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Evaluation of the anxiolytic effect of the methanol stem extracts of *Cissus quadrangularis*

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the anxiolytic activity of *C. quadrangularis* in animal models. Stem extract of *C. quadrangularis* possesses a significant anxiolytic activity equal to that of the diazepam. Therefore, *C. quadrangularis* was found to be a promising anxiolytic agent in animal models.

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ABSTRACT

Objective: To investigate the potentials of the stem of *Cissus quadrangularis* (*C. quadrangularis*) in the control of anxiety and related motor coordination effects in mice using experimental models.

Methods: The methanol extract of the stem of *C. quadrangularis* was studied in mice using elevated plus maze, hole board, open field and stair case tests. Acute toxicity and phytochemical analysis were also carried out.

Results: The methanol extract (100, 200, 300 and 400 mg/kg) exhibited significant anxiolytic effects, as evident by significant ($P<0.05$) increase in the number of crossings at 100, 200 and 300 mg/kg dose and number of rearing at 200, 300 and 400 mg/kg dose in open field behavior test. Time spent in the open arms and number of entrances in the open arms increased significantly ($P<0.05$, $P<0.01$) at the dose of 200 and 300 mg/kg in elevated plus maze test. *Post hoc* analysis showed that *C. quadrangularis* at the dose of 200 and 300 mg/kg significantly ($P<0.05$) increased the number of steps taken and number of head dips. Significant ($P<0.05$) reduction in time duration on the bar and number of rearing were observed at the dose of 200, 300 and 400 mg/kg. The acute toxicity test revealed an oral LD₅₀ above 5000 mg/kg, while phytochemical studies showed the presence of phytosterols, terpenoids, saponins, flavanoids, tannins, carbohydrates and aminoacids.

Conclusions: The stem extracts of *C. quadrangularis* is anxiolytic in nature, which contribute to its use in traditional medicine as anxiolytic.

KEYWORDS

Exploratory behavior, Hole board, Spontaneous motor activity, Stair case, Antianxiety, *Cissus quadrangularis*

1. Introduction

India is a country of vast biodiversity and traditional knowledge for using herbal medicines to cure many ailments in various cultures and tribes^[1]. Anxiety and stress disorders are among the most common of all chronic diseases. The prevalences of these disorders are increasing in many countries, and these disorders have a much earlier age of onset than other chronic conditions. Since the introduction of benzodiazepines in the 1960s, they have been the most

commonly prescribed treatment for anxiety, remaining the mainstay of pharmacological treatment in anxiety disorders. However, they have prominent side effects, such as sedation, myorelaxation, ataxia, and amnesia, and they can cause pharmacological dependence^[2]. Thus, new therapies for the treatment of anxiety disorders are necessary, and the study of medicinal plants could provide new therapeutic options^[3].

Cissus quadrangularis L. (*C. quadrangularis*), a succulent vine from Asia and Africa, has been known for its therapeutic uses since ancient times. The plant

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is used in the treatment of osteoporosis, asthma, cough, hemorrhoids, and gonorrhoea^[4]. Phytochemical studies of *C. quadrangularis* have shown the presence of several phytochemical constituents, such as ascorbic acid, flavonoids, and triterpenoids^[5]. *C. quadrangularis* Linn. has been widely used as traditional medicine in Africa and Asia including Thailand for the treatment of hemorrhoid, osteoporosis and scurvy^[6]. Plant extract inhibits cyclooxygenase, 5-lipoxygenase enzyme activity and proinflammatory mediators^[7]. It possesses antiinflammatory, antibacterial, antiviral and antiulcerogenic properties^[8–11]. *C. quadrangularis* ethanol extract upregulates superoxide dismutase, glutathione peroxidase and endothelial nitric oxide synthase expression in hydrogen peroxide-injured human ECV304 cells^[12]. The effects of *C. quadrangularis* on the bone marrow mesenchymal stem cell proliferation and osteogenic differentiation were also studied^[13]. Pharmacological studies revealed that plant was directly used for treating bone fracture^[14,15]. Neuropharmacological effects of the methanol root extract of *C. quadrangularis* were reported by Viswanatha Swamy *et al*^[16]. The present study was designed to study anxiolytic activity of the stem extracts of *C. quadrangularis* to scientifically validate the extract which is already in common use.

