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Involvement of 5-HT_{1A} and $GABA_A$ receptors in the anxiolytic-like effects of *Cinnamomum cassia* in mice

Hyun-Sook Yu, Seok-Yong Lee, Choon-Gon Jang *

Department of Pharmacology, College of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea

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Abstract

An elevated plus maze (EPM) test was used to determine if the 5-HT_{1A}, $GABA_A$, and benzodiazepine receptors play a role in the anxiolytic-like effects of a 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) in mice. A single treatment with *C. cassia* (750 mg/kg, p.o.) significantly increased the number of entries into and the time spent in the open arms of the EPM compared with the controls. A repeated treatment with *C. cassia* (100 mg/kg, 5 days, p.o.) significantly increased the time spent in the open arms of the EPM. Moreover, WAY 100635, (+)-bicuculline, and flumazenil blocked the effect of *C. cassia*. However, there were no changes in the locomotor activity and horizontal wire test observed in any group compared with the controls. Taken together, these results show that *C. cassia* has no adverse effects, such as myorelaxant effects, and might be an effective anxiolytic agent by regulating the serotonergic and GABAergic system.

Keywords: Elevated plus maze; Anxiolytic-like effect; *Cinnamomum cassia*; 5-HT_{1A} receptor; $GABA_A$ receptor; Benzodiazepine receptor

1. Introduction

Anxiety disorders are common mental diseases of the central nervous system and present proliferating health problems worldwide. Anxiety disorders present in a number of forms, although probably all share a number of common neurological circuits. While certain psychological treatments are of proven efficacy (Beck, 1988). Pharmacotherapy remains the most widespread and efficacious treatment, especially in severe cases (Sandford et al., 2000). Anxiety disorders are thought to be linked to an overactivity of the central serotonergic system (Briley et al., 1990; Kawahara et al., 1993).

The 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor is viewed as a relevant target for the treatment of psychiatric disorders, notably anxiety and depression (File, 1996). 5-HT_{1A} receptors are located at the presynaptic and postsynaptic sites (Blier et al., 1993). The somatodendritic autoreceptor, when activated by systemic stimulation, is believed to exert anxiolytic-like effects and to reduce 5-HT release both in the cell body and in the terminal regions of the serotonergic neurons (Laaris et al., 1997). The other 5-HT_{1A} receptor is localized postsynaptically to the serotonergic neurons in the hippocampus, septum, amygdala, and cortex, where it increases signal transfer, which leads to an inhibition of the firing activity (Okazawa et al., 1999).

GABA is a major inhibitory transmitter in the central nervous system. The γ-aminobutyric acid type A ($GABA_A$) receptor, the chloride ion channel complex and the central benzodiazepine receptors located on the neuronal membranes within this complex have been suggested to play an important role in the regulation of the stress and anxiety states (Short and Maier, 1993; Johnson et al., 1998).

The benzodiazepine binding site and $GABA_A$ receptor are structurally and functionally coupled (Sieghart, 1995). Benzodiazepines (BDZs) have become the primary pharmacological treatment for generalized anxiety disorder. However, BDZs is often associated with tolerance development and withdrawal symptoms, which poses a risk of relapse upon discontinuation (Lader, 1995; Ballenger, 2001). Many studies have examined the use of native plants for more specific, lower cost treatments with fewer harmful effects (Hui et al., 2002; Kim et al., 2004; Jung

* Corresponding author. Tel.: +82 31 290 7780; fax: +82 31 292 8800.
E-mail address: jang@skku.ac.kr (C.-G. Jang).

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et al., 2005, 2006), with the aim of developing potent functional foods and medicinal substances with anxiolytic-like effects without adverse effect.

Cinnamomum cassia (*C. cassia*) has been traditionally used to treat dyspepsia, gastritis, blood circulation disturbances and inflammatory disease in both Eastern and Western countries. *C. cassia* has been reported to have significant antiallergic, antiulcerogenic, antipyretic, and analgesic activity (Kurokawa et al., 1998; Huss et al., 2002). However, the anxiolytic-like effects of *C. cassia* have not been reported. Therefore, this study investigated the effects of *C. cassia* on anxiety.

