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The anxiolytic effect of the chemical constituent of the roots of *Cordia myxa* (Boraginaceae)

Dabole Bernard, Matcheme Mathieu, Abdou Tchoukoua, Ngatanko Abaissou Hervé, Foyet Harquin Simplice Harquin, Meli Alain and Loura Benoît

Abstract
Neurodegenerative pathology such as anxiety was studied on one of the compounds that were isolated from the root of *Cordia myxa*, a plant belonging to the Boraginaceae family. The investigation of the ethyl acetate extract of the root of *Cordia myxa* had led to the isolation of Alphitolic acid (Betulinic dihydroxy-2α, 3β acid) compound 1 and stigmasterol (Stigmasta-5,22-dien-3β-ol) compound 2.The isolation and characterization of these compounds were carried out respectively by using column chromatography and NMR (1D, 2D). The anxiolytic activity of the compound 1 (1mg/kg, 5mg/kg and 10mg/kg, i.p) were evaluated in mus musculus mice of the swiss strain, the behavioral assays included Open Field Test Activity and Light-Dark transition test. Compound 1 significantly ($^{\delta\delta}P< 0.01$, $^{\delta\delta\delta}P< 0.001$ and $^{\delta}P<0,05$) decreased the anxiety on several parameters, taking benzodiazepine (2mg/kg; Roche, France, i.p) as reference.

Keywords: *Cordia myxa*, anxiolytic activity, alphitolic acid

Introduction
Through its mediators, stress can lead to acute or chronic pathological, physical and mental conditions in individuals with a vulnerable genetic, constitutional and/or epigenetic background [1]. About 6.8 million people die every year as because of neurological disorders [2]. Neuropsychiatric troubles are multifactorial disorders with a multitude of causes such as environmental causes, stress, genetic, molecular and biochemical alteraction [3]. Recent epidemiological data show that chronic stress condition is highly associated to psychiatric mental health impairment: burnout, mood disorders (anxiety, depression), sleep disorders, behavioral disorders (drug addiction, alcohol) [4]. Many drugs are useful in the treatment of neuropsychiatric diseases but secondary effects inaccessibility for some patients, and the high cost of these products for the poor population makes their use problematic [5]. The therapy of neuropsychiatric diseases used traditionally the different parts of curative plants. Nevertheless, these treatments use decoctions and infusions of these plants or mixtures of several plants [6].
Cordia myxa species belongs to the plant family Boraginacea. They are from tropical and subtropical region. It is a medium tree with twisted trunk, which can go up to 12m high. Phytochemical screening carried out on *Cordia myxa* indicated the presence of fatty acids, steroids, carbohydrates, flavanones, flavanones glycosides, triterpenoids, diterpenoids, pyrrolizidine alkaloids, glycosides and saponins [7]. Traditionally, the species *cordia myxa* has several virtues, the plant parts are reputed for many medicinal properties, such as diuretic and laxative, and as a cure in diseases of lungs and spleen, coughs, helminthiasis, leprosy, and skin diseases, their leaves and bark are consumed to treat dyspepsia, fever and diarrhea [7,8]. In Cameroon, they used *Cordia myxa* as firewood; moreover, they used it for the treatment of neurodegenerative diseases with symptoms of anxiety and depression.
Therefore, the objective of the present work was to provide a scientific base for the traditional used of *Cordia myxa* extract of the root by evaluating the anxiolytic activity based on the Opend-Field-Activity-Test (OFT) and Light-Dark-Transition test (LDB) of the chemical constituent isolated and characterized from this extract.

Materials and methods
Plant material
Cordia myxa, commonly known as lasora, belongs to the plant family Boraginacea. Its synonyms are *Cordia latifolia*, *Cordia obliqua* and *Cordia dichotoma* [7].

