

## Involvement of Histaminergic System in the Anxiolytic-Like Activities of *Morus alba* Leaves in Mice

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The aim of this study was to identify the effects of 85% methanolic extract of *Morus alba* leaves (EMA), which is a traditional herb, in mice. The effects of EMA on the anxiolytic-like behaviour were studied using the elevated plus maze (EPM) and hole-board test. To elucidate the mode of action of the anxiolytic-like effects of EMA, the mice were subjected to the co-administration of EMA (200 mg/kg, *per os* (*p.o.*)) and either antagonist. EMA (at 200 or 400 mg/kg) significantly increased the percentages of time-spent in the open arms and entries into the open arms of the EPM *versus* vehicle-treated control group ( $p < 0.05$ ). Moreover, in the hole-board test, EMA (200 and 400 mg/kg) significantly increased the number of head-dips *versus* vehicle-treated control group ( $p < 0.05$ ). However, there were no changes in the locomotor activity and myorelaxant effects in any group compared with the vehicle-treated control group. In addition, the anxiolytic-like effects of EMA were abolished by thioperamide (10 mg/kg, intraperitoneally (*i.p.*)), which is a histamine H<sub>3</sub> receptor antagonist. Moreover, results from reverse transcription polymerase chain reaction (RT-PCR) also revealed that the amygdala histidine decarboxylase mRNA expression levels in EMA (200 mg/kg)-treated group were significantly higher than those in the vehicle-treated controls ( $p < 0.05$ ). These results suggest that EMA might prove to be an effective anxiolytic agent and that EMA acts *via* the histaminergic system in central nerve system.

**Key words** anxiety; *Morus alba*; elevated plus-maze; histamine; thioperamide

Mulberry tree (*Morus alba* L., Moraceae) is cultivated in China, Japan and Korea and is an important herbal material in traditional Chinese medicine. For example, the root bark of mulberry trees has long been used for anti-inflammatory, diuretic, antitussive, and antipyretic purposes in Oriental medicine.<sup>1)</sup> Moreover, fruit extract of *M. alba* was reported to modulate the monoamine oxidase activity during exercise and to promote the capability of physical activities.<sup>2)</sup> In addition to those herbal materials from *M. alba*, the leaves of this plant are one of the well-known traditional Chinese medicinal herbs and have been traditionally used to cure or prevent diabetic hyperglycemia.<sup>3,4)</sup> Based on those traditional usages, researchers reported that nitric oxide synthase positive neurons were decreased by the treatment with *M. alba* extracts in the various hypothalamic areas in the streptozotocin-induced diabetic rat brain.<sup>4)</sup> Interestingly, those hypothalamic areas, including the paraventricular nucleus and ventromedial hypothalamic nucleus are rich in histaminergic neurons originating from the tuberomammillary body.<sup>5)</sup> These findings suggest a possibility that the leaves of *M. alba* may affect the activity of the histaminergic nervous system. Moreover, several clinically useful anxiolytics have been found to affect the turnover rate of brain histamine in the rat brain. For example, diazepam inhibits histamine turnover by acting on  $\gamma$ -aminobutyric acid (GABA)<sub>A</sub> receptors<sup>6)</sup> and also inhibits histamine release in the rat striatum.<sup>7)</sup> Although earlier report has suggested that

methanolic extract of *M. alba* L. has anxiolytic effects mediated through the GABA<sub>A</sub>-benzodiazepine mechanism,<sup>8)</sup> there was no evidence to prove it. Furthermore, there have been no pharmacological studies upon of the effects of leaves of *M. alba* on the histaminergic system.

Therefore, we hypothesize that the leaves of *M. alba* administered *in vivo* may affect histaminergic transmission directly or indirectly in the brain and may result in anxiogenic or anxiolytic-like behaviours. If the leaves of *M. alba* act as an agonist of the histamine H<sub>1</sub> receptor or an antagonist of histamine H<sub>2</sub> or H<sub>3</sub> receptors, anxiety-like behaviours will be observed.<sup>9)</sup> To test this hypothesis, elevated plus-maze (EPM) and hole-board tests were employed to investigate whether the leaves of *M. alba* specifically targets histaminergic systems in the mouse brain.

### MATERIALS AND METHODS

**Materials** Quercetin, thioperamide, WAY-100635, and flumazenil were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). *M. alba* leaves were obtained from a herbalist supplier in Seoul, Korea, and voucher specimens (DHUHMP-09-04) were maintained. Diazepam was supplied by the local pharmaceutical company (Daewon Pharm. Co., Ltd.). The material was authenticated by Prof. Yong Han Kim of Department of Herbal Medicinal Pharmacology, College of Herbal Bio-industry, Daegu Haany University. All other materials were of the highest grade and were obtained from standard commercial

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sources.

