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Compared *in vitro* anthelmintic effects of eight tannin-rich plants browsed by goats in the southern part of France

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Abstract. The search for alternative solutions to chemical treatments is nowadays prompted by the emergence and widespread diffusion of resistance to anthelmintics in populations of gastrointestinal nematodes in sheep and goats. Experimental evidence is accumulating to suggest that tannin-rich plants have anthelmintic properties and may therefore represent a possible alternative option to control nematodes. However, most data have been acquired on legume forages. In contrast, information on plants browsed by goats (or sheep) remains scarce. The anthelmintic properties of a range of concentrations of 8 plant extracts: chestnut (*Castanea sativa*), pine tree (*Pinus sylvestris*), heather (*Erica erigena*), genista (*Sarothamnus scoparius*), brambles (*Rubus fructicosus*), oak tree (*Quercus robur*), hazel bush (*Corylus avellana*) and ash tree (*Fraxinus excelsior*) composing browse in the South of France have been examined on the 3 main nematode species of small ruminants (i.e. *Teladorsagia circumcincta*, *Haemonchus contortus* and *Trichostrongylus colubriformis*), using two *in vitro* assays: (i) the larval migration inhibition assay on third-stage larvae (L3); and (ii) the adult worm motility inhibition assay. The tannin contents of the plant were measured according to the method of the European Pharmacopea. The results indicated that total tannin contents ranged from 24.7% DM to 1.5%. All plants showed some anthelmintic activity in both stages. However, variations in efficiency were observed depending on the parasitic stages (L3 *vs* adult worms) or species. Overall, the most consistent results were found with plant extracts possessing the highest tannin content (oak tree, hazelnut, chestnut and brambles respectively).

Keywords. Natural anthelmintic – Tannins – Gastrointestinal nematodes – Small ruminants.

Effets anthelminthiques* in vitro *de huit plantes riches en tannins broutées par des chèvres dans la partie sud de la France

Résumé. *La recherche de solutions alternatives aux anthelminthiques chimiques est désormais un impératif pour gérer les nématodes gastrointestinaux parasites des petits ruminants compte tenu de la diffusion constante des résistances aux anthelminthiques dans les populations de vers. Plusieurs données récentes suggèrent que des plantes riches en tannins possèdent des propriétés anthelminthiques et pourraient représenter une solution alternative pour la maîtrise de ce parasitisme. Cependant, ces données ont surtout été acquises sur des légumineuses fourragères. Les informations sur les propriétés antiparasitaires de plantes de parcours ou de sous-bois exploitées par les chèvres ou les moutons restent rares. Les effets d'une gamme de concentrations d'extraits de 8 plantes [châtaignier (*Castanea sativa*), pin sylvestre (*Pinus sylvestris*), bruyère arborescente (*Erica erigena*), genêt à balai (*Sarothamnus scoparius*), ronce (*Rubus fructicosus*), chêne (*Quercus robur*), noisetier (*Corylus avellana*), frêne (*Fraxinus sp.*)] présentes sur les couverts pastoraux du sud de la France ont été examinés, sur 3 espèces parasites de petits ruminants, en utilisant deux tests* in vitro *: (i) le test d'inhibition de migration larvaire sur les larves infestantes ; et (ii) le test d'inhibition de motilité des vers adultes. Les teneurs en tannins des plantes, mesurées selon la méthode de la Pharmacopée Européenne, indiquent des valeurs allant de 24,7% à 1,5% de la MS. Tous les extraits ont montré une activité anthelminthique sur les 2 stades. Cependant, de fortes variations d'efficacité ont été notées selon le stade (L3 ou vers adultes) ou l'espèce concernée. De manière générale, les résultats les plus constants ont été trouvés avec les plantes présentant les plus fortes teneurs en tannins (chêne, noisetier, châtaignier, ronces).*

Mots-clés. *Anthelminthiques naturels – Tannins – Nématodes gastrointestinaux – Petits ruminants.*

I – Introduction

Nematode gastrointestinal parasitism remains a major threat to efficient production in small ruminants. Up to now, the control of these parasitic diseases has largely relied on the repeated use of anthelmintics (AHs). However, the prevalence of AH resistance in worm populations is constantly increasing, especially in small ruminants and this phenomenon challenges the usual mode of control based on chemical drugs (Jackson and Coop, 2000). In addition, the increasing demand of consumers to limit the use of chemical substances in farm industry merits consideration. These two main reasons underpine the current search for alternative or rather complementary solutions to chemotherapy to control gastrointestinal parasitism in sheep and goats. Amongst these solutions, the AH properties presented by tanniniferous plants and their potential use to limit infections have received recent attention (see reviews by Kahn and Díaz-Hernández, 2000; Hoste *et al.*, 2006).

