

## Anxiolytic-like Effects of *Polygala tenuifolia* Willdenow Using the Elevated Plus Maze and Hole-board Apparatus in Mice

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**Abstract** – The purpose of this study was to characterize the putative anxiolytic-like effects of the aqueous extract of the root of *Polygala tenuifolia* (AEPT) using an elevated plus maze (EPM) and hole-board apparatus in mice. The AEPT was orally administered at 50, 100, 200 or 400 mg/kg to ICR mice, 1 h before the behavioral evaluation in the EPM, respectively. Control mice were treated with an equal volume of saline, and positive control mice with buspirone (2 mg/kg). Single treatments of the AEPT significantly increased the percentage of time spent and arm entries into the open arms of the EPM versus saline controls ( $P < 0.05$ ). Moreover, there were no changes in the locomotor activity and myorelaxant effects in any group compared with the saline controls. In the hole-board test, single treatments of the AEPT (200 and 400 mg/kg) significantly increased the number of head-dips versus saline controls ( $P < 0.05$ ). In addition, the anxiolytic-like effects of the AEPT were blocked by WAY 100635 (0.3 mg/kg, i.p), a 5-HT<sub>1A</sub> receptor antagonist not by flumazenil, a GABA<sub>A</sub> antagonist. These results indicate that *P. tenuifolia* is an effective anxiolytic agent, and suggest that the anxiolytic-like effects of *P. tenuifolia* is mediated via the serotonergic nervous system.

**Keywords** □ Anxiety, *Polygala tenuifolia*, elevated plus-maze, WAY 100635, flumazenil

### INTRODUCTION

Since the introduction of benzodiazepines in the 1960s, they have remained the most commonly prescribed treatment for anxiety. Although these compounds are the mainstay of drug treatment for anxiety disorders, they have many side-effects such as sedation, myorelaxation, ataxia, amnesia, and pharmacological dependence (Lader and Morton, 1991). Research has been conducted to identify safer, more specific, and perhaps lower cost therapies (Carlini, 2003). A number of studies have demonstrated that the newer anxiolytic agents, such as chlor-diazepoxide, diazepam, and buspirone, exert their actions through an interaction with multiple receptors within the CNS, such as GABAergic or serotonergic receptors.

Various traditional herbal remedies have also been suggested to possess activity at multiple sites within the CNS (Chung et

al., 1995). For example, the root of *Polygala tenuifolia* Willdenow (Polygalaceae), a traditional oriental medicine, is known to have sedative, antipsychotic, cognitive improving/neuroprotective, and anti-inflammatory effects on the central nervous system (Shin et al., 2004). Phytochemical studies revealed that *P. tenuifolia* contains polygalitol, tenuigenin, polygalasaponin, and xanthone derivatives (Ikeya et al., 1991). Sapogenins were firstly isolated from this plant in 1947 and some of the polygalasaponins showed considerable inhibitory activity against the action of cAMP (Nikaido et al., 1982). Recently, Kawashima et al. (2004) reported that 3,4,5-trimethoxycinnamic acid exhibits anti-stress actions by the suppression of norepinephrine content in locus coeruleus induced by corticotrophin-releasing hormone. Until now, however, there were no reports on the anxiolytic effects of *P. tenuifolia*.

The purpose of this study was to characterize the anxiolytic-like activity of the aqueous extract of *P. tenuifolia* (AEPT). Its anxiolytic effects were examined using the elevated plus-maze (EPM) and the hole-board apparatus in mice, respectively. And we examined the myorelaxant effects using a horizontal wire

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test in mice. In addition, this study also investigated which nervous systems are involved in the anxiolytic-like effects of the AEPT through the co-administration of AEPT and either flumazenil or WAY 100635.

## MATERIALS AND METHODS

### Animals

Male ICR mice, weighing 25-30 g, were purchased from the Orient Co., Ltd. of the Charles River branch (Seoul, Korea). The animals were housed 5 or 6 per cage, allowed access to water and food ad libitum, and maintained under a constant temperature ( $23 \pm 1^\circ\text{C}$ ) and humidity ( $60 \pm 10\%$ ) under a 12-h light/ dark cycle (light on 07.30 - 19.30 h). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea.

### Materials

Buspirone, WAY 100635, and flumazenil were purchased from the Sigma Chemical Co. (USA). The roots of *P. tenuifolia* were obtained from a herbal supplier in Seoul, Korea, and voucher specimens (KHUOPS-04-31) were deposited at the herbarium of the College of Pharmacy, Kyung Hee University (Seoul, Korea). The material was authenticated by Prof. C.S. Yook of the Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University. All the other materials were of the highest grade commercially available.

