ABSTRACT: In the present study, the antinociceptive profiles of *Viola tricolor* L. (*V*. *tricolor* L.) extract were examined in ICR mice. *V*. *tricolor* L. extract administered orally (200 mg/kg) showed an antinociceptive effect as measured by the tail-flick and hot-plate tests. In addition, *V*. *tricolor* L. extract attenuated the writhing numbers in the acetic acid-induced writhing test. Furthermore, the cumulative nociceptive response time for intrathecal (i.t.) injection of substance P (0.7 μg) was diminished by *V*. *tricolor* L. extract. Intraperitoneal (i.p.) pretreatment with yohimbine (α2-adrenergic receptor antagonist) attenuated antinociceptive effect induced by *V*. *tricolor* L. extract in the writhing test. However, naloxone (opioid receptor antagonist) or methysergide (5-HT serotonergic receptor antagonist) did not affect antinociception induced by *V*. *tricolor* L. extract in the writhing test. Our results suggest that *V*. *tricolor* L. extract shows an antinociceptive property in various pain models. Furthermore, this antinociceptive effect of *V*. *tricolor* L. extract may be mediated by α2-adrenergic receptor, but not opioidergic and serotonergic receptors.

*Key Words:* *Viola tricolor* L., Anti-Nociception, Inflammatory Pain, α2-adrenoceptor

INTRODUCTION

Heartsease, also known as wild *Viola tricolor* L. (pansy, Violaceae), has a long history in phytotherapy. The therapeutic activity of *V*. *tricolor* L. has been identified in treating various skin conditions, such as eczema, seborrhea, impetigo, acne, catarrh of the respiratory tract, and whooping cough. It is also helpful in cases of cradlecap in babies. The herb is employed in treating frequent and painful urination in conditions such as cystitis. Due to the high concentration of rutin in the herb, it may be employed to prevent bruising and broken capillaries, to check the build up of fluid in the tissues and to help to reduce blood pressure (Baloyte *et al*., 1973; Bisset and Wichtl, 2001). It can gently alter the functioning of nerves, and the immune system. It is helpful in cases of nightmares, insomnia, and distressed sleep with frequent night awakenings. The herb of wild pansy may be very successfully used after surgery to prevent reoccurring tumors (McGuflin *et al*., 1997).

However, the effect of this herb on pain is unclear. Therefore, in this study, we attempted to characterize antinociceptive profiles and mechanisms of *V*. *tricolor* L. extract in various pain models.

MATERIALS AND METHODS

These experiments were approved by the University of Hallym Animal Care and Use Committee (Registration Number: Hallym 2009-05-01). All procedures were conducted in accordance with the ‘Guide for Care and Use of Laboratory Animals’ published by the National Institutes of
Health and the ethical guidelines of the International Association for the Study of Pain.

1. Experimental animals
   Male ICR mice (MJ Co., Seoul, Korea) weighing 20-25 g were used for all the experiments. Animals were housed 5 per cage in a room maintained at 22±0.5 ℃ with an alternating 12 hr light-dark cycle. Food and water were available ad libitum. The animals were allowed to adapt to the laboratory for at least 2 hr before testing and were only used once. Experiments were performed during the light phase of the cycle (10:00-17:00).

2. Oral administration, and intraperitoneal (i.p.) and intrathecal (i.t.) injections
   Oral administration was performed with gauge in a volume of 500 μl/25 g body weight. I.p. injection was conducted to unanesthetized mice with volume of 2.5 μl. The i.t. administration was performed following the method of Hylden and Wilcox (Hylden and Wilcox, 1980; 1981) using a 30-gauge needle connected to a 25 μl Hamilton syringe with polyethylene tubing. The i.t. injection volume was 5 μl and the injection site was verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of the injected dye in the spinal cord. The dye injected i.t. was distributed both rostrally and caudally but with short distance (about 0.5 cm from the injection site) and no dye was found visually in the brain. The success rate for the injections was consistently found to be over 95%, before the experiments were done.

