Original Research

Effect of *Ephedra altissima* stems extract on behavior in the mouse

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Abstract

Herbal products are being paid particular attention by people around the world since they are natural, effective and without side effects. In modern medicine, some drugs prescribed to the patients are derived from medicinal plants. Thus, the aim of this study is to investigate the central nervous effects of methanol extract of *Ephedra altissima* stems, in Albino mice. Doses of 500, 1500 and 3000 mg/kg of the methanol extract were administered in sub-acute doses (three doses), intraperitoneally at 24, 5.0 and 1.0 hours before scoring. General locomotor behavior profile, antidepressant activity and anxiolytic activity were studied. The results revealed that the methanol extract of *Ephedra altissima* at 500, 1500, and 3000 mg/kg produced central nervous system depression, a remarkable decrease in the immobility time (forced swimming maze test), no anxiolytic-like effect by using elevated plus maze model of anxiety. These results suggest that methanol extract of *Ephedra altissima* exhibits a central depressant activity, antidepressant like activity and mild antipsychotic activity without anxiolytic-like effect in tested experimental animal models with the doses used. Therefore, it can be concluded that *Ephedra altissima* extract may have a central depression, antidepressant-like activity with no effect as anxiolytic like mood.

Keywords: *Ephedra altissima*, forced swimming maze, Irwin test, mice, plus maze

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Introduction

Medicinal plants have served as a rich source of new molecules with pharmacological properties that fill an essential gap in the search for superior therapeutic agents [1]. Search for novel pharmacotherapy from medicinal plants for psychiatric illnesses has progressed significantly in the past decade. Some plants are well known for their central effects such as antidepressant, analgesic, anticonvulsant, anti-muscarinic and anxiolytic effects. Many of today's conventional drugs originated directly or indirectly from plants; many valuable psychoactive drugs, such as yohimbine, ephedrine, tubocurarine and galanthamine were discovered through the study of indigenous remedies. There is a pressing need for the development of herbal medicines which will be safe and have lesser side effects [2]. Ephedra species member of Ephedraceae family is one of the oldest medicinal plants known to humankind. It is mainly found in North Africa, Middle East, the Indian subcontinent, Southeast Asia, North and South-western America [3]. This plant is used in Chinese and Indian folkloric medicine for centuries as a remedy for the treatment of nasal congestion, allergic rhinitis, acute coryza, common cold, sinusitis, cough and asthma [4]. *Ephedra altissima* is high climbing joint fir, is one of the species found in Libya locally known as Alanda; this species is climbing much-branched shrubs, about 2 m long, which grows predominantly in north-western Libya mainly in Nafusa mountain. This species extends across western North Africa to the Canary Islands [5]. Phytochemical analysis has demonstrated that the principal chemical components of this species are alkaloids, phenols, flavonoids, essential oils, terpenoids, tannins, coumarins, saponins, sterols and glycosides [6]. Moreover, Ephedra species possess several biological

properties such as antioxidant [7], anti-inflammatory [8], antimicrobial [9], analgesic [10], cytotoxic [11], antiasthmatic [12], hepatoprotective [13], antiulcer [14], antihyperglycemia and antihyperlipidemia [15]. Thus, the aim of this study is to determine the neuropharmacological activities of *ephedra altissima* stem extract, also we tried to evaluate the anxiolytic and antidepressant activities after administration of stem methanolic extract to understand its neurobehavioral characteristic.

Materials and Methods

Plant collection and preparation of the extract

The fresh stems of *Ephedra altissima* were collected from Rabtah, Nafusa Mountain, Libya. The plant was authenticated by Prof. Mohamed Abuhadra, Department of Botany, Faculty of Science, University of Tripoli, Libya. A specimen was deposited in the herbarium section. The plant was shade dried and coarsely powdered. The coarse powder was subjected to extraction with series of solvents of increasing polarity n-hexane, ethyl acetate then methanol, respectively, by cold maceration, then concentrated using a rotary evaporator at 60 °C to yield semi-solid mass extracts.

