Brief Report

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The ethanol extract of *Cirsium japonicum* increased chloride ion influx through stimulating $GABA_A$ receptor in human neuroblastoma cells and exhibited anxiolytic-like effects in mice

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ABSTRACT: The aim of the present study was to evaluate the anxiolytic effects of the ethanol extract of *Cirsium japonicum* (CJ) in mice. The extract was orally administered at dosages of 50, 100, 200, or 400 mg/kg of body weight. The CJ-induced behavioral changes were assessed using the open-field and elevated-plus maze test. The ethanol extract of CJ did not affect overall locomotor activity of mice in the open-field test, however, it showed increase exploration in the unprotected center zone, which is thought to reflect anxiolytic-like effects. Furthermore, the CJ extract (100 and 200 mg/kg) significantly increased the percentage of time spent in the open arms of the elevated plus-maze, indicating the anxiolytic effects of the substance. This anxiolytic effects of the extract were comparable to that of the benzodiazepine, diazepam. To further characterize the anxiolytic activities of CJ, its action on human neuroblastoma cells were assessed. The CJ extract dose-dependently increased chloride ion (Cl^-) influx, which was blocked by co-administration of the $GABA_A$ receptor competitive antagonist, bicuculline, suggesting a $GABA_A$ receptor – Cl^- channel mechanism of action. Taken altogether, the present study demonstrates that the ethanol extract of CJ has anxiolytic effects, probably mediated through GABAergic neurotransmission.

Keywords: *Cirsium japonicum*, anxiolytic, benzodiazepine, GABA, anxioselective

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1. Introduction

Anxiety is a psychological and physiological condition characterized by emotional, cognitive, somatic, and behavioral factors. It is considered as normal reaction to stressors and is important for survival, since it alerts us to danger and prepares us to cope with imminent situations (*1*). However, when anxiety is overwhelming, it may fall under the classification of anxiety disorder. Anxiety disorders are the most common psychiatric diagnosis in general population which has an estimated lifetime prevalence of about 16% and a 12-month prevalence of 11% (*2*). Anxiolytics or anti-anxiety agents are drugs used to relieve anxiety and manage its related psychological and physical symptoms. Thus drugs usually act as depressors of the central nervous system (CNS) (*1,2*). Presently, benzodiazepines (*e.g.* diazepam) are one of the drugs of choice in treating/controlling anxiety. However, it is prescribed with special considerations due to its common side effects (*1*). With the hope of finding an alternative treatment for anxiety with fewer side effects, many researchers have delved into the study of herbal medicines or plant extracts (*3,4*). Currently, studies on herbal medicine as complementary and alternative medicine (CAM) for mild to moderate anxiety disorders are becoming popular and accepted worldwide (*4,5*).

Cirsium japonicum (CJ) is a perennial herb native to China, Japan, and Korea belonging to Compositae family (*6*). It has been classified in the Japanese and Chinese Pharmacopeia and was used in various preparations as an antihemorrhagic, antihypertensive, and uretic agent. CJ has also been traditionally used as an antioxidant, antidiabetic, and antimicrobial (*7-10*). There are several studies about the pharmacological effects of CJ, but very limited information is available about its psychopharmacological effects. A recent notable study with CJ shows that it has antidepressant property, causing significant decrease in depression associated with pre-menstrual syndrome (*11*).

Considering the above-mentioned effects, we decided to assess whether CJ has anxiolytic properties.

Thus, the goal of the present study was to evaluate the psychopharmacological activities of CJ, specifically its probable anxiolytic effect. The ethanol extract of CJ was dissolved in water and were orally administered to mice in dosages of 50, 100, 200, or 400 mg/kg of body weight. Then, behavioral changes consequential to CJ treatment were observed in the open-field and the elevated plus-maze test. The open-field test provides a unique opportunity to systematically assess novel environment exploration, general locomotor activity, and provide an initial screening for anxiety-related behavior, through exploration on the unprotected center area (*12*). Furthermore, CJ's effects were evaluated in the elevated plus-maze test, the most widely used and well-established paradigm in assessing anxiety-like behavior in rodents (*13*). The effect of one of the most commonly used and prescribed anxiolytic agent, the benzodiazepine diazepam, was also evaluated and used as a reference drug. Moreover, to further characterize the anxiolytic effects of CJ, its action on the γ-aminobutyric acid (GABA) receptor-chloride ion (Cl^-) channel complex was assessed in human neuroblastoma cells (*1,12,14*).

