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Ethanol Extract of Polygalae Radix Augments Pentobarbital-Induced Sleeping Behaviors through $GABA_A$ergic Systems

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Abstract – Polygalae radix (PR) has traditionally been used as a sedative and anti-stress agent in oriental countries for a long time. PR which contains many ingredients is especially rich in saponins. This study was performed to investigate whether ethanol extract of PR enhances pentobarbital-induced sleep behaviors. In addition, possible mechanisms also were investigated. PR inhibited locomotor activity in mice. PR increased sleep rate and sleep time by concomitant administration with sub-hypnotic dose of pentobarbital (28 mg/kg). PR prolonged total sleeping time, and shortened sleep latency induced by pentobarbital (42 mg/kg). In addition, PR increased intracellular chloride concentration in primary cultured neuronal cells. The expression level of glutamic acid decarboxylase (GAD) were increased, and γ-aminobutyric acid $(GABA)_A$ receptors subunits were modulated by PR, especially increasing γ-subunit expression. In conclusion, PR augments penobarbital-induced sleep behaviors through activation of $GABA_A$ receptors and chloride channel complex.
Keywords – Polygalae radix, Locomotor, Sleeping, Glutamic acid decarboxylase (GAD), Chloride influx, $GABA_A$ receptors

Introduction

Polygalae Radix (PR) is the dried root of *Polygala tenuifolia* Willd, which has been used as a traditional medicine of expectorant, tonic, tranquillizer, and antipsychotic agent (Chung *et al*., 2002; May *et al*., 2013). PR also was widely used in the prescription to treat mental diseases, for example, amnesia, neurasthenia, palpitation, and insomnia (Chung *et al*., 2002; Ikeya *et al*., 2004; Zhao *et al*., 2013). The dried root of this plant chemically contains polygalitol, tenuigenin, polygalasaponin, oligosaccharides, and xanthone derivatives (Jin and Park, 1993; Sun *et al*., 2000; Wang *et al*., 2003). Accumulating studies indicate that PR would be useful for the treatment of Alzheimer's disease, as alleviating various symptoms of cognitive deficits and facilitating learning and memory, especially in China, Japan, and Korea (Ikeya *et al*., 2004; Lee *et al*., 2010). Cognition was improved by PR, via regulation of cholinergic marker enzyme activities and the antioxidant defense systems (Lee *et al*., 2010). Triterpenoid saponins with neuroprotective effects have been identified (Yabe *et al*., 2003). Furthermore, polygalasaponins could enhance learning and memory, possibly through the improvement of synaptic transmission, the activation of the mitogen-activated protein (MAP) kinase cascade and the enhancement of brain-drived neurotrophic factor (BDNF) level (Gomes *et al*., 2011).

On the other hand, polygalasaponin possesses evident anxiolytic and sedative-hypnotic activities, and has a relatively safe dose range in folk medicine (Yao *et al*., 2010). In addition, oral administration of 3,4,5-trimethoxycinnamic acid (TMCA), one of the constituents in Onji, prolonged sleeping time induced by hexobarbital in mice to exhibit sedative action (Kawashima *et al*., 2004).

Insomnia symptoms appear to be one of the most frequent sleep complaints in the general population with an estimated prevalence varying from 10 to nearly 60% depending on regions and ages (Ohayon and Shapiro, 2002). Individuals reporting disturbed sleep are more likely to report emotional distress and recurrent health problems (Edinger *et al*., 2000; Morin and Gramling, 1989). This is not surprising since it has been shown that sleep deprivation has great impact on the daytime life of healthy subjects; alertness, attention, concentration, cognitive abilities, memory, mood and pain. γ-Aminobutyric acid $(GABA)_A$ receptors complex has been known to play important role in the modulation of sleep, and the increase and decrease in pentobatbital-induced sleeping time can

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be useful tool for examining influences on GABAergic systems. They have been limited in clinical uses becuase of side effects such as dependence and tolerance (Kim *et al*., 2012; Sivam *et al*., 1982) .

The present study was examined whether ethanol extract of PR enhaces sleeping behaviors induced by pentobarbital. In addition, chloride channel, and expressions of GAD and $GABA_A$ receptors sutypes were measured after administration of PR to know possible mechanisms via $GABA_A$ergic systems.

