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Vanillin attenuates the ethanol withdrawal syndrome and ethanol withdrawal induced anxiety by regulating the neurochemical balance

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Abstract

Ethanol abuse is a major public issue globally and withdrawal of ethanol after chronic exposure contributes to the development of behavioural changes. The present study evaluates vanillin effect against the ethanol withdrawal syndrome (EWS) and the associated anxiety. Rats were exposed to ethanol for 21 days at 7.2% concentration maximum with drinking water in a modified liquid diet. Vanillin at doses of 100 and 200 mg/kg were administered 30 min prior to ethanol withdrawal, and behavioural changes were observed at 1st, 2nd, 4th, 6th and 12th h of ethanol withdrawal. Moreover, the locomotor activity was assessed using the astrophotometer and level of anxiety by the elevated plus maze. The level of neurotransmitters and mRNA expression of corticotropin-releasing factor (CRF) and corticotropin releasing factor receptor 1 (CRFR1) were estimated in brain tissue of vanillin treated EWS rats. There was a significant improvement in the ethanol withdrawal behaviour in the vanillin treated group compared to EWS rats. The locomotor activity and level of anxiety was observed to be reduced significantly ($p < 0.01$) in the vanillin treated group compared to EWS rats. Treatment with vanillin ameliorates the altered level of γ-aminobutyric acid (GABA), dopamine and glutamate and level of corticosterone in ethanol withdrawal rats. mRNA expression of CRF and CRFR1 was reduced significantly ($p < 0.01$) in brain tissue of the vanillin treated group compared to the EWS group of rats. In conclusion, data reveal that treatment with vanillin shows a beneficial effect against EWS and ethanol withdrawal associated anxiety by regulating CRF/CRFR1 expression.

Key words: *vanillin, ethanol, anxiety, corticosterone, behaviour.*

Introduction

Dependence on alcohol is the major health and public issue throughout the globe and occurs due to abuse of alcohol for a long period [9]. Withdrawal of ethanol abruptly in an individual on chronic alcohol leads to the development of the ethanol withdrawal syndrome with symptoms such as convulsions, tremors, tachycardia, sweating, delirium, insomnia, hallucination, vomiting, nausea, elevation of the pulse rate and blood pressure and agitation, which is associated with anxiety [17]. These withdrawal symptoms vary from mild to severe, which causes death and cardiac arrest in approximately 10% of individuals [20]. The literature suggests that these symptoms start from 6 h of ethanol withdrawal, relapse of ethanol use occurs due to negative emotional response, i.e. symptoms, including anxiety [3]. There are several neurotransmitters such as γ-aminobutyric acid (GABA) and NMDA, which alter in ethanol consumption. Moreover, upregulation of N-methyl-D-aspartate (NMDA) receptor and downregulation of GABA occurs in ethanol dependence and also dopamine involved in the activation of reward circuit [6]. Withdrawal of ethanol contributes to alteration of these neurochemicals and glutamate and GABA minergic system are the possible targets

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for the management of the ethanol withdrawal syndrome. Moreover, corticotropin-releasing factor (CRF) is sensitized in ethanol withdrawal syndrome (EWS), which promotes anxiety too [12]. There are several conventional drugs like disulfiram, naltrexone, acamprosate and benzodiazepine used for management of EWS, however these drugs have several limitations viz. weight loss, irritability, sleep disturbance, headache, emotional stress and anxiety [5]. Thus, there is a need for the development of an alternative therapy for the management of EWS.

Molecules from herbal sources have shown a promising effect against neurological disorders including EWS. Vanillin chemically is a phenolic aldehyde, a natural flavouring agent isolated from vanilla beans [16]. Vanilla has been reported for its anti-microbial, antifungal, neuroprotective, anti-inflammatory, antioxidant, anticancer and immunomodulator activity [1,11,13,15,24,26]. Vanillin hydrogel promotes the wound healing by regulating the inflammatory cytokines [7]. Vanillin has shown potential antioxidant activity as it regulates scavenging of reactive oxygen species (ROS) [14] and it protects neuroinflammation by reducing inflammatory cytokines (interleukin 6 [IL-6], nuclear factor κB [NF-κB] and tumour necrosis factor α [TNF-α]) and oxidative stress [13]. Vanillin shows anti-depressant property and also protects neurodegeneration by regulating GABA receptor [22]. Moreover, vanilla was traditionally used for the management of depression and anxiety in the 17th century as a home remedy. Thus, the presented report evaluates the effect of vanillin against EWS.

