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Anxiolytic-like effect of asiaticoside in mice

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Abstract

The putative anxiolytic activity of asiaticoside was examined in male mice by using a number of experimental paradigms of anxiety, with diazepam being as a positive anxiolytic control. In the elevated plus-maze test, diazepam (1 and 2 mg/kg) or asiaticoside (5 or 10 mg/kg) increased the percentage of entries into open arms and of time spent on open arms. In the light/dark test, as with 1 mg/kg diazepam, asiaticoside (10 and 20 mg/kg) increased the time spent in the light area and the movement in the light area without altering the total locomotor activity of the animals. In the hole-board test, asiaticoside at 10 mg/kg significantly increased head-dipping counts and duration as well as diazepam (0.3 mg/kg). Thus, these findings indicated that asiaticoside exhibited an anxiolytic-like effect. Further studies will be required to assess the generality of present findings to other species and behavioural paradigms.

Keywords: Asiaticoside; Diazepam; Anxiolytic; Elevated plus-maze; Light/dark box; Hole-board test; Mice

1. Introduction

Asiaticoside, a major pentacyclic triterpenoid saponin component of *Centella asiatica* (L.) Urh, is described to have wound healing, antiulcer, antioxidant and anti-inflammatory activities. Guo et al. (2004) reported that the mechanisms by which asiaticoside exerted its antiulcer and anti-inflammatory effects may be associated with inhibition of NO synthesis by its inhibitory effects on inducible nitric oxide synthase (iNOS).

C. asiatica has been used for centuries in Ayurvedic and traditional Chinese medicine to alleviate symptoms of depression and anxiety. As the dietary supplements, *C. asiatica* is used to treat sleep disorders by patients with mental health conditions. Studies in the rat have shown that long-term pretreatment with *C. asiatica* enhanced elevated plus-maze performance, and attenuated the acoustic startle response (ASR). Furthermore, a double-blind, placebo-controlled study was undertaken to evaluate the effect of *C. asiatica* on the ASR in humans, and the preliminary findings suggested that *C. asiatica* had anxiolytic activity in humans as revealed by the ASR (Bradwejn et al., 2000). In addition, Chatterjee et al. (1992) reported that extract of *C. asiatica* inhibited significantly gastric ulceration induced by cold and restraint stress (CRS) in Charles–Foster rats, and could enhance gamma-aminobutyric acid (GABA) concentrations in the brain of the rat. However, Pretreatment with bicuculline methiodide (specific $GABA_A$-antagonist) at the dose level of 0.5 mg/kg im, reversed the antiulcerogenic activity of extract of *C. asiatica.*

These findings could be explained by the presumption that asiaticoside, major active constituent of *C. asiatica*, may interact with the receptor of gamma-aminobutyric acid (GABA). It is well-known that $GABA_A$ receptors mediate the anxiolytic effect of benzodiazepines and that GABA in the CNS exerts an inhibitory effect on stress-induced ulcerogenesis.

Based on the above suppose, we have investigated here its anxiolytic potential by using animal models sensitive to clinically effective anti-anxiety compounds.

2. Material and methods

2.1. Animals

Male Swiss mice (bred at the Experimental Animal center of Shenyang Pharmaceutical University) weighing 16–18 g were housed in groups of five in polycarbonate cages (cage size: 25 × 14 × 12 cm) for at least 10 days prior to testing. The

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Table 1
Effects of acute asiaticoside on the behavior of male mice in the elevated plus-maze test

Drug	Dose (mg/kg)	Latency to enter open arm (s)	Open arm entries	Closed arm entries	Total arm entries	Open arm time (s)
Vehicle	–	148.0±30.4	3.7±1.1	13.4±1.0	17.1±1.7	32.1±9.6
Diazepam	0.5	63.7±30.9*	7.9±1.8	13.5±0.7	21.4±1.4	75.7±14.9
	1	28.8±10.5**	8.3±1.4	12.1±1.2	20.4±2.0	87.3±14.0*
	2	22.8±8.7***	11.1±2.1**	12.0±1.1	23.1±2.4	102.5±17.8**
Asiaticoside	5	32.0±9.7**	8.1±1.2	12.9±1.0	21.0±1.0	83.7±12.4*
	10	47.4±17.7**	7.0±1.4	9.8±0.8	16.8±1.1	76.7±12.8
	20	62.6±30.8*	5.9±1.2	10.1±0.8	16.0±1.5	67.6±12.2
	40	73.0±18.1	6.0±0.9	12.0±1.1	18.0±1.4	59.5±10.2

