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Study of the chemical components of *Peganum harmala* and evaluation of acute toxicity of alkaloids extracted in the Wistar albino mice

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Abstract
The purpose of this study is to realize a phytochemical screening and determine the acute toxicity of *Peganum harmala* species. The plant samples were collected from Harmalia region, north-eastern Algeria. The results showed the presence of certain chemical compounds such as: alkaloids, flavonoids, saponins, reducing compounds, tannins and volatile oils in all parts of the plant. Triterpenes or sterols and anthraquinone were present only in fruits and seeds. A total absence of coumarins and cardenolides was recorded in all parts of the plant. The quantitative extraction of alkaloids by the titration method showed that the seeds contain the highest proportion of alkaloids (3.94%). Study of the acute toxicity of total alkaloids from seeds (TAS) of male Albino-Wister mice by the intraperitoneal route showed that the alkaloid is a moderately toxic substance (lethal dose 50%: 350 mg / kg BW). Observation of clinical changes such as convulsion, agitation, tachycardia, shortness of breath, drowsiness, decrease in locomotors activity and anorexia, during the treatment period (14 days) which confirms the hypothesis that alkaloids have an effect on the central nervous and respiratory systems.

Keywords: Peganum harmala, phytochemical, alkaloids, acute toxicity, Algeria.

1. Introduction
Plants and plant extracts have been used since the dawn of civilization by mankind. The use of ethnobotanical preparations for various reasons justified or not, is still continued by various cultures all around the world. Considering structural and biological diversity of terrestrial plants, they offer a unique renewable resource for the discovery of potential new drugs and modern medicine has developed a rational strategy for drug discovery which involves the study of plants and plant materials based on their ethnobotanical usage [1], including the plant *Peganum harmala*, which belongs to the family of Zygophylaceae, distributed mainly in the Mediterranean region, also found in Central Asia, North Africa and also cultivated in America and Australia [2]. It is rich in alkaloids of type β-carboline and contains up to 2 - 7% total alkaloids [3]. Several studies have shown various biological activities and pharmacological characteristics of its seeds such as hypothermia [4], hallucinogen factor [5], antidepressant [6], inhibitor of the enzyme monoamine oxydase (MAO) [3] , antibacterial, antifungal and anti-virus [7,8]. It is effective for the treatment of dermatosis disease [9], its leaves are used as an antinociceptive [10].However, it causes abortion in rats [11].
The objective of this work is insufficient information on the study of plant's *P.harmala* that grows in eastern Algeria, we examined phytochemical to detect secondary metabolites and determine the level of alkaloids in all the different parts of the plant, and record clinical observations and calculating the value of the LD50 of total alkaloids from seeds (TAS) by intraperitoneal route in the case of acute toxicity in the mice.

2. Materials and methods
2.1. Plant material collection and identification
Different plant parts such as roots, leaves, stems, flowers, fruits and seeds of *P.harmala* were collected from the Harmalia region (South east of the town of Ain M'lila, Algeria) .The samples were harvested during the months:

March, May, July and September in2011. The botanical identity of the plant was confirmed by Dr. Y. Halis, and a voucher specimen (No. 0118/HBPA) was deposited at the Herbarium of Laboratory of Bio-molecules and Plant Amelioration, Larbi Ben M'hidi University of Oum El Bouaghi, Algeria. The plant parts were dried in air at room temperature and stored for one month in dark bottle amber.

GPS coordinates

The coordinates of the region of Harmlia (as Google earth) are:

- Latitude: 35 ° 55'30 .95 "N
- Longitude: 6 ° 37'20 .19 "E
- Elevation: 795m

2.2. Phytochemical analysis

2.2.1 Phytochemical screening

Phytochemical tests were performed on different extracts prepared from each sample dry powder of the roots, leaves, stems, flowers, fruits and seeds, using three solvents of different polarities: water, ethanol and diethyl ether. The detection method of the different families of chemical compounds co-existing is a precipitation reaction or staining reagents. These reactions result in the appearance of turbidity, flocculation or a color change that may depending on the intensity of the result of the concentration of certain constituents [12, 13].