The vast majority of the *in vivo* and *in vitro* studies examining the effects of tannin-rich (TR) plants against the gastrointestinal nematodes have been performed on legume forages (Hoste *et al.*, 2006). Although tannin-rich woody plants and bushes are largely distributed, particularly in the Southern European countries, only a limited number of *in vitro* studies have addressed the question of their potential direct anthelmintic properties despite the fact that: (i) these plants compose the environment of production systems exploiting rangelands or pastures; (ii) their high palatability, particularly by goat and deer (Papachristou *et al*., 2005); and (iii) some *in vivo* evidence on anthelmintic activity when ruminants consume browse in rangeland environment either in tropical (Kabasa *et al.*, 2000; Kahiya *et al.*, 2003) or in temperate conditions (Hoste *et al.*, 2004; Osoro *et al.*, 2007a,b).

The main objective of the present study was to compare the anthelmintic effects of 5 additional woody plant extracts, on the most prevalent nematode species, i.e. *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Such plants compose part of the rangeland vegetation in the Southern part of France and are usually browsed by goats. The results will be compared to those of previous data published on brambles, hazel nut and oak tree (Paolini *et al.*, 2004).

II – Materials and methods

Two *in vitro* assays, i.e. the Larval Migration Inhibition (LMI) assay on third-stage larvae (L3) and the Adult Motility Inhibition assay (AMI) were performed to examine the effects of tanniniferous plants on 3 nematode species: *Tel. circumcincta*, *H. contortus* and *T. colubriformis*. The AH properties of 8 plant extracts were examined: chestnut (*Castanea sativa*), pine tree (*Pinus sylvestris*), heather (*Erica erigena*), genista (*Sarothamnus scoparius*), brambles *(Rubus fructicosus*), oak tree (*Quercus robur*), hazel tree (*Corylus avellana*) and ash tree (*Fraxinus excelsior*) which are part of browse in Southern France. Rye-grass (*Lolium perenne*) was used as negative controls due to very low tannin content.

The plants were collected in the fields in the southern part of France at the end of spring. For rye-grass, genista, heather and brambles, the stems and leaves were collected. For the trees, only the leaves were collected. Five grams of each dried plant in powder were extracted by maceration in 100 ml of 30% ethanol at 60°C for 2 h. After filtration and solvent evaporation, the extract was concentrated under low pressure at 40°C, frozen and lyophilized to obtain a grounded sample, maintained at 4°C. To prepare the extract solutions applied in the bioassays, the powders were dissolved in phosphate buffer saline (PBS 0.1M, pH 7.2) and serially diluted immediately prior to incubation. The tannin contents were measured according to the method of the European Pharmacopea (2001) based on the binding between tannins and proteins of a standardised skin powder. The percentages of tannins were expressed by reference to a pyrogallol standard.

For LMI tests, the nematode ensheathed L3 were obtained from donor goats infected with pure strains of each species. For each plant, 1000 microlitres of a larval solution, concentrated at 2000 L3/ml, were added to microtubes containing either PBS (negative control), levamisole at 0.5% (anthelmintic control) or plant extract at concentrations of 300, 600 and 1200 µg/ml. The

incubations were carried out for 3 h at 20°C. After repeated washings with PBS, the L3 were retrieved in 800 µl PBS and added to inserts equipped with a 20 µm mesh, positioned in a conical tube, with the mesh just above PBS. Three replicates were run for each plant concentration and for the controls. After 3 h, the inserts were retrieved and the number of L3 having actively migrated were counted. The percentage of migration was calculated as [(T – M) / T × 100], where T is the total number of L3 deposited in the sieve and M the number of L3 in PBS, after migration.

The adult worms for AMI were obtained from goats experimentally infected with each of the 3 nematode species. Four weeks after infection, the animals were euthanised. Immediately after death, the small intestine or the abomasum was taken to collect the worms, using a modified Baermann method, with saline at 37°C. After 2 h, the worms having migrated to the saline were collected and placed in 24-(for *T. colubriformis* or *Tel. circumcincta*) or 48-(for *H. contortus*) multiwell plates. One ml of plant extracts at different concentrations (75, 150, 300, 600 or 1200 µg/ml) in PBS were added to the wells. Levamisole (positive anthelmintic control) and PBS (negative control) were also included on each plate. The measurements were performed in triplicates. The supernates were changed every 24 h. At 6, 24 and 48 h, the motility of the adult worms was noted by careful observation under a stereomicroscope at magnification x40. A motility index was calculated as the ratio between the number of immobile worms/total number worms in the 3 wells per concentration.

