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Anxiolytic Effects of Acute Injection of Hydro-Alcoholic Extract of Lettuce in the Elevated Plus-Maze Task in Rats

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Background: Anxiety is a physiological state characterized by cognitive, somatic, emotional, and behavioral components. There is some evidence in traditional medicine for the effectiveness of lettuce (*Lactuca sativa*) in the treatment of anxiety in humans.
Objectives: The present study investigated the effects of a hydro-alcoholic extract of lettuce on rat behavior in the elevated plus-maze (EPM) and the results compared with the effects of diazepam.
Materials and Methods: Adult male Wistar rats weighing 200 – 240 g were used in the present study. Seven different groups of rats received an intraperitoneal (IP) injection of lettuce extract (50, 100, 200 mg/kg), diazepam (0.3, 0.6, or 1.2 mg/kg), or vehicle (control group), 30 minutes before entering the EPM test. The total distance covered by the animals, the percentage of entries into the open arms of the EPM, the time spent in the open arms, and the number of entries into the closed arms were recorded for a 5 minutes duration.
Results: An IP injection of both diazepam and lettuce extract before an EPM trial significantly increased the percentage of open arm entries and the time spent in the open arms. Diazepam decreased the total distance covered by the animals and the number of closed arm entries, whereas lettuce extract had no effect on these parameters. Locomotor activity was not significantly changed by the lettuce extract.
Conclusions: Acute administration of lettuce extract has an anxiolytic profile in rats similar to that of anxiolytic diazepam at low dose. Future investigations are essential for better understanding of the anxiolytic properties and neurobiological mechanisms of lettuce extract.

*Keywords:*Anxiety; Lettuce (*Lactuca sativa*); Anti Anxiety Agents; Diazepam; Rat

1. Background

Anxiety is among the most common, and most treatable mental disorders. Emotional, cognitive, behavioral, and physical components can all be present in anxiety. Anxiety affects one-eighth of the total population throughout the world, and it has become an important area of research in psychopharmacology during this decade (1). Benzodiazepines are the major class of compounds used in anxiety and they have remained the most commonly prescribed treatment for anxiety (2). However, the realization that benzodiazepines present a narrow safety margin between an anxiolytic effect and those causing unwanted side effects has prompted considerable research in order to evaluate new compounds with less undesirable side effects (3, 4).

Lactuca sativa, a plant commonly known as lettuce, is a member of the Compositae family (5). Lettuce is an important leafy vegetable, which is also known for its medicinal properties (6). The major components present in lettuce extract are 15-oxalyl and 8-sulfate conjugates of guaianolide sesquiterpene lactones, lactucin, deoxylactucin, and lactucopicrin (7). Anticonvulsant and sedative-hypnotic effects have been demonstrated for the leaves of this plant (5, 8). The whole plant has been used in the treatment of stomach problems to stimulate digestion, to enhance appetite and relieve inflammation (8, 9), via the anti-inflammatory activities of triterpene lactones (10). An extract of lettuce seed contains triterpenoids, saponins and simple phenols, and possesses anti-nociceptive and anti-inflammatory effects (8). Lettuce extract has also been shown to possess large amounts of phytochemicals that include flavonoids and polyphenols which have the potential to serve as antioxidants (6, 8, 11). The elevated plus-maze (EPM) is one of the most widely used models of animal anxiety, and it was developed based on the observation that rats avoid open elevated alleys, and on the assump-

Implication for health policy/practice/research/medical education:
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tion that this avoidance is generated by fear (12). An extensive study by Pellow et al. (1985) validated this test through the use of behavioral, physiological and pharmacological approaches (13).

2. Objectives

There is evidence for the anxiolytic activity of lettuce extract in chronic oral feeding in mice (6, 14). In order to extrapolate these findings more precisely to the treatment of anxiety, research using behavioral methods in rat species is useful. On the basis of these considerations, this study was designed to characterize the anxiolytic-like activity of a hydro-alcoholic extract prepared from lettuce leaves, using an EPM following an acute intraperitoneal (IP) injection.

