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# Anxiolytic-like Effects of Methanol Extract of *Zizyphi Spinosi Semen* in Mice

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**Abstract** – *Zizyphi Spinosi Semen* (ZSS), a traditional Chinese folk medicine, has been used for treatment of insomnia and anxiety. This experiment was performed to investigate the anxiolytic-like effect of methanol extract of ZSS (MEZSS) in mice by using the experimental paradigms of anxiety and compared with that of a known anxiolytic, diazepam. In the elevated plus-maze test, it showed that MEZSS (100 mg/kg, p.o.) and diazepam (2.0 mg/kg, p.o.) increased the percentage of time spent on the open arms and the number of open arms entries. MEZSS (50, 100 and 200 mg/kg, p.o.) and diazepam (0.5 mg/kg, p.o.) significantly increased the number of head dips compared with that of control group in the hole-board test. However, MEZSS has no effect on decreasing the locomotor activity, while diazepam (2.0 mg/kg, p.o.) significantly inhibited locomotor activity. MEZSS did not decrease the strength force in the grip strength test, either. In addition, GABAergic involvements were also investigated to understand the possible mechanisms. $GABA_A$ receptors subunits and glutamic acid decarboxylase (GAD) were not over expressed, compared with that of the saline group. We also found that MEZSS did not increase chloride influx in cultured cerebellar granule cells. It is concluded that MEZSS might have anxiolytic-like effects, but these effects might not be mediated by GABAergic transmission.

**Keywords** □ methanol extract of *Zizyphi Spinosi Semen* (MEZSS), anxiolytic-like effect, elevated plus-maze, hole-board, locomotor, grip strength, GABA subunits, GAD, chloride influx.

## INTRODUCTION

Anxiety affects one-eighth of the total population worldwide and has become an important area of research interest in psychopharmacology (Eisenberg *et al.,* 1998). Benzodiazepines have been used for the treatment of several forms of anxiety although these compounds have well-known their side-effects such as sedation, muscle relaxation, amnesia and dependence (Jordan *et al.,* 1996). However, many researchers have been evaluated new compounds with less undesirable effects from herbs (Griffiths *et al.*, 1987).

*Zizyphi Spinosi Semen* (ZSS), the dried seed of *Zizyphus jujuba Mill var. spinosa* (Rhamnaceae), has been known to contain many pharmacologically active components (Peng *et al.,* 2000). It has been used as an analgesic, tranquilizer and anticonvulsant in oriental countries such as Korea and China for over 2500 years (Li *et al*., 2005) and has been prescribed for the treatment of insomnia and anxiety in Asia (Lee *et al.,* 2004). Recently it was reported that ZSS significantly increase sleep time induced by pentobarbital (Adzu *et al*., 2002). It was reported that Sanjoin-Tang showed anxiolytic-like effects and water extract of ZSS also increased open arm entries and spent time in open arms in high doses (Ahn *et al.*, 2004). In this experiment, we are interested in whether the methanol extract of ZSS (MEZSS) might exert anxiolytic-like effects using more experimental paradigms of anxiety. In addition, GABAergic involvements were also investigated to understand the possible mechanisms.

## MATERIALS AND METHODS

### Animals

Male ICR mice (Samtako, Korea) weighing 20-25g, in groups of 10-12, were used throughout the experiments. Animals were housed in acrylic cages (45×60×25 cm) with water and food available *ad libitum* under an artificial 12-h light/dark cycle (light on at 7:00) and at a constant temperature (22 ± 2 °C). Mice were housed in the departmental room for 1 week

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before testing to ensure adaptation to the new environment.

**Experimental compound and drugs**

ZSS (300 g) were extracted three times in a reflux condenser for 24 h each with 2 L of 70% methanol. The solution was combined, filtered through Whatman No. 1 filter paper, and concentrated using a rotary vacuum evaporator followed by lipophilization. The yield was about 10% (w/w). The extraction was dissolved in 0.9% physiological saline with 1% carboxymethylcellulose (CMC). Diazepam was purchased from Myung-In Pharm. Co., Ltd. (Kyunggi-Do, Korea) and was dissolved in 0.9% physiological saline before testing. All other chemicals used for molecular experiments were obtained from Sigma Chemical Co. All the experimental compound and drug were oral administered to the animals 30 min prior to the behavior experiment.

