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Catalpol and Mannitol, Two Components of *Rehmannia glutinosa*, Exhibit Anticonvulsant Effects Probably via $GABA_A$ Receptor Regulation

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Abstract

Epilepsy is a brain disorder that affects millions of people worldwide and is usually managed using currently available antiepileptic drugs, which result in adverse effects and are ineffective in approximately 20-25% of patients. Thus, there is growing interest in the development of new antiepileptic drugs with fewer side effects. In a previous study, we showed that a *Rehmannia glutinosa* (RG) water extract has protective effects against electroshock- and pentylenetetrazol (PTZ)-induced seizures, with fewer side effects. In this study, the objective was to identify the RG components that are responsible for its anticonvulsant effects. Initially, a number of RG components (aucubin, acteoside, catalpol, and mannitol) were screened, and the anticonvulsant effects of different doses of catalpol, mannitol, and their combination on electroshock- and chemically (PTZ or strychnine)-induced seizures in mice, were further assessed. Gamma-aminobutyric acid (GABA) receptor binding assay and electroencephalography (EEG) analysis were conducted to identify the potential underlying drug mechanism. Additionally, treated mice were tested using open-field and rotarod tests. Catalpol, mannitol, and their combination increased threshold against electroshock-induced seizures, and decreased the percentage of seizure responses induced by PTZ, a GABA antagonist. GABA receptor binding assay results revealed that catalpol and mannitol are associated with GABA receptor activity, and EEG analysis provided evidence that catalpol and mannitol have anticonvulsant effects against PTZ-induced seizures. In summary, our results indicate that catalpol and mannitol have anticonvulsant properties, and may mediate the protective effects of RG against seizures.

Key Words: *Rehmannia glutinosa*, Catalpol, Mannitol, Anticonvulsant, Epilepsy, Gamma-aminobutyric acid (GABA)

INTRODUCTION

Epilepsy, characterized by recurrent and unpredictable seizures, is one of the most common neurological disorders (Fisher *et al*., 2005) that can develop in any person at any age (Hauser, 1992). Its causes vary, and each patient's seizure symptoms are different. The exact mechanism of epilepsy is still unknown, and approximately 50 million people worldwide struggle with its management. Additionally, approximately 20-25% of the people diagnosed with epilepsy do not respond to the currently available antiepileptic drugs, and present recurring seizures as well as adverse side effects (Begley *et al*., 2000; Fisher *et al*., 2000; Schmidt and Schachter, 2014). In a survey study on more than 10,000 epilepsy patients, up to 50% of them reported various side effects after taking antiepileptic drugs (Fisher *et al*., 2000). Consequently, there is growing interest in the development of new drugs with fewer side effects such as natural or herbal products (Quintans Júnior *et al*., 2008; Woo *et al*., 2011).

Rehmannia glutinosa (RG) has been widely used in traditional Chinese medicine to treat a variety of health issues. For example, it has been reported to exhibit anti-oxidative, anti-

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inflammatory, and anti-tumor activities (Kim *et al*., 1999, 2005; Baek *et al*., 2012). In a previous study, we demonstrated that in mice, its water extract had anticonvulsant effects against electroshock- and chemically (pentylenetetrazol, PTZ)-induced seizures, without any side effects such as sedation or muscle relaxation (Kim *et al*., 2017). RG contains more than 70 compounds (Zhang *et al*., 2008), including iridoid compounds (e.g., catalpol, dihydrocatalpol, and aucubin), phenethyl alcohol glycosides (e.g., acteoside, and isomaltoside), and saccharides (e.g., mannitol, stachyose, and raffinose), which are its major components (Tomoda *et al*., 1971; Zhang *et al*., 2008; Lee *et al*., 2011). Therefore, the purpose of this study was to screen and determine the RG component(s) associated with the anticonvulsant effects of its extract.

Several RG components, including aucubin, acteoside, catalpol, and mannitol, were screened to determine whether they have anticonvulsant effects, and catalpol, mannitol, and their combination were chosen for further assessment. Recently, using LiCl/pilocarpine, electroencephalography (EEG), or extracellular field recording in brain slice, some studies have reported that catalpol or mannitol might have anticonvulsant effects (Haglund and Hochman, 2005; Serafini, 2017; Gao *et al*., 2018), however, their roles remain unclear. In this study, their protective effects as well as that of their combination against electroshock- or chemically (PTZ or strychnine)-induced seizures, were evaluated in mice. Their chemical structures are shown in Table 1. Additionally, gamma-aminobutyric acid (GABA) A receptor binding assay and electroencephalography (EEG) analysis were performed to identify the potential underlying mechanisms, based on the results of the chemically-induced seizure experiments. Open-field and rota-rod tests were also performed to evaluate the psychopharmacological or side effects of catalpol, mannitol, and their combination.