The aim of this study was to determine the putative anxiolytic-like effects of the 50% EtOH extract of *C. cassia*. The anxiolytic-like, locomotor behavior, and myorelaxant effects of the extract were examined using the elevated plus maze (EPM), locomotor activity test, and horizontal wire test, respectively. Moreover, this study also examined which neurotransmitter systems are involved in the anxiolytic-like effects of the *C. cassia* through the co-administration of *C. cassia* and WAY 100635, (+)-bicuculline, or flumazenil.

2. Materials and methods

2.1. Animals

Male ICR mice, 22–26 g, were purchased from the MJ Co., Ltd. (Seoul, Korea). The animals were housed 10 per cage, allowed access to food and water ad libitum, and maintained under a constant temperature (23 ± 1 °C) and humidity (55 ± 5%) under a 12-h light/dark cycle (light on 7:00–19:00). All the experiments involving animals were carried out in accordance with the NIH Guidelines of Laboratory Animals, and the Institutional Animal Care and Use Committee of Sungkyunkwan University approved the protocol.

2.2. Drugs and chemicals

The 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) was obtained from Sam-Jin Pharmaceutical Co. Ltd. (Hwasung, Korea). WAY 100635, (+)-bicuculline, and Tween 80 were purchased from Sigma (St. Louis, MO, USA) and flumazenil was purchased from Tocris (Tocris, UK). Diazepam was supplied by local pharmaceutical company (Myung In Pharm. Co., Ltd, Korea).

2.3. Sample preparation and drug administration

The stem barks of *C. cassia* were purchased in Kyungdong market, Seoul, Korea. The plant was identified by Dr. Gap-Jin Oh, Sam-Jin Pharmaceutical Co. Ltd, Korea. A voucher specimen (#SJ-51357) was deposited at the herbarium of the Sam-Jin Pharmaceutical Co. The dried powdered stem barks (300 g) of *C. cassia* were extracted twice (each time for 3 h followed by heating) with a mixture of EtOH and water (1:1) in a reflux apparatus. The extract was concentrated to dryness under vacuum. The yield of the 50% ethanolic mixture extract was 5.8% (w/w, 17.4 g).

The 50% EtOH extract of *C. cassia* was freshly dissolved in distilled water containing 0.7% carboxyl methylcellulose. Flumazenil was dissolved and suspended in a solvent containing 99% distilled water and 1% Tween 80 with sonication, and administered. WAY 100635 and (+)-bicuculline were dissolved in a 0.9% physiological saline solution. Diazepam was diluted with 0.9% physiological saline solution containing 1% Tween 80. Vehicle control group was treated with distilled water containing 0.7% carboxyl methylcellulose. All samples were prepared freshly on test day and administered in a volume 0.1 ml/10 g of body weight of mouse.

