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RESEARCH ARTICLE

EFFECT OF THREE EXTRACTS OF TUNISIAN LEGUME FORAGE ON THE EXSHEATHMENT PROCESS OF *HAEMONCHUS CONTORTUS* INFECTIVE STAGE

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ABSTRACT

The use of tanniferous plants was suggested as a possible alternative option for controlling gastrointestinal nematodes of small ruminants. This experiment evaluated the possible *in vitro* effects of three phenological stages (Bloom, Seeds formation, Seeds) of Tunisian fabacae (*Hedysarum carnosum* Desf.) against *Haemonchus contortus* third stage larvae. The larval exsheathment assay (LEA) was used to determine the proportions (%) of exsheathment of three plant acetonic extracts at different concentrations (1200, 600, 300, 150 µg/ml). To confirm the role of tannins in the anthelmintic effects of extracts, polyvinylpolypyrolidone (PVPP) was used as deactivating chemical tannins. *H. carnosum* (S) showed the highest content of TP, TT and BA. The % of exsheathment was phenological stage, dose and incubation time-dependent ($P<0.001$). As the phenological stage advanced, the % of exsheathment decreased. After the addition of PVPP, the restoration of L3 exsheathment to values similar to those of control indicates that tannins are probably the main secondary compounds involved in anthelmintic effects. However, *in vivo* assays are necessary to confirm these results.

INTRODUCTION

Worldwide, the parasitic infections with gastrointestinal nematodes (GIN) remain a major concern for welfare, health and small ruminant production. The control of these diseases has been relied to the use of commercial anthelmintic drugs. However, this technique is nowadays facing several limits. The first one is the development of anthelmintic resistance in worm populations (Jackson and Coop, 2000; Kaplan, 2004). The second one is the enhanced concern of consumers on the possible residues in food products or environmental consequences. The exploitation of bioactive plants, rich en secondary metabolites such as tannins, could offer accessible, sustainable and environmentally alternative solution for the control of GIN (Hoste *et al*., 2006, 2011). Some studies have explored the anthelmintic properties of legume forage such as *Lotus pedunculatus*, *Lotus corniculatus* (Hoste *et al*., 2006), *Onobrychis viciifolia* (Paolini *et al*., 2005; Heckendorn, 2007; Manolaraki *et al*., 2010) or *Hedysarum coronarium* (Niezen *et al*., 2002, 1998, 1995). *Hedysarum carnosum* Desf. is a Tunisian fabacea resistant to drought and salinity. This study aimed (i) to test the *in vitro* anthelmintic activity of the acetonic extract of *H. carnosum* against *Haemonchus contortus* third stage larvae (ii) to verify the phonological stage effect on the AH activity (iii) to confirm tannins AH activity, a tannins inhibitor, the polyvinylpolyrrolidone (PVPP) was used (Alonzo-Diaz *et al*, 2008).

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MATERIALS AND METHODS

Plant materials and preparation of plant extracts

In spring 2011, *Hedysarum carnosum* Desf. was collected from Kairouan area (Tunisia) at different phenological stages (PS): bloom (B), seeds formation (SF), seeds (S). The geographical remoteness is: latitude 35°40′38.76″N, longitude 10°08′21.59″E, altitude 68 m. The chemical composition of the collected samples was determined (Table 1).

After being freeze-dried and ground to pass through a 1 mm screen, 10 g of each sample was shaking in 70:30 acetone: water (v/v) solution for 1 hour in a water bath (32-35°C). The acetone was removed under low pressure at a temperature below 35°C and the aqueous solution was washed three times with 100 ml of dichloromethane to remove chlorophyll and lipids. The remaining fraction was frozen then freeze-dried for 24 h and kept at 4°C in air-tight containers until used in the *in vitro* biological assay.