MATERIALS AND METHODS

Drugs & materials

Catalpol, acteoside, and aucubin were purchased from ChemFaces (Wuhan, China), and mannitol was obtained from Sigma-Aldrich Inc (St. Louis, Mo, USA). Each component was dissolved in physiological saline and subcutaneously (s.c.) administered to the test animals. PTZ, strychnine, and diazepam were obtained from Sigma-Aldrich Inc., diluted in physiological saline, and administered intraperitoneally (i.p.). [^{3}H]-SR95531 used in the GABA receptor binding assay was obtained from Perkin Elmer (Waltham, MA, USA).

Animals

Five-week-old male ICR mice (20-25 g) obtained from Hanlim Laboratory Animals Co. (Hwaseong, Korea), were used in this study. They were housed in each group under a 12 h/12 h light/dark cycle (7 AM-7 PM) at constant temperature and humidity (22 ± 2°C, 55 ± 5%). Food and water were provided *ad libitum*, with the exception of the eve of treatment. 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 (Seoul, Korea).

Measurement of electroshock-induced seizure threshold

The mice were divided into the following treatment groups: control (vehicle), catalpol, mannitol, acteoside, and aucubin (a series of dosages: 1-20 mg/kg), and diazepam (2 mg/kg), and the treatment drugs (catalpol, mannitol, acteoside, aucubin, vehicle, all s.c., and diazepam, i.p.) were administered 30 min before the test. To induce seizures as defined in previous studies, (Yoon *et al*., 2011; Kim *et al*., 2017), the mice were given electroshocks using an electroconvulsion device (ECT Unit 57800, Ugo Basile, Gemonio, Italy; 50 Hz frequency, 0.5 ms duration). Briefly, the condition of each mouse was characterized as "present" (complete tonic extension with overt hind limb extension) or "absent" (no seizure), and the staircase method was used to determine seizure threshold. For example, if a mouse showed complete tonic convulsion with hind limb extension, the next mouse was electroshocked with a 2 mA decrease in current intensity. If the mouse did not show any convulsion, the next was electroshocked with a 2 mA increase in current intensity. Thus, a convulsive current (CC)-relationship curve was generated for each treatment group, and the CC that induced seizures in 50% of the animals (CC_{50}) was determined.

Anticonvulsant potency against PTZ-induced seizures

PTZ can induce seizures by antagonizing GABA (Pellmar and Wilson, 1977). The mice were randomized into groups, and treated with vehicle, catalpol, mannitol, catalpol and mannitol combined, or diazepam, 30 min before PTZ (70 mg/kg) administration, immediately after which, each mouse was observed for 20 min. Seizures were assessed using the "all or none" method, as previously described (Kim *et al*., 2017). The percentage of mice that showed seizure responses was estimated in each treatment group.

Anticonvulsant potency against strychnine-induced seizures

Strychnine is a convulsion-inducing drug that antagonizes glycine (Larson and Beitz, 1988). As described in the PTZ-induced seizure tests, mice were randomly assigned into groups treated with either vehicle, catalpol, mannitol, their combination, or diazepam 30 min before strychnine (1 mg/kg) administration, immediately after which, each mouse was monitored in an observation cage for 20 min. The percentage of animals that showed seizure responses in each treatment group was determined and compared with that of the control group.

GABA receptor binding assay

[^{3}H]-SR95531, a $GABA_A$ antagonist was used for $GABA_A$ receptor binding detection. All processes were performed as previously reported, with slight modifications (Heaulme *et al*., 1987). Briefly, after treatment, cerebral cortex extracted from 5-week-old ICR mice were homogenized and centrifuged at 20,000×*g* at 4°C for 20 min, and the protein samples obtained (2.4 mg) were used for $GABA_A$ receptor detection. The samples were incubated with 10 nM [^{3}H]-SR95531 (4 Ci/mmol) and 50 μL of test samples in a final volume of 200 μl, for 2 h at room temperature. Each test molecule was added in serial concentrations, from 10^{-10} to 10^{-6} M, in the presence of 10 nM [^{3}H]-SR95531. Thereafter, they were filtrated using a GF/C microfiber filter. Subsequently, the samples were washed 3 times in binding buffer, and a thin layer chromatography paper was used to ensure proper drying of the GF/C filter membranes. The level of [^{3}H]-SR95531, which is indicative of nonspecific binding, in the GABA (1.0 mM) incubated samples was mea-

sured using the Wallac 1450 MicroBeta® TriLux liquid scintillation counter (Perkin Elmer, MA, USA). Half maximal inhibitory concentration (IC_{50}) values for the tested molecules were converted to K_i values using the Cheng-Prusoff equation [K_i=IC_{50}/(1+[L]/K_d)] (K_i, absolute inhibition constant; [L], concentration of labeled ligand; K_d, the dissociation constant of labeled ligand for the receptor) (Cheng and Prusoff, 1973).