2.4. Elevated plus maze test

The anxiolytic-like behaviors were measured using the elevated plus maze. The plus maze was elevated to a height of 50 cm from the floor and consisted of two open arms (30 × 5 cm) and two closed arms (30 × 5 cm) with 15 cm walls, extending from the central platform (2.5 × 2.5 cm) in a dimly lit room (40 lux) with a video camera suspended above the maze to record the experiment. The arms were connected to a central square to give the apparatus the appearance of a plus sign. The maze floor and walls were constructed from opaque polyvinyl plastic, and the open arms had a low (0.5 cm) edge. Each mouse was placed on the center of the platform facing an open arm. The animals were tested individually and only once for a 5 min period. The maze floor was cleaned after each trial so as to remove any residue or odors using 10% ethanol. The measurements were taken using the video-based Ethovision System and analyzed: the number of entries into the open arm and closed arms and the time spent in each arm in the EPM. The 50% EtOH extract of *C. cassia* or control were administered orally 1 h before testing. In the acute experiment, the 50% EtOH extract of *C. cassia* (250, 500, and 750 mg/kg,) was orally administered to mice. In the chronic experiment, the mice were orally administered for 5 days, once a day *C. cassia* at 50, 75, and 100 mg/kg. In a separate antagonism experiment, the mice were subjected to the co-administration of 50% EtOH extract of *C. cassia* 750 mg/kg and WAY 100635 (0.1, 0.3, and 1.0 mg/kg, i.p.), (+)-bicuculline (0.1, 0.3, and 1.0 mg/kg, i.p.), and flumazenil (5, 10, and 20 mg/kg, i.p.). Thirty minutes before the *C. cassia* treatment (750 mg/kg, p.o.), the mice were administered WAY 100635 (0.1, 0.3, and 1.0 mg/kg) and (+)-bicuculline (0.1, 0.3, and 1.0 mg/kg) intraperitoneally. Thirty minutes after the *C. cassia* treatment (750 mg/kg, p.o.), the mice were administered flumazenil (5, 10, and 20 mg/kg) intraperitoneally. The mice served as positive control were administered diazepam (2 mg/kg, i.p.) 30 min before EPM test.

2.5. Locomotor activity test

Testing was carried out in opaque black plastic boxes (30 × 30 × 30 cm) using a computer-based video-tracking system (NeuroVision, Pusan National University, Pusan, Korea). The mice were placed in the center of the apparatus to evaluate the locomotor test 1 h after being treated with the single (250, 500, and 750 mg/kg, p.o.) and repeated (50, 75, and 100 mg/kg, p.o.)

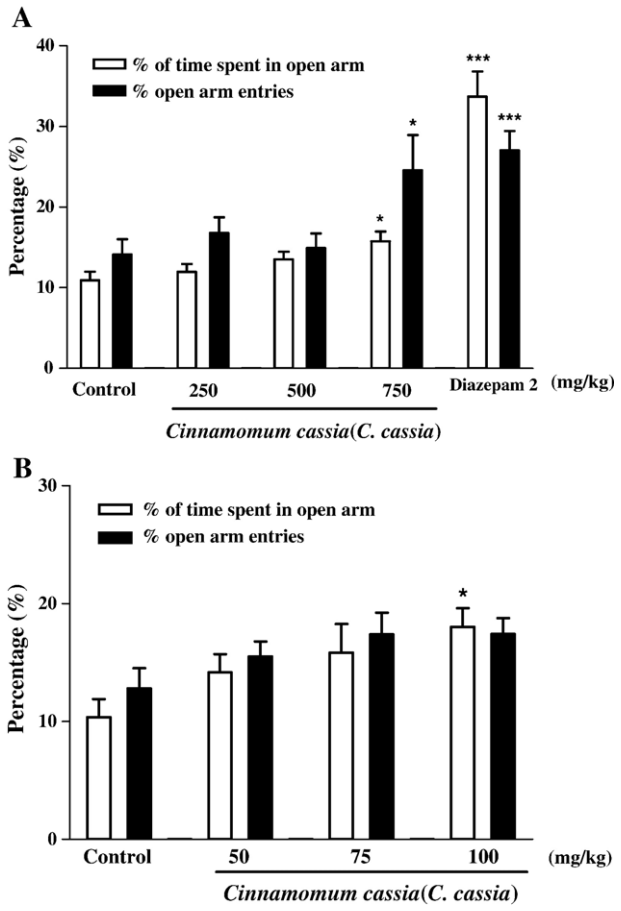

Fig. 1. Effect of the single (A) and repeated (B) treatment with the 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) on the percentage entries into and the time spent in the open arms of the elevated plus maze over a 5 min test period. Each bar indicates the mean±S.E.M. of 9–10 mice. The *P* values for group comparisons were obtained using one-way ANOVA followed by the Student–Newman–Keuls test (*, $P<0.05$ and ***, $P<0.001$ compared with the vehicle treated control group).

administration of *C. cassia*. The locomotor activity was recorded for 5 min and expressed as the total ambulatory distance (cm).

