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Effect of Five Mediterranean Shrubs Extracts on Larval Exsheathment of *Haemonchus contortus*

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ABSTRACT

Background: Mediterranean shrub species cover more than 70% of the total area in Tunisia and in summer when the herbaceous species have wilted, they constitute feeding resource for livestock. The use of tanniniferous shrubs seems to be a good alternative to control gastrointestinal nematodes infections in small ruminants. This study evaluated the *in vitro* anthelmintic (AH) effect of *Ceratonia siliqua* (*C.siliqua*), *Periploca angustifolia* Labill. (*P. angustifolia*) and *Medicago arborea* (*M. arborea*) against *Haemonchus contortus* third stage larvae (L3).

Methods: The larval exsheathment assay (LEA) was used to determine the proportions (%) of exsheathment of five acetonic extracts at different concentrations (1200, 600, 300, 150 µg/ml). To confirm the role of tannins in the AH effects of extracts, polyvinyl polypyrolidone (PVPP) was used as deactivating chemical tannins.

Result: The highest % L3 exsheathed was recorded for *M. arborea* (55.01%) and the lowest value was founded for *C. siliqua* and *P. angustifolia* leaves (16.26%). Our results were concentration-dependent (P<0.001). The % of exsheathment increased as the time of incubation increased (P<0.001). *P. angustifolia* pods recorded the lowest EC50 value (P<0.05). After PVPP addition, all the acetonic extracts showed a restoration of L3 exsheathment values similar to control values (P<0.001).

Key words: *Haemonchus contortus*, Larval exsheathment assay, Shrubs, Tannins.

INTRODUCTION

Haemonchus contortus is one of the most Gastrointestinal nematode (GIN) pathogens encountered in small ruminants because of its high prevalence and pathogenicity (O'Connor *et al.*, 2006). The control of this disease has been relied to the use of synthetic anthelmintic (AH). However, the repeated use of chemical AH is nowadays facing several limits. Such as, the worldwide development of AH resistance in small ruminant (Kaplan, 2004) and the increasing concern of consumers about possible drug residues in food products and in the environment. The exploitation of bioactive metabolites in plants seems to be a sustainable and an alternative solution for the control of GIN. Indeed, it seems to modulate the biology of nematode parasites, either by reducing the parasitic infection in the host or the pasture contamination (Hoste *et al.*, 2006).

Some studies have focused on the AH properties of legume forage. The aims of the current study were to verify (i) the *in vitro* AH effect of three Mediterranean woody shrubs against *Haemonchus contortus* third stage larvae using LEA and (ii) to verify tannins AH activity, a tannins inhibitor, the polyvinyl polyrrolidone (PVPP) was used.

MATERIALS AND METHODS

Plants samples

Five samples from three different plants were collected in spring-summer using the diagonal Methods (Theau *et al.*, 2010): *C.siliqua* (leaves and pods), *P.angustifolia* (leaves and pods) and *M.arborea*: (pods). They were collected from two regions in central Tunisia:

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- ✓ El Nathour (latitude 36°07′ 12.91″ N, longitude 10°05′ 40.25″ E, altitude 114 m) for *C. siliqua* .
- ✓ Saouaf (latitude 36°13′ 53.07″ N, longitude 10°10′ 25.21″ E, altitude 158 m) for *P.angustifolia* and *M. arborea*.

Preparation of plant extracts

After being freeze-dried and grounded at 1 mm screen, 10 g of each shrub 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.

Chemical composition

Representative samples of shrubs were dried at 60°C for 72 h and then grounded at 1 mm screen for chemical analysis composition. Ash, crude protein (CP) and crude fiber (CF) contents were quantified according to AOAC (2000) and acid detergent fiber (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) were analyzed according to Van Soest *et al.* (1991).

Polyphenolic compounds and biological activity

The Folin-Ciocalteu method described by Makkar (2003) was used to quantify the concentrations of total polyphenols (TP) and total tannins (TT) in the shrub samples. For each 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 (g-equiTA).