**Elevated plus-maze test**

The elevated plus-maze apparatus consists of four arms (30×5 cm) elevated 45 cm above the floor, with each arm positioned at 90° relative to the adjacent arms. The two enclosed arms had 30 cm walls and to facilitate grip on the open arms these included a raised edge of 0.25 cm. Open and closed arms were connected via a central area (5×5 cm) to form a plus sign. The maze floor was constructed of black Plexiglas and the wall of the enclosed arms was constructed of clear Plexiglas (Chen *et al*., 2003). Four 25-W red fluorescent lights arranged as a cross at 100 cm above the maze were used as the source of illumination and the video camera was suspended above the maze record movements for analysis. Mice were randomly assigned (with a slight adjustment for matched body-weight) to experimental groups. Diazepam was administered orally 30 min prior to the test. MEZSS was administered orally 60 min prior to the test. Test commenced by placing a mouse on the central platform of the maze facing an open arm. The number of entries into and the time spent on each of the two types of arms were recorded during the 5-min trial. An arm entry was defined as all four paws having crossed the dividing line between an arm and the central area. The plus-maze was thoroughly cleaned with 70% methanol after each trial. Mice were randomly allocated to the following groups: control (normal saline with 1% CMC, diazepam (2.0 mg/kg), and MEZSS (25, 50 and 100 mg/kg),

**Hole-board test**

The hole-board apparatus (Ugo Basile, Italy) consisted of gray Perspex panels (40×40 cm, 2.2 cm thick) with 16 equidistant holes each 3cm in diameter spaces on the floor. Photocells below the surface of the hole measured the number of head-dips. The board was positioned 15 cm above a table. Mice were randomly allocated to the following groups: control (normal saline with 1% CMC (p.o.), diazepam (0.5 mg/kg, p.o.), and MEZSS (50, 100 and 200 mg/kg, p.o.), Each mouse was individually placed on the center of the board facing away from the observer and allowed to freely roam about the apparatus after the oral administration of diazepam 30 min and MEZSS 60 min prior to the testing, respectively. The number of head dips on the hole-board was counted for 5 min (Da Silva and Elisabetsky, 2001). After each trial, the floor of the apparatus was wiped absolute methanol to remove traces of previous paths. This test session also was recorded with a camera mounted vertically above the hole-board test

**Locomotor activity**

Since the plus-maze experiment was affected by changes in locomotor activity, an additional experiment was carried out with the specific aim of monitoring the activity. Separately from the experiment above, spontaneous locomotor activity was measured automatically with a tilting-type ambulometer (AMB-10, O'Hara, Japan). Each mouse was placed in the activity cage (20 cm in diameter, 18 cm in height) and after an adaptation period of 10 min, the test compound administration protocol was implemented. Diazepam (2 mg/kg) and MEZSS (25, 50 and 100 mg/kg) were administered orally 30 min and 60 min prior to the experiment, respectively. Ambulatory activity was measured for 1 hour after oral administration of the agents. Tilting typed locomotor activity was measured.

**Grip strength**

Accordingly, the first 5 sessions of training were limited to handing each animal for 5 min. then, there were 10 training sessions during which animals were held around the midsection, facing the handle of the grip strength meter (GSM , designed by TSE-Systems and distributed by Scipro, Inc.), and one forearm was manually restrained by the experiment. When the unrestrained forepaw is brought into contact with the handle, the animals reliably grasp the bar, and the animal is then gently pulled away form the device (Kehl *et al.,* 2000). The GSM then measure the maximal force before the animal releases the bar. Each testing session was performed for both forepaws three times 60min and 30 min after the administration of MEZSS (25, 50 and 100 mg/kg) and diazepam (2 mg/kg), respectively. Means of three trails were calculated.