Table 1. Chemical composition of plant samples

Compound	*Hedysarum carnosum Desf.*		
	Bloom	Seeds Formation	Seeds
DM (%)	16.60	19.4	17.69
Ash (% DM)	16.88	18.07	19.22
CP (% DM)	13.45	16.65	15.64
NDF (% DM)	30.12	33.31	49.17
ADF (% DM)	16.01	18.78	32.21
ADL (% DM)	4.09	4.31	11.06

Quantification of polyphenolic compounds and Biological Activity of plant extracts

Folin-Ciocalteu method

This method described by (Makkar, 2003) was used to quantify the concentrations of total polyphenols (TP) and total tannins (TT) in the plant samples. For each plant sample, we measured polyphenols without and with addition of PVPP (Sigma Aldrich Ltd), then we determined TT by difference between TP measured without PVPP and non TT measured with PVPP. The quantification of TP and TT was done in three replicates, made at 725 nm using of a spectrophotometer (UV-visible Spectronic Unicum, Genesys 8). A tannic acid standard curve was performed and total phenols and total tannins were expressed as g-equivalent tannic acid/100 g DM, referred to as g-equiTA.

Butanol-HCl assay

The condensed tannins (CT) of each plant samples were determined by the butanol-HCl method (Makkar, 2003). In test tubes, we deposited 0.05 ml of tannin extract, 0.45 ml of 70:30 acetone:water (v/v) solution, 3 ml of butanol-HCl and 0.1 ml of ferric reagent. After covering their open sides, the tubes were boiled for 60 min. A blank containing the reagents without extract was used as a control. They were then cooled and absorbances at 550 nm were measured on a spectrophotometer **(**UV-visible Spectronic Unicum, Genesys 8). Concentrations of CT were expressed as g - equivalent of leucocyanidin /100 g of DM.

Radial Diffusion Method

The Biological Activity (BA) of tannins was quantified using the Radial Diffusion Method (Hagerman and Bulter, 1978). This Technique is based on the property of tannins to form insoluble complexes with protein. We used Bovine Serum Albumin BSA (Sigma Aldrich Ltd) and tannic acid (Sigma Aldrich Ltd) as standards. The activity was expressed as g-equiTA.

Larval exsheathment assay (LEA)

The larval exsheathment assay was artificially performed (Bahaud *et al*., 2006 on the infective stage larvae (L3) of *Haemonchus contortus* (INRA goat strain, France) with extracts of each plant at different doses (1200, 600, 300, 150 µg/ml). One thousand ensheathed L3 were incubated for 3h at 20°C. After incubation, the larvae were washed and centrifuged (1000 rpm at 20°C during 3mn) three times in phosphate buffer saline solution (PBS: 0.1M phosphate, 0.05M NaCl, pH 7.2). Then, the larvae were subjected to an artificial exheathment process by contact with a sodium hypochloride solution (2%, w/v) and sodium chloride solution (16.5%, w/v) diluted 1-400 in PBS. The kinetics of larvae exsheathment were measured at 20 min intervals for 60 min under microscopic observation at a magnification of ×100. PBS was used as a negative control. 4 replicates were considered for each plant extract. In order to check the role of tannins in the anthelmintic effects of extracts an inhibitor of tannins, polyvinylpolypyrolidone (PVPP: Sigma Aldrich Ltd) was used.

Statistical analyses

Data were subjected to analysis of variance using SPSS Statistics 20. The model included Phenological stage (Bloom, seeds formation, seeds), dose of extract, time of incubation and all their interactions. The Duncan test was used to detect differences between treatments and values biochemical analysis and % of LEA are reported means with corresponding standard deviation (SD).

The effective concentration for 50% inhibition (EC50) ratios for each plant extract for the LEA was calculated with the PoloPLUS 2002-2003 (Probit and Logit Analysis). EC50 was obtained by non-linear regression analysis of 4 replicates for each of 5 dilutions (PBS, 150, 300, 600, 1200 µg/ml).