### Sample preparation

The *P. tenuifolia* extract was prepared by boiling in 10 volumes of water for 120 min. The aqueous solution obtained was filtered, concentrated in a water bath under vacuum, frozen and lyophilized (Eyela, model FDU-2000, Japan) to yield the aqueous extract (yield 15.3%), which was then stored at  $-20^\circ\text{C}$  until required. AEPT was freshly dissolved in saline and orally administered. Flumazenil were suspended with a 10 % aqueous solution of Tween-80 for the intraperitoneal injection. Buspirone and WAY 100635 were dissolved in saline.

### Spontaneous behavior in the open field test

Testing was carried out in clear black Plexiglas boxes ( $40 \times 40 \times 40$  cm) equipped with the video-based Ethovision System (Noldus, Wageningen, The Netherlands). The mice were placed in the center of the apparatus to evaluate horizontal locomotor

activity 1 h after being treated with AEPT (50, 100, 200, and 400 mg/kg) and video-recorded for 5 min. The horizontal locomotor activity is expressed in terms of the total ambulatory distance and the frequency of rearing.

### Elevated plus-maze test

The EPM for mice consisted of two perpendicular open arms ( $30 \times 7$  cm) and two enclosed arms ( $30 \times 7$  cm) with 20 cm high walls, extending from the central platform ( $7 \times 7$  cm). The open and closed arms were connected by a central square,  $7 \times 7$  cm, to give an apparatus of a plus sign appearance. The floor and walls of the maze were constructed from the dark opaque polyvinylplastic. The maze was raised to a height of 50 cm above the floor level in a dimly lit room (40 Lux) and a video camera was suspended above the maze to record the movements for analysis (Lister, 1987; Pellow and File, 1986). Each mouse was placed at the center of the platform, its head facing an open arm. The animals were tested individually and only once for 5 min. The maze was cleaned after each trial so as to remove any residue or odors. The following measurements were taken and analyzed using the video-based Ethovision System: the number of entries into the open or closed arms, the time spent in each arm, and the total distance moved in the EPM. All the experiments were carried out between 10:00 and 16:00 o'clock.

One hour after the AEPT treatment (50, 100, 200 and 400 mg/kg, p.o.), the mice were placed in the EPM. The mice in the control group were given the vehicle solvent only, and the animals were tested individually once only for 5 min. In a separate antagonism study, the mice were subjected to the co-administration of AEPT (400 mg/kg, p.o.) and either WAY 100635 (0.3 mg/kg, i.p.) or flumazenil (10 mg/kg, i.p.) 1 h and 30 min prior to testing, respectively. The mice were treated with buspirone (2 mg/kg, i.p.) 1h before EPM test and used as the positive controls.

### Hole-board test

The hole-board apparatus (Ugo Basile, Italy) consisted of gray Perspex panels ( $40 \times 40$  cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor. Photocells below the surface of the holes measured the number of head-dips. The board was positioned 15 cm above a table. The method was adapted from Takeda et al. (1998). Mice were transported to the dimly lit laboratory at least 1 h before testing. Each animal was individually placed singly in the center of the board facing away from the observer and its behavior was recorded for 5

min. The number of head-dips was recorded. Mice were orally administered AEPT (50, 100, 200 and 400 mg/kg, po) 1 h prior to the testing.

### Horizontal wire test

A horizontal wire test was carried out by treating the mice with AEPT (50, 100, 200 and 400 mg/kg, p.o.) according to a slight modification of the method reported by Bonetti *et al.* (1982). Briefly, the mice were lifted by the tail and allowed to grasp a horizontally strung wire (1 mm diameter, 15 cm long, and placed 20 cm above the table) with their forepaws, after which they were then released. The number of mice from each treatment group that did not grasp the wire with their forepaws or actively grasped the wire with at least one hind paw within a 10 sec period was recorded. A myorelaxant drug would impair the ability of the mice to grasp the wire, and muscle relaxation is commonly associated with sedation.

### Statistics

The values are expressed as means  $\pm$  S.E.M. The data was analyzed by a one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test for the multiple comparisons. Statistical significance was set at  $P < 0.05$ .