2.6. Horizontal wire test

Immediately after the EPM test, the horizontal wire test was carried out. The mice were lifted by the tail and allowed to grasp a horizontally strung wire (1 mm diameter, 30 cm long, and placed 20 cm above the table) with their forepaws, and then released. The number of mice from each treatment group that did not grasp the wire with the forepaws or actively grasp the wire with at least one hind paw within 10 s was recorded. A myorelaxant drug would impair the ability of the mice to grasp the wire. Muscle relaxation is commonly associated with sedation (Hui et al., 2002).

2.7. Statistical analysis

The values are expressed as the mean±S.E.M. The percentage of time spent in the open arms was calculated using the following formula: time spent in the open arms/(time spent in the open arms+time spent in the closed arms+time spent in the center zone)×100. The percentage of entries into the open arms was calculated using the same method. The data was analyzed by one-way analysis of variance (ANOVA) followed by a Newman–Keuls test using Prism 3.0 (Graphpad Software, Inc). Statistical significance was set at $P<0.05$.

3. Results

3.1. Effect of C. cassia in the elevated plus maze test

As shown in the vehicle treated group, the mice typically avoided spending time in or entering the open arms. A single treatment with *C. cassia* (750 mg/kg, p.o.) significantly increased the percentage of entries into and the time spent in the open arms compared with the controls (Fig. 1A; $P<0.05$). The diazepam-treated (2 mg/kg, i.p.) positive control group showed increase of the percentage of entries into and the time spent in the open arms compared with the vehicle treated group (Fig. 1A; $P<0.001$). The repeated treatment with *C. cassia* (100 mg/kg, p.o.) significantly increased the percentage of time spent in the open arms compared with the controls (Fig. 1B; $P<0.05$).

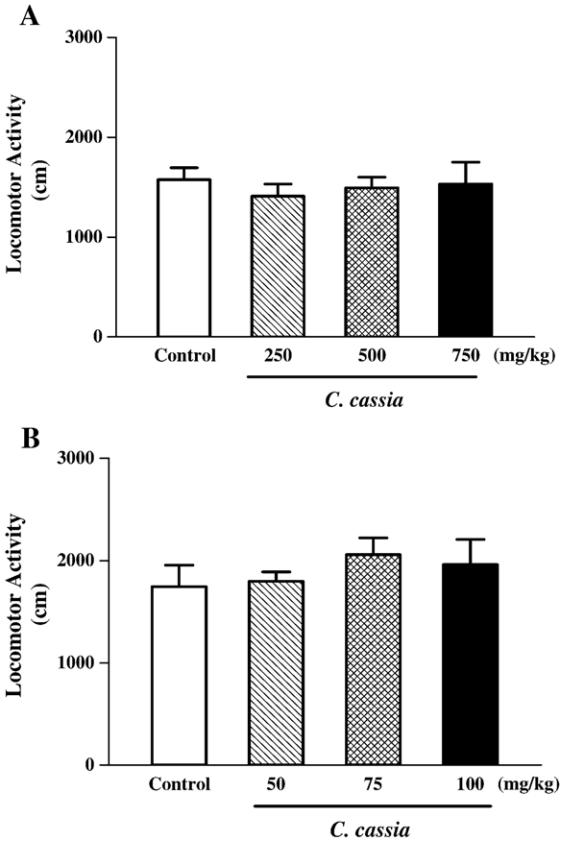

Fig. 2. Effect of a single (A) and repeated (B) treatment with the 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) on the locomotor activity test. The locomotor activity was recorded for 5 min and expressed as the total ambulatory distance (cm) 1 h after *C. cassia* was administered to mice. Each bar represents the mean±S.E.M. of 9 mice.