**Animals** Male ICR mice at 5 weeks of age, weighing 23–25 g, were purchased from the Orient Co. (a branch of Charles River Laboratories, Seoul) and housed in the University Animal Care Unit for 1 week prior to experimentation. The animals were housed 5 per cage, allowed free access to water and food, and maintained under constant temperature ( $23\pm1^\circ\text{C}$ ), humidity ( $60\pm10\%$ ) and a 12-h light/ dark cycle (lights on from 07:00–19:00h). Animal treatment and maintenance were conducted in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985) and with the Animal Care and Use Guidelines of Daegu Haany University, Korea.

**Sample Preparation** A methanolic extract of the *M. alba* leaves (EMA) was prepared with 85% methanolic solution under a sonicator ( $25^\circ\text{C}$ ) for 1 h. Then, the extract solution obtained was filtered using a Whatman No. 1 filter paper and was concentrated on a water bath under vacuo. It was then, frozen and lyophilised (Eyela, model FDU-2000, Japan) to yield methanolic extracts (yield 3.4%), which were then stored at  $-20^\circ\text{C}$  until required.

**Ultra Performance (UP) LC Quantitative Analysis** Reverse-phase UPLC was performed on the Waters Acquity UPLC H class (Milford, Massachusetts, U.S.A.), consisting of a Quarternary Solvent Manager pump, Sample Manager-TN and PDA detector. Empower 3 pro software (Milford, Massachusetts, U.S.A.) was used for UPLC data analysis. Chromatographic separation was accomplished on a Waters C18 reverse phase column (Acquity BEH  $50\times1.0\text{mm}$  i.d.,  $1.7\mu\text{m}$ ) at  $30^\circ\text{C}$  and monitored at 350 nm. A gradient solvent system consisted of methanol (solvent A) and water (solvent B) was used from 5% (solvent A): 95% (solvent B) to 100% (solvent A): 0% (solvent B) for 20 min at a flow rate of  $0.5\text{mL}/\text{min}$ . The standard stock solutions were prepared by dissolving standards in 100% methanol to set a final concentration of  $10\text{mg}/\text{mL}$ . After ultracentrifugation at 9000 rpm and filtration through a syringe filter ( $0.45\mu\text{m}$ , Pall Co.),  $2\mu\text{L}$  of sample was injected 6 times. The average retention time of quercetin was 9.3 min. The calibration curve of quercetin was drawn with ( $R^2$ ) of quercetin was as follows:  $y=48143x-23093$ ;  $R^2=0.999935$  in standard concentrations ranging from 1 to  $500\mu\text{g}/\text{mL}$ . Using these equations, the quantity of quercetin in the EMA was calculated and determined as  $14.80\pm0.48\mu\text{g}/\text{g}$ .

**EPM Test** The EPM for mice consisted of two perpendicular open arms ( $30\times7\text{cm}$ ) and two enclosed arms ( $30\times7\text{cm}$ ) with 20 cm high walls, extending from the central platform ( $7\times7\text{cm}$ ). The open and closed arms were connected by a central square ( $7\times7\text{cm}$ ), to give an apparatus of a plus sign in the shape of a plus sign. The floor and walls of the maze were constructed from the dark opaque polyvinyl plastic. The maze was raised to a height of 50 cm above the floor level in a dimly illuminated room (20 Lux) and a video camera was suspended above the maze to record the movements for analysis.<sup>10,11</sup> Each mouse was placed at the centre of the platform, its head facing an open arm. The animals were tested individually and only for 5 min. The maze was cleaned after each trial to remove any residue or odours. The following measurements were taken and analysed 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 of the experiments were performed between

10:00 and 16:00.

One hour after the EMA treatment (50, 100, 200 and  $400\text{mg}/\text{kg}$ , *per os* (*p.o.*)), the mice were placed in the EPM. EMA was suspended in a 10% Tween80 solution (v/v). The mice in the control group were given the vehicle solvent only, and the animals were tested individually for 5 min. In a pilot study, we observed that EMA exhibited anxiolytic-like activity 1 h after oral administration and diazepam exhibited similar activity 30 min after intraperitoneal administration. Therefore, we selected these time points for the present study. The doses of EMA employed in the present study were based on the traditional dosage (5–9 g).<sup>12</sup> In a separate antagonism study, the mice were subjected to the co-administration of EMA ( $200\text{mg}/\text{kg}$ , *p.o.*) and either WAY-100635 ( $0.3\text{mg}/\text{kg}$ , intraperitoneally (*i.p.*)),<sup>13</sup> flumazenil ( $10\text{mg}/\text{kg}$ , *i.p.*),<sup>14</sup> or thioperamide ( $10\text{mg}/\text{kg}$ )<sup>15</sup> 1 h and 30 min prior to testing. The mice were treated with diazepam ( $1\text{mg}/\text{kg}$ , *i.p.*) 30 min before EPM test and used as the positive controls.

**Hole-Board Test** The hole-board apparatus (Ugo Basile, Italy) consisted of gray Perspex panels ( $40\times40\text{cm}$ , 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 the table. The method was adapted from.<sup>16</sup> Mice were transported to the dimly lit laboratory at least 1 h before testing. Each animal was individually placed alone in the centre of the board facing away from the observer and its behaviour was recorded for 5 min, including the number of head-dips was recorded. Mice were orally administered EMA (50, 100, 200 and  $400\text{mg}/\text{kg}$ ) 1 h prior to the testing.

