Avicenna Journal of Phytomedicine (AJP)

Original Research Article

Anxiolytic-like effect of ethanolic extract of *Argemone mexicana* and its alkaloids in Wistar rats

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Article history:
Received: Aug18, 2015
Received in revised form: Oct 14, 2015
Accepted: Dec 20, 2015
Vol. 6, No. 4, Jul-Aug 2016, 476-488.

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Keywords:
Argemone mexicana
Alkaloids
GABAA receptor antagonist
Anxiolytic-like effect

Abstract

Objective: *Argemone mexicana* is a Papaveracea plant; some reports have shown their antibacterial, anti-cancer, sedative and probably anti-anxiety properties. From their aerial parts, flavonoids and alkaloids have been isolated, which are intrinsically related to some actions on the central nervous system. The aim of this study was to evaluate the anxiolytic-like effects of the plant, using its ethanolic extract and alkaloid-enriched extract obtained from fresh leaves.

Material and Methods: Phytochemical screening was carried out together with evaluation of antioxidant capacity and the enrichment of alkaloids present in the extract. Subsequently, 100 and 200 mg/kg doses of ethanolic extract and alkaloid-enriched extract (200 µg/kg) were intraperitoneally administered to female Wistar rats, which were exposed to elevated plus maze (EPM) test. Picrotoxin (1 mg/kg), a non-competitive gamma-aminobutyric acid A ($GABA_A$) chloride channel antagonist, was used in experimental procedures to evaluate if this receptor is involved in the anxiolytic-like effects of *A. mexicana*. To discard motor effects associated with the treatments, the rats were evaluated by the locomotor activity test.

Results: Only the ethanolic extract at 200 mg/kg and alkaloid-enriched extract (200 µg/kg) produced anxiolytic-like effects similarly to diazepam 2 mg/kg on EPM test, without affecting locomotor activity. Meanwhile, the administration of picrotoxin blocked anti-anxiety effect of alkaloid-enriched extract of the plant.

Conclusion: These results showed that *A. mexicana* is a potential anxiolytic agent and we suggest that this effect is mediated by the $GABA_A$ receptor. These effects are related to the presence of alkaloids.

Please cite this paper as:
Arcos-Martínez AI, Muñoz-Muñiz OD, Domínguez-Ortiz MA, Saavedra-Vélez MV, Vázquez-Hernández M, Alcántara-López MG. Anxiolytic-like effect of ethanolic extract of *Argemone mexicana* and its alkaloids in Wistar rats. Avicenna J Phytomed, 2016; 6 (4): 476-488.

Introduction

Argemone mexicana (Papaveraceae) is a prickly plant with intense yellow flowers like poppies. This plant, commonly known as "chicalote" grows between 0.3 and 1.2 meters (Hakim, 1954; Schwarzbach and Kadereit, 1999). The aerial part of the plant (without seeds) is used to treat malaria (Graz et al., 2010), dropsy and icterus, has analgesic and anti-parasitic activities and is considered as an antidote for snake bites (Bhattacharjee et al., 2006; Rahman et al., 2011). The plant contains several kinds of secondary metabolites such as polyphenols, tannins, saponins, flavonoids and alkaloids (Bhalke and Gosavi, 2009a; Ji et al., 2011).

Due to their antioxidant activity, flavonoids and polyphenols are usually recommended as a complementary therapy in order to treat several chronic disorders, which are intrinsically related to anti-inflammatory processes (Nijveldt et al., 2001; Kanadaswami et al., 2005). This kind of compounds and the alkaloids may play an important role in certain neurological diseases (Wink, 2000; Hovatta et al., 2010). Although many alkaloids are sufficiently lipophilic to enter the cells, they can also interact with proteins or lipids at the cell periphery such as neuroreceptors and ion channels. In the central nervous system (CNS) disorders, anxiety is a universal and generally adaptive response to a threat or danger, but in certain circumstances it can become maladaptive (House and Stark, 2002). Its regulation is integrally associated with the function of various neurotransmitter systems, mainly the gamma-aminobutyric acidergic system (Sanders and Shekhar, 1995; Uusi-Oukari and Korpi, 2010). In this regard, the elevated plus maze (EPM) test is a well validated (Pellow et al., 1985) and widely used test of anxiety in which a rat or mouse is faced with a choice of open elevated arms for those enclosed by a high wall (File et al.; 1999). This test is commonly used to define the anxiolytic potential of drugs and plant extracts (Gilhotra and Dhingra, 2010), where diazepam (an anxiolytic drug) is frequently used as a pharmacological control in a dose range of 1-3 ml/kg (Fernández-Guasti et al., 2001; Contreras et al., 2014; Rodríguez-Landa et al., 2014). The study of effect of alkaloids and flavonoids present in the ethanolic extract of *A. mexicana* is important due to the implications it could have on CNS.