Significant differences in means for the LMI rates between treatments and at the different concentrations were assessed using a general linear model (GLM) procedure. For AMI, for each treatment (plant extract and dose), the number of immobile worms was recorded depending on time and concentrations. The survival curve was analysed by applying the non parametric, stratified Cox regression test. Both tests were performed against the PBS control with Systat 9 software (SPSS Ltd).

III – Results and discussion

1. Measurements of total tannins in extracts

The contents of total free tannins found in the various plants were respectively: rye-grass (0.27%), genista (1.48%), pine tree (2.48%), ash tree (4.50%), oak tree (5.30%), hazelnut tree (14.20%), brambles (7.40%), heather (19%) and chestnut tree (24.7%).

2. Measurements of anthelmintic activities with the two bioassay procedures

Examples of results obtained with *T. colubriformis* with extracts of genista, pine tree, heather and chestnut are illustrated for LMI and AMI respectively on Figs 1 and 2.

A general summary of the results obtained with the 2 bioassays on the 3 nematodes with the 8 browse extracts plus the rye grass ones are provided in Table 1. No difference to PBS values were observed with the negative control (rye-grass extracts). This suggests that the other significant effects observed were not due to any artefact related to a possible contamination of samples with chemicals during the preparation.

Each of the tannin-rich plant extracts has shown, to some extent, significant results on the 3 nematode species. For 4 plant species (pine tree, chestnut, heather and genista), these results confirm those acquired with another *in vitro* test examining the possible interference with L3 exsheathment (Bahuaud *et al.*, 2006) although in this case, another mechanism is probably involved. Overall, the results tend thus to confirm the hypothesis of a direct anthelmintic activity associated with extracts of tanniniferous plants composing browse in the Southern part of France. These *in vitro* data also confirm the results of *in vivo* studies from the same area indicating that a higher use of browse by infected goats was associated with a lower level of gastrointestinal parasitism (Hoste *et al.*, 2001, 2004). In addition, the *in vitro* efficacy of heather against *H. contortus* and *T. colubriformis* was also in agreement with results of *in vivo* studies measuring the AH effect of heather supplementation in goats (Osoro *et al.*, 2007a,b).

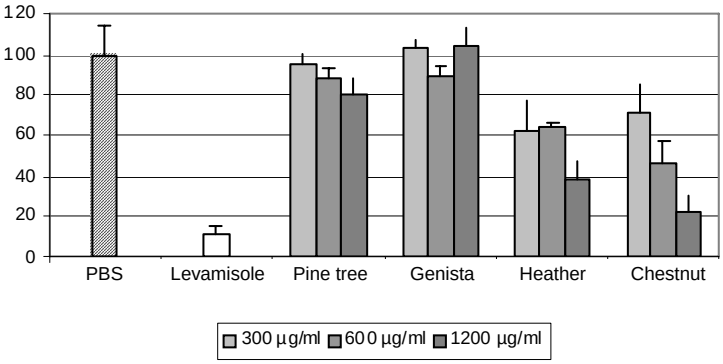

Fig. 1. Results of LMI assay performed on infective larvae of *T. colubriformis* by reference to control PBS values. Significant, dose dependent effects were observed with pine tree, heather and chestnut. Genista extracts did not result in any anthelmintic activity in the concentrations tested.

