



In vitro anthelmintic activity of Tunisian fabacae (*Hedysarum coronarium* L., ecotype Bikra 21) against *Haemonchus contortus*

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Abstract

The use of bioactive tanniferous plants was suggested as an alternative to control gastrointestinal nematodes (GIN) infections in small ruminants. This study aimed to evaluate the anthelmintic effect of acetonic extract of *Hedysarum coronarium* (Ecotype Bikra 21, sulla) as fresh or dried biomass (hay) in the infective larvae (L3) of *Haemonchus contortus*. The larval exsheathment assay (LEA) was used to determine the proportions (%) of exsheathment of two plant acetonic extracts at different concentrations (1200, 600, 300, 150 µg/ml). To confirm the role of tannins in the anthelmintic effects of extracts, polyvinylpyrrolidone (PVPP) was used as deactivating chemical tannins. The results indicated that the proportion of exsheathment was dose-dependent. Fresh sulla had the highest levels of total tannins (TT), total phenols (TP), condensed tannins (CT) and the highest biological activity (BA). It also stopped nearly the exsheathment process. Tannins are involved in anthelmintic (AH) effects because of the restoration of L3 exsheathment to values similar to those of controls after the addition of PVPP. It is concluded that *in vivo* investigation should be conducted to confirm the AH activity of sulla.

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Introduction

Gastrointestinal nematode (GIN) remains a major health and welfare problem affecting the productivity and health of in ruminants. *Haemonchus contortus* is one of the most GIN pathogens encountered in small ruminants because of its high prevalence and pathogenicity (Urquhart *et al.*, 1996; O'Connor *et al.*, 2006). For more than 50 years, the control of GIN parasitism has been based on the administration of chemical anthelmintic (AH). However, the repeated use of synthetic AH is facing some limits. The worldwide diffusion of AH resistance in small ruminant (Jackson and Coop, 2000; Kaplan, 2004) and the increasing concern of consumers about chemical drug residues in food and in the environment explain the need for alternative and sustainable options (Waller, 1997). The use of bioactive compounds in many plants, in particular condensed tannin (CT) containing plants, have presented AH activity against GIN nematodes of sheep and goats (Hoste *et al.*, 2006). *In vitro* methods are widely used to test the AH activity of various plant species as their ease to establish, rapid and cost-effective. Artificial larval exsheathment assay (LEA) has been developed by Bahuaud *et al.* (2006) to test plants extract on larval exsheathment. Some studies focused on the AH properties of Mediterranean woody species (Manolaraki *et al.*, 2010; Aissa *et al.*, 2013), tropical plants (Alonso-Diaz *et al.*, 2008) and legume forages such as *Onobrychis viciifolia* (Paolini *et al.* 2005; Heckendron, 2007; Manolaraki *et al.*, 2010) or *Hedysarum coronarium* L. (Niezen *et al.*, 1995, 1998, 2002). *Bikra 21*, an ecotype of *H. coronarium* L. is largely cultivated in Tunisia.

The objectives of the current study were (i) to assess the *in vitro* AH effect of *H. coronarium* used as fresh biomass or hay against *Haemonchus contortus* using LEA and (ii) to confirm tannins AH activity, a tannins inhibitor, the polyvinylpyrrolidone (PVPP) was used (Alonso-Diaz *et al.*, 2008).

Materials and methods

Preparation of plant extracts

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In spring 2011, *Hedysarum coronarium* L., ecotype *Bikra 21* (*sulla*) was cultivated in the experimental farm of the High Institute of Agricultural Sciences of Chott-Mariem (Sousse, Tunisia). The geographical remoteness is: latitude 35°54'53.57"N, longitude 10°33'36.80"E, altitude 1.03 Km. One part of the *sulla* plot was used as fresh forage at full bloom stage and a second part at the same stage was air-dried to make hay. 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 forage 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.

Determination of polyphenolic compounds and Biological Activity of plants 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 Unicam, 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 Unicam, 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 (Bahuaud *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 exsheathment 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, polyvinylpolypyrrolidone (PVPP: Sigma Aldrich Ltd) was used.

Statistical analyses

Data were subjected to analysis of variance using SPSS Statistics 20. The model included forage type (fresh or air-dried), 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

Polyphenolic compounds and Biological Activity of plants extracts

The concentration of the polyphenolic compounds and the biological activity of the two plants extracts are reported in Table 2. Fresh sulla extracts exhibited the highest concentrations of TP, TT, CT and the highest BA.

Table 1. Chemical composition of plant samples.

