. 2). On days 4, 7, and 10, ethanol administration alone (Vehicle+Ethanol) significantly increased locomotor activity, as indicated by the ### symbol (p<0.001 vs. Vehicle+Saline), confirming the development of sensitization. However, treatment with hesperidin across all dosages (4, 8, and 16 mg/kg) marked decreased these ethanol-induced locomotor counts, with statistical markers *** denoting p<0.001 in comparison to the Vehicle+Ethanol group. This suggests a strong inhibitory effect of hesperidin on ethanol-induced hyperlocomotion. Notably, hesperidin's effect was comparable to ondansetron, a known anti-sensitization agent, highlighting its potential as a therapeutic candidate. In addition, hesperidin alone (with saline) did not produce significant changes in locomotor activity, indicating that its modulatory effect is specific to ethanol-induced stimulation. Overall, hesperidin effectively reduces the development of ethanol-induced sensitivity to locomotor stimuli in a dose-dependent manner, supporting its neurobehavioral protective role [20,21].

Influence of hesperidin chronic treatment on the expression of sensitization to locomotor stimulant effect of ethanol

The figure shows how long-term hesperidin therapy affects the expression phase of ethanol-induced locomotor sensitization (Fig. 3). Animals treated with ethanol alone (Vehicle + Ethanol) exhibited a notable elevation in locomotor counts (P<0.001 vs. Vehicle+Saline, indicated by ###), confirming the robust expression of sensitization after repeated ethanol exposure. However, chronic administration of hesperidin produced a dose-dependent attenuation of this hyperlocomotor activity. Hesperidin at 4 mg/kg considerably decreased the ethanol-induced counts (*p<0.05), with greater reductions observed at 8 mg/kg (**p<0.01) and 16 mg/kg (***p<0.001), showing a strong suppressive effect at higher doses. Ondansetron+Ethanol also demonstrated a significant reduction in locomotor activity, similar to hesperidin. Importantly, hesperidin alone (with saline) did not affect baseline locomotion, indicating its specific role in mitigating ethanol-induced sensitization rather than producing sedation or motor suppression. These results demonstrate the potential therapeutic role of hesperidin in preventing relapse or ethanol-triggered behavioral sensitization by consistently suppressing the expression of ethanol-induced locomotor sensitization.

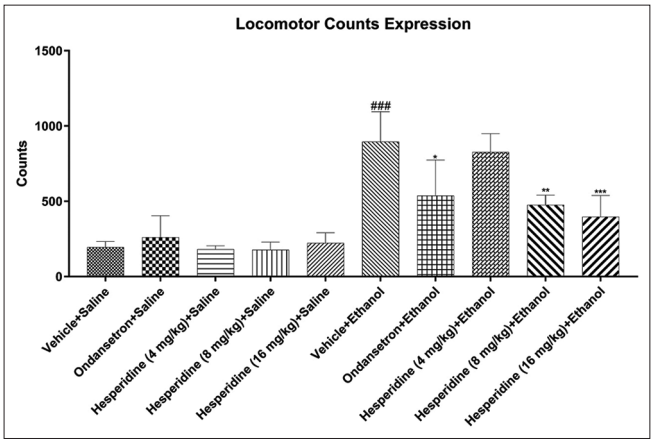

Fig. 3: Influence of hesperidin chronic treatment on the expression of sensitization to locomotor stimulant effect of ethanol. The data presented is mean±standard deviation (n=6). One way analysis of variance followed by Tukey's multiple comparison test was used for the analysis. Statistical significance was considered at *p<0.05, **p<0.01, ***p<0.001 as compared to Vehicle+Ethanol and #p<0.05, ##p<0.01, ###p<0.001 as compared to Vehicle+Saline in all cases

Effect of hesperidin on blood ethanol concentration EILS

The graphs (Fig. 4) that are displayed assess how hesperidin affects the activity of ADH, a crucial enzyme in the metabolism of ethanol [22] under saline and ethanol-treated conditions in the model of EILS. In the groups treated with saline (left panel), ADH activity remained relatively stable across all treatments, including hesperidin at 4, 8, and 16 mg/kg, as well as ondansetron and the vehicle control, suggesting that hesperidin alone does not significantly affect basal ADH levels.

In the ethanol-treated groups (right panel), a general increase in ADH activity is observed, which is expected as ethanol metabolism stimulates ADH expression. Interestingly, the co-administration of hesperidin at all doses maintained ADH activity levels comparable to the ethanol control group (Vehicle + Ethanol), with minor variations. There was no significant enhancement or suppression of ethanol-induced ADH activity by hesperidin or ondansetron, indicating that hesperidin does not directly modulate ethanol metabolism through ADH pathway. Therefore, the observed behavioral effects of hesperidin in EILS are likely independent of its influence on blood ethanol concentration or ADH-mediated ethanol clearance.

Influence of hesperidin and ethanol treatment on motor coordination in mice

The rota rod test is used in Fig. 5 to illustrate how ethanol and hesperidin affect Swiss albino mice's motor coordination. Latency to fall (measured in seconds) was used for the assessment.