2. Materials and Methods

2.1. *Animals*

The male ICR mice (20-25 g) used in the present study were obtained from Hanlim Laboratory Animals Co. (Hwasung, Korea). They were housed in groups in a temperature-controlled (22 ± 2°C) and humidity-controlled (55 ± 5%) animal room on a 12/12 h light/dark (7:00-19:00 h light) schedule. Food and water were freely available, except the night before and during the experiments. Mice were allowed to acclimatize to the laboratory setting, for at least 7 days, before the commencement of any experiments. Eight to ten animals were used in each experimental group. Animal treatment and maintenance were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23 revised 1985) and the Animal Care and Use Guidelines of Sahmyook University, Korea.

2.2. *Drugs and materials*

Samples of aerial part of CJ were obtained from Cheju province of Korea, and were examined by botanical experts. The dried sample (10 g dry weight) was subjected to ethanol extraction (900 mL) by shaking for 25 h at room temperature. The obtained ethanol extract was filtered then freeze-dried. Diazepam, bicuculline, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The CJ extract and diazepam were diluted with sterile distilled water before administration. *N*-(6-Methoxyquinolyl) acetoethylester (MQAE) was purchased from Invitrogen Co. (Carlsbad, CA, USA). Bicuculline was dissolved in DMSO, with a maximum concentration of 0.1%. CJ extract was orally administered to mice in dosages of 50, 100, 200, or 400 mg/kg. One mg/kg diazepam was intraperitoneally (*i.p.*) injected to mice belonging to the reference group. Animals in the control group received the vehicle (distilled water).

2.3. *Psychopharmacological evaluation*

2.3.1. *Open-field test*

Mice were placed in an open-field arena, consisting of a square plexiglas container (42 × 42 cm) with a field bordered by 42 cm high sidewalls. Mice were orally pre-treated with CJ, or vehicle, or diazepam (*i.p.*), 30 min before the commencement of the test. Prior to recording, animals were first habituated to the open-field for 2 min to remove the bias of novelty. The observed parameters were moved distance on open-field arena and unprotected center zone, was recorded for 10 min (*12*). Ethovision (Noldus, Netherlands) system was used to record animal movement.

2.3.2. *Elevated plus-maze (EPM) test*

The elevated plus-maze box and arms were made of plastic. The plus maze consisted of four arms that comprised of two open arms (30 × 6 cm in mice) and two closed arms (30 × 6 cm in mice) enclosed by 20 cm high walls. Each arm had a delimited central area of 6 × 6 cm. The entire maze was elevated to a height of 50 cm above the floor. Mice were orally pre-treated with CJ, vehicle or diazepam (*i.p.*), 30 min before placement on the EPM. To begin a test session, mice were placed in the center of the maze facing one of the open arms. An entry into an arm was defined as the animal placing all four paws over the line marking that area. The observed parameters were (*i*) time spent in the open arms and (*ii*) number of entries into the open arms during the 5 min test period (*13*). The percentage of open arm entries (100 × open/total entries) was calculated for each animal.

2.3.3. *Assay of Cl^- influx*

Human neuroblastoma SH-SY5Y cells (Korean Cell Line Bank, Korea) were cultured in minimum essential medium (MEM) (Invitrogen Co.) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich Co.) in a humidified incubator of 95% air and 5% CO_2 at 37°C.