Material and methods

Animal

Male Wistar rats (200-225 g) were housed under controlled conditions (temperature: 25 ±2°C, humidity: 55 ±5%) with a 12 h light/dark cycle as per the guidelines. Exposure to ethanol and all behavioural experiments involved in EWS were carried out in other separate and isolated laboratories, which have the same environmental conditions with the colony room. All the animal experimentation was approved by the Animal Ethics Committee of the Ganzhou People's Hospital, China (JGZ20220015).

Chemicals

Vanillin was procured from Merck Ltd., Beijing, China. Enzyme-linked immunosorbent assay (ELISA) and assay kits were purchased from Thermo Fisher Scientific Inc., Beijing, China. Primers used in quantitative real-time PCR (qRT-PCR) were procured from Bio-Rad Laboratories India Pvt. Ltd., Haryana, India.

Experimental

All the rats were housed individually, and ethanol was given in the modified liquid diet. No extra chow or water was supplied to the rats. The composition of the modified liquid diet with ethanol was in cow milk 925 ml, 25-75 ml ethanol (96.5% ethyl alcohol), vitamin A 5000 IU and sucrose 17 g [25]. All the rats were given modified liquid diet without ethanol for 7 days for the habituation. Then, liquid diet with 2.4% ethanol was administered for 3 days. The ethanol concentration was increased to 4.8% for the following 4 days and finally to 7.2% for 14 days. Liquid diet was freshly prepared daily and presented at the same time of the day (9:30 h). The weight of the rats was recorded every day, and daily ethanol intake was measured and expressed as g per kg per day. Control rats (n = 8) were pair fed with an isocaloric liquid diet containing sucrose as a caloric substitute for ethanol.

Evaluation of the ethanol withdrawal syndrome

Ethanol was withdrawn and replaced with isocaloric ethanol-free diet at 9:30 h at the end of 21st day of protocol. All the animals were then assigned into four groups randomly (n = 8 for each group). The control group received ethanol free modified diet; the EWS group received ethanol with modified diet; and vanillin 100 mg/kg and 200 mg/kg group received ethanol with modified diet and 30 min prior to ethanol withdrawal animals received vanillin 100 mg/kg and 200 mg/kg, p.o. Ethanol withdrawal symptoms such as locomotor hyperactivity, agitation, tremor, tail stiffness, stereotyped behaviour and wet dog shakes were assessed by placing the rat in an open field apparatus at 1st, 2nd, 4th, 6th and 12th h of ethanol withdrawal. Moreover, body weight and amount of ethanol consumption were estimated in each group of rats.

Evaluation of anxiety by the elevated plus maze

The elevated plus maze was used to determine the level of anxiety in the rodent model as per reported method. The elevated plus maze consists of two open and two enclosed arms, each with an open roof, elevated 40-70 cm from the floor. The model is based on rodents' aversion to open space. In EPM this translates to a restriction of movement to the enclosed arms. Anxiety reduction in the plus-maze was indicated by an increase in the proportion of time spent in the open arms (time in open arms/total time in open or closed arms) and increase in the proportion of entries into the open arms (entries into open arms/total entries into open or closed arms). The total number of open arm