3. Materials and Methods

3.1. Experimental Animals

Male Wistar rats weighing 200-240 g were purchased from the Razi Institute, Tehran, Iran. These animals were transported to a room adjacent to the test laboratory 72 hours before the test. They were housed in groups of five per cage, under a 12:12 dark/light cycle (lights on at 07:00 h) at 23°C ± 1°C and given free access to food and water. Groups of 10 rats were randomly assigned to different treatment groups and tested in varying order. Animals were divided into seven groups: control group, diazepam (0.3, 0.6, 1.2 mg/kg IP), and lettuce groups (50, 100, 200 mg/kg IP). Animals were tested repeatedly under the same experimental conditions. All experiments were carried out in a quiet room under controlled light conditions between 11:00 a.m. and 3:00 p.m. Behavioral observations took place in soundproof rooms during the same period of the day to reduce the confounding influence of diurnal variation in spontaneous behavior. Each animal was tested only once.

All animals received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals', prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985) and the study was also approved by the local ethics committee of Hamadan University of Medical Sciences. The minimum number of animals and duration of observation required to obtain consistent data were employed.

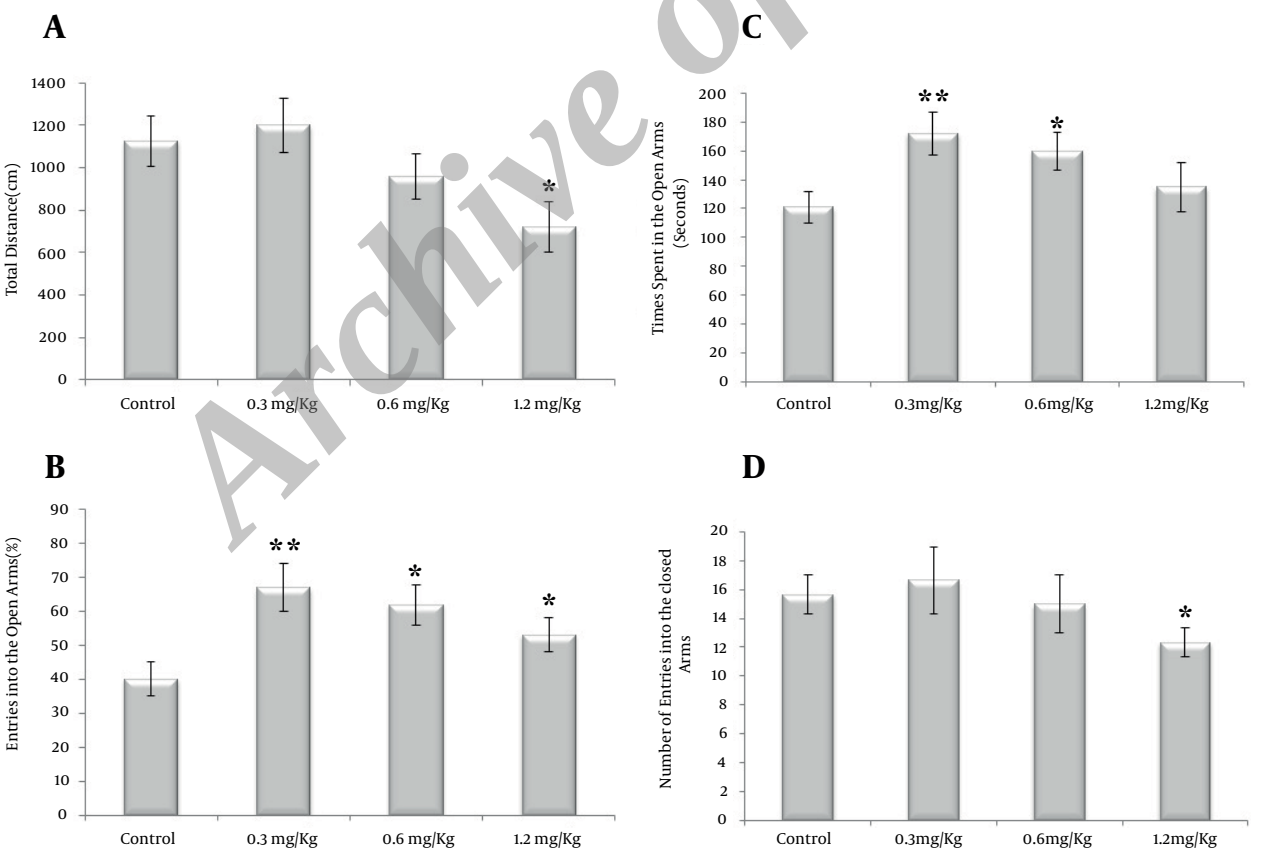

Figure 1. The Effects of Diazepam (0.3, 0.6, 1.2 mg/kg IP) on the Total Distance Covered by Rats (A), the Percentage of Entries into the Open Arms of the EPM (B), Time Spent in the Open Arms (C) and the Number of Closed Arm Entries (D) During the 5 Minutes Test Session. Data represent means ± SEM. *: $P < 0.05$, **: $P < 0.01$

