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Original Research Article

Anxiety-like behavior associated with alcohol withdrawal syndrome in mice and possible antagonistic effect of *Polycephalomyces nipponicus* aqueous extract

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Abstract

Purpose: *To investigate the effect of Polycephalomyces nipponicus (P. nipponicus) extract on ethanol withdrawal syndrome in mice.*
Methods: *Male Institute of Cancer Research (ICR) mice were divided into 3 groups of 10 animals per group. Mice were intraperitoneally injected with ethanol (2 g/kg/day) or normal saline solution 0.9 % (0.05 mL/kg) as control for 10 consecutive days. Anxiety-like behavior associated with alcohol withdrawal syndroms (AWS) was assessed at 12, 24, and 36 h after the last dose of ethanol or normal saline using the light-dark box, open field, and elevated plus maze tests. Thereafter, the time that showed the most prominent AWS was chosen to determine the effect of P. nipponicus extract. P. nipponicus extract (600 mg/kg, orally) or diazepam (4 mg/kg, i.p., as a positive control), was administered 1 h before the tests.*
Results: *Polycephalomyces nipponicus extract significantly mitigated anxiety-like behavior in alcohol-withdrawn mice across all evaluated models ($p < 0.05$). The results suggest that P. nipponicus reduced alcohol withdrawal syndrome, especially anxiety-like behavior.*
Conclusion: *This study provides evidence that P. nipponicus may be useful in the treatment of alcohol withdrawal syndrome, especially anxiety-like behavior. This study shows the potential of P. nipponicus as a new intervention for alleviating neurochemical imbalances linked to alcohol withdrawal.*

Keywords: *Polycephalomyces nipponicus, Ethanol, Anxiety, Alcohol withdrawal syndrome*

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INTRODUCTION

Ethanol, the principal component of alcoholic beverages, is the most consumed recreational beverage and a significant substance of abuse among adult population. This prevalence, however, comes with alcohol-related deaths ranking as the third leading cause of mortality on a global scale [1]. The perils associated with excessive alcohol consumption extend beyond immediate health risks and development of dependence in many individuals. When the habitual intake of alcohol is abruptly halted or significantly reduced in dependent individuals, a distinct syndrome called alcohol withdrawal syndrome (AWS) emerges.

Alcohol withdrawal syndrome is characterized by

a continuum of signs and symptoms indicative of central nervous system (CNS) hyperexcitability. This heightened neural activity serves as a compensatory response to the depressive effect induced by prolonged alcohol consumption [2]. Severity and duration of symptoms vary widely, and it is influenced by factors such as quantity and duration of alcohol consumption, individual differences, and overall health [3]. Typical manifestations of AWS encompass a spectrum ranging from more common symptoms such as anxiety, shakiness, sweating, vomiting, fast heart rate, hyperthermia, and hyperventilation, to the more severe expressions of neural hyperexcitation, including hallucinations, delusions, and grand mal seizures [4].

Development of alcohol dependence and AWS are involved with a wide range of neurochemical systems, including glutamate, γ-amino butyric acid (GABA), monoamines and various neuromodulators and ion channels [2]. Many pieces of evidence implicate adenosine, one of the central neuromodulators, in the pathophysiology of many psychiatric disorders, including alcohol use disorder [5]. Adenosine also plays a crucial role in regulating neuronal activity and modulating signaling by other neurotransmitters, which are key players in various facets of alcohol use disorders [6].

Polycephalomyces nipponicus is an insect pathogenic fungus that exhibits significant potential as a source of natural antioxidants and antibacterial agents, because of its high total phenolic and flavonoid content [7]. It is enriched with adenine and adenosine, making it a compelling candidate for investigation of its impact on AWS. This study investigated the potential of *P. nipponicus* extract as a novel intervention for mitigating anxiety-like behavior associated with alcohol withdrawal in mice, which might offer a promising avenue for future therapeutic strategies in alcohol use disorders.

EXPERIMENTAL

P. nipponicus extract

The mycelia of *P. nipponicus* isolate Cod-MK1201 were identified and extracted as previously described [7]. The fungal mycelium was collected, dried at 50 °C overnight, and then ground into powder with a mortar and pestle. The dried powdered mycelium was mixed with sterile distilled water (100 mg/mL), sonicated, centrifuged, and filtered through a 0.2-µm filter before use.

