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Original Article

# Anxiolytic effect of an extract of *Salvia miltiorrhiza* roots in rats

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**Abstract**

*Background*: Preparations from roots of *Salvia miltiorrhiza*, a herb widely used in traditional Chinese medicine, have been reported to induce a series of central effects, including sedation. In the wake of this ethnopharmacological information, the present study was designed to assess the anxiolytic potential of an extract of *S. miltiorrhiza* roots.
*Methods*: To this end, rats were acutely treated with *S. miltiorrhiza* extract (0, 50, and 100 mg/kg; i.g.) and exposed to the Elevated Plus Maze (EPM) test. The effect of treatment with *S. miltiorrhiza* extract on Stress-Induced Hyperthermia (SIH; a physiological response to stressful events) was also evaluated.
*Results*: Treatment with 100 mg/kg *S. miltiorrhiza* extract produced robust anxiolytic effects at the EPM test; specifically, it increased (a) percent of entries into open arms, (b) percent of time spent in open arms, (c) total number of head dips, (d) number of unprotected head dips, and (e) number of end-arm explorations in open arms, without any alteration in spontaneous locomotor activity. Treatment with 100 mg/kg *S. miltiorrhiza* extract also suppressed SIH response. The anxiolytic effects produced by 100 mg/kg *S. miltiorrhiza* extract were comparable to those exerted by acute treatment with 1.5 mg/kg (i.p.) of the reference compound, diazepam.
*Conclusion*: These data demonstrate the ability of an extract of *S. miltiorrhiza* roots to produce anxiolysis in two different rodent models of "anxiety".


*Keywords:* Anxiety; Rats; *Salvia miltiorrhiza*

## 1. Introduction

*Salvia miltiorrhiza* Bunge (Labiatae) is a native plant of China; it also grows in Japan, Korea, and Mongolia. Its dried roots, often referred to as Danshen, are listed in the Chinese Pharmacopoeia and constitute one of the most commonly used herbal remedies in Chinese folk medicine.[1] Preparations containing *S. miltiorrhiza* roots are used to treat a range of pathologies, including coronary heart disease, angina pectoris, cerebrovascular diseases, ischemic stroke, hepatitis, hyperlipidemia, diabetes, Alzheimer's disease, Parkinson's disease, and insomnia.[2–4] The use of herbal preparations containing *S. miltiorrhiza* roots has recently been expanding also in several Western Countries.[2–4] The major, biologically-active chemical constituents of *S. miltiorrhiza* roots are diterpene pigments – of orange-red color and with a phenanthrenequinone structure – such as tanshinone I, tanshinone IIA, tanshinone IIB, cryptotanshinone, and miltirone.[2,3]

The traditional use of *S. miltiorrhiza* roots as a "calming" or sedative remedy[3] suggests that they may exert anxiolytic effects. With the intent of contributing towards clarifying

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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whether *S. miltiorrhiza* roots produce anxiolysis, the present study evaluated the effect of an extract of *S. miltiorrhiza* roots in two validated rodent models of anxiety: the Elevated Plus Maze (EPM) test and the Stress-Induced Hyperthermia (SIH) test. To our knowledge, no study has experimentally evaluated the anxiolytic potential of extracts of *S. miltiorrhiza* roots. Therefore, the present study was designed with the intent of (a) proving experimentally several reports from traditional medicines on the "calming", or anxiolytic, properties of herbal preparations containing *S. miltiorrhiza* roots and (b) providing experimental bases for subsequent investigations aimed at identifying the underlying neural substrates and the active ingredients involved.

EPM is one of the most widely used procedures to assess anxiety-related behaviors in rodents. It is based on the innate unconditioned fear of rodents for open, heightened, and unprotected environments (resulting in avoidance) and their preference for sheltered, enclosed, and dark spaces (resulting in approach).[5–7] Specifically, the EPM test measures the conflict of rats and mice between the instinct to explore the open, unprotected arms of the maze and the proclivity to explore its closed, safe arms. Administration of anxiolytic compounds (e.g., benzodiazepines) markedly increased the proportion of time spent in and number of entries into the open arms of the maze; conversely, administration of anxiogenic compounds (e.g., pentylenetetrazole, yohimbine, and caffeine) suppressed the proportion of explorations in the open arms in favor of those in the closed arms.[5] Validity and reliability of the EPM procedure have further been improved by recording of several behavioral, anxiety-related measures (e.g., exploration and activity in the distal portion of the open arm; head dips below the level of the open arm) and measures of risk-assessment responses [e.g., stretch-attend postures (SAPs)].[6,7] These additional measures, along with the initial spatiotemporal measures, provide a comprehensive and ethologically-based evaluation of the rat behavior and, in pharmacological tests, of the effect of the tested drug.[6,8]