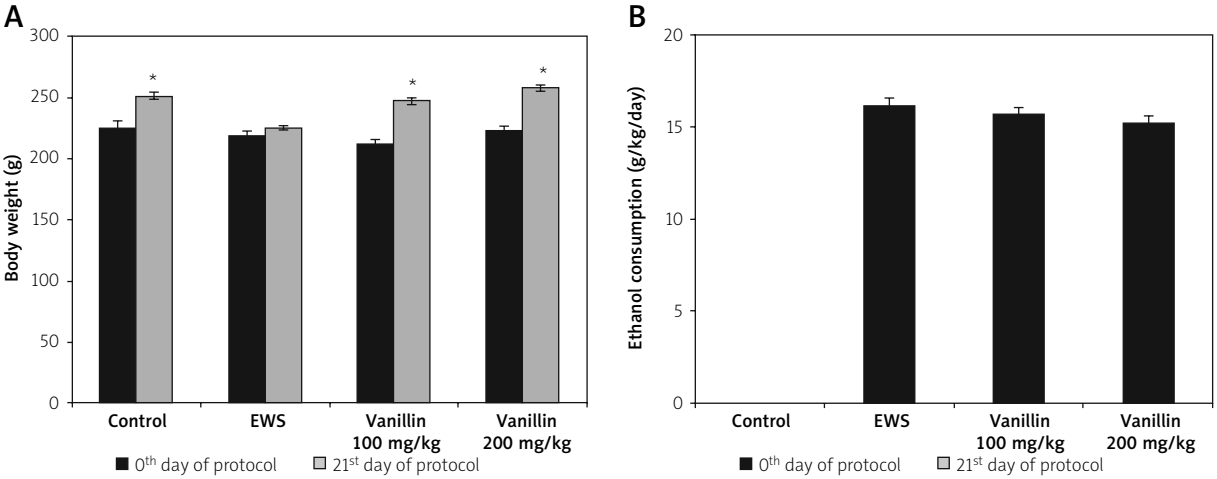


Fig. 1. Effect of vanillin on ethanol consumption and body weight in ethanol withdrawal syndrome rats. **A**) Effect of vanillin on change in body weight on 0th and 21st day of ethanol exposure protocol. **B**) Effect of vanillin on ethanol consumption in 7.2% of ethanol exposure rats. Mean ±SEM (n = 8); *p < 0.05 compared to 0th day of protocol.

entries and number of closed arm entries were usually employed as measures of general activity or anxiety.

Evaluation of locomotor activity

Locomotor behaviour was assessed by recording each animal for 5 min using the actophotometer. Animals doing the locomotion cut the beam of light which falls on the photocell and was recorded digitally.

Preparation of brain tissue homogenate

Blood was withdrawn from retro orbital plexus and plasma was separated from the blood by its centrifugation for 10 min at 2000 × g. All the animals were sacrificed using cervical dislocation and isolated brain tissue was homogenized in 0.1 M phosphate buffer (pH = 7.4). Brain tissue homogenate was centrifuged for the period of 15 min at 3000 rpm and supernatant was used for the estimation of biochemical parameters.

Estimation of dopamine, glutamate, and GABA

The level of glutamate, GABA, and dopamine in brain tissue homogenate using their respective assay kits as per the directions given by the manufacturer of kits. Moreover, the level of CORT was estimated in the plasma using the ELISA kit.

qRT-PCR analysis

Trizol reagent was used to isolate total RNA from the brain tissue homogenate and the RT-PCR kit was used to produce cDNA from RNA. Reverse transcription was achieved, and the mixture was incubated for 15 min at 42°C and then inactivated by heating the same for 5 s at 85°C and removing the gDNA. The qPCR system contained a 20 µl total volume composed of forward (0.4 µl) and reverse primers (10 µmol/l), 2× TransStart®Tip Green qPCR Supermix (10 µl), cDNA template (1 µl), and sufficient H_2O. The PCR conditions were maintained for 30 s at 94°C for denaturation, followed by 5 s at 94°C, 15 s at 60°C, and 10 s at 72°C for 45 cycles. The CT values of the samples were determined, and relative expression was represented by 2-ΔΔCT.

Primer	CRF
Forward	GGTGGACTACATCTACCAAGGC
Reverse	GATTGTCTCGGATGTGGTGGAC
Primer	CRFR1
Forward	GATGTTTGGAGAGGGCTGCT
Reverse	CCAAGCGACGATAATGGGGA

Statistical analysis

Results were expressed as mean ±SEM (n = 8). The statistical analysis was performed using one-way ANOVA followed by Dunnett test for multiple comparisons (GraphPad Prism software, ver. 6.1; USA). The level of statistical significance was set at p < 0.05.

Results

Effect of vanillin on body weight and ethanol consumption

Consumption of ethanol and body weight was observed in the vanillin treated ethanol withdrawal

syndrome rats as shown in Figure 1A, B. There was no significant change in the weight observed among all the groups on 0th day of protocol. Body weight in control, vanillin 100 mg/kg and vanillin 200 mg/kg groups was significantly enhanced on 21st day of protocol ethanol administration. There was no significant change in body weight of the EWS group on 21st day of protocol compared to 0th day of ethanol administration (Fig. 1A). Ethanol consumption was observed in each group of rats and there was no alteration in the ethanol consumption among groups (Fig. 1B).