Animals

Male outbred Mlac: Institute of Cancer Research (ICR) mice, 6 - 8 weeks, weighing 25 - 35 g were purchased from the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. Mice were allowed to acclimatize in a specified room under standard housing conditions (22 ± 1 °C, 12 h light/dark cycle with lights on at 06:00 and off at 18:00). Ethical approval was obtained from the Animal Research Ethics Committee, Khon Kaen University, Thailand (approval no. IACUC-KKU-53/2565, reference number 660201.2.11/366), and adhered to the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health [8].

Design

The mice received intraperitoneal injections with either normal saline solution 0.9 % (n = 10) or ethanol (2 g/kg/day; n = 10) for 10 consecutive days. Anxiety-like behavior as a sign of AWS in mice was tested at 12, 24, and 36 h after the last dose of ethanol using the light-dark box (LDB) test, open field test (OFT), and elevated plus maze (EPM) test (Figure 1 A). The time that showed the most prominent sign of AWS was selected to further study the effect of *P. nipponicus* on anxiety-like behavioral alterations resulting from ethanol withdrawal. Every animal received two treatments. The initial treatment consisted of once daily intraperitoneal (IP) injections of ethanol (2 g/kg) for 10 consecutive days. A total of 30 mice were equally and randomly assigned to three groups (n = 10). Group 1 served as the control (ethanol withdrawal and received normal saline), group II received diazepam (4 mg/kg following ethanol withdrawal), and Group 3 received *P. nipponicus* (600 mg/kg following ethanol withdrawal) one hour before behavioural assessments. (Figure 1 B).

Light-dark box test

The apparatus used for the light/dark transition test comprised a cage partitioned into two parts with a door. The compartments measuring one-third for the dark compartment and two-thirds for the light chamber, have external dimensions of W27 x L46 x H30 cm. The two chambers were connected by a small opening (dimensions: W7 x H7 cm). The mice were positioned in the center of the light compartment, facing the dark side.

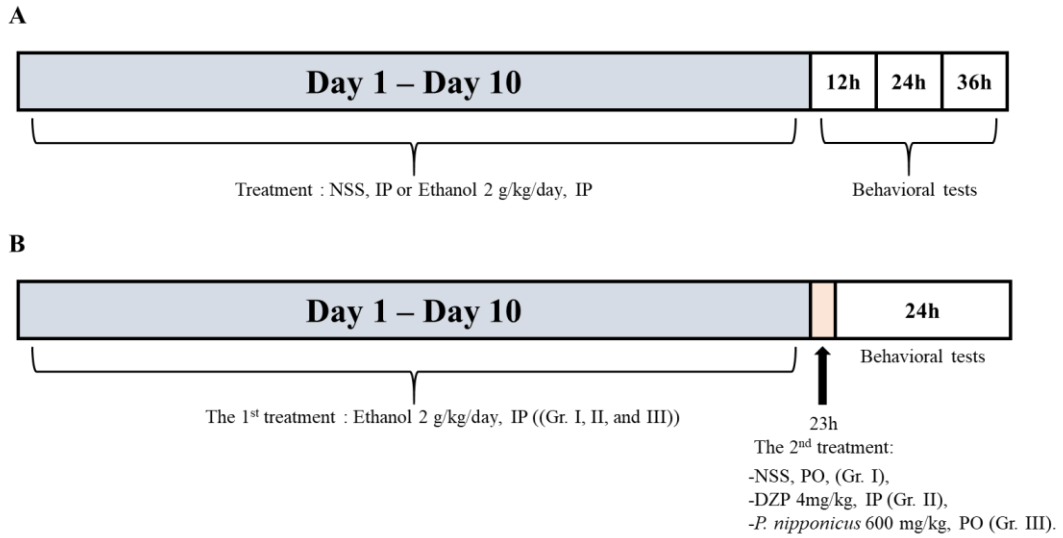

Figure 1: Experimental methodologies (A) to ascertain the optimal time interval to observe AWS. and (B) to determine the effect of *P. nipponicus* and DZP on AWS. ***Key:*** PO: per os/per oral, IP: intraperitoneal

Mice were allowed to move freely between the two chambers with the door open for 5 min. After that, behavioral patterns such as the duration of time spent in the light compartment (with all four limbs), and the frequency of crossings from dark to light. An increased duration in the light compartment and a greater frequency of crossings indicated less anxiety-like behaviors [9].