The roots of *cordia myxa* were harvested in Kossel Danneel (Guider), a locality located in the Northern Region of Cameroon. The botanical identification was made by Dr. Froumsia Mouksia, Department of Biological Sciences, University of Maroua, Maroua, Cameroon. One Voucher specimen (Nº 6410/HEFG) was deposited at the Herbarium of School for the Training of Specialists in Wildlife Management of Garoua, Garoua, Cameroon.

Extraction and isolation

The powdered roots (3.50 kg) were extracted successively with hexane and ethyl acetate at the room temperature for 72 hours. Evaporation of solvent yielded a dark residue (76 g) which was submitted to column chromatography on silica gel (SiO_2, 0.063-0.200m) with a system of gradient solvents of increasing polarity (hexane, hexane-ethyl acetate). Gradient elution using hexane-EtOAc [2:8] yielded compound 1 (152.5mg) and hexane-EtOAc [8:2] afforded compound 2.

Animals

The evaluation of the anxiolytic activity of the compound 1 was carried out on male anxious mice of the genus mus musculus, of swiss strain, aged 8 to 12 weeks and weighing 22 to 26g. These mice were purchased from the National Veterinary Laboratory (LANAVET), Garoua, Cameroon. They were kept under standard conditions at the Neuroscience Laboratory, Department of Biological Sciences, University of Maroua, Maroua, Cameroon. These mice were grouped by a group of five animals in polypropylene cages and subjected to natural light. The diet was a classical laboratory diet with satiated drinking water; the animals were acclimatized to laboratory conditions for 7 days before the beginning of experiment. Pharmacological tests are usually performed by screening according to the method described by Trease and Evens [9], which consists of determining the effective dose for further pharmacological testing. However, the anxiolytic effect of the compound 1 is evaluated by Light-Dark transition test (LDB) according to the method of Gong [10] and Open Field Test Activity (OFT) according to the method of Foyet [11] and Lee [12]. For each test, the animals will be divided into 5 batches, including batch 1 of negative control (7 mice), batch 2 for the positive control (7 mice, 2mg/kg), batch 3 for dose 1 (7 mice, 1mg/kg), batch 4 for dose 2 (7 mice, 5mg/kg) and batch 5 for dose 3 (7 mice, 10mg/kg).

Statistical analysis

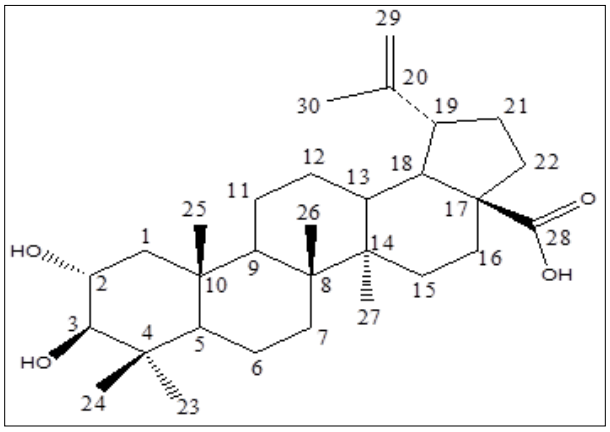
All the results were expressed as ± SEM. The data were analysed by one and two ANOVA followed by bonferoni's and Dunnett's multiple comparison test as the post test. All analysis were performed using the software Graph Pad Prism version 6.00 for Windows, Graph pad Software, San Diego, California, USA, http:// www. Graph pad.com/.

Chemical

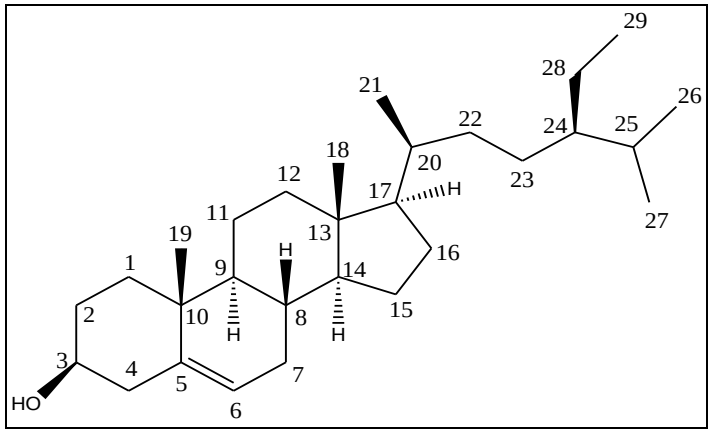
The French brand benzodiazepine was used as the reference compound. The different doses of compound 1 and the reference compound are all administered to mice by the intraperitoneal route (i.p).