**$GABA_A$ receptor subunits expression**

Primary cultures of cerebellar neurons enriched in granule cells were prepared from cerebella of 8-day-old Sprague–Dawley rats as previously described (Houston and Smart, 2006). After culture for 8 days, these cells express functional $GABA_A$ receptor, with an expression pattern similar to that apparent in the cerebellum during postnatal development but different from that observed in the adult rat cerebellum. Briefly, cells were plated ($1\times10^6$ cells per 0.2 ml) in 96 microplates or ($2\times10^6$ cells per 2 ml) in 60-mm dishes that had been coated with poly-D-lysine (10 μg/ml) (Sigma, St. Louis, MO) and were cultured in basal Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 2 mM glutamine, gentamicin (100 μg/ml), antibiotic-antimycotic solution (10 ml/l) (Sigma) and 25 mM KCl; such a high concentration of potassium was necessary to induce persistent depolarization, which promotes the survival of granule cells. Cytosine arabinofuranoside (final concentration, 10 μM, Sigma) was added to cultures 18-24 h after plating to inhibit the proliferation of non-neuronal cells.

Cells were maintained for a total of 8 days in culture and long-term treatment with MEZSS was therefore initiated accordingly. MEZSS was dissolved in ethanol and diluted sequentially in culture medium to a final concentration; control cells were treated with solvent alone at the same dilution as that experienced by the drug-treated cells (0.1% v/v). The culture medium was completely replaced every day with fresh medium containing the appropriate drug.

After treatment, cells were harvested and treated with lysis buffer. The extracts were centrifuged at 20,000×g for 20 min. Equal amount of proteins were separated on a SDS/12% polyacrylamide gel, and transferred to a nitrocellulose membrane (Hyboud ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). The blots were blocked for 2 h at room temperature with 5 % (w/v) non-fat dried milk in a Tris-buffered saline (10 mM Tris (pH 8.0) and 150 mM NaCl) solution containing 0.05% Tween-20. The membrane was incubated with the specific antibodies, rabbit polyclonal antibodies against $GABA_A$ receptor subunit (1:500) (Santa Cruz Biotechnology Inc.), for 6 h at room temperature. The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). The immunoreactive proteins were detected using the ECL western blotting detection system

**Glutamic acid decarboxylase (GAD) expression**

Cells were maintained for a total of 8 days in culture and long-term treatment with MEZSS was therefore initiated accordingly. MEZSS was dissolved in ethanol and diluted sequentially in culture medium to a final concentration; control cells were treated with solvent alone at the same dilution as that experienced by the drug-treated cells (0.1% v/v). The culture medium was completely replaced every day with fresh medium containing the appropriate drug.

After treatment, cells were harvested and treated with lysis buffer. The extracts were centrifuged at 20,000×g for 20 min. Equal amount of proteins were separated on a SDS/12% polyacrylamide gel, and transferred to a nitrocellulose membrane (Hyboud ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). The blots were blocked for 2 h at room temperature with 5 % (w/v) non-fat dried milk in a Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% Tween-20. The membrane was incubated with the specific antibodies, rabbit polyclonal antibodies against GAD (1:500) (Santa Cruz Biotechnology Inc.), for 6 h at room temperature. The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). The immunoreactive proteins were detected using the ECL western blotting detection system.

**Intracellular chloride influx**

The intracellular $Cl^-$ concentration of cerebellar granule cells was estimated using the $Cl^-$ sensitive fluorescence probe MQAE according to the method of West and Molloy with a slight modification (West *et al.*,1996) The buffer (pH 7.4) used contained the following: 2.4 mM $HPO^{2-}_4$, 0.6 mM $H_2PO^-_4$, 10 mM HEPES, 10 mM D-glucose and 1 mM $MgSO_4$. A variety of MQAE-loading conditions were assessed. The cells were incubated overnight in a medium containing 10 mM MQAE (Dojindo, Japan). After loading, the cells were washed three times in the relevant $Cl^-$ containing buffer. The buffer was replaced with buffer with or without the compounds or control. Repetitive fluorescence measurements were initiated immediately using a FLUOstar (excitation wavelength: 320 nm; emission wavelength: 460 nm; BMG LabTechnology, Germany). The data is presented as the relative fluorescence Fo/F, where Fo is the fluorescence without $Cl^-$ ions and F is the fluorescence as a function of time. The Fo/F values were directly proportional to $[Cl^-]i$

### Statistical analysis

The results are presented as the mean±S.E.M. and the significance of the effects of the compounds was assessed using analysis of variance (ANOVA). In case of significant variation, the individual values were compared with Dunnett's test.