SIH test measures the innate increase in mammal body temperature, mediated by the autonomic nervous system, in response to exposure to situations perceived as threatening or distressing.[9] Experimentally, individual body temperature of rats and mice is measured twice, before (T1) and after (T2) exposure to a given stressor: the difference in body temperature ($\Delta T = T2 - T1$) is defined as SIH response. SIH response is pharmacologically manipulable (at least in terms of its reduction), as several anxiolytic compounds (e.g., benzodiazepines) have been reported to produce its suppression.[10] In the present study, the SIH test was associated to the EPM test: the anxiogenic and stress-induced stimuli producing SIH comprised indeed all those interventions required to perform the EPM test [i.e.: rat move from the homecage to the waiting cage (together with temporary isolation); rat move from the housing room to the waiting room; rat move from the waiting room to the testing room; rat exposure to the EPM].

The anxiolytic effect of the *S. miltiorrhiza* extract was compared to that of the benzodiazepine, diazepam, that was included in the experimental design as standard anxiolytic compound.

## 2. Methods

The experimental procedures employed in the present study were (a) in accordance with European Directive no. 2010/63/EU and subsequent Italian Legislative Decree no. 26, March 4, 2014, on the "Protection of animals used for scientific purposes".

### 2.1. Animals

Male Wistar rats ($n = 53$) (Envigo, San Pietro al Natisone, Italy), of 6–7 weeks of age and weighing approximately 250 g, were used. Rats were housed 3/cage in standard plastic cages with wood chip bedding. The animal facility was under a reversed 12:12 h light–dark cycle (lights on at 9:00 p.m.), at a constant temperature of 22 ± 2 °C and relative humidity of approximately 60%. Behavioral tests were conducted during the first 4 h of the dark phase of the light/dark cycle. Food pellets (standard rodent chow; Envigo, San Pietro al Natisone, Italy) and tap water were always available (24 h/day), with the sole exception of the 2 h before administration of *S. miltiorrhiza* extract, when food pellets were removed so that rats had empty stomachs at the time of extract infusion. Over the two weeks preceding the start of each experiment, rats underwent daily 10-min sessions during which they were extensively habituated to handling and intragastric infusion (experiment testing *S. miltiorrhiza* extract), or intraperitoneal injection (experiment testing diazepam).

### 2.2. Apparatus

The EPM apparatus (Med Associates, St. Albans, VT, USA) was made of black plexiglas and consisted of 4 arms [12(w) × 60(l) cm] positioned to form a *plus* sign. Two opposite facing arms had 50(h)-cm walls and open roof (closed arms), whereas the other two opposite facing arms had no walls (open arms), but only a 0.5(h)-cm border. The arms were connected by a 12 × 12-cm central square. The entire maze was elevated 50 cm above the floor. A custom-made, computer-controlled, video-tracking system was used to record rat behavior. The EPM apparatus was located in a soundproof room adjacent to both waiting and testing rooms.

Body temperature was monitored by means of a rectal thermometer for rodents (Keithley Instruments, Cleveland, OH, USA) with 0.1 °C accuracy.

### 2.3. Experimental procedure

Two independent experiments were conducted, one testing *S. miltiorrhiza* extract and one testing diazepam. On the test day of each experiment, rats were divided into three (experiment testing *S. miltiorrhiza* extract) or two (experiment testing diazepam) groups, carefully matched for body weight.

Baseline (T1) body temperature was taken immediately before pharmacological treatment. At T1, two consecutive recordings (30 s apart) were performed in each rat; the value assigned to each rat was given by the average of these two recordings.

Immediately after T1 recording of body temperature, rats were treated with 0 ($n = 11$), 50 ($n = 11$), and 100 ($n = 11$) mg/kg *S. miltiorrhiza* extract or 0 ($n = 10$) and 1.5 ($n = 10$) mg/kg diazepam. *S. miltiorrhiza* extract (Danshen Extract Powder, Xi'an Honson Biotechnology Co. Ltd., Xi'an, China) (i) was prepared from dried roots of *S. miltiorrhiza*, extracted with ethanol, and (ii) contained 10.1% tanshinone IIA. *S. miltiorrhiza* extract was dissolved in a 1:1 mixture of Tween 80 and PEG 600. The solution was kept under continuous agitation, at room temperature and in a dark container, for at least 12 consecutive hours before administration. *S. miltiorrhiza* extract was administered acutely and intragastrically (by means of a metal gavage) at the infusion volume of 2 ml/kg. Diazepam (injectable Valium®, Roche, Monza, Italy) was dissolved in a 1:1 mixture of saline and PEG 600. Diazepam was administered acutely and intraperitoneally at the injection volume of 2 ml/kg.