Results and Discussion

The compound 1 crystallizes as a white powder in the Hex-AcEt [2:8] solvent system. It is soluble in MeOH and gives a purplish-red coloration in the Libermann-Buchard test, showing that our molecule has a triterpene skeleton. The ^1H NMR spectrum of compound 1 shows us in strong fields six (06) methyl between δH 0.77-1.67ppm characteristic of pentacyclic triterpene. A five singlet to conch having a chemical shift of 0.77ppm (3H, s, Me-26); 0.91ppm (3H, s, Me-24); 0.96ppm (3H, s, Me-25); 0.98ppm (3H, s, Me-23); 1.00ppm (3H, s, Me-27); 1.67ppm (3H,s, Me-30). We have two ethylene protons resonating at 4.69 and 4. 56ppm (H-29), these are exomethylene proton. We also observe a multiplet at 2.31ppm, which corresponds to the proton H-19(1H, m). These spectroscopic data correspond to the chemical displacements of compounds of the dihydroxylup-20(29)-ene type, thus pentacyclic triterpenes of the lupane series [13]. Towards the strong fields, we observes a singlet at 1.67ppm corresponding to the protons of the methyl group at C-30 and the chemical shift of 3,60ppm corresponding to the chemical shift of the proton in position 2(1H, ddd). The analysis of its ^{13}C NMR spectrum confirms the presence of the triterpene skeleton of lupane type by the olefinic signals observed at δc152.0ppm(C-20) and 110.1ppm(C-29) and the signal at 180.4ppm to that of the carbonyl of the carboxylic acids. Two signals at 84.4ppm and 69.7ppm are attributable to C-3 and C-2 respectively and are consistent with the biosynthesis of pentacyclic triterpene [14] carrying hydroxyl groups. The DEPT 135 spectrum combined with the ^{13}C NMR spectrum allows us to distribute these different carbons such as: six(06) methyl groups with chemical shift δc 29.1(C-23); 19.5(C-30); 17.8(C-24); 17.2(C-25); 16.6(C-26); 15.0(C-27); ten(10) methylenes at δc 19.5(C-6); 22.2(C-11); 26.8(C-12); 30.8(C-16); 31.7(C-15); 35.4(C-7); 38.2(C-21); 46.4(C-22); 48.6(C-1); seven(07) methynes δc 69.7(C-2); 84.2(C-3); 56.8(C-5); 51.9(C-9); 48.5(C-18); 39.6(C-13); 39.6(C-19); seven(07) quaternary carbons δc 39.5(C-4); 40.5(C-10); 57.5(C-17); 152.0(C-20); 180.4(C-28). In view of the above, it appears that the raw formula of our molecule is $C_{30}H_{50}O_3$, a formula corresponding to the family of pentacyclic triterpenes possessing six unsaturation. The *J*-modulated HSQC spectrum presents 7 quaternary carbons, 7methines, 10 methylenes and 6 methyl.The set of all our data compared to the values in the literature (Table1) allows us to confirm alphitolic acid as the structure of the compound 1 [15] The compound 2 was identified as β-sitosterol [16].



Compound 1



Compound 2

Fig 1: Chemical structure of compounds 1 and 2.

Table 1: ^{13}C and ^{1}H NMR (MeOD, 500MHz) spectral data of compound 1 compared to the literature [15].