## RESULTS

### Effects of MEZSS on the elevated plus-maze

Behaviors observed in the elevated plus-maze confirmed the anxiolytic activity of diazepam reported previously (Cha *et al*., 2005; Dalvi *et al*., 1999; Fernandes *et al*., 1999). As the positive control, diazepam 2 mg/kg increased open arm entries (24.72%, $p<0.05$) and time spent on open arms (32.39%, $p<0.01$) compared with the saline group. MEZSS (100 mg/kg) increased the percentage of open arm entries (23.08%, $p<0.05$) and time spent on open arms (27.37%, $p<0.05$), compared with that of the saline animals (Fig. 1).

### Effects of MEZSS on the head dips

In the hole-board test, both of the diazepam (0.5 mg/kg) and MEZSS (50, 100 and 200 mg/kg) significantly increased the head dips, compared with that of saline group. Diazepam 0.5 mg/kg increased head dips (31.72%, $p<0.001$) and MEZSS (50, 100 and 200 mg/kg) increased head dips (46.69%, $p<0.001$, 51.04%, $p<0.001$, 53.33%, $p<0.001$) compared with the saline group (Fig. 2).

### Effects of MEZSS on the locomotor activity.

Locomotor activity was significantly decreased by diazepam (2 mg/kg) (73.11%, $p<0.001$) compared with the saline group. However; MEZSS at the doses of 25, 50 and 100 mg/kg did not affect locomotor activity in the test animals (Fig. 3).

### Effects of MEZSS on the grip strength

In the grip strength meter test, diazepam (2.0 mg/kg) significantly decreased the strength force (3.71 g/body weight, $p<0.001$) compared with the control group (4.66 g/body weight). But, MEZSS has no effect in decreasing the strength force at the dose of 25 (4.74 g/body weight), 50 (4.85 g/body weight) and 100 mg/kg (4.70 g/body weight) (Fig. 4).

### Effects of MEZSS on the subunits expression of $GABA_A$ receptors

The effects of treatment of cerebellar granule cells for 5 days with 64 μg/ml MEZSS on the abundance of $GABA_A$ receptor

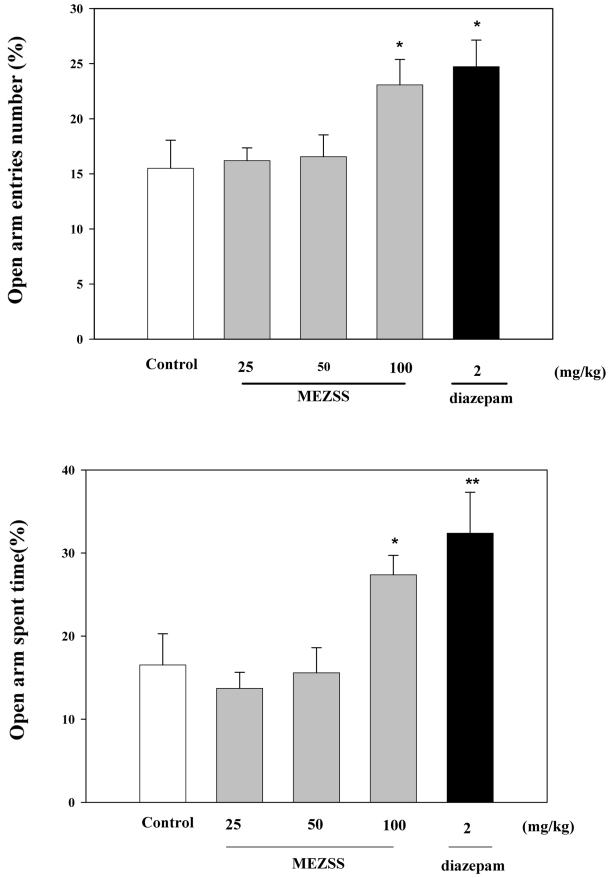


**Fig. 1.** Effects of MEZSS on the percentage of open arm entries and time spent in open arms on the elevated plus-maze in mice (n=9~11). Open arm entries and spent time in open arms by elevated plus-maze were measured for 5 min. Data are expressed as mean ± S.E.M. *P<0.05, **P<0.01, compared with the saline-treated group, respectively.