Values represent mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ drug vs. control groups (one-way ANOVA followed by two-tailed Dunnett' t-test). $n=9–10$.

conditions of the breeding room were controlled (temperature 22±2 °C, relative humidity 60±10%, 12-h reversed light-dark cycle, lights on 19:00). Food and water were freely available with the exception of the brief test periods. Animals were handled gently every day for seven days. All mice were experimentally naive.

The experiments were performed following approval of the Committee of Experimental Animal Administration of the University and were in accordance with the National Institutes of Health Guide of the Care and Use of Laboratory Animals.

2.2. Drugs and treatments

Asiaticoside was purchased from Guangxi Changzhou Natural Products Development Co., Ltd. (Nanning, China). Diazepam was purchased from Hubei Pharmaceutical Factory (Hubei, China). Tween-80 was obtained from Shenyang Dongxing Reagent Factory (Shenyang, China). Diazepam and asiaticoside were both ultrasonically dispersed in distilled water containing Tween-80 (0.5%). All drugs were prepared immediately before use and were administered PO in a volume of 10 ml/kg body weight. Control group received the corresponding vehicle.

3. Behavioral tests

3.1. Elevated plus-maze test

The elevated plus-maze consists of four arms (30×5 cm) elevated 45 cm above the floor, with each arm positioned at 90° relative to the adjacent arms. The two enclosed arms had 30 cm walls and to facilitate grip on the open arms these included a raised edge of 0.25 cm. Open and closed arms were connected via a central area (5×5 cm) to form a plus sign. The maze floor was constructed of black Plexiglas and the wall of the enclosed arms was constructed of clear Plexiglas (Chen et al., 2003). Four 25-W red fluorescent lights arranged as a cross at 100 cm above the maze were used as the source of illumination. Mice ($n=9–10$ per group) were randomly assigned (with a slight adjustment for matched body-weight) to eight experimental groups (vehicle-control, 0.5, 1, 2 mg/kg diazepam or 5, 10, 20, 40 mg/kg asiaticoside). Drug was administration PO 60 min prior to the test. Testing commenced by placing a mouse on the central platform of the maze facing an open arm. The number of entries into and the time spent on each of the two types of arms were recorded during the 5-min trial. An arm entry was defined as all four paws having crossed the dividing line between an arm and the central area. The plus-maze was thoroughly cleaned after each trial.

3.2. Light/dark transition test

The light/dark box consists of two compartments: one light area (27 L×27 W×27 H cm, 400 lx) illuminated by 100-W desk lamp was painted white, and the other dark area (18 L×27 W×27 H cm, 4 lx) was painted black. The floor of the light area was divided into nine equal squares (9×9 cm) by black lines and the dark area was divided into six equal squares (9×9 cm) by white lines. The two compartments were separated by a partition with a tunnel (7.5×7.5 cm) to allow passage from one compartment to the other (Mi et al., 2005). Mice ($n=9–10$ per group) were randomly assigned (with a slight adjustment for matched body-weight) to six experimental groups (vehicle-control, 1, 2 mg/kg diazepam or 10, 20, 40 mg/kg asiaticoside). Drug was administration PO 60 min prior to the test. The experiments were performed between 09:00 and 14:00, i.e. in the middle of the dark phase. Animals were placed in the center of the dark area facing the wall opposite to the tunnel (Costall et al., 1989). The following parameters were recorded during 5 min: 1) latency time for the first crossing to the lit compartment, 2) the number of crossings between the light and the dark compartment, 3) the total time spent in the illuminated part of the cage, 4) the overall movements (squares entered) in both areas.