3. Assessment of antinociception and experimental protocols
   All assessments for measuring antinociceptive properties of V. tricolor L. extract were carried out by blinded observers.

4. Tail-flick and hot-plate tests
   Antinociception was determined by the tail-flick (D’Amour and Smith, 1941) and the hot-plate paw-flicking tests (Eddy and Leimbach, 1953). For the measurement of the tail-flick latency, mice were gently held with one hand with the tail positioned in the apparatus (EMDIE Instrument Co., Maidens, VA, USA, Model TF6) and the tail-flick response was elicited by applying radiant heat to the dorsal surface of the tail. The intensity of radiant heat was adjusted so that the animal flicked its tail within 3 to 5 sec. For the hot-plate test, mice were individually placed on the 55 ℃ hot-plate apparatus (Itec Life Science, Woodland Hills, CA, USA, Model 39 Hot Plate) and then, the reaction time starting from the placement of the mouse on the hotplate to the time of licking the front paw was measured. Basal latency for the hot-plate test was approximately 9 sec. Animals were pretreated orally once with vehicle (control) or V. tricolor L. extract at 200 mg/kg doses 30 min prior to performing the tail-flick or hot-plate tests.

5. Acetic acid-induced writhing test
   For the writhing test (Koster et al., 1959), 1% acetic acid was injection i.p. and then, the animals were immediately placed in an acrylic observation chamber (20 cm high, 20 cm diameter). The number of writhes was counted during 30 min after the injection of acetic acid. A writhes was defined as a contraction of the abdominal muscles accompanied by an extension of the forelimbs and elongation of the body. Animals were pretreated orally once with vehicle (control) or V. tricolor L. extract at 200 mg/kg doses 30 min prior to performing the acetic acid-induced writhing test.

6. Substance P-induced nociceptive behavioral test
   Vehicle (control) or 200 mg/kg of V. tricolor L. extract was pretreated orally 30 min prior to performing i.t. injection of substance P (0.7 μg/5 μl). Immediately after i.t. injection with substance P, the mice were placed in an observation chamber (20 cm high, 20 cm diameter) and their nociceptive behavioral responses were recorded during 30 min. The cumulative response time of licking, stretching and biting episodes directed toward the lumbar and caudal region of spinal cord were measured with a stop-watch timer (Hylden and Wilcox, 1981).

7. Pretreatment of antagonists
   At first, mice were pretreated i.p. with either saline, naloxone (5 mg/kg), methysergide (5 mg/kg), or yohimbine (5 mg/kg), 10 min before oral administration of vehicle as a control or a fixed dose of V. tricolor L. extract (200 mg/kg). And then, the writhing response was tested 30 min after the treatment with either vehicle or V. tricolor L. extract (Choi et al., 2003; Park et al., 2009; Suh et al., 1996; 1996; 1999).

8. Drugs
   Acetic acid, substance P, naloxone, methysergide and
9. Statistical analysis

Data were presented as the mean±SEM. The statistical significance of differences between groups was assessed with one-way ANOVA with Bonferroni’s post-hoc test using GraphPad Prism version 4.0 for Windows Vista (GraphPad Software, San Diego, CA, USA); P<0.05 was considered significant.

Results

1. Effect of Viola tricolor L. extract on the tail-flick and hot-plate paw-licking responses

As revealed in Fig. 1a and b, oral treatment of V. tricolor L. extract at the dose of 200 mg/kg led to 62.5% and 46.6% increased latencies of the tail-flick and hot-plate paw-licking responses compared to the control group of mice. The sedative effect was manifested, when the mice were treated with V. tricolor L. extract orally at the dose of 200 mg/kg. However, there were no paralysis and motor changes.