Immediately after pharmacological treatment, rats were singly housed in waiting cages and moved from the housing room to the waiting room. Sixty minutes after administration of *S. miltiorrhiza* extract and 30 min after administration of diazepam, rats were moved from the waiting room to the testing room (where the EPM was located) and exposed to the EPM.

Each rat was placed on the central square of the EPM, facing one of the two open arms. EPM test duration was 5 min. During the EPM test, the following 8 variables were recorded: (a) number of entries into closed arms; (b) number of entries into open arms (then expressed as percent of total number of entries into both arms); (c) time spent in open arms (then expressed as percent of total time spent in both arms); (d) total number of head dips; (e) number of protected head dips (defined as those head dips occurring from closed arms); (f) number of unprotected head dips (defined as those head dips occurring in open arms); (g) number of end-arm explorations in open arms; (h) number of SAPs.

Entry into a given arm was scored once the rat had all 4 paws in that arm.[7] Head dips were scored anytime the rat had moved the head below the level of the maze floor.[7] End-arm explorations in open arms were defined as the rat reaching, with all 4 paws, the last 10-cm portion of an open arm.[11] SAPs were defined as the rat being motionless but stretching to its full length with the forepaws toward a stimulus (i.e., an open arm), keeping the hindpaws in the same place, and then resuming the initial position.[7]

Number of entries into closed arms was recorded as measure of spontaneous locomotor activity.[7,12,13] Percent of entries into open arms, percent of time spent in open arms, total number of head dips, number of unprotected head dips, and number of end-arm explorations in open arms were recorded as measures of anxiety-related behaviors.[7,12,13] Number of protected head dips was recorded as measure of decision-making and assessment of height and openness from the central square.[13] Number of SAPs was recorded as measure of risk assessment.[6,14]

At the end of each single test, the maze was cleaned thoroughly before exposure of the following rat.

Immediately after termination of the EPM test (i.e., 65 min after infusion of *S. miltiorrhiza* extract or 35 min after injection of diazepam), body temperature was re-recorded (T2); T2 recording of body temperature was performed in the same way as T1 recording.

### 2.4. Statistical analysis

Data from the EPM test were initially tested for normality using the D'Agostino and Pearson test. In the experiment testing *S. miltiorrhiza* extract, when normally distributed, data were analyzed by 1-way ANOVA, followed by the Newman–Keuls test for *post hoc* analysis; when not normally distributed, data were analyzed by the Kruskal–Wallis test, followed by the Dunn's test for *post hoc* analysis. In the experiment testing diazepam, when normally distributed, data were analyzed by the 2-tailed Student *t* test; when not normally distributed, data were analyzed by the 2-tailed Mann–Whitney test.

In both "*S. miltiorrhiza* extract" and "diazepam" experiments, data on SIH response were analyzed by 2-way (treatment; time) ANOVA for repeated measures on the factor "time", followed by the Newman–Keuls test for *post hoc* comparisons.

## 3. Results

### 3.1. Elevated plus maze

#### 3.1.1. Locomotor activity

Treatment with *S. miltiorrhiza* extract did not alter basal locomotor activity, as indicated by the lack of any difference in number of entries into closed arms between vehicle-treated rat group and the two *S. miltiorrhiza* extract-treated rat groups [$F(2,30) = 0.92$, $p > 0.05$] (Fig. 1A).

Similarly, no difference in number of entries into closed arms was observed between vehicle- and diazepam-treated rat groups ($p > 0.05$, Student *t* test) (Table 1).

#### 3.1.2. Anxiety-related behaviors

ANOVA indicated that treatment with *S. miltiorrhiza* extract significantly altered the percent of entries into open arms [$F(2,30) = 11.30$, $p < 0.0005$] (Fig. 1B). Treatment with 100 mg/kg *S. miltiorrhiza* extract produced an approximately 3-fold increase, in comparison to vehicle treatment, in percent of entries into open arms ($p < 0.0005$, Newman–Keuls); conversely, treatment with 50 mg/kg *S. miltiorrhiza* extract was ineffective.