3.3. Hole-board test

The hole-board apparatus consists of Perspex box (60×60×35 cm) with four equidistant holes 2 cm in diameter in the floor. The floor of the box was positioned 12 cm above the ground and divided in to nine (20×20 cm) squares. Mouse were placed singly in the center of the hole-board, and during a 5-min trial the following measures were recorded: the number of head-dips, the time spent head-dipping, the number of rears, the latency to the first head-dipping and the total locomotor activity. A head dip was scored if both eyes disappeared into the hole (Moreira et al., 2000). Mice were randomly allocated to the following groups ($n=9–10$ per group): vehicle control, diazepam (0.1, 0.3, 0.6, 1.2 mg/kg), asiaticoside (5, 10, 20, 40 mg/kg). At the end of each test the animal was removed and the box was cleaned.

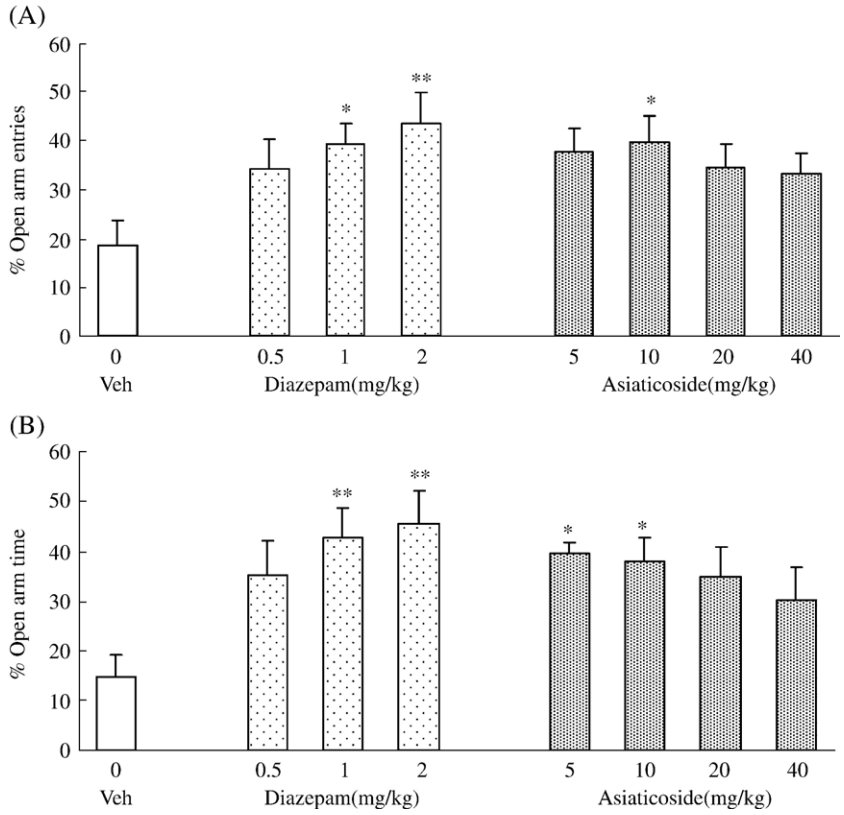

Fig. 1. Effects of asiaticoside and diazepam in the elevated plus-maze in male mice. (n=9–10). Results are expressed as means±SEM. Mice were given a 5-min test 60 min after PO with diazepam (0.5, 1, 2 mg/kg) or asiaticoside (5, 10, 20 and 40 mg/kg). Significant differences calculated from two-tailed Dunnett' t-test are expressed by *P<0.05, **P<0.01 drug vs. corresponding vehicle after one-way ANOVA.

In the above three tests, the animals were transferred in their home cages from the animal unit to the experimental room 1 h before each test session. After the habituation period to the laboratory the mice were subjected to the test. All the apparatus were cleaned thoroughly between trials to remove any trace of odor. Test sessions were recorded via an overhead video camera linked to a monitor and video-recorder in an adjacent room, and all behavioral recordings were carried out with the observer unaware of the treatment of the mice.