Positions	Alphitolic acid		MM5(MeOH, 500Hz)	
	δc(ppm)	δH(m, J en Hz)	δc(ppm)	δH(m, J en Hz)
1	46.7	0.90(dd, 12,3-3,1); 1.99(dd, 12, 4-4,6)	48.6	0.79(t, 12.0); 1.97(dd, 12.4-4.6)
2	69.2	3.74(ddd, 11,4-9,5-4,6)	69.7	3.60(td, 10.3-5.1)
3	83.9	3,04(1H, dl, J=9,6Hz	84.4	2.87(1H, dl, J= 9.6Hz)
4	39.2	-	39.5	-
5	55.4	0,85(dm, 9,1)	56.8	0.75(dl, 6.5)
6	18.2	1, 45(m); 1,57(m)	19.5	1.37(t, 8,5); 1.48(m)
7	34.2	1,44(m)	35.4	1.33(m); 1.36(m)
8	40.7		41.9	
9	50.4	1,40(dd, 12,9-2,2)	51.9	1.31(dd, 15.7-3.4)
10	38.5	-	40.5	-
11	37.0	1,52(td, 8,5-2,2); 2,02(m)	22.2	1.24(dd, 11.8-3.5); 1.42(dd, 12.6-2.4)
12	25.3	1,09(dt, 13,0-4,5); 1,77(m)	26.8	0.99(dd, 12.9-4.6); 1.67(dd, 13.7-2.6)
13	38.3	2,24(ddd, 12,8-12,7-3,8)	39.6	2.20(td, 11.9-3.4)
14	42.5	-	43.6	-
15	29.6	1,23(dt, 13,5-6,4-3,3); 1,58(m)	31.7	1.13(dt, 13.3-3.0); 1.46(td, 14.9-3,5)
16	32.1	1,48(m); 2,32(dt, 12,9-6,6-3,1)	30.8	1.37(tl, 8.5); 2.22(dt, 12.9-3.4)
17	56.3	-	57.5	-
18	49.2	3.55(1H, m)	48.5	3.01(1H, m)
19	46.8	3,05(dt, 10,5-3,6)	50.4	2.31(td, 10.7-4.9)
20	150.2	-	152.0	-
21	30.5	1,46(m); 2,04(m)	38.2	1.89(m) ; 1.42(m)
22	20.9	1,35(td, 12,6-4,4); 1,52(td, 12,6-2,4-2,0)	46.4	2.41(t, 6.9); 1.73(td, 10.7-4.9)
23	28.4	1.06(3H, s)	29,1	0.98 (3H,s)
24	16.5	0.85(3H, s)	17,8	0.91(3H, s)
25	17.3	0.94(3H, s)	16,6	0.96(3H, s)
26	16.1	0.98(3H, s)	17,2	0.77(3H, s)
27	14.6	1.03(3H, s)	15,0	1.00(3H, s)
28	181.2	-	180,4	-
29	109.7	4.67(d, 2,9) ; 4.78(sl)	110,1	4.70(d, 2,1); 4.59(sl)
30	19.3	1.74(3H, s)	19,5	1.67 (3H, s)