**Horizontal Wire Test** Immediately after the EPM test in the single treatment of EMA, a horizontal wire test was carried out by treating the mice with EMA (50, 100, 200 and  $400\text{mg}/\text{kg}$ , *p.o.*) according to a slightly modified version of the method reported by Bonetti *et al.*<sup>17</sup> Briefly, the mice were lifted by the tail and allowed to grasp a horizontally strung wire (1 mm diameter, 15 cm length, and placed 20 cm above the table) with their forepaws, after which they were 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 s 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.

**Locomotor Activity in the Open Field Test** Testing was conducted in clear black Plexiglas boxes ( $40\times40\times40\text{cm}$ ) equipped with the video-based Ethovision System (Noldus, Wageningen, the Netherlands). The mice were placed in the centre of the apparatus to evaluate horizontal locomotor activity 1 h after being treated with EMA (50, 100, 200 and  $400\text{mg}/\text{kg}$ ); the mice were and video-recorded for 5 min. Horizontal locomotor activity was expressed as total ambulatory distance and the frequency of rearing.<sup>18</sup>

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)** For RT-PCR, the mice not subjected to behavioral tests were sacrificed and the amygdala was rapidly dissected on an ice-cold glass plate 1 h or 30 min after the final administration of EMA to detect the levels of expressed histidine decarboxylase (HDC) mRNA. Isolated mice amygdala were homogenized in 1 mL Trizol Reagent (Molecular Research Center, OH, U.S.A.) and incubated in the same solution for

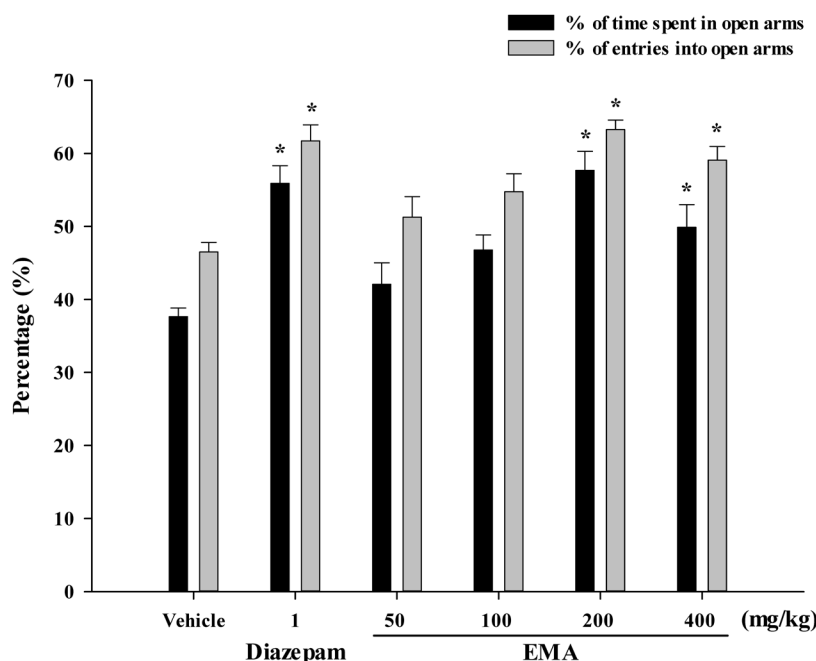


Fig. 1. Effect of Treatment of the 80% Methanolic Extract of *M. alba* Leaves (EMA) on the Percentages of the Time Spent in and the Number of Entries into the Open Arms of the Elevated Plus-Maze over a 5 min Test Period in the Mice

Mice were administered EMA (50, 100, 200 or 400 mg/kg, *p.o.*). Each bar represents the mean  $\pm$  S.E.M. of 10 mice. *p* values for the group comparisons were obtained by one way ANOVA followed by Student–Newman–Keuls test (\**p* < 0.05 compared with the vehicle-treated control group).

5 min at RT and then stored at  $-70^{\circ}\text{C}$  until used. For RNA extraction, 0.2 mL of chloroform was added per ml of extract and the samples were further incubated for 3 min at RT. They were then centrifuged at  $15000\times g$  for 15 min and the supernatant was incubated with isopropyl alcohol (0.5 mL/mL) at RT for 10 min. Samples were then centrifuged at  $15000\times g$  for 15 min at RT and the RNA pellet was washed once with 85% ethanol. RNA was redissolved in 10  $\mu\text{L}$  RNase-free water and stored at  $-70^{\circ}\text{C}$  until used. To remove any residual contaminating DNA, RNA was incubated with 1  $\mu\text{L}$  DNase (RQ-DNase, Promega, Alexandria, New South Wales, Australia) at  $37^{\circ}\text{C}$  for 30 min, after which 90  $\mu\text{L}$  of RNase-free water was added and the RNA was extracted a second time with Trizol Reagent and chloroform as described above. The final RNA pellet was dissolved in 10  $\mu\text{L}$  RNase-free water.