In this sense, Anarthe and Chaudhari (2011) have reported that ethyl acetate (EtOAc) and ethanolic (EtOH) extracts of *A. mexicana* (200 mg/kg) decreased the motor activity and grip strength of rats evaluated on rotarod test, which suggested a depressant activity on CNS; also, it increased the sedative effect in pentobarbitone-induced sleep test. For this reason, they suggested that the plant possesses analgesic, anxiolytic and sedative effects. In this paper, we are presenting our results of the behavioral study based on EPM test with the ethanolic extract and alkaloid-enriched extract of *A. mexicana* in ovariectomized Wistar rats.

Materials and Methods

Plant material

Plant was collected in Alto Lucero, Veracruz-México in January 2012 (during full flowering stage). A taxonomist confirmed the taxonomic classification and a voucher specimen (CIB 14658) was deposited in the Instituto de Investigaciones Biológicas, Herbarium of Universidad Veracruzana.

Preparation of organic extracts

About 2.15 kg of aerial part of plant were cut into small pieces, dried at room temperature and extracted by sequential maceration with different solvents. Three crude extracts were obtained and solvent was removed using a rotary evaporator and reduced pressure (hexane 5.9 g; AcOEt 77.0 g; EtOH 53.7 g). Crude extracts were kept in darkness in glass vials at room temperature for further use. Phytochemical

screening of the plant extracts was undertaken using standard qualitative methods (color test and/or Thin Layer Chromatography, TLC). Two milligrams of each extract were dissolved in chloroform (5 ml) before application to TLC plates (2×5 cm). The revealing agents were: Dragendorff solution (for alkaloids), $FeCl_3$ 1% in ethanol (for flavonoids), $ZnCl_2$ (for sapogenins), perchloric acid (for sterols) and NaOH (for quinones) (Kaufman et al., 2006).

Isolation of alkaloids from ethanolic extract

Two grams of ethanolic extract were stirred vigorously with 35 ml of a mixture of chloroform–ethyl ether–ethylic alcohol (68:24:8) for 10 min. After that, 5 ml of dilute ammonia was added, and the flask was shaken for 1 hr and allowed to stand for 8 hr with occasional shaking. To this, 10 ml of distilled water was added, and the mixture was shaken vigorously. The organic phase was washed into a separator funnel with a few milliliters of a mixture of ether-chloroform (1:1), and then, shaken with 5 ml of H_2SO_4 0.5 N, for a complete extraction of alkaloids. The combined acidic extract was filtered and made alkalize with dilute ammonia solution. The liberated alkaloids were extracted with chloroform and washed with a mixture of chloroform-ethanol (1:1). Finally, solvent was completely removed using a rotary evaporator.

Quantification of total alkaloids

Samples of 10 and 20 mg/ml (in triplicate) were taken and pH was maintained between 2-2.5 using dilute HCl. Then, 2 ml of Draggendorf´s reagent (DR) was added to it, and the precipitated formed was centrifuged. The centrifuged was continued until complete precipitation by adding DR. After centrifugation, the centrifuged was completely decanted. The obtained precipitate was further washed with alcohol and, the filtrate was discarded. Solid residue was then treated with 2 ml of disodium sulfide solution (1%) and the dark brown precipitate was centrifuged. Subsequently, 2 drops of disodium sulfide were added to complete precipitation. Residue was dissolved in 2 ml concentrated nitric acid, with warming if necessary. This solution was diluted to 10 ml in a volumetric flask with distilled water. Then, 41.6 µl was pipetted out, and 208 µl thiourea solutions (3%) were added to it. The absorbance was measured at 435 nm. Results were expressed as total alkaloids (µg/ml) by using the following equation:

Absorbance = 0.0198 [Total alkaloids (µg/ml)]-0.0606, which was obtained from bismuth nitrate stock solution 10%.

Series of dilutions of the stock solution were made by pipetting out 1, 2, 3, 4, 5, 6, 7, 8, and 9 ml and were placed into 10 ml volumetric flasks and diluted to volume with distilled water. A sample containing 41.6 µl of this solution was taken, and 208 µl thiourea solutions were added to it. The absorbance value of the yellow solution was measured at 435 nm against colorless reagent (blank reagent).

