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Anxiolytic-Like Effects of *Chrysanthemum indicum* Aqueous Extract in Mice: Possible Involvement of $GABA_A$ Receptors and $5\text{-}HT_{1A}$ Receptors

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Abstract

Chrysanthemum indicum Linne is an ancient herbal medicine used to treat bone and muscle deterioration, ocular inflammation, headache, and anxiety in Korea, China, and Japan. Furthermore, tea derived from *Chrysanthemum indicum* Linne has been used to treat anxiety by facilitating relaxation and curing insomnia. However, no reports exist on the anxiolytic-like effects of *Chrysanthemum indicum* Linne water extract (CWE) in mice. In the present study, we investigated the anxiolytic-like effects of CWE using the elevated plus-maze (EPM) test in mice. CWE, at a dose of 500 mg/kg (p.o.), significantly increased the time spent in the open arms of the EPM compared to a vehicle-injected control group. Moreover, the effect of CWE (500 mg/kg) was blocked by bicuculline (a selective $GABA_A$ receptor antagonist) and WAY 100635 (a selective $5\text{-}HT_{1A}$ receptor antagonist). Taken together, these findings suggest that the anxiolytic-like effects of CWE might be mediated by the $GABA_A$ receptor and the $5\text{-}HT_{1A}$ receptor.

Key Words: *Chrysanthemum indicum* Linne, Anxiolytic-like effects, Elevated plus maze, $GABA_A$ receptor, $5\text{-}HT_{1A}$ receptor

INTRODUCTION

Anxiety disorder, one of the most common psychiatric disorders affecting all age groups (Singh and Singh, 2002), encompasses several different forms of a type of mental illness characterized by abnormally and pathologically excessive fear and anxiety (Shin and Liberzon, 2010). The National Institute of Mental Health reported that anxiety disorders are a serious medical illness that affect approximately 19 million adults in the USA (Cryan and Holmes, 2005). It is difficult to overstate the fact that the rapidly increasing prevalence of anxiety disorders creates a burden on health systems around the world. Furthermore, anxiety disorder, classified as a mood disorder, not only causes significant disruption in psychological well-being, but also increases the risk of cardiovascular morbidity and mortality (Albert *et al.*, 2005). For these reasons, the development of an anxiolytic agent is important.

Researchers have been searching for effective and safe agents with fewer side effects than existing anxiolytic agents, such as benzodiazepines, barbiturates, and antidepressants. Recently, many studies have focused on specific targets of brain neurotransmitter systems such as $GABA_A$ receptor and $5\text{-}HT_{1A}$ receptor (Bailey and Toth, 2004; Clènet *et al.*, 2005; Jiang *et al.*, 2009).

The GABAergic system, the primary inhibitory neurotransmitter system, is thought to play an important role in anxiety disorders. Several pharmacologic agents, primarily with benzodiazepine structures, have been used to target the GABAergic system (Lydiard, 2003). $GABA_A$ receptors (ionotropic) and $GABA_B$ receptors (metabotropic) are widely expressed in the central nervous system. The $GABA_A$ receptor is known to be more associated with the acute stress response, and anxiolytic agents used clinically, such as benzodiazepines, target this receptor (Rudolph and Möhler, 2006). $GABA_A$ receptor agonists have shown anxiolytic-like effects in animal models of anxiety disorder (Rodgers and Dalvi, 1997), while a $GABA_A$ receptor antagonist, bicuculline, increased anxiety in rats tested by the widely-used elevated plus maze (EPM) (Miller *et al.*, 2010).

The brain serotonergic system, one of the well-characterized neurotransmitter systems related to emotion, is involved in mediating various behaviors included appetite, insomnia, depression, and anxiety disorders. The serotonergic system acts through at least 14 distinct receptor subtypes, exhibiting

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various complex physiological effects. Although the roles of individual receptor subtypes are not completely understood, many researchers have focused on the 5-HT_{1A} receptor subtype in particular (Heisler *et al.*, 1998). Specifically, some studies suggest that a selective 5-HT_{1A} receptor antagonist, WAY 100635, has an anxiolytic effect on rodents in the EPM (Cheeta *et al.*, 2000) and several studies suggest that WAY 100635 reversed an anxiogenic-like effect in an animal model (Mello *et al.*, 2005).