Experiments were performed according to the methods of Yoon *et al*. (*15*). Briefly, cells were washed twice and suspended at a concentration of 4×10^5

cells/ ml in Hank's solution. For loading MQAE into the cells, cells were incubated with the dye overnight in a final concentration of 5 mM at room temperature. Fluorescence (excitation wavelength set as 365 nm, the emission wavelength at 450 nm) was monitored in a well-stirred cuvette. Experiments were performed at room temperature to minimize fluorescence dye loss. Data are presented as relative fluorescence F/F_0, where F_0 is minimize fluorescence without Cl^- ions and F is the fluorescence as a function of time. The F/F_0 was directly proportional to $[Cl^-]_i$. All fluorescence values were corrected for background fluorescence which was separately determined using HEPES-buffered KSCN solution containing 5 µM valinomycin to maximally quench the MQAE ion-selective signal. In separate experiments the F_0 value was determined by bathing the cells with Cl^--free (KNO_3) solution containing 10 mM tributyltin and 10 mM nigericin.

2.4. *Statistical analysis*

Data are expressed as mean ± the standard error of the mean (SEM). For statistical evaluation of data, one-way analysis of variance (ANOVA) was used. When statistically significant differences were found, Dunnett test was used as a post-hoc test, to determine the statistical difference between groups. Differenced were considered statistically significant when $p < 0.05$.

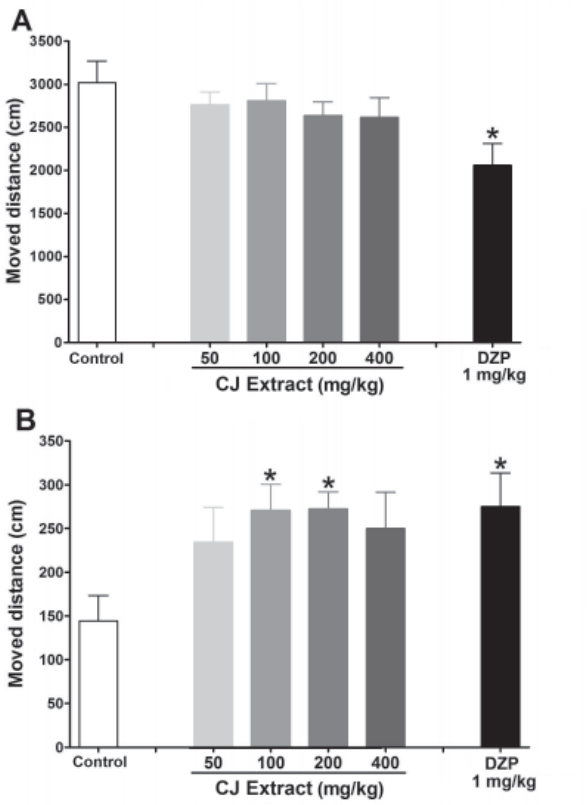

Figure 1. Effects of ethanol extract of CJ on locomotor activity in mice. Each bar represents the mean ± SEM (n = 8-10) of the moved distance in arena **(A)** and center zone **(B)**, for 10 min. * $p < 0.05$ significantly different from the control group. DZP, diazepam.

3. Results

3.1. *Open-field test*

Figure 1 shows the distance moved in the open-field arena and unprotected center area of animal treated with the CJ extract, diazepam, or vehicle (control group). Ambulatory activity was significantly decreased by diazepam ($q = 3.16$, $p < 0.05$) as compared to the control group, indicative of its sedative effects (*16*). On the other hand, the CJ extract did not significantly affect ambulatory activity in the open-field arena. In addition, diazepam significantly increased exploratory time at the unprotected center zone ($q = 2.84$, $p < 0.05$). This increase in exploration in the center area was thought to reflect the anxiolytic property of diazepam (*17*). Similarly, the CJ extract showed a significant increase in exploratory activity in the center area of the open-field test at doses of 100 ($q = 2.75$, $p < 0.05$) and 200 ($q = 2.78$, $p < 0.05$) mg/kg.

