



## Protection Against Electroshock- and Pentylentetrazol-induced Seizures by the Water Extract of *Rehmannia glutinosa* can be Mediated through GABA Receptor-chloride Channel Complexes

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**Abstract** – Epilepsy is a brain disorder that affects millions of people worldwide. It is characterized by recurrent and unpredictable seizures that are usually controlled with antiepileptic/anticonvulsive drugs. However, most antiepileptic drugs produce various side effects such as tolerance and sedation. Thus, there is a growing interest for alternative anticonvulsive drugs, preferably from natural or herbal sources. In this study, we evaluated the anticonvulsive effects of *Rehmannia glutinosa* (RG). The anticonvulsive effect of RG extract was evaluated using electroshock- and chemical-induced seizure tests in mice. To identify its probable mechanism of action, the effects of RG extract on Cl<sup>-</sup> influx was measured *in vitro*. We found that RG extract has anticonvulsive effects against electroshock-induced seizures, as indicated by an increased seizure threshold in mice. The RG extract also decreased the percentage of seizure responses induced by the GABAergic antagonist, pentylentetrazole. These results suggest that the anticonvulsive effects of RG extract are mediated through a GABAergic mechanism. In support of this mechanism, our *in vitro* test showed that RG extract increases intracellular Cl<sup>-</sup> influx. Furthermore, RG extract did not show sedative and/or muscle relaxant effects in the open-field and rota-rod tests. Altogether, these results confirm that RG extract could be a herbal anticonvulsant and a potential alternative for clinical use.

**Keywords** – *Rehmannia glutinosa*, Anticonvulsant, Epilepsy, GABA, Electroshock-induced seizure, Chemical-induced seizure

### Introduction

Epilepsy is a brain disorder characterized by recurrent and unpredictable seizures.<sup>1</sup> A patient with epilepsy experiences epileptic seizures as a result of abnormal, excessive, or hypersynchronous activity of neurons in the brain.<sup>1,2</sup> Approximately 50 million people suffer from epilepsy worldwide, and approximately 2.4 million new patients are diagnosed yearly. Epilepsy is usually controlled with antiepileptic drugs (e.g., acetazolamide, benzodiazepines, and phenobarbital), and in rare cases, when antiepileptic drugs are ineffective, surgical therapy is used.<sup>3</sup> Unfortunately, 20 - 30% of patients with epilepsy show resistance to current antiepileptic drugs.<sup>4</sup> Most

antiepileptic drugs produce diverse side effects such as sedation, cognitive impairment, and tremors.<sup>5</sup> In addition, about three-fourths of patients in developing countries do not receive proper treatment owing to the lack of information and cost issues.<sup>6</sup> To overcome these issues, the development of effective and accessible antiepileptic drugs is needed.

There is a growing interest for new antiepileptic drugs from natural or herbal products.<sup>7-9</sup> Herbal products are preferred alternatives because they usually have fewer side effects than currently available anticonvulsants. For these reasons, our team is searching for herbal products that have anticonvulsive activities with fewer side effects. In our search, we have found that an herbal mixture containing *Rehmannia glutinosa* (RG) decreases the level of seizures in patients with epilepsy.<sup>10</sup> Another mixture called Samulanshintang, which also includes RG, significantly decreased chemically (i.e., pentylentetrazole, PTZ)-induced seizures in mice.<sup>11</sup> These studies suggest

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that RG has anticonvulsive activities. However, to our knowledge, no study has identified the effects of RG per se on seizures or convulsions.

RG is a perennial herb that belongs to the family Orobanchaceae. RG is grown in Europe and North America as an ornamental garden plant. In Asia, it is regarded as an herbal plant and is one of the 50 fundamental herbs used in traditional Chinese medicine (TCM).<sup>12</sup> RG is used for various conditions and has been reported to have beneficial effects on the immune, cardiovascular, blood, and central nervous systems; it has also been reported to have anti-tumor, anti-inflammatory, and antioxidative activities.<sup>13-20</sup> Despite its various biological benefits, the anticonvulsive activity of RG has not been evaluated. Thus, the aim of the present study was to evaluate the anticonvulsive effects of RG. For this, the RG extract was administered to mice, and its protective effects against electroshock- or chemical-induced seizures were assessed. To elucidate its possible mechanism of action, the effect of RG extract on  $\text{Cl}^-$  ion influx in human neuroblastoma cells was also studied. In addition, behavioral tests were conducted (i.e., open-field and rotarod tests) to assess the psychopharmacological effects or side effects of RG extract.