Electroencephalography recording

EEG recording was used to evaluate the effects of catalpol and mannitol on brain electrical wave activities after PTZ administration, and all processes were performed in compliance with the manufacturer's instructions (Sirenia software v1.7.6, Pinnacle Technology Inc., KS, USA). Briefly, immediately after PTZ administration, each mouse was anesthetized with Zoletil (50 mg/kg, im), and the skull surface was exposed. The head mount was placed on the dry skull and stabilized with 4 screws. An epoxy resin was applied between the screw head and the holes of the head mount, and dental cement was used to fix the head mount and screws to the skull. After surgery, the mice were allowed 5 d for recovery and 2 d for habituation to the EEG recording environment before the initiation of the recordings. The mice were then administered catalpol (10 mg/kg), mannitol (20 mg/kg), and vehicle subcutaneously, or diazepam (2 mg/kg) intraperitoneally. Immediately, EEG was recorded for 40 min using the Sirenia software (Pinnacle Technology Inc.). After the first recording with each pre-treatment, the mice were administered PTZ (50 mg/kg, i.p.), and immediately, the EEG was recorded for 40 min. The total power of each EEG wavelength (delta 0.5-3.99 Hz, theta 4-7.99 Hz, and alpha 8-12.99 Hz) was used to evaluate the effect of each pre-treatment.

Locomotor activity test

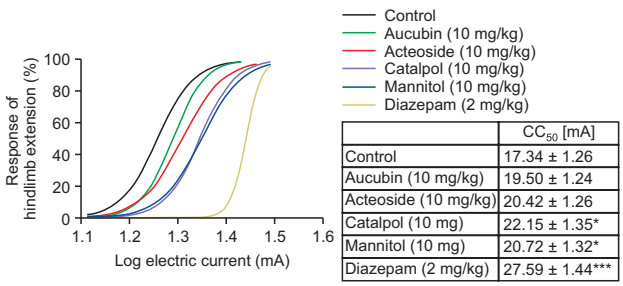
The effect of catalpol and mannitol on mouse locomotor activity was assessed using the open-field test. Each mouse was placed at the center of a black, Plexiglas chamber (42×42×42 cm), and its locomotor activity recorded 30 min after the administration of catalpol (10 mg/kg), mannitol (20 mg/kg), catalpol (10 mg/kg) and mannitol (20 mg/kg) combination, diazepam (2 mg/kg), or vehicle. A computerized system (Ethovision System, Noldus, Wageningen, Netherlands) was used to record the distance moved (cm) and movement durations (s) for 12 min, wherein 2 min constituted the habituation phase, and the remaining 10 min was for analysis.

Rota-rod test

The effect of catalpol and mannitol on mouse balance and motor coordination was assessed using a rota-rod device (Ugo Basile, Varese, Italy) rotating at a fixed velocity of 36 rounds per min. All mice were trained for 3 min on the rotating rod 24 h prior to the test, and on the test day, they were treated with catalpol (10 mg/kg), mannitol (20 mg/kg), catalpol (10 mg/kg) and mannitol (20 mg/kg) combination, diazepam (2 mg/kg), or vehicle 30 min before the test. Latency time [s] (until the first fall) and falling frequency were recorded for 10 min.

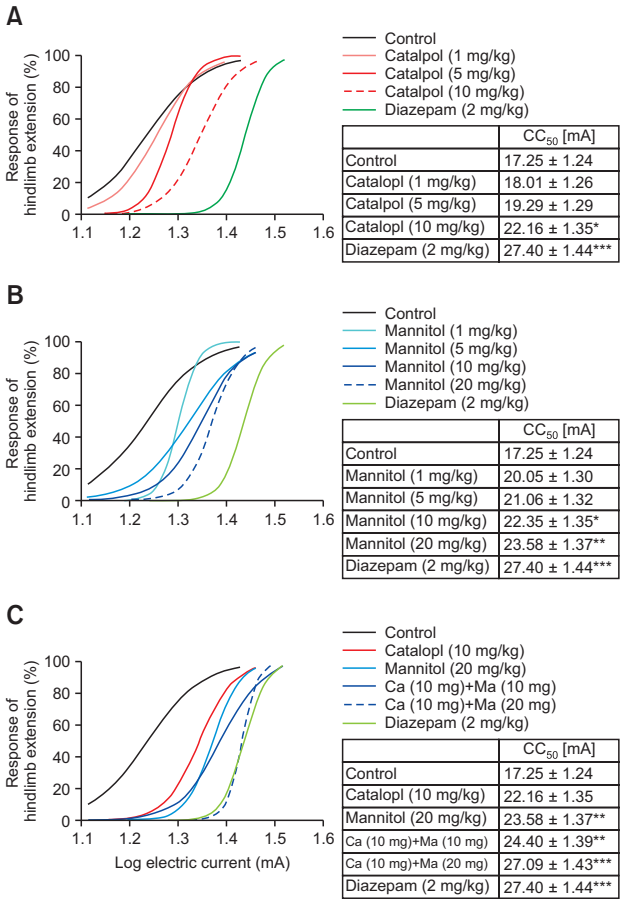
Statistical analysis

All data were expressed as mean ± SEM. With the exceptions of binding assay and EEG data that were analyzed using two-way analysis of variance (ANOVA), all data were analyzed using one-way ANOVA. When a statistically significant difference between groups was found, the Bonferroni post-hoc test was applied. Analyses were performed using GraphPad Prism