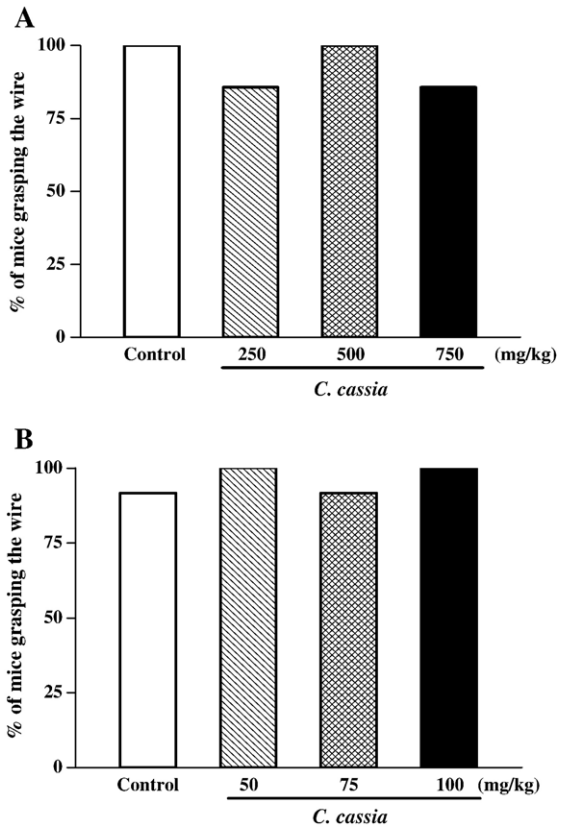

Fig. 3. Effect of the single (A) and repeated (B) treatment with the 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) in the horizontal wire test. The performance of the mice in the horizontal wire test was examined immediately after the elevated plus maze test in the single and repeated treatment with the 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*). Each bar represents the mean±S.E.M. of 9–10 mice.

3.2. Effect of C. cassia on the locomotor activity test

A locomotor activity test was carried out to differentiate between the possible stimulatory effects of the tested drugs on the modulation of the exploratory behavior. As shown in Fig. 2, a single treatment (250, 500, and 750 mg/kg) of *C. cassia* produced no significant changes in total ambulatory distances (1411±120.4 cm, 1493±108.1 cm, and 1531±220.6 cm, respectively) compared with the vehicle treated control group (1574±121.7 cm). The repeated treatment (50, 75, and 100 mg/kg) of *C. cassia* produced no significant changes (1797±91.2 cm, 2058±163.9 cm, and 1959±246.8 cm, respectively) in the total ambulatory distances compared with the vehicle treated control group (1746±208.9 cm).

3.3. Effect of the C. cassia on the horizontal wire test

As shown in Fig. 3, a single (250, 500, and 750 mg/kg) treatment of *C. cassia* showed no significant changes in the percentage of mice of grasping the wire (85.7, 100, and 85.7%, respectively) compared with the vehicle treated control group (100%). The repeated (50, 75, and 100 mg/kg) treatment of *C. cassia* showed no significant changes in the percentage of mice of grasping the wire (100, 91.6, and 100%, respectively) compared with the vehicle treated control group (91.6%). *C. cassia* did not elicit a myorelaxant effect.

3.4. Effect of WAY 100635 on the anxiolytic-like activity of C. cassia

The *C. cassia* (750 mg/kg) treated mice were co-administered WAY 100635, a 5-HT_{1A} receptor antagonist, in order to determine if the anxiolytic effects of *C. cassia* may involve the serotonergic neurotransmitter system, especially the 5-HT_{1A} receptors. As shown in Fig. 4, the effects of *C. cassia* (750 mg/kg) significantly increased the percentage of entries and the time spent ($P<0.01$) in the open arms ($P<0.05$). WAY 100635 (0.3 and 1.0 mg/kg) significantly blocked the anxiolytic-like effects of *C. cassia* (750 mg/kg) ($P<0.05$).

3.5. Effect of (+)-bicuculline on the anxiolytic-like activity of C. cassia

The *C. cassia* (750 mg/kg) treated mice were co-administered (+)-bicuculline, a competitive antagonist of $GABA_A$ receptor in order to determine if the anxiolytic effects of *C. cassia* occur through the GABAergic neurotransmitter system, particularly the GABA site of the $GABA_A$ receptors. As shown in Fig. 5, the effects of *C. cassia* (750 mg/kg) significantly increased the percentage of entries into ($P<0.01$) and the time spent in the open arms ($P<0.05$). (+)-Bicuculline (0.1, 0.3, and 1.0 mg/kg) significantly blocked the anxiolytic-like effects of *C. cassia* (750 mg/kg) ($P<0.05$, $P<0.01$).