Open field test

The open-field apparatus consisted of a square arena (60 x 60 square arena, 50 high walls). For analysis, the open field was divided into two areas: the center (20 x 20 cm) and the outer peripheral area. In the beginning, the mice were placed in the center of the open field box and were left to explore the apparatus for 5 min. The time spent in the central area, as a measurement of anxiety-related behavior was recorded. Thereafter, behaviors such as duration spent in the central area (with all four legs) and frequency of entries in the center area were recorded. The more time spent in the center area and a high number of entries indicated lower levels of anxiety-like behavior [10].

Elevated plus maze (EPM) test

The EPM apparatus consisted of a plus-shaped maze elevated and comprised two open arms (30 x 10 cm) positioned opposite each other and perpendicular to two closed arms of the same size with 5 cm high side walls and end wall, along with a central region (10 x 10 cm) that connects all the arms. In this test, the mice were placed on the central platform facing a closed arm. The number of times spent in the open arms was used as a measure of anxiety-like behavior. The elevated plus maze test was documented with a video camera. The frequency of entries into the open arms or closed arms, and the time spent in the open arms were measured as indices of anxiety-like behavior. Increased duration in the open arms and/or higher number of entries into the open arms indicated less anxiety-like behavior [11].

Statistical analysis

Data was analyzed using the Statistical Packages for Social Sciences (SPSS 21.0 IBM, Armonk, NY, USA). Measurement data were expressed as mean ± standard error of means (SEM), and compared using one-way analysis of variance (ANOVA) accompanied by the Tukey post-hoc test. A two-tailed Student t-test was utilized in comparison between 2 groups. $P < 0.05$ was considered statistically significant.

RESULTS

Behavioral effect of ethanol withdrawal

Study group showed significantly lower time spent in the light area and frequency of crossings at all times compared to control group ($p < 0.05$) using the DLB (Table 1), and OFT (Table 2). Furthermore, study group showed significantly lower time spent in the open arm and in the frequency of entries in the open arms at all times compared to control group ($p < 0.05$). However, there was a significant increase in the number of entries in the closed arms at 24 h, but not at 12 h and 36 h in study group compared to control

group ($p < 0.05$; Table 3). Therefore, to ascertain the effect of *P. nipponicus* on anxiety-like behavior associated with AWS and in comparison, with diazepam, 24 hours following the last ethanol dose was selected.

P. nipponicus extract on ethanol withdrawal-induce anxiety-like behavior

Light dark box (LBD)

Diazepam treatment resulted in significantly higher time spent in the light compartment (Figure 2 A) and in the number of crossings (Figure 2 B; $p < 0.05$). Furthermore, administration of *P. nipponicus* (600 mg/kg, orally) significantly increased the time spent in the light part (Figure 2 A) and in the number of crossings (Figure 2 B) compared to control group (NSS; $p < 0.05$).

Open field

Treatment with DZP significantly increased the time spent in the center area (Figure 3 A) and number of entries in the center area (Figure 3 B; $p < 0.05$). However, *P. nipponicus* at 600 mg/kg showed significant increase in time spent in the central area (Figure 3 A) and in the number of entries in the center area (Figure 3 B) compared to control group (NSS; $p < 0.05$).

Table 1: Dark-light box test following ethanol withdrawal in mice

Time (h)	Time spent in the light part (s)		No. of crossing (light » dark)	
	Control	***Ethanol***	***Control***	***Ethanol***
12	102.40±3.15	56.11±3.74*	14.30±0.86	9.11±1.05*
24	90.40±2.60	29.67±3.58*	10.90±1.22	5.44±0.65*
36	91.20±2.60	21.24±1.52*	10.20±0.92	4.89±0.45*

Note: *$P < 0.05$ vs control group (n = 10)

Table 2: Open field test following ethanol withdrawal in mice

Time (h)	Time spent in the center area (s)		No. of crossing	
	Control	***Ethanol***	***Control***	***Ethanol***
12	14.00±1.28	9.33±0.47*	11.30±0.68	6.33±0.50*
24	12.44±0.56	5.38±1.03*	9.70±1.16	3.67±0.37*
36	13.33±1.03	4.11±0.89*	8.20±0.67	2.89±0.39*

Note: *$P < 0.05$ vs control group (n = 10)