	CC_{50} [mA]
Control	17.34 ± 1.26
Aucubin (10 mg/kg)	19.50 ± 1.24
Acteoside (10 mg/kg)	20.42 ± 1.26
Catalpol (10 mg)	22.15 ± 1.35*
Mannitol (10 mg)	20.72 ± 1.32*
Diazepam (2 mg/kg)	27.59 ± 1.44***

Fig. 1. Effects of the components of *Rehmannia glutinosa* (RG) on seizures induced by electroshock in mice (n=15-20/group). Animals were treated with vehicle, catalpol, mannitol, acteoside, aucubin (s.c.), or diazepam (i.p.). The numbers in the box represent the CC_{50} values with 95% confidence intervals. *p<0.05, ***p<0.001; significantly different from the control group. CC_{50}, convulsive current 50.



	CC_{50} [mA]
Control	17.25 ± 1.24
Catalopl (1 mg/kg)	18.01 ± 1.26
Catalpol (5 mg/kg)	19.29 ± 1.29
Catalopl (10 mg/kg)	22.16 ± 1.35*
Diazepam (2 mg/kg)	27.40 ± 1.44***

	CC_{50} [mA]
Control	17.25 ± 1.24
Mannitol (1 mg/kg)	20.05 ± 1.30
Mannitol (5 mg/kg)	21.06 ± 1.32
Mannitol (10 mg/kg)	22.35 ± 1.35*
Mannitol (20 mg/kg)	23.58 ± 1.37**
Diazepam (2 mg/kg)	27.40 ± 1.44***

	CC_{50} [mA]
Control	17.25 ± 1.24
Catalopl (10 mg/kg)	22.16 ± 1.35
Mannitol (20 mg/kg)	23.58 ± 1.37**
Ca (10 mg)+Ma (10 mg)	24.40 ± 1.39**
Ca (10 mg)+Ma (20 mg)	27.09 ± 1.43***
Diazepam (2 mg/kg)	27.40 ± 1.44***

Fig. 2. Effects of catalpol and mannitol on seizures induced by electroshock in mice (n=30/group) after treatment with (A) various dosages of catalpol, (B) various dosages of mannitol, or (C) catalpol and mannitol combination. The numbers in the box represent CC_{50} values with 95% confidence intervals. *p<0.05, **p<0.01, ***p<0.001; significantly different from the control group. CC_{50}, convulsive current 50.

v7 (GraphPad, St. Louis, MO, USA). $p<0.05$ was considered statistically significant.

RESULTS

Effects of RG components on electroshock-induced seizures in mice

Fig. 1 shows the effect of each tested RG component against electroshock-induced seizures in mice. The positive control group (treated with diazepam) exhibited significantly increased seizure threshold (27.59 ± 1.44 mA, $p<0.001$) compared with the control group (18.13 ± 1.26 mA). All RG components increased the seizure threshold: aucubin (19.50 ± 1.29 mA), acteoside (20.42 ± 1.31 mA), catalpol (22.15 ± 1.35 mA, $p<0.05$), and mannitol (22.34 ± 1.35 mA, $p<0.05$). Notably, catalpol and mannitol treatments resulted in higher seizure thresholds than the other RG components, and based on this observation, they were considered for further assessment of anticonvulsant properties.

Catalpol and mannitol treatments confer protection against electroshock-induced seizures in a dose-dependent manner

Fig. 2A shows the effects of a series of catalpol and mannitol dosages on electroshock-induced seizures. Compared with the control group (17.25 ± 1.24 mA), catalpol increased the seizure threshold in mice in a dose-dependent manner: 1 mg/kg (18.01 ± 1.265 mA), 5 mg/kg (19.29 ± 1.29 mA), and 10 mg/kg (22.16 ± 1.35 mA, $p<0.05$). Similarly, compared with the control group, mannitol increased the seizure threshold in a dose-dependent manner: 1 mg/kg (20.05 ± 1.30 mA), 5 mg/kg (21.06 ± 1.32 mA), 10 mg/kg (22.35 ± 1.35 mA, $p<0.05$), and 20 mg/kg (23.58 ± 1.37 mA, $p<0.01$) (Fig. 2B). The administration of the catalpol (10 mg/kg) and mannitol (20 mg/kg) combination resulted in a further increase in seizure threshold (27.09 ± 1.43 mA, $p<0.001$, Fig. 2C).