Amygdalal RNA (1  $\mu\text{L}$ ), isolated as described above, was mixed with an equal volume of oligo-dT (50  $\mu\text{M}$ ) and deoxyribonucleotide triphosphates (10 mM; dNTP) and 7  $\mu\text{L}$  of RNase-free water, then denatured by incubation for 5 min at  $65^{\circ}\text{C}$ . This mix was then placed on ice for 1 min. For the synthesis of amygdalal cDNA by reverse transcription, DiaStar<sup>TM</sup> RT Kit (SolGent, Korea) were added and the mix was incubated at  $50^{\circ}\text{C}$  for 60 min, then at  $85^{\circ}\text{C}$  for 5 min to inactivate the reverse transcriptase. Samples were cooled on ice, incubated with 1  $\mu\text{L}$  RNase (SolGent, Korea) at  $37^{\circ}\text{C}$  for 20 min to digest the template RNA.

The amygdalal cDNA was amplified by PCR using synthetic specific primers to HDC, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follows :

HDC

5'-GAT CAG ATT TCT ACC TGT GG-3' (sense)

5'-GTG TAC CAT CAT CCA CTT GG-3' (antisense)

GAPDH

5'-GCC AAG GTC ATC CAT GAC AAC-3' (sense)

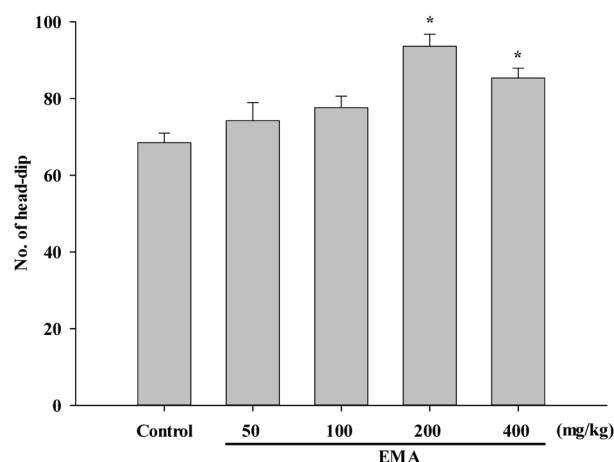


Fig. 2. Effects of *M. alba* Leaves (EMA) on the Changes in Head-dipping Behaviours in Mice Tested on the Hole-Board

Mice were administered EMA (50, 100, 200 or 400 mg/kg, *p.o.*). Each bar represents the mean  $\pm$  S.E.M. of 10 mice. *p* values for group comparisons were obtained by one way ANOVA followed by Student–Newman–Keuls test (\**p* < 0.05 versus the vehicle-treated control).

5'-AGT GTA GCC CAG GAT GCC CTT-3' (antisense).

In separate reactions, specific primers (1  $\mu\text{L}$  of 10  $\mu\text{M}$ ) were added to 5  $\mu\text{L}$  of cDNA, in a final volume of 50  $\mu\text{L}$  PCR reaction mix (2 mM dNTP; 2 mM  $\text{MgCl}_2$ ; 1U *Taq* DNA Polymerase). Amplification was initiated by denaturation for 5 min at  $95^{\circ}\text{C}$  and then annealing of 40 cycle for 40 s at  $57^{\circ}\text{C}$ , and subsequent elongation for 60 s at  $72^{\circ}\text{C}$ . The amplified PCR products were analyzed by 1% agarose gel electrophoresis in Tris-acetate–ethylenediaminetetraacetic acid (EDTA) (TAE) buffer and stained with EcoDye<sup>TM</sup> DNA staining solution (SolGent, Korea). The agarose gels were examined under UV light using a gel documentation system (DAIHAN Scientific, Korea).