Determination of total phenolic content

The total phenolic concentration was determined using the Folin-Ciocalteu´s reagent according to Spanos and Wrosltad (1990). To 2.74 µl of each sample (1 mg/ml, three replicates), 137.36 µl of 1/10 dilution of Folin-Ciocalteu´s reagent and 109.89 µl of Na_2CO_3 (7.5%, w/v) were added and incubated at 45 °C for 15 min. The absorbance of all samples was measured at 750 nm using a micro plate reader spectrophotometer. Results were expressed as gallic acid equivalent (µg/ml) by using the following equation, which was obtained from standard gallic acid graph (range 0.2-3 µg/ml):

Absorbance = 0.3059 [GAE (µg/ml)]-0.0137.

Total flavonoids

Ferric trichloride was used for flavonoids determination (Chang et al., 2002). 1.6 ml aliquot of different concentrations of ethanolic extract (1.6, 8

and 16 mg/ml) was separately mixed with 1.5 ml of methanol, 80 µl of ferric trichloride (10%), potassium acetate (1 M) and 2.24 ml of distilled water. After keeping the mixture at room temperature for 30 min, the absorbance of the maximum reaction was measured at 415 nm. The calibration curve was prepared by using quercetin as standard at concentrations of 25-100 µg/ml of quercetin equivalents (QE) per ml of methanol.

Absorbance = 0.0028 [QE (µg/ml)]-0.0369.

Determination of antioxidant capacity

Determination of ferric reducing/antioxidant power assay (FRAP)

Assay was carried out according to the method of Benzie and Strain (1996). The FRAP reagent was freshly prepared from acetate buffer (3.1 g sodium acetate trihydrate and 16 ml of acetic acid made up to 1 l; pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM iron (III) chloride solution at a ratio of 10:1:1 (v/v), respectively. A total of 12.5 µl sample extract was added to 225 µl of the FRAP reagent and 12.5 µl of water. The sample was well mixed. The absorbance was measured at 593 nm by using a microplate reader spectrophotometer. Samples were measured in three replicates. Standard curve for iron (II) sulfate solution (100, 200, 400, 600, 800 and 1000 µmol/mL) was prepared by using the similar procedure. The results were expressed as µmol Fe (II) /100 g extract sample. Absorbance = 0.0015 [Fe^{+2} (µmol/ml)]-0.1365

Free radical scavenging by the use of DPPH radical

The DPPH radical scavenging capacity of the ethanolic extract was measured according the method described by Brand-Williams et al. with modifications by Miliauskas et al. (2004). DPPH radicals have a maximum absorption at 517 nm, which disappears with reduction by an antioxidant compound. The DPPH radical solution in methanol (9×10^{-5} M) was freshly prepared, and 2.9 ml of this solution was mixed with 100 µl of ethanolic extract at several concentrations (250, 500 and 1000 µg/ml). The samples were incubated for 30 min at 37 °C in a water bath, and decrease in absorbance at 517 nm was measured (AE). A blank sample containing 100 µl of methanol in the DPPH radical solution was prepared, and its absorbance was measured (AB). Radical scavenging activity was calculated using the following formula:

% Inhibition = [(AB-AE)] /AB × 100

Animals

In this study, we used sixty-six adult female Wistar rats (200-250 g). All animals were housed in a room under a 12 hr/12 hr light/dark cycle (light on at 07:00), grouped as six per cage at an average temperature of 25 °C (± 1 °C) and had free access to water and food *ad libitum*. The behavioral tests were conducted during the light phase, between 8:00 and 12:00 hr. All of the rat procedures followed the principles of animal care based on the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and Mexican Official Standard for the Care and Use of Laboratory Animals (NOM-062-ZOO-1999, 2001).

Ovariectomy

The animals were anaesthetized with ethyl ether and a ventral incision made to expose and remove the ovaries (Olson and Bruce, 1986). After suturing muscles and skin, animals were allowed a 2-week recovery period after which, they were randomly assigned to different groups that consisted of six animals (Martínez-Mota et al., 2000; Fernández-Guasti et al., 2001; Estrada-Camarena et al., 2002; Walf and Frye, 2008; Fedotova, 2012).

Treatments

The vehicle (VEH) consisted of 5% tween-80, 5% polyethylene glycol and 90% saline solution (NaCl 0.9%). Ethanolic extract of *A. mexicana* (AMX_{100} and AMX_{200}; 100 and 200 mg/kg, respectively), diazepam (DZP, 2 mg/kg), alkaloids (ALK, 200 µg/kg) and picrotoxin (PTX, 1 mg/kg) were dissolved in vehicle solution.