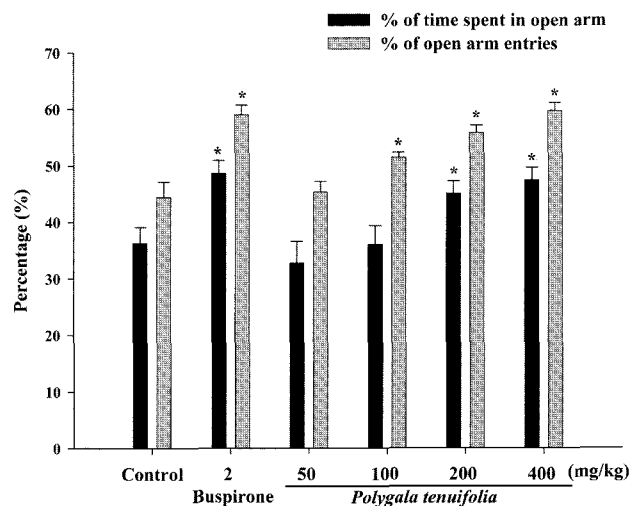
## RESULTS

### Effect of AEPT on the locomotor activity test

A locomotor activity test was performed to differentiate between the possible stimulatory effects of the tested drugs on the modulation of exploratory behavior. AEPT (50, 100, 200, and 400 mg/kg) caused no significant changes in either the total ambulatory distances or rearing frequencies compared with the saline control group (data not shown).

### Effect of AEPT in the EPM

The mice in the saline-treated group typically avoided spending time on or entering into the open arms. The percentage of time spent in the open arms was significantly increased in the AEPT-treated mice (200 and 400 mg/kg) compared with saline treated group (Fig. 1;  $P < 0.05$ ). In addition, there was also significantly increased in the percentage of open arm entries in the AEPT-treated mice (200 and 400 mg/kg) compared with saline treated group (Fig. 1;  $P < 0.05$ ). However, no significant change was observed in terms of percentage of time spent or the open arm entries at doses of 50 and 100 mg/kg of AEPT. The buspirone-treated (2 mg/kg) group, as a positive



**Fig. 1.** Effect of a single treatment of the aqueous extract of *Polygala tenuifolia* Willdenow. (*P. tenuifolia*) on the percentage of the time spent in and the number of entries into the open arms of the elevated plus-maze over a 5 min test period in the mice. Each bar represents mean  $\pm$  S.E.M. of 10-12 mice.  $P$  values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test ( $*P < 0.05$  as compared with the saline-treated control group).

control, the percentage of time spent and arm entries into the open arms were significantly increased compared with the saline-treated group ( $P < 0.05$ ).

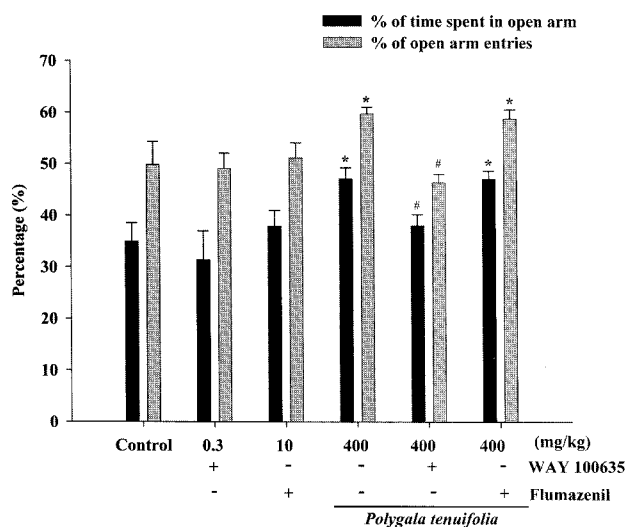
### Effect of WAY 100635 and flumazenil on the anxiolytic-like activity of AEPT

In order to determine if the anxiolytic-like effect of AEPT is exerted via the serotonergic or GABAergic nervous system, AEPT (400 mg/kg) treated mice were subjected to a co-treatment with either WAY 100635, a 5-HT<sub>1A</sub> receptor antagonist, or flumazenil, a GABA<sub>A</sub> receptor antagonist. As shown in Fig. 2, the anxiolytic-like effects of AEPT were abolished by WAY 100635 (0.3 mg/kg) but not by flumazenil (10 mg/kg). Also, there were no significant differences compared with control group in the WAY 100635-treated or flumazenil-treated group.

### Hole-board test

The effect of AEPT on the changes in head-dipping behavior in mice is shown in Fig. 3. AEPT-treated mice manifested significant increases in the number of head-dips at doses of 200 and 400 mg/kg. However, there were no significant increases at dose of 50 and 100 mg/kg of AEPT.