ANOVA indicated that treatment with *S. miltiorrhiza* extract significantly altered the percent of time spent in open arms [$F(2,30) = 9.55$, $p < 0.001$] (Fig. 1C). Treatment with 100 mg/kg *S. miltiorrhiza* extract produced an approximately 4-fold increase, in comparison to vehicle treatment, in percent

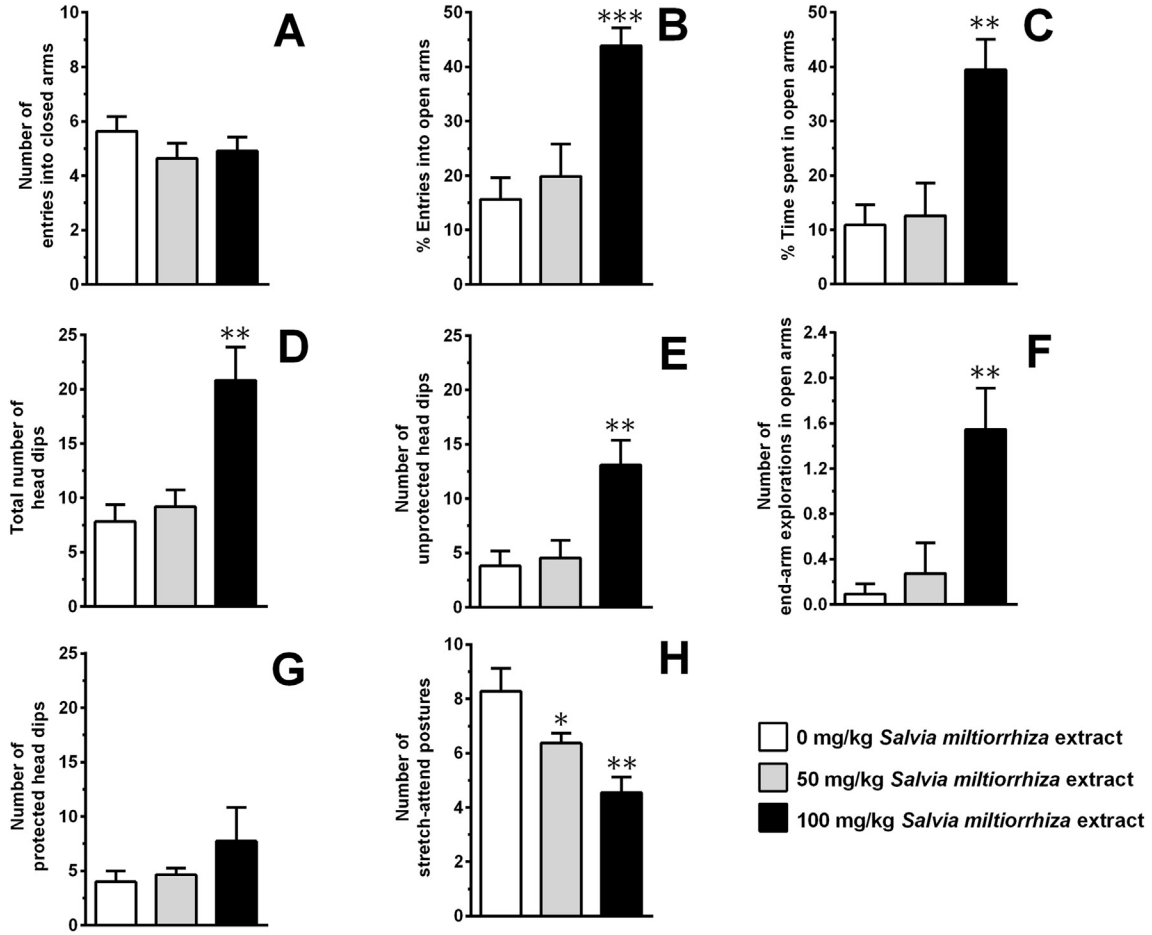


Fig. 1. Effect of the acute administration of two different doses of an extract of *Salvia miltiorrhiza* roots on measures of (i) spontaneous locomotor activity [number of entries into closed arms (panel A)], (ii) "anxiety" [percent of entries into open arms (panel B); percent of time spent in open arms (panel C); total number of head dips (panel D); number of unprotected head dips (panel E); number of end-arm explorations in open arms (panel F)], (iii) decision-making [number of protected head dips (panel G)], and (iv) risk assessment [number of stretch-attend postures (panel H)] in Wistar rats exposed to the Elevated Plus Maze (EPM) test. *Salvia miltiorrhiza* extract was administered intragastrically 60 min before the EPM test. The EPM test lasted 5 min. Each bar is the mean ± SEM of $n = 11$ rats. *: $p < 0.05$, **: $p < 0.005$, and ***: $p < 0.0005$ in comparison to the rat group treated with 0 mg/kg *Salvia miltiorrhiza* extract (Newman–Keuls or Dunn's test).

of time spent in open arms ($p < 0.005$, Newman–Keuls test); conversely, treatment with 50 mg/kg *S. miltiorrhiza* extract was ineffective.