3.6. Effect of flumazenil on the anxiolytic-like activity of C. cassia

The *C. cassia* (750 mg/kg) treated mice were subjected to a co-administration with flumazenil, benzodiazepine site of

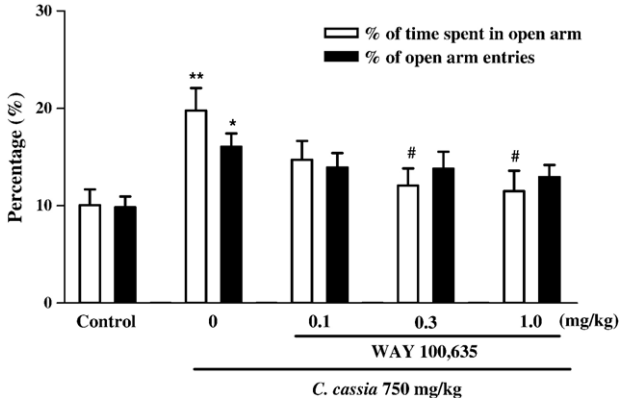

Fig. 4. Anxiolytic-like effects of 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) were blocked by WAY 100635. *C. cassia* was administered orally to the mice at 750 mg/kg per mouse. Thirty minutes before oral administration, WAY 100635 (0.1, 0.3, and 1.0 mg/kg) or vehicle were administered intraperitoneally; $N=9–10$ mice per group. The data is expressed as the mean±S.E.M. of the percentage of entries into and the time spent in the open arms of the elevated plus maze over a 5 min test period. *P* values for the group comparisons were obtained using one-way ANOVA followed by a Student–Newman–Keuls test (*, $P<0.05$ versus the vehicle treated controls, #, $P<0.05$ as compared to the corresponding effect of the agonist).

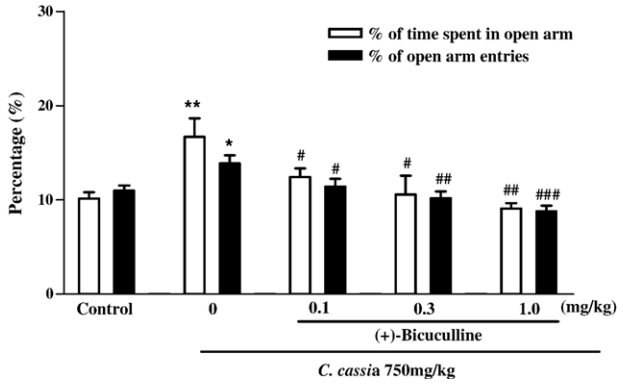

Fig. 5. Anxiolytic-like effects of the 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) were blocked by (+)-bicuculline. *C. cassia* was administered orally at 750 mg/kg per mouse. Thirty minutes before the last oral administration, (+)-bicuculline (0.1, 0.3, and 1.0 mg/kg) or vehicle were administered intraperitoneally; $N=9\sim10$ mice per group. The data is expressed as the means ± S.E.M. of the percentage of entries into and the time spent in the open arms of the elevated plus maze over the 5 min test period. *P* values for the group comparisons were obtained by one-way ANOVA followed by Student–Newman–Keuls test (*, $P<0.05$ versus the vehicle treated controls, $^{\#}$, $P<0.05$ as compared with the corresponding effect of agonist).

$GABA_A$ receptor antagonist in order to determine if the anxiolytic effects of *C. cassia* occur through the GABAergic nervous system, particularly the benzodiazepine site of the $GABA_A$ receptors. As shown in Fig. 6, the effects of *C. cassia* (750 mg/kg) significantly increased the percentage of entries ($P<0.05$) and the time spent in the open arms ($P<0.05$). Flumazenil (5, 10, and 20 mg/kg) significantly blocked the anxiolytic-like effects of *C. cassia* (750 mg/kg).