3.2. *EPM test*

Figure 2 shows the percentage of time spent and entries in the open arm of the plus-maze. As expected, diazepam significantly increased percentage of entries ($q = 6.14$, $p < 0.001$) and time spent ($q = 7.04$, $p < 0.001$) in the open arms of the maze, demonstrating

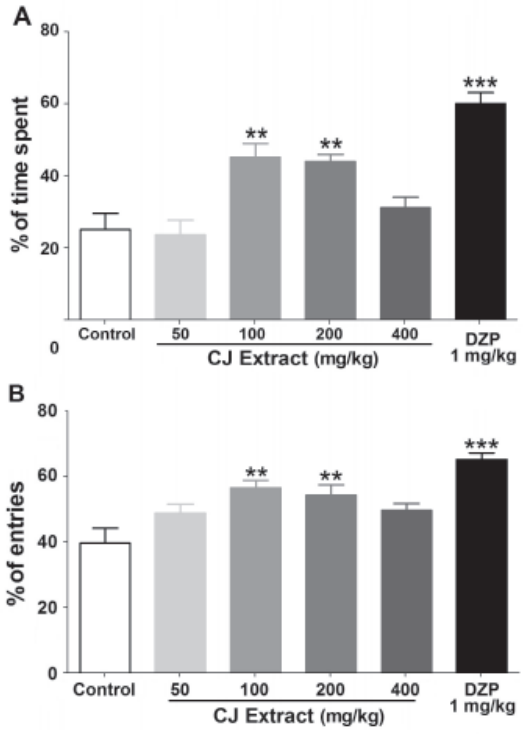

Figure 2. Effects of ethanol extract of CJ on elevated plus maze test in mice. Each bar represents the mean ± SEM (n = 8-10) of the percentage of time spent **(A)** or entries **(B)** into the open arm of the maze. ** $p < 0.01$, *** $p < 0.001$ significantly different from the control group. DZP, diazepam.

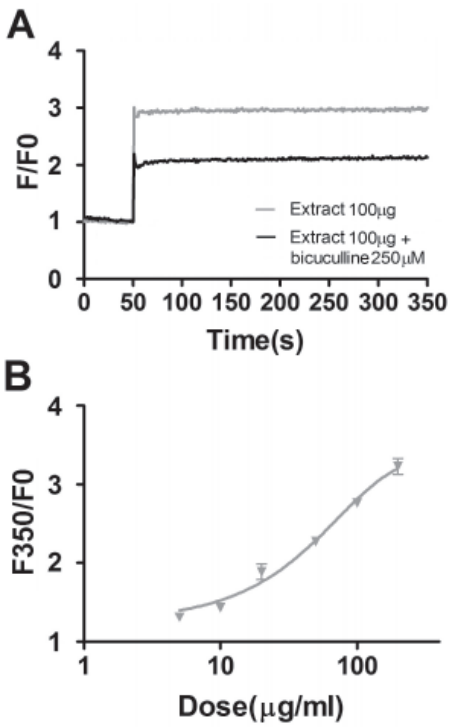

Figure 3. Effects of ethanol extract of CJ or CJ plus bicuculline on [Cl^-] influx in neuroblastoma cells. Fluorescence was monitored in the excitation wavelength at 365 nm and the emission wavelength at 450 nm using Cl^- sensitive indicator, MQAE. Contents of influx Cl^- were expressed as a peak (a.u.).

its anxiolytic effects (*18*). The CJ extract also showed profound increase in percentage of time spent [100 (q = 4.05, $p < 0.01$), 200 mg/kg (q = 3.82, $p < 0.01$)] and entries [100 (q = 4.06, $p < 0.01$), 200 mg/kg (q = 3.53, $p < 0.01$)], in the open arm of the plus maze.

3.3. *Intracellular Cl^- measurement assay*

Figure 3 shows the action of the CJ in cultured human neuroblastoma cell. Treatment of CJ extract increased Cl^- ion influx in human neuroblastoma cells, in a dose-dependent manner. Furthermore, this CJ-induced increment in the Cl^- influx was significantly blocked by the specific antagonist of the $GABA_A$ receptor, bicuculline (Figure 3).

4. Discussion

The present study shows that the ethanol extract of CJ produces anxiolytic-like effects in animals, as evaluated by the open-field test and the elevated plus-maze test. Furthermore, the CJ extract induced Cl^- ion influx in human neuroblastoma cells, suggesting that its anxiolytic effects are probably mediated through a GABAergic mechanism of action.