Experimental

Plant materials and extraction – The dried Polygalae radix (500 g) was purchsed from a herb market in Cheongju city. The plant was identified by the herbaium of College of Pharmacy, Chungbuk National University, which a voucher specimen was deposited (CBNU201105-PT). It was extracted 2 times with 50% ethanol (1 L) at room temperature for 24 hours. This extract was evaporated under reduced pressure and then freeze-dried to acquire an ethanol extract. The total yield of the extraction was 7% (35 g).

Reagents and chemicals – The Cl^- sensitive fluorescence probe N-(ethoxycarbonyl-methyl)-6-methoxy quinolinium bromide (MQAE), Muscimol were purchased from Sigma-Aldrich co. (St. Louis, MO, USA). Pentobarbital sodium was purchased from Hanlim Pharm. Co., Ltd. (Seoul, Korea). Fetal bovine serum (FBS) and DMEM were obtained from GIBCO (Grand Island, NY, USA). The specific rabbit polyclonal antibodies agonist $GABA_A$ receptors subunits or gluamic aid decarboxylase $(GAD)_{65}$ and the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase were obtained from Abcam (Cambridge, UK). Chemiluminescent HRP substrate was purchased from Millipore Co. (Billerica, MA, USA).

Animals – The animals used were ICR male mice (purchased from Samtako, Korea), weighing 25 - 28 g, in groups of 10-12 heads. The mice were housed in acrylic cages (45 × 60 × 23 cm) with water and food available ad libitum under an artificial 12 h light/dark cycle (light on at 7:00 am) and at a constant temperature (22 + 2 °C). To ensure adaptation to the new environment, the mice were kept in the departmental holding room for 1 week before testing. All of the behavioral experiments were performed between 10:00 and 18:00. All of the experiments involving animals were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985), and the Institutional Animal Care and Use Committee of Chungbuk National University approved the protocol.

Locomotor activity – 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 minute. The extracts (1.0, 2.0 and 5.0 mg/kg, p.o) were administered to the mice 1 hour prior to the experiment, and ambulatory activity was measured for 1 hour after the administration of the agents.

Pentobarbital-induced sleep – Ten to twelve mice were used for each treatment group. All experiments were carried out between 1:00 and 5:00 pm. Animals were food-deprived for 24 h, prior to the experiment. Pentobarbital sodium and muscimol were diluted in 0.9% physiological saline, and administered to each mouse intraperitoneally (i.p.) to induce sleep. PR was suspended in physiological saline and was administered orally (p.o.) to the mice (0.1 mg/10 g). Muscimol as a positive control drug was administered 30 min prior to administration of pentobarbital. Pentobarbital was given to animals placed in a box 1 h after the oral administration of test drugs. Those animals that stopped moving in the box within 10 minute after pentobarbital injection were immediately transferred to another box. Those individuals that stayed immobile for more than 3 minute were judged to be asleep. The time that elapsed from receiving pentobarbital until each animal lost its righting reflex when positioned delicately on its back represented the latency to onset of sleep. The animals were observed constantly, and the time of awakening, characterized by righting of the animal, was noted. The sleeping time was defined as the time taken for the animal to regain spontaneous movement after being trasferred to the second box. Animals that failed to fall asleep within 10 min after pentobarbital administration were excluded from the experiments (Darias *et al*., 1998; Wolfman *et al*., 1996).

Cell culture – Primary cultures of cerebellar neurons enriched in granule cells were prepared from cerebella of 8 days old Sprague-Dawley rats as previously described (Zhu and Baker, 1996). After 8 days cell culture, cells express functional $GABA_A$ receptors, with an expression pattern similar to that of the cerebellum during postnatal development, but different from the pattern observed in the adult rat cerebellum (Follesa *et al*., 2002). Briefly, cells were plated (1×10^5 cells per well) in 96 well microplates that had been coated with poly-L-lysine (50 μg/mL; Sigma, St. Louis, MO, USA) and were cultured F12 mixture media supplemented with 10% heat-inactivated fetal bovine serum, gluamin, gentamicin (100 μg/mL), antibiotic-antimycotic solution (10 μg/mL), and

potassium chloride (25 mM); a high concentration of potassium was necessary to induce persistent depolarization, which promotes the survival of granule cells. Cytosine arabinofuranoside (final concentration, 10 μM was added to culture 18~24 h after plating, to inhibit the proliferation of non-neuronal cells.