2. Materials and methods

2.1. Plant materials

The stem part of *C. quadrangularis* used in this work was collected from nursery of Saurashtra University in Rajkot City (Gujarat, India) in October 2007 and dried in shade. The plant was identified and authenticated by Prof. Vishal Muliya, Department of Botany, Christ College, Rajkot. A voucher specimen of the plant has been deposited in the Herbarium–cum–Museum of the University, Department of Pharmaceutical Sciences, Saurashtra University (Rajkot, India).

2.2. Preparation of extract

Stems of *C. quadrangularis* were cleaned with tap water, dried and finally powdered. This powdered plant material (1 kg) was extracted in a Soxhlet extraction apparatus with 700 mL absolute methanol until all methanol soluble constituents were extracted out with methanol. The solution was filtered, solvent was removed under vacuum and the solid mass obtained was stored at 4 °C in amber color bottle until use. The yield of the methanol extract was 10.2% (w/w).

2.3. Phytochemical screening

The chemical constituents of the extracts were identified by qualitative chemical tests using the procedures of Kokate *et al*^[17].

2.4. Animals

Swiss albino mice (20–30 g) of either sex, bred at the Animal House, Saurashtra University, Rajkot, were used in the present study. Animals were housed in polypropylene cages in air conditioned quarters at (22±2) °C, relative humidity (50±20) %, light (10 h): dark (14 h) cycle. Mice were given standard pellet diet and water in sufficient quantity. All the protocols were conducted with prior approval from Institutional Animal Ethics Committee (IAEC) of Saurashtra University, Rajkot, India.

2.5. Acute toxicity test

Healthy adult Swiss mice of either sex weighing between 20–25 g were randomly divided into five groups of 6 mice per group. Group 1, 2, 3, 4 and 5 received 200, 500, 1000, 2000 and 5000 mg/kg *i.p.* methanol extracts of *C. quadrangularis*. The study was carried out according to OECD (Organization for Economic Cooperation and Development 2001) guideline number AOT–425^[18]. The mice were observed for 2 h for behavioral, neurological and autonomic profiles and for any lethality or death for the next 3 d.

2.6. Experimental design

Adult Swiss albino mice of either sex were randomly divided into six groups of 6 mice per group. Fresh drug solutions were prepared using 0.5% w/v sodium carboxy methyl cellulose solution at the time of drug administration. Group 1 received vehicle only and the treatments were given in a volume of 10 mL/kg body weight, Group 2 received standard treatment with diazepam (1 mg/kg, *i.p.*), Groups 3, 4, 5 and 6 received methanol extracts (100, 200, 300 and 400 mg/kg, *i.p.*). Mice were tested in dark cycle and habituated to injection prior to test day.

2.7. Open field behavior test

Exploratory behavior was evaluated in an open–field paradigm^[19]. The open field was made of plywood and consisted of a floor (96×96 cm) with high walls. Drugs were administered to different groups as mentioned above. Thirty minutes later, each animal was placed at one corner of the apparatus and for the next 5 min, the ambulations (number of squares crossed) and number of rearing were manually recorded.

2.8. Elevated plus maze model of anxiety

The elevated plus maze is a well established animal model for testing anxiolytic drugs^[20]. Drugs were administered to different groups and 30 min later, each mouse was placed at the center of the elevated plus maze with its head facing the open arms. During a 5 min experiment, the behavior of the mice was recorded in terms of the number of entries into the open arms, and average time spent by the mice in the open arms.

Every precaution was taken to ensure that no external stimuli, other than the height of plus maze, could invoke anxiety in the animals.

2.9. Muscle relaxant activity

The test is used to evaluate the activity of drugs interfering with motor coordination using rotarod method^[21]. In this test, mice were placed on a horizontal rotating rod and animals remaining on the rod for 3 min or more in two successive trials were selected. Mice in group of 6 each, received vehicle or the extract of *C. quadrangularis i.p.*, and 30 min later were placed on the rotating rod. The animal was placed with the four paws on a 2.5 cm diameter bar, 25 cm above the floor, which was turning at 12 r/min. The time of duration on the bar was measured for 1 min, for each animal.

2.10. Staircase test

The test was carried out essentially as previously described^[22]. Thirty minutes before testing mice was dosed with vehicle or 100, 200, 300 and 400 mg/kg *i.p.* methanol extract or 1 mg/kg *i.p.* diazepam. Mice were subsequently individually placed on the bottom level facing away from the stairs. The number of steps ascended and rearing made in a 3 min period were recorded, and the ratio of steps to rears calculated. In addition, the numbers of faecal boli and urination spots produced by mice at the end of the test were counted. The apparatus was wiped with a wet paper towel and dried between animals.