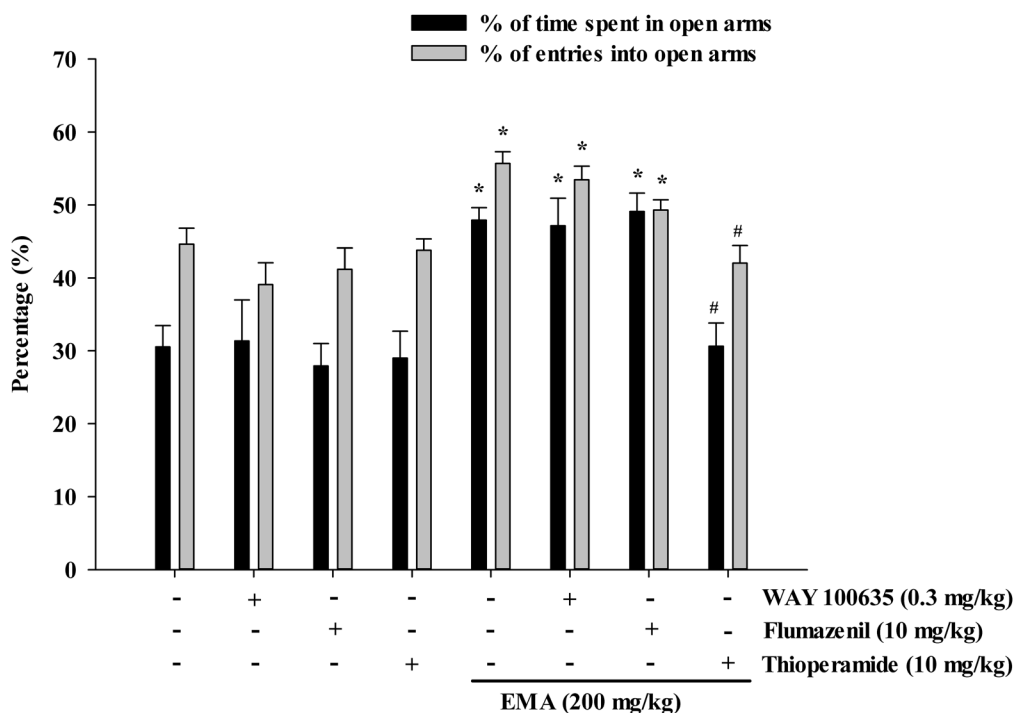


Fig. 3. Anxiolytic-Like Effects of *M. alba* Leaves (EMA) Were Blocked by Thioperamide but Not by WAY 100635 or Flumazenil

The data are 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 EMA (200 mg/kg), EMA (200 mg/kg)+WAY 100635 (0.3 mg/kg), flumazenil (10 mg/kg), or thioperamide (10 mg/kg) (30 min prior testing, i.p.), or saline;  $n=10$  mice per group.  $p$  values for the group comparisons were obtained by two way ANOVA followed by Student–Newman–Keuls test (\* $p<0.05$  versus the vehicle-treated control, # $p<0.05$  compared with the EMA-administered group).

GAPDH was used to as an internal loading control.

**Statistical Analysis** Values are expressed as the mean  $\pm$  S.E.M. Data were analysed by a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons. For the antagonism study, the interactions between the agonist and antagonist were analysed separately with a two-way ANOVA [factors: agonist versus antagonist]; pairwise comparisons for the assessment of the antagonist influence on the agonist effects were conducted by using Tukey's test. Statistical significance was set at  $p<0.05$ .

## RESULTS

**Effects of EMA Treatment in the EPM** The percentages of time spent in the open arms were significantly increased in the EMA-treated mice (200 or 400 mg/kg) compared with vehicle-treated group ( $57.7 \pm 2.6\%$  or  $49.9 \pm 3.1\%$ , respectively, versus  $37.6 \pm 1.2\%$ , Fig. 1;  $p<0.05$ ). Also, the percentages of numbers of entries into open arms were significantly increased in the EMA-treated mice (200 or 400 mg/kg) compared with vehicle-treated group ( $63.3 \pm 1.3\%$  or  $59.1 \pm 1.9\%$ , respectively, versus  $46.5 \pm 1.3\%$ , Fig. 1;  $p<0.05$ ). However, no significant change was observed in terms of the percentages of time spent in open arms and number of entries into open arms at 50 and 100 mg/kg of EMA. In the diazepam-treated (1 mg/kg) group, which was a positive control, the percentages of time spent in open arms and number of entries into the open arms were significantly increased compared with the saline-treated group ( $55.9 \pm 2.4\%$  and  $61.7 \pm 2.2\%$ ,  $p<0.05$ ). Thereafter, we adopted 200 mg/kg dosing for further studies.

**Effects of EMA Treatment in Hole-Board Test** The effects of EMA on the changes in head-dipping behaviour

in mice are shown in Fig. 2. EMA-treated mice manifested significant increases in the number of head-dips at doses of 200 and 400 mg/kg ( $93.6 \pm 3.2$  or  $85.4 \pm 2.6$ , respectively, versus  $68.5 \pm 2.5$ , Fig. 2;  $p<0.05$ ). However, there were no significant increases at doses of 50 and 100 mg/kg of EMA.

**Effects of WAY-100635, Flumazenil and Thioperamide, on the Anxiolytic-Like Activity of EMA** To determine which nervous system is involved in the anxiolytic effect of EMA, the EMA (200 mg/kg) treated mice were subjected to a co-treatment with either WAY-100635, which is a 5-HT<sub>1A</sub> receptor antagonist, or flumazenil, which is a GABA<sub>A</sub> receptor antagonist. As shown in Fig. 3, the anxiolytic-like effects of EMA were not antagonised either by WAY-100635 (0.3 mg/kg) or flumazenil (10 mg/kg). However, the anxiolytic-like effects of EMA (200 mg/kg) were blocked by thioperamide (10 mg/kg), which is a H<sub>3</sub> receptor antagonist.

**Effect of EMA on the Locomotor Activity Test and Horizontal Wire Test** False-positive results might be argued in these behavioural tasks with drugs that stimulate locomotion.<sup>19)</sup> However, as summarised in Table 1, no alterations in locomotor activity or rearing frequencies were observed in mice administered EMA with the doses of 50, 100, 200 or 400 mg/kg in the open-field test compared with vehicle-treated animals.