Chrysanthemum indicum Linne, a perennial herb belonging to the Asteraceae family, is widely distributed in eastern Asia. Traditionally, *Chrysanthemum indicum* was used as a folk remedy to treat the deterioration of bone and muscle, ocular inflammation, and headache. Furthermore, tea of *Chrysanthemum indicum* Linne has been used to treat anxiety by facilitating relaxation and curing insomnia. Recently, some studies have suggested that *Chrysanthemum indicum* has anti-inflammatory effects and anti-apoptotic effects *in vitro* and *in vivo* (Chen *et al.*, 2008; Cheon *et al.*, 2009). However, the anxiolytic-like effect of *Chrysanthemum indicum* has not yet been reported.

The aim of this study was to investigate the effects of *Chrysanthemum indicum* on anxiety. For this purpose, we examined whether *Chrysanthemum indicum* Linne water extract (CWE) has anxiolytic-like effects in mice by using the EPM test. Furthermore, we studied the possible mechanisms by which CWE contributes to the anxiolytic-like effects in the EPM test.

MATERIALS AND METHODS

Animals

Male ICR mice (four-weeks-old, weighing 24-27 g) were purchased from DaeHan Biolink (Eumseong, Korea). Eight to ten animals were housed per cage. They were allowed access to water and food ad libitum, and maintained at constant temperature (23±1°C) and humidity (55±5%) under a 12 h light/dark cycle (lights on 07:00-19:00 h) for one week before the experiments began. Mice were divided randomly into groups. All experiments were conducted in accordance with the NIH Guidelines on the Care and Use of Laboratory Animals, and our protocol was approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University.

Drugs and chemicals

(+)-Bicuculline and WAY 100635 (Sigma, St. Louis, MO, USA) were used in the EPM test. Thirty minutes before the CWE oral administration, (+)-bicuculline (0.3 and 1 mg/kg), WAY 100635 (0.3 and 1 mg/kg), or saline was administered intraperitoneally. Behavioral experiments were performed 1 h after the CWE administration. We chose dose and time point of (+)-bicuculline and WAY 100635 injentions based on the previous study (Yu *et al.*, 2007).

CWE preparation and drug administration

Chrysanthemum indicum Linne flowers were purchased at Kyungdong market, Seoul, Korea. The dried, powdered flowers (300 g) of *Chrysanthemum indicum* were extracted three times (each time for 3 h followed by ultrasonic extraction) after adding 4 L of distilled water. Extract solutions were concentrated using a vacuum concentrator (EYELA, Tokyo, Japan). The yield of the extract was 43 g (w/w). The aqueous extract of *Chrysanthemum indicum* was freshly dissolved in distilled water. (+)-Bicuculline and WAY 100635 were dissolved in a physiological 0.9% saline solution. The vehicle control group was treated with distilled water or saline. All samples were freshly prepared before the test and administered in a volume of 0.1 ml/10 g of body weight per mouse.

Quantitative HPLC determination of chlorogenic acid

Samples were analyzed using the Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA), equipped with a quaternary solvent delivery system, an autosampler, and a DAD detector. Separations were carried out on a J'sphere ODS-H80 column (250×4.6 mm, 4 μm, YMC Co., Ltd. Japan). Chlorogenic acid was detected at 40°C and 310 nm in isocratic elution mode using methanol (A) and 0.1% phosphoric acid in water (B). The elution profile was as follows: 0-20 min., 20% A in 80% B. Chlorogenic acid was prepared at 1 mg/ml and 10 μl was injected as an external standard. The CWE quantification assay was performed in triplicate.

Elevated plus maze test (EPM)

The EPM test was performed according to the method described by Lister, with modifications (Lister, 1987). The standard plus maze consisted of two open arms (30×5 cm) and two closed arms with a wall (30×5×5 cm) connected to a central zone (5×5 cm) to form a cross. It was elevated to a height of 50 cm above the floor. A video camera was suspended above the maze to record the experiment. The maze floor and walls were constructed from opaque polyvinyl plastic, and the open arms had a low (0.5 cm) edge. The mouse was placed on the central zone facing an open arm. The maze floor was cleaned thoroughly between trials using 10% ethanol. The time spent in the open arm and number of open arm entries with four paws was recorded for a 5-min period using the video-based Ethovision 3.1 system. This test was performed under light (20 ± 5 lux) to encourage closed-arm entries. The parameters were calculated using the following formula: percentage of time spent in the open arm (%) = (time spent in the open arms/sum of time spent in each arm)×100; percentage of open arm entries (%) = (number of entries in the open arms/total number of entries)×100.