2.2.1.1 Preparation of ethanolic extract

The ethanolic extract was prepared by a mixture of 50 g of each sample dry powder (roots, leaves, stems, flowers, fruits and seeds) in 250 ml of ethanol under reflux for 1h. The extract is filtered by using Whatman filter paper. The filtrate was used for phytochemical screening.

Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids.

- *Detection of tannins:* The alcohol extract (1mL) was added to 2 mL of water and 2-3 drops of diluted solution of ferric chloride. The appearance of a green or blue-green color indicates the presence of tannins [14].
- *Detection of alkaloids:* 20 mL of the extract was added to 5 mL of hydrochloric acid HCl (10%). At this acidic medium heated in a water bath, was added a volume of ammonium hydroxide solution (10%) until obtain a medium of pH= 9 which was extracted with diethylic ether and concentrate with a rotary evaporator. The residue will be taken in 0.5 mL of HCl (2%), divide into two equal parts. The first was treated with a few drops of Mayer's reagent and the second with Wagner's reagent. Observation: turbidity or precipitation [15].
- *Detection of sterols and triterpenes:* The residue of ethanol extract was dissolved in 0.5 mL acetic anhydride and then in 0.5 mL of chloroform. Then 1 mL of concentrated sulphuric acid is added (Liebermann-Burchard reaction). At the contact zone of the two liquids a brownish red ring was formed denoting the presence of sterols and triterpenes.
- *Detection of reducing compounds:* 2 mL of aqueous solution was added at 1 mL of the alcoholic solution and 20 drops of Fehling's solution, heat the solution. A brick red precipitate denotes the presence of reducing compounds [14].
- *Detection Test of coumarins:* 15mL of HCl (10%) was added to 25mL of ethanolic solution, and heated under reflux for 30 min and strain the mixture. The residue was extracted with 15 mL of ether in triplicate. Divide the filtrate into two equal parts, evaporate the first in a rotary evaporator, dissolve the residue in 1mL of water and treat with 0.5mL NH_4OH (10%), examined under ultra-violet light, fluorescence intensity indicates the presence of coumarins. The second one was used as control [16].
- *Detection of Anthraquinones:* About 8 mL of the extract was boiled with 10 % HCl for few minutes in water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% ammonia was added to the mixture and heated. Formation of rose-pink color indicates the presence of anthraquinones.

2.2.1.2 Preparation of diethyl ether extract

The extract was prepared by a mixture of 50 g of each sample dry powder (roots, leaves, stems, flowers, fruits and seeds) in 250 mL of diethyl ether under reflux for 1h. The extract is filtered by using Whatman filter paper. The filtrate was used for phytochemical screening:

- *Detection of volatile oils*: The residue obtained after evaporation of 20mL of ethereal solution was dissolved in ethanol and concentrated. a residual aroma reveal a positive test[17].
- *Detection of alkaloids bases:* The resulting residue obtained was dissolved after evaporation of 10 ml of the ethereal solution in 1.5 mL of HCl 2% and add 1-2 drops of Mayer or Wagner reagent. The appearance of yellowish white precipitate indicates the presence of alkaloid bases [15].