## Experimental

**General experimental procedures** – Pentylentetrazole (PTZ), strychnine, and diazepam were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). N-(6-methoxyquinolyl) acetoethyl ester (MQAE) was purchased from Invitrogen Co. (Carlsbad, CA, USA). RG extracts were dissolved in sterile distilled water and were given to animals orally. Diazepam, PTZ, and strychnine were diluted in physiological saline and were administered intraperitoneally (i.p.).

**Plant materials** – RG was obtained from Kyungdong mart (Seoul, Korea). The herb was harvested in Gyeong-sangbuk-do, Korea in October 2014, and identified by Prof. Dong Sool Yim, Sahmyook University, Seoul, Korea. Voucher specimens of RG were deposited in Sahmyook University, Korea. To produce RG water extract, the root of RG was added to a 10-fold volume of double-distilled water and boiled until the volume was reduced by one-half. The extract was filtered with Whatman No. 1 filter paper, concentrated on a water bath under reduced pressure, and then lyophilized (Elela, model FD-5N). For RG ethanol extract, the root of RG was extracted with 20 volumes of 80% ethanol at 70°C for 3 h. The extract was filtered, concentrated with a rotary evaporator,

and then lyophilized.

**Animals** – The male ICR mice (20 - 25 g) used in this study were obtained from Hanlim Laboratory Animals Co. (Hwaseong, Korea). Animals were housed in groups in an animal room with controlled temperature and humidity ( $22 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$ ). Food and water were provided *ad libitum*, except the night before the experiment. The animal room was kept on a 12 h/12 h light/dark cycle (7AM–7PM). All animals were acclimatized to the housing conditions for 7 days prior to the experiment. Animal treatment and maintenance were performed 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.

**Electroshock-induced seizure model** – To induce seizures, mice were given electroshocks (50 Hz frequency, 0.5 ms duration) generated by an electroconvulsion device (ECT Unit 57800, UgoBasile, Gemonio, Italy) delivered through ear clips. Mice were divided into groups [control (distilled water), diazepam (2 mg/kg), RG groups (100 mg/kg RG ethanol extract), (50, 100, 200, and 400 mg/kg RG water extract)] with 20 mice per group. RG extracts or distilled water were orally administered 60 min before the test. Diazepam was administered i.p. 30 min before the test. Seizures were judged as “absent” (i.e., no seizure) or “present” (i.e., complete tonic extension with overt hindlimb extension). The staircase method was used to determine the seizure threshold.<sup>21</sup> In this method, one animal’s response to a given current intensity determined the subsequent current intensity for the next animal. If the first animal showed complete tonic extension with hindlimb extension, the current intensity has decreased by 2 mA for the next animal. If the first animal showed no convulsions, the current intensity has increased by 2 mA for the next one. This allowed us to generate a convulsive-current (CC) relationship curve for each group. The convulsive current 50 ( $\text{CC}_{50}$ ) that induces seizures in 50% of the animals represents the seizure threshold.

**Pentylentetrazole (PTZ)-induced seizure** – PTZ is a convulsion-inducing drug that acts through the GABA receptor.<sup>22</sup> Sixty mice were randomly assigned to 6 groups (10 per group). Each animal was orally treated with RG water extract (50, 100, 200, or 400 mg/kg), distilled water (1 ml/kg), or diazepam (2 mg/kg, i.p.) 60 min (30 min for the diazepam group) prior to administration of PTZ (70 mg/kg, i.p.). Immediately after PTZ administration, animals were placed individually in observation cages, and the presence (all, 100%) or absence (none, 0%) of seizures (i.e., overt hind limb extensions) was assessed for 20 min.

The percentage of seizure responses or the percent of animals that showed seizure responses in each group were analyzed and compared with the control group.