Table 3: Elevated plus maze test done following ethanol withdrawal in mice

Time (h)	Time in the open arms (sec)		No. of open arms entries		No. of closed-arms entries	
	Control	***Ethanol***	***Control***	***Ethanol***	***Control***	***Ethanol***
12	54.25±3.92	33.25±2.17*	5.86±0.74	4.38±0.66*	4.06±0.58	4.75±0.82
24	56.22±2.45	23.90±4.67*	5.57±0.81	2.74±0.42*	3.83±0.70	5.71±0.57*
36	56.75±2.68	26.10±3.12*	5.63±0.50	2.75±0.65*	4.57±0.37	4.83±0.60

Note: *$P < 0.05$ vs control group (n = 10)

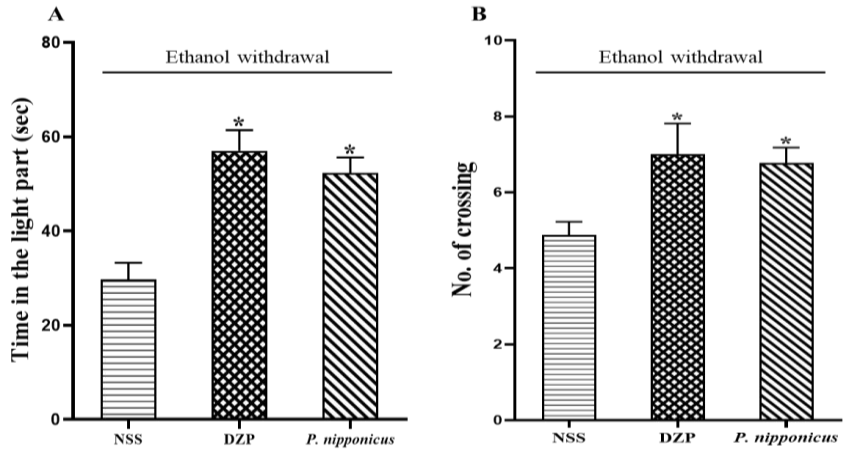

Figure 2: Effect of *P. nipponicus* and DZP on anxiety-related behaviors induced by ethanol withdrawal in mice tested by LDB. A: time spent in the light part (s), B: number of crossings. **Key:** *$P < 0.05$ vs control (NSS)

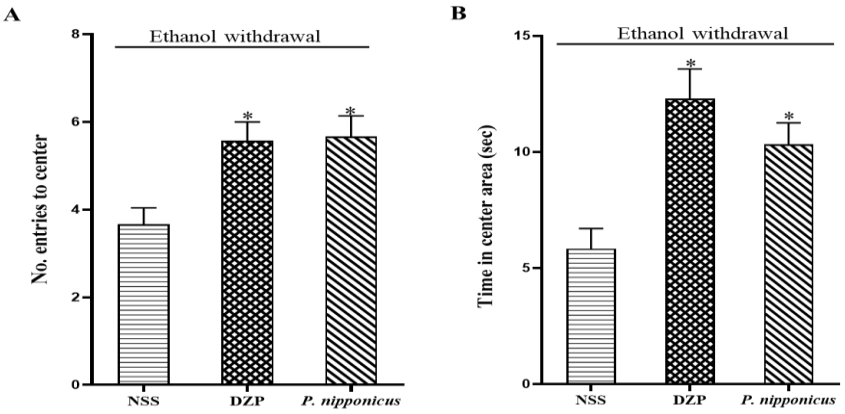

Figure 3: Effect of *P. nipponicus* and DZP on anxiety-related behaviors induced by ethanol withdrawal in mice tested by OFT. A: time in the center area (s), B: number of entries to the center. **P* < 0.05 vs control (NSS)