RESULTS AND DISCUSSION

Polyphenolic compounds and Biological Activity of plants extracts

The plant extract with the highest quantity of TP, TT and BA was *H. carnosum* (S). In contrast, the extract of *H. carnosum* (B) had the lowest levels of the polyphenolic compounds and the biological activity. The *H. carnosum* (SF) extract showed the highest quantity of CT (Table 2). These results are contradictory to those reported by Pilluza *et al*. (2000) for *H. coronarium* but in agreement with those reported by Theodoridou *et al*. (2011) for sainfoin. According to Iason *et al*. (1995) reported by Aufrère *et al*. (2012) that the luminous intensity and the hydric stress can include an increase of CT content.

Table 2. Mean values (±S.D.) of total phenols, total tannins, condensed tannins and biological plant samples

Compound	*Hedysarum carnosum Desf.*		
	Bloom	Seeds formation	Seeds
Total phenols[1]	0.95±0.05	1.08±0.08	1.29±0.31
Total tannins[1]	0.14±0.05	0.53±0.48	0.55±0.640
Condensed tannins[2]	0.02±0.001	0.42±0.004	0.102±0.005
Biological activity[1]	0.29±0.06	0.60±0.11	0.61±0.07

1: Expressed as g equivalent tannic acid /100g of DM
2: Expressed as g equivalent of leucocyanidin /100g of DM

Larval exsheathment assay

The results of statistical analyses of LEA with and without PVPP are presented in Table 3. The main effects and the interaction (Phenological stage x Dose x Time) were significant ($P<0.001$).

Kinetics of L3 exsheathment at different doses

Figures 1a., 1b. and 1c. show the results of kinetics of the larval exsheathment assay without PVPP. The percentages of exsheathed L3 after 60 min of contact with the sodium

hypochlorite solution for PBS were 94.87, 100 and 72.84 for *H.carnosum* B, SF and S, respectively. At 150, 300 and 600 µg/ml, the acetonic extracts of the three plant samples had low impacts on the % of exsheathment of *H. contortus*. The highest level of inhibition of the exsheathment process was observed for *H.carnosum* (S) at the concentration 1200 µg/ml. Our results are similar to those reported by (Brunet *et al*. (2007) and Manoloraki (2011) for sainfoin and by Aïssa *et al*. (2015) for *H.coronarium*.

Table 3. Results of the statistical analyses of the Larval exsheathment assay without and with PVPP

	LEA	
	Without PVPP	With PVPP
Effects	P-value	P-value
Phenological stage	<0.001	<0.001
Dose of extract	<0.001	<0.001
Time of incubation	<0.001	<0.001
Phenological stage x Dose x Time	<0.001	<0.001

The addition of PVPP to the plant extracts restored values of the % of exsheathment close to the control values for *H.carnosum* at the phenological stage Bloom, seeds formation and seeds (Figure 2a., 2b. and 2c.). Indeed, Aïssa *et al*. (2015) and Alonzo-Diaz *et al*. (2008) showed a restoration of L3 exsheathment to values similar to PBS. These results suggest a major role of tannins of *H.carnosum*, especially CT which is known for its ability to bind with protein, in anthelmintic activity (Aïssa *et al*., 2015; Bravo, 1998; Manolaraki *et al*., 2010; Molan *et al*., 2004; Waterman, 1999).

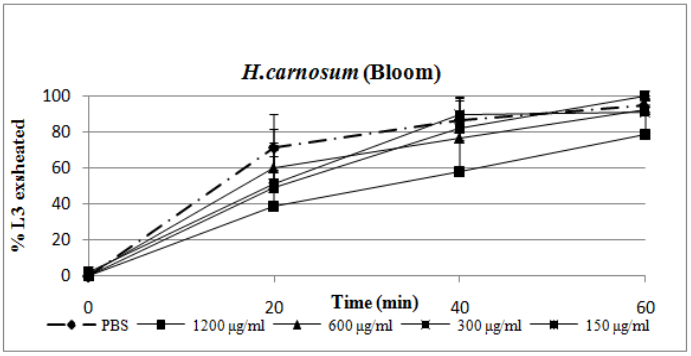
Phenological stage effect

For the assay without and with PVPP, the % of exsheathment varied (P<0.001) between the phenological stage. Indeed, as the PS advanced, the % of exsheathment decreased (Table 4).