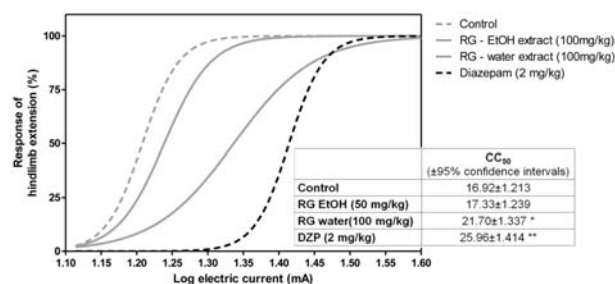
**Strychnine-induced seizure model** – Strychnine induces convulsions by antagonizing glycine.<sup>23</sup> As in the PTZ-induced seizure model, 60 mice were randomly assigned to 6 groups and were treated with RG water extract (50, 100, 200, or 400 mg/kg), diazepam, or distilled water. After administration of strychnine (1 mg/kg), animals were placed individually in observation cages and the presence (all, 100%) or absence (none, 0%) of seizures was assessed for 20 min. The percent of seizure responses was recorded and compared with that of the control group.

**Assay of  $\text{Cl}^-$  influx** – The intracellular  $\text{Cl}^-$  was measured based on the study by West and Molloy.<sup>24</sup> SH-SY5Y human neuroblastoma cells were used to monitor the changes in intracellular  $\text{Cl}^-$ , using MQAE as the  $\text{Cl}^-$  sensitive indicator. The cells were suspended in Hank's solution at a concentration of  $4 \times 10^5$  cells/ml and then incubated with MQAE (5 mM) overnight at room temperature to load the dye into the cells. The cells were washed with Hank's solution.

Fluorescence was monitored in a cuvette with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The fluorescence intensity ratio (i.e.,  $F_0/F$ ) was used in this experiment.  $F_0$  indicates the initial fluorescence value (i.e., at 0 s), and  $F$  is the fluorescence value at a given time. The  $F_0/F$  value is directly proportional to the concentration of  $\text{Cl}^-$ . The fluorescence values were corrected by subtracting background fluorescence that was measured with HEPES-buffered KSCN solution that included 5  $\mu\text{M}$  valinomycin as a quench.

**Locomotor activity test** – An open-field test was conducted to evaluate the sedative or locomotor-impairing effects of RG. Each mouse was placed in the center of a black, Plexiglass chamber ( $42 \times 42 \times 42$  cm) 60 min after administration of RG (50, 100, 200, and 400 mg/kg) or 30 min after administration of diazepam (2 mg/kg). Animals were given a 2-min habituation, and then a computerized system (Ethovision System, Noldus, the Netherlands) recorded the distance moved (cm) and movement durations (s) for 10 min.

**Rota-rod test** – A rota-rod test was used to assess the balance and motor coordination of animals after administration of RG water extract. The rota-rod (UgoBasile, Varese, Italy) was fixed at a speed of 36 rpm. All animals were trained for 3 min on the rotating rod, 24 h prior to the test. Animals were treated with distilled water, RG



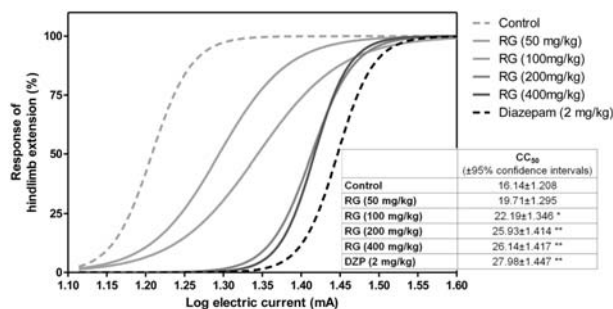
**Fig. 1.** Effects of *Rehmannia glutinosa* (RG) water extract and ethanol extract on seizures induced by electroshock in animals ( $n = 10$  per group). The numbers in the box represent  $\text{CC}_{50} \pm 95\%$  confidence intervals (\* $p < 0.05$  versus control; \*\* $p < 0.01$  versus control; DZP, diazepam).

extracts, or diazepam before the start of the experiment. Latency time (i.e., time of first fall in sec) and the frequency of falling were recorded for 10 min.