4. Discussion

C. cassia has been reported to have significant antiallergic, antiulcerogenic, antipyretic, anaesthetic, and analgesic activities (Kurokawa et al., 1998; Huss et al., 2002). It is commonly used to treat inflammation, tumors, stomachic, pyretic and against influenza virus or microorganism, and as analgesic (Kurokawa et al., 1998; Lee and Ahn, 1998; Lee et al., 1999). However, to our knowledge, there are no reports of the anxiolytic-like effects of *C. cassia* or what neurotransmitter system is largely involved in.

The EPM is an etiologically valid animal model of anxiety because it uses natural stimuli, such as a fear of a new, brightly lit open space and the fear of balancing on a relatively narrow raised surface (Imaizumi and Onodera, 2000; Jung et al., 2006). Generally, an anxiolytic agent increases the number of entries into and the time spent in the open arms of the EPM. In accordance with previously published reports, diazepam increased the percentage time spent in open arms and open arm entries (Helton et al., 1996; Eguchi et al., 2001). In the present experiments, diazepam (2 mg/kg, i.p.) significantly increased the percentage of time spent in the open arms and open arm entries of the EPM. The single and repeated treatment of the *C. cassia* increased the percentage of time spent in the open arms as well as the percentage of open arm entries of the EPM.

The 5-HT_{1A} receptors play important roles in the mediation of 5-HT neurotransmission in the central nervous system, and changes in their functional state are implicated in human anxiety. Buspirone and a series of structurally related 5-HT_{1A} ligands were anxiolytic in the clinic (Wheatley, 1988). Buspirone, a somatodendritic 5-HT_{1A} partial agonist, inhibits serotonergic system activity by acting on the somatodendritic autoreceptors and possesses anxiolytic effects at lower doses (Sharp et al., 1989). WAY100635, a somatodendritic 5-HT_{1A} antagonist, enhances serotonergic system activity via complete blockade of the somatodendritic autoreceptors and possesses anxiogenic activity. (Collinson and Dawson, 1997). We observed that the anxiolytic-like effect of *C. cassia* was significantly blocked by WAY100635 at 0.3 and 1.0 mg/kg.

The $GABA_A$ receptors including benzodiazepine site mediate the fast inhibitory neurotransmission in the mammalian central nervous system. $GABA_A$ receptors possess binding sites for several drugs, such as anxiolytics, anticonvulsants, general anesthetics, barbiturates, ethanol, and neurosteroids, which are known to elicit at least some of their pharmacological effects via the $GABA_A$ receptors (Johnston, 1996; Mehta and Ticku, 1999; Korpi et al., 2002). These receptors are potently inhibited by the competitive antagonists bicuculline and SR95531 and the plant alkaloid picrotoxin (Delaney and Sah, 1999). We observed that the anxiolytic-like effect of *C. cassia* was significantly blocked by (+)-bicuculline at 0.1, 0.3, and 1.0 mg/kg.

The benzodiazepines receptor agonists are commonly prescribed for the treatment of anxiety, sleep, and seizure disorders for over 40 years. It has been reported that 4-hyroxybenzaldehyde (100 mg/kg) of phenolic compounds has anxiolytic-like effects and probably works via the activation of the benzodiazepine site of the $GABA_A$ receptors in the central nervous system because those effects were blocked by flumazenil (Jung et al., 2006). In our experiment, we also observed that the anxiolytic-like effect of *C. cassia* was significantly blocked by flumazenil at 5, 10, and 20 mg/kg. The anxiolytic-like effects of *C. cassia* (750 mg/kg)