Statistical analysis

All data are expressed as the mean ± SEM and were analyzed with Prism 5.0 software (Graphpad Software, Inc., San Diego, CA, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test in order to detect inter-group differences. A p-value<0.05 was considered statistically significant.

RESULTS

Determination of the chlorogenic acid content in CWE

Chlorogenic acid, the functional component of CWE, was analyzed by HPLC. HPLC analysis of the standard substances showed that the retention time of chlorogenic acid was 11.44 min (Fig. 1). The chlorogenic acid content in CWE was determined from the liner regression equation of the calibration graph and was found to be 0.035%.

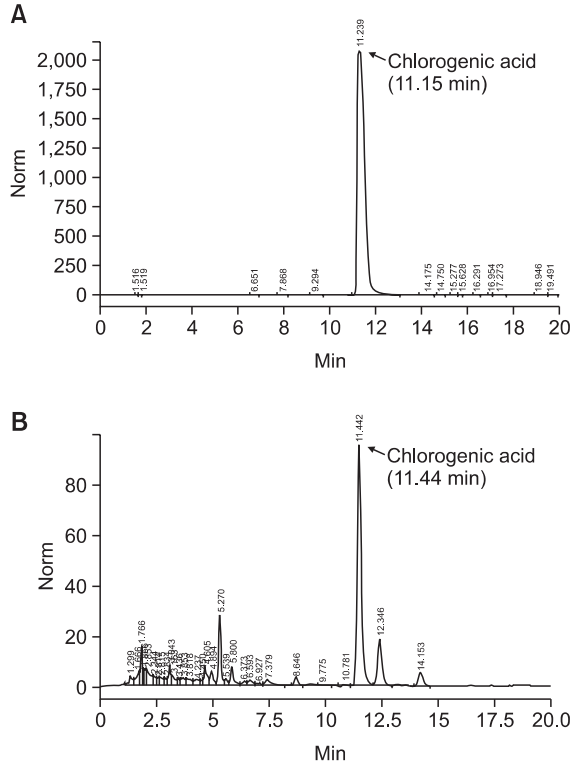

Fig. 1. Representative HPLC chromatogram of (A) chlorogenic acid standard and (B) aqueous extract of Chrysanthemum indicum (CWE). The retention times of chlorogenic acid in the CWE fraction and the chlorogenic acid standard were found to be 11.44 and 11.15 min, respectively. The chlorogenic acid content in the CWE was determined from the linear regression equation extracted from the calibration graph for this compound. Our CWE was determined to be 0.035% chlorogenic acid.

Effect of CWE in the elevated plus maze test

The control group mice typically avoided spending time in the open arms because of the height above the floor and the light. Fig. 2 shows that acute treatment with CWE (500 mg/kg, p.o.) markedly increased the time spent in the open arms compared with the vehicle group ($F_{(3,21)}$=3.268, p<0.05; p<0.05). The times spent in the open arms of the mice treated with vehicle, and 125 mg/kg, 250 mg/kg, and 500 mg/kg CWE were 25.42 ± 5.6, 51.54 ± 8.5, 53.13 ± 9.1, and 74.79 ± 17.7 sec, respectively. Based on the dose-activity data from 3 doses (low, medium, and high) of CWE, we have chosen a most effective dose which is 500 mg/kg to investigate antagonism study.

Effect of GABA receptor antagonism on the anxiolytic profile of CWE

As shown in Fig. 3, the administration of 500 mg/kg CWE significantly increased the time mice spent in the open arms compared with the vehicle group (p<0.05). This effect of CWE was blocked by pretreatment with the $GABA_A$ receptor antagonist, bicuculline, at 0.3 mg/kg and 1 mg/kg ($F_{(3,41)}$=4.051, p<0.05; p<0.05 and p<0.01, respectively). The actual times spent in the open arms of the mice group treated with vehicle, 500 mg/kg CWE, and 0.3 mg/kg or 1 mg/kg bicuculline were 39.37 ± 4.9, 69.81 ± 8.1, 46.73 ± 9.0, and 30.34 ± 8.0 sec, respectively.

