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Reversible Nematostatic Effect of *Peganum harmala* L. (Nitrariaceae) on *Meloidogyne javanica*

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ABSTRACT

Meloidogyne javanica is considered as the most damaging nematode of vegetables in Morocco. Eco-friendly bionematicides are urgently required for its control. In *vitro* experiments were carried out to assess the direct effect of bioproducts of *P. harmala* against *M. javanica*. The bioassay showed extracts to be nematotoxic. Aqueous extracts of *P. harmala* exhibited reversible nematostatic activity. The estimated ID50 of the most active product in methanolic extracts was 368 ppm. HPLC-MS of the methanolic etract revealed that total content of major alkaloids of *P. harmala* was approximately 12.162±0.637mg/g. Harmine (8.514 ± 0.521 mg/g) is the dominant alkaloid. In conclusion, *P. harmala* has a reversible nematostatic activity on second stage juveniles of *M. javanica*. The effect of *P. harmala* is due to its possession of a high content of β-carboline alkaloids, which warrant further experimentation. Bioproducts from *P. harmala* should be exploited through formulations for management of the root knot nematode.

KEYWORD

Peganum harmala, Phytonematodes, *in vitro*, nematicide, alkaloids.

INTRODUCTION

Plant parasitic nematodes are responsible for immense yield losses estimated at US$100 billion annually worldwide (Abd-Elgawad and Askary, 2015). The main cause of the losses, are root knot nematodes of the genus *Meloidogyne*, highly destructive pests in tropical and subtropical crop production regions (Sikora *et al.*, 2018). *Meloidogyne javanica*, a very common specie of the genus, is highly polyphagous which is widely distributed in Morocco and is highly prevalent in southern regions. Management of root knot nematodes is based on synthetic nematicides, though, efficacy declines with continued use, which compromises long-term suppression of nematodes. Further, increased awareness of the impacts of these chemicals on environmental and human health, are resulting in increased restrictions on their use. Botanical nematicides are safe, cheap and environmentally friendly options. Plant-derived extracts in particular have been reported as nematotoxic against the root knot nematode *M. javanica* in *in vitro* and pot experiments (Oka *et al.*, 2014; Kepenekci and Saglam, 2018). *Peganum harmala* L. is a perennial herbaceous plant, growing on semi-arid and predesertic areas of Morocco, and is distributed across North Africa, the Middle East, the Mediterranean Sea regions, India and Pakistan. It has also been introduced and naturalized in parts of South Africa, Australia and the USA (Herraiz *et al.*, 2010). Seeds of *P. harmala* are used in Moroccan traditional medicine in a powdered form for maceration, decoction or infusion to treat different human diseases (Bellakhdar, 1997).

The plant is renowned for its high toxicity against animals and several pests and pathogens (Mahmoudian *et al.*, 2002). Extracts of the plant demonstrated a wide range of biological activities with various properties, which are of an antioxidant, antiviral, antifungal, insecticidal and antibacterial nature (Hayet *et al.*, 2010; Akhtar et al., 2018; Danial *et al.*, 2018; Ibrahim *et al.*, 2018). Active compounds of *P. harmala* are several alkaloids, β-carbolines and the quinazoline derivatives vasicinone and vasicine (Mirzaie *et al.*, 2007). These compounds are mostly responsible for biological activities of the species (Lamchouri *et al.*, 2010). β-carbolines alkaloids in particular such as: harmine, harmaline, harmalol and harman are the most renowned alkaloids isolated from *P. harmala*. These alkaloids possess several biological properties and are antibacterial (Danial *et al.*, 2018). In addition to being antifungal and insecticidal (Rharrabe *et al.*, 2007; Nenaah, 2010), aqueous extracts of *P. harmala* were reported to have a nematicidal effect in *in vitro* and in *vivo* experiments against root knot nematodes (El Allagui *et al.*, 2007; Mayad *et al.*, 2013; Abood, 2017).

Previous work reporting lethal effects on root knot nematodes in *in vitro* experiments associates mortality of juveniles with immobility during incubation, which does not provide accurate results on the actual effect of extracts on nematodes. This study reports for the first time the nature of the effect of *P. harmala*

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extracts on *M. javanica* by using Meldola blue vital stain. Objectives of this study are (I) determining the direct effect of different extracts from *P. harmala* on second stage juveniles of *M. javanica*, (II) reporting the major compounds of seeds and (III) determining the nature of the effect (lethal or nematostatic) of the extracts on the root knot nematode.