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 SpectronicUnicum, Genesys 8). Concentrations of CT were expressed as g -equivalent of leucocyanidin/100 g of DM.

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 *H. contortus* (INRA goat strain, France) with extracts of each shrub at different doses (1200, 600, 300, 150 µg/ml). One thousand ensheathed L3 were incubated for 3 h at 20°C. After incubation, the larvae were washed and centrifuged (1000 rpm at 20°C during 3 mn) 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 was 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 shrub extract. In order to check the role of tannins in the anthelmintic effects of extracts an inhibitor of tannins, PVPP was used.

Statistical analyses

Data were subjected to analysis of variance using SPSS Statistics 20. The model included shrub type, 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. 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

Chemical composition

Carob leaves exhibited the highest concentrations of CP and ADF ($P<0.05$) than pods (Table 1). For carob leaves, Silanikove *et al.* (1996) reported higher DM, ash and NDF contents; however, the CP level was similar (9%). Concerning carob fruits, the CP, NDF and ADF contents were slightly lower than those founded by Obeidat *et al.* (2010). The contents of chemical constituents (ash, CP, ADL) of *P.angustifolia* leaves were higher than pods ($P<0.05$).

For *M. arborea*, Ventura *et al.* (1999) founded similar ash value, lower NDF content and higher ADF rate.

This chemical composition variation's can be due to several parameters such as: soil and air moisture, species and plant organ harvested (Jarrige *et al.*, 1995).

Polyphenolic compounds and biological activity

C. siliqua leaves showed the highest polyphenolic compounds and BA ($P<0.05$) than *C. siliqua* pods (Table 2). In comparison to values reported by Manolaraki *et al.* (2010), TP and TT values of carob were higher for leaves and lower for pods, CT rates of carob leaves and pods were lower and the carob leaves BA was similar.

P. angustifolia pods presented highest TP and TT contents than *P. angustifolia* leaves ($P<0.05$). For CT and BA values, the tendency was reversed ($P<0.05$). This difference may be due to assay methods that do not measure all the same entities (Aufrère *et al.*, 2012). It should be noted that the highest values in CT and BA were recorded for leaves. Indeed, Aufrère *et al.*, (2012) reported that the majority of tannins were accumulated in young leaves and the secondary compounds rates varied according pedoclimatic conditions (water stress, light intensity), species, locality and the plant exploited organ's of the plant.

Shrub's effect on larval exsheathement assay

The effect of shrub was demonstrated without PVPP (P<0.001) and with PVPP, a tannin inhibitor (P=0.004) (Table 3). The highest % L3 exsheathed was recorded for *M. arborea* (55.01%) and the lowest value was founded for *C. siliqua* and *P. angustifolia* leaves (16.26%). The % L3 exsheathed values for carob pods were higher than carob leaves. The leaves and pods of *P. angustifolia* showed close values for %L3 exsheathed and also inhibition.

The % L3 exsheathed values founded for carob pods were higher than for leaves. *P. angustifolia* leaves and pods showed close values for the % L3 exsheathed. The recorded value was equivalent to 39.20% for *M. arborea*.

The highest inhibition was recorded for *C. siliqua* leaves and *P. angustifolia* leaves and pods. This strong inhibition

Table 1: Chemical composition of shrubs.

		DM	Ash	CP	NDF	ADF	ADL
		%			%DM		
C. siliqua	Leaves	42.94^{b}	4.70	9.01^{a}	35.11^{b}	23.34^{a}	10.22
	Pods	87.94^{a}	2.76	5.59^{b}	47.83^{a}	13.93^{b}	7.15
	P-value	<0.001	0.207	0.05	0.015	0.011	0.214
	SEM	2.198	0.913	0.950	2.513	1.472	1.312
P. angustifolia	Leaves	31.51^{b}	12.54^{a}	7.87	42.28	26.75^{b}	13.40^{a}
	Pods	25.14^{a}	4.52^{b}	5.91	44.54	36.31^{a}	0.66^{b}
	P-value	0.015	0.007	0.438	0.323	0.021	<0.001
	SEM	2.189	2.380	1.313	2.052	1.826	0.421
M. arborea	Pods	34.34	9.25	17.78	47.27	23.17	7.40