Table 4. Effect of phenological stage on % of exsheathment on the infective stage larvae of *Haemonchus contortus* for the different phenological stage

		Bloom	Seeds formation	Seeds	ESM
% of exsheathment	Without PVPP	56.13^{c}	48.49^{b}	13.78^{a}	1.51
	With PVPP	53.77^{b}	59.64^{c}	39.16^{a}	1.93

^{a,b,c}Means in the row with different superscripts differ (P<0.001).



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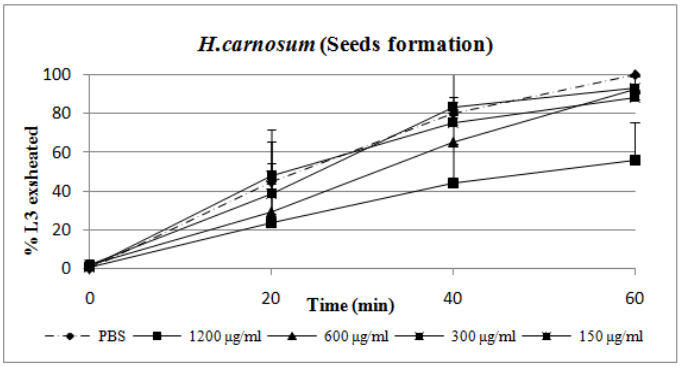

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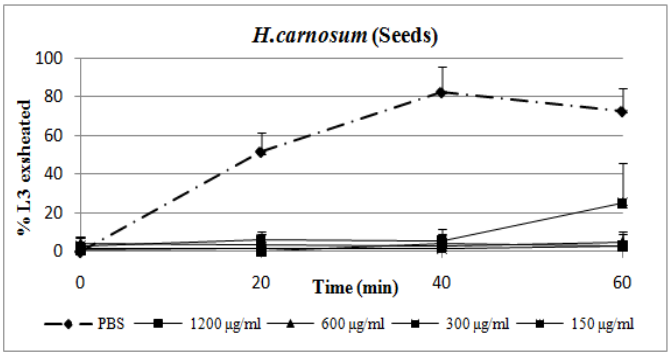

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Figure 1. Kinetics of exsheathment on the infective stage larvae (L3) of *Haemonchus contortus* of *Hedysarum carnosum*: bloom (a), seeds formation (b), seeds (c) without PVPP.

Table 5. Effect of doses on % of exsheathment on the infective stage larvae of *Haemonchus contortus* for the different doses

		PBS	150	300	600	1200	1200+PVPP	ESM
		µg/ml						
% of exsheathment	Without PVPP	57.19^{c}	40.24^{b}	37.72^{b}	35.98^{b}	26.2^{a}	-	1.95
	With PVPP	64.30^{c}	-	-	-	33.15^{a}	55.13^{b}	1.93

^{a,b,c}Means in the row with different superscripts differ (P<0.001).

Table 6. Effect of incubation times on % of exsheathment on the infective stage larvae of *Haemonchus contortus*

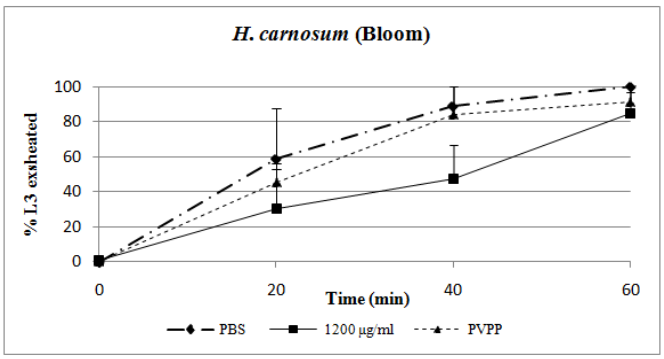
		0	20	40	60	ESM
		Min				
% of exsheathment	Without PVPP	1.51^{a}	33.15^{b}	55.52^{c}	65.65^{d}	1.74
	With PVPP	1^{a}	45.46^{b}	72.48^{c}	84.5^{d}	2.22

^{a,b,c,d}Means in the row with different superscripts differ (P<0.001).