Behavioral tests

Elevated plus maze

The apparatus was constructed of wood and consisted of two opposite open (50 × 10 cm, length × width) and closed arms (50 × 10 × 40 cm, length × width × height) set in a "plus" configuration. The arms were connected by a central 10 × 10 cm square, and thus, the maze formed a "plus" shape. The entire maze was elevated 50 cm from the floor and lit by dim light. Changes in the time spent on the open arms indicated changes in anxiety (Pellow et al., 1985), and the percentage of closed arm entries is the best measure of locomotor activity (File, 1993). The rats were individually placed at the center of the maze, facing an open arm (Walf and Frye, 2007; Contreras et al., 2014). Additionally, the anxiety index was calculated according to Cohen et al. (2013) as follows:

Anxiety Index = 1 − [([Open arm time/Test duration] + [Open arms entries/Total number of entries])/2].

The time spent in closed arms was not measured because the rat's movement through the central platform made it difficult to separate the time spent in the closed arms from the time spent in the central platform (Wada and Fukuda, 1991).

Locomotor activity test

To evaluate the effects of the treatments on spontaneous locomotor activity and discard the possibility of hypoactivity or hyperactivity attributable to the treatments that could influence performance in the elevated plus maze, a 5-min locomotor activity test was performed after the elevated plus maze test (Contreras et al., 2014). We used an opaque Plexiglas cage (44×33×20 cm), with the floor divided into 12 squares (11×11 cm each) for a 5-min videotaped locomotor activity session. Square crossings were counted when an animal moved from one square to another with its hind legs. After each test, the cage was carefully cleaned with a solution containing ammonia 0.5%, ethanol 15%, extran 10%, isopropyl alcohol 5%, Pinol® 19% and water 50.5% (Gutiérrez-García and Contreras, 2009).

Ethanolic extract and alkaloids effects as assessed by elevated plus maze test

Thirty female rats were assigned into five groups of six animals each: VEH, DZP (2 mg/kg), *A. mexicana* EtOH extracts: AMX_{100} (100 mg/kg), AMX_{200} (200 mg/kg) and ALK (200 µg/kg). All of the chemicals were administered intraperitoneally (i.p.) at a volume of 1 ml/kg. The experiment consisted of testing AMX_{100}, AMX_{200}, and mixtures enriched in ALK extracted from plant in EPM test. The rats were first tested by using EPM test (5 min) and then by the locomotor activity test (5 min). The elapsed time between the injections of substances and the beginning of the behavioral tests was 1 hr.

Interaction between picrotoxin and alkaloids by using the elevated plus maze test

Pretreatment

The doses and pretreatment schedules were based on a previous study that effectively antagonized the behavioral effects produced by anxiolytic drugs: PTX 1 mg/kg (Rodríguez-Landa et al., 2013). PTX was administered intraperitoneally in an equivalent volume of 1 ml/kg 30 min before the extracts, including the VEH.

Experimental groups

The EPM test included six independent groups (n = 6) that received six different treatments: VEH-VEH, VEH-ALK, PTX-

VEH, PTX-ALK, PTX-ALK, PTX-DZP and VEH-DZP. ALK, DZP and VEH were intraperitoneally injected 60 min before the behavioral tests. The locomotor activity test was conducted for <5 min after the EPM test.

Statistical analysis

The data obtained from behavioral tests were analyzed using the one-way analysis of variance for independent groups, and the Student Newman Keuls post hoc test. Results are expressed as mean ± standard error, and only $p \leq 0.05$ was considered significant.

Results

Phytochemical screening

Phytochemical screening confirmed the presence of alkaloids, terpenoids, sterols, steroids, flavonoids and quinones in the extracts. Due to the similarity among profiles of secondary metabolites presents in each extract, we decided to work only with the ethanolic extract in order to simplify the study for a minimum use of animals and experiments. Since the ethyl acetate and methanolic extracts had similar profiles, also Anarthe and Chaudhari (2011) found similar activities when the ethyl acetate and methanolic extracts were used in their work.

Total phenolic content, alkaloids and flavonoids in the ethanolic extract of *A. mexicana*

Total content of polyphenols, flavonoids and alkaloids were quantified in the ethanolic extract, the results are shown in Table 1.

Table 1. Total contents of alkaloids, flavonoids and phenols in the ethanolic extract of *Argemone mexicana*

	Alkaloids[a]	Flavonoids[b]	Phenols[c]
Ethanolic extract	0.96 ± 0.04	190.90 ± 26.70	12.31 ± 1.20

Data are expressed as mean ± standard deviation (n=3). [a]mg/g of extract, [b]meq of quercetin/g of extract, [c]mg GAE/g of extract.