Compound	Fresh sulla	Sulla Hay
Dry matter (%)	11.5	88.6
Ash (% DM)	14.1	7.8
Crude protein (% DM)	16.6	8.7
Neutral detergent fiber (% DM)	55.1	71.2
Acid detergent fiber (% DM)	35.2	46.1
Acid detergent lignin (% DM)	9.5	14.9

Larval exsheathment assay

The results of statistical analyses of LEA with and without PVPP are presented in Table 3. The main

effects of dose extract and incubation time were significant for LEA without and with PVPP, however the main effect of forage type (fresh vs hay) was

significant ($P < 0.01$) for LEA with PVPP. Only, interactions for the three main factors were not significant for LEA without and with PVPP. The interaction "Forage types x Time" was not significant for LEA with PVPP.

Kinetics of L3 exsheathment at different doses

Fig. 1A. and 1B. shows the results of kinetics of the larval exsheathment assay without PVPP. For PBS, over 86% of *H. contortus* L3 were exsheathed at 60 min after contact with sodium hypochlorite solution. For both forages types (fresh and dried sulla), the acetonc extract of *H. coronarium* at 150 and 300

$\mu\text{g/ml}$ had a low effect on the % of exsheathment of *H. contortus*. At 600 $\mu\text{g/ml}$, the % of exsheathment of fresh sulla (19.4 %) was lower compared to hay sulla (53.12 %). At 1200 $\mu\text{g/ml}$, a high level of inhibition of larval exsheathment was observed for sulla hay whereas for fresh sulla, a total inhibition of exsheathment (2.67 %) was observed. After the addition of PVPP, the inhibitory effect on larval exsheathment of extract of fresh sulla was completely reversed: 100 % of exsheathed larvae (Fig. 2A.). Moreover, the % of exsheathment was only 60.87 % for hay sulla after the addition of PVPP (Fig. 2B.).

Table 2. Mean values (\pm S.D.) of total phenols, total tannins, condensed tannins and biological activity for fresh and dried Sulla.

Compound	Fresh sulla	Sulla Hay
Total phenols ¹	1,30 \pm 0,114	0.45 \pm 0.029
Total tannins ¹	0,27 \pm 0,260	0.02 \pm 0.010
Condensed tannins ²	0.373 \pm 0.02	nd
Biological activity ¹	0,87 \pm 0,065	0.25 \pm 0.081

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

2: Expressed as g equivalent of leucocyanidin /100g of DM

nd: not detected.

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

Effects	LEA	
	Without PVPP	With PVPP
Forage types	P-value 0.091	P-value <0.01
Dose	<0.0001	<0.0001
Time	<0.0001	<0.0001
Forage types x Dose	<0.0001	<0.01
Forage types x Time	<0.0001	0.341
Dose x Time	<0.0001	<0.0001
Forage types x Dose x Time	0.713	0.058

Dose extract effect

The % of exsheathment varied ($P < 0.001$) between doses. As the doses increased, the % of exsheathment decreased to 44.58, 35.03, 14.34 and 5.57 for the doses 150, 300, 600 and 1200 $\mu\text{g/ml}$, respectively (Table 4). After the addition of PVPP, the % of exsheathment was similar to that of PBS and higher than that of the dose 1200 $\mu\text{g/ml}$.

Incubation time effect

Table 4 reports the % of exsheathment without and

with PVPP at different times of incubation. The % of exsheathment varied substantially between times ($P < 0.001$). As the time of incubation increased, the % of exsheathment increased for LEA without and with PVPP (Table 5).

Effective concentration for 50% inhibition

EC₅₀ of *H. contortus* was different ($p < 0.05$) for plant extract. The highest value of EC₅₀ was obtained with fresh sulla (Table 6).

Table 4. 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
		µg/ml					
% of exsheathment	Without PVPP	52.95 ±38.8 ^a	44.58 ±33.7 ^b	35.03 ±26.9 ^c	14.34 ±16.8 ^d	5.57 ±7.2 ^e	-
	With PVPP	50.32 ±38 ^a	-	-	-	5.47 ±7.3 ^b	55.43 ±36.4 ^a

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

Table 5. Effect of incubation times on % of exsheathment on the infective stage larvae of *Haemonchus contortus*.

		0	20	40	60
		min			
% of exsheathment	Without PVPP	2.7±2.1 ^d	23.87±20.8 ^c	37.02±31.6 ^b	58.4±32.4 ^a
	With PVPP	2±1.6 ^d	33.83± 27.4 ^c	50.77± 38 ^b	61.7±41.6 ^a

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

Discussion

Hedysarum coronarium (Sulla, ecotype Bikra 21), a bioactive plant, is a temperate tannin – containing legume forages. After haymaking, an important drop was observed for the values of polyphenolic

compounds when compared to fresh sulla. This could be the result of the loss of leaves induced by the material handling and the effect of sun exposure of the biomass (Aufrère *et al.*, 2012).