It has been observed that in saline treated group and all treatment groups (vehicle, ondansetron, and hesperidin at 4, 8, and 16 mg/kg), the latency time was found to be similar with no significant differences. This observation clearly suggested that hesperidin does not impact or enhance the motor coordination in mice under normal conditions

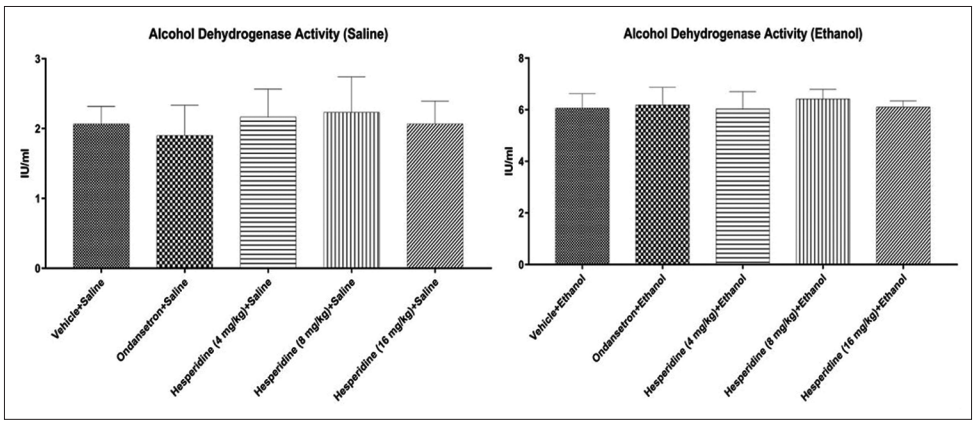

Fig. 4: Effect of hesperidin on blood ethanol concentration ethanol-induced locomotor sensitization. The data presented is mean±standard deviation (n=6). One-way analysis of variance followed by Tukey's multiple comparison test was used for the analysis

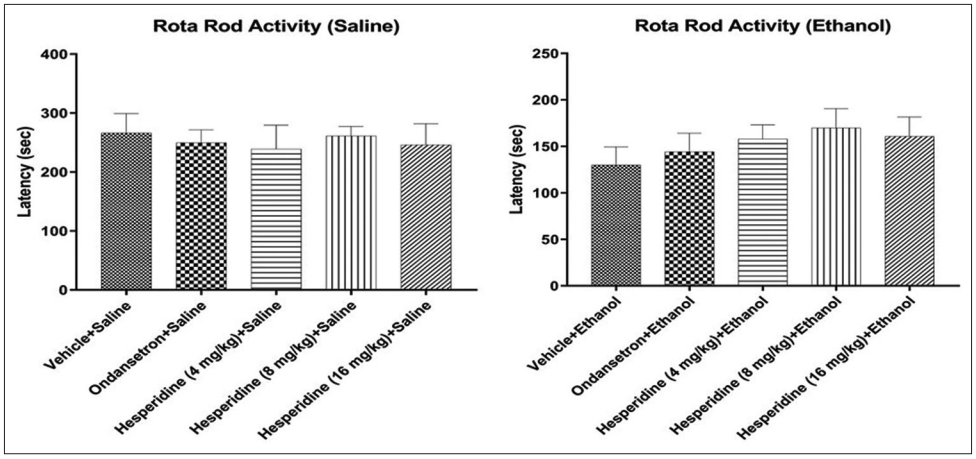

Fig. 5: Effect of hesperidin and ethanol on motor coordination of Swiss albino mice using rota rod test. The data presented is mean±standard deviation (n=6), and one-way analysis of variance followed by Tukey's multiple comparison test was used for the analysis

(without ethanol). Similar observation was also found when hesperidin was given in ethanol treated groups. This study clearly showed no effect of hesperidin (at 4, 8, and 16 mg/kg) when assessed following ethanol or saline in the coordination task. Hesperidin did not show any muscle relaxant activity, so locomotion was not reduced when used individually or in combination with ethanol.

CONCLUSION

Hesperidin dose dependently inhibits the locomotor sensitization to ethanol. Further, hesperidin inhibits the acquisition and expression of locomotor sensitization brought on by ethanol. Study confirmed that hesperidin *per se* does not influence motor coordination or locomotor activity. It is also found that hesperidin does not influence blood ethanol concentration in mice. From above results, it is found that hesperidin dose dependently inhibits the rewarding effects of ethanol. Hence, hesperidin can be drug of choice for treating ethanol addiction and or drug addiction and can be drug of choice for pharmacotherapy of the drug dependence or drug abuse. However, further research is needed to recognize the molecular mechanisms, particularly the receptor systems and signaling pathways that may be modulated by hesperidin which leads to its protective effects in ethanol dependence.

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AUTHOR CONTRIBUTION

Dr. Kailasam Koumaravelou designed the study and planned the work with its aim and objectives, reviewed the manuscript, and edited the article. Mr. Krushna Zambare performed the work, collected data, and wrote the manuscript.

CONFLICT OF INTEREST

Authors report no conflict of interest.

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