ANOVA indicated that treatment with *S. miltiorrhiza* extract significantly altered the total number of head dips [$F(2,30) = 10.85$, $p < 0.0005$] (Fig. 1D). Treatment with 100 mg/kg *S. miltiorrhiza* extract produced an approximately 2.5-fold increase, in comparison to vehicle treatment, in total number of head dips ($p < 0.005$, Newman–Keuls test); conversely, treatment with 50 mg/kg *S. miltiorrhiza* extract was ineffective.

ANOVA indicated that treatment with *S. miltiorrhiza* extract significantly altered the number of unprotected head dips [$F(2,30) = 8.33$, $p < 0.005$] (Fig. 1E). Treatment with 100 mg/kg *S. miltiorrhiza* extract produced an approximately 3.5-fold increase, in comparison to vehicle treatment, in number of unprotected head dips ($p < 0.005$, Newman–Keuls test); conversely, treatment with 50 mg/kg *S. miltiorrhiza* extract was ineffective.

Kruskal–Wallis test indicated that treatment with *S. miltiorrhiza* extract significantly altered the number of end-arm explorations in open arms ($\chi^2 = 15.98$, df = 2, $p < 0.005$) (Fig. 1F). Treatment with 100 mg/kg *S. miltiorrhiza* extract produced an approximately 15-fold increase, in comparison to vehicle treatment, in number of end-arm explorations in open arms ($p < 0.005$, Dunn's test); conversely, treatment with 50 mg/kg *S. miltiorrhiza* extract was ineffective.

Treatment with diazepam produced clear anxiolytic effects. Specifically, in comparison to vehicle treatment, diazepam administration resulted in: (a) an approximately 3-fold increase in percent of entries into open arms ($p < 0.005$, Student *t* test); (b) an approximately 6.5-fold increase in percent of time spent in open arms ($p < 0.005$, Student *t* test); (c) an approximately 4.5-fold increase in total number of head dips ($p < 0.0005$, Student *t* test); (d) an approximately 9.5-fold increase in number of unprotected head dips ($p < 0.005$, Student *t* test); (e) occurrence of end-arm explorations in open arms ($p < 0.05$, Mann–Whitney test) (Table 1).

Table 1
Effect of the acute administration of 1.5 mg/kg diazepam on measures of (i) spontaneous locomotor activity (number of entries into closed arms), (ii) "anxiety" (percent of entries into open arms; percent of time spent in open arms; total number of head dips; number of unprotected head dips; number of end-arm explorations in open arms), (iii) decision-making (number of protected head dips), and (iv) risk assessment (number of stretch-attend postures) in Wistar rats exposed to the Elevated Plus Maze (EPM) test.

| | Diazepam | |
|---|---|---|
| | 0 mg/kg | 1.5 mg/kg |
| Locomotor activity | | |
| Number of entries into closed arms | 4.90 ± 0.55 | 4.80 ± 0.59 |
| Anxiety-related behaviors | | |
| Percent of entries into open arms | 14.05 ± 4.41 | 39.72 ± 6.32** |
| Percent of time spent in open arms | 6.18 ± 1.86 | 41.60 ± 10.17** |
| Total number of head dips | 4.40 ± 0.54 | 19.50 ± 3.18*** |
| Number of unprotected head dips | 1.40 ± 0.43 | 13.00 ± 2.87** |
| Number of end-arm explorations in open arms | 0.00 ± 0.00 | 1.90 ± 0.72* |
| Decision-making | | |
| Number of protected head dips | 3.00 ± 0.39 | 6.50 ± 1.88 |
| Risk assessment | | |
| Number of stretch-attend postures | 5.80 ± 0.89 | 5.30 ± 1.03 |

Diazepam was administered intraperitoneally 30 min before the EPM test. The EPM test lasted 5 min. Each figure is the mean ± SEM of $n = 10$ rats. *: $p < 0.05$, **: $p < 0.005$, and ***: $p < 0.0005$ in comparison to the rat group treated with 0 mg/kg diazepam (2-tailed Student $t$ test or 2-tailed Mann–Whitney test).