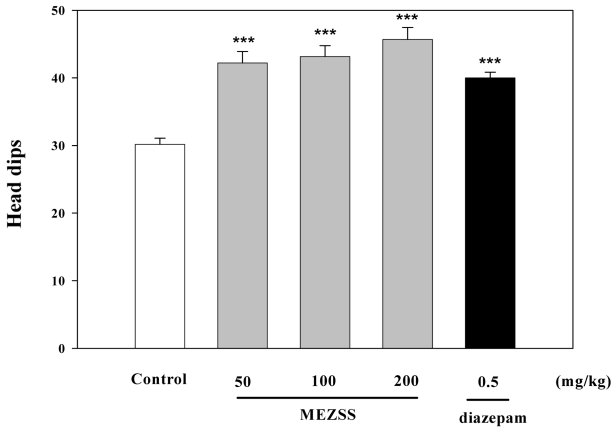


**Fig. 2.** Effects of MEZSS on head-dips in hole-board test in mice (n=10~12). Head-dips by hole-board test were measured for 5min. Data are expressed as mean ± S.E.M. ***P<0.001, compared with that of saline-treated group, respectively.

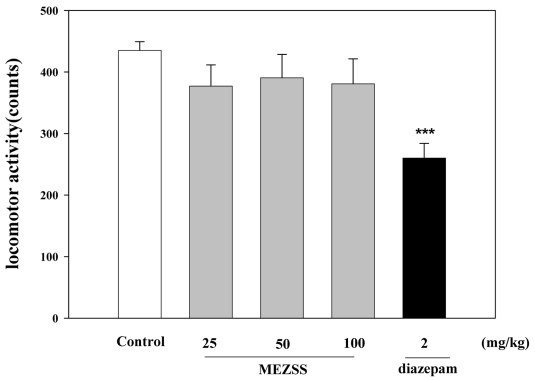


**Fig. 3.** Effects of MEZSS on Locomotor activity test in mice (n=9~11). Ambulation activity was measured for 1h. Data are expressed as mean ± S.E.M. ***P<0.001, compared with that of saline-treated group, respectively.

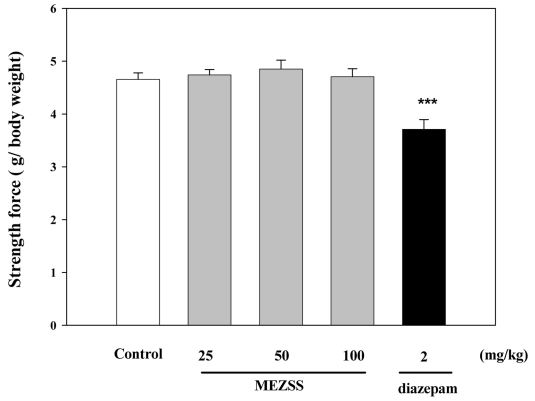


**Fig. 4.** Effects of MEZSS on grip strength meter test in mice (n=9~11). Data are expressed as mean ± S.E.M. ***P<0.001, compared with that of saline-treated group, respectively.

subunits were examined. MEZSS treatment did not influence amount of the α-, β- and γ-subunit expression (Fig. 5).

**Effects of MEZSS on the expression of GAD.**

The effects of treatment of cerebellar granule cells for 5 days with 64 μg/ml MEZSS on the abundance of GAD were examined. MEZSS treatment did not affect abundance of GAD (Fig. 6).

**Effects of MEZSS on the intracellular chloride influx.**

MEZSS did not increase chloride influx in this experiment, compared with that of saline group (Fig. 7).