Moreover, at 5 mg/kg, diazepam significantly decreased the percentage of mice grasping the wire (Fig. 4). In contrast, EMA (50, 100, 200 and 400 mg/kg) did not decrease the percentage of mice grasping the wire compared with vehicle-treated control group, indicating a lack of myorelaxation at these doses.

**Effect of EMA on HDC mRNA Expression Levels in the Amygdala Region** To investigate the effects of EMA on the



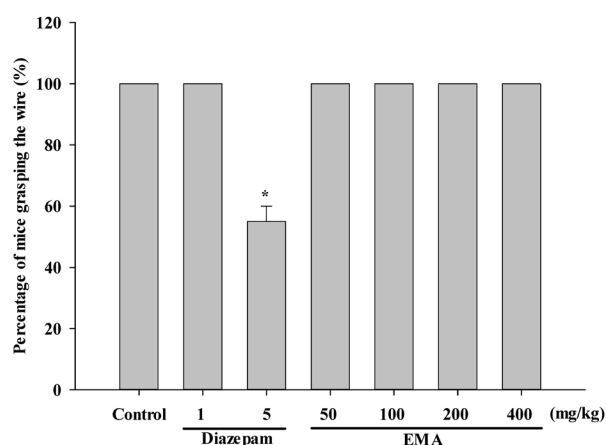


Fig. 4. Performance of Mice in the Horizontal Wire Test 1 h after Oral Administration of Control, Diazepam (1 and 5 mg/kg), or *M. alba* Leaves (EMA)

Data represent percentage of mice grasping the wire after administration of EMA. Each bar represents the mean  $\pm$  S.E.M. of 10 mice. *p* values for group comparisons were obtained by one way ANOVA followed by Student–Newman–Keuls test (\**p* < 0.05 versus the vehicle-treated control).

Table 1. Locomotor Activity Effects of the Methanolic Extract of the Leaves of *Morus alba* L. (EMA) on Open Field Test in Mice

	Total distance moved (cm/5 min)	Frequency of rearing (No.)
Control	2283.8 $\pm$ 171.0	99.1 $\pm$ 4.2
EMA (mg/kg)		
50	2202.3 $\pm$ 67.1	105.4 $\pm$ 4.8
100	2289.7 $\pm$ 111.5	106.3 $\pm$ 3.2
200	2376.1 $\pm$ 145.9	99.5 $\pm$ 3.9
400	2065.1 $\pm$ 87.8	100.0 $\pm$ 5.2

Spontaneous locomotor behaviour was measured in the open-field test for 5 min as described in Materials and Methods. Animals were orally treated with EMA (50, 100, 200 or 400 mg/kg) 1 h before the test. Control animals were treated with the same volume of vehicle. Data are expressed as the means  $\pm$  S.E.M. (*n* = 9–10/group).

release of HDC which is a molecule crucial for anxiolytic-like behavior, we conducted RT-PCR analyses after the administration of EMA. Results from RT-PCT also revealed that the amygdala HDC mRNA expression levels in EMA (200 mg/kg)-treated group were significantly higher than those in the vehicle-treated controls (*p* < 0.05, Fig. 5).

## DISCUSSION

In this study, the effects of EMA were examined in animal models of anxiety using the elevated plus-maze, hole-board test, horizontal wire test and locomotor activity, which are classic models for screening anxiety and myorelaxant activity. The main findings of this study were that EMA treatment significantly increased the time spent in the open arms and the frequency of open arm entries and that these effects were antagonised by thioperamide, suggesting the involvement of the central histaminergic neurons in the central nervous system (CNS). In the hole-board test, the treatment with EMA significantly increased the number of head-dips compared with the vehicle-treated controls. Moreover, no changes in locomotor activity or myorelaxant effects were observed. Therefore, we suggest that EMA has an anxiolytic-like effect due to hista-

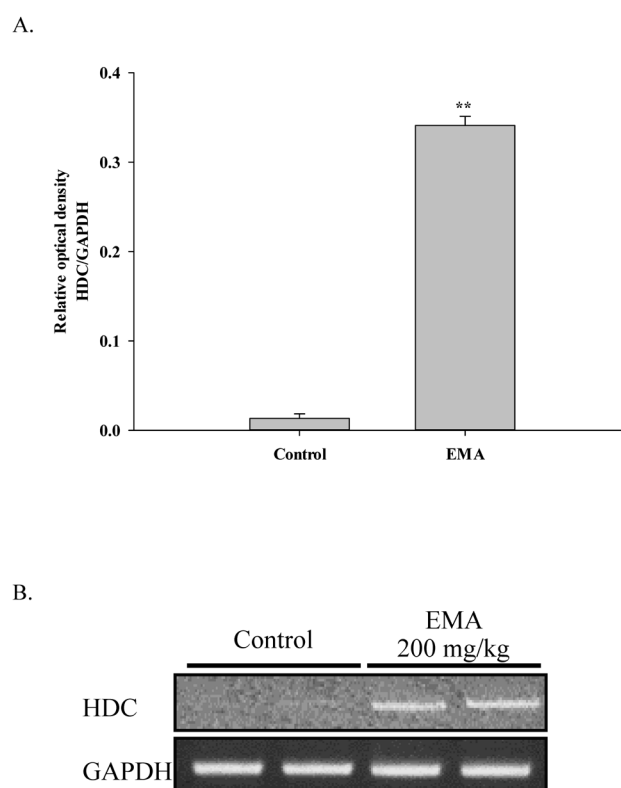


Fig. 5. Effects of Administration of Methanolic Extract of the Leaves of *Morus alba* L. (EMA) on Histidine Decarboxylase (HDC) Expression in the Amygdala of Mice