Measurement of intracellular Cl^- influx – The intracellular Cl^- concentration $[Cl^-]i$ of cerebellar granule cells was estimated using Cl^- sensitive fluorescence probe MQAE according to the method of West and Molloy, with a slight modification (West and Molloy, 1996). The buffer (pH 7.4) contained the following components: 2.4 mM HPO_4^{2-}, 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 medium containing 10 mM MQAE (Sigma, St. Louis, MO, USA). After loading, the cells were washed three times in the appropriate Cl^- containing buffer or Cl^- free buffer. The buffer was replaced with buffer with or without the compounds or Cl^- free buffer. Repetitive fluorescence measurements were initiated immediately using a FLUOstar plate reader (Excitation wavelength: 320 nm, emission wavelength: 460 nm; BMG Lab Technology, Germany). The data is presented as the relative fluorescence F_0/F, where F_0 is the fluorescence without Cl^- and F is the fluorescence as a function of time. The F_0/F values were directly proportional to $[Cl^-]i$.

Western blot of $GABA_A$ receptors subtypes and GAD – The cultured cerebellar neurons were primay cultured for 8 days. PR extract was treated in the primary cultured cerebellar neurons. PT was dissolved in distilled water (DW) and diluted sequentially in culture medium to final concentrations of 5, 10 μM. The culture medium was completely replaced with fresh medium containing the appropriate drug. After treatment of PR, the cells were harvested and treated with lysis buffer. The extracts were centrifuged at 13,000 × g at 4 ºC for 10 min and the supernatant was recovered. The concentration of protein in the supernatant was determined, and then the supernatant was used for western blot analysis. The concentration of total protein was determined by the modified Lowry method using bovine serum albumin as a standard. The samples were stored at –20 ºC. Twenty five micrograms of protein was added to each lane, and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 12% polyacrylamide gels. Proteins were transferred to PVDF membranes (Hybond-P, GE Healthcare, Amersham, UK) using a semidry transfer system. The blots were blocked for 2 h at the room temperature with 5% (w/v) BSA (applied to all primary antibodys excepting for GADPH) and 5% (w/v) skim milk (Only applied to GADPH) in Tris-buffered saline solution (TBS) containing 0.1% Tween 20. Immunoblots were incubated with one of the following primary antibodies: rabbit anti-$GABA_A$ α1 polyclonal antibody (diluted 1 : 2,500 in TBS containing 0.1% Tween 20, 5% BSA Abcamplc, Cambridge, UK), rabbit anti-$GABA_A$ β1 polyclonal antibody (diluted 1 : 2,500 in TBS contain 0.1% Tween 20, 5% BSA), rabbit anti-$GABA_A$ γ1 polyclonal antibody (diluted 1 : 2,500 in TBS containing 0.1% Tween 20, 5% BSA) and rabbit anti-glutamic acid decarboxylase (GAD) polyclonal antibody (diluted 1 : 2,500 in TBS containing 0.1% Tween 20, 5% BSA). Blots were then washed and incubated with the horseradish peroxidase-conjugated second antibodies: goat anti-rabbit IgG (diluted 1:3,000 in TBS containing 0.1% Tween 20, 1% BSA). Immunoreactive bands were developed with a BM chemiluminescence detection kit (Roche Diagnostics, Mannheim, Germany). The quantitative analysis of detected bands was performed with densitometric scanning, and all values were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GADPH) in the sample, which was measured as follows. All of the immunoblots were stripped, incubated with sheep anti-GADPH, subsequently incubated with rabbit anti-rabbit IgG, and developed to confirm equal protein loading.

Statistical analysis – All statistical analysis were conducted using SigmaStat software (SPSS Inc., Chicago, IL, USA). After ANOVA testing, post hoc comparisons were performed using Holm-sidak test. A p-value of less than 0.05 was considered to be significant. The values are expressed as the mean ± SEM.

Results and Discussion

PR (1, 2 and 5 mg/kg, p.o) were orally administered to animals prior to 1 h and ambulatory activity was measured for 1 hour. PR (2 and 5.0 mg/kg) decrease the locomotor activity, showing sedative effects (Fig. 1). PR (5 mg/kg) enhanced sub-hypnotic pentobarbital (28 mg/kg)-induced sleep behaviors, increasing the numbers of fall asleep and sleeping time, similar to muscimol (Table 1). PR significantly increased the total sleeping time and shortened sleep latency induced by pentobarbital (42 mg/kg) (Fig. 2 and 3). PR increased intracellular chloride concentration in primary cultured cells (Fig. 4). Expressions of GAD65/67 and γ-subunits of GABA receptors were increased. However, α-subunits were decreased, and no efffect on β-subunits expression (Fig. 5 and 6).