Effect of vanillin on symptoms of ethanol withdrawal syndrome

The effect of vanillin was determined on the behavioural changes such as agitation, tremor, rearing, tail stiffness, grooming, sniffles, and stereotyped behaviour of ethanol withdrawal syndrome rats as shown in Figure 2. There was a significant ($p < 0.01$) increase in the number of agitation, tremor, rearing, tail stiffness, grooming, sniffles and stereotyped behaviour changes at 1st, 2nd, 4th, 6th and 12th h of ethanol withdrawal in EWS compared to the control group of rats. However, treatment with vanillin 100 and 200 mg/kg ameliorates the altered behavioural changes in EWS rats.

Effect of vanillin on the locomotor activity

Actophotometer was used to estimate the effect of vanillin on locomotor activity of EWS rats as shown in Figure 3. There was a significant ($p < 0.01$) increase in locomotor activity of the EWS group compared to the control group of rats. However, locomotor activity was observed to be reduced in vanillin 100 and 200 mg/kg treated groups compared to the EWS group of rats.

Effect of vanillin on the level of anxiety

The level of anxiety was determined by estimating the percentage of entries to open and closed arms in vanillin treated EWS rats using the elevated plus maze

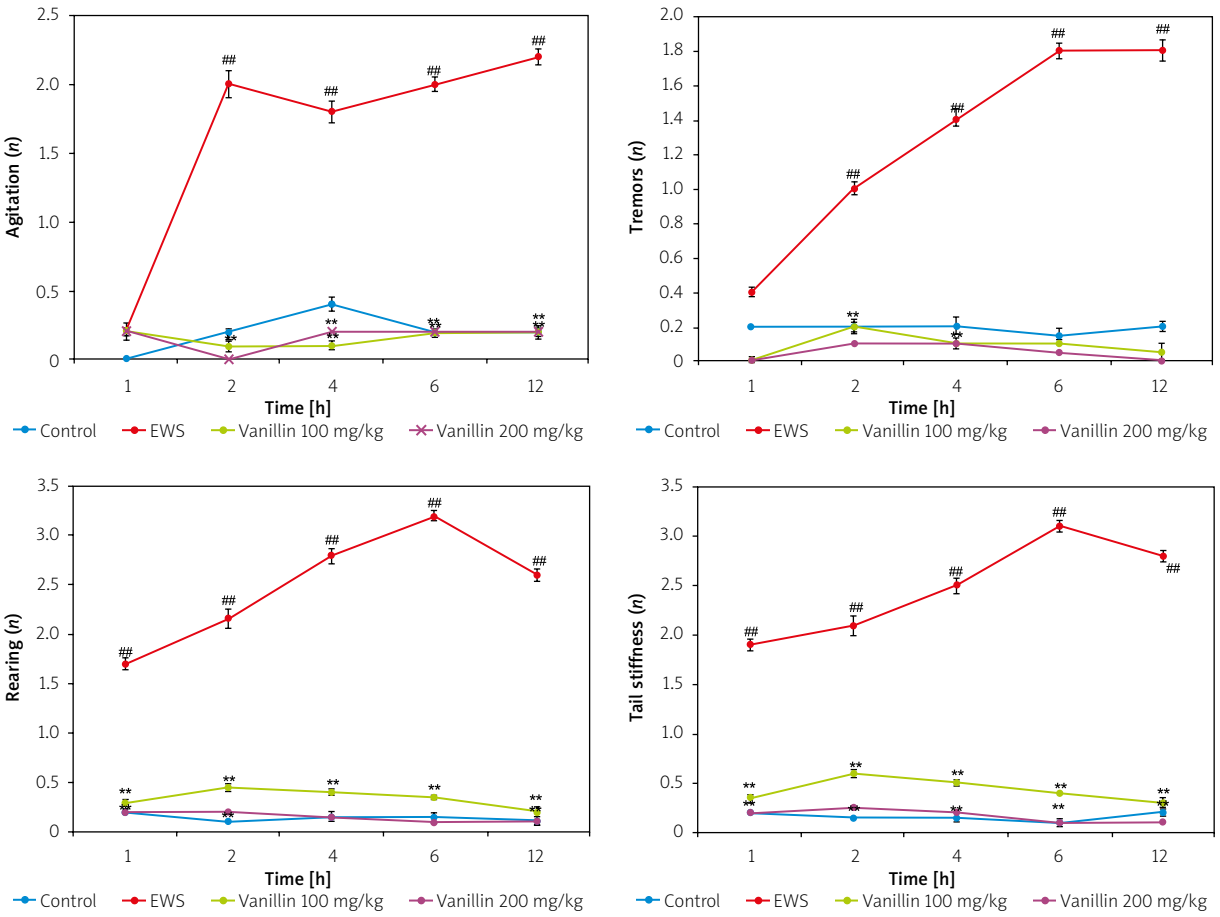
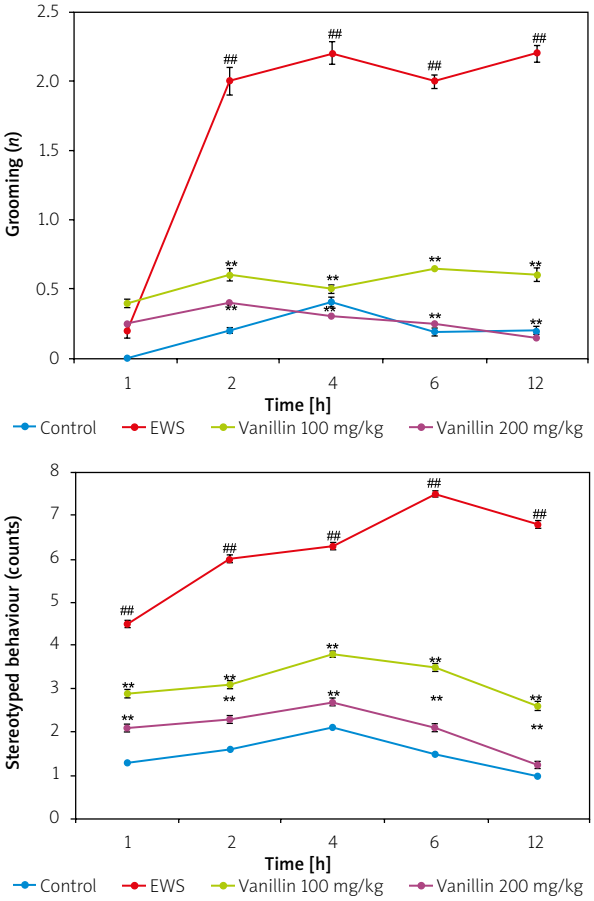