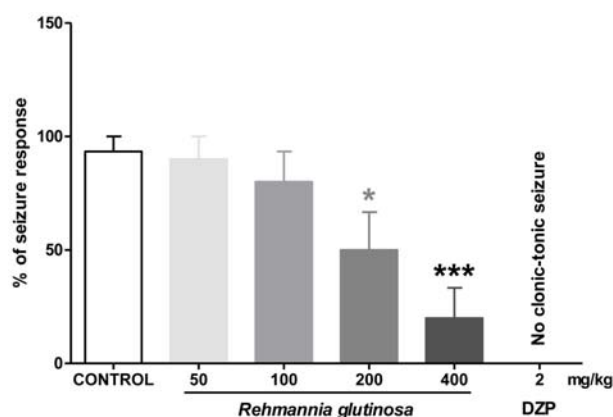
**Statistical analysis** – All data are expressed as mean  $\pm$  standard error of the mean (SEM). Data were analyzed with one-way analysis of variance (ANOVA). When significant variation between groups was found, the Dunnett's test was used as a post-hoc test. Analyses were performed using GraphPad Prism Version 5.02 software (CA, USA).  $p < 0.05$  was considered statistically significant.

## Result and Discussion

In the present study, we evaluated the anticonvulsive activity of RG extract against electroshock- and chemical-induced (i.e., PTZ- and strychnine-induced) seizures in mice. Electroshock-induced convulsion test is a widely used method to identify the potential anticonvulsive property of various substances in rodents.<sup>25</sup> Current antiepileptic drugs such as phenytoin, carbamazepine, and diazepam showed protective effects against electroshock-induced seizures.<sup>26</sup> In our preliminary experiment, we observed that the water extract of RG had greater protective effects (i.e., a greater increase in seizure threshold) against electroshock-induced seizures than the ethanol extract of RG did (Fig. 1). Based on this information, we proceeded to examine the effects of various dosages of RG water extract against electroshock-induced seizures. Our results showed that RG water extract, at dosages of 100, 200, and 400 mg/kg, significantly increased the seizure threshold of mice in this test (Fig. 2), confirming that RG water extract has anticonvulsive effects. As expected, diazepam also showed significant anticonvulsive effects against electroshock-induced seizures. The findings of the electroshock-induced seizure test were supported by the results of the PTZ-induced convulsion test. The PTZ-induced convulsion

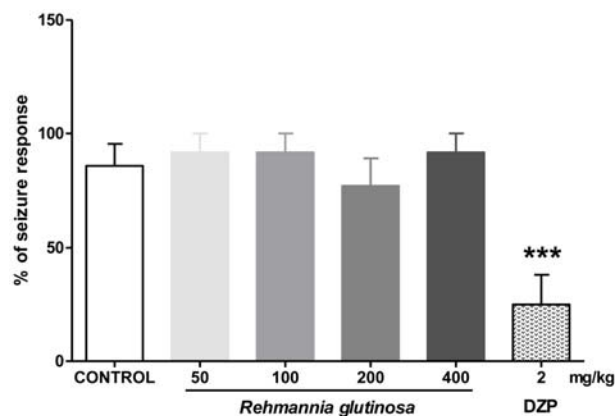


**Fig. 2.** Effects of RG water extract on seizures induced by electroshock in animals ( $n = 20$  per group). The numbers in the box represent  $CC_{50} \pm 95\%$  confidence intervals (\* $p < 0.05$  versus control; \*\* $p < 0.01$  versus control; DZP, diazepam).



**Fig. 3.** Effects of RG water extract on seizures induced by PTZ in animals ( $n = 15$  per group). Each bar represents mean  $\pm$  SEM of percent seizure response (\* $p < 0.05$  versus control; \*\*\* $p < 0.001$  versus control; DZP, diazepam).

test is also used to screen anticonvulsive activity of new drugs.<sup>25</sup> PTZ is a GABA neuron inhibitor.<sup>27</sup> GABA is the major inhibitory neurotransmitter in the mammalian brain. When GABA is antagonized, as in PTZ treatment, neurons become disinhibited, which may lead to excessive stimulation and ultimately to seizures. Treatment with RG water extract dose-dependently reduced the percentage of seizure responses induced by PTZ in mice (Fig. 3). One-way ANOVA showed significant differences in the percentage of seizure responses among the experimental groups [ $F(5, 64) = 16.72, p < 0.001$ ]. Diazepam completely inhibited the seizure response induced by PTZ. These results suggest that the anticonvulsive effect of RG is probably mediated through a GABA-related mechanism. However, the RG water extract was ineffective against strychnine-induced seizures in mice (Fig. 4). Strychnine induces seizures through competitive antagonism of the glycine receptor.<sup>23</sup> Glycine is another inhibitory neurotransmitter in the central nervous system.<sup>28</sup> Diazepam acts