The anxiolytic and sedative-hypnotic activities of

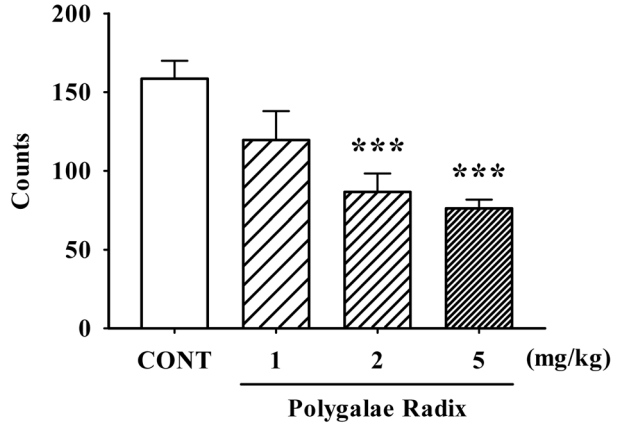

Fig. 1. Effects of PR extract on locomotor activity. PR extract decreased locomotor activity in mice. Values are expressed as the mean ± SEM. ***P < 0.005, compared with that of the control. Ten to twelve mice were used in each group.

Table 1. Effects of Polygalae radix on sleep by sub-hypnotic dose of pentobarbital (28 mg/kg, ip)

Group	Dose (mg/kg)	No. falling asleep/total	Sleep time (min)
Control	0	5/15	22.3 ± 1.7
Muscimol	0.2	13/14**	40.2 ± 4.1***
PR	1.0	6/14	27.0 ± 4.0
	2.0	6/14	27.6 ± 3.2
	5.0	10/13*	33.9 ± 3.6*

Values are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.005, compared with that of control.

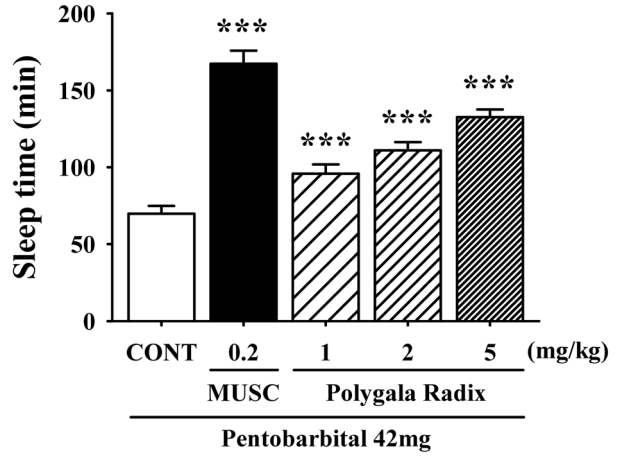

Fig. 2. Prolongation effects of PR extract on pentabarbital induced sleep. PR extract increased pentobarbital-induced sleep. Values are expressed as the mean ± SEM. **P < 0.01, ***P < 0.005, compared with that of the control. For detail, refere Fig. 1.

polygalasaponins extracted from *Polygala tenuifolia* Willdenow (*Polygalaceae*) were previously determined in mice using hole-board, elevated plus maze, open field,

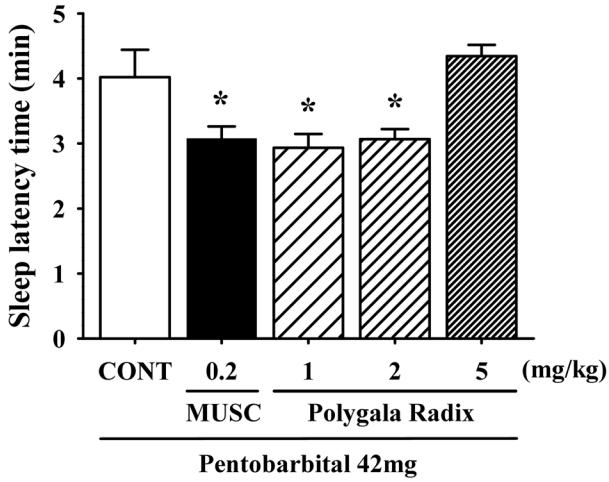

Fig. 3. Effects of PR extract on pentobarbital-sleep latency in mice. PR extract did not reduce sleep latency. Values are expressed as the mean ± SEM. *P < 0.05, compared with that of the control. For detail, refere Fig. 1.