Fig. 2. Effect of vanillin on change in the symptoms of ethanol withdrawal syndrome rats. Mean ±SEM ($n = 8$); ##$p < 0.01$ compared to the control group; **$p < 0.01$ compared to the EWS group.



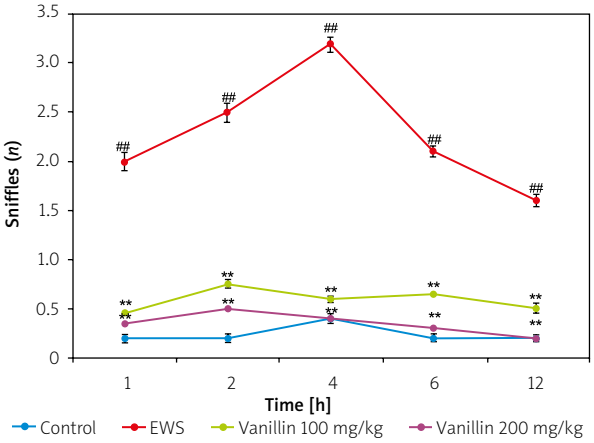


Fig. 2. Cont. $^{\#\#}p < 0.01$ compared to the control group; $^{**}p < 0.01$ compared to the EWS group.

as shown in Table I. There was a significant ($p < 0.01$) reduction in percentage of open arm entries and increase in percentage of closed arm entries in EWS rats compared to the control group of rats. However treatment with vanillin attenuates the altered percentage of open and closed arm entries in EWS rats.

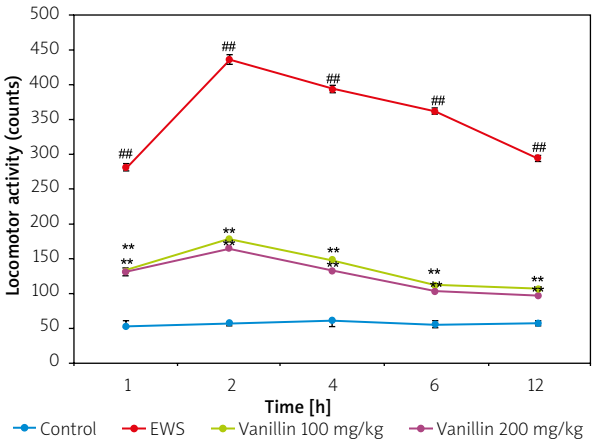


Fig. 3. Effect of vanillin on the locomotor activity of ethanol withdrawal syndrome rats using actophotometer. Mean ±SEM ($n = 8$); $^{\#\#}p < 0.01$ compared to the control group; $^{**}p < 0.01$ compared to the EWS group.