3.2. Drugs

Lettuce leaves were collected and identified in the Botanic Institute of Hamadan University of Medical Sciences. The plant material was dried at 40°C with air circulation, then ground and extracted with 70% ethanol by percolation at room temperature. The extracts were dried at 40°C under vacuum, and finally freeze-dried. The pharmacological assays were carried out with aqueous suspensions of the dried extract. The doses are expressed as mg of dried extract/kg per rat. Diazepam (Sigma-Aldrich, Germany) was diluted to 2 mg/20 mL, with 0.9% NaCl containing ethanol (372 mg/20 mL) (Sigma-Aldrich). Different concentrations of the lettuce extract were prepared by dissolving the extracts in 0.9% NaCl containing ethanol to form a homogeneous suspension.

3.3. Elevated Plus Maze

Anxiolytic activity was measured using the elevated plus-maze test. This test has been widely validated to measure anxiety in rodents (13, 15-25). Briefly, the apparatus consisted of two open arms (50 × 10 cm each), two enclosed arms (50 × 10 × 50 cm each), and a central platform (10 × 10 cm), arranged in such a way that the two arms of each type were opposite to each other. The maze was elevated 100 cm above the floor. Thirty minutes after an IP injection of the extract (50, 100, 200 mg/kg), diazepam (0.3, 0.6 and 1.2 mg/kg), or specific vehicle (0.9% NaCl containing ethanol as a control group), each animal was placed at the center of the maze facing one of the enclosed arms. During the five-minute test period, the number of open and enclosed arm entries, plus the time spent in the arms (26), and the distance travelled as measures of locomotor activity were recorded (27). Entry into an arm was defined as the point when the animal placed all four paws onto the arm. Animal behaviors in the experimental sessions were recorded by a video camera located above the maze, interfaced with a monitor and a computer in an adjacent room. This apparatus allowed the measurement of activity or inactivity, time and distances covered in each part of the maze during a 5 minutes period of time. After the test, the maze was carefully cleaned with a wet tissue paper (10% ethanol solution).

3.4. Statistical Analysis

Calculation of the distances covered in each part of the maze, total time spent in each of the open arms,