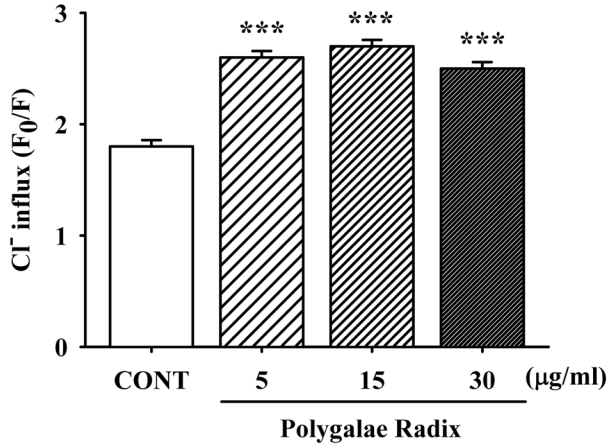

Fig. 4. Effects of PR extract on Cl^- influx in primary cultured hippocampus cells. PR extract increased Cl^- influx in neuroal cells. Values are expressed as the mean ± SEM. ***P < 0.005, compared with that of the control.

and pentobarbital-induced hypnosis tests. From these experiments, we found similar results which PR increased pentobarbital-induced sleep in agreement with previous study. PR increased the rate of sleep onset and prolonged sleep time induced by sub-hypnotic pentobarbital, and incresed total sleep and shortened sleep latency. Therefore, PR would be useful for the treatemtn of insomnia, similar to muscimol. We continued to understand the possible mechanisms whether these effects could be mediated by $GABA_A$ergic systems. $GABA_A$ receptors possess different biding sites such as GABA, benzodiazepine and barbiturate. $GABA_A$ receptors form heteromeric GABA-gated Cl^- channels assembled from a large family of subunits genes.

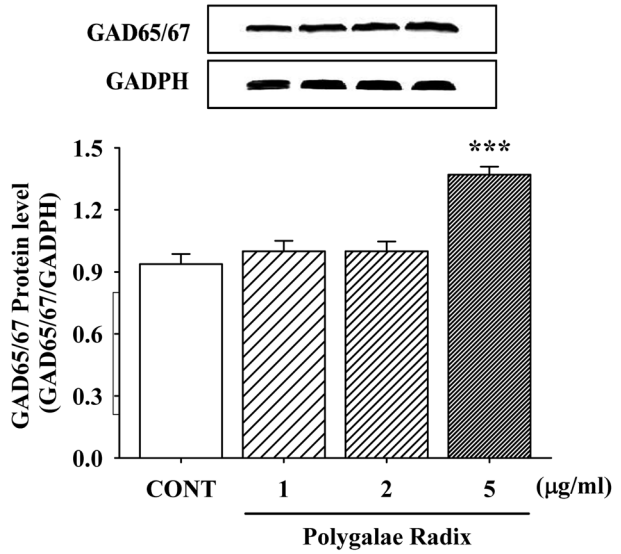

Fig. 5. Expressions of GAD by PR extract. PR extract increased GAD expression. Values are expressed as the mean ± SEM. ***P < 0.005, compared with ththat of the control.

$GABA_A$ receptors linked chloride channels are opened after binding GABA to give a net inward flux of negative Cl^- ions (outward current), hyperpolarizing the membrane and reducing neuronal firing (Macdonald and Olsen, 1994). Muscimol and other $GABA_A$ receptors agonists potentiated Cl^- influx as well as potentiated pentobarbital or other agonists induced Cl^- influx when being used simultaneously (Chistina Grobin *et al*., 1999). To investigate the detailed mechanisms involved in the prolongation of the pentobarbital induced sleeping time caused by PR, We measured Clinflux in the primary cultured cerebellar granule cells. PR produced significant increase in Cl^- influx, results similar to those of pentobarbital. This suggests that PR might act on $GABA_A$ receptors to induce Cl^- channel opening. In addition, the activation of GAD also plays an important role in GABAergic systems. GAD activation increases GABAergic transmission because GABA is generated from glutamate by the action of GAD. It was reported that GAD activating agents induce sedative/hypnotic actions shortly ((Ma *et al*., 2009a; Ma *et al*., 2008; Ma *et al*., 2009b). Researchers proved that the pharmacological profile and different behaviors of $GABA_A$ receptors agonists also depend upon its subunit composition (Rudolph *et al*., 2001; Rudolph and Knoflach, 2011). GAD activation and $GABA_A$ receptors γ-subunits could be the molecular target for PR, which potentiates sleep. GAD65 is responsible for the vesicular GABA production, because this isoform is directly involved in GABA transmission at the synapse (Buddhala *et al*., 2009).