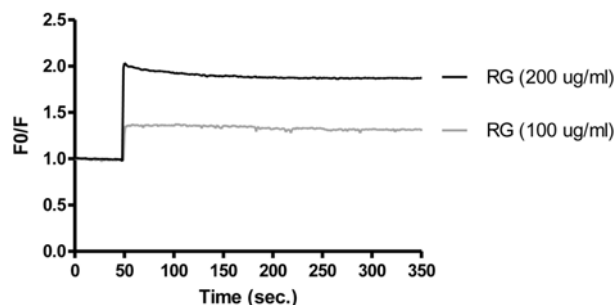
**Fig. 4.** Effects of RG water extract on seizures induced by strychnine in animals ( $n = 12 - 14$  per group). Each bar represents mean  $\pm$  SEM of percent seizure response (\*\*\* $p < 0.001$  versus control; DZP, diazepam).

through both GABA and glycine receptors,<sup>29</sup> thus it was observed to have protective effects against strychnine-induced seizure. Based on these findings, the results of the strychnine-induced seizure test demonstrate that the anticonvulsive effects of RG extract are not glycine receptor-mediated. Similar results (i.e., protective effects against electroshock- and PTZ-induced seizures but not strychnine-induced seizures) were observed in a study using Felbamate.<sup>26</sup> Felbamate is a currently used antiepileptic drug that is reported to have a dual mechanism of action—enhancement of GABAergic responses and inhibition of NMDA receptors.<sup>30</sup> Based on this observation, there is a possibility that, instead of a GABA/glycine mechanism, RG extract might act through a GABA/NMDA-related mechanism. This topic might be an interesting focus in future studies. Regardless, the present findings indicate that RG extract has anticonvulsive effects, and that these effects are probably GABA-mediated.

As aforementioned, substances that are effective against PTZ-induced seizures usually enhance GABAergic neurotransmission.<sup>26,27</sup> For instance, diazepam produces anticonvulsive effects by binding to GABA receptors.<sup>31</sup> This binding opens the ion channel in the receptor, allowing  $Cl^-$  ions to permeate into the postsynaptic neuron to consequently reduce the excitability or inhibit the activity of the neuron.<sup>26,32</sup> Considering these, we examined the effects of RG extract on  $Cl^-$  influx in human neuroblastoma cells, *in vitro*. The results show that RG water extract (100 and 200  $\mu g/ml$ ) increases intracellular  $Cl^-$  influx (Fig. 5). This result confirms the notion that the anticonvulsive effect of RG extract is mediated through GABAergic neurotransmission.

Another goal of the current study is to determine the

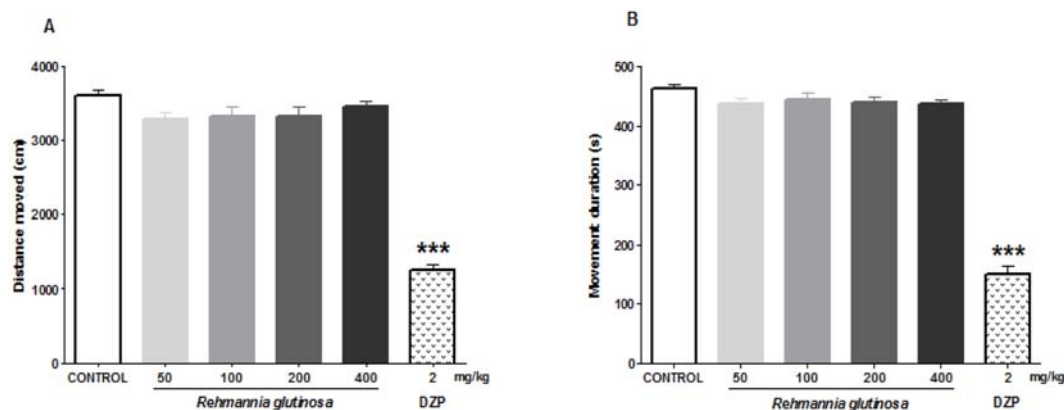
psychopharmacological effects or side effects of RG extract. The common side effects of anticonvulsive drugs are sedation, fatigue, and muscle relaxation.<sup>5</sup> To examine whether RG extract induces these effects in mice, we