Antioxidant capacity

Antioxidant capacity was quantified by the FRAP and DPPH assays. Based on our results, ethanolic extract exhibited a moderate antioxidant activity index (AAI) calculated from DPPH assay (Table 2); however, the results observed in the FRAP test were in the same direction as those reported by Ji et al. (2011), who described an inhibitive effect of corrosion of mild steel in acidic media when extracts of *A. mexicana* were used as a coating.

Table 2. Antioxidant capacity of *Argemone mexicana*

	FRAP μmol Fe^{+2}/ml	DPPH (IC_{50}) μg/ml	AAI*
Ethanolic extract	838.3 ± 78.7	1218.5	0.008

Data are expressed as mean ± standard deviation (n=3). DPPH radical solution in methanol (100 μM, 39.58 μg/ml) by using (33μg/ml) of plant extract. *AAI = Final concentration of DPPH (μg/ml)/IC_{50} (μg/ml) (Scherer and Godoy, 2009).

Ethanolic extract and alkaloids effects as assessed by elevated plus maze test

The analyses of the percentage of open arm entries ($F_{(4, 25)} = 7.194$, $P < 0.001$) revealed significant differences. Student Newman Keuls post hoc test indicated that AMX_{200}, ALK and DZP significantly increased the percentage of open arm entries compared to VEH (Figure 1a). Treatment with AMX_{200}, ALK and DZP statistically increased the time spent on the open arms ($F_{(4, 25)} = 10.477$, $P < 0.001$) compared to VEH group (Figure 1b). The anxiety index was significantly lower in the groups treated with AMX_{200}, ALK and DZP ($F_{(4, 25)} = 8.496$, $P < 0.001$) than VEH (Figure 1c). No statistical difference was observed among AMX_{200}, ALK and DZP. AMX_{100} had no anxiolytic-like effects compared with DZP. Finally, treatments did not affect the ambulatory activity in the locomotor activity test ($F_{(4, 25)} = 1.788$, $P = 0.163$); hence, this test allowed to discard locomotor problems caused by the treatments (Figure 2).