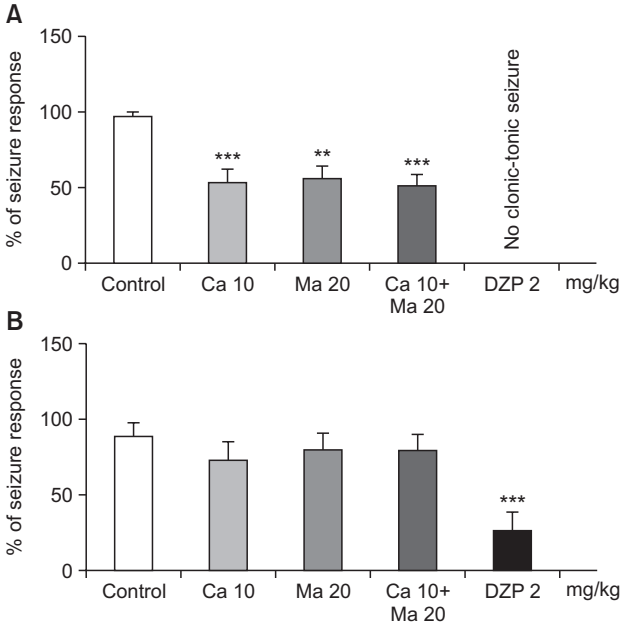


Fig. 3. Effects of catalpol and mannitol on chemically-induced seizures (PTZ or strychnine) in mice (n=30/group). (A) Effects of catalpol, mannitol, and their combination on seizures induced by PTZ. (B) Effects of catalpol, mannitol and the combination on seizures induced by strychnine. Each bar represents mean ± SEM of the percentage of seizure responses in each group. $^{**}p<0.01$, $^{***}p<0.001$; significantly different from the control group. Ca, catalpol; DZP, diazepam; Ma, mannitol; PTZ, pentylenetetrazol; SEM, standard error of the mean.

Effects of catalpol and mannitol on chemically (PTZ or strychnine)-induced seizures in mice

Fig. 3A shows the effects of catalpol and mannitol on PTZ-induced seizures in mice. One-way ANOVA showed significant differences in the percentage of seizure responses of the treatment groups. Catalpol [$F(4, 153)=13.43$, $p<0.001$], mannitol [$F(4, 153)=13.43$, $p<0.01$], and their combination [$F(4, 153)=13.43$, $p<0.001$] decreased the percentage of PTZ-induced seizure responses. Diazepam completely inhibited PTZ-induced seizure responses.

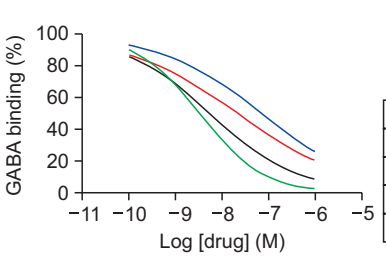
In Fig. 3B, the effects of catalpol and mannitol on strychnine-induced seizures are illustrated. One-way ANOVA showed significant differences in the percentage of seizure responses of the experimental groups [$F(4, 77)=5.53$, $p<0.001$]. However, post-hoc analysis revealed significant effects only in the diazepam-treated group ($p<0.001$), and not in catalpol- or mannitol-treated groups.

Assessment of catalpol and mannitol in the GABA receptor-binding assay

GABA, catalpol, and mannitol showed dose-dependent GABA receptor binding (Fig. 4). Two-way ANOVA found significant group differences in the IC_{50} of GABA receptor binding (%) of the different molecules tested [$F(3, 40)=184.9$, $p<0.001$]. The IC_{50} for GABA, catalpol, mannitol, and diazepam was 5.62 ± 0.09 nM, 73.62 ± 0.08 nM, 22.26 ± 0.12 nM, and 3.32 ± 0.07 nM, respectively, and their K_i values were 2.18 ± 0.03 nM, 28.56 ± 0.03 nM, 8.64 ± 0.05 nM, and 1.29 ± 0.03 nM, respectively.

Effects of catalpol and mannitol on EEG in mice

After catalpol, mannitol, vehicle, or diazepam administration to mice, their delta (0.5-3.99 Hz), theta (4-7.99 Hz), and alpha (8-12.99 Hz) waves were evaluated. Fig. 5 shows the EEG before and after PTZ treatment in each condition. Two-way ANOVA detected significant group differences in the delta



	IC50 [nM]
GABA	5.62 ± 0.09
Catalpol	73.62 ± 0.08
Mannitol	22.26 ± 0.12
DZP	3.32 ± 0.07

Fig. 4. Effects of catalpol and mannitol on GABA receptor binding (%) in brain (n=3 per material and dosage). [^{3}H]-SR95531, a GABAa antagonist was used to detect GABA receptor binding (%). GABA, catalpol, mannitol, and diazepam were added at concentrations of 10^{-10} to 10^{-6} M in the presence of 10 nM [^{3}H]-SR95531. DZP, diazepam; GABA, gamma-aminobutyric acid.