2.2.1.3 Preparation of aqueous extract

- The extract was prepared by a mixture of 50 g of each sample dry powder (roots, leaves, stems, flowers, fruits and seeds) in 250 mL of distilled water under reflux for 1h. The extract is filtered by using Whatman filter paper. The filtrate was used for phytochemical screening.
- *Detection of tannins*: 1mL of the aqueous solution was treated with 1mL of water and 1-2 drops of dilute solution of ferric chloride. The appearance of a dark green color or blue-green indicates the presence of tannins [14].
- *Detection of cardenolides:*
  Keller-killiani Test: filtrate is mixed with 10 mL of (chloroform and ethanol). The organic phase is evaporated till dryness, and precipitate is dissolved of 3 mL of glacial acetic acid containing traces of $FeCl_3$ and 1 mL of conc. H_2SO_4 were added. A reddish-brown colour formed at the junction of two layers and the upper layer turned bluish green.
  Baljet test: 1mL of baljet agent (9.5ml of Picric acid mixed with 0.5 of NaOH in ethanol) is added to 1mL the neat alcoholic extract. The non-appearance of orange color reveals the presence of cardenolides is added to the extract and made alkaline, gives a stable orange color.
- *Detection of saponins*: 2 mL of the aqueous solution was added to a little of water and then stir in a strong way [16]. Persistent foam confirmed the presence of saponins. Abandon the mixture for 20 minutes and classify content saponins:
  - No foam = Negative test.
  - Foam less than 1 cm = weakly positive test.
  - Moss 1-2 cm = positive test.
  - Foam over 2 cm = very positive test.

2.2.2 *Alkaloids extraction*

100 g of each powder parts of plant (roots, leaves, flowers, steams, fruits and seeds) was macerated in ethanol (70%), alcoholic extract was evaporated to one fifth of the initial volume by rotary evaporator, after, 20 mL of hydrochloric acid HCl (0.1N) was added to the concentrated extract obtained is then filtered and extracted twice with 20 mL of chloroform, the latter was treated twice with 10 mL of hydrochloric acid (0.1 N), then we add ammonia NH_3 (0.1 N) to obtain an extract of pH = 9, then adding 30 mL of chloroform, according to the classical method [18]. The operation is repeated 3 times then evaporates, residues were dissolved in 20 mL of hydrochloric acid (0.02 N) and titrated with potassium hydroxide KOH (0.02 N) using methyl red as an indicator, then calculating the percentage of alkaloids seeds and roots of the plant by the formula:

$$\%\text{Alkaloïds} = \frac{\text{Volume of acid}(0.02\ \text{N}) - \text{base volume consumed}(0.02\ \text{N})}{\text{Powder mass (plant)}} \times 0.0046 \times 100$$

2.3 Experimental Animals

Male Wistar albino mice of weights 25-35gms, obtained from the animal house of the Pasteur Institute in Algiers, were used for the acute toxicity study. They were housed in stainless steel wire mesh cages up to a maximum of 8 per cage, in a well-ventilated room with 12 h light/dark cycle, with free access to clean drinking water and food (standard rat feed). The animals are acclimated to the conditions of the pet shop at the faculty of natural sciences and life at Constantine University for one week before experimentation.

2.4 Acute Toxicity Testing

The extract of (TAS) of *P.harmala* to be tested is dissolved in distilled water and administered at different doses intraperitoneally in a dose per group. The mice were weighed, identified by a marking and divided into 7 groups of 8 animals, one of which is a control group. The 6 groups of mice were treated with simple application

and successively with the following doses: 100, 200, 300, 400, 500, 600 mg/kg body weight (BW), while mice in the control group received distilled water. Food was provided to the mice approximately an hour after treatment.

- *Clinical observations:* the animals were observed 30 min after dosing, followed by hourly observation for 8h and once a day for the next 14 days. All observations were systematically recorded with individual records being maintained for each animal. Surviving animals were weighed and visual observations for mortality, behavioral pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period.
- *Determination of* lethal dose *(LD50)*: The LD50 was determined according to probit method (method of least squares, method Finney) using the [Software StatPlus® Professional] (Ver.2009 Build 5.8.4.3 ©2010) and the method described by Behrens and Karber (1935) (19). The results were calculated with the formulation below:

$$\mathbf{LD50 = LD100 - \sum (a.b)/n}$$

n =number of mice in each group.
a=the difference between two consecutive doses.
b=the arithmetic average deaths caused by two consecutive doses.