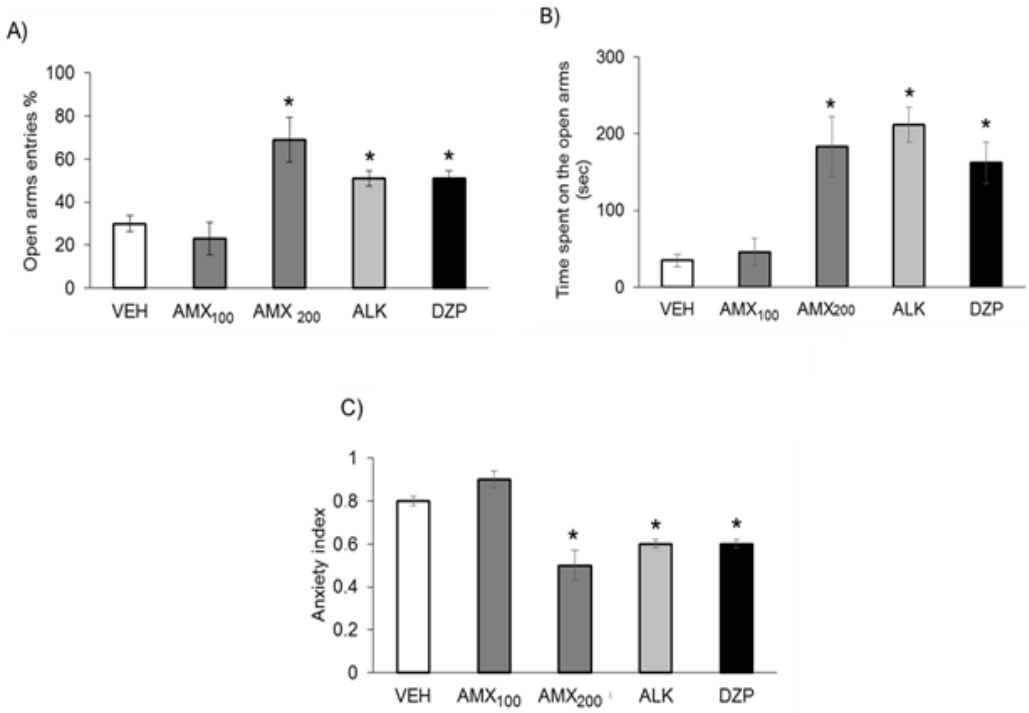

Figure 1. Elevated plus maze test. Percentage of open arm entries (A) and the time spent on the open arms (B) significantly increased in AMX_{200}, ALK and DZP. Anxiety index was significantly reduced in AMX_{200}, ALK and DZP (C). Hence, AMX_{200} and ALK exert anxiolytic effects similar to diazepam. $^{*}P < 0.05$ vs VEH group. Each bar represents mean ± standard error values. One-way ANOVA and Student Newman Keuls *post hoc* test were used for statistical analysis. VEH: vehicle, AMX_{100} and AMX_{200}: ethanolic extract of *Argemone mexicana* (100 and 200 mg/Kg, respectively), ALK: alkaloids mixtures (200 µg/ml) and DZP: diazepam (2 mg/kg); n = 6 for each group.

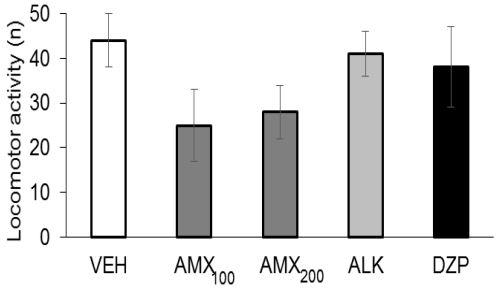

Figure 2. Locomotor activity test. No significant differences were found among subgroups as assessed by one-way ANOVA. Each bar represents mean ± standard error values. VEH: vehicle, AMX_{100} and AMX_{200}: ethanolic extract of *A mexicana* (100 and 200 mg/kg, respectively), ALK: alkaloids mixtures (200 µg/ml) and DZP: diazepam (2 mg/kg); n = 6 for each group.

Interaction between picrotoxin and alkaloids by using the elevated plus maze test

In this behavioral test, the analysis of the percentage of open arm entries ($F_{(5, 30)} = 8.034$, $P < 0.001$) and the time spent on the open arms ($F_{(5, 30)} = 33.557$, $P < 0.001$) indicated significant differences. *Post hoc* test revealed that 200 µg/kg of ALK, the same as DZP, increased these behavior variables as compared to VEH group; however, these effects were cancelled by the noncompetitive $GABA_A$ chloride channel antagonist PTX only in the ALK group ($F_{(5, 30)} = 8.034$, $P < 0.001$; Figures 3a and 3b). Moreover, anxiety index was lower in the groups that received ALK or DZP in comparison with VEH group (Figure 3c). Interaction between PTX and DZP also decreased the anxiety index similar to a single dose of DZP ($F_{(5, 30)} = 20.005$, $p<0.001$). No statistically significant differences were observed among the treatments in the locomotor activity test ($F_{(3, 23)} = 2.426$, $p = 0.096$; Figure 4).