DM- Dry matter; CP- Crude protein; CF- Crude fiber; ADF- Acid detergent fiber; NDF- Neutral detergent fibre; ADL- Acid detergent lignin. SEM- Standard error of the means. $^{a, b}$ - Means in the same column with different superscripts differ (P<0.05).

Table 2: Mean values (±S.D.) of total phenols, total tannins, condensed tannins and biological activity for shrubs.

		TP[1]	TT[1]	CT[2]	BA[1]
C. siliqua	Leaves	11.63±0.14^{a}	6.11±0.28^{a}	0.17±0.01^{a}	8.97±0.99^{a}
	Pods	3.58±0.10^{b}	2.07±0.08^{b}	0.10±0.01^{b}	0.70±0.05^{b}
	P-value	0.005	0.010	0.049	0.005
	SEM	1.015	0.615	0.029	1.065
P. angustifolia	Leaves	6.09±0.25^{b}	0.72±0.06^{b}	0.99±0.05^{a}	2.42±0.23^{a}
	Pods	9.01±0.17^{a}	2.19±0.19^{a}	0.19±0.01^{b}	2.00±0.17^{b}
	P-value	0.05	0.012	0.016	0.041
	SEM	1.164	0.238	0.141	0.267
M. arborea	Pods	1.29±0.20	0.15±0.25	0.00±0.00	1.66±0.11

[1]: Expressed as g equivalent tannic acid /100g of DM. [2]: Expressed as g equivalent of leucocyanidin /100g of DM. TP - Total phenols; TT -Total tannins; CT- Condensed tannins; BA- Biological activity. SEM- Standard error of the means. $^{a, b}$- Means in the same column with different superscripts differ (P<0.05).

Table 3: Effect of Shrub's on the percentage of exsheathment on the infective stage larvae of *Haemonchus contortus*.

		% L3 exsheathed		
		Without PVPP	With PVPP	Inhibition
C. siliqua	Leaves	16.26^{c}	34.33^{b}	73.18^{a}
	Pods	40.53^{b}	51.74^{a}	24.60^{b}
P. angustifolia	Leaves	16.26^{c}	38.34^{b}	71.70^{a}
	Pods	19.07^{c}	39.55^{b}	74.46^{a}
M. arborea	Pods	55.01^{a}	39.20^{b}	12.62^{b}
P-value		<0.001	0.004	<0.001
SEM		1.199	1.480	2.268

SEM- Standard error of the means.
$^{a, b, c}$- Means in the same column with different superscripts differ (P<0.05).

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

Dose	% L3 exsheathed	
µg/ml	Without PVPP	With PVPP
PBS	58.13^{a}	60.81^{a}
150	29.74^{b}	-
300	24.28b^{c}	-
600	18.80c^{d}	-
1200	16.39^{d}	10.19^{c}
1200+PVPP	-	49.94^{b}
P-value	<0.001	<0.001
SEM	1.199	1.480

SEM- Standard error of the means. ^{a,b,c,d}- Means in the same column with different superscripts differ (P<0.05).

could be due to their high content of secondary compounds and mainly to their high BA content. Indeed, *C.siliqua* leaves and *P.angustifolia* leaves and pods showed an inhibition higher than 50% so it may be considered as a bioactive plant with an interesting anthelmintic power.

Dose extract effect on larval exsheathement assay

Our results were concentration-dependent (P<0.001). As the doses increased, the % of exsheathment decreased to 29.74, 24.28, 18.80 and 16.39 for the doses 150, 300, 600 and 1200 µg/ml, respectively (Table 4). After addition of PVPP, the exsheathment trend reversed and was closer to that of PBS. Previous studies showed the same trend (Alonzo-Diaz *et al.*, 2008; Manolaraki *et al.*, 2010; Oliveira *et al.*, 2011).