(A) Quantitative analysis of the expression of HDC mRNA and (B) RT-PCR for HDC mRNA and its quantitative analysis in the amygdala 1 h after EMA (200 mg/kg, *p.o.*) administration. Control animals were treated with the same volume of saline. Values are expressed as means  $\pm$  S.E.M. (*n* = 4/group). \**p* < 0.05, compared with the control group.

mine receptor activation and that it has no adverse effects, such as myorelaxant effects.

Histaminergic neurons, which originate from the tuberomammillary nucleus in the posterior hypothalamus, project diffusely throughout the CNS and have implicated in the regulation of many functions, including emotion, sleep/wake, thermoregulation, memory, and other homeostatic processes.<sup>20,21</sup> Recently, it has been reported that the activation of the histamine H<sub>1</sub> receptor has anxiogenic effects. Additionally, a histamine releasing agent, compound 48/80, exhibits angiogenic properties.<sup>22,23</sup> Those previous reports suggest that the histaminergic system plays an important role in the anxiety behaviour. Moreover, we hypothesised that EMA might exhibit its effects through affecting through altering histaminergic neurotransmission because it affected various hypothalamic functions including reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase and Neuropeptide Y immunoreactivities. We investigated this hypothesis whether the anxiolytic-like properties of EMA would be antagonised by thioperamide, which is a histamine H<sub>3</sub> receptor antagonist.

In the present study, the administration of EMA increased the percentages of entries into open arms and time spent in the open arms. Although Yadav *et al.*<sup>8</sup>) demonstrated that intraperitoneal administration with methanolic extract of *M. alba* L. induced a motor depressant effect, indicating a significant skeletal muscle relaxant and sedative effect, based on the results of their behavioural experiments, EMA did not alter

spontaneous behaviour and exerted no significant myorelaxant effect at the chosen dosage regimen as measured using the horizontal wire test. Total distances of movement on the EPM were not changed by EMA treatment *versus* the vehicle-treated control group (data not shown). These observations indicate that the anxiolytic effect of EMA is not simply the result of either a general stimulation of locomotor activity or exploratory behaviour consequent to exposure to a novel environment. Interestingly, the increase of the percentage of entries into open arms by the EMA treatment was significantly decreased in the vehicle-treated group by administration of thioperamide. Moreover, the percentage of time spent in the open arms showed a similar pattern. The  $H_3$  receptor was initially described as a presynaptically located autoreceptor regulating histamine synthesis and release of histamine from neuronal synaptic vesicles.<sup>24)</sup> Thioperamide is a selective antagonist of the  $H_3$  receptor and increases histamine release by blocking presynaptic  $H_3$  autoreceptor.<sup>25)</sup> Therefore, the antagonistic effects of thioperamide on the EMA in the EPM suggest that EMA has an antagonistic activity in the histaminergic synaptic cleft. However, we could not identify the exact properties of EMA—namely, whether EMA acts as a  $H_1$  receptor antagonist, a  $H_2$  receptor antagonist or a  $H_3$  receptor agonist. Each of the three is likely to inhibit the effects of thioperamide in the EPM. If EMA exhibits its effects through as a  $H_3$  receptor agonist, those effects in the EPM might be potentiated by the treatment of *R*- $\alpha$ -methylhistidine, which is a specific agonist of the  $H_3$  receptor. However, we did not observe any changes of the percentage of entries into open arms or the percentage of spent time in open arms with *R*- $\alpha$ -methylhistidine (data not shown). Yuzurihara *et al.*<sup>9)</sup> reported that the activation of the  $H_2$  receptor inhibits the anxiety in mice. These previous observations and our results mean that EMA exhibits its anxiolytic-like behaviour *via* blockade of the  $H_1$  receptor. However, further research is needed to clarify these issues.