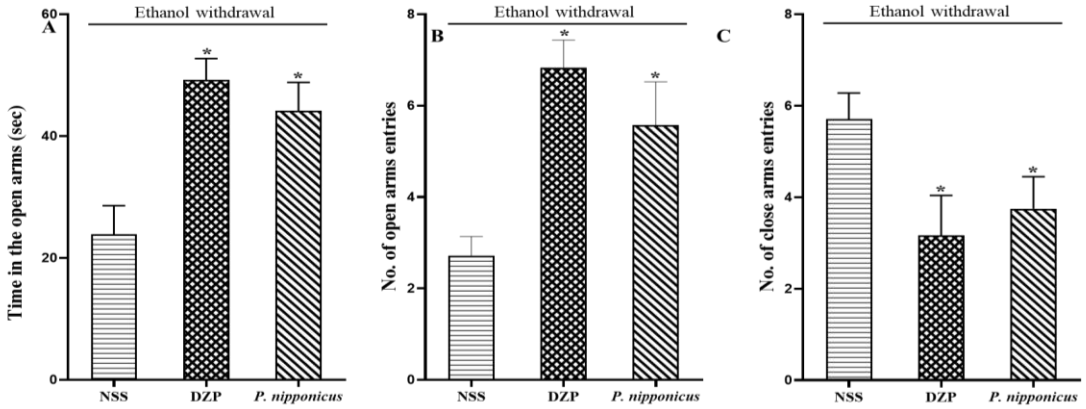

Figure 4: Effect of *P. nipponicus* and DZP on anxiety-related behaviors induced by ethanol withdrawal in mice tested by EPM. A: the time spent in the open arms (s). B: number of open arms entries. C: number of close arms entries. **P* < 0.05 vs control group (NSS)

Elevated plus maze

Treatment with DZP significantly increased the time and number of entries in the open arms, and the number of entries in the closed arm (Figure 4 A - C; $p < 0.05$). However, treatment with *P. nipponicus* at 600 mg/kg resulted in a significant increase in time spent in the open arms, number of entries in open arms, and the number of entries in the closed arm (Figure 4 A - C) compared to control group (NSS; $p < 0.05$).

DISCUSSION

Alcohol withdrawal syndrome (AWS) may develop within 6 to 24 h after abrupt discontinuation or decrease in alcohol consumption [12]. This study demonstrated that ethanol withdrawal significantly induced anxiety-like behavior in all tests. Diazepam was used as a positive control and mitigated the effect of ethanol withdrawal in the light-dark box, open field and elevated plus maze tests. Pre-treatment with *P. nipponicus* significantly alleviated the effect of the ethanol-induced anxiogenic effect induced by ethanol withdrawal. Results from this study suggested that *P. nipponicus* (its main compound being adenosine), significantly reduced the effect of ethanol in anxiety-like behavior of AWS. Generally, ethanol consumption may influence the adenosine neuromodulation system by elevating adenosine levels, hence enhancing the activation of adenosine receptors in the brain. The adenosine modulation system regulates mood, memory [13], and impairment of motor performance [14].

There are several potential reasons for the anti-anxiety and anti-depressive benefits of *P. nipponicus*. Since *P. nipponicus* is primarily composed of adenosine [15], it may help modulate the adrenergic system. Some studies suggest that ethanol may elevate adenosine levels and inhibit cellular uptake by type 1 equilibrative nucleoside transporter (ENT-1), resulting in a blockade by modulating equilibrium

among adenosine receptors, so influencing other neurotransmitters involved in anxiety [16]. This disturbs the equilibrium of the adenosine modulation system in the brain, leading to detrimental symptoms related to depression [15]. In addition, *P. nipponicus* has many active ingredients, including adenine and adenosine [17].

Adenosine is a modulator that has a pervasive effect [18], and it plays an important role in regulating neuronal activity and modulation signaling by other neurotransmitters, such as GABA, glutamate, and dopamine [15]. Also, ethanol enhances adenosine signaling by inhibiting type 1 equilibrative nucleoside transporter (ENT1), thereby elevating adenosine concentrations in the synaptic cleft. Chronic exposure to ethanol induces neuroadaptations in the densities of adenosine A1 and A2A receptors, perhaps contributing to ethanol dependence and neurotoxicity [19]. *P. nipponicus* extract, which contains adenosine, therefore has advantages in the treatment of mood disorders induced by ethanol. Therefore, *P. nipponicus* helps in reducing anxiety-like behavior in AWS. However, the extract mechanisms need to be further investigated.

CONCLUSION

Oral administration of *P. nipponicus* extract reduces alcohol withdrawal-induced anxiety in a similar extent as diazepam. Although the exact mechanism of action of *P. nipponicus* still needs further investigation, this study shows the potential of *P. nipponicus* as a new intervention for alleviating the neurochemical imbalances linked to alcohol withdrawal.

DECLARATIONS

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Ethical approval

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Khon Kaen University, Thailand; record number IACUC-KKU-53/2565, reference number 660201.2.11/366.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. JS, KP and CL conceived and supervised the project; AS and KS prepared the extract; SR, JK and KS performed the experiments; SR, JS, KP and CL analyzed the data; SR, JS and KP wrote the manuscript, reviewed, and modified the paper. All authors agree to be accountable for all aspects of work, ensuring integrity and accuracy.