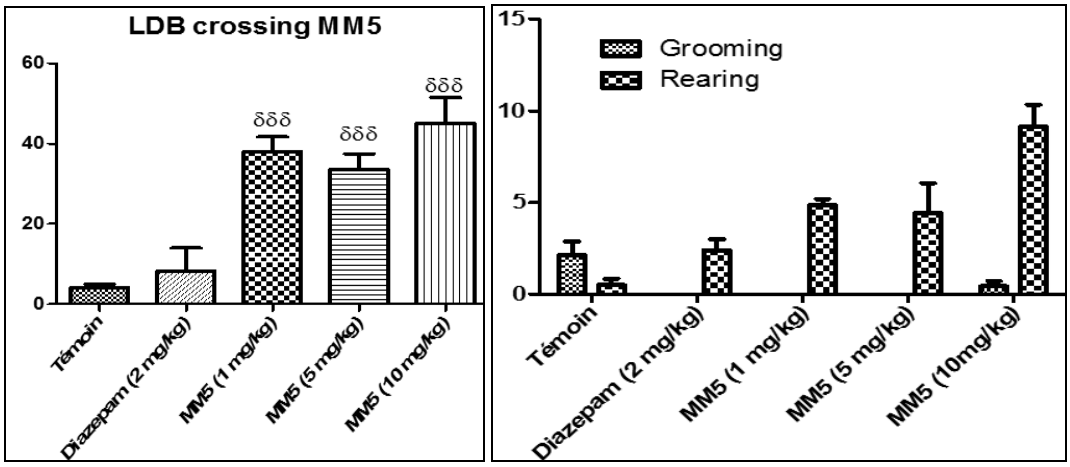
To evaluate the anxiolytic activity of the compound 1(alphitolic acid) at different doses (1mg/kg, 2mg/kg and 5mg/kg), we used the Ligh-Dark-Transition Test, one of the most widely used pharmacological tests to evaluate the anxiolytic activity of a compound [10] and Open-Field-Activity Test (OPF). These tests consists of evaluating the pharmacological test on anxiety, is a white polywood and measures 72.72cm walls [17].

To perform this test, the animals are divided into 5 batches, including the negative control (7 mice); the 2nd batch for the positive control (7 mice, 2mg/kg); 3rd batch for dose 1(7 mice, 1mg/kg); the 4th batch for dose 2 (7 mice, 5mg/kg); and the 5th batch for dose 3 (7 mice, 10mg/kg). These batches of mice will receive the different doses of compound 1 orally30min after the test [11].

Results of Ligh-Dark-Transition Test

For this pharmacological test, the parameters highlighted are box times, number of lines crossed, "grooming" and "rearing".

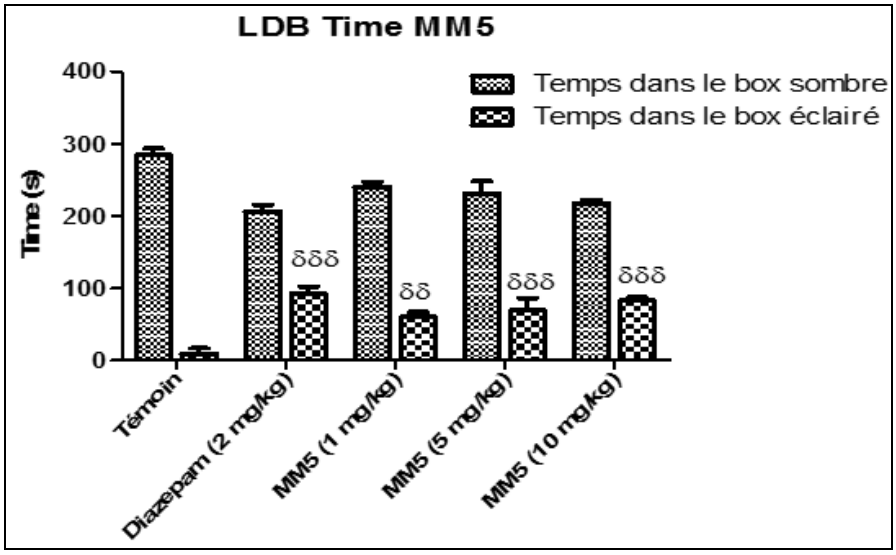
Administration of compound 1 at different doses, 1mg/kg, 5mg/kg and 10mg/kg resulted in a significant increase ($\delta\delta\delta P‹0.0001$) in the number of crossings between the two boxes of the maze compared to those of the negative and positive control lot (Figure 2a). Rodents spend all their time rearing that grooming. For those in the control lot, rodents spend most of their time grooming than standing on their hind legs. Treatment with Diazepam (2mg/kg) results in an increase in the number of rearing and total cancellation of grooming. Similarly for those given compound1 at 1mg/kg and 5mg/kg. However, at 10mg/kg compound 1, there was an increase in the number of rearing and a decrease in the number of grooming (Figure 2b).