## DISCUSSION

In this study, the effects of MEZSS were performed in ani-

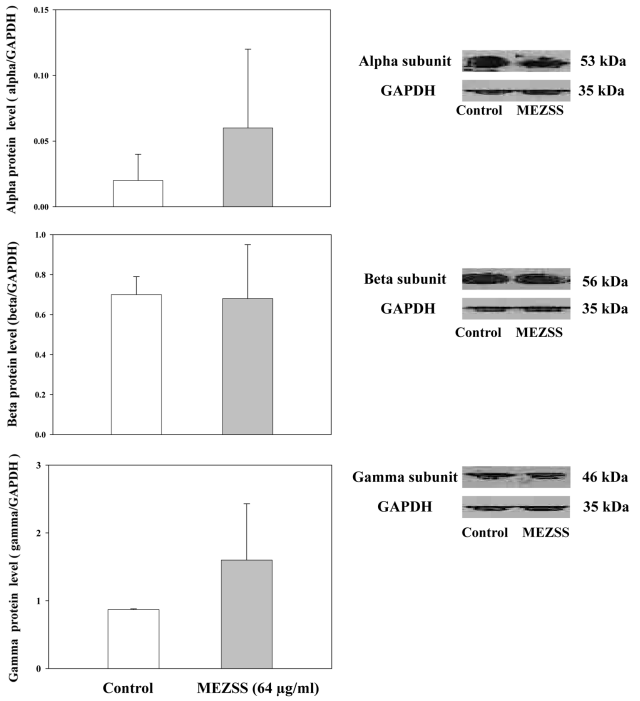


**Fig. 5.** Expression of GABA receptor subunits in primary cultured cerebellar granule cells. Each column represents the mean with S.E.M.

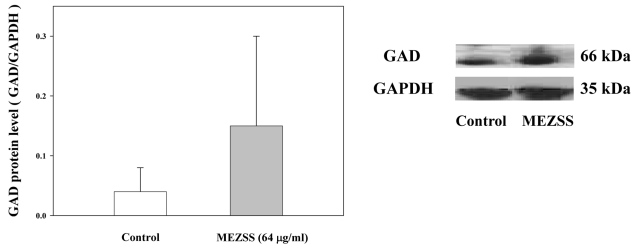


**Fig. 6.** GAD in primary cultured cerebellar granule cells. Each column represents the mean with S.E.M.

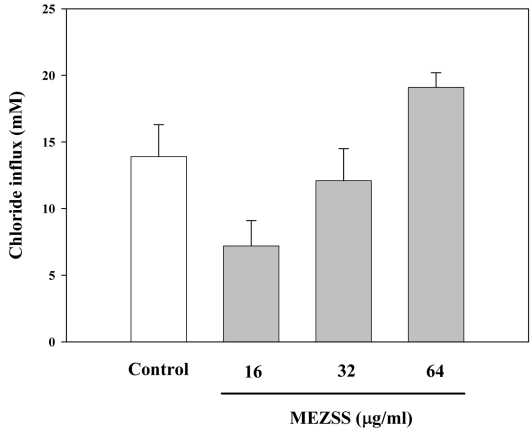


**Fig. 7.** Intracellular chloride influx. Each column represents the mean with S.E.M.

mal models of anxiety, such as elevated plus-maze, hole-board test, which are classical models for screening central nervous system actions providing in formation about myo-relaxant activity and anxiety. One of the most widely used animal models for screening putative anxiolytic is the elevated plus-maze, in which rodents show an avoidance of exposed open areas of the maze, which are presumed to be the most aversive, and a preference for sections enclosed by protective walls (Weiss *et al*., 1998). Conventional anxiety indices in the elevated plus-maze test comprise percent open arm entries and percent time spent in these areas in the maze, with anxiolytic generally increasing and anxiogenic decreasing these measures.

ZSS has been used as an analgesic, tranquilizer and anticonvulsant for centuries and is one of the herbs widely used in Korea and China due to the central nervous system (CNS) calming effect. In these experiments, MEZSS showed to have anxiolytic-like effects. The results of the present study demonstrate that MEZSS (100 mg/kg) increased open arm entries and spent time in open arm in the elevated plus-maze model. In addition, the hole-board test provides a simple method for measuring the response of an animal to an unfamiliar environment and is widely used to assess emotionality, anxiety and /or responses to stress in animals. Head-dipping behavior was sensitive to changes in the emotional state of the animal, and suggested that the expression of an anxiolytic state in animals may be reflected by an increase in head-dipping behavior (Takeda *et al*., 1998). In our experiments, MEZSS (25, 50 and 100 mg/kg) increased the head dips in the hole-board test which also showed the anxiolytic-like effects.