MATERIAL AND METHODS

Plant Material

Seeds and aerial parts of *P. harmala* were harvested in Agadir during July from plants grown in black plastic bags on a specific substrate (1/3 peat: 1/3 clay: 1/3 sand). After desiccation of plant matter for one week at 40 °C, it was reduced to a powder and stored in the dark at room temperature (25°C).

Preparation of Plant Extracts

The fraction containing polar molecules was prepared using maceration in water and soxhlet apparatus with methanol. Aqueous extract was obtained by macerating plant powder in distilled water under agitation of the solution for 24 hours, after 10 minutes of ultrasonication and filtration it was stored at a temperature of 4°C.

For methanolic and hexanic extracts, the dry plant powder obtained from the aerial parts of *P. harmala* was extracted with hexane, chloroform, ethyl acetate and finally methanol. Whereas seed extracts were prepared after passage of methanol followed by hexane. Extracts obtained by Soxhlet extraction were concentrated by evaporation under reduced pressure using a rotary evaporator. The dry residue was used for the preparation of different concentrations used for nematol-ogical bioassays and chemical analysis. The dry hexane was dissolved in distilled water using Tween 20 at 0.2% while the dry methanol extract was dissolved in distilled water only after exposure to ultrasonication. All obtained solutions were stored at +4 °C in a refrigerator.

Oil Emulsion

The oil of *P. harmala* was obtained by cold extraction from seeds. This product was used for preparing an emulsion of oil in water 2% and was stabilized by Tween 20 at 0.2%. The solution was stirred for two hours in the dark and filtered through Whatman paper. The filtrate was used as a stock solution for the preparation of different concentrations used for trials *in vivo* and *in vitro*.

Inoculum of Root Knot Nematode *M. javanica*

Infested tomato roots with *M. javanica* were washed thoroughly under tap water. Then, roots were cut and ground in a blender in a solution of sodium hypochlorite NaOCl at 1% for 2 min to release the eggs from the gelatinous mass. The mixture was filtered through a column of sieves (meshes ranging from 250µm to 40µm). The fraction collected with a 40µm sieve containing the eggs of *M. javanica* was deposited to hatch on cellulosic paper to allow migration of second stage juveniles, after which they were collected in suspension. After 48h, the J2 in suspension were staged under a light microscope and counted.

In vitro Evaluation of Nematotoxic Potential of Extracts of *P. harmala*

Second stage juveniles (J2) of *M. javanica* were suspended in Petri dishes in sterile distilled water to give methanolic extracts of final concentrations of 0, 10, 20, 50 and 100 µg/ml. Each treatment was performed in three replicates. The Petri dishes were incubated at room temperature (20 °C ± 2) for 72 hours. The J2 considered intoxicated by the extract are immobile and the rate of toxicity (Tox) of J2 was calculated every 24 hours for the period of incubation and corrected relative to control treatment (distilled water) using the formula (Abbott, 1925):

$$Tox(\%) = ((Tr - Tm) / (100 - Tm)) * 100 \quad (1)$$

where Tr = percentage of immobile J2 in the extract and Tm = percentage of immobile J2 in distilled water.

Meldola Blue Staining Bioassay

J2 of *M. javanica* were exposed for 72 hours to three concentrations of aqueous extracts prepared previously from *P. harmala* seeds at 0.5, 1 and 5% (V/V). The assay was performed using identical conditions of the preceding experiment, with the exception of use of Meldola blue staining (Ogiga and Estey, 1974). After incubation at room temperature (23 °C + 2) for 72 hours, in each sample, we added three drops of Meldola blue dye, to allow dead nematodes to stain (purple). The petri dish contents were passed through a 5µm filter to retain a clear solution of nematodes. Stained and non-stained immobile nematodes were counted under 40× magnifications and expressed in percentage of death and paralysed larvae to assess respectively the mortality (M) and nematostatic percentage (Nr) of larvae after the incubation period and fixed relative to the control according to the Abbott formula (1) given above. We performed two-way ANOVA for *in vitro* tests and one-way ANOVA for the Meldola blue staining bioassay with the Newman and Keuls test ($P \leq 5\%$) for means classification.