3.4. Statistical analyses

All analyses were performed using the software SPSS V11.5 for windows. All data were represented as mean±SEM values. Data were analyzed by one-way ANOVA. Whenever ANOVA was significant, further comparisons between vehicle-and drug-treatment groups were performed using the Dunnett's t-tests. The level of statistical significance adopted was P<0.05.

4. Results

4.1. The elevated plus-maze test

The effects on the behavior of mice in the elevated plus-maze are summarized in Table 1 and Fig. 1. ANOVA for latency to enter open arm yielded [F(7,69)=3.646, P<0.01], open arm entries yielded [F(7,69)=2.358, P<0.05], for time spent in the open arm [F(7,69)=2.622, P<0.05], for total arm entries [F(7,69)=2.520, P<0.05], for the ratio open/total time [F(7,69)=2.832, P<0.05], for the ratio open/total entries [F(7,69)=2.160, P<0.05] even though the follow-up Dunnett's t-test did not show differences between control and drug conditions in total

Table 2
Effects of acute asiaticoside on the behavior of male mice in the light/dark transition test

Drug	Dose (mg/kg)	Time in light area (s)	Number of transition	Latency (s)	Movements in light area	Overall movement
Vehicle	–	95.6±7.3	17.1±1.8	27.8±7.7	32.3±4.8	88.6±8.7
Diazepam	1	144.9±14.9**	19.7±2.5	19.3±5.3	58.2±6.6**	109.8±9.6
	2	122.0±7.8	25.8±2.4**	20.3±3.1	75.0±4.0***	151.7±4.4***
Asiaticoside	10	135.5±6.0*	20.2±0.9	13.0±2.4	48.8±1.9*	94.5±3.9
	20	133.0±7.7*	18.6±1.4	19.6±5.4	49.4±3.9*	98.2±8.4
	40	128.9±12.1	19.8±2.0	25.8±5.6	46.4±4.4	92.3±7.5

Values represent mean±SEM. * P<0.05, **P<0.01, ***P<0.001 drug vs. control groups (one-way ANOVA followed by two-tailed Dunnett' t-test). n=9–10.

Table 3
Effects of acute asiaticoside on exploratory behavior in male mice tested on the hole-board test

Drug	Dose (mg/kg)	Head-dip latency (s)	Head-dip counts	Head-dip duration (s)	Locomotion	Rearing counts
Vehicle	–	84.8±19.8	10.5±1.3	17.1±3.2	79.1±6.6	10.5±1.2
Diazepam	0.1	68.3±10.2	12.6±1.0	27.2±3.5	82.4±6.3	16.7±2.8
	0.3	71.0±15.2	16.1±0.8*	40.2±3.0***	88.7±7.1	13.1±3.0
	0.6	57.1±8.8	14.5±0.8	35.3±3.7**	89.6±6.6	17.2±1.8
	1.2	82.0±15.4	12.8±1.2	25.7±3.5	122.0±10.0**	23.6±3.0**
Asiaticoside	5	90.3±14.5	13.1±1.5	29.9±4.0	88.0±8.0	16.3±2.7
	10	72.3±10.8	16.8±1.6**	37.2±4.3**	92.1±6.6	16.1±2.4
	20	66.5±10.9	14.8±1.6	35.1±4.3**	82.6±6.0	17.8±2.8
	40	55.7±8.3	13.2±1.0	32.9±2.4*	80.0±8.8	16.9±2.6

Values represent mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ drug vs. control groups (one-way ANOVA followed by two-tailed Dunnett' t-test). $n=9–10$.

arm entries. Contrasts with control groups revealed that diazepam reduced latency to enter an open arm from 0.5 to 2 mg/kg ($P<0.05$, $P<0.01$and $P<0.001$ respectively), and the doses of 1 and 2 mg/kg increased time spent in the open arm ($P<0.05$ and $P<0.01$, respectively), the ratio open/total entries ($P<0.05$ and $P<0.01$, respectively) and the ratio open/total arm time (both $P<0.05$). Asiaticoside, significantly reduced latency to enter open arm from 5 to 20 mg/kg ($P<0.01$, $P<0.01$and $P<0.05$ respectively), increased the ratio open/total arm time at doses of 5 and 10 mg/kg (both $P<0.05$), the ratio open/total entries at dose of 10 mg/kg ($P<0.05$) and time spent in the open arm at dose of 5 mg/kg ($P<0.05$).