Animals

Healthy male Swiss albino mice of 25 - 35 gm bred in the animal house of Faculty of Pharmacy-University of Tripoli, Libya. The animals were placed in a standard laboratory environment (room temperature 25 °C ± 2 °C; 12 hours' light/dark cycle) and were supplied with an adequate amount of standard food pellet diet and water. Before conducting the experiments, the animals were kept in the laboratory for at least one day before testing to acclimate to a new environment.

Chemicals and drugs

N-hexane, ethyl acetate, methanol and Tween 80 were obtained from Sigma-Aldrich, Steinheim, Germany, imipramine hydrochloride was obtained from Novartis Pharma AG, KurtkÖy Istanbul and diazepam was obtained from Roche, Basel, Switzerland.

Experimental protocol

Standard drugs and extract doses were freshly prepared on the day of the experiment. Mice were divided into six groups (n = 6): group 1 (control group), mice were treated with 1% tween 80 (5 ml/kg) as the vehicle. Groups 2, 3, 4 (test groups) were administered methanolic extract in 500, 1500, and 3000 mg/kg, respectively; group 5 was administered diazepam (2 mg/kg, as standard for anxiolytic activity); group 6, mice was administered imipramine (10 mg/kg, as standard for antidepressant activity). Sub-acute administration was adopted (three doses, i.p.) at 24, 5.0 and 1.0 hours before scoring. All drugs were administered as a homogenous suspension in 1% tween 80. All the animal experiments were carried out after ethical approval from ethics committee of university of Tripoli (25-2020).

The Neuropharmacological activities of methanolic extract of *E. altissima* were estimated by Irwin test, elevated plus-maze test and forced swimming maze test.

Experimental analysis

General Pharmacological Observations (Irwin Test): The qualitative effects of the methanolic extract on behaviour and physiological function were investigated by using the procedure described by Irwin. Irwin's test is a test that assesses the behaviour and autonomic response of rodents after pre-treatment with a compound. In addition to the parameters assessed, the test provided data on the lethality of the extract or compound in 24 hours' period [16].

Elevated plus maze test: Elevated plus-maze (EPM) is a rodent model of anxiety that is used as a screening test for a putative anxiolytic or anxiogenic compounds and as a general research tool in neurobiological anxiety research. The apparatus composed of two open arms (30 cm x 05 cm) and two close arms (30 cm x 05 cm with side walls of 15 cm) that extended from a common central platform (5 x 5 cm). The apparatus was elevated to a height of 45 cm above the floor level [17]. Mice were gently handled by the right hand and placed on the central platform of the maze facing the close arm. Different parameters were scored to evaluate anxiolytic effect and spontaneous motor activity in the elevated plus-maze, which included: time spent by the mouse on each of the arms, lines crossing on close or open arms and the number of entries into close or open arms. An arm entry was defined as the entry of all the four paws into the arm [18]. The total number of lines crossings and the total number of arm entries were calculated; it expresses the spontaneous motor activity [19, 20]. Anxiety measure was calculated by dividing the time spent on close arms by the total time of the test [20]. The duration of the test was four minutes.

Forced swimming test: The forced swimming test (FST) is used extensively to evaluate the antidepressant efficacy of new compounds that prevent the depressive-like condition. This method is based on observing the animals exposed to a condition of swimming forcefully that they become immovable and lethargic after a period of an energetic activity (struggling) and generating only the movements required to keep their heads above the water [21]. The mice were placed individually in glass cylinders (height 27 cm with diameter 15 cm) filled with water to a height of 16 cm (maintained at 23 – 25 ^{0}C).

The duration of the test was six minutes. Behaviour parameters (duration of immobility) were recorded during the last four minutes of the six minutes testing period [22]. Immobility behaviour is defined as the animal floated on the surface with front paws together and made only those movements with hind limbs that were necessary to keep float [23].

Statistical analysis: All data were presented as mean ± SEM value. Statistical analysis tested by using one-way Analysis of variance (ANOVA) followed by LSD post-hoc test for multiple comparisons using SPSS Version 20. The statistically significant level was set at a *p* value < 0.05.

