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Original Article

A Lack of tolerance to the anxiolytic action of *Echium amoenum*

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Abstract

The anxiolytic effect of the flower of *Echium. amoenum* was shown in several experimental studies in mice. The present study was aimed to determine whether tolerance develops to anxiolytic action of *E. amoenum* in mice. NMRI male mice were injected intraperitoneal with hydroalcoholic extract (12.5, 25 and 50 mg/kg) or saline once each day (8 am) for period of 7 days and then tested on light/dark box model. Anxiolytic effect was determined by light/dark box and elevated plus-maze. According to the results, hydroalcohoic extract of *E. amoenum* when given both acutely and chronically (7 days) at 25 and 50 mg/kg, significantly increased the time in the illuminated zone. The number of transitions in the light/dark apparatus, however, was not significantly altered by the tested doses of the plant. Diazepam at 0.5 and 1 mg/kg produced anxiolytic effect in both model of anxiety, namely, the light/dark box and elevated plus-maze. No tolerance was developed to the anxiolytic effect of *E. amoenum* extract after 7 days of treatment. Our results suggest that one week treatment with extract of the *E. amoenum* does not produce tolerance to its anxiolytic action. Longer period of treatment using implant procedure is probably necessary to cause molecular changes in order to induce tolerance.

Keywords: Anxiety; *Echium amoenum;* Elevated plus-maze; Light dark box; Tolerance

INTRODUCTION

Borage (*Echium amoenum*) is a wild annual herb that belongs to Boraginaceae family which grows in large parts of Europe, Mediterranean region, and also in northern parts of Iran (1). The flowers and the leaves of borage are used for medicinal purposes in various parts of the world. The flowers and the leaves of borage are used medicinally in the West for the treatment of stress and circulatory heart diseases, pulmonary complaints, as poultice for inflammatory swellings, as a diuretic (due to potassium nitrate), as a laxative, emollient and demulcent (2,3). The decoct of dried violet-blue petal of *E. amoenum* (known in Iran as Ox-tongue) has been widely advocated for a variety of effects such as demulcent, anti-inflammatory and analgesic, anxiolytic, sedative and other psychiatric illnesses in folk medicine of Iran (1,4). Phytochemical studies on the petals of *E. amoenum* have revealed the presence of many chemicals such as rosmarinic acid, anthocyanidine, flavonoids, g-linolenic acid and trace amount of alkaloids, a clear lemon-yellow volatile oil with d-cadinene as the major component (5-7).

The anxiolytic effect of the flower of *E. amoenum* was shown in two separate experimental studies in mice (8,9). In both studies and indeed in most of other studies that have been carried out on *E. amoenum*, the extract was given acutely. It is custom to the native people that this plant extract must be consumed as long as the stress and anxiety exists. Therefore it is not quite clear whether the effectiveness of the plant extract declines with period of usage. As most people use the decoct of the plant over a long period of time, this study was aimed to determine whether tolerance develops to anxiolytic properties of the plant extract after several days of administrations. Tolerance to the synthetic anxiolytic drugs such as benzodiazepines limit their use and hence replacement with herbal remedies without such short come could provide real cure over long time treatment (10).

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The aim of the current study was therefore to compare the acute and chronic action of *E. amoenum* extract in light/dark box and the elevated plus-maze models of anxiety in mice.

MATERIALS AND METHODS

Preparation of the plant material

Flowers of *E. amoenum* were collected from North of Iran. For preparation of hydroalcoholic extract, air-dried and powdered flowers of the plant (200 g) were macerated with 600 ml of ethanol and water (8:2) for 48 h. The extract was then shaken, filtered and evaporated in a rotating evaporator under reduced pressure to give a residue (5.5 g). The residue was dissolved in normal saline for final suitable concentrations.

Animals

Male NMRI mice (Pasteur institute, Tehran, Iran) weighing 25-30 g were housed in cages of six at room temperature in a 12 h light/dark cycle. Food and water were available *ad libitum*. Tests were performed only after the mice had acclimated to the above environment. All experiments were conducted between 09:00 and 13:00 every day to avoid any temporal factor (e.g., circadian rhythm). Each animal was used for only one experimental condition. All experiments were carried out in accordance with international guideline outlined in the Guide for the Care and Use of Laboratory Animals (11).