3. Results and discussion

3.1 Result

Phytochemical analysis

Qualitative analysis carried out for ethanolic, diethyl ether and aqueous extracts of the aerial and earth parts of the plant showed the presence of most Active constituents (Tables 1, 2 and 3).

Table 1: Tests carried out on ethanolic extracts

Parts / Compounds	Roots	Stems	Flowers	Leaves	Fruits	Seeds
Flavonoids						
- Alkaline reagent	+	+	+	+	+	+
- Lead acetate	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Triterpenes and sterols (Liebermann-Burchard reaction)	-	-	-	-	+	+
Reducing compounds	+	+	+	+	+	+
Coumarins	-	-	-	-	-	-
Anthraquinones	-	-	-	-	+	+

Phytochemical examination of the different parts of the plant *P.harmala* revealed the presence of flavonoids, alkaloids, saponins, volatile oils, tannins and reducing compounds. A total absence of coumarins and cardenolides in all parts of the plant is recorded, while the triterpenes or sterols and anthraquinones are present only in seeds and fruits

Table 2: Tests carried out on diethyl ether extracts

Parts / Compounds	Roots	Stems	Flowers	Leaves	Fruits	Seeds
Volatile oils	+	+	+	+	+	+
Alkaloids bases	+	+	+	+	+	+

Table 3: Tests carried out on aqueous extracts

Parts / Compounds	Roots	Stems	Flowers	Leaves	Fruits	Seeds
Tannins	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Cardenolides	-	-	-	-	-	-

Key: - : absence; +: presence

Figure 1shows that the alkaloids rate varies from one part to another, the highest percentage recorded in the seeds, roots and fruits with rates of 3.94%, 2.93% and 2.74 %, respectively. Record the lowest percentage of alkaloids with leaves, stems and flowers with rates of 1.69%, 1.14% and 0.71%, respectively.

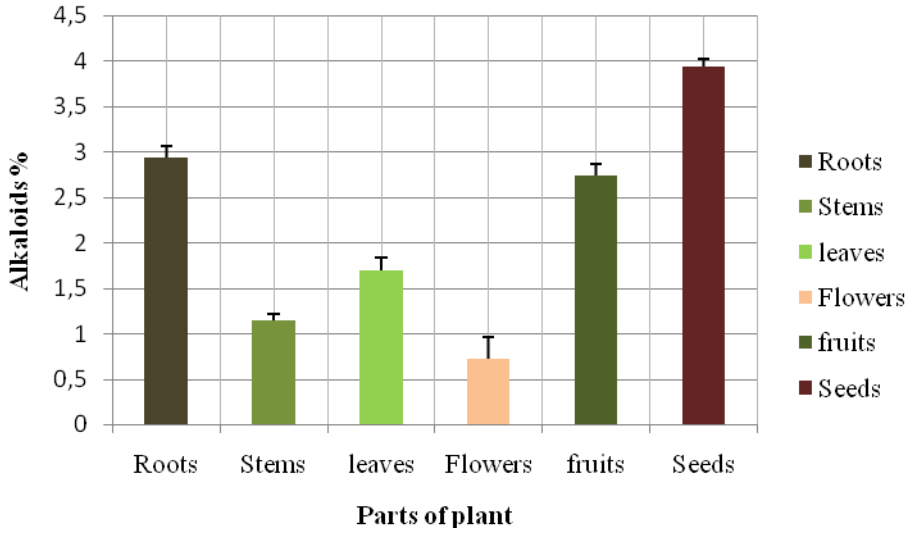

Figure 1: Rate of alkaloids in different plant parts of *P.harmala*

Behavioral Observation

Since the beginning of treatment, we recorded a clinic card, characterized by acute symptoms with higher doses, in the form of strong convulsion, agitation, tachycardia and difficulty breathing. Secondary symptoms characterized by a decrease in locomotor activity of mice, drowsiness and anorexia. Recording the death of the 15th minute to 24 hours, after 72 hours, surviving animals regain normal behavior similar to the control group.