Table 7. EC50 of *Haemonchus contortus* in the larval exsheathment assay

	Phenological stage	EC50	Limit at 0.95	
		µg/ml	lower	upper
H. carnosum	Bloom	>1200^{b}	>1200	>1200
	Seeds formation	>1200^{b}	1180.1	>1200
	Seeds	70.28^{a}	12.30	129.50

^{a,b}Means in the row with different superscripts differ (P<0.05).



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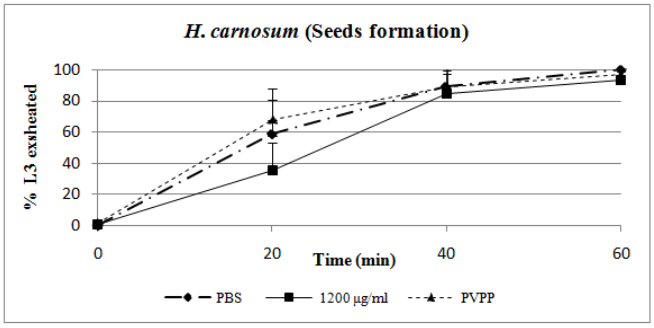

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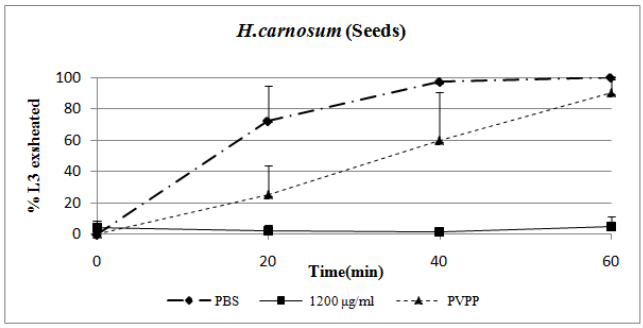

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Figure 2. Kinetics of exsheathment on the infective stage larvae (L3) of *Haemonchus contortus* of *Hedysarum carnosum*: bloom (a), seeds formation (b), seeds (c) with PVPP

Extract dose effect

The % of exsheathment varied (P<0.001) between doses. Before the addition of PVPP, the % of exsheathment decreased to 40.24, 37.72, 35.98 and 26.2 for the doses 150, 300, 600 and 1200 µg/ml, respectively (Table 5). So, more the extract dose increased more the % of exsheathment decreased. After the addition of PVPP, the % of exsheathment was higher than that of the dose 1200 µg/ml and closer to that of PBS. A dose-dependent relationship with the % of exsheathment was reported for *H.coronarium* (Aïssa *et al*., 2015) and for sainfoin (Brunet *et al*., 2007; Manolaraki, 2011).

Incubation time effect

The results of the % of exsheathment without and with PVPP reported in Table 6 showed differences (P<0.001) between incubation times. For LEA without and with PVPP, the % of exsheathment increased as the time of incubation increased. Aïssa *et al*. (2015) reported that the LEA was incubation time-dependent for *H.coronarium*.

Effective concentration for 50% inhibition

Table 7 reportes the results of the effective concentration for 50% inhibition. EC50 of *H.contortus* was different (p<0.05) for plant extracts. The highest EC50 was obtained for *H.carnosum* (S). The EC50 reported by Aïssa *et al*. (2015) for *H.coronarium* (fresh sulla) was lower than our result for *H.carnosum* (S). This difference could be explained by the content and the chemical structure of condensed tannins (Hoste *et al*., 2006).

Conclusion

The results of this study suggested that *H. carnosum* (seeds) extract can be used to control *H.contortus*. However, *in vivo* tests must be conducted to confirm these results. Therefore, further studies are needed to better understand the nature of the secondary compounds responsible for this effects and its mode of action on parasite.

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