[F (3, 34)=6.002, p<0.01], theta [F (3, 31)=8.046, p<0.001], and alpha waves [F (3, 32)=4.032, p<0.05]. Only mice in the vehicle group showed a significant increase in delta (p<0.001) and theta (p<0.001) waves after PTZ administration, while catalpol-, mannitol-, or diazepam-treated mice did not present any increased delta and theta waves. For alpha waves, there were no significant differences among the different treatments in response to PTZ. Additionally, Bonferroni post-tests found significant differences between catalpol-treated mice and vehicle-treated mice after PTZ administration in delta (p<0.001), theta (p<0.001), and alpha waves (p<0.01). Mannitol-treated mice also showed significant effects compared to the control group after PTZ treatments in delta (p<0.01), theta (p<0.01), and alpha waves (p<0.01). These results are similar as the ones of DZP-treated mice.

Effects of catalpol and mannitol on the locomotor activity in mice

Fig. 6 shows the total distance moved (cm) and the movement duration (s) of the mice in the open-field test after treatment with catalpol, mannitol, catalpol and mannitol combination, diazepam, or vehicle. One-way ANOVA showed significant differences in the distance moved [F (4, 37)=15.81, p<0.001] and in the movement duration [F (4, 42)=48.74, p<0.001] among the experimental groups. However, catalpol, mannitol, and their combination did not significantly alter the locomotor activity of mice, compared with the control group. On the other hand, diazepam significantly decreased the distance moved (p<0.001) and the movement duration (p<0.001) of mice, indicating alterations in locomotor activity or sedation.

Effects of catalpol and mannitol on motor balance and coordination in the rota-rod test

Fig. 7 shows the latency to first fall (s) and falling frequency of the mice subjected to the rota-rod test after catalpol-, mannitol-, catalpol and mannitol combination, diazepam-, or vehicle treatment. One-way ANOVA detected no significant group differences in latency time [F (4, 47)=2.269, p=0.07]; however, significant group differences in the falling frequency were noted [F (4, 42)=3.562, p<0.05] among the experimental groups. However, catalpol, mannitol, and their combination did not significantly alter the latency to first fall or falling frequency, compared with the control group, while the diazepam group presented significant (p<0.01) decreases.

DISCUSSION

The objective of this study was to identify the RG component(s) that mediates the anticonvulsant effects of its

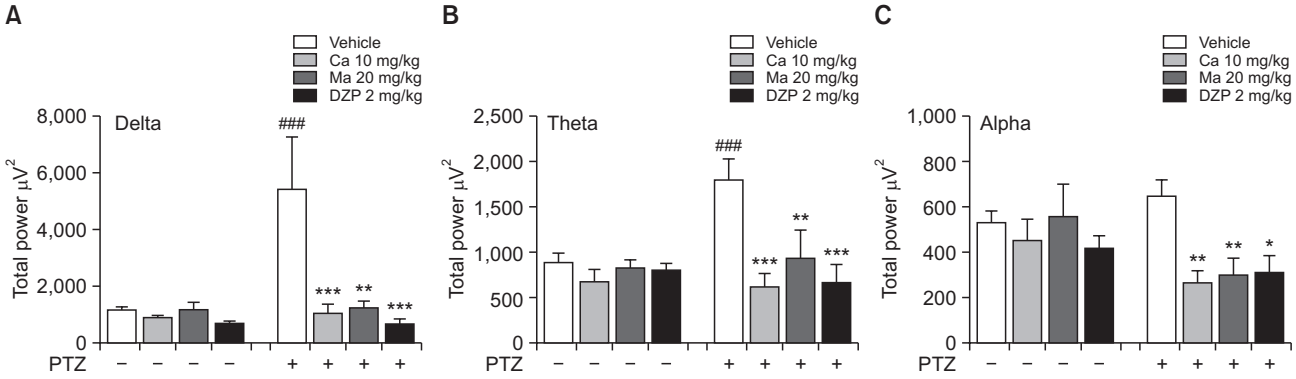


Fig. 5. Effects of catalpol and mannitol on EEG activities in mice (n=6/group). Each bar represents mean ± SEM of the total power of (A) delta, (B) theta, and (C) alpha waves for 30 min after treatment with vehicle, catalpol, mannitol, or DZP and for another 30 min after administration of PTZ (50 mg/kg). *p<0.05, **p<0.01, ***p<0.001; significantly different from the control group after PTZ administration. ###p<0.001; significantly different from the control group without PTZ treatments. Ca, catalpol; DZP, diazepam; EEG, electroencephalographic; Ma, mannitol; PTZ, pentylenetetrazol; SEM, standard error of the mean.