Results

General behavioural profile –Irwin: Irwin's test results are presented in **Table 1**. Treatment of mice with different doses of extract produced a reduction of alertness and motor activity, which is represented by spontaneous activity and reactivity. The depressing effect was most intense at dose 3000 mg/kg.

Table 1: Neuropharmacological profile of methanolic stem extract of *Ephedra altissima* in mice - Irwin observations

Observations		Control group	Extract treated groups					
			500 mg/kg		1500 mg/kg		3000mg/kg	
			30 min.	24hr	30 min.	24hr.	30 min.	24 hr.
Behavioral	Alertness	4	3.08±0.83*	4	3±0.0*	4	1.33±0.21*	4
Awareness	Visual placing	4	4	4	4	4	4	4
	Passivity	0	0.66±0.21*	0	0.83±0.17*	0	1.50±0.22*	0
	Stereotype	0	0	0	0	0	0	0
Mood	Grooming	4	4	4	4	4	4	4
	Vocalization	0	0	0	0	0	0	0
	Restlessness	0	0	0	0	0	0	0
	Irritability	0	0	0	0	0	0	0
	Fearfulness	0	0	0	0	0	0	0
Motor activity	Spontaneous activity	4	3.4±0.20*	4	3.08±0.08*	4	1.33±0.21*	4
	Reactivity	4	3.4±0.20*	4	3.08±0.08*	4	1.33±0.21*	4
	Touch response	4	4	4	4	4	4	4
	Pain response	4	4	4	4	4	4	4
Neurological CNS Excitation	Startle response	0	0	0	0	0	0	0
	Tremors	0	0	0	0	0	0	0
	Twitches	0	0	0	0	0	0	0
	Convulsions	0	0	0	0	0	0	0
Posture	Body posture	4	4	4	4	4	4	4
	Limb posture	4	4	4	4	4	4	4
Motor incoordinatio n	Staggering gait	0	0	0	0	0	0	0
	Abnormal gait	0	0	0	0	0	0	0
	Righting reflex	4	4	4	4	4	4	4
Muscle tone	Limb tone	4	4	4	4	4	3.58±0.15*	4
	Grip strength	4	4	4	4	4	3.58±0.15*	4
	Body tone	4	4	4	4	4	4	4
	Abdominal tone	4	4	4	4	4	4	4
Reflexes	Pinna reflex	4	4	4	4	4	4	4
Autonomic	Writhing	0	0	0	0	0	0	0
	Palpebral opening	4	3.1±0.44*	4	3.25±0.25*	4	3.33±0.11*	4
	Urination	0	0.7±0.21*	0	1.16±0.16*	0	1.16±0.16*	0
	Salivation	0	0	0	0	0	0	0
	Piloerection	0	0	0	0	0	0	0
	Skin color	4	4	4	4	4	4	4
	Lacrimation	0	0	0	0	0	0	0
Dead	Dead number	0	0	0	0	0	0	0

All values are expressed as mean ± S.E.M. (n = 6). ∗p < 0.05 versus control group. Control Basel score 4 = normally found sign, < 4 = subnormal effect, > 4 = supernormal effect. Control Basel score 0 = normally not found sign, > 0 = abnormal effect.

The passivity of mice was increased with the three doses. A mild muscle relaxant effect was observed only with the higher dose of methanol extract 3000 mg/kg, which resulted from the decrease in the limb tone and grip strength. The palpebral opening was slightly reduced with the three doses. No deaths were recorded over the 24 h observation period.

The anxiolytic effect of *E. altissima* using elevated plus-maze model: Anxiety measure decreased after administration of diazepam ($p < 0.05$) compared to the control-treated group. The extract treated groups at doses 500 and 1500 mg/kg did not show any significant changes in anxiety measure, the total lines crossed and the total number of entries ($p > 0.05$) compared to the control group. Anxiety measure was significantly increased, while the total lines crossed and the total number of entries was significantly decreased at a dose of 3000 mg/kg ($p < 0.05$). Extract treated groups of 500, 1500 mg/kg showed a significant decrease in anxiety measure compared to the extract-treated group of 3000 mg/kg ($p < 0.05$); while the total lines crossed and the total number of entries were increased in the extract-treated group of 500 mg/kg compared to the extract-treated group of 3000 mg/kg ($p < 0.05$) (**Table 2**).