2.11. Hole-board test

The hole-board apparatus consisted of gray perspex panels (40 cm×40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor^[23]. The board was positioned 15 cm above a table. Photocells below the holes measured the number of head-dips. Animals were placed singly in the center of the board facing away from the observer and animal behavior and head-dip numbers were recorded for 5 min.

2.12. Statistical analysis

The results were expressed in terms of mean±SEM and analyzed by ANOVA followed by Dunnett's *post hoc* test with a significance level of $P < 0.05$ using graph pad software (Version 3.01).

3. Results

3.1. Phytochemical screening

The results of the phytochemical screening of *C. quadrangularis* extracts have been presented in Table 1.

3.2. Acute toxicity study

The *i.p.* administration of the extract (5000 mg/kg) did not cause any mortality and during the observation period of 3 d post dosing no abnormal clinical signs were observed in treated mice throughout the observation period. It did not induce any adverse effects on the body weight gain of mice treated at 5000 mg/kg. No gross pathological alterations were detected in the treated mice at terminal necropsy. Thus LD₅₀ of *Cissus* extract was found to be more than 5000 mg/kg.

Table 1

Phytochemical screening of *C. quadrangularis* extract.

Test	Methanol extract
Carbohydrates	+
Steroids/terpenes	+
Glycosides	–
Amino acids	+
Saponins	+
Phytosterols	+
Polyphenolic compounds/tannins	+
Flavonoids	+
Alkaloids	–
Anthraquinones	–

+: positive, -: negative.

3.3. Open field behavior test

The methanol extract of *C. quadrangularis* showed significant effects as evident by increase in the number of crossings compared to controls using open field test, in mice. Methanol extract at the dose of 300 mg/kg, *i.p.* produced most significant increase in the number of crossings (66.670±4.072), which was comparable with standard drug diazepam (75.830±4.354) (Table 2). However, methanol extract at the dose of 400 mg/kg did not produced significant increase in the number of crossings but significantly reduced number of rearing.

Table 2

Effect of *C. quadrangularis* on open field behavior test in mice.

Treatment	Dose (mg/kg)	No. of square crossed	No. of rearing
Control	Vehicle	27.330±2.390	28.830±3.660
Diazepam	1	75.830±4.354**	9.500±1.648*
Methanol extract	100	44.500±3.622*	25.330±1.994
Methanol extract	200	58.500±4.137*	18.830±2.167*
Methanol extract	300	66.670±4.072**	12.170±2.330**
Methanol extract	400	32.170±3.135	10.830±1.470**

Values are expressed as mean±SEM, n=6. *: $P < 0.05$, when compared with control group, **: $P < 0.01$, when compared with control group.

3.4. Elevated plus maze model of anxiety

A possible anxiolytic activity of *C. quadrangularis* methanol extracts were assessed by the elevated plus maze test. Results (Table 3) showed that the time spent in the open arms and the numbers of entries in the open

Table 3Effect of *C. quadrangularis* on elevated plus maze performance in mice.

Treatment	Dose (mg/kg)	Time spent in open arm (s)	Time spent in closed arm (s)	No. of entries to open arm	No. of entries to closed arm
Control	Vehicle	30.500±1.408	214.170±11.077	6.330±1.022	18.833±1.249
Diazepam	1	75.700±3.648**	113.170±7.735**	16.500±1.565**	9.167±1.138**
Methanol extract	100	38.300±6.453	181.830±6.973*	11.500±1.335	15.830±1.662
Methanol extract	200	51.500±5.427**	161.500±5.078**	12.330±1.498*	11.330±1.282**
Methanol extract	300	63.500±1.839**	147.000±6.088**	17.170±1.424**	13.167±1.621*
Methanol extract	400	45.667±2.765*	185.500±4.566*	9.667±1.542	15.170±1.869

Values are expressed as mean±SEM, n=6. *: P<0.05, when compared with control group, **: P<0.01, when compared with control group.

arms were significantly increased and time spent in the closed arms and the numbers of entries in the closed arms were significantly decreased as compared to controls. On the contrary, methanol extract 200 and 300 mg/kg, *i.p.* significantly increased the time of duration in the open arms (51.500±5.427) and (63.500±1.839), as well as the number of entrances in the open arms (12.330±1.498) and (17.170±1.424), respectively, these effects were comparable with standard drug diazepam. Methanol extract at the dose of 400 mg/kg showed less significant effect in time spent in open and closed arm compare to standard diazepam and not produced any significant effect in number of entries in open and closed arm.