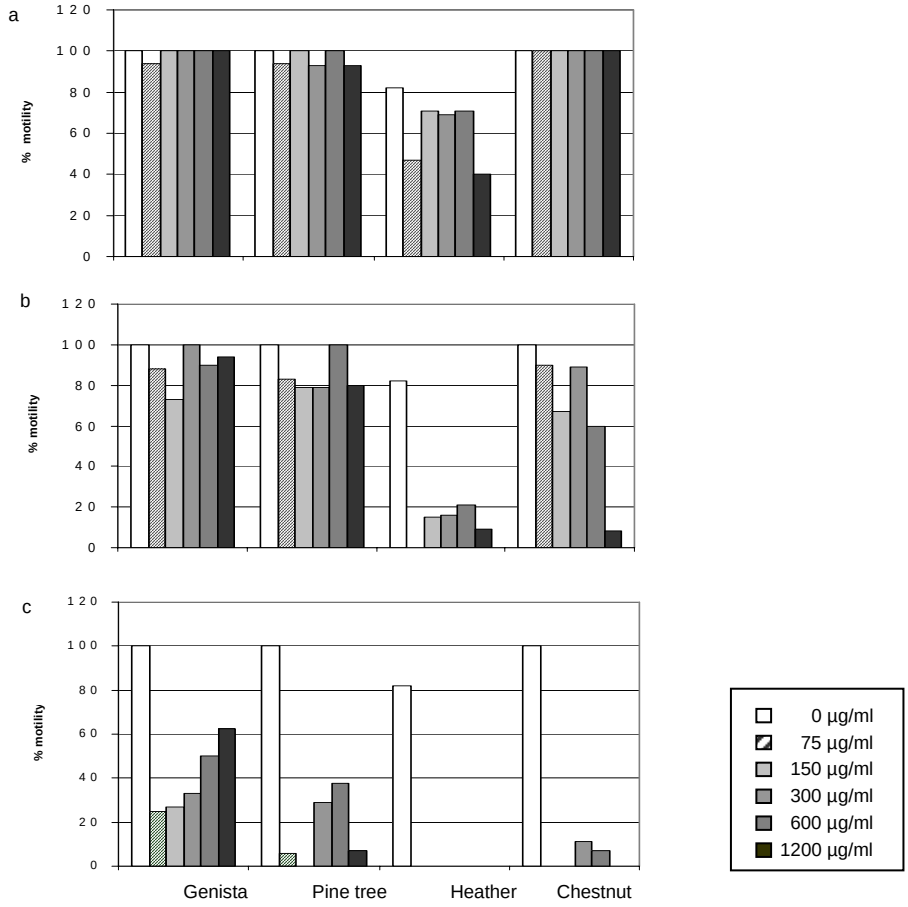

Fig. 2. Effects of genista, pine tree, heather and chestnut on adult *T. colubriformis* motility after: a) 6 h, b) 24 h and c) 48 h incubation with extracts at concentrations of 0, 75, 150, 300, 600, 1200 µg/ml.

Table 1. Summary of the main anthelmintic effects observed with the 8 plant extracts based on the LMI (L3) and the AMI (adult worms) assays. The signs * indicate statistical difference to controls (P < 0.05). NS indicates non significant results and NP that the specific assays were not performed

	T. colubriformis		*H. contortus*		*T. circumcincta*	
	L3	**Adult**	**L3**	**Adult**	**L3**	**Adult**
Rye-grass	NS	NS	NS	NS	NS	NS
Oak tree	NS	*	*	*	*	*
Hazel tree	NS	*	*	*	*	*
Brambles	*	*	NS	*	*	*
Pine tree	*	*	NS	*	NS	NS
Genista	NS	*	NS	*	*	NS
Heather	*	*	*	*	NS	NS
Chestnut tree	*	*	*	*	*	NS
Ash tree	*	NP	*	NP	*	NP

Not all parasite species or stages showed the same susceptibility to the AH activity of plant extracts (see Table 1). Such differences in susceptibility have also been described in previous *in vivo* experiments (Athanasiadou *et al.*, 2001). They have been attributed to possible differences in the concentrations of free tannins between the abomasum and the small intestine. However, the hypothesis of real differences in susceptibility to tannins depending on the parasite species has also to be evoked since modulations in the response to extracts of the same plant have been found in other *in vitro* experiments (Bahuaud *et al.*, 2006; Brunet and Hoste, 2006).

Furthermore, variations in AH activity have also been observed depending on the plant extracts. It is worth to note that the less consistent results on the various parasite stages and/or species have been obtained with the extracts presenting the lowest level of tannins (genista, pine tree). This observation supports the hypothesis that tannin content is one modulating factor in the AH activity of plants. Using a similar source of tannins, both *in vivo* studies with quebracho (Athanasiadou *et al.*, 2001) and *in vitro* data on sainfoin (Manolaraki *et al.*, 2007) have confirmed this hypothesis. Moreover, for legume forages, the influence of the nature of condensed tannins on AH efficacy has also been evoked, especially the role of the ratio between different types of condensed tannins, prodelphinidins and procyanidins (Molan *et al.*, 2003). In the case of browse plants, similar factors may also be involved. In addition, due to the high diversity of plant secondary compounds in these plants, other biochemical components could also be responsible for part of the AH activity. To exploit these plants for their AH effects in farm systems, further basic information and better understanding of the role of these different factors is essential, as well as avoiding the possible antinutritional consequences.

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