4.2. The light/dark-transition test

Results of the light/dark test are shown in Table 2. ANOVA indicated a significant effect on time in the light area [$F(5,52)=3.104$, $P<0.05$], the number of inter-compartment transitions [$F(5,52)=2.440$, $P<0.05$], the number of line crossings in the light area [$F(5,52)=10.514$, $P<0.001$], the overall number of line crossings [$F(5,52)=10.831$, $P<0.001$], but not for the latency to enter the light area [$F(5,52)=1.053$]. Comparisons between the vehicle control group and experimental groups (Dunnett's t-test) indicated that diazepam had significantly increased the number of line crossings in the light area at doses of 1 and 2 mg/kg (both $P<0.01$), the time spent in the light area at dose of 1 mg/kg ($P<0.01$), the number of transitions between the two compartments and the overall number of line crossings at dose of 2 mg/kg ($P<0.01$ and $P<0.001$, respectively). Asiaticoside, at the doses of 10 and 20 mg/kg, significantly increased the time spent in the light area and the number of line crossings in the light area (both $P<0.05$).

4.3. The Hole-board test

Hole-board measures are summarized in Table 3. ANOVA demonstrated significant effects on head-dip counts [$F(8, 77)=2.560$, $P<0.05$], head-dip duration [$F(8, 77)=4.186$, $P<0.01$], squares crossed [$F(8, 77)=3.221$, $P<0.01$] and rear counts [$F(8, 77)=2.278$, $P<0.05$]. Control and experimental groups were compared with Dunnett's t-test procedure. Diazepam induced significant increase in head-dip counts at dose of 0.3 mg/kg ($P<0.05$), head-dip duration at doses of 0.3 and 0.6 mg/kg ($P<0.001$ and $P<0.01$, respectively), squares crossed and rear counts at 1.2 mg/kg (both $P<0.01$). Asiaticoside, from 10 to 40 mg/kg, caused significant increase in head-dip duration ($P<0.01$ in three cases), and the dose of 10 mg/kg could also increase head-dip count ($P<0.01$). Both diazepam and asiaticoside failed to significantly reduce head-dip latency.

5. Discussion

The results of the present study demonstrate that asiaticoside has an anxiolytic-like effect in the elevated plus-maze, light/dark box and hole-board test.

One of the most widely used animal models for screening putative anxiolytics is the elevated plus-maze, in which rodents show an avoidance of exposed open areas of the maze, which are presumed to be the most aversive, and a preference for sections enclosed by protective walls (Weiss et al., 1998). Conventional anxiety indices in the elevated plus-maze test comprise percent open arm entries and percent time spent in these areas in the maze, with anxiolytics generally increasing and anxiogenics decreasing these measures. In the present study, 0.5–2 mg/kg diazepam dose-dependently reduced latency to enter open arm, increased the absolute number and percent number of open arm entries, the absolute time as well as the percent time spent on open arms. Asiaticoside significantly reduced latency to enter open arm, increased the percent number of open arm entries and the percent time spent on open arms, without altering the number of total arm entries. The number of open arm entries and the time spent on open arms tended to be increased following asiaticoside, however, these changes did not reach statistical significance. Both diazepam and asiaticoside did not significantly change the number of closed arm entries, a parameter thought to reflect locomotor activity.

Light/dark box is also widely used in rodents as a model for screening anxiolytic or anxiogenic drugs, which is designed to exploit the tendency of rodents to explore a novel environment when confronted with the aversive properties of a brightly lit area. Anxiolytics have been found to increase locomotion and time spent in the light zone, whereas anxiogenics decrease them (Imaizumi et al., 1994).