Table 6. EC₅₀ of *Haemonchus contortus* in the Larval exsheathment assay.

Forage Type	EC ₅₀ (µg/ml)	Limit at 0.95	
		lower	upper
Fresh sulla	356.651 ^a	278.792	438.247
Hay sulla	822,458 ^b	580,813	>1200

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

Cadbiau *et al.* (2009) reported lower values of TP and CT for fresh sulla. However, Waghorn *et al.* (2002) reported higher level of CT (68 g/kg DM). This difference may be due to assay methods that do not measure all the same entities (Aufrère *et al.*, 2012). Our result for the LEA was dose-dependent. Brunet *et al.* (2007) and Manolaraki (2011) reported that the LEA of sainfoin extracts was dose-dependent. Brunet *et al.* (2007) presented the same tendency than our results in the kinetics of exsheathment. Indeed, the more the dose extract increased the more the % of exsheathment decreased. Paolini *et al.* (2003), Paolini *et al.* (2005) and Heckendorn (2007) obtained positive results with sainfoin hay suggesting that AH activity was preserved after haymaking of *H.coronarium*.

After addition of PVPP, Oliveira *et al.* (2011) and

Alonzo-Diaz *et al.* (2008) noted that the % of exsheathment was completely reversed similarly to our findings. The restoration of L3 exsheathment to values similar to PBS, after PVPP addition, indicates that tannins of *H. coronarium* tannins are involved in the AH effects against *H. contortus*. Bravo (1998), Waterman (1999) and Molan *et al.* (2003, 2004) mentioned that CT had the properties to form complexes with macromolecules, including parasite proteins. Therefore, this could explain the relationship between dose - response and the existence of a threshold for AH activity (Brunet *et al.*, 2007).

The results of this study indicate that *H.coronarium* cultivated in Tunisia can be used as an alternative option to reduce GIN. Moreover, the haymaking reduced this AH activity.

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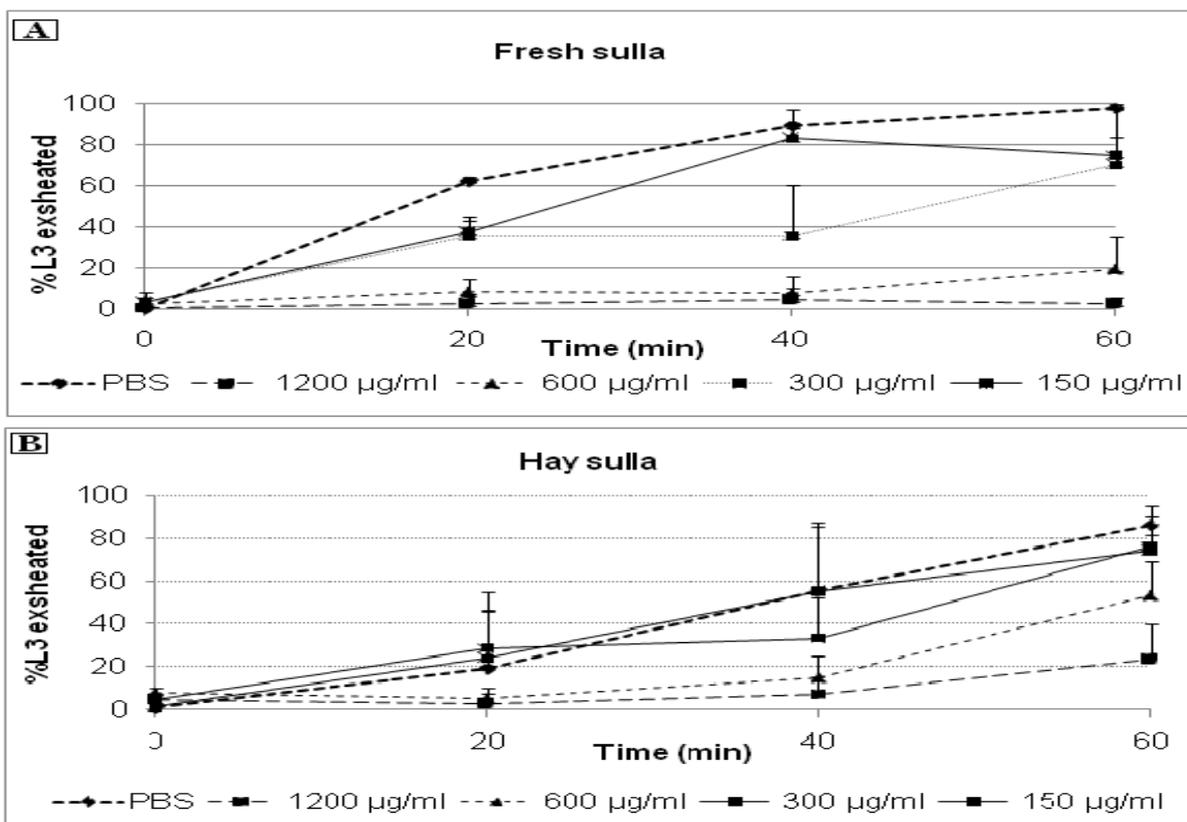