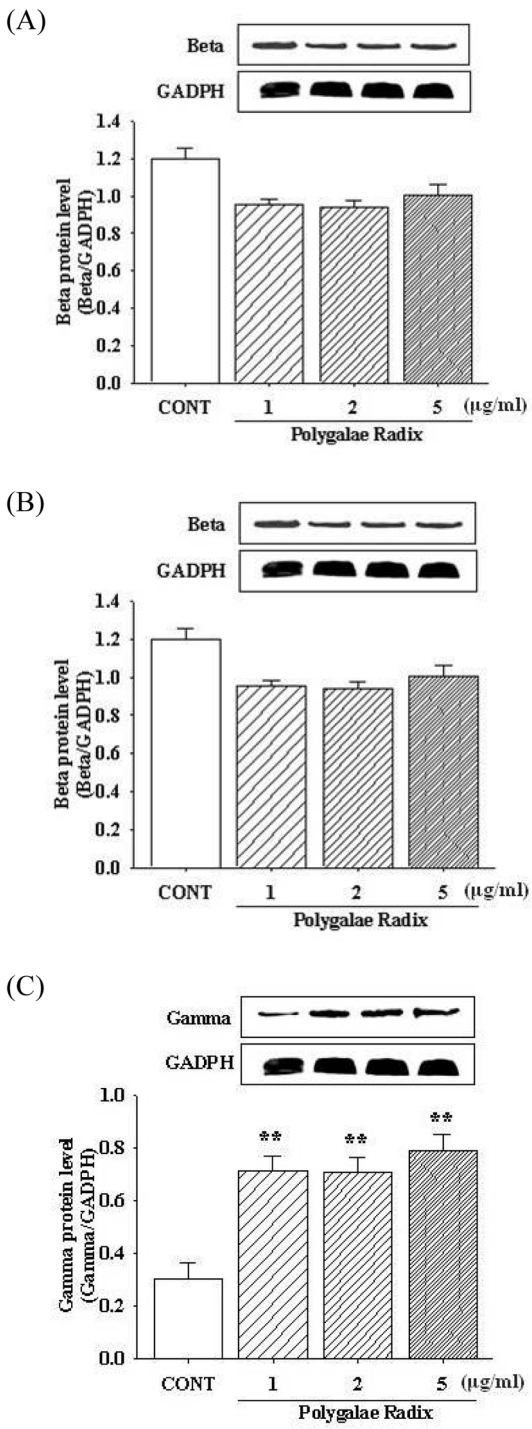

Fig. 6. Expressions of $GABA_A$ receptors subunits by PR extract. PR extract decreased expression of α-(A) and β-(B) subunits, but increased γ-(C) subunits. Values are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, compared with that of the control.

Many efforts have been done to develop sleep aids from phytomedicines. There have been some usefull remedies in western countries such as St John's wort

(*Hypericum perforatum*), Kava Kava (*Piper methysticum*) and Valerian (*Valeriana officinalis*) (Barnes *et al*., 2001), (Pallesen *et al*., 2002; Wheatley, 2001). However, although Chinese medicies have traditionally been used as remedies for insomnia, medicinal drugs have not yet been developed as sleep aids. Here, these studies inform that PG would be a good candidate as a sleep aid.

In conclusion, PR enhanced hypnotic effects in pentobarbital-treated mice. $GABA_A$ receptors and chloride channel complex might be involved in the mechanisms of these effects. It has been known that polygalasaponin of PR possesses anxiolytic and sedative-hypnotic activities in many kinds of animal models (Yao *et al*., 2010). In addition, TMCA, a component of Onji exhibits sedative effects by suppressing norepinephrine content in locus coeruleus (Kawashima *et al*., 2004). So, further investigation is needed in order to understand which might be active compounds of PR.