Several lines of evidence indicate that the central histaminergic activity is regulated by serotonergic or GABAergic neurotransmission with regard to anxiety-like behaviours. Buspirone, which is a 5-HT<sub>1A</sub> receptor agonist, has been used as an anxiolytic drug and has been found to decrease the turnover rate of brain histamine in mice and rats.<sup>26)</sup> The effects of histamine turnover rate and extracellular histamine level were also affected by the administration of diazepam, which is a GABA<sub>A</sub> agonist.<sup>6,7)</sup> These previous observations suggest that the anxiolytic properties of EMA might be associated with the 5-HT<sub>1A</sub> receptor or GABA<sub>A</sub> receptor. We tested these possibilities using flumazenil, which is a GABA<sub>A</sub> antagonist, and WAY-100635, a 5-HT<sub>1A</sub> receptor antagonist. Neither flumazenil nor WAY-100635 showed any changes in the percentage of entries into open arms and the percentage of time spent in the open arms, as shown in the previous reports.<sup>14,27)</sup> With the administration of flumazenil, the increases of the percentages of entries into open arms and time spent in the open arms were not changed compared with those of EMA treatment. Moreover, the similar results were also obtained with the treatment of WAY-100635. Taken together, these results suggest that the anxiolytic-like effects of EMA are selective and are mediated through the  $H_1$  receptor, and not the 5-HT<sub>1A</sub> or the GABA<sub>A</sub> receptor.

HDC is the enzyme converts L-histidine to histamine.<sup>28)</sup> Meguro *et al.*<sup>29)</sup> reported that HDC activity is a good marker

for the central histaminergic neuron system. In previous report, HDC knockout animals exhibit altered mast cell development and increased bone formation<sup>30)</sup> the irreversible inhibitor of HDC,  $\alpha$ -fluoromethyl-[S]-histidine, which produces histamine loss in a tissue-dependent fashion,<sup>31)</sup> reduced locomotor activity in the open-field and an avoidance learning test.<sup>32)</sup> In addition, HDC knockout mice also showed reduced exploratory behavior in the open-field, reduced locomotor activity in the home cage in the dark, increased measures of anxiety on the elevated plus-maze, impairments in long-term object recognition memory, and enhanced motor coordination on the rotarod.<sup>32–36)</sup> In the present study, the increase in expression levels of HDC mRNA in the amygdala region after EMA administration suggests that the anxiolytic-like activity are dependent on HDC expression in the amygdala.

In the present study, the exact reasons why the higher dose of EMA (400mg/kg) was less effective than that of lower doses (200mg/kg) are unclear. However, it is possible that these phenomena were observed because any neurotransmitter autoreceptor of any neurotransmitters. Similar bell-shaped dose–response curves have been reported after psychiatric treatment for drugs such as buspirone, cannabidiol and *etc.*—other.<sup>37,38)</sup> Although the reason for these bell-shaped curves in the EPM is still obscure, the same bell-shaped trends have been observed previously in the reports on agmatine<sup>39,40)</sup> and GMP,<sup>41)</sup> which are endogenous modulatory compounds that produce their anxiolytic-like effects. Most of the anxiolytic effects are due to the amount of neurotransmitters in the synaptic cleft. Although we did not measure the neurotransmitters contents in the synaptic cleft, we assumed that EMA could also influence on neurotransmitter concentration in the synaptic cleft. Therefore, we consider that the bell-shaped dose-dependent curves for EMA in the present studies resulted from its influences on neurotransmitters and receptors. Further studies are needed to clarify these issues.

Until now, it has not been clear which constituent of EMA exerts its anxiolytic-like effects. Several reports exist on compounds isolated from the leaves of *M. alba* including flavone derivatives, unsaturated acid, and lignan glycoside.<sup>42)</sup> Of these constituents, quercetin, which is a flavonoid compound, is known to be contained high amounts.<sup>43)</sup> Moreover, quercetin is reported to have antagonistic activity to the histamine  $H_2$  receptor at a high dosage (200mg/kg).<sup>44)</sup> If quercetin is the main contributory compound in this anxiolytic-like behaviour, EMA should have a high concentration of quercetin. Our unpublished data also showed that quercetin exhibited its behavioural properties *via* the GABA<sub>A-p</sub> receptor, rather than histaminergic receptor (data not shown). Although this is speculative, the main contributory compounds might not be quercetin but may be other species. At present, we do not conclude that the histaminergic nervous system is the only system involved in the anxiolytic-like effects of EMA. We are currently investigating the major component(s) of EMA involved in the anxiolytic-like effect and the exact receptor systems associated with the effects.

Although benzodiazepines remain the mainstay of drug treatment in anxiety disorders, safer and more specific anxiolytics are needed because those compounds have prominent side-effects, such as sedation, myorelaxation, ataxia, amnesia, and pharmacological dependence.<sup>45)</sup> Natural anxiolytic agents are featured in such research because herbs have been used

to treat psychiatric disorders and generally have fewer harmful effects.<sup>46)</sup> The potential clinical benefits of some herbal remedies commonly used in psychiatric practice have been addressed in earlier reviews.<sup>47–51)</sup> Until now, we have investigated the anxiolytic agents from the herbal materials. Because the leaves of *M. alba* have been consumed for generations in Eastern Asia, it is unlikely that the leaves have side effects that are severe enough to prevent their pharmacological activities alone or in combination with other agents.

In summary, the present results demonstrate that the methanolic extract of *M. alba* leaves exerts an anxiolytic-like effect and that histaminergic neurotransmission is involved in that effect. Although the underlying mode of action remains to be elucidated and the findings of herb effects may not be clinically useful outcomes in patients or in humans, the findings of this study may be important in confirming the medicinal action of the leaves of *M. alba*.

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