The restoration of L3 exsheathment to values similar to PBS, after PVPP addition, indicates that tannins of these shrubs are involved in the AH effects against *H.contortus*.

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

Time	% L3 exsheathed	
mn	Without PVPP	With PVPP
0	2.86^{d}	1.47^{c}
20	27.29^{c}	41.08^{b}
40	39.21^{b}	56.30^{a}
60	48.42^{a}	64.22^{a}
P-value	<0.001	<0.001
SEM	1.199	1.480

SEM -Standard error of the means.$^{a, b, c, d}$-Means in the same column with different superscripts differ (P<0.05).

Incubation time effect on larval exsheathement assay

The result of incubation time effect on the % of exsheathment without and with PVPP was reported in Table 5. The % of exsheathment increased as the time of incubation increased (P<0.001) as reported by Aïssa *et al.* (2015).

Interaction effects

Table 6 summarized data from the LEA. The interaction (shrub×time×dose) was significant (P<0.001). For PBS, the %L3 exsheathed of *H.contortus* ranged from 91.51% for *C.siliqua* leaves to 96.43% for *P.angustifolia* pods at 60 min. At the highest concentration, the %L3 exsheathed at 60min ranged from 0.00% to 93.75% for *C.siliqua* leaves and *M.arborea*, respectively. Indeed, AH was important for *C.silique* and *P.angustifolia* (leaves and pods). These results are in agreement with other studies that reported shrub's AH activity (Alonso-Diaz *et al.*, 2008; Oliveira *et al.*, 2011). The variability of the AH effect is mainly due to variability in their phenolic compounds at the quantitative and qualitative level (Manolaraki *et al.*, 2010).

Table 6: Larval Exsheathment values of infective third-stage larvae of *Haemonchus contortus* of shrubs without PVPP (mean ±S.D.).

		Dose	0	20	40	60
		µg/ml	mn			
C. siliqua	Leaves	PBS	0.67±1.35	54.19±26.82	86.53±9.91	91.51±12.91
		150	3.65±3.15	1.19±2.38	19.29±13.99	26.04±26.43
		300	4.53±5.05	5.00±5.77	2.13±2.46	8.40±7.61
		600	2.94±5.88	5.24±0.88	4.36±3.20	8.13±13.00
		1200	2.78±3.56	1.04±2.08	6.34±4.34	0.00±0.00
	Pods	PBS	2.86±3.83	62.56±14.31	66.50±22.71	92.65±7.71
		150	1.15±1.43	20.34±16.07	55.38±22.41	93.16±6.47
		300	1.31±1.66	35.11±37.22	80.69±9.68	76.69±13.23
		600	4.36±4.41	46.25±14.93	34.93±20.83	78.64±32.56
		1200	2.11±1.59	17.13±14.31	11.61±12.59	40.15±25.73
P. angustifolia	Leaves	PBS	4.55±3.44	56.46±29.95	78.02±13.54	96.18±4.81
		150	3.03±2.06	7.95±6.65	2.81±3.29	32.34±32.41
		300	2.88±2.55	4.93±7.26	3.06±3.76	6.14±4.37
		600	4.80±5.72	6.67±8.16	0.00±0.00	4.10±3.16
		1200	1.63±1.89	0.00±0.00	6.95±5.65	2.72±3.16
	Pods	PBS	1.97±2.33	70.51±15.25	93.55±5.90	96.43±7.14
		150	1.31±2.63	12.87±6.68	30.53±20.67	18.76±12.47
		300	3.59±3.41	3.90±4.56	7.93±5.68	6.59±4.94
		600	10.21±15.82	5.90±6.84	3.55±4.22	3.85±4.44
		1200	1.67±3.33	2.96±2.12	3.14±2.26	2.10±2.44
M. arborea	Pods	PBS	1.69±1.98	52.61±27.11	76.77±33.84	92.75±12.50
		150	2.42±1.73	75.21±18.83	90.78±12.31	96.43±7.14
		300	1.21±1.40	58.86±25.45	76.23±23.43	96.43±7.14
		600	1.80±2.17	31.65±27.92	62.09±44.49	96.55±42.82
		1200	2.36±2.05	52.61±27.11	76.77±33.84	93.75±12.50

