TITLE: ***IN VITRO* EFFECTS OF THREE WOODY PLANT AND SAINFOIN EXTRACTS ON THIRD-STAGE LARVAE AND ADULT WORMS OF THREE GASTROINTESTINAL NEMATODES.**

V. PAOLINI [1]*, I. FOURASTE [2] and H. HOSTE [1].

[1]: Unité Mixte de Recherches 1225 INRA/DGER, "Interactions Hôte Pathogène". Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, F31076 Toulouse. France.
[2]: Faculté des Sciences Pharmaceutiques, Université Paul Sabatier Toulouse III, 35 Chemin des Maraîchers, F31062 Toulouse. France.

*Corresponding author: V.PAOLINI

Unité Mixte de Recherches 1225 INRA/DGER, " Interactions Hôte Pathogène ".
Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, F31076 Toulouse. France.
Email: v.paolini@envt.fr Tel: +(33) 05-61-19-38-75 Fax: +(33) 05-61-19-32-43.

Running title : *In vitro* effects of tanniferous plants on nematodes

SUMMARY

Most studies on the effects of tanniferous plants on nematodes have examined forages but have neglected the woody plants. Therefore, *in vitro* effects of extracts from 3 woody plants (*Rubus fructicosus*, *Quercus robur*, *Corylus avellana*) have been tested on trichostrongyles and compared to sainfoin, a legume forage. Because some *in vivo* results indicated that the effects of tannins differed depending on the parasitic species and/or stages, the effects were measured on third-stage larvae (L3) and adult worms of *Teladorsagia circumcincta, Haemonchus contortus* and *Trichostrongylus colubriformis*. The effects of plant extracts varied according to the plant sources, the parasite species and stages. For the woody plants, significant inhibitory effects were obtained on both stages of abomasal species. Results for *T. colubriformis* were more variable. Effects of sainfoin extracts were significant on *T. colubriformis* and *H.contortus* L3, and on abomasal adult worms. In order to assess the implications of tannins, polyethylene glycol (PEG), an inhibitor of tannins, was added to hazel tree, oak and sainfoin extracts. Without PEG, significant inhibitory effects on L3 and adult worms were confirmed. After addition of PEG, the larval migration and motility of adult worms were restored in most cases. These results confirm variations in effects depending on factors related to plants or parasites and suggest that tannins are partly responsible for the effects.

Key words: *In vitro* methods , Nematode, Tannins, Woody plants, Sainfoin

INTRODUCTION

Gastrointestinal parasitism with nematodes 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. However, the prevalence of anthelmintic resistances in nematode populations is constantly increasing, especially in goats (Jackson & Coop, 2000). The prevalence of resistances in this last host species now severely impairs the control of trichostrongyloses. In addition, in lactating animals, the use of antiparasitic chemical molecules is drastically restricted. These reasons, plus the increasing demand of consumers to limit the use of chemical substances in farm industry, explain the current research seeking alternative or complementary solutions to chemotherapy for the control of gastrointestinal parasitism. Amongst these solutions, the possibilities offered by tanniferous plants have been explored in several studies (Kahn & Diaz-Hernandez, 2000). The initial results have been obtained from field studies, showing that the consumption of tanniferous forages by parasited sheep affected the egg output and the worm burdens (Niezen *et al*. 1998a, b, 2002). In addition, some *in vivo* assays in experimentally infected sheep, using quebracho extracts as a rich source of condensed tannins, tended to confirm the results obtained in natural infections (Athanasiadou *et al*. 2000a, b, 2001). Data in goats are scarce, but have tended to confirm the results reported in sheep (Paolini *et al*. 2003a,b,c; Min *et al*. 2003). In addition, in this host species, results from assays under controlled conditions suggested differences in effect of quebracho depending on nematode species and parasitic stages (Paolini *et al*. 2003 a,c).

Major differences in metabolism, digestive physiology and immune response exist between the two small ruminant species. It is now recognized that results obtained in sheep should not be directly transposed to goats. One of the main difference between the two hosts is also related to their feeding behaviour. Goats are usually considered as natural browsers whereas sheep are grazers (Gordon, 2003). Consequently, it is usually acknowledged that goats are more tannin-tolerant than sheep and exhibit a better ability to exploit the vegetation of rangelands (Silanikove et *al*. 1996; Landau *et al*. 2000; Silanikove, 2000). Despite this adaptation, the vast majority of studies performed on the effects of condensed tannins on the gastrointestinal parasites, in goats as in sheep, were performed using tanniferous

forages. Only two studies in goats showed that the consumption of woody plants composing the browse and containing tannins could significantly modulate nematode infections (Kabasa, Opuda-asibo & Ter meulen, 2000; Kahiya, Mukaratirwa & Thamsborg, 2003). Similarly, all the *in vitro* assessments of the effects of tanniferous plants on nematodes have been restricted to extracts from forages.