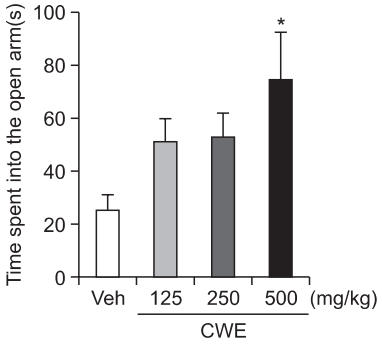

Fig. 2. Effects of single treatment with CWE in the EPM test. Time spent in the open arms was measured for 5 min. Significant differences were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*p<0.05 compared with the vehicle group). Data are the mean ± SEM (N=6).

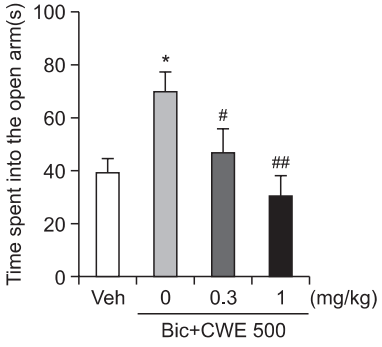

Fig. 3. Anxiolytic-like effects of CWE were blocked by bicuculline. CWE was administered orally at 500 mg/kg per mouse. Bicuculline (0.3 and 1 mg/kg) or vehicle was administered 30 minutes before the oral administration of CWE; N=9-13 mice per group. Data are expressed as the mean ± SEM of the time spent in the open arms over the 5-min test period. Significant differences were identified by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*p<0.05 compared with the vehicle group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 compared with the group administered 500 mg/kg CWE).

Effect of 5-HT_{1A} receptor antagonism on the anxiolytic profile of CWE

The mice treated with CWE (500 mg/kg) were concurrently administered WAY 100635, a 5-HT_{1A} receptor antagonist, in order to determine if the anxiolytic effects of CWE may involve the serotonergic neurotransmitter system, particularly the 5-HT_{1A} receptors. As shown in Fig. 4, CWE (500 mg/kg) significantly increased the time spent in the open arms ($F_{(3,40)}$=4.148, p<0.05; p<0.05), and WAY 100635 (0.3 and 1 mg/kg) significantly blocked the anxiolytic-like effects of 500 mg/kg CWE (p<0.05). The actual times spent in the open arms for groups treated with vehicle, 500 mg/kg CWE, and 0.3 mg/kg or 1 mg/kg WAY 100635 were 39.68 ± 5.9, 79.43 ± 14.4, 36.44 ± 8.1, and 42.98 ± 9.4 sec, respectively.

DISCUSSION

The findings presented here support the idea that CWE has an anxiolytic-like effect through the GABAergic and se-

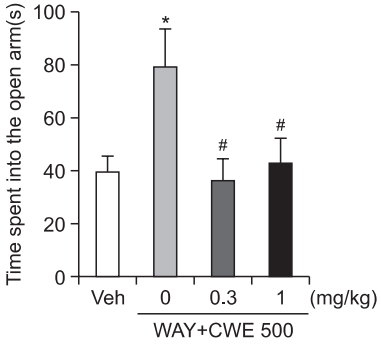

Fig. 4. The anxiolytic-like effects of CWE were blocked by WAY 100635. CWE was administered orally at 500 mg/kg per mouse. Thirty minutes after the last oral administration, WAY 100635 (0.3 and 1 mg/kg) or vehicle was administered intraperitoneally; N=10-12 mice per group. Data are expressed as the mean ± SEM of the time spent in the open arms of the elevated plus maze. P-values for group comparisons were obtained using one-way ANOVA followed by the Newman–Keuls multiple comparison test (*p<0.05 compared with the vehicle group; #p<0.05 compared with the 500 mg/kg CWE-treated group).

rotonergic systems. To investigate the anxiolytic-like effects of CWE in mice and to elucidate possible mechanisms, we used the EPM test with a $GABA_A$ receptor antagonist and a 5-HT_{1A} receptor antagonist. In our study, CWE-administered mice showed a dose-dependent increase in the time spent in the open arm of the maze, an effect that was markedly blocked by a $GABA_A$ receptor antagonist and by a 5-HT_{1A} receptor antagonist.