Light/dark box test

The light/dark paradigm was based on the design of Crawley and Goodwin with slight modifications (12). The test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior in response to novel environment and light. An increase in the time spent in the illuminated area is thought to reflect anxiolytic-like responding. The apparatus consisted of two compartments: an open topped rectangular box (45 × 27 × 30 cm high), is divided into small area (18 × 27 cm) and a large (27 × 27 cm) area with an opening door (7.5 × 7.5 cm) located in the center of the partition at floor level. The close-topped small compartment was painted black and illuminated by a dim red light, whereas the open-topped large compartment was painted white and brightly illuminated. The test was conducted in a sound-attenuated room. Mice were placed individually in the middle of the light compartment facing the doorway separating the two compartments. A 5-min test was given during which the latency to enter the brightly lit area with all four paws and the number of transitions between the two compartments was recorded. Between each trial, the maze was wiped clean with a damp sponge and dried with paper towels. Anxiolytic activity was evaluated by time spent in the illuminated compartment, and the number of transitions.

Elevated plus-maze

Anxiolytic activity was measured using the elevated plus-maze apparatus as described by Lister (13). The maze consisted of two open (30 × 5 × 0.2 cm) and two closed (30 × 5 × 15 cm) arms, extending from a central platform (5 × 5 cm) and elevated to a height of 45 cm above the floor. The entire maze was wooden and painted black. Experiments were conducted in a quiet room that was illuminated by only a dim light. Mice were administered a single i.p. dose of diazepam, vehicle or extract 30 min before their placement on the EPM. At the start of the session, the mouse was placed on the center of the maze facing an open arm, and the number of entries and the time spent in closed and open arms were recorded during a 5 min observation period. Arm entries were defined as entry of all four paws into an arm. The percentage of open arm entries (100 × open/total entries) and open arm time (100 × open/open + closed arm time) was calculated for each animal. Between each trial, the maze was wiped clean with a damp sponge and dried with paper towels.

Statistics

All data are expressed as mean ± SEM. The differences among multiple groups were first analyzed by ANOVA. When a statistical significance was detected, Dunnett's t test was used to determine statistical significance between multiple testing groups and the

corresponding control. Student's t test was used to evaluate the statistical significance between two groups. These statistical comparisons were analyzed using SigmaStat software (San Jose, CA). $P<0.05$ was considered significant.

RESULTS

Light/dark box test

A) Acute action of the E. amoenum

In the light/dark box, three doses of diazepam (0.5, 1 and 1.5 mg/kg) were tested first to find out the minimum effective dose of diazepam on this system (data not shown). Diazepam at 1 mg/kg was found to significantly increase both measured parameters, i.e. the number of transition in the light/dark box and also the time in the illuminated zone ($P<0.05$, Fig. 1A and 1B). Based on our previous studies, three doses (12.5, 25 and 50 mg/kg) of hydroalcoholic extract of *E. amoenum* were evaluated for the anxiolytic activities (8). As shown in Fig. 1A, *E. amoenum* at the doses of 25 and 50 mg/kg significantly increased the time spent by mice in the illuminated side by 181% and 209%, respectively ($P<0.05$). The number of transitions in the light/dark apparatus, however, was not significantly altered by the tested doses of the plant (Fig. 1B).