The objectives of the current study were therefore to screen the effects of woody plants consumed in rangelands by goats, on three of the most common species of nematodes: *Teladorsagia circumcincta, Haemonchus contortus* and *Trichostrongylus colubriformis*. In addition, the effect of one fodder crop, the sainfoin, was tested for comparison. Sainfoin was selected due to previous positive results acquired in infected goats (Paolini, Dorchies & Hoste, 2003b). Because of the differences in results depending on the parasitic stages observed from *in vivo* controlled studies, this screening also aimed at comparing the actions of extracts on both third-stage larvae and adult worms. Last, these *in vitro* tests also aimed at verifying a direct action of tannins from woody plants and sainfoin, as suggested previously *in vivo* with quebracho and *in vitro* with other legume forages (Athanasiadou *et al*. 2000a, b; Paolini *et al*. 2003a, c; Molan *et al*. 2000a, b).

MATERIALS AND METHODS

Experimental design

In vitro experiments were conducted to test the effects of tanniferous plants on the motility of the ensheated third-stage larvae and on the motility of adult worms of the three most common nematodes in small ruminants: *T. circumcincta, H.contortus* and *T.colubriformis*. The different plants which were tested could be divided into two groups: a forage legume, sainfoin (*Onobrychis vicifolia*), and three woody plants composed of brambles (*Rubus fructicosus*), oak (*Quercus robur*) and hazel tree (*Corylus avellana*). Lucerne (*Medicago sativa*) and rye-grass (*Lolium perenne*) were used as controls because of their low percentages of tannins (Niezen *et al*. 2002; Molle *et al*. 2003).

Preparation of plant extracts

The plants were collected in the fields in the southern part of France at the end of spring. For sainfoin, lucerne, rye-grass and brambles, the stems and leaves were collected. For oak and hazel tree, the samples were limited to the leaves. Five grams of each plant sample were reduced to powder by crushing, extracted by 100 ml of water at 90°C for 2 h. The filtrate was concentrated under low pressure at low temperature (40°C), frozen and lyophilized for 24h to obtain a grounded dry sample (in powder). Plant extract solutions applied to third-stage larvae and adult worms were prepared as follows. The powders were dissolved in phosphate buffer saline (PBS; 0.1M phosphate, 0.05M NaCl; pH 7.2) and serially diluted immediately prior to incubation. The range of dilutions which were examined were 300, 600 and 1200 g/ml for the bioassay on larval migration inhibition and 75, 150, 300, 600 and 1200 µg/ml for the assay on the adult worms.

Condensed tannin analyses

Total condensed tannin contents from each plant extract were measured by the butanol-HCl method described by Jackson *et al*. (1996). The condensed tannins used as a standard were the commercial quebracho extracts, from the bark of the tropical tree *Schinopsis spp*., manufactured by UNITÀN s.a.i.c.a (Buenos Aires, Argentina).

Assay procedures

Larval migration inhibition bioassay (LMI)

The larval migration inhibition (LMI) bioassay developed by Wagland *et al*. (1992) and modified by Rabel, Mc Gregor & Douch (1994) was used to determine the inhibiting effects of plant extracts at the different concentrations on *T.circumcincta, H.contortus* and *T.colubriformis*. The infective larvae were obtained respectively from donor sheep infected with a pure strain of either *H.contortus* or *T.colubriformis* and a donor goat infected with *T.circumcincta*.

The concentrations of plant extracts used for incubations were 300, 600 or 1200 µg/ml. Five hundred microlitres of a larval solution concentrated at 2000 L3/ml (~1000; third-stage of larvae, L3) were added to microtubes containing either PBS without condensed tannins, anthelmintic control (levamisole at 0.5% concentration) or a range of plant extract concentrations for each plant species. All incubations were

carried out for 3h at 20°C. Thereafter, the L3 were washed and centrifugated (at 5000 rpm during 5 minutes) three times in PBS. After the last washing, the L3 were retrieved in 800 µl of PBS and added to inserts equipped with a 20 µm mesh, positioned in a conical tube, with the mesh just above PBS. The 20 µm mesh size was selected in order to ensure that migration of larvae through the sieves was an active phenomenon. Three replicates were run for each plant concentration as well as for the PBS and anthelmintic controls. After 3h at room temperature, the inserts were retrieved. According to Wagland (1992) and Rabel, Mc Gregor & Douch (1994), the number of L3 present in the PBS, i.e. those which have actively migrated through the mesh, were counted under a stereomicroscope at magnification X 40. The percentage of migration was calculated as $\frac{T - M}{T} \times 100$

where T is the total number of L3 deposited in the sieve and M the number of L3 present in PBS, after migration.