The open-field test is a simple way to measure general locomotor activity, willingness to explore as well as anxiety-like behaviors (*12*). In the present study, diazepam (1 mg/kg) caused significant decrease in exploratory activity, which is expected because of its known sedative properties (*16*). Treatment of the CJ extract did not profoundly alter general locomotion demonstrating that the CJ might not have, or might have very minimal, sedative properties. In addition, the animal's exploration in the center zone of the open-field was also evaluated. Activity in the center zone of the open-field can be a tool to evaluate the anxiolytic properties of a substance (*12*). Normally animals explore on the peripheral area and tend to remain close to the walls of the open-field, if a drug/substance has anxiolytic properties it would promote central zone exploration (*19*). Diazepam, a known anxiolytic drug, significantly increased central-zone exploration. This increase in center zone activity was also observed in animals treated with the CJ extract (100 and 200 mg/kg), reflecting a probable anxiolytic effects of CJ (Figure 1B). This anxiolytic-like activity of CJ extract, in the open-field test, was further supported by the findings in the EPM. The EPM test is the most widely used and accepted paradigm to measure anxiety-related behavior in rodents and considered to be a valid animal model of anxiety because it utilizes natural anxiogenic stimuli complementary to those observed in humans (*13,20*). Diazepam exhibited its established anxiolytic effects by significantly increasing the time spent and entries in the open arms of the maze. Increase in the proportion of time spent and entries into the open arms of the maze indicates reduction of anxiety (*17*). In a highly comparable manner, the CJ extract (100 and 200 mg/kg) significantly increased time spent and entries into the open arms of the maze. Taken altogether, our behavioral results suggest that the ethanol extract of CJ has anxiolytic properties comparable to that of diazepam, but with less sedative effects.

It can be observed from the results that the anxiolytic effects of CJ are not dose-dependent. Due to the limitations of the present study we cannot fully explain the exact reason behind this result, and that we can only infer based on previous reports/studies. Some studies on the anxiolytic effects of plant extracts, reported somewhat similar results (less effects on higher doses). This was attributed to the complex pharmacokinetic and pharmacodynamics of plant extracts (*21,22*). Plant extracts are composed of various components mostly with unknown pharmacological and dose-response data (*21*). It is possible that with higher dose complex interactions occur (probably between CJ and other bodily systems), which then altered the anxiolytic effects of the extract. Somewhat in support, is the *in vitro* finding (discussed below) wherein dose-dependent effects were observed when the CJ extract was administered directly to cultured human neuroblastoma cells, detouring some pharmacokinetic processes. It would be beneficial to investigate the individual effect of the extract in order to elucidate the

agents responsible for its anxiolytic effects. However, the present study is limited, thus further studies are needed to adequately address these issues. Nevertheless, the present results can be helpful for therapeutic decisions and with respect to toxicology.

It is thought that anxiety is a product of an imbalance in the brain; primarily between the excitatory (glutamate) and inhibitory (GABA) forces. In a normally functioning brain there is a delicate balance between excitatory and inhibitory forces, when this equilibrium is disrupted it would cause abnormalities in functioning (*23*). Wierońska *et al*. (*23*) asserted that this was the case in anxiety, the harmony between excitatory *versus* inhibitory forces in the brain is disrupted, such that GABA levels are decreased resulting to decrease inhibition leading to over-excitation. Indeed, this might be true because of the fact that GABA is the brain's principal modulatory (inhibitory) neurotransmitter, playing a very significant role in maintaining/regulating homeostatic milieu or equilibrium (*24*). Furthermore, supporting this hypothesis is the mechanism of action of one of the most commonly prescribed anxiolytic drug, the benzodiazepines (diazepam). Benzodiazepines alleviate anxiety by potentiating GABA neurotransmission, counterbalancing the over-excitation observed in anxiety (*25*). Specifically, it binds at the allosteric (benzodiazepine) site in the $GABA_A$ receptor resulting to a facilitation of the opening of its Cl^- sensitive ion channel (*24,26*). Based on all of these, we have decided to evaluate the effects of the CJ extract at the GABA receptor by measuring Cl^- influx in human neuroblastoma cells. Indeed, our results showed that the CJ extract facilitated Cl^- influx in a dose dependent manner (Figure 3). In addition, this increase in Cl^- influx was significantly blocked by co-administration of bicuculline, a specific and competitive antagonist of the $GABA_A$ receptor (*27*). These findings suggest that the anxiolytic-like effects of CJ might be mediated through a $GABA_A$ receptor-Cl^- ion channel mechanism of action.