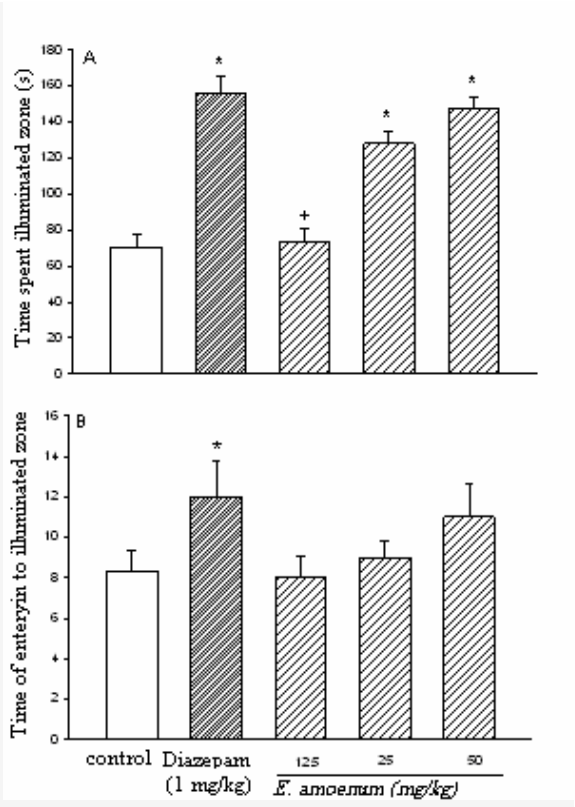

Fig. 1. Effect of single treatment of diazepam and extract of *E. amoenum* on (A) the time spent in the illuminated section and (B) the number of transitions in the light/dark box apparatus over a 5 min period of time. Various doses of *E. amoenum* (12.5, 25 and 50 mg/kg, i.p.) or vehicle were injected 30 min prior to test. Bar represents mean ± SEM (n=6). P values for group comparisons were made using One-way ANOVA followed by post hoc Dunnett's test (*$P<0.05$ versus the vehicle treated control group and $^{+}P<0.05$ versus the diazepam treated group).

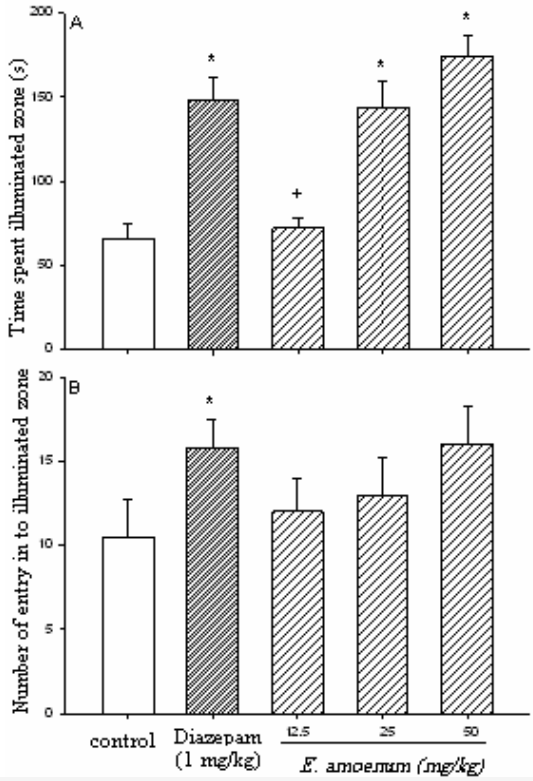

Fig. 2. Effect of chronic treatment (1 week) with diazepam *E. amoenum* extract on (A) the time spent in the illuminated section and (B) the number of transitions in the light/dark box apparatus over a 5 min period of time. Various doses of *E. amoenum* (12.5, 25 and 50 mg/kg, i.p.) or vehicle were injected 30 min prior to test. Bar represents mean ± SEM (n=6). P values for group comparisons were made using One-way ANOVA followed by post hoc Dunnett's test (*$P<0.05$ versus the vehicle treated control group and $^{+}P<0.05$ versus the diazepam treated group).

Table 1. Behavioral parameters recorded in the plus-maze after treating mice with acute and after 7 days diazepam treatment

Treatment	Acute		Chronic	
	Open arm times (%)	Open arm entries (%)	Open arm times (%)	Open arm entries (%)
Control	20 ± 5.5	35 ± 0.9	22 ± 6.0	38 ± 1.7
Diazepam (0.5 mg/kg)	49 ± 7.1*	54 ± 1.7	47 ± 9.4*	60 ± 4.1
Diazepam (1 mg/kg)	58 ± 5.6*	65 ± 1.6*	57 ± 12.0*	70 ± 2.7*

Effects of acute and chronic diazepam treatment on the percentage of time spent in the open arms and the percentage of arm entries of the elevated plus-maze during a 5 min test in mice. Diazepam or saline, were injected 30 min prior to test. Data are presented as mean values (± SEM) from group of 6 mice. *P<0.05 compared with vehicle-treated control.

B) Chronic action of E. amoneum

In a similar fashion to its acute action, the plant extract at 25 and 50 mg/kg increased the time spent in the illluminated zone of the apparatus (Fig. 2A). One week daily administration of the *E. amoneum* extract did not significantly alter the number of transitions in the light/dark box (Fig. 2B).