Calculation of lethal dose LD50

By the Behrens–Karber's method

After administration of doses ranging from (100-600) mg / kg (BW) of (TAS) of the plant *P. harmala* by the intraperitoneal route to different groups of mice, the mice mortality percentage and function of the dose are listed in table 4.

Table 4: Mortality percentage after 24 hours from intraperitoneal administration of different doses of TAS.

Groups	**1**	**2**	**3**	**4**	**5**	**6**
Dose (mg/kg)	100	200	300	400	500	600
Number of animals	8	8	8	8	8	8
number of mortality	0	1	3	5	7	8
% of mortality	0	12.5	37.5	62.5	87.5	100
Difference successive doses (a)	100	100	100	100	100	-
Arithmetic average deaths caused by two	0.5	2	4	6	7.5	-
a x b	50	200	400	600	750	-

$LD50 = LD100 - \sum(a.b)/n$

LD50 = 350 mg/kg PW.

The graph in Figure 2 is constructed from the values of Table 4, which determines the value of LD 50 of (TAS) which is equal to 350 mg / kg BW.

*By probit method***:** Based on the [Software StatPlus® Professional] (Ver.2009 Build 5.8.4.3 ©2010), we also calculated the LD50 using the method of probit (least squares method and method of Finney)

The relationship between doses of (TAS) of the plant *P. harmala* and the mortality rate of mice calculated by probit analysis method of least squares (normal distribution) Table 5 and Figure 3 and the method of Finney (lognormal distribution) Table 6 and Figure 4, where LD50 values are estimated at 350 mg/kg BW and 328.48 mg/kg BW consecutively. The LD50 value was estimated at 350mg/kg BW with the Behrens–Karber's method

Table 4 and Figure 2. The three methods are in good agreement and this value indicates that the TAS *P.harmala* is moderately toxic

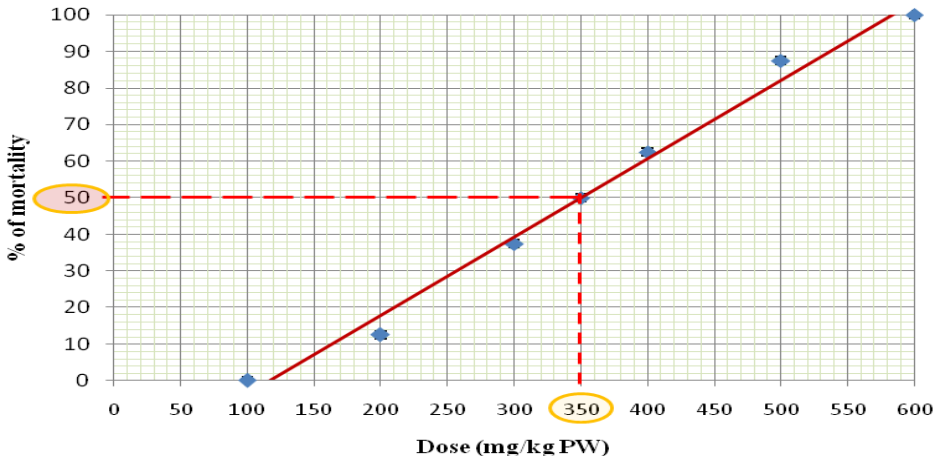

Figure2: Percentage of mortality as a function of the dose of (TAS) administered intraperitoneally

3.2. Discussion

The phytochemical study of the plant *P. harmala* extracts has shown that this herb contains: flavonoids, saponins, tannins, compounds reducers, volatile oils, anthraquinones, triterpenes and sterols and alkaloids. Indeed, several authors have shown that different types of chemical compounds highlighted in the extracts of this plant have therapeutic effects such as: narcotic, anthelmintic, malaria and in some cases against rheumatism [9, 20, 21].