### Horizontal wire test

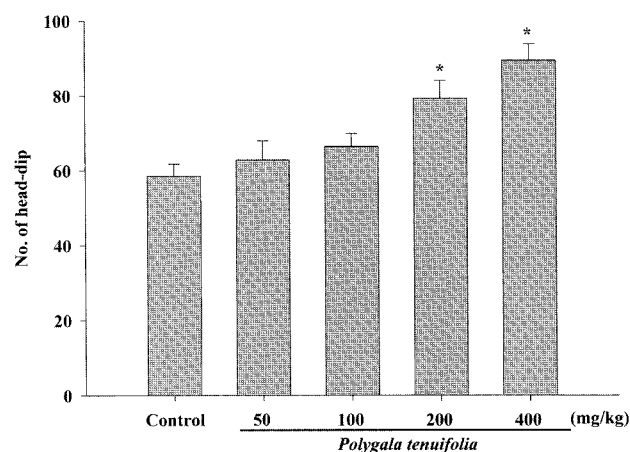


**Fig. 2.** Anxiolytic-like effects of *Polygala tenuifolia* Willdenow. (*P. tenuifolia*) were blocked by WAY 100635 but not by flumazenil. The data is expressed as the mean ( $\pm$  S.E.M.) of the percentage of the time spent in and the number of entries into the open arms of the elevated plus-maze, 1 h after the oral administration of AEPT (400 mg/kg), AEPT (400 mg/kg) + WAY 100635 (0.3 mg/kg) or flumazenil (10 mg/kg) (30 min prior testing, i.p.), or saline; N = 10-12 mice per group. *P* values for the group comparisons were obtained by one way ANOVA followed by Student Newman-Keuls test (\* $P$ <0.05 versus the saline-treated control, # $P$ <0.05 as compared with the AEPT-treated group).

At 5 mg/kg, diazepam significantly decreased the percentage of mice grasping the wire (Fig. 4). In contrast, AEPT (50, 100, 200 and 400 mg/kg) did not compromise the mice grasping the wire compared with saline control group, indicating a lack of myorelaxation at these doses.

## DISCUSSION

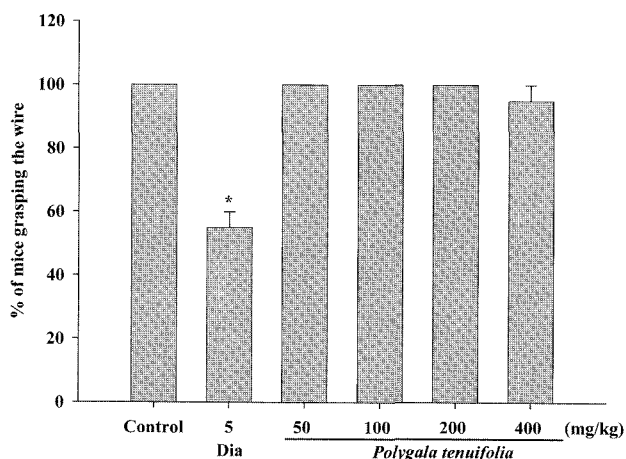
The dried root of *P. tenuifolia* is used in traditional Chinese, Japanese, and Korean medicine as a sedative, antipsychotic, cognitive improving/neuroprotective, and anti-inflammatory therapeutic agent (Shin et al., 2004). Chen et al. (2004) reported that the extract of *P. tenuifolia* contributes to the beneficial effect on memory and behavioral disorders produced by lesioning of the nucleus basalis magnocellularis in rats. In addition, it was reported that the components of *P. tenuifolia* could penetrate the blood-brain barrier and improve cognitive impairment through elevating cholinergic neurotransmission by blocking acetylcholine hydrolysis and could protect neurons against glutamate, amyloid  $\beta$  and C-terminal fragments, indicating that *P. tenuifolia* and its components might be effective in treatment



**Fig. 3.** Effects of *Polygala tenuifolia* Willdenow. (*P. tenuifolia*) on the changes in head-dipping behaviors in mice tested on the hole-board. Mice were administered AEPT (50, 100, 200 or 400 mg/kg, po). Each bar represents the mean  $\pm$  S.E.M. of 10-12 mice. *P* values for group comparisons were obtained by one way ANOVA followed by Student Newman-Keuls test (\* $P$ <0.05 versus the saline-treated control).

of Alzheimer's disease (Park et al., 2002). However, to our knowledge no study has investigated the anxiolytic effects of AEPT or determined which neuronal mechanism is primarily involved in.