Antidepressant activity of *E. altissima* using forced swim maze model: The duration of mobility along with immobility showed by animals of standard as well as treated groups, in 240-second swimming session, as compared with that of the normal control group. Administration of extract at doses of 1500 and 3000 mg/kg produced a significant reduction in the duration of immobility and expanded the mobility time compared to the control group ($p < 0.05$). While there was an insignificant reduction of the duration of immobility produced by extract at dose 500 mg/kg ($p > 0.05$). Administration of the standard imipramine showed a reduction in the duration of immobility compared to the control group **(Table 3)**.

Table 2: Effects of methanolic extract of *E. altissima* in elevated plus-maze model

Treatment	Dose	Anxiety measure	Total lines crossed	Total number of entries
Tween 80 1%	5ml/kg	0.898±0.0352	49.50±9.369	8.17 ± 2.182
Diazepam	2mg/kg	0.696 ± 0.0765*	52.00 ± 15.591	9.67 ± 3.412
MEEA 1	500mg/kg	0.734 ± 0.0973[a]	45.83 ± 8.097[a]	8.50 ± 1.688[a]
MEEA 2	1500mg/kg	0.901 ± 0.0362[a]	25.00 ± 8.748	4.17 ± 1.447[a]
MEEA 3	3000mg/kg	0.982 ± 0.0091*	5.67 ± 1.498*	1.17 ± 0.167*

Each value is presented as the mean ± S.E.M., (n = 6). MEEA = Methanol extract of Ephedra altissima stems. *$p < 0.05$ significantly different compared to control group, [a]$p < 0.05$ significantly different compared to Extract 3000 mg/kg treated group.

Table 3: Effects of *E. altissima* methanolic extract on the duration of immobility in forced swim maze model

Treatment	Dose	Duration of immobility (seconds)
Tween 80, 1%	5 ml/kg	120.02 ± 11.46
Imipramine	10 mg/kg	75.03 ± 19.44*
MEEA1	500 mg/kg	110.30 ± 10.22[a]
MEEA2	1500 mg/kg	75.70 ± 12.09*
MEEA3	3000 mg/kg	55.80 ± 12.85*

Each value is presented as the mean ± S.E.M.; (n = 6). MEEA = Methanol extract of Ephedra altissima stems. *$p < 0.05$ significantly different compared to control group, [a] $p < 0.05$ significantly different compared to extract 3000 mg/kg treated group.

Discussion

The present study demonstrates that *E. altissima* methanolic extract exerted central depression, sedation, mild muscle relaxation and mild antipsychotic like effect. The extract also showed no anxiolytic-like effect but a sedative-like effect. Decrease in behavioural despair significantly after extract treatment, this suggesting antidepressant-like-activity. The phytochemical screening found that the active constituents of the same samples of the current study were found to be alkaloids, saponins, tannins, flavonoids, terpenoids, carbohydrates, cardiac glycosides, and coumarins [6]. Many types of research showed that plants containing phenols, flavonoids, saponins, and tannins are useful in many CNS disorders [24]. It has been reported that *E. altissima* contains tannins, alkaloids, flavonoids, triterpenoids, phenols, sterols, and saponins [6]. The active constituents in ephedra species include ephedrine, pseudoephedrine, apigenin, quercetin, rutin, isoquercetine, kaempferol, linalool, hesperidin, luteolin, fucosterol and ß-Sitosterol and others [25].