3.5. Muscle relaxant activity

In the rotarod test, there was significant reduction in time of duration on the bar after administration of methanol extract of *C. quadrangularis* (200, 300 and 400 mg/kg, *i.p.*), as compared to controls (Table 4). It was found that, the methanol extract of *C. quadrangularis* at the dose of 400 mg/kg, *i.p.* exhibited a marked reduction in time of duration on the bar (24.500±3.314), as related to standard diazepam (25.170±3.331). There was significant reduction in motor coordination in mice and mice were unable to hold on the rotating rod.

Table 4Effect of *C. quadrangularis* on muscle relaxant activity in mice using rotarod method.

Treatment	Dose (mg/kg)	Time of duration on the bar (s)
Control	Vehicle	46.830±3.516
Diazepam	1	25.170±3.331**
Methanol extract	100	46.170±5.839
Methanol extract	200	32.330±2.390*
Methanol extract	300	29.830±3.291*
Methanol extract	400	24.500±3.314**

Values are expressed as mean±SEM, n=6. *: P<0.05, when compared with control group, **: P<0.01, when compared with control group.

3.6. Stair case test

The number of steps taken by mice in the staircase test was significantly influenced by drug treatments. *Post hoc* analysis showed that *C. quadrangularis* at dose of 100 mg/kg

(P<0.05), 200 mg/kg (P<0.01) and 300 mg/kg (P<0.05) increased the number of steps taken, while other doses of 400 mg/kg, did not significantly alter this parameter (Table 5). The numbers of rears performed by the mice were significantly reduced at dose of 200, 300 and 400 mg/kg (Table 5). Neither the number of faecal boli, nor urine spots produced by mice in the test were significantly affected by the drug treatments.

Table 5Effect of *C. quadrangularis* on stair case test in mice.

Treatment	Dose (mg/kg)	No. of step climbed	No. of rearing
Control	Vehicle	8.667±1.308	29.330±3.263
Diazepam	1	21.833±2.626**	10.167±1.740**
Methanol extract	100	16.500±1.996*	23.500±2.291
Methanol extract	200	19.830±2.428**	18.670±2.404*
Methanol extract	300	17.670±2.092*	19.833±2.626*
Methanol extract	400	7.500±1.565	8.330±2.060**

Values are expressed as mean±SEM, n=6. *: P<0.05, when compared with control group, **: P<0.01, when compared with control group.

3.7. Hole-board test

Similar to diazepam 1 mg/kg, *C. quadrangularis* at dose of 200 and 300 mg/kg (Table 6) increased significantly the number of head dips (41.830±3.754) and (38.670±3.283), respectively, as compared to saline treated control (26.170±2.982).

Table 6Effect of *C. quadrangularis* on hole board test in mice.

Treatment	Dose (mg/kg)	No. of head dips
Control	Vehicle	26.170±2.982
Diazepam	1	43.830±3.081**
Methanol extract	100	31.670±3.095
Methanol extract	200	41.830±3.754**
Methanol extract	300	38.670±3.283*
Methanol extract	400	21.670±2.011

Values are expressed as mean±SEM, n=6. *: P<0.05, when compared with control group, **: P<0.01, when compared with control group.

4. Discussion

Phytochemical studies on stems of *C. quadrangularis* Linn. revealed the presence of amino acids, carbohydrates, steroids, glycosides, saponins, phytosterols, tannins, polyphenols, triterpenoids, and flavonoids. A sedative and anxiolytic actions

was already shown by other triterpenoid compounds, such as those present in *Centella asiatica* Linn.^[24], as assessed by the elevated-plus-maze test in rodents.

In the present work, the anxiolytic effects of *C. quadrangularis*, were investigated on classic animal models, such as elevated plus maze, open field, hole board, stair case and rotarod tests. Animal models of anxiety are typically based on exposure of animals to a stressful condition (a potential or actual threatening situation) and a specific test for measuring behavioral and physiological responses. The elevated plus maze test is considered one of the most widely validated tests for assaying new benzodiazepine-like anxiolytic agents. This test is based on the observation that rodents tend to avoid elevated areas and, therefore avoidance of the open arms in elevated plus maze test is interpreted as anxiety behavior. Our results showed that *C. quadrangularis* at dose of 200 and 300 mg/kg were able to increase significantly all the parameters in the elevated plus maze test, as compared to the control group. Similar results were also observed with the diazepam treated group at a recognized anxiolytic dose (1 mg/kg), suggesting an anxiolytic-like effect from *C. quadrangularis*.