Study of quantitative evaluation of alkaloids has shown that the highest percentage was in the seeds, fruits and roots which are considered as storage areas final synthesis products**.**

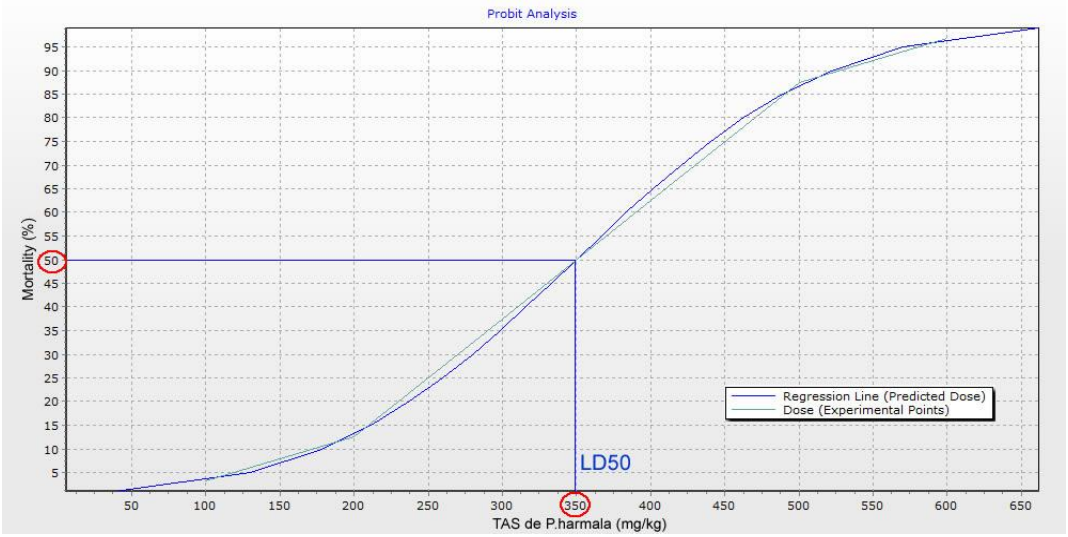

Figure 3: Analysis by the probit method (Least squares [Normal Distribution]), LD50 = 350 mg/ kg (Software StatPlus2009; 5.8.4.3)

Table 5: Regression Statistics to calculate LD50 (Least squares) [Software StatPlus] (2009; 5.8.4.3)

LD (lethal dose) 50	**350**	LD (lethal dose) 50 Standard Error	**33,4851**
LD50 LLD (low lethal dose)	281,7065	LD50 ULD (upper lethal dose)	418,2934
Beta	0,0074	Intercept	2,3869
Beta Standard Error	0,0017		
LD (lethal dose) 10	178,3243	LD (lethal dose) 16	216,059272
LD (lethal dose) 84	483,9407	LD (lethal dose) 90	521,675683
LD (lethal dose) 100	550,9110		

Table 6: Regression Statistics to calculate LD50 (Finney Method) [Software StatPlus] (2009; 5.8.4.3)

LD50	328,4817	LD50 Standard Error	35,0924
LD50 LLD	258,4716	LD50 ULD	392,5942
Log10[LD50]	2,5165	Standard Error	0,04630
Beta	6,3724	Intercept	-11,0363
Beta Standard Error	1,6518		