Many anxiolytic drugs affect locomotor activity. Similar to diazepam, typical GABA/benzodiazepine receptor agonist, but MEZSS still showed anxiolytic-like effects without affecting locomotor activity at lower doses (25, 50 and 100 mg/kg), indicating that the extract exerts anxiolytic-like and hypnotic effects at different doses. However, diazepam significantly inhibited locomotor activity. In the grip strength meter test, MEZSS also had no effect in decreasing the strength force which showed that MEZSS has no effect in muscle relaxation. Therefore, it suggested that MEZSS showed better profile than diazepam.

GABA systems are the most ubiquitous mechanisms on anxiolytic effects. MEZSS has no significant effect in influencing the amount of the α-, β- and γ-subunit expression, and MEZSS treatment did not affect abundance of GAD. MEZSS did not increase chloride influx significantly when treated the cerebella granule cells, compared with that of saline group. Therefore, it showed that anxiolytic-like effects of MEZSS might not be mediated by GABAergic transmission. On the hand, it was reported that anxiolytic-like effects of ZSS were blocked by pindolol, a 5-$HT_{1A}$ receptor antagonist. They suggested that anxiolytic-like ZSS might be mediated by serotonergic mechanisms (Ahn *et al.*, 2004). In agreement with previous report, MEZSS did not inhibited strength force and locomotor activity, which were involved in the GABAergic mechanisms.

Various traditional herbal medicines have also been suggested to possess anxiolytic activity. Some of them such as St. John's wort and ginseng have been introduced for clinical treatment of anxiety (Friede and Freudenstein, 2002; Bhattacharya and Mitra, 1991; Park *et al*., 2005; Cha *et al.*, 2005). In addition, it is also reported that the saponin-containing fraction of the leaves of Albizzia lebbeck from India have anxiolytic effects (Rex *et al*., 2002; Heinrich and Gibbons, 2001). Research also has focused on the development of drugs with fewer side effects such as sleeping, muscle relaxation and drug dependence.

To summarize, all the data presented here indicate that methanol extract of ZSS induce anxiolytic-like effects in the plus-maze test and hole-board test while has hypnotic or muscle-relaxant activity at doses that do not influence locomotor activity and strength force. Further investigation is needed for MEZSS derivatives with strong pharmacological actions and their possible mechanisms.

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## REFERENCES

Adzu, B., Amos, S., Dzarma, S., Wambebe, C. and Gamaniel, K. (2002). Effect of Zizyphus spina-christi wild aqueous extract on the central nervous system in mice. *J. Ethnopharmacol*. **79**, 13–16.

Ahn, N.Y., Jung, J.W., Oh, H.R., Shin, J.S., Hyeon, S.Y., Lee, B.K., Cheong, J.H., Ryu, J.H. (2004). Anxiolytiv-like effects of Sanjoin-Tang extracts and its ingredients in the elevated plus-maze in mice. *J. Appl. Pharmacol.,* **12**, 151-156.

Bhattacharya, S.K. and Mitra, S.K. (1991). Anxiolytic activity of Panax ginseng roots: an experimental studies. *J. Ethnopharmacol.* **43**, 87-92.

Cha, H.Y., Park, J.H., Hong, J.T., Yoo, H.S., Song, S., Hwang, B.Y., Eun, J.S. and Oh, K.W. (2005). Anxiolytic-like effects of ginsenosides on the elevated plus-maze model in mice. *Biol. Pharm. Bull*. **28**, 1621-1625.

Chen, S.W., Xin, Q., Kong, W.X., Min, L. and Li, J.F. (2003). Anxiolytic-like effect of succinic acid in mice. *Life Sci.* **773**, 3257-3264.