Effect of vanillin on neurochemicals

There are several biochemical parameters such the level of neurotransmitters in brain tissue and the corticosterone level in the plasma which were estimated in EWS rats using ELISA as shown in Figure 4A, B. The level of GABA and dopamine was reduced significantly ($p < 0.01$) and there was an increase in concen-

Table I. Vanillin ameliorates the level of anxiety in ethanol withdrawal syndrome induced anxiety using the elevated plus maze

No.	Group	Open arm entry (%)	Closed arm entry (%)
1	Control	36.25 ±1.62	63.75 ±2.32
2	EWS	10.95 ±1.02$^{\#\#}$	89.05 ±3.16$^{\#\#}$
3	Vanillin 100 mg/kg	22.87 ±1.54**	77.13 ±2.83**
4	Vanillin 200 mg/kg	31.40 ±1.76**	68.60 ±2.52**

Data were expressed as mean ±SEM (n = 8); $^{\#\#}p < 0.01$ compared to the control group; $^{**}p < 0.01$ compared to the EWS group

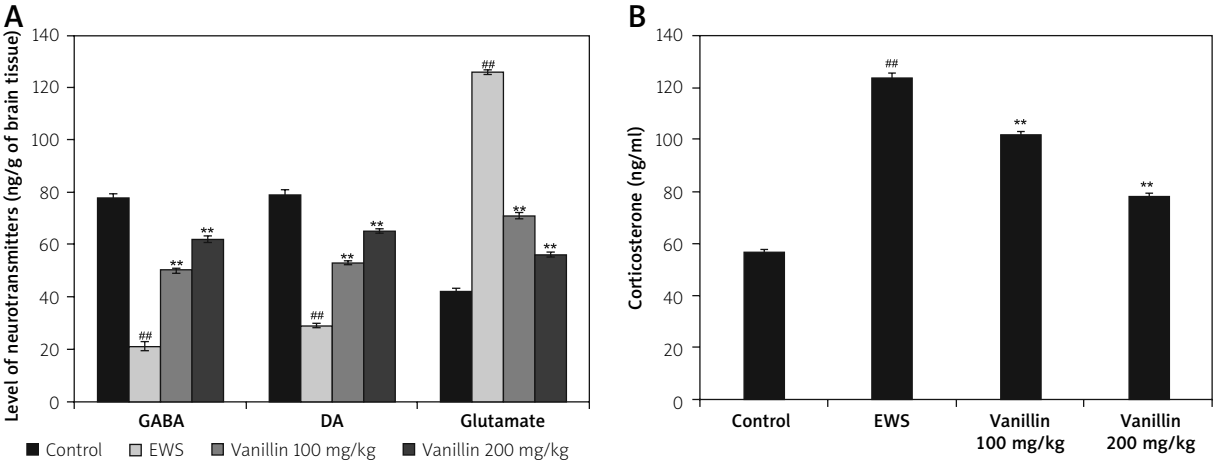


Fig. 4. Effect of vanillin on the biochemical parameters in ethanol withdrawal syndrome rats. **A**) Effect of vanillin on the level of neurotransmitters in the brain tissue homogenate of ethanol withdrawal syndrome rats. **B**) Effect of vanillin on the level of corticosterone in the plasma of ethanol withdrawal syndrome rats. Mean ±SEM (n = 8); $^{\#\#}p < 0.01$ compared to the control group; $^{**}p < 0.01$ compared to the EWS group.

tration of glutamate in the brain tissue of EWS group compared to the control group of rats. Treatment with vanillin attenuates the altered level of dopamine, GABA and glutamate in the brain tissue homogenate of ethanol withdrawal syndrome rats (Fig. 4A). The corticosterone level was enhanced ($p < 0.01$) significantly in EWS group compared to the control group of rats. There was a significant reduction in concentration of corticosterone in the plasma of the vanillin treated group compared to the EWS group of rats (Fig. 4B).