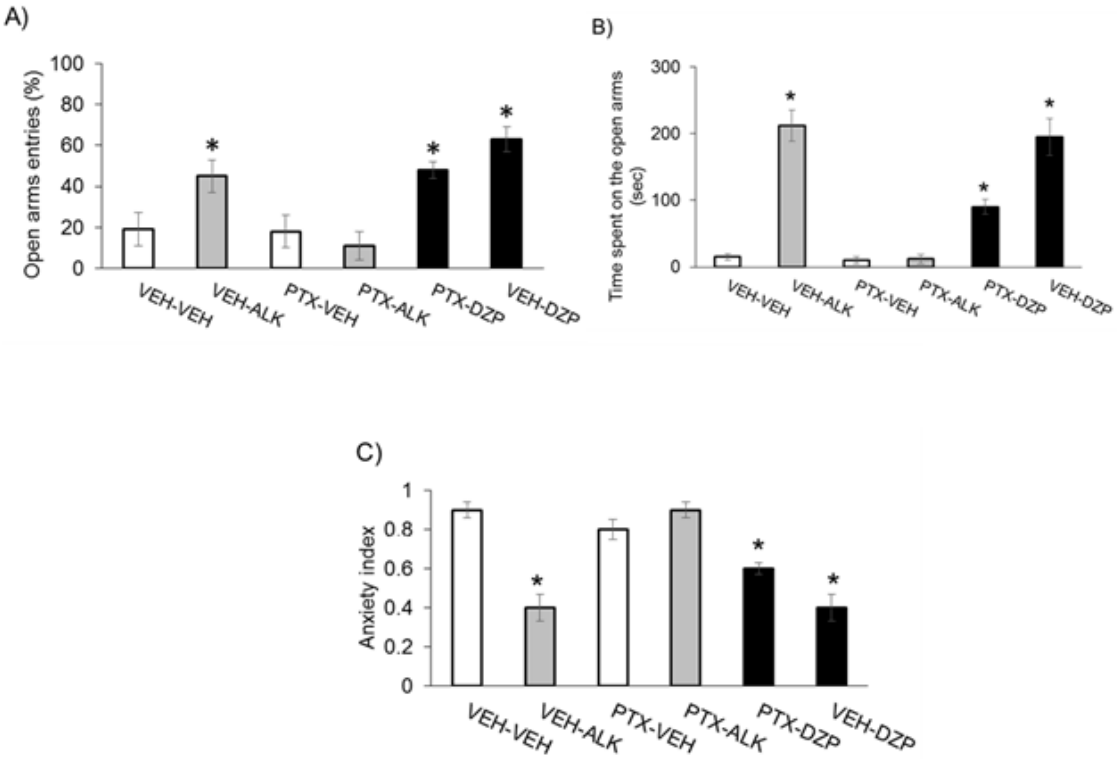

Figure 3. Elevated plus maze test. Picrotoxin blocked the anxiolytic-like effect of ALK on percentage of open arm entries (A) and the time spent on the open arms (B), but no significant changes in the DZP group were observed. Anxiety index was significantly reduced in ALK and DZP groups; however, picrotoxin cancelled this effect only when it was administered with ALK, but not with DZP (C). Each bar represents mean ± standard error values. VEH: vehicle, AMX_{100} and AMX_{200}: ethanolic extract of *Argemone mexicana* (100 and 200 mg/kg, respectively), ALK: alkaloids mixtures (200 μg/ml) and DZP: diazepam (2 mg/kg); n = 6 for each group.

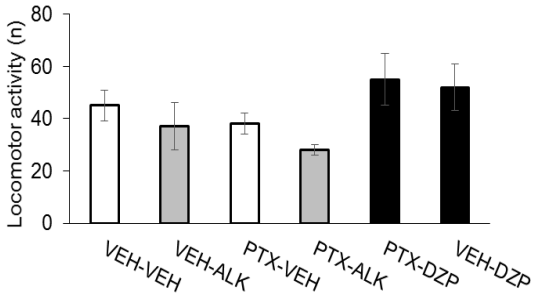

Figure 4. Locomotor activity. No significant differences were found among subgroups, one-way ANOVA. Each bar represents mean ± standard error values. VEH: vehicle, AMX_{100} and AMX_{200}: ethanolic extract of *Argemone mexicana* (100 and 200 mg/kg, respectively), ALK: alkaloids mixtures (200 μg/ml) and DZP: diazepam (2 mg/kg); n = 6 for each group.

Discussion

The present study explored the anxiolytic-like effect of different doses of ethanolic extract of *A. mexicana* (100 and 200 mg/kg) and the alkaloid-enriched extract (200 μg/ml) in EPM test, and the results were compared with the effects of diazepam. Phytochemical screening was carried out and it was consistent with other previously reported works (Bhalke et al., 2009b; Dash and Murthy, 2011); however, no studies have quantified the amount of different metabolites (alkaloids, flavonoids, terpenes, etc.) in *A. mexicana* extracts. Some reports have suggested that flavonoids and alkaloids could act as protective compounds in organism due to their antioxidant properties, which prevent the attack of free radicals, leading to long-term beneficial effects such as anti-diabetic, anti-neoplastic, anti-depressant and some anxiolytic-like actions (Wink, 2000). It has also been reported that the aqueous extract obtained from fresh leaves, results in a 92% protection of the steel sheets from acid corrosion compared to untreated steel sheets; this result

suggests that the metabolites present in the plant have great antioxidant properties (Ji et al., 2011). Contrary to expectations, our results showed that each gram of the ethanol extract contains a number of phenolic compounds, flavonoids and alkaloids (12.13 meq GA/g, 0.17% and 0.1%, respectively) which are considerably low as compared to other commonly used medicinal plants for the treatment of anxiety, such as *Hypericum perforatum* (2-4% of flavonoids) or *Passiflora incarnata* (2% of alkaloids) (Navarro et al., 2008).

Several reports show that flavonoids and alkaloids affect the CNS (Dash et al., 2011; Kennedy and Wightman, 2011). In this sense, our observations in the FRAP assay were in agreement with Ji and collaborators (2011), so that the reducing power of EtOH extract was high; in contrast, the DPPH test did not show a high activity (Table 2); however, Perumal et al. (2010) showed that EtOH extract of the root of this plant exhibited a good free-radical scavenging activity compared to ascorbic acid. For this reason, we consider that the analyzed EtOH extract has a regular antioxidant capacity and it is possible that long-term use might have a protective effect on CNS, but it requires more rigorous and specific studies.