It has been reported that natural flavonoids possess a selective and relatively mild affinity for benzodiazepine receptors; it was found to be ligands for γ-aminobutyric acid type A receptor in the CNS, which led to the hypothesis that they act as benzodiazepine-like molecules. Their behavioural effects in animal models of anxiety, sedation and convulsion [26] support this. Some flavonoids might also produce their tranquilizing activity by acting on serotonergic receptors. Both serotonergic and GABA-ergic systems are activated by groups of metabolites, such as flavonoids, terpenes, and sterols [27, 28]. Data from Irwin test are also used to assess the safety pharmacology of drugs [29]. This test data of the current study showed that the methanolic extract decreased alertness, spontaneous activity and reactivity, indicating that the extract has a CNS depressant effect. Since, locomotors activity is a measure of the level of excitability of the CNS [30], this decrease in spontaneous motor activity could attribute to the sedative effect of the ephedra extract constituents; this effect was dose and time-dependent. High sedation was observed using a high dose of 3000 mg/kg *E. altissima* extract; the sedative effect in our current study could be through GABA-ergic mechanism related to benzodiazepine-like components that activate $GABA_A$ receptors.Benzodiazepine receptor activation leads to stimulate GABA receptors binding to GABA neurotransmitter released result in a sedative effect [31]. Another mechanism could be through the serotonergic system, due to antagonism of $5\text{-}HT_{1A}$ [32] or $5\text{-}HT_{2A/2C}$ receptors [33]. In neurological profile, the grip strength and limb tone were slightly reduced with the high dose of *E. altissima* extract, indicating a mild muscle relaxation effect. Mild significant narrowing of the palpebral fissure was observed, with all the doses of the extract, in addition to the reduction of spontaneous motor activity suggesting an antipsychotic like effect [34]. Continuous observation for 24 hours after the test, revealed no physical signs of toxicity or lethality with all tested doses. This suggests that the LD50 is greater than 3000 mg/kg. After 24 hours of observation, the scores are back to normal, this indicates that the effects of the active constituents are reversible. In an elevated plus-maze test, the substance has an anxiolytic effect generally increases the time and proportion of entrance into the open arms by the treated animals that are exposed to EPM [35]. The test is based on the natural aversion of rodents to open spaces. An anxiolytic-like effect appears as decreased open arm avoidance because reduced anxiety removes the interference of fear with exploratory tendencies and hence drives the greater exploration of the open arms [36].

The present results showed that the treatment with *E. altissima* extract at dose 3000 mg/kg significantly decreased the time spent in open arms and percentage of entries into open arms compared to the control group. The decreased in the number of entries and time spent in open arms although this is usually interpreted as an anxiogenic effect, the extract administration also decreased the number of closed arms entries as well as the total number of entries, suggesting a sedative-like effect, more than an anxiogenic effect. The total lines crossed highly decreased with high dose (3000 mg/kg) indicating a decrease in spontaneous motor activity, also due to sedative effect; there were no significant changes with small doses of the extract. This finding suggests that *E. altissima* extract possesses sedative activity at high doses used in this study. Ephedra contains active constituents reported to have anxiolytic-like effects when studied using extracts of different plants [37] suggesting that the sedative effect may have masked any anxiolytic action. Anxiolytics possess a biphasic profile, showing facilitation of exploratory behavior and anxiolytic activity at low doses and an inhibition and sedative activity at high doses as observed with diazepam and clorazepate [38]. Therefore, the effect of *E. altissima* is probably related to benzodiazepine-like components, that act whereby $GABA_A$ receptors. The number of evidence point out the flavonoids is responsible for these effects, the presence of flavonoids like apigenin, quercetin, rutin, isoquercetin, Luteolin,

hesperidin, and kaempferol, suggesting the involvement of GABA-ergic receptors [39 - 41]. Increasing the dose produce mild sedation and a reduction in ambulatory locomotors activity, it activates the $GABA_A$ receptor by interacting with the benzodiazepine binding site [42, 43]. Flumazenil partly antagonized this effect [44]. A study was conducted on the methanolic extract of *Tilia americana* upon testing the anxiolytic effect in male mice using EPM. It found that a mixture of the flavonoids quercetin, rutin, and isoquercetin is responsible for the anxiolytic and sedative-like activity. This effect is produced through the activation of GABA/BDZ and $5HT_{1A}$ serotonergic receptors, which were inhibited in the presence of flumazenil and WAY100635 (5-HT_{1A} antagonist) [45].