Dalvi, A. and Rodgers, R.J. (1999). Behavioral effects of diazepam in the murine plus-maze: flumazenil antagonism of enhanced head dipping but not the disinhibition of open-arm avoidance. *Pharmacol. Biochem. Behav.* **62**, 727-734.

Da Silva, A. L. and Elisabetsky, E. (2001). Interference of propylene glycol with the hole-board test. *Braz. J. Med. Biol. Res.* **34**, 545-7.

Eisenberg, D.M., Davis, R.B., Ettner, S.L. Appel, S. Wilkey, S. Van Rompay, M. and Kessler, R.C. (1998). Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *Complementary Therapies in Medicine*. **7**, 1569-1575.

Friede, M. and Freudenstein, J. (2002). Antidepressant and anxiolytic effect of St. John's wort extract ZE-117. *Eur. Psychiatry*. **17**, 96.

Fernandes, C., Arnot, M.I, Irvine, E.E., Bateson, A.N., Martin, I.L. and File, S.E. (1999). The effect of treatment regimen on the development of tolerance to the sedative and anxiolytic effects of diazepam. *Psychopharmacology (Berl)*. **145**, 251-259.

Griffiths, R.R., Ator, N.A., Roache, J.D. and Lamb, R.J. (1987). Abuse liability of triazolam: experimental measurements in animals and humans. *Psychopharmacol. Ser.* **3,** 83-87.

Heinrich, M. and Gibbons, S. (2001). Ethnopharmacology in drug discovery an analysis of its role and potential contribution. *J. Pharm. Pharmacol*. **53**, 425-432.

Houston, C.M. and Smart, T.G.. (2006), CaMK-II modulation of GABA(A) receptors expressed in HEK293, NG108-15 and rat cerebellar granule neurons. Eur. J. Neurosci., **24**, 2505-2515.

Jordan, A.D., Kordik, C.P., Reitz, A.B. and Sanfillipo, P.J. (1996). Novel anxiolytic agents-1994-to present. *Expert Opin Ther Pat*. **6**, 1047-1060.

Kehl, L.J., Trempe, T.M. and Hargreaves, K.M. (2000). A new animal model for assessing mechanisms and management of muscle hyperalgesia. *Pain*. **85**, 333-343.

Lee, S.M., Park, J.G., Lee, Y.H., Lee, C.G.., Min, B.S., Kim, J.H. and Lee, H.K. (2004). Anti-complementary activity of triterpenoides from fruits of Zizyphus jujuba. *Biol Pharm Bull.* 27, 1883-6.

Li, J.W., Ding, S.D. and Ding, X.L. (2005). Comparison of antioxidant capacities of extracts from five cultivars of Chinese jujube. *Process Biochemistry*, **40**, 3607-3613.

Park, J.H., Cha, H.Y., Seo, J.J., Hong, J.T., Han, K. and Oh, K.W. (2005). Anxiolytic-like effects of ginseng in the elevated plus-maze model: Comparison of red ginseng and sun ginseng. *Prog. Neuropsychopharmacol. Biol. Psychiatry*. **29**, 895-900.

Peng, W.H., Hsieh, M.T., Lee, Y.S., Lin, Y.C. and Liao, J. (2000). Anxiolytic effect of seed of Ziziphus jujuba in mouse models of anxiety. *Journal of Ethnopharmacology*. **72,** 435-441.

Rex, A., Morgenster, E. and Fink, H. (2002). Anxiolytic-like effects of Kava-Kava in the elevated plus-maze test-a comparison with diazepam. *Prog. Neuropsychopharmacol. Biol. Psychiatry*. **26**, 855-860.

Takeda, H., Tsuji, M. and Matsumiya, T. (1998). Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and /or anxiolytic state in mice. *European Journal of Pharmacology*. **350**, 21-29.

Weiss, S.M., Wadsworth, G., Fletcher, A. and Dourish, C.T. (1998). Utility of ethological analysis to overcome locomotor confounds in elevated plus-maze of anxiety. *Neurosci. Behav. Rev*. **23**, 265-271.

West, M.R., Molloy, C.R. (1996). A microplate assay measuring chloride ion channel activity. *Anal Biochem.* **241**, 51-8.