RESULTS AND DISCUSSION

In vitro Evaluation of Nematotoxic Potential of Extracts of *P. harmala*

The J2 of *M. javanica* reacted differently in different treatments (Table 1). After 24 hours of incubation, extracts of the fresh and dry aerial parts and aqueous and methanolic extracts of seeds caused remarkable toxicity (over 44%) notably at 2% concentration. The methanolic and aqueous extracts of the seeds were the most nematotoxic at 2%. Toxicity (expressed as a percentage of immobile J2) ranges between 94.33% and 97%. After 72 hours, methanol and hexane extracts were toxic from 0.02% and the rate of immobile J2 was in the range of 38.33 to 40%. At the highest dose tested, all products from seed or aerial parts led to inhibition of J2 and showed no differences. The toxicity was between 70% (hexane extract) and 98.33% (methanol extract). Tween 0.2% showed no significant difference with the control (distilled water). Of the juveniles exposed to extracts for 72 hours, almost all juveniles regained mobility for all extracts except in the oil emulsion of *P. harmala* at 2%. The percentage of these juveniles showed no significant difference with the control treatment. This indicates that the observed toxicity of extracts resulted in a reversible nematostatic effect. In oil emulsion, the number of recovered

Table 1: Toxicity of extracts of *P. harmala* at different concentrations against J2 of *M. javanica* during 72 hours incubation (DAP: dried aerial parts; FAP: fresh aerial parts)

Treatments	Concentration (%)	24h	48h	72h	Mobile J2 after washing
Aqueous extract (FAP)	00,20	05g,h*	05,33i	09g,h	96,33a
	01,00	36,33e,f	42e,f,g	44,33d,e,f	94,33a
	02,00	44,33d,e	69b,c,d	61,33b,c,d,e	96,33a
	05,00	50,33c,d,e	**94,67a**	80,33a,b,c	97a
Aqueous extract (seeds)	00,20	5,33g,h	7,33i	8,67g,h	96a
	01,00	57,33c,d	34g,h	46d,e,f	96,33a
	02,00	**94,33a**	84,67a,b	62,33b,c,d,e	93,33a
	05,00	**95,33a**	90,67a	**85a,b**	95,33a
Aqueous extract (DAP)	00,20	13g,h	17,67h,I	17,67f,g,h	95,67a
	01,00	28f,g	40,33f,g	57b,c,d,e	96,33a
	02,00	**75b**	52,33d,e,f	73,67a,b,c,d	94,67a
	05,00	**63,33b,c**	60c,d,e,f	71a,b,c,d,e	96a
Methanolic extract (seeds)	00,02	14,33g,h	17,33h,I	40d,e,f	94,33a
	00,20	51,33c,d,e	61,67c,d,e	81a,b,c	92,33a
	02,00	**97a**	91,33a	**98,33a**	96,67a
Hexane extract (Seeds)	00,02	27f,g	32,67g,h	38,33e,f,g	97,67a
	00,20	27f,g	32g,h	50c,d,e,f	95a
	02,00	22,67f,g	78a,b,c	70a,b,c,d,e	96a
Oil emulsion	00,02	2,33g,h	7,33i	17,33 f,g,h	97a
	00,20	4g,h	7,33i	20,33f,g,h	91,33a
	02,00	25,67f,g	45e,f,g	**89a,b**	**79,67b**
Distilled water		2g,h	2,33i	4h	97b
Tween20	00,20	3,g,h	4i	5,33h	95b

* Numbers followed by the same letter in the same column are not significantly different according to Newman and Keuls test (P ≤ 5%).

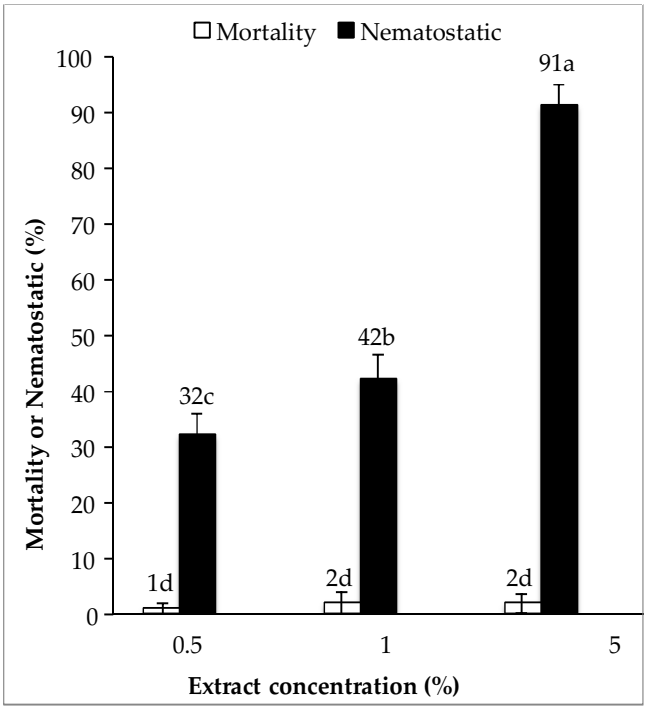

Fig.1: Mortality and nematostatic percentages of second stage juveniles (J2) of *M. javanica* after 72h of exposure to *P. harmala* extract

individuals was relatively small compared to the control. The *P. harmala* oil emulsion induced an irreversible nematostatic or lethal effect.