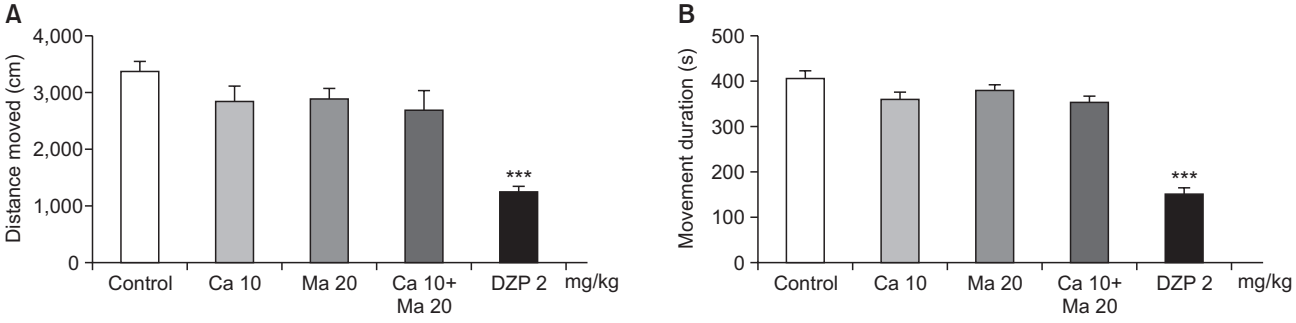


Fig. 6. Effects of catalpol, mannitol and their combination on locomotor activity in mice (n=15/group). Each bar represents mean ± SEM of (A) the total distance moved and (B) movement duration, recorded for 10 min. ***p<0.001; significantly different from the control group. Ca, catalpol; DZP, diazepam; Ma, mannitol; SEM, standard error of the mean.

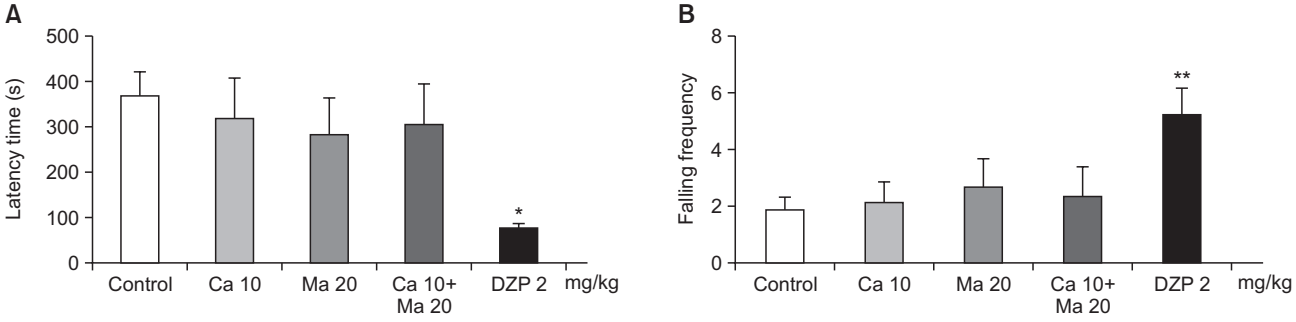


Fig. 7. Effects of catalpol, mannitol and their combination on the rota-rod test in mice (n=15/group). Each bar represents mean ± SEM of (A) latency time and (B) falling frequency, recorded for 10 min. **p*<0.05, ***p*<0.01; significantly different from the control group. Ca, catalpol; DZP, diazepam; Ma, mannitol; SEM, standard error of the mean.

water extract. The study revealed that catalpol and mannitol, which are major RG components, have anticonvulsant properties, as evidenced by the increased threshold against both electroshock- and PTZ-induced seizures, and by the normalized theta and delta waves after PTZ administration in mice. Catalpol and mannitol showed anticonvulsant properties similar to that of the RG water extract (Kim *et al*., 2017), indicating that these components may play key roles in RG's anticonvulsant effects.