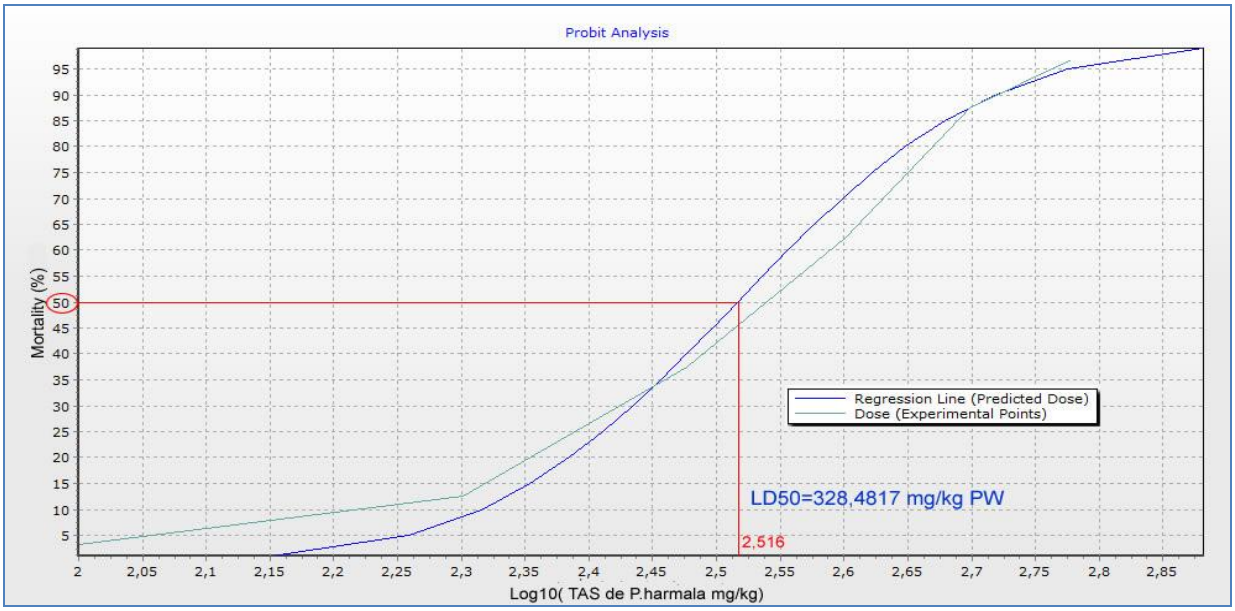

Figure 4: Analysis by the probit method (Finney Method [Log-normal Distribution]), LD50 = 328.48 mg/ kg (Software StatPlus2009; 5.8.4.3)

The clinic map male mice treated by (TAS) of *P. harmala* is characterized by a relatively rapid appearance of symptoms, due to the rapid absorption of alkaloids (indole) of *P. harmala* [22], including tachycardia, probably due to blockage of muscarinic M_2 leads to a suppression of vagal tone [23], difficulty breathing, severe agitation and convulsions by reaching the central nervous system. It was shown by bellakhdar (1997) that the alkaloids are responsible for the paralysis of the nervous system that causes death by respiratory arrest in vertebrates, that translates a blockade of brain GABA (Gamma Amino Butyric Acid) and therefore their mediation inhibitory, producing a stimulating effect that is responsible for the increase in muscle tone and a maximum of convulsions and neuronal excitability [22]. Moreover, alkaloids exert an inhibitory action **on** central dopaminergic system inducing sedation and paradoxical sleep disturbances [24].
The symptoms observed (Inactivity, tachycardia, agitation, difficulty breathing) in mice treated by (TAS) of P.harmala is similar to that observed in Cattle, donkeys and camels, especially in a period of drought [25-27].
In view of the results of the LD50 equal to 350 mg / kg BW, where the DL50 between 50 and 500 mg / kg BW, so the (TAS) of *P.harmala* is categorized product moderately toxic according to the classification of the level of toxicity of Hodge and Sterner [28].

Conclusion

The phytochemical study revealed the presence of major bioactive chemical constituents in different extracts of *P. harmala*. The plant under investigation can be a potential source of useful drugs. However, further studies are required to isolate the pure active principal from the crude plant extracts for proper drug development. The highest rate of crude alkaloids was recorded with seeds compared to other parties. TAS administered intraperitoneally is moderately toxic. The study of the effects of (TAS) on the central nervous system and Respiratory system is also of interest. This plant is commonly used in traditional medicine, deserves to be used with caution.

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