The forced swimming test is a widely accepted behavioural model for assessing pharmacological antidepressant activity [17, 46]. Characteristic behaviour scored in this test is termed immobility, reflecting behavioural despair as seen in human depression [47]. The antidepressant effects of methanolic extracts of *E. altissima* and imipramine were studied by observing the changes in the duration of immobility in the forced swim test. The extract produces a dose-dependent decrease in the duration of immobility. Most of the medicinal plants exerted antidepressant effects through synaptic regulation of serotonin, noradrenaline and dopamine [48]. It was found that the effect was produced by activation of the 5-HT_{1A} receptor and α_2-adrenoceptors [49]. Rutin, a flavonoid compound isolated from *Schinus molle*, exerted an antidepressant effect in mice, probably through increasing noradrenaline and serotonin in the synaptic gap [50]. Effect on the serotonergic system and MAO-A activity was found with the administration of quercetin 4'-O-glucoside or quercetin using Swiss albino mice of both sexes. These substances produced antidepressant-like effects in mice using FST [51]. Another flavonoid analyzed is hesperidin, which has an antidepressant effect on the Tail suspension test similar to fluoxetine and imipramine [52]. Also, it may be through enhancing 5-HT levels and decreasing 5-HT turnover in some of the brain regions [53].

In another study evaluated the effect of apigenin isolated from *Circium japonica* on the MAO enzyme. It also stimulated the uptake of L-tyrosine, a noradrenalin precursor [54]. In another work, administration of different doses of phytosterol and fucosterol to male mice produced antidepressant effects using FST and tail suspension test, achieving an effect with comparable efficacy of fluoxetine, a standard dose in humans. The antidepressant mechanism is mediated by increasing monoamines and reducing the rate of 5-HT metabolism [55]. Extract exerted effects similar to those of fluoxetine using FST and tail suspension test finally, the effects on monoamine levels in mice brains confirm that sterols modify the serotonergic and noradrenergic systems but do not impact the dopaminergic system [56]. The doses of ephedrine needed to induce locomotors activity are in the range of dopamine-releasing concentration of the drug. Therefore, it is suggested that ephedrine acts indirectly possibly through the inhibition of uptake and increase the release of dopamine [57]. At higher doses, the release of norepinephrine causes anxiety, restlessness, and insomnia. Prolonged use of ephedra may lead to neurotoxicity, resulting in depletion of brain monoamines [58]. Ephedrine alkaloids are CNS stimulants that produce excitation of the CNS. A study conducted to test the behavioural effects of *Ephedra nebrodensis* Tineo, which contains ephedrine alkaloids; mice were injected i.p. with 200 mg/kg of the extract, the extract produced an amphetamine-like action, manifested as an increase in locomotors activity, rearing and stereotype behaviour [59]. The increase in locomotion could be due to the increase in dopamine release [60]. Based on the available data, it has been assumed that *E. altissima* lack ephedrine-type alkaloids, according to a literature survey of Ephedra species, it was found that not all these species contain alkaloids [3]. Ephedrine and pseudoephedrine were not detected in *E. altissima* and *E. foemina*, despite claims to the contrary [61 - 63]. If ephedrine-type alkaloids found, their contents are scarcas well as CNS depressant effects [64] of Ephedra species [25] and in the total extract they probably are present in a greater quantity than ephedrine, also, it was demonstrated that Ephedra contains flavonoids and proanthocyanidols making their pharmacological activities less intense [65].

Conclusion

The present study showed that the methanolic stem extract of *E. altissima* possesses a central inhibitory effect in mice; however, it has potent antidepressant-like activity, mild antipsychotic-like activity and devoid of anxiolytic activity at studied doses.

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Conflict of Interest

All authors declare no conflict of interest.

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