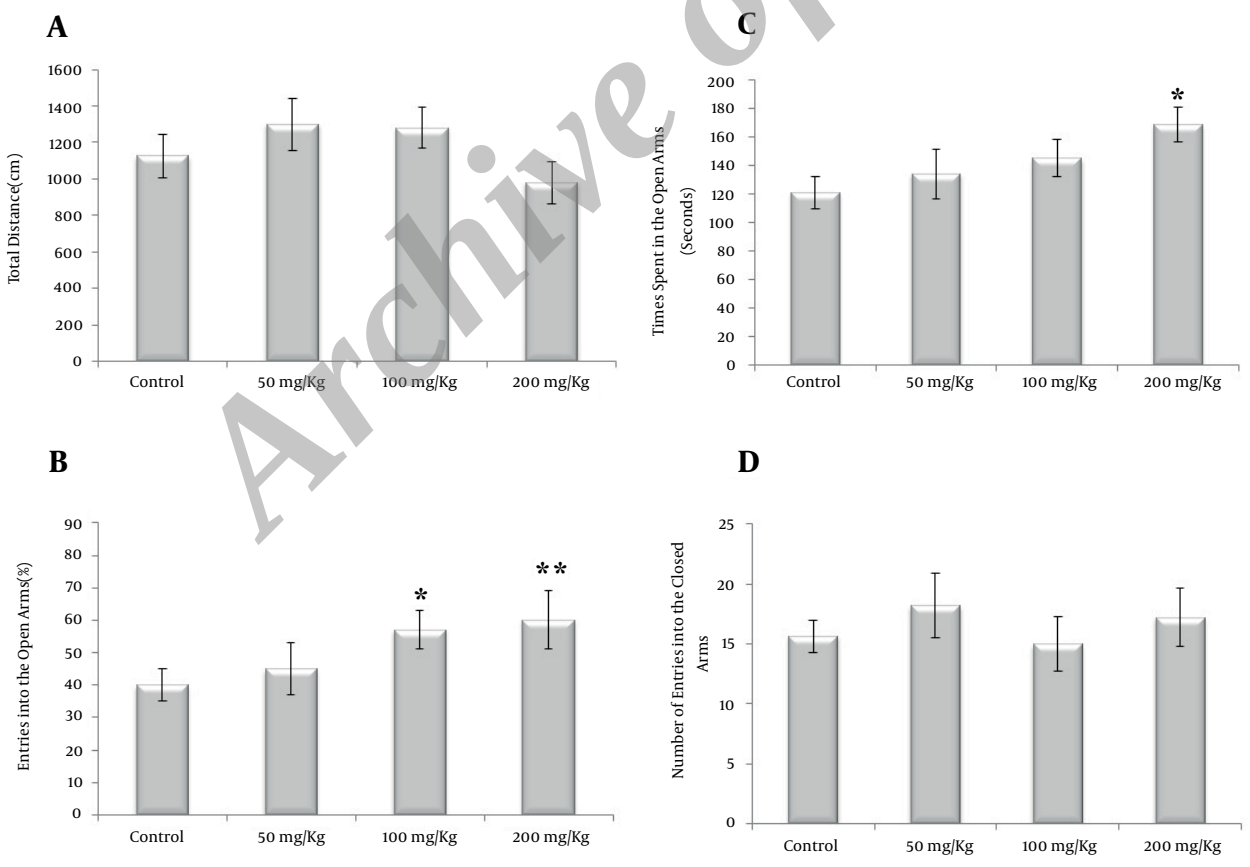

Figure 2. The Effects of Lettuce Extract (50, 100, 200 mg/kg IP) on the Total Distance Covered by the Rats (A), the Percentage of Entries into the Open Arms of the EPM (B), Time Spent in the Open Arms (C) and the Number of Closed Arm Entries (D) During the 5 Minutes Test Session. *: $P < 0.05$, **: $P < 0.01$

percentage of entries into the open arms compared to total entries, and number of entries into the closed arms of EPM were performed using computerized analysis. The statistical analysis of data were performed by one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc analysis. In all cases the differences were considered to be significant if $P < 0.05$.

4. Results

The results showed that the total distance covered by high dose diazepam (1.2 mg/kg) treated rats, during the 5 minutes test, was significantly ($P < 0.05$) different from the controls (Figure 1 A). The ANOVA revealed the significant effects of diazepam treatment on the percentage of entries into the open arms (Figure 1 B), and on the total time spent in the open arms (Figure 1 C). Diazepam showed a significant increase in open arms exploration at concentrations 0.3 ($P < 0.01$) and 0.6 mg/kg ($P < 0.05$), but interestingly not at 1.2 mg/kg (Figure 1 B, C). The number of closed arms entries was significantly ($P < 0.05$) different for the group that received 1.2 mg/kg of diazepam, but the number of closed arm entries was not significantly different for the groups that received 0.3 or 0.6 mg/kg of diazepam (Figure 1 D).

The total distance covered by the lettuce extract treated rats during the 5 minutes test was not significantly different from the controls (Figure 2 A). Acute doses of lettuce extract revealed significant effects on the percentage of entries into the open arms (Figure 2 B), and on the total time spent in the open arms (Figure 2 C). A Tukey's post-hoc analysis revealed the significant effects of lettuce extract treatment on the percentage of entries into the open arms (100 mg/kg, $P < 0.05$ and 200 mg/kg, $P < 0.01$) and the total time spent in the open arms (200 mg/kg, $P < 0.05$). Lettuce extract showed no significant increase in open arms exploration in concentrations of 50 mg/kg. The number of closed arms entries was not significantly different for groups that received lettuce extract (Figure 2 D).

5. Discussion

The elevated plus-maze is probably the most widely used model of animal anxiety (28, 29). Rats exposed to an EPM tend to avoid the open arms and prefer to stay in the enclosed arms. Therefore, drugs that elicit a decrease in the time spent in the open arms are considered to be anxiogenic. An increase in the time and the proportion of the entrances into the open arms without a change in locomotor activity is regarded as a powerful marker for an anxiolytic substance effect (13). The results of our study showed that acute treatment with a single IP injection of lettuce extract increased the time of open arms exploration, similar to the effects observed after the reference anxiolytic drug diazepam at low dose. This effect was not induced by changes in motor activity, at these doses, since the total distance covered by the rats and the number of closed arms entries were not modified by the lettuce extract. The high dose of 200 mg/kg induced the most marked effects and led to a change in the classic anxiety-related behavioral parameters. These results could indicate anxiolytic-like activity to the lettuce leaves extract. Diazepam was applied as an anxiolytic positive control drug (30, 31).