Table 7: Larval Exsheathment values of infective third-stage larvae of *Haemonchus contortus* of shrubs with PVPP (mean ±S.D.).

		Dose	0	20	40	60
		µg/ml	mn			
C. siliqua	Leaves	PBS	1.56±3.12	63.54±30.08	82.50±20.61	92.62±9.53
		1200	1.85±3.70	1.92±3.85	5.28±6.11	0.00±0.00
		1200+PVPP	2.08±4.17	48.41±20.99	37.01±24.75	75.13±26.17
	Pods	PBS	1.47±2.94	72.50±22.17	97.73±4.54	100±0.00
		1200	1.79±3.57	13.46±15.85	46.31±28.96	59.03±35.82
		1200+PVPP	2.59±3.39	53.73±21.38	87.20±20.42	85.10±14.86
P. angustifolia	Leaves	PBS	0.00±0.00	55.28±42.04	83.54±13.95	96.87±6.25
		1200	0.00±0.00	0.00±0.00	2.50±5.00	4.54±9.09
		1200+PVPP	0.00±0.00	51.25±34.25	78.54±8.67	87.50±25.00
	Pods	PBS	0.00±0.00	59.17±27.27	77.50±7.39	91.67±16.66
		1200	0.00±0.00	0.00±0.00	0.00±0.00	1.09±2.17
		1200+PVPP	6.28±4.22	66.67±23.57	73.33±46.19	97.50±5.00
M. arborea	Pods	PBS	1.56±3.12	63.54±34.08	82.50±20.61	92.62±9.53
		1200	0.00±0.00	29.10±19.84	25.51±20.35	23.16±12.79
		1200+PVPP	2.87±3.35	37.65±9.74	52.28±29.28	56.55±18.17

Table 8: EC50 of *Haemonchus contortus* in the larval exsheathment assay.

		EC50	Limit at 0.95	
		µg/ml	Lower	Upper
C. siliqua	Leaves	58.72[b]	5.55	111.00
	Pods	1132.86[a]	803.28	>1200
P. angustifolia	Leaves	53.95[a]	12.20	97.77
	Pods	16.12[a]	1.29	41.98
M. arborea	Pods	954.08	702.82	>1200

[a, b]-LS-Means in the same column with different superscripts differ (P<0.05).

After PVPP addition, all the extracts of the studied shrubs showed a restoration of the L3 exsheathment values similar to the control values (P<0.001) (Table 7). Alonso-Diaz *et al.* (2008) and Oliveira *et al.* (2011) also noted that the % of exsheathment was completely reversed. This suggests a major role of tannins, especially CT. Molan *et al.* (2003) mentioned that CT have the property of forming complexes with macromolecules including proteins and in our case the proteins of parasites (Manolaraki *et al.*, 2010).

Effective concentration for 50% inhibition

EC50 was different (p<0.05) for plant extract (Table 8). *C. siliqua* leaves showed a lower EC50 than pods. However, the EC50 of *P. angustifolia* pods and leaves were similar. *M. arborea* had an EC50 around 954.08µg/ml. More EC50 was lowest and the shrub sample's was more effective against the exsheathment of L3 larvae, helping to prevent animal infestation.

CONCLUSION

C. siliqua and *P. angustifolia* showed the highest polyphenolic compounds and biological activity and they recorded the highest inhibition. So, this study confirmed the AH potential of these shrubs. Therefore, further *in vivo* assays must be conducted to confirm these findings to better use of these plants in livestock.

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