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<sub>A</sub> receptor and 5-HT<sub>1A</sub> 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<sub>A</sub> receptors (ionotropic) and GABA<sub>B</sub> receptors (metabotropic) are widely expressed in the central nervous system. The GABA<sub>A</sub> 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<sub>A</sub> receptor agonists have shown anxiolytic-like effects in animal models of anxiety disorder (Rodgers and Dalvi, 1997), while a GABA<sub>A</sub> 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

**Key Words:** *Chrysanthemum indicum* Linne, Anxiolytic-like effects, Elevated plus maze, GABA<sub>A</sub> receptor, 5-HT<sub>1A</sub> 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<sub>A</sub> receptor and 5-HT<sub>1A</sub> 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<sub>A</sub> receptors (ionotropic) and GABA<sub>B</sub> receptors (metabotropic) are widely expressed in the central nervous system. The GABA<sub>A</sub> 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<sub>A</sub> receptor agonists have shown anxiolytic-like effects in animal models of anxiety disorder (Rodgers and Dalvi, 1997), while a GABA<sub>A</sub> 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
various complex physiological effects. Although the roles of individual receptor subtypes are not completely understood, many researchers have focused on the 5-HT<sub>1A</sub> receptor subtype in particular (Heisler <i>et al.</i>, 1998). Specifically, some studies suggest that a selective 5-HT<sub>1A</sub> receptor antagonist, WAY 100635, has an anxiolytic effect on rodents in the EPM (Cheeta <i>et al.</i>, 2000) and several studies suggest that WAY 100635 reversed an anxiogenic-like effect in an animal model (Mello <i>et al.</i>, 2005).

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

The aim of this study was to investigate the effects of <i>Chrysanthemum indicum</i> on anxiety. For this purpose, we examined whether <i>Chrysanthemum indicum</i> 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 injections based on the previous study (Yu <i>et al.</i>, 2007).

**CWE preparation and drug administration**

<i>Chrysanthemum indicum</i> Linne flowers were purchased at Kyungdong market, Seoul, Korea. The dried, powdered flowers (300 g) of <i>Chrysanthemum indicum</i> 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 <i>Chrysanthemum indicum</i> 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 404 nm 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 linear regression equation of the calibration graph and was found to be 0.035%.