### 3.1.3. Decision-making

ANOVA indicated that treatment with *S. miltiorrhiza* extract failed to alter the number of protected head dips [$F(2,30) = 1.07$, $p > 0.05$] (Fig. 1G). Data suggest the presence of a tendency toward an increase in the number of protected head dips in the rat group treated with 100 mg/kg *S. miltiorrhiza* extract, in comparison to vehicle-treated rat group.

Treatment with diazepam exerted no more than a tendency toward an increase in the number of protected head dips ($p > 0.05$, Student $t$ test) (Table 1).

### 3.1.4. Risk assessment

ANOVA indicated that treatment with *S. miltiorrhiza* extract significantly altered the number of SAPs [$F(2,30) = 8.86$, $p < 0.001$] (Fig. 1H). In comparison to vehicle treatment, administration of 50 and 100 mg/kg *S. miltiorrhiza* extract reduced the total number of SAPs by approximately 25% ($p < 0.05$, Newman–Keuls test) and 45% ($p < 0.005$, Newman–Keuls test), respectively.

Conversely, treatment with diazepam failed to alter the number of SAPs ($p > 0.05$, Student $t$ test) (Table 1).

### 3.2. Stress-induced hyperthermia

ANOVA indicated a significant effect of treatment with *S. miltiorrhiza* extract [$F(2,30) = 5.30$, $p < 0.05$], a highly significant effect of time [$F(1,30) = 33.57$, $p < 0.0001$], and a significant "treatment × time" interaction [$F(2,30) = 4.19$, $p < 0.05$] on SIH response. Specifically, vehicle-treated rats displayed a clear SIH response: after exposure to the sequence of stressful events, mean body temperature in vehicle-treated rats increased from 37.2 °C (T1) to 37.6 °C (T2) ($\Delta T = 0.4$ °C; $p < 0.001$, Newman–Keuls test) (Fig. 2). Treatment with 100 mg/kg *S. miltiorrhiza* extract resulted in a virtually complete inhibition of SIH response, as body temperature averaged 37.3 °C [$p < 0.005$ (Newman–Keuls test)], comparing T2 body temperature in vehicle- and 100 mg/kg *S. miltiorrhiza*-treated rats]; conversely, treatment with 50 mg/kg *S. miltiorrhiza* extract was ineffective (Fig. 2).

ANOVA indicated a highly significant effect of treatment with diazepam [$F(1,18) = 44.31$, $p < 0.0001$], a highly significant effect of time [$F(1,18) = 106.35$, $p < 0.0001$], and a highly significant "treatment x time" interaction [$F(1,18) = 142.33$, $p < 0.0001$] on SIH response. After exposure to the sequence of stressful events, mean body temperature in vehicle-treated rats increased from 37.1 °C (T1) to 37.9 °C (T2) ($\Delta T = 0.8$ °C; $p < 0.0005$, Newman–Keuls test) (Table 2). Treatment with diazepam resulted in a complete inhibition of SIH response, as body temperature averaged 37.0 °C [$p < 0.0005$ (Newman–Keuls test)], comparing T2 body temperature in vehicle- and diazepam-treated rats] (Table 2).

## 4. Discussion

Data from both tests (EPM and SIH) consistently indicate that acute treatment with an extract of *S. miltiorrhiza* roots exerted robust anxiolytic effects in rats. The results of the