We decided to use the EPM test because it is a validated behavioral model for selective identification of anxiolytic and anxiogenic effects of drugs in rats (Pellow et al., 1985; Hogg, 1996). Normally, exploratory behavior of rats has a strong trend toward the closed arms, this represents a protection zone, compared to the open arms, or exposed areas (Pellow et al., 1985). In animals exposed to the maze for the first time, benzodiazepines reliably increase the percentage of time spent on the open arms and the percentage of entries into the open arms (File et al., 1999).

The results of behavioral evaluation of *A. mexicana* showed anxiolytic-like effects similarly to diazepam on EPM test, without altering locomotor activity test, discarding a possible sedative effect. Also, 2 mg/kg of diazepam is considered an anxiolytic dose, which does not disrupt general motor activity (Fernández-Guasti et al., 2001; Carro-Juárez et al., 2012; Contreras et al., 2014; Rodríguez-Landa et al., 2014.). This finding is important because some substances with stimulant or sedative effects could affect the locomotor activity of animals in behavioral tests (Contreras et al., 1998, 2014; Furlan and Brandão, 2001; Ramos et al., 2008; Gutiérrez-García and Contreras, 2009).

Alkaloid mixture (Singh et al., 2011; Priya and Rao, 2012) was isolated from the crude EtOH extract in order to testing their anxiolytic-like effect on EPM test. The alkaloid-enriched extract was administered to independent groups of animals at a dose of 200 µg/kg, which is equivalent to a single dose of 200 mg/kg of EtOH crude extract (Table 1). Our results showed that 200 mg/kg of ethanolic extract and alkaloids increased the percentage of open arm entries and the time spent on the open arms which are indicatives of anti-anxiety effects on EPM test (Pellow et al., 1985; Hogg, 1996). Also, these treatments reduced the anxiety index, a parameter that unifies the results of the number of entries to the open arms and the time spent in these spaces similarly to diazepam; therefore, alkaloids are involved in the anxiolytic-like effects observed in this work (Figures 1 and 3).

Alkaloids like protopine, coptisine and tetrahydroberberine present in the plant, affect the CNS (Brahmachari et al., 2013), specifically through inhibition of serotonin and noradrenaline transporters, inhibition of MAO enzyme, and by direct bounding with dopamine and GABA receptors (Kardos et al., 1986; Ro et al., 2001; Xu et al., 2006).

When antagonism with picrotoxin was evaluated, we found that the anxiolytic-like effects observed with the alkaloid-enriched extract on EPM test were canceled by this non-competitive antagonist at $GABA_A$ receptor; so that, the

percentage of open arm entries and the time spent on the open arms were decreased similarly to vehicle group. The anxiety index only was increased in the interaction between picrotoxin and alkaloids; this effect could be explained by the blocking effect of picrotoxin on chloride ion channels but not by the binding site of the benzodiazepines on $GABA_A$ receptor (Girish et al., 2013). Other studies have demonstrated that protopine alkaloid has antidepressant-like effects on the tail suspension test in mice through inhibition of serotonin and noradrenaline transporters (Xu et al., 2006) and has affinity for $GABA_A$ receptor (Kardos et al., 1986). Also, chloride channels participate in the actions of substances with anxiolytic activity (Gee et al., 1988; Deng et al., 2007; Zorumski et al., 2013). In summary, *Argemone mexicana* contains several kinds of metabolites with a regular free-radical scavenging activity and a good reductive capacity. Our results demonstrated that this plant exerts anxiolytic-like effects due to its alkaloids and this action is probably mediated through $GABA_A$ receptor chloride channels.

Acknowledgement

The authors thank Mercedes Acosta Avilés from Bioterio de la Facultad de Medicina of the Universidad Veracruzana for obtaining the experimental animals and Janeth Gallegos Estudillo from Instituto de Ciencias Básicas of the Universidad Veracruzana for her help. This study was partially supported by a grant from the Consejo Nacional de Ciencia y Tecnología, México (CONACyT: Project No. 225199) and Programa para el Desarrollo Profesional Docente (PRODEP, PROMEP:103.5/13/7135). A.I. Arcos-Martínez received fellowship from CONACyT (Reg. 223532).

Conflict of interest

The authors declare that there is no conflict of interests.

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