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References

Barnes, J., Anderson, L.A., and Phillipson, J.D., St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties. *J. Pharm. Pharmacol.* **53**, 583-600 (2001).

Buddhala, C., Hsu, C.C., and Wu, J.Y., A novel mechanism for GABA synthesis and packaging into synaptic vesicles. *Neurochem. Int.* **55**, 9-12 (2009).

Chistina Grobin, A., Inglefield, J.R., Schwartz-Bloom, R.D., Devaud, L.L., and Morrow, A.L., Fluorescence imaging of GABAA receptor-mediated intracellular [Cl^-] in P19-N cells reveals unique pharmacological properties. *Brain Res*. **827**, 1-11 (1999).

Chung, I.W., Moore, N.A., Oh, W.K., O'Neill, M.F., Ahn, J.S., Park, J.B., Kang, U.G., and Kim, Y.S., Behavioural pharmacology of polygalasaponins indicates potential antipsychotic efficacy. *Pharmacol. Biochem. Behav.* **71**, 191-195 (2002).

Darias, V., Abdala, S., Martin-Herrera, D., Tello, M.L., and Vega, S., CNS effects of a series of 1,2,4-triazolyl heterocarboxylic derivatives. *Pharmazie* **53**, 477-481 (1998).

Edinger, J.D., Glenn, D.M., Bastian, L.A., and Marsh, G.R., Slow-wave sleep and waking cognitive performance II: Findings among middle-aged adults with and without insomnia complaints. *Physiol. Behav.* **70**, 127-134 (2000).

Follesa, P., Porcu, P., Sogliano, C., Cinus, M., Biggio, F., Mancuso, L., Mostallino, M.C., Paoletti, A.M., Purdy, R.H., Biggio, G., and Concas, A., Changes in GABAA receptor gamma 2 subunit gene expression induced by long-term administration of oral contraceptives in rats. *Neuropharmacol*. **42**, 325-336 (2002).

Gomes, C., Smith, S.C., Youssef, M.N., Zheng, J.J., Hagg, T., and Hetman, M., RNA polymerase 1-driven transcription as a mediator of BDNF-induced neurite outgrowth. *J. Biol. Chem*. **286**, 4357-4363 (2011).

Ikeya, Y., Takeda, S., Tunakawa, M., Karakida, H., Toda, K., Yamaguchi, T., and Aburada, M., Cognitive improving and cerebral protective effects of acylated oligosaccharides in Polygala tenuifolia. *Biol. Pharm. Bull*. **27**, 1081-1085 (2004).

Jin, B.Y. and Park, J., [Studies on the alkaloidal components of Polygala tenuifolia willd]. *Zhongguo Zhong Yao Za Zhi* **18**, 675-677, 702-673 (1993).

Kawashima, K., Miyako, D., Ishino, Y., Makino, T., Saito, K., and Kano, Y., Anti-stress effects of 3,4,5-trimethoxycinnamic acid, an active constituent of roots of Polygala tenuifolia (Onji). Biol Pharm Bull **27**, 1317-1319 (2004).

Kim, J.W., Kim, C.S., Hu, Z., Han, J.Y., Kim, S.K., Yoo, S.K., Yeo, Y.M., Chong, M.S., Lee, K., Hong, J.T., and Oh, K.W., Enhancement of pentobarbital-induced sleep by apigenin through chloride ion channel activation. *Arch. Pharm. Res*. **35**, 367-373 (2012).

Lee, M.R., Yun, B.S., Park, S.Y., Ly, S.Y., Kim, S.N., Han, B.H., and Sung, C.K., Anti-amnesic effect of Chong-Myung-Tang on scopolamine-induced memory impairments in mice. *J. Ethnopharmacol.* **132**, 70-74 (2010).

Ma, H., Kim, C.S., Ma, Y., Nam, S.Y., Kim, D.S., Woo, S.S., Hong, J.T., and Oh, K.W., Magnolol enhances pentobarbital-induced sleeping behaviors: possible involvement of GABAergic systems. *Phytother. Res*. **23**, 1340-1344 (2009a).