Anxiety models are grouped into two types involving either conditioned (potentiated startle) or unconditioned (social interaction and light/dark exploration tests) anxiety, and the EPM test is an excellent way to study unconditioned and spontaneous behavior (Rodgers and Dalvi, 1997). The EPM test, which may be the most popular model for measuring anxiety in animals, is based on the strong conflict between rodents' proclivity toward dark, enclosed alleys (approach) and an unconditioned fear of brightly lit areas, heights or open spaces (avoidance) (Walf and Frye, 2007). In the EPM test of mice treated with CWE alone, CWE increased the time spent in the open arms in a dose-dependent manner. This finding suggests that CWE may alleviate unconditioned anxiety.

The $GABA_A$ receptor is known to be a mediator of unconditioned anxiety. $GABA_A$ receptors contain several subunits (α1-6, β1-3, γ1-3, δ, ε, π, ρ1-3, θ) encoded by 19 different genes (Luscher *et al.*, 2011). Specifically, benzodiazepines, a well-known class of anxiolytic agents that bind to only α2-containing $GABA_A$ receptors, reduce unconditioned anxiety in mice whereas benzodiazepines that bind to both α1- and α2-containing $GABA_A$ receptors are involved in conditioned fear (Smith *et al.*, 2012). Our findings show that the CWE-induced anxiolytic effect is inhibited by bicuculline, a competitive $GABA_A$ receptor antagonist, in an unconditioned anxiety model, which suggests that the anxiolytic-like effect of CWE may involve $GABA_A$ receptors that only contain a2 subunits.

The 5-HT_{1A} receptor is also known as a modulator of unconditioned anxiety. Several studies have reported that 5-HT_{1A} receptor antagonists have an anxiolytic effect in anxiety models (Rodgers and Cole, 1994; Griebel *et al.*, 1999; Starr *et al.*, 2007). In contrast, 5-HT_{1A} receptor antagonists also have an anxiogenic effect in rodents undergoing the EPM test (Collinson and Dawson, 1997; Peng *et al.*, 2004). This difference seems to be caused by the location of the 5-HT_{1A} receptor in the central nervous system. Peng and colleagues (2004) demonstrated that an agonist of postsynaptic 5-HT_{1A} receptors has anxiogenic effects, as does WAY 100635, an antagonist of presynaptic 5-HT_{1A} receptors or autoreceptors. These findings are consistent with our observation that WAY 100635 inhibited the anxiolytic-like effect of CWE, suggesting that CWE may exert anxiolytic-like effects through the activation of presynaptic 5-HT_{1A} receptors. Unlike the Fig. 3, however, Fig. 4 did not showed dose dependent effects. A possibility of the reason why WAY 100635 did not reduced CWE effects dose- dependently is that 0.3 and 1 mg/kg of WAY 100635 have maximum effect. Reasons for WAY 100635 effects are needed further study.

However, the specific substance that causes the anxiolytic-like effects of CWE has not yet been identified. For this reason, we investigated the contents of CWE using quantitative HPLC analysis with chlorogenic acid as a standard compound. We found that CWE contains 0.035% chlorogenic acid, a functional ingredient. Several studies reported that chlorogenic acid has anticarcinogenic activity and anti-oxidative activity (Tanaka *et al.*, 1990; Kwon *et al.*, 2010). Furthermore, a recent study reported that chlorogenic acid has anxiolytic effects on anxiety-related behaviors (Bouayed *et al.*, 2007). Our observation suggests that chlorogenic acid as well as other polyphenol components contained in CWE may be responsible for the anxiolytic-like effects of CWE.

In summary, our findings show that a single treatment with CWE confers anxiolytic effects in the mouse EPM test. These effects are blocked by both bicuculline and WAY 100635. Taken together, our results suggest that CWE has anxiolytic-like effects mediated by regulation of the $GABA_A$ and 5-HT_{1A} receptors.

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