In preliminary study of the light/dark-transition test in which mice were placed in the center of the lit area, we found that the mice showed two types of avoidance response (active vs.

passive avoidance), which corresponds to two different latencies. Half the control group had short latency followed by an extended length of time in the dark box and very few transitions. It may be assumed that the mice fled the brightness of the box. The other half of the control group had long latency (even >180s) with inhibition of locomotor and exploratory activities (unpublished data). Due to two different latencies, the time spent in the light area, which includes latency time for the first crossing to the dark compartment, could not appropriately reflect the emotional state of the mice. So in the present study, animals were placed in the center of the dark area. As shown in the table, the control group had stable latency, and both diazepam and asiaticoside reduced the latency to enter the light area, although no significant level was reached.

The number of transitions between the two compartments was a controversial parameter. Crawley and some authors reported increases in transitions between the two compartments by rodents after anxiolytics administration, while others reported no significant changes after treatment with anxiolytics. Furthermore, Lepicard et al. (2000) reported that the number of transitions reflected both anxiety and exploration, whereas the time spent in the light area was a stronger indication in the study of anxiety. This conclusion is based on the fact that the time spent in the light area provided the most consistent dose-effect results (Young and Johnson, 1991). These observations are in good agreement with our results. The diazepam (2 mg/kg) enhanced transitions and the overall number of line crossings, without inducing significant increase in the time spent in the light area. The diazepam (1 mg/kg) and asiaticoside (10 and 20 mg/kg) prolonged the time spent in the light area and induced a selective increase in the movement in the light area which was not due to a generalized increase in motor behavior because total activity remained unchanged.

The hole-board has gained popularity as a model of anxiety, offering "a simple method for measuring the response of an animal to an unfamiliar environment, with advantages that several behaviors can be readily observed and quantified in this test" (Takeda et al., 1998). Based on previous reports, Takeda et al. indicated that head-dipping behavior was sensitive to changes in the emotional state of the animal, and suggested that the expression of an anxiolytic state in animals might be reflected by an increase in head-dipping behavior (Takeda et al., 1998). In agreement with previous findings, in mice low doses of benzodiazepines (0.3 mg/kg diazepam in this study) will frequently increase the number and duration of head-dip in the hole-board test (Lister, 1987a). On the other hand, decreases in exploration can also be observed with concomitant increase in locomotor activity following moderate doses of benzodiazepines (1.2 mg/kg diazepam in this study) (Lister, 1987a,b). In the current study, the 10 mg/kg dose of asiaticoside significantly increased head-dip counts and duration indicating an anxiolytic effect.

Our preliminary study showed that asiaticoside did not produce the anxiolytic effect at doses lower than 5 mg/kg or higher than 40 mg/kg such as 80 mg/kg on the elevated plus-maze (unpublished data). Asiaticoside exhibited an inverted U-shaped dose-response curve for the percentage of open arm entries based on the present and preliminary set of data. The bell-shaped dose-response curve, observed in our experiment in animal anxiety models, is characteristic of a number of drugs and substances with anxiolytic properties, such as, pentobarbital (Vogel et al., 1971), buspirone (Przegalinski et al., 1989), L-701324 an antagonist at glycineB receptors (Przegalinski et al., 1998) and LY 354740, a group II mGluR agonist (Klodzinska et al., 1999). As indicated by the indices which reflect locomotor activity, such as closed arm entries in the elevated plus-maze, overall movement in the light/dark box and locomotion in the hole-board test, the lack of an anxiolytic effect at high doses did not seem to prevented by motor-disturbing effect. The pharmacological mechanism that might account for the anxiolytic effect of asiaticoside has yet to be determined. Recently, we found that asiaticoside antagonized the convulsion induced by the noncompetitive $GABA_A$ receptor antagonist, picrotoxin (unpublished data). This suggests that the interaction between asiaticoside and $GABA_A$ receptors may be important for the anxiolytic effect of asiaticoside.

To summarize, all the data presented here indicate that asiaticoside could induce anxiolytic-like effect in the plus-maze, light/dark box and hole-board test in mice though its mechanism is still unclear and required to be further investigated.

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