2. Effect of Viola tricolor L. extract on the nociceptive behavior induced by acetic acid and substance P

V. tricolor L. extract attenuated the acetic acid-induced writhing numbers (Fig. 2a). Treatment with V. tricolor L. extract at the dose of 200 mg/kg led to 54% decrease in the acetic acid-induced writhing response compared to the control group of mice. In vehicle-treated control mice, i.t. injection of substance P (0.7 μg) caused acute, immediate behavioral responses, i.e., licking, scratching and biting the lumbar or caudal region, which lasted about 30 min. As shown in Figs. 2b, cumulative nociceptive response times for i.t. administration of substance P was significantly diminished by 61 %.

3. Effect of opioidergic, serotonergic and adrenergic system on the inhibition of writhing response induced by Viola tricolor L. extract

We examined the possible involvement of opioidergic, serotonergic and adrenergic system in the V. tricolor L. extract-induced antinociception. The pretreatment with naloxone (opioid receptor antagonist, Fig. 3a) or methysergide (serotonin receptor antagonist, Fig. 3b) did not affect V. tricolor L. extract-induced antinociception. However, the

yohimbine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). V. tricolor L. (300 g) was dissolved in 80% ethanol (1,500 ml) and extracted as refluxing for 3 hours, and then the extract was filtered for obtaining A. This process was repeated again once to obtain B from residue. A and B were mixed. This mixture was decompressed and dried for using as V. tricolor L. extract. V. tricolor L. extract, naloxone, methysergide and yohimbine were dissolved in saline. All drugs were prepared just before use.
blockade of $\alpha_2$-adrenergic receptor with systemic pre-administration of yohimbine abolished the *V. tricolor* L. extract-induced inhibition of the writhing response (Fig. 3c). The treatment of naloxone, methysergide or yohimbine itself did not affect the writhing response (Fig. 3).

**Discussion**

In the present study, we found that *V. tricolor* L. extract...
administered orally produces antinociception in various pain models. The tail-flick response is believed to be a spinally mediated reflex and the paw-licking hot-plate response is a more complex supraspinally organized behavior (Chapman et al., 1985). Moreover, Grumbach (1966) has shown that the effectiveness of analgesic agents in the tail-flick pain model is highly correlated with relief of human pain. Our results demonstrate that V. tricolor L. extract causes to prolong the tail-flick and hot-plate response latencies, indicating the increase of nociceptive threshold.

We also examined the effect of V. tricolor L. extract on the acetic acid-induced writhing test. I.p. injection of acetic acid can produce the peritoneal inflammation (acute peritonitis), which cause a response characterized by contraction of the abdominal muscles accompanying an extension of the forelimbs and elongation of the body. This writhing response is considered as a visceral inflammatory pain model (Koster et al., 1959; Vyklicky, 1979). In the present study, we clearly showed the antinociceptive effect of V. tricolor L. extract in an acetic acid-induced writhing test. Furthermore, it has been reported that i.t. injection of substance P in mice can also elicit nociceptive responses, consisting of biting, scratching and licking the caudal parts of the body (Hylden and Wilcox, 1981; Cumberbatch et al., 1994). We found in the present study that V. tricolor L. extract was also effective in attenuating substance P-induced nociceptive responses. These results suggest furthermore that V. tricolor L. extract may exert their antinociceptive effect via the central sites, possibly spinally mediated mechanisms.

The roles of opioid, serotonergic and adrenergic receptors in the regulation of modulation of nociceptive processing have been demonstrated in many previous studies. For example, it is well known that opioid receptors are involved in the antinociception (Schnauss and Yaksh, 1984; Yaksh, 1979; 1984). Also, it has been reported that α2-adrenergic receptors by spinal injection of methysergide or yohimbine antagonize the antinociception induced by morphine administered supraspinally (Yaksh, 1979; Jensen and Yaksh, 1984; Wigdor and Wilcox, 1987). We observed in the present study that α2-adrenergic receptor, but not opioidergic and serotonergic receptors, appear to be involved in orally administered V. tricolor L. extract-induced antinociception.

In conclusion, our results suggest that V. tricolor L. extract shows an antinociceptive property in various pain models. Furthermore, this antinociceptive effect of V. tricolor L. extract may be mediated by α2-adrenergic receptor, but not opioidergic and serotonergic receptors.

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