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REFERENCES

1. *Pervin Z, Stephen JM. Effect of alcohol on the central nervous system to develop neurological disorder: pathophysiological and lifestyle modulation can be potential therapeutic options for alcohol-induced neurotoxication. AIMS Neurosci 2021; 8: 390–413.*
2. *Becker H, Mulholland P. Neurochemical mechanisms of alcohol withdrawal. Handb Clin Neurol 2014; 125: 133–156.*
3. *Jesse S, Bråthen G, Ferrara M, Keindl M, Ben-Menachem E, Tanasescu R, Brodtkorb E, Hillbom M,*

Leone MA, Ludolph AC. Alcohol withdrawal syndrome: mechanisms, manifestations, and management. Acta Neurol Scand 2017; 135: 4–16.

4. *Metten P, Schlumbohm JP, Huang LC, Greenberg GD, Hack WR, Spence SE, Crabbe JC. An alcohol withdrawal test battery measuring multiple behavioral symptoms in mice. Alcohol Fayettev N 2018; 68: 19–35.*
5. *Ruby CL, Adams C, Knight EJ, Nam HW, Choi DS. An essential role for adenosine signaling in alcohol abuse. Curr Drug Abuse Rev 2010; 3: 163–174.*
6. *Haun HL, Olsen ACK, Koch KE, Luderman LN, May CE, Griffin WC. Effect of caffeine on alcohol drinking in mice. Alcohol Fayettev N 2021; 94: 1–8.*
7. *Sangdee A, Sangdee K, Seephonkai P, Jaihan P, Kanyaphum T. Colony characteristics, nucleoside analog profiles, and genetic variations of medicinal fungus Polycephalomyces nipponicus (Ascomycetes) isolates from Northeast Thailand. Int J Med Mushrooms 2017; 19: 445–455.*
8. *National Institutes of Health. Guide for the care and use of laboratory animals. 8th ed. National Academies Press (US); 2011. doi:10.17226/12910.*
9. *Bourin M, Hascoët M. The mouse light–dark box test. Eur J Pharmacol 2003; 463: 55–65.*
10. *Ferraz-de-Paula V, Stankevicius D, Ribeiro A, Pinheiro ML, Rodrigues-Costa EC, Florio JC, Lapachinske SF, Moreau RL, Palermo-Neto J. Differential behavioral outcomes of 3,4-methylenedioxymethamphetamine (MDMA-ecstasy) in anxiety-like responses in mice. Braz J Med Biol Res Rev Bras Pesqui Medicas E Biol 2011; 44: 428–437.*
11. *Komada M, Takao K, Miyakawa T. Elevated plus maze for mice. J Vis Exp JoVE 2008; 22: 1088.*
12. *Mirijello A, D'Angelo C, Ferrulli A, Vassallo G, Antonelli M, Caputo F, Leggio L, Gasbarrini A, Addolorato G. Identification and management of alcohol withdrawal syndrome. Drugs 2015; 75: 353–365.*
13. *Pinheiro BG, Luz DA, Cartágenes S de C, Fernandes L de MP, Farias SV, Kobayashi NHC, Fontes-Júnior EA, Ferreira SG, Cunha RA, Prediger RD, et al. The role of the adenosine system on emotional and cognitive disturbances induced by ethanol binge drinking in the immature brain and the beneficial effect of caffeine. Pharm 2022; 15: 1323.*
14. *Möykkynen T, Korpi ER. Acute effect of ethanol on glutamate receptors. Basic Clin Pharmacol Toxicol 2012; 111: 4–13.*
15. *Coelho JE, Alves P, Canas PM, Valadas JS, Shmidt T, Batalha VL, Ferreira DG, Ribeiro JA, Bader M, Cunha RA, et al. Overexpression of adenosine A2A receptors in rats: effect on depression, locomotion, and anxiety. Front Psychiat 2014; 5: 67.*
16. *Fritz BM, Companion M, Boehm SL. "Wired," yet intoxicated: modeling binge caffeine and alcohol co-consumption in the mouse. Alcohol Clin Exp Res 2014; 38: 2269–2278.*
17. *Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci. 2001; 24: 31–55.*
18. *Butler TR, Prendergast MA. Neuroadaptations in adenosine receptor signaling following long-term ethanol exposure and withdrawal. Alcohol Clin Exp Res 2012; 36: 4–13.*
19. *Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 2001; 24: 31–55.*