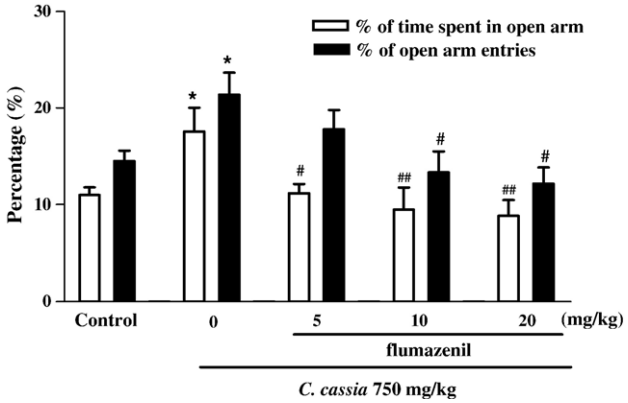

Fig. 6. The anxiolytic-like effects of the 50% EtOH extract of *Cinnamomum cassia* (*C. cassia*) were blocked by flumazenil. *C. cassia* was administered orally at 750 mg/kg per mouse. Thirty minutes after the last oral administration, flumazenil (5, 10, and 20 mg/kg) or vehicle were administered intraperitoneally; $N=9\text{–}10$ mice per group. The data is expressed as the mean ± 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 over 5 min test period. *P* values for the group comparisons were obtained using one-way ANOVA followed by the Student–Newman–Keuls test (*, $P<0.05$ versus the vehicle treated controls, $^{\#}$, $P<0.05$ as compared to the corresponding effect of agonist).

were blocked by WAY 100635, (+)-bicuculline, and flumazenil. Therefore, both the 5-HT_{1A} and $GABA_A$ receptors systems might mediate the anxiolytic-like effects of *C. cassia*.

Total distances of movement on the locomotor activity test were also unchanged by the *C. cassia* treatment compared with the vehicle controls. In the horizontal wire test, no significant myorelaxant effect was observed after administering the *C. cassia* extract. This suggests that the anxiolytic-like effect of *C. cassia* is selective, and not the result of either the general stimulation of the locomotor activity or an exploratory behavior consequent to exposure to a novel environment.

We scheduled that flumazenil was intraperitoneally administered to mice 30 min after the *C. cassia* treatment different from WAY 100635 and (+)-bicuculline administration schedule. The ex vivo occupancy of flumazenil is dependent on incubation time. Occupancy measured in crude brain homogenates using the ex vivo method was time dependent with a 3 mg/kg dose giving occupancies of 77% and 12% using 0.5 or 60 min ex vivo incubations times, respectively (Delaney and Sah, 1999; Li et al., 2006). Due to the fact that flumazenil has a rapid dissociation from the benzodiazepine receptor we intraperitoneally administered this compound to mice 30 min after *C. cassia* treatment.

At present, it is not known which constituents of *C. cassia* are involved in its anxiolytic-like effects via 5-HT_{1A} and $GABA_A$ receptors. Yoon et al. (2005) reported that cinnamic acid of phenylpropanoid compounds contained in *C. cassia* has anxiolytic-like effect using EPM test. p-Coumaric acid, caffeic acid, and ferulic acid of cinnamic acid derivatives have anxiolytic-like effects of the EPM in mice and do not affect the locomotor activities and myorelaxant effect. Yoon et al. (2005) also reported that each derivative does not change $GABA_A$ receptor binding and the anxiolytic-like effects of other phenylpropanoid compounds may be mediated via monoaminergic neurotransmitter system such as serotonergic, dopaminergic, or noradrenergic system. However, further studies will be needed to determine if which the main active compound of *C. cassia* has the anxiolytic-like effect.

Taken together, the main findings of this study were that *C. cassia* significantly increased the percentage of entries into and the time spent in the open arms in a mouse using the EPM test, and these effects were antagonized by WAY 100635, (+)-bicuculline, and flumazenil. However, there were no changes in locomotor activities or myorelaxant effects. Accordingly, it is suggested that *C. cassia* possesses an anxiolytic-like effect that is mediated by 5-HT_{1A} and $GABA_A$ receptor activation and has no adverse effect, such as myorelaxant effects.

Acknowledgements

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