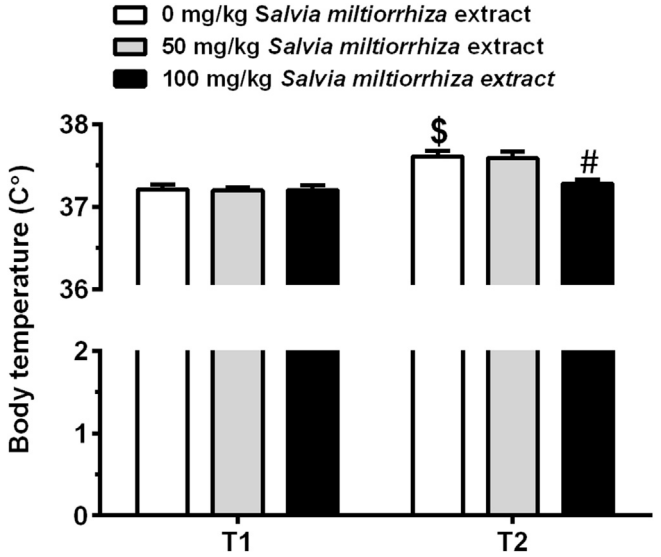


Fig. 2. Effect of the acute administration of two different doses of an extract of *Salvia miltiorrhiza* roots on stress-induced hyperthermia (measure of "anxiety") in Wistar rats exposed to a sequence of stressful events, comprising all those interventions required to perform the Elevated Plus Maze (EPM) test. *Salvia miltiorrhiza* extract was administered intragastrically 60 min before the EPM test. Rat body temperature was recorded twice: at T1 (baseline; immediately before treatment with *Salvia miltiorrhiza* extract) and T2 (immediately after termination of the EPM test, i.e. 65 min after treatment with *Salvia miltiorrhiza* extract). At both T1 and T2, two consecutive recordings (30 s apart) were performed in each rat; the value assigned to each rat was given by the average of these two recordings. Each bar is the mean ± SEM of $n = 11$ rats. $^{\$}$: $p < 0.001$ in comparison to the same rat group at T1 (Newman–Keuls test); $^{\#}$: $p < 0.005$ in comparison to the rat group treated with 0 mg/kg *Salvia miltiorrhiza* extract at T2 (Newman–Keuls test).

Table 2
Effect of the acute administration of 1.5 mg/kg diazepam on stress-induced hyperthermia (measure of "anxiety") in Wistar rats exposed to a sequence of stressful events, comprising all those interventions required to perform the Elevated Plus Maze (EPM) test.

| | Diazepam | |
|---|---|---|
| | 0 mg/kg | 1.5 mg/kg |
| T1 (°C) | 37.06 ± 0.03 | 37.09 ± 0.05 |
| T2 (°C) | 37.89 ± 0.06* | 37.03 ± 0.06** |

Diazepam was administered intraperitoneally 30 min before the EPM test. Rat body temperature was recorded twice: at T1 (baseline; immediately before treatment with diazepam) and T2 (immediately after termination of the EPM test, i.e. 35 min after treatment with diazepam). At both T1 and T2, two consecutive recordings (30 s apart) were performed in each rat; the value assigned to each rat was given by the average of these two recordings. Each figure is the mean ± SEM of $n = 10$ rats. *: $p < 0.0005$ in comparison to the same rat group at T1 (Newman–Keuls test); **: $p < 0.0005$ in comparison to the rat group treated with 0 mg/kg diazepam at T2 (Newman–Keuls test).

EPM experiment indicate *anti*-anxiety behaviors in both primary, spatiotemporal measures, as treatment with *S. miltiorrhiza* extract increased (a) percent of entries into open arms and (b) percent of time spent in open arms. At the dose of 100 mg/kg *S. miltiorrhiza* extract this increase was remarkably large, with both variables averaging 40–45%. These figures suggest that treatment with 100 mg/kg *S. miltiorrhiza* extract rendered the two opposite environments of the EPM (open and closed arms) almost equivalent in terms of the rats' emotional response; in other words, treatment with 100 mg/kg *S. miltiorrhiza* extract produced anxiolytic effects strong enough to induce the rats to approach and explore the heightened and unprotected open arms (otherwise mostly avoided) in a manner highly comparable to that by which they approached and explored the dark and sheltered closed arms. Importantly, the above differences do not seem to be the mere consequence of non-specific alterations in the rats' general motor activity or state of well-being, as none of the two doses of *S. miltiorrhiza* extract altered the number of entries into the closed arms, a validated and reliable measure of spontaneous locomotor activity in rats and mice exposed to the EPM.[7,12,13] To summarize, treatment with *S. miltiorrhiza* extract altered the *quality*, rather than the *quantity*, of exploratory behavior of the rats exposed to the EPM.

Additional elements in favor of the anxiolytic effect of *S. miltiorrhiza* extract are provided by the analysis of several ethological measures of anxiety. These measures include (a) total number of head dips, (b) number of unprotected head dips, and (c) number of end-arm explorations in open arms; these measures complement with the two above spatiotemporal measures, providing a more complete profiling of the rat behavior at the EPM test. Treatment with 100 mg/kg *S. miltiorrhiza* extract markedly increased the frequency of all three different behaviors, confirming that this dose of *S. miltiorrhiza* extract was capable of producing robust anxiolysis at the EPM test.