Elevated plus-maze

Acute and chronic effects of diazepam

In the elevated plus-maze, the behaviour observed confirmed the anxiolytic activity of diazepam as reported previously (14). Table 1 shows the anxiolytic activity of diazepam at 0.5 and 1.0 mg/kg after a single injection and 7 days treatment. Acute and chronic injection of diazepam at both doses of 0.5 and 1 mg/kg significantly increased the time spent in the open arms (P<0.05, Table 1).

DISCUSSION

The objective of this study was to evaluate the anxiolytic effect of hydroalcoholic extract of *E. amoenum* after acute and chronic doses in light/dark box model of anxiety. The anxiolytic action of hdyroalcoholic extract of *E. amoenum* was previously demonstrated in elevated plus-maze model of anxiety (8). Similar findings were also observed when we tested the plant extract acutely on the light/dark box model anxiety. In this study, the anxiolytic action of the plant extract was demonstrated at two doses smaller than 50 mg/kg. Doses larger than 50 mg/kg were not used as in previous works, doses above 50 mg/kg have caused severe sedative effects (8).

Both acute and chronic exposure to diazepam resulted in increased percentage of transition in the light/dark box and the time spent in the illuminated zone. The acute anxiolytic action of diazepam is consistent with the results of numerous previous studies, where diazepam and other benzodiazepines were shown to produce robust anxiolytic effects in a variety of anxiolytic screening procedures, including conflict model (15), elevated plus-maze procedures (16), other non-punishment procedures (17,18), and drug discrimination models (19). These findings suggest that tolerance to the anxiolytic effects of diazepam was not observed following chronic exposure. This could be due to either length of treatment or the dose. There are a wide variation in anxiolytic dose of diazepam varying from 0.5-3 mg/kg. It could be possible that the higher dose and longer duration of treatment is require to produce tolerance to anxiolytic effects.

In a similar fashion to diazepam, chronic administration of the plant extract significantly increased the time spent in the illuminated zone of the apparatus indicating the absence of tolerance to the anxiolytic action of the *E. amoenum*. Compared with vehicle controls, animals treated with the plant extract exhibited enhanced percentage of illuminated time after the challenge doses with 25 and 50 mg/kg. The number of transitions in to the light/dark box, however, did not seem to be affected by either acute or chronic treatment of the *E. amoenum*. This type of behavior was only confined to the plant extract, i.e. diazepam at the given dose of 1 mg/kg did manage to increase the number light/dark box transition after the challenge dose of 1 mg/kg. The lack of reduction in the anxiolytic effects of the E. amoenum challenge

again pointed to lack of tolerance development. While it is possible that the acute sedative effects of the plant extract may interfere with anxiety-related behaviors, such action could not occur at 25 mg/kg dose. Reduction in locomotor activity by this plant extract was previously shown to occur at doses above 50 mg/kg (8). Data from previous studies have shown that sedative effect to *E. amoenum* could occur at 50 mg/kg or above.

Previous research has reported that the dose and the duration of drug exposure may play a role in the detection of tolerance to the anxiolytic effects (20, 21). It is possible that continuous exposure to this low dose for a longer duration may have resulted in the development of tolerance to the anxiolytic effects of *E. amoenum*. It is documented that with diazepam for example, when animals are treated for 21 days (0.3 or 1 mg/kg), tolerance was observed and higher daily dose of diazepam (3 mg/kg) for the same period of time did not produce tolerance to diazepam's anxiolytic action (20). Longer period of treatment together with the right dose of a drug in some cases is required to make the necessary adaptation for the insensitivity to drugs appears.

Fluctuation in the body dose of the drug could also affect the development of tolerance. To prevent daily rise and fall in drug concentration in the body, it is best to use the drugs in the form of implant. However, with the plant extract this procedure can not routinely be done. The period of treatment and the doses of *E. amoenum* extract in this study were based on our previous experience. While setting up the model, we noticed that doses larger than 100 mg/kg were not tolerated by the animals. A maximum anxiolytic effect was observed at 50 mg/kg.

CONCLUSION

In conclusion our results suggest that one week treatment with extract of the *E. amoenum* does not produce tolerance to its anxiolytic action. Longer period of treatment using implant procedure is probably necessary to cause molecular changes in order to induce tolerance. Similar analogy also applies to diazepam, i.e. longer duration of treatment with variable doses of diazepam should be applied to induce tolerance.

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