Electroshock-induced seizure tests in rodents are commonly used to assess the potential anticonvulsant activities of molecules under examination (Löscher *et al*., 1991). Catalpol and mannitol exhibited greater anticonvulsant activities than other RG components (acteoside and aucubin). As shown in Fig. 1, the CC_{50} value of acteoside was similar to that of mannitol. However, other preliminary results on acteoside were not significant (data not shown). In electroshock-induced seizure tests, the anticonvulsant effects of catalpol and mannitol increased in a dose-dependent manner. Additionally, the CC_{50} value after the combined administration of catalpol and mannitol was similar to those obtained after diazepam or RG water extract treatment (Kim *et al*., 2017). In support of this result, another study also showed that catalpol and mannitol treatment significantly reduced PTZ-induced seizure responses, and the reductions were similar to those brought about by a 200 mg/kg RG water extract treatment (Kim *et al*., 2017). PTZ is a non-competitive GABA antagonist that induces seizures via $GABA_A$ receptor inhibition (Deyn *et al*., 1989; Huang *et al*., 2001). Some $GABA_A$ receptor agonists such as diazepam and phenobarbital, which activate $GABA_A$ receptors, are known to be effective anticonvulsants (Hansen *et al*., 2004). In response, the activated $GABA_A$ receptors reduce the excitability or inhibit the activity of postsynaptic neurons (Rogawski and Porter, 1990). In this study, diazepam administration did not induce responses to PTZ-induced seizure, and catalpol and mannitol administration reduced PTZ-induced seizures (Fig. 3), indicating that the anticonvulsant effects of catalpol and mannitol may be related to $GABA_A$ receptors. On the other hand, catalpol and mannitol did not reduce seizure responses induced by strychnine, a glycine antagonist (Larson and Beitz, 1988), while they were reduced by diazepam. Glycine is an inhibitory neurotransmitter that acts in parts of the central nervous system, including the spinal cord and brainstem (Lynch, 2004). Its activated receptors produce an inhibitory postsynaptic potential, and strychnine induces seizures by acting as its strong antagonist. Consequently, the results of this study indicate that the anticonvulsant properties of catalpol and mannitol might be associated with $GABA_A$ receptors, rather than glycine receptors.

Supporting the results of the chemically-induced seizure test, the GABA receptor binding assay showed that catalpol and mannitol had affinity for $GABA_A$ receptors (Fig. 4). Actually, their K_i values were similar to those of diazepam, with regards to $GABA_A$ receptors (Berezhnoy *et al*., 2004; Tan *et al*., 2009). These results indicate that the anticonvulsant effects of catalpol and mannitol on electroshock- and PTZ-induced seizures could be mediated via $GABA_A$ receptor activity. However, the combination of catalpol and mannitol had an additive effect (combination index, CI=0.94) on electronic-induced seizures (Chou and Talalay, 1984), but not on PTZ-induced seizures. Based on these all results, it can be suggested that the anticonvulsant properties of catalpol and mannitol might be mediated by other pathways as well as $GABA_A$ receptor activities. For example, Gao *et al*. (2018) reported that catalpol decreased LiCl/pilocarpine-induced seizure responses and altered Nrf2-Keap1-ARE expression.

The anticonvulsant effects of catalpol and mannitol were further supported by the EEG results (Fig. 5). Recently, several studies have reported that PTZ induces an increase of 1 to 7 Hz in the EEG of animals (Lüttjohann *et al*., 2009; Grauncke *et al*., 2016; Pontes *et al*., 2016; Hamoy *et al*., 2018). Lüttjohann *et al*. (2009) reported that the spike frequency changed from 2-3 Hz at the initiation of a PTZ-induced seizure phase to 6-7 Hz in the middle of the seizure phase. This study demonstrated that the catalpol and mannitol pre-treated groups did not present changes in delta (0.5-3.99 Hz) and theta (4-7.99 Hz) waves after PTZ administration, while the vehicle group showed increased delta and theta waves, in agreement with previous studies (Lüttjohann *et al*., 2009; Grauncke *et al*., 2016; Pontes *et al*., 2016; Hamoy *et al*., 2018). The effects of catalpol and mannitol on EEG were similar to those of diazepam.

Additionally, catalpol and mannitol did not induce any changes in the locomotor activity, balance, and motor coordination of their treatment groups; however, diazepam reduced locomotor activity and decreased the rota-rod latency time (Fig. 6, 7). This result is consistent with that of our previous study on RG water extract (Kim *et al*., 2017), and indicates that catalpol and mannitol do not induce side effects such as sedation and muscle relaxation.

In summary, catalpol and mannitol exhibited protective effects against electroshock- and PTZ-induced seizures, similar to those of diazepam and RG water extract, a result further supported by the detection of mice EEG changes after PTZ administration. Using the $GABA_A$ receptor binding assay, this study also demonstrated that the anticonvulsant effects of catalpol and mannitol may be associated with $GABA_A$ receptor activity. Thus, catalpol and mannitol have anticonvulsant properties, and may mediate the protective effects of RG against seizures. Additionally, catalpol and mannitol does not produce side effects such as sedative or muscle relaxant effects. These findings suggest that catalpol and mannitol could be considered as a new anticonvulsant with minimal side effects.

CONFLICT OF INTEREST

The authors confirm that they do not have any conflicts of interest.

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