Analysis of data on risk assessment provides additional, interesting details on the pharmacological profile of the tested *S. miltiorrhiza* extract. Risk assessment stands for a repertoire of behaviors that animals execute to gather information when facing a potentially dangerous situation, such as a novel and aversive environment. In the EPM test, risk assessment mainly consists in SAPs, i.e. the forward elongations of head and shoulders from the central square or a closed arm and toward an open arm, followed by retraction to the original, "safe" position; SAPs are made with the intent of assessing the precariousness of the opposing environment. Besides being representation of a decision-making process, SAPs incorporate elements of "anxiety", as suggested by the numerous pharmacological data reporting their suppression after treatment with well-established anxiolytic agents.[12,15,16] In the present study, treatment with both doses of *S. miltiorrhiza* extract reduced, in a dose-related fashion, the number of SAPs; these data can be interpreted proposing that, due to the anxiolytic effect of *S. miltiorrhiza* extract, body elongations from the closed arms toward the open arms, with rapid returns to the original positions, were replaced – in large part – by full entries into and explorations of the open arms.

SIH test produced consistent results: treatment with 100 mg/kg *S. miltiorrhiza* extract completely abolished the increase in rat body temperature secondary to the stressful events associated to rat exposure to the EPM test. These data confirm, and extend to a physiological response to stressful and anxiogenic events, that the *S. miltiorrhiza* extract tested in the present study possesses *anti*-stress and anxiolytic properties in rats.

Notably, the anxiolytic effect of *S. miltiorrhiza* extract resulted to be highly comparable, at both EPM and SIH tests, to that exerted by diazepam, a reference anxiolytic compound in preclinical behavioral pharmacology. In the present study, diazepam was tested at a dose (1.5 mg/kg, i.p.) in the range of those well-known to effectively suppress several anxiety-related behaviors in rats without altering basal motor activity and coordination.[5,17–20] The similarity in magnitude of the anxiolytic effect of *S. miltiorrhiza* extract and diazepam confers further relevance to the behavioral pharmacological profile of *S. miltiorrhiza* extract.

In close agreement with the results of the present study, a recent study found that non-sedative doses of an essential oil obtained from the aerial parts of *S. miltiorrhiza* exerted robust anxiolytic effects in rats exposed to EPM and Social Interaction tests.[21] These data, together with those of the present study, suggest that all parts of *S. miltiorrhiza* – both aerial parts[21] and roots – are sources of agents with anxiolytic action in rats. Notably, none of the diterpenes proposed as active ingredients of preparations from *S. miltiorrhiza* roots was contained in that specific essential oil of the aerial parts.[21] This suggests that *S. miltiorrhiza* contains a large variety of active compounds with anxiolytic potential.

Additional, indirect support to the anxiolytic profile of preparations from *S. miltiorrhiza* roots may come from the results of a previous study demonstrating that acute administration of miltirone, one of the main active ingredients of *S. miltiorrhiza* roots, produced anxiolytic effects in mice exposed to a punishment-induced conflict test.[22] Miltirone has been reported to behave as partial agonist at the benzodiazepine

binding site,[22−24] providing a clue on the mechanism of its anxiolytic action.

The results of the present study also provide a possible key to the interpretation of different lines of experimental evidence on the reducing effect of extracts of *S. miltiorrhiza* roots on multiple alcohol-related behaviors. Acute and repeated treatment with different extracts of *S. miltiorrhiza* roots has indeed been reported to reduce alcohol drinking (including relapse-like drinking) and operant self-administration of alcohol in rats.[25−29] These results have been collected entirely in Sardinian alcohol-preferring (sP) rats, a rat line selectively bred for high alcohol preference and consumption.[30] Besides alcohol preference, sP rats display inherent predisposition to anxiety-related behaviors, as demonstrated using several different experimental procedures, including EPM.[30] Notably, voluntarily consumed alcohol markedly reduced these anxiety-related behaviors,[31,32] suggesting that anxiolysis is likely one of the alcohol effects that drive sP rats to seek and consume large amounts of alcohol. Accordingly, it is conceivable that the anxiolytic effects of extracts of *S. miltiorrhiza* roots substituted for those of alcohol, making alcohol seeking and drinking less urgent and resulting in the observed reductions in alcohol drinking and self-administration. If theoretically transposed to humans, these data suggest that *S. miltiorrhiza* extracts would represent a potentially effective, therapeutic option for those alcoholic patients with comorbid anxiety and alcohol use disorder.

In conclusion, the results of the present study indicate that acute treatment with an extract of *S. miltiorrhiza* roots exerted robust anxiolytic effects in two experimental procedures validated to detect anxiety-related behaviors in rats.

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