Fig. 1. Kinetics of exsheathment on the infective stage larvae (L3) of *Haemonchus contortus* of fresh sulla and hay sulla without PVPP.

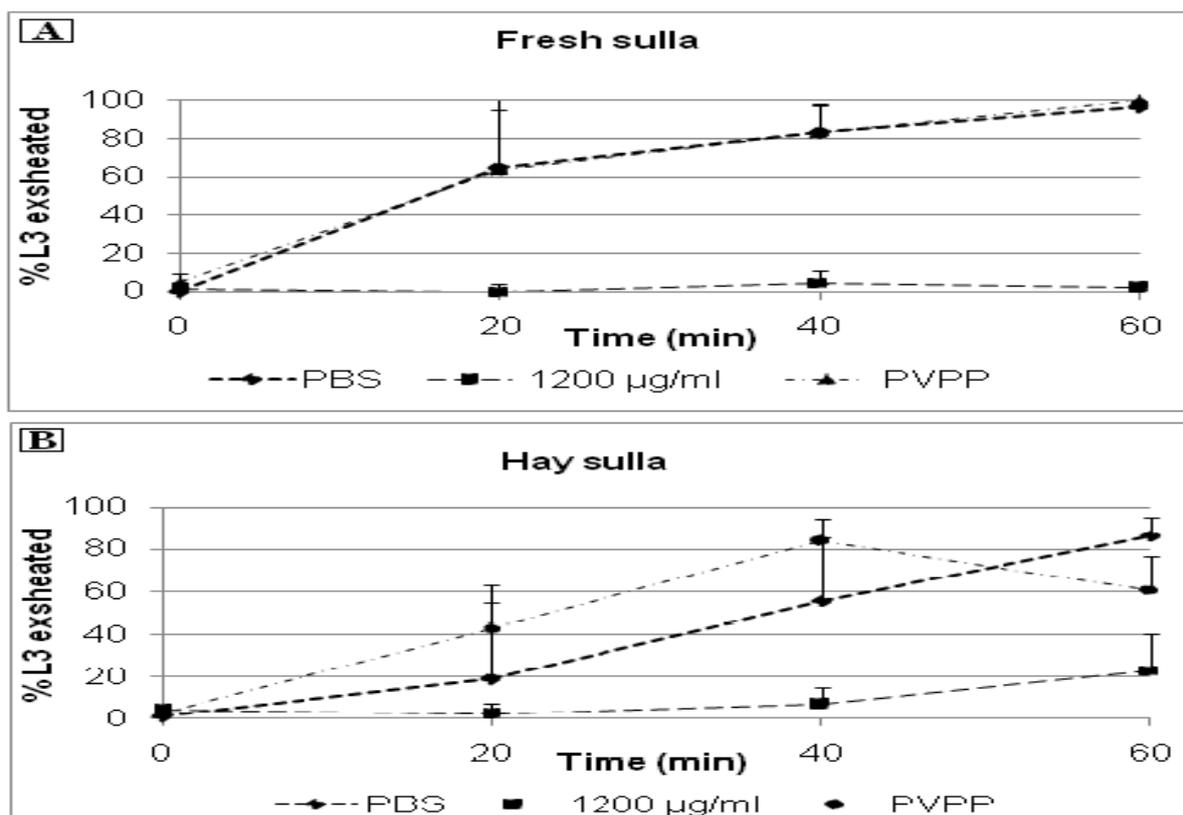


Fig. 2. Kinetics of exsheathment on the infective stage larvae (L3) of *Haemonchus contortus* of fresh sulla and hay sulla with PVPP.

Conclusion

The larval exsheathment was affected by extract dose and time of incubation. These promising findings be confirmed by *in vivo* studies.

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