Diazepam is known to be anxiolytic in humans and produce reductions in anxiety-like behavior in several animal models of behavior (13, 32-34). In this method, a high dose of diazepam does not induce an anxiolytic effect, because it reduces animal locomotion activity as a result of its powerful sedative effect. Therefore, EPM is not a suitable method to assess the anxiolytic activity of a high dose of diazepam, so other anxiety survey methods, like the Shuttle Box should be used.

Plants had been used for medicinal purposes, long before recorded history (35), and their utilization in medication is still well-disseminated around the world (36, 37). Various types of herbal medicines have been used as anxiolytics in different parts of the world (3). In the present study, lettuce extract decreased the level of anxiety in animals. According to this finding, this plant extract is indicated as a significant source of natural antioxidants, which has the capacity to decrease anxiety, and this might be helpful in preventing the progression of anxiety (6). However, the components responsible for antianxiety activity were unclear until a few years ago. In this scenario, a dried extract of lettuce leaf was administered orally to mice for a duration of 15 or 30 days and locomotor and anxiolytic activities were performed. A hydro-alcohol extract of lettuce leaf that is rich in polyphenols and other secondary metabolites is a potent anxiolytic agent (6, 14). In earlier studies the HPLC analysis of the polyphenols of *Lactuca sativa* revealed the presence of chlorogenic acid, vanillin, epicatechin, caffeic acid, rutin hydrate, sinapic acid, quercetin-3-rhamnoside, p-coumeric acid and quercitin. The presence of these components in the extract may promote anxiolytic activity (14). Rutin is known to have anti-anxiety properties (38, 39) and this can have effects on the serotonin and GABA systems. GABA is widely known to be involved in the etiology of anxiety, hence the short term effectiveness of diazepam, a GABA agonist, in relieving anxiety.

Oxidative stress has been implicated in depression, anxiety disorders and high anxiety levels (40). Flavonoids and phenolic compounds, which are widely distributed in plants, have been reported to exert free radical scavenging abilities, anti-inflammatory, anticarcinogenic, and anxiolytic properties (40, 41). Recently, the antioxidant activity of lettuce has been reported to prevent chronic diseases related to oxidative stress, such as cancer (8, 11). Tocopherols, known collectively as vitamin E, are lipid soluble antioxidants synthesized by plants and other photosynthetic organisms (42, 43). One of these plants is lettuce (44). According to the results of one ex-

periment, lettuce clearly showed a beneficial effect on lipid metabolism and on tissue oxidation. Lettuce consumption increases total cholesterol end-product excretion and improves antioxidant status due to high levels of antioxidants (vitamins C, E and carotenoids). It has been reported that lettuce intake significantly increased both ascorbic acid and α-tocopherol plasma levels, which contributes to improved plasma antioxidant capacity within 2 hours of consumption. Other lipid-soluble antioxidants (lutein and vitamin E) may also improve plasma antioxidant capacity (45).

In conclusion, our results demonstrate that acute administration of lettuce extract has an anxiolytic effect in rats. Our findings also favor the position that conventional EPM measurements are sufficient and reliable for detecting the anxiolytic-like effects of lettuce extract. However, the exact mechanism(s) and the active compound(s) involved in these effects need to be clarified in future studies. Future work should be focused on the neurobiological mechanisms of action and possible interactions of lettuce extract with classical neurotransmitters and neuromodulators.

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Authors' Contributions

Study concept and design: Komaki, Khaledi Nasab and Shahidi; acquisition of data: Khaledi Nasab and Komaki; analysis and interpretation of data: Komaki and Khaledi Nasab; drafting of the manuscript: Komaki, Shahidi and Sarihi; critical revision of the manuscript for important intellectual content: Komaki, Sarihi and Ghaderi; statistical analysis: Komaki, Khaledi Nasab and Salehi; administrative, technical, and material support: Ghaderi and Salehi; study supervision: Komaki and Shahidi.

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