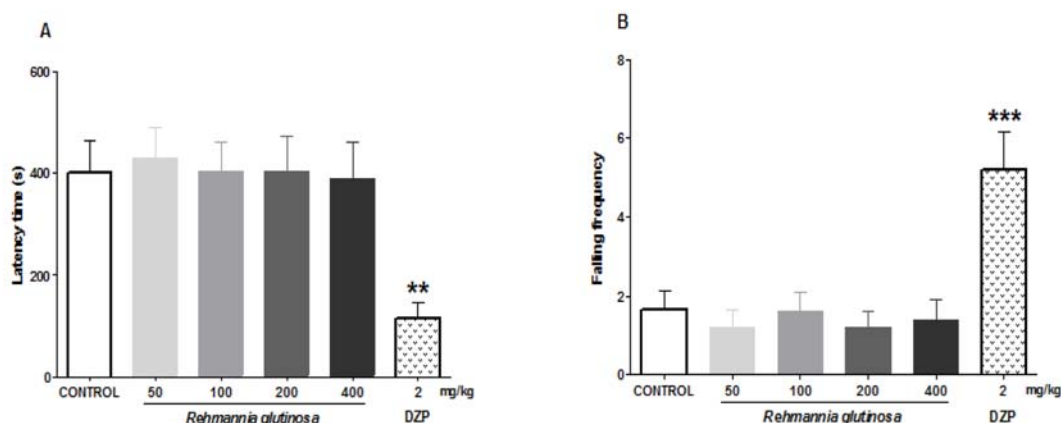
**Fig. 5.** Effects of RG water extract on  $\text{Cl}^-$  influx in neuroblastoma cells. Fluorescence was monitored with an excitation wavelength of 365 nm and an emission wavelength of 450 nm, using the  $\text{Cl}^-$ -sensitive indicator, N-(methoxyquinolyl) acetoethyl ester (MAQE). Contents of influx  $\text{Cl}^-$  ion were expressed as a peak (a.u.).

utilized the open-field (OFT) and rota-rod tests. The OFT, also called the locomotor activity test, is the most widely used method to examine the sedative effects of substances in rodents. Anticonvulsive drugs such as diazepam show sedative effects in this test.<sup>31</sup> In concordance with these studies, diazepam treatment significantly decreased the locomotor activity of mice in the present study (Fig. 6). On the other hand, RG water extract did not alter locomotor activity of the mice. This result indicates that RG extract does not have locomotor impairing and/or sedative effects. Similarly, the water extract of RG did not alter latency and falling frequency in the rota-rod test (Fig. 7). Drugs that have muscle relaxant effects or those that alter the balance and coordination of animals (e.g., diazepam) decrease the latency to first fall and increase falling frequency in this test. Collectively, the results indicate that RG water extract does not have sedative and muscle relaxant effects.

In summary, we have found that the water extract of



**Fig. 6.** Effects of RG water extract on locomotor activity in animals ( $n = 10$  per group). Each bar represents mean  $\pm$  SEM of total distance moved (A) and the movement duration (B) for 10 min (\*\* $p < 0.001$  versus control; DZP, diazepam).



**Fig. 7.** Effects of RG water extract on the rota-rod test in animals ( $n = 10$  per group). Each bar represents mean  $\pm$  SEM of the latency time (A) and falling frequency (B) for 10 min (\*\* $p < 0.01$  versus control; \*\*\* $p < 0.001$  versus control; DZP, diazepam).

RG has protective effects against electroshock- and PTZ-induced seizures. We also found that this extract increases  $\text{Cl}^-$  ion influx *in vitro*, indicating that its effect might be mediated through GABAergic neurotransmission. Furthermore, we have found that RG does not produce sedative or muscle relaxant effects. These findings suggest that RG water extract could be a potential anticonvulsive alternative drug with minimal side effects.

### Acknowledgments

This research was supported by the Bio-Synergy Research Project of the Ministry of Science, ICT and Future Planning through the National Research Foundation (NRF-2014M3A9C4066465).

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Received November 5, 2016

Revised January 13, 2017

Accepted January 13, 2017