Ma, Y., Han, H., Nam, S.Y., Kim, Y.B., Hong, J.T., Yun, Y.P., and Oh, K.W., Cyclopeptide alkaloid fraction from Zizyphi Spinosi Semen enhances pentobarbital-induced sleeping behaviors. *J. Ethnopharmacol*. **117**, 318-324 (2008).

Ma, Y., Ma, H., Eun, J.S., Nam, S.Y., Kim, Y.B., Hong, J.T., Lee, M.K., and Oh, K.W., Methanol extract of Longanae Arillus augments pentobarbital-induced sleep behaviors through the modification of GABAergic systems. *J. Ethnopharmacol*. **122**, 245-250 (2009b).

Macdonald, R.L. and Olsen, R.W., GABAA receptor channels. *Annu. Rev. Neurosci*. **17**, 569-602 (1994).

May, B.H., Lu, C., Lu, Y., Zhang, A.L., and Xue, C.C., Chinese herbs for memory disorders: a review and systematic analysis of classical herbal literature. *J. Acupunct. Meridian. Stud*. **6**, 2-11 (2013).

Morin, C.M. and Gramling, S.E., Sleep patterns and aging: comparison of older adults with and without insomnia complaints. *Psychol. Aging* **4**, 290-294 (1989).

Ohayon, M.M. and Shapiro, C.M., Tenses of insomnia epidemiology. *J Psychosom Res* **53**, 525-527 (2002).

Pallesen, S., Bjorvatn, B., Nordhus, I.H., and Skjerve, A., [Valerian as a sleeping aid?]. *Tidsskr Nor Laegeforen* **122**, 2857-2859 (2002).

Rudolph, U., Crestani, F., and Mohler, H., GABA(A) receptor subtypes: dissecting their pharmacological functions. *Trends Pharmacol. Sci*. **22**, 188-194 (2001).

Rudolph, U. and Knoflach, F., Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. *Nat. Rev. Drug Discov.* **10**, 685-697 (2011).

Sivam, S.P., Nabeshima, T., and Ho, I.K., Acute and chronic effects of pentobarbital in relation to postsynaptic GABA receptors: a study with muscimol. *J. Neurosci. Res*. **7**, 37-47 (1982).

Sun, X., Shi, S., and Yang, G., [Studies on chemical constituents of fat oil of Polygala tenuifolia]. *Zhong Yao Cai* **23**, 35-37 (2000).

Wang, H., Tong, Y.X., Ye, W.C., and Zhao, S.X., [Studies on chemical constituents in roots of Polygala tenuifolia]. *Zhongguo Zhong Yao Za Zhi* **28**, 828-830 (2003).

West, M.R. and Molloy, C.R., A microplate assay measuring chloride ion channel activity. *Anal. Biochem.* **241**, 51-58 (1996).

Wheatley, D., Kava and valerian in the treatment of stress-induced insomnia. *Phytother. Res*. **15**, 549-551 (2001).

Wolfman, C., Viola, H., Marder, M., Wasowski, C., Ardenghi, P., Izquierdo, I., Paladini, A.C., and Medina, J.H., Anxioselective properties of 6,3'-dinitroflavone, a high-affinity benzodiazepine receptor ligand. *Eur. J. Pharmacol*. **318**, 23-30 (1996).

Yabe, T., Tuchida, H., Kiyohara, H., Takeda, T., and Yamada, H., Induction of NGF synthesis in astrocytes by onjisaponins of Polygala tenuifolia, constituents of kampo (Japanese herbal) medicine, Ninjin-yoei-to. *Phytomed*. **10**, 106-114 (2003).

Yao, Y., Jia, M., Wu, J.G., Zhang, H., Sun, L.N., Chen, W.S., and Rahman, K., Anxiolytic and sedative-hypnotic activities of polygalasaponins from Polygala tenuifolia in mice. *Pharm. Biol*. **48**, 801-807 (2010).

Zhao, Z., Fang, M., Xiao, D., Liu, M., Fefelova, N., Huang, C., Zang, W.J., and Xie, L.H., Potential antiarrhythmic effect of methyl 3,4,5-trimethoxycinnamate, a bioactive substance from roots of polygalae radix: suppression of triggered activities in rabbit myocytes. *Biol. Pharm. Bull*. **36**, 238-244 (2013).

Zhu, S. and Baker, R.C., Effects of inhalation anesthetics of Kainate-induced glutamate release from cerebellar granule cells. *Life Sci*. **58**, 1359-1366 (1996).

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