(a) **(b)**

Fig 2: These histograms illustrate the number of transitions made by the animals between the two boxes of the maze (Figure2a), the number of grooming and rearing in LDB. Each bar chart represents the mean ± ESM, N=7, the two-variable ANOVA test followed by bonferoni's post-test (Figure2b) single-variable ANOVA followed by Dunnett's multiple comparison post-test was used ($^{\delta\delta\delta}P<0,001$(Figure2a), all in comparison to the control.

The parameter highlighted here is the time taken by the animals in the two boxes of the maze. According to the figure 3 below, the animal exposed in the lighted box transits almost immediately to the dark box to take refuge. Then, the animals of the negative control lot spend of time in the dark box. Conversely, in the animals given Diazepam (2mg/kg) and the different doses of compound 1, we all noted a significant decrease in time spent in the dark stall, coupled with a significant increase in time spent in the light stall ($^{\delta\delta\delta}P<0,001$) for those in the positive control group, and those given 5mg/kg, 10mg/kg compound 1 and ($^{\delta\delta}P<0, 01$) for those given 1mg/kg.

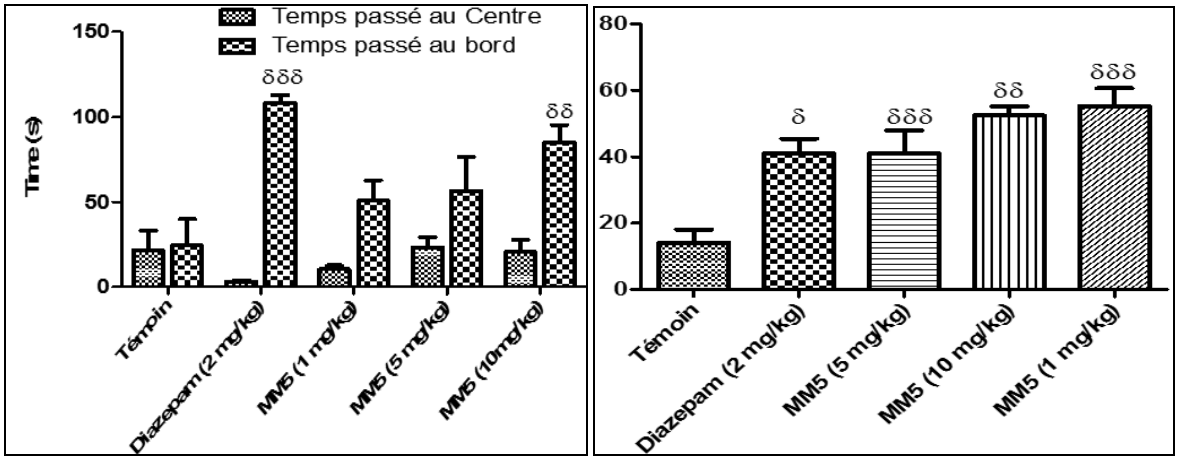


(c)

Fig 3: Time taken by the animals in the dark and light stall. Each histogram represents the mean ± ESM, N=7, the bivariate ANOVA test followed by Bonferoni's post-test was used; $^{\delta\delta}P<0,01$; $^{\delta\delta\delta}P<0,001$ compared to control time.

Results Open-Field-Activity Test (OPF).

The animals introduced in the centre of Open-Field polywood explore the environment by spending their time in the centre and at the edge of the polywood. Those in the control lot spend almost identical amounts of time in the centre and at the edge of the Open-Field. Those from the positive control group who administered Diazepam at a dose of 2mg/kg resulted in a significant increase ($^{\delta\delta\delta}P<0.001$) in time spent at the edge of the polywood compared to the centre. But rodents given compound 1 at different doses showed a significant ($^{\delta\delta}P<0.01$) increase in time spent at the edge at 10mg/kg (Figur 4a). In addition, the effect of compound 1 on exploratory activity in the open area is shown in figure 4b. The animals in the control batch did not explore the area to any great extent. Diazepam treatment (2mg/kg) resulted in a significant ($^{\delta}P<0.05$) increase in the number of lines crossed compared to the vehicle. Different doses of compound 1 also resulted in a significant increase ($^{\delta\delta\delta}P<0.001$) for the 5mg/kg and 1mg/kg doses, ($^{\delta\delta}P<0.01$) for the 10mg/kg dose in the number of lines crossed compared to the control.