Effect of vanillin on mRNA expression of CRF/CRFR1

The effect of vanillin was estimated based on the mRNA expression of CRF and corticotropin releasing factor receptor 1 (CRFR1) was estimated in brain tissue homogenate of ethanol withdrawal syndrome rats using qRT-PCR as shown in Figure 5. mRNA expression of CRF and CRFR1 was enhanced significantly in brain tissue homogenate of EWS group than control group of rats. There was significant reduction in mRNA expression of CRF and CRFR1 in brain tissue of vanillin treated group than EWS group of rats.

Discussion

Habituation or chronic ethanol consumption is one of the major public health problems. The ethanol withdrawal syndrome (EWS) is developed after withdrawal of alcohol in an individual on chronic ethanol consumption [21]. People with chronic ethanol consumption commonly develop physical dependence, its withdrawal causes development of behavioural changes and anxiety. These changes negatively motivate the individual to revert to the use of alcohol [4]. These behavioural changes develop due to alteration in the level of biochemical parameters such as neurotransmitters and endocrine hormones including corticosterone [10]. Evidence reveals that NMDA receptor is upregulated and GABA-A receptor is downregulated in the ethanol withdrawal condition [19]. Modulating GABA and glutamate level are the possible targets for the management of EWS. Vanillin is reported to show potential

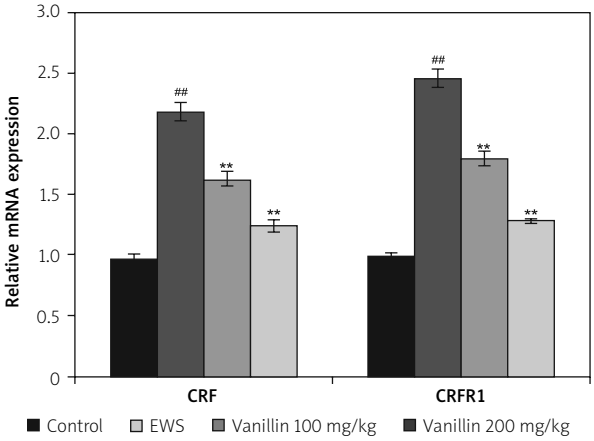


Fig. 5. Effect of vanillin on the mRNA expression of CRF/CRFR1 in the tissue homogenate of ethanol withdrawal syndrome rats using qRT-PCR. Mean ±SEM (n = 8); $^{\#\#}p < 0.01$ compared to the control group; $^{**}p < 0.01$ compared to the EWS group.

anti-depressant activity by modulating GABA and also protects neurodegeneration [22]. Thus, the presented report determines the beneficial effects against EWS.

Ethanol withdrawal syndrome characterized with several symptoms such as stereotyped behaviour, grooming, sniffles, tail stiffness, tremors and agitation [17] and the present study also supports it. The literature reveals that administration of ethanol at 9-15 g/kg/day for more than four consecutive days causes dependence and its withdrawal alters the behavioural signs [8]. Data of the presented report suggest that ethanol exposure reached 14.9-16.9 g/kg/day in the ethanol administered group and changes in behaviour were observed in the negative control group compared to the control group. Modulation of these altered behavioural changes could be achieved for the treatment of EWS. Results of the study suggest that treatment with vanillin attenuates the altered behavioural changes in EWS. Neurotransmitters such as GABA, dopamine and glutamate regulate the behaviour of an individual and chronic ethanol consumption modulates these neurotransmitters [2]. Ethanol withdrawal alters the level of these neurotransmitters and attenuation of these altered levels of neurotransmitters treats the EWS. Treatment with vanillin attenuates the altered level of GABA, dopamine, and glutamate in the brain tissue of EWS rats.

Anxiety is another common behavioural change occurring in the ethanol withdrawal condition. Corticosteroid is one of the major hormones called an anxiety inducer, commonly associated with EWS. The literature suggests that the level of corticosterone increases in EWS [18], so anxiety is commonly associated with it. Moreover, corticosterone release enhances due to an increase in corticotropin releasing factor (CRF) and due to upregulation of corticotropin releasing factor receptor 1 (CRFR1) [23]. Data of the study also support it and treatment with vanillin reduces the level of corticosterone, CRF and CRFR1 in EWS rats.

Conclusions

In conclusion, data of the study reveal that treatment with vanillin attenuates the behavioural changes in EWS rats by regulating neurotransmitters and modulates the EWS associated anxiety by regulating the level of corticosterone. Results of the present study reveal that vanillin could be used clinically for the management of EWS.

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Disclosure

The authors report no conflict of interest.

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