An anxiolytic agent increases the frequency of entries into the open arms and increases the time spent in open arms of the EPM. The EPM is considered to be an etiologically valid animal model of anxiety because it uses natural stimuli, such as a fear of a new, brightly lit open space and a fear of balancing on a relatively narrow raised surface (Dawson and Tricklebank, 1995). In this study, the buspirone treatment prolonged the percentage of time spent in the open arms and the percentage of open arm entries (Fig. 1). The AEPT treatment also prolonged the percentage of time spent in the open arms as well as the percentage of open arm entries without altering the spontaneous behavior at the chosen dose regimen. The total distances of movement on the EPM were also unchanged by the AEPT treatment versus the saline controls (data not shown). In the hole-board test, the number of head-dips was dose-dependently increased by AEPT treatment (Fig. 3). In addition, the horizontal wire test, no significant myorelaxant effect was observed after administering the AEPT (Fig. 4). These observations suggest that the anxiolytic-like effect of AEPT is selective, and not the result of either a general stimulation of the locomotor activity or an exploratory behavior consequent to exposure to a novel environment.



**Fig. 4.** Performance of mice in the horizontal wire test 1h after oral administration of control, diazepam (Dia, 5 mg/kg), or AEPT. Data represent percentage of mice grasping the wire after administration of AEPT. Each bar represents the mean  $\pm$  S.E.M. of 10-12 mice. *P* values for group comparisons were obtained by one way ANOVA followed by Student Newman-Keuls test (\**P*<0.05 versus the saline- treated control).

5-HT<sub>1A</sub> receptors play important roles in the mediation of 5-HT neurotransmission in the CNS, and changes in their functional state are implicated in human anxiety. Indeed, local microinjection of 5-HT<sub>1A</sub> receptor agonists directly into the dorsal raphe nucleus, to stimulate somatodendritic 5-HT<sub>1A</sub> autoreceptors, reproduces anxiolytic effects (Schreiber and De Vry, 1993). Moreover, these phenomena can also be induced by systemic administration (Remy *et al.*, 1996). Therefore, 5-HT<sub>1A</sub> autoreceptors in the dorsal raphe nucleus are probably a key target whereby 5-HT<sub>1A</sub> receptor agonists exert their anxiolytic properties. In addition, postsynaptic 5-HT<sub>1A</sub> receptors located in limbic forebrain areas might also be involved, as some have reported a reduction in anxiety-driven behavior in rats that have been administered 5-HT<sub>1A</sub> receptor agonists directly into the hippocampus (Kataoka *et al.*, 1991). We observed that AEPT (400 mg/kg) treatment produced good anxiolytic-like activities by EPM and hole-board test. Furthermore, these anxiolytic-like behaviors were completely blocked by WAY 100635, a specific 5-HT<sub>1A</sub> receptor antagonist (Fig. 2). It is generally accepted that GABA<sub>A</sub> agonist also has anxiolytic properties. However, the anxiolytic-like effect of AEPT was not blocked by flumazenil, a GABA<sub>A</sub> antagonist. Thus, we concluded that the anxiolytic-like activity of AEPT was mediated via the activation of the 5-HT<sub>1A</sub> receptor and that AEPT could be useful as a selective 5-HT<sub>1A</sub> receptor agonist. However, we could not find which receptors, presynaptic or postsynaptic receptors, were involved in those activities.

Until now, it is not known which constituent of AEPT exerts this anxiolytic-like effect. This plant has been shown to contain polygalasaponin, tenuigenin, polygalitol, and xanthone derivatives. Previously, it was reported that polygalasaponin extracted from *P. tenuifolia* (Sakuma and Shoji, 1981) reduced apomorphine-induced climbing behavior, which is one of the most widely used to predict serotonin and dopamine antagonist properties *in vivo* (Chung *et al.*, 2002). In addition, 3,4,5-trimethoxycinnamic acid, a phenylpropanoid, isolated from *P. tenuifolia* exhibits anti-stress actions by the suppression of norepinephrine content in locus coeruleus induced by corticotrophin-releasing hormone (Kawashima *et al.*, 2004). Recently, we observed that GABAergic nervous system is involved in the anxiolytic-like effects of phenylpropanoid (Yoon *et al.*, unpublished data). Collectively, it is likely that the anxiolytic-like effects of AEPT may be exhibited by saponin derivatives not by phenylpropanoid compound. Further studies are required to clarify this issue.

In summary, the present results demonstrate that the *P. tenuifolia* has an anxiolytic-like effect, and suggest that this effect may be mediated the serotonergic nervous system. However, the nature of its underlying mode of action remains to be elucidated. Although the findings of herb effects may not in general provide clinically useful outcomes in patients or in normal humans, the findings of this study may be important because they confirm the validity of *P. tenuifolia* as a medicinal plant.

## ACKNOWLEDGMENTS

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