In order to further corroborate the anxiolytic activity observed in the elevated plus maze test, we also used the hole-board test, in which exploration is also gradually inhibited by anxiety. Similar to elevated plus maze, this test is also useful for modeling anxiety and anxiolytic agents have been shown to increase the number of head dips^[25]. Our results showed that *C. quadrangularis* at dose of 200 and 300 mg/kg significantly increased the number of head dips, indicating the anxiolytic-like effect.

The tests described above are only predictive of a narrow spectrum of behavioral patterns. Consequently, additional tests are recommended to control for possible confounding factors, e.g. locomotor activity. The open-field test allows measurements by which it is possible to evaluate autonomic effects of drugs and general activity of animals. The experimental findings clearly demonstrated that the methanol extract of *C. quadrangularis* at dose of 100, 200 and 300 mg/kg elicited a significant increase in number of squares crossed and the dose of 200, 300 and 400 mg/kg significant decrease in number of rearing compare control. This test utilizes behavioral changes in rodents exposed to a novel environment and has been used to detect anxiogenic and anxiolytic activity under identical situations.

The increased muscle tone is a common feature of anxiety states in humans. Thus the extract was tested for its effect on muscle coordination and balance by performing rotarod test in mice. Time of duration on the bar markedly decreased at dose of 200, 300 and 400 mg/kg in comparison to control. The observed changes were found to be statistically significant.

In the staircase paradigm, step-climbing is purported to reflect exploratory or locomotor activity, while rearing behavior is an index of anxiety state. Our result showed that methanol extract at dose of 100, 200 and 300 mg/kg increase the number of steps taken and the dose of 200, 300 and 400 mg/kg reduce the number of rearing.

Our findings showed that the animals treated with *C.*

quadrangularis 100 mg/kg did not induce changes in locomotion of mice in the open-field arena, while dose of 200 and 300 mg/kg showed an evident increase in this parameter. Taken together, our results observed in elevated plus maze, hole board, stair case and open-field tests suggest that *C. quadrangularis* 200 and 300 mg/kg has anxiolytic like effects without inducing significant sedative action in animals. *C. quadrangularis* at the dose of 100 and 400 mg/kg not showed significant anxiolytic effect.

In conclusion, the methanol stem extract of *C. quadrangularis* at doses of 200 and 300 mg/kg possesses a significant anxiolytic activity equal to that of the diazepam. Although further phytochemical and clinical investigations are necessary, these results are promising since *C. quadrangularis* might be considered as an alternative for the treatment of sedative and anxiety disorders to other medications currently used.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Anxiety and stress disorders are among the most common of all chronic diseases. The prevalences of these disorders are increasing in many countries, and these disorders have a much earlier age of onset than other chronic conditions. Therefore, new therapies for the treatment of anxiety disorders are necessary all over the world. Medicinal plants such as *C. quadrangularis* could provide new therapeutic options.

Research frontiers

The present research work depicts to study anxiolytic activity in animal models of the stem extracts of *C. quadrangularis* to scientifically validate the extract which is already in common use. To do this, acute toxicity and phytochemical analysis were carried out.

Related reports

This plant is used for the treatment of various diseases such as osteoporosis, asthma, cough, hemorrhoids, and gonorrhoea. Phytochemical studies of *C. quadrangularis* found constituents such as flavonoids, triterpenoids, stilbene derivatives, resveratrol, piceatannol, pallidol, perthenocissin, and phytosterols.

Innovations and breakthroughs

C. quadrangularis is used for the treatment of various diseases such as osteoporosis, asthma, cough, hemorrhoids, and gonorrhoea. In Thailand, this plant is used for the treatment of hemorrhoid, osteoporosis and scurvy. Moreover some studies demonstrated that, plant extract inhibits cyclooxygenase, 5-lipoxygenase enzyme activity and proinflammatory mediators. It possesses anti-inflammatory, antibacterial, antiviral and antiulcerogenic properties. In the present study, authors have demonstrated the anxiolytic activity of *C. quadrangularis* in animal models.

Applications

From the literature survey it has been found that this plant is safe to humans. This scientific study support and suggest the use the stem extracts of *C. quadrangularis* of this plant as some agents with commonly used anxiolytic disorders.

Peer review

This is a valuable research work in which authors have demonstrated the anxiolytic activity of *C. quadrangularis* in animal models. Stem extract of *C. quadrangularis* possesses a significant anxiolytic activity equal to that of the diazepam. Therefore, *C. quadrangularis* was found to be a promising anxiolytic agent in animal models.

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