The Meldola Blue staining bioassay allowed the assessment of the nematostatic activity potential of *P. harmala* aqueous extract on J2 of *M. javanica* through determination of paralyzed proportion of exposed larvae. The recorded percentage of paralyzed larvae was high and increased significantly with concentration. It ranged from 32% to 91% at 0.5 and 5% of aqueous extract respectively. While the Mortality percentage was low (1 to 2 %) and remained stable between concentrations (Fig. 1).

The study of the correlation between concentration and toxicity of extracts on J2 after 72 hours of incubation revealed significance of the methanolic extract. High significance was found between the aqueous extract and oil emulsion of seeds effect and for both fresh and dried aqueous extracts of aerial parts of *P. harmala*. The correlation was very strong for the aqueous extract of the seeds and aerial parts as well as for the oil emulsion (Table 2). The study of the dose-response relationship between J2 of *M. javanica* and tested extracts showed that ID90 varies depended on the type of extract. The lowest ID90 recorded was 0.37% for the methanol extract of the seeds and the highest was 12.85% for the aqueous extract of the dry aerial parts of *P. harmala*.

Table 2: Values of Pearson correlation coefficient between the concentration of extracts and *in vitro* toxicity of J2 of *M. javanica* with ID90 and ID50 after 72 hours incubation

Treatments	Correlation Coefficient "r"	ID90 (%)	ID50 (%)	Slope
Aqueous extract (FAP)	00,90**	8,62	1,41	1,63±0,11
Aqueous extract (Seeds)	00,93**	8,53	1,37	1,71±0,13
Aqueous extract (DAP)	00,74**	12,85	0,99	1.152 ±0,83
Methanolic extract	00,70*	0,37	0,05	1.48±0,09
Oil emulsion	00,98**	2,27	0,72	2,57±0,24

* Significant correlation

** Highly significant correlation

Seed analysis in *P. harmala*

Among the four alkaloid molecules targeted, only harmol was not detected in seeds. This may be related to the presence of the compound in trace molecules existing below the detection limits by the method used or was due to reduction of the harmol to harmalol. Harmine was the most abundant molecule (8.514 ± 0.521 mg / g dry matter) followed by harmalol (2.774 ± 0.164 mg / g). The harmaline concentration was of the order of (0.874 ± 0.016 mg / g). The total content of these compounds was approximately 12.2% (Table 3).

Table 3: The quantity (Q) and time of retention (TR) of total harmaline (Hline), harmine (Hine), harmalol (Hlol) and harmol (Hol) in the seeds of *P. harmala* L.

Alkaloids	Hlol	Hol	Hline	Hine	Major alkaloids
TR (min)	1.98	2.21	2.48	3.1	
Q (mg/g)	2.774±0.164	-	0.874±0.016	8.514±0.521	12.162±0.637

The present work shows that *P. harmala* tested extracts have a reversible nematostatic effect on second stage juveniles of *M. javanica*. In contrast to previous studies reporting a lethal effect of *P. harmala* extracts on *Meloidogyne* spp. (El Allagui 2007; Jassim and Abou Foul 2009; Saeed *et al.*, 2015; Abood 2017).

This biological effect could be attributed to bioactive compounds in *P. harmala*, such as those shown below (Table 3), several alkaloids interact with different neuroreceptors as well as cause monoamine oxidase (MAO) inhibition (Herraiz *et al.*, 2010; Moloudizargari *et al.*, 2013), resulting in increased serotonin activity. Loss of coordination with paralysis and respiratory paralysis by harmaline was reported (Chen *et al.*, 2005) in vertebrates. Previous studies associated this effect with potent reversible MAO inhibitory effect (Herraiz *et al.*, 2010) and with inhibitive activity against acetylcholinesterase (Zheng *et al.*, 2009).

CONCLUSION

P. harmala extracts are highly toxic to second-stage juveniles of *M. javanica* by inducing a direct reversible nematostatic effect. Further investigations for agroecological use of these botanicals in management of root knot nematodes should be included in future research directions *in vitro* and in field, to clarify their optimum use.

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