(a) **(b)**

Fig 4: Each bar chart represents the mean ± ESM, N=7, the bivariate ANOVA test followed by Bonferoni's post-test was used (Figure4a), the univariate ANOVA test followed by Dunnett's multiple comparison post-test was used (Figure 4b); $^{\delta}P<0.05$; $^{\delta\delta P}<0.01$); $^{\delta\delta\delta}P<0.001$) compared to control time.

Discussion of results on anxiety

Benzodiazepine was introduced as a medication about 50 years ago for the treatment of various forms of anxiety. Its toxic and undesirable effects led to the search for beneficial treatments that are less toxic and less expensive. The study of the anxiolytic property of the compound 1 isolated from the roots of *Cordia myxa* (Boraginaceae) on different models of anxious animals, appears from these analyses that in anxiety tests for LBD, the administration of Diazepam and the isolated compound 1 at doses of 1; 5; and 10mg/kg significantly decreased the time spend in the dark box. Similarly, the isolated compound 1 also significantly increased the degree of exploration of the lightening box while significantly increasing the number of rearing. In addition, the opened area, the administration of Diazepam and the isolated compound 1 resulted in a reduction in the time spent in the centre while increasing the time spent at the edge. Similarly, the isolated compound 1 also increased the level of animal exploration in the maze. With regard to these properties, on the observation of the parameters studied, we can say that the compound 1 isolated from the roots of *Cordia myxa* possesses anxiolytic properties. These results are similar to those of Foyet and his collaborators working on the aqueous extracts from the trunk bark of *Alafia-multiflora*, produced a significant reduction in grooming time and time spent at the Opened-Field test centre [12]. The grooming explains the stress in rodents, the time spent in the centre by the animals generally explains that the animal is anxious. Similarly, rearing generally indexes locomotor activity and the number of lines crossed indicates central nervous system stimulation [18]. Our results are in agreement with those of Lee and his collaborators in 2007, working on aqueous extracts of *Acanthus-montanus* root. Administration of these extracts to mice significantly increased the rearing number and the exploratory activity of these animals [11]. All these results show that the different doses administered to the animals present anxiolytic effects. Compound 1 is a steroid with anxiolytic activity similar to Diazepam. It would act via the gamma-aminobutyric acid receptor complex $(GABA)_A$.

Conclusions

The present study investigate the anxiolytic effect in male anxious mice of the genus mus musculus, of swiss strain, of alphitolic acid isolated from the roots of *cordia myxa* (Boraginaceae). Two compounds, alphitolic acid and stigmasterol were isolated from the roots of *Cordia myxa* and characterized, using chromatography methods and NMR (1D, 2D). The behavioral assays of anxiolytic effect included Open Field Test Activity (OFT) and Light-Dark transition test (LDB). In the anxiety tests for LBD, the administration of Diazepam and the isolated compound 1 at doses of 1; 5; and 10mg/kg significantly decreased the time spend in the dark box. The isolated compound 1 also significantly increased the degree of exploration of the lightening box and increasing the number of rearing. In the opened area, the administration of Diazepam and the isolated compound 1 resulted in a reduction in the time spent in the centre while increasing the time spent at the edge. The isolated compound 1 also increased the level of animal exploration in the maze. With regard to these properties, on the observation of the parameters studied, we can say that the compound 1 isolated from the roots of *Cordia myxa* possesses anxiolytic properties. The anxiolytic properties exhibited by compound 1 is more than those exhibited by the reference.

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