In the present study we have observed a somewhat incongruent result in the sedative effects of diazepam and the CJ extract. Diazepam produced anxiolytic effects coupled with sedative effect. On the other hand, the CJ extract showed comparable anxiolytic effects but without profound sedative effects. Although, anxiolytic and sedative effects usually go hand in hand, there are reports which implied that anxiolytic and sedation are two dissociable things (*28*). In fact researchers have identified specific areas in the $GABA_A$ receptor wherein these effects are mediated. Sedation is mediated in the (α_1 and/or α_5) subunit of the GABA receptor, while anxiolytic is mediated in the (α_2 and/or α_3) subunit (*24*). Benzodiazepines (diazepam) targets all of these subunits, thus it manifest both sedative and anxiolytic effects (*29*). It is possible that the CJ extract acts on the subunits of the $GABA_A$ receptor which mediates anxiety, but not on the subunits which mediates sedation. This might probably explain the anxiolytic effects, void of significant sedative effects, manifested by the CJ extract. This particular effect may be beneficial, because the sedative effects associated with anxiolytic agents may sometimes be undesirable and even dangerous. Sedative drugs may cause deterioration of person's optimal daily functioning (*e.g.* work, school) or even predispose one to fatal harm (*e.g.* driving, falls, *etc*.) (*30*). This is the reason why scientists are on the search to find an anxioselective drug; and based on the present results CJ might be a promising candidate.

5. Conclusion

The results from present study provide evidences for the anxiolytic effect of the ethanol extract of CJ. In addition, CJ's anxiolytic effects are void of profound sedation suggesting that CJ is a promising anxioselective drug.

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References

1. Meyer JS, Quenzer LF. Psychopharmacology: Drugs, the brain, and behavior. In: Anxiety Disorders (Donini G, Emerson K, Sydney C, Via M, eds.). Sinauer Associates, Inc., Sunderland, MA, USA, 2005; pp. 411-438.
2. Kessler RC, Chiu WT, Demler O, Merinkangas KR, Walters EE. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry. 2005; 62:617-627.
3. Rex A, Morgenstern E, Fink H. Anxiolytic-like effects of kava-kava in the elevated plus maze test – a comparison with diazepam. Prog Neuropsychopharmacol Biol Psychiatry. 2002; 26:855-860.
4. Lakhan SE, Vieira KF. Nutritional and herbal supplements for anxiety and anxiety-related disorders: Systematic review. Nutr J. 2010; 9:42.
5. World Health Organization. The World Health Fact sheets 2008 – Media centre: Traditional medicine *http://www.who.int/mediacentre/factsheets/fs134/en/* (accessed November 15, 2012).
6. China Pharmacopoeia Committee. Pharmacopoeia of the People's Republic of China. Chemical Industry Press, Beijing, China, 2000; 1:42.
7. Han JY, Ahn SY, Kim CS, Yoo SK, Kim SK, Kim HC, Hong JT, Oh KW. Protection of apigenin against kainate-induced excitotoxicity by anti-oxidative effects. Biol Pharm Bull. 2012; 35:1440-1446.
8. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. VII. On the antihemorrhagic principle in Cirsium japonicum DC. Chem Pharm Bull (Tokyo). 1987; 35:861-864.

9. Liao Z, Wu Z, Wu M. *Cirsium japonicum* flavones enhance adipocyte differentiation and glucose uptake in 3T3-L1 cells. Biol Pharm Bull. 2012; 35:855-860.
10. Yin J, Heo SI, Wang MH. Antioxidant and antidiabetic activities of extracts from *Cirsium japonicum* roots. Nutr Res Pract. 2008; 2:247-251.
11. Chung MS, Kim GH. Effects of *Elsholtzia splendens* and *Cirsium japonicum* on premenstrual syndrome. Nutr Res Pract. 2010; 4:290-294.
12. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. Eur J Pharmacol. 2003; 463:3-33.
13. Pellow S, File SE. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. Pharmacol Biochem Behav. 1986; 24:525-529.
14. Engin E, Treit D. The effects of intra-cerebral drug infusions on animals' unconditioned fear reactions: A systematic review. Prog Neuropsychopharmacol Biol Psychiatry. 2008; 32:1399-1419.
15. Yoon SY, dela Peña IC, Shin CY, Son KH, Lee YS, Ryu JH, Cheong JH, Ko KH. Convulsion-related activities of *Scutellaria flavones* are related to the 5,7-dihydroxyl structures. Eur J Pharamcol. 2011; 659:155-160.
16. Masur J, Märtz RM, Carlini EA. Effects of acute and chronic administration of cannabis sativa and (−) δ9-*trans*-tetrahydrocannabinol on the behavior of rats in an open-field arena. Psychopharmacologia. 1971; 19:388-397.
17. Han H, Ma Y, Eun JS, Li R, Hong JT, Lee MK, Oh KW. Anxiolytic-like effects of sanjoinine A isolated from Zizyphi Spinosi Semen: Possible involvement of GABAergic transmission. Pharmacol Biochem Behav. 2009; 92:206-213.
18. Yu HS, Lee SY, Jang CG. Involvement of 5-HT_{1A} and $GABA_A$ receptors in the anxiolytic-like effects of *Cinnamomum cassia* in mice. Pharmacol Biochem Behav. 2007; 87:164-170.
19. Simon P, Dupuis R, Costentin J. Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behav Brain Res. 1994; 6:59-64.
20. Dawson GR, Tricklebank MD. Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol Sci. 1995; 16:33-36.
21. Ahmed M, Azmat A, Azeem MA. Dose-response curve of Somina (herbal preparation): A study on frog heart. Pak J Pharmacol. 2004; 21:19-22.
22. Rabbani M, Sajjadi SE, Mohammadi A. Evaluation of the anxiolytic effect of *Nepeta persica* Boiss. in mice. Evid Based Complement Alternat Med. 2008; 5:181-186.
23. Wierońska JM, Stachowicz K, Nowak G, Pilc A. Anxiety Disorders: The Loss of Glutamate-GABA Harmony in Anxiety Disorders (Kalinin VV, ed.). InTech University Campus STeP Ri Slavka Krautzeka Rijeka, Croatia, 2011; pp. 135-158.
24. Stahl SM. Stahl's essential psychopharmacology: Neuroscientific basis and practical applications. In: Anxiety Disorders and Anxiolytics (Muntner N, Grady M, eds.). 3rd ed., Cambridge University Press, Cambridge, UK, 2000; pp. 721-772.
25. Li K, Xu E. The role and the mechanism of γ-aminobutyric acid during central nervous system development. Neurosci Bull. 2008; 24:195-200.
26. Whiting PJ. The GABA-A receptor gene family: New targets for therapeutic intervention. Neurochem Int. 1999; 34:387-390.
27. Delaney AJ, Sah P. GABA receptors inhibited by benzodiazepines mediate fast inhibitory transmission in the central amygdala. J Neurosci. 1999; 19:9698-9704.
28. Basile AS, Lippa AS, Skolnick P. Anxioselective anxiolytics: Can less be more? Eur J Pharmacol. 2004; 500:441-451.
29. Derry JM, Dunn SM, Davies M. Identification of a residue in the γ-aminobutyric acid type A receptor alpha subunit that differentially affects diazepam-sensitive and -insensitive benzodiazepine site binding. J Neurochem. 2004; 88:1431-1438.
30. Stenbacka M, Jansson B, Leifman A, Romelsjö A. Association between use of sedatives or hypnotics, alcohol consumption, or other risk factors and a single injurious fall or multiple injurious falls: A longitudinal general population study. Alcohol. 2002; 28:9-16.

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