Effects on adult worms

Adult worms were obtained from goats which were experimentally infected with a pure strain of either *T. colubriformis, H. contortus* or *T.circumcincta.* Four weeks after infection, the animals were euthanised with pentobarbital injection. Immediately after death, the small intestine or the abomasum was collected, opened, the lumen content was retrieved and the mucosa washed gently. The worms were collected using a modified Baermann method, with saline solution at 37°C. After 2h, the worms having migrated to the saline were collected and quickly placed in 24-(for *T.colubriformis* or *T.circumcincta*) or 48-(for *H.contortus*) multiwell plates. One ml of the different concentrations (from 75 to 1200 µg/ml) of plant extracts diluted in PBS (with penicillin and streptomycin 4%), were added to the wells. Levamisole (concentrations from 0.125% to 1%) as an anthelmintic control and PBS controls were included on each plate. All the measurements were performed in triplicates. The mean numbers of worms per well were respectively 11,51 (± s.d.= 4,9) for *H.contortus* ; 14,43 (±s.d.= 6,28) for *T.circumcincta*; and 26,36 (± s.d.= 10,17) for *T.colubriformis*. The supernatants were changed every 24h. The motility of adult worms was noted by careful observation under a stereomicroscope at magnification X40 after 6, 24, 48h for *T.colubriformis* and *H.contortus*. The observation was extended to 72h for *T.circumcincta*. At each time, a motility index was calculated as

the ratio between the number of immobile worms / total number worms in the 3 wells per concentration.

Addition of polyethylene glycol (PEG)

PEG is a specific inhibitor of tannins (Jones & Mangan, 1977). In order to ascertain the role of tannins in the *in vitro* effects on the two parasitic stages of the three species of nematodes, an additionnal assay was performed. Sainfoin was used as an example of tanniferous legume forage. Hazel tree and oak leaves were selected from the woody plants due to their high tannin contents. In addition, extracts of rye-grass were used to provide negative control, since this plant has a very low tannin contents. Incubations were made with the 4 plant extracts at 1200 µg/ml with or without polyethylene glycol (PEG provided by SIGMA; Av.Mol.Wt:3350; 1 µg / µg plant extract). Levamisole and PBS controls were used. The methods used to assess the effects on the L3 and on the adult worms were similar to those previously described.

Statistical analyses

LMI bioassay

Significant differences in means for the LMI rates between treatments and at the different concentrations were assessed using a general linear model (GLM) procedure with Systat 9 software (SPSS Ltd, 1999).

Assay on adult worms

For each treatment (plant extract and dose), the number of immobile worms was recorded depending on time and concentrations on the three wells. The survival curve was analysed by applying the non parametric, stratified Cox regression test, using Systat 9 software (SPSS Ltd, 1999).

RESULTS

Condensed tannin analyses

The different percentages of condensed tannins for the plant extracts were expressed according to a quebracho standard. The percentages of condensed tannins from the negative controls were 0.4% for lucerne, and 1.1% for the rye-grass extracts. For the sainfoin, the percentage of condensed tannins was 3.2%. For the

three woody plants, the percentages of condensed tannins were respectively 7.4% for bramble, 5.3% for oak leave, and 14.2% for hazel tree leave extracts.

Larval migration inhibition bioassay (LMI)

Effects of plant extracts

For *T.circumcincta* (Fig.1A), the percentage of migration for the PBS control was 67%. With levamisole at 0.5%, only 2.65% of the larvae were able to migrate. Overall, the plant extracts reduced the migration of larvae. The effect was significant for oak extracts (P<0.01) and close to significance for hazel tree (P<0.06) and brambles (P<0.07). There was no effect of lucerne and sainfoin. A significant dose-effect for oak (P<0.02) and bramble (P<0.03) extracts was also observed.

Fig.1A

For *H.contortus* (Fig.1B), the migration rate for the PBS controls was 58%. With anthelmintic at 0.5%, only 2% of migration was observed. Extracts of oak, sainfoin and hazel tree showed a significant, inhibitory effect (respectively P<0.01 for oak and hazel tree, P<0.02 for sainfoin) on the migration of L3 when compared to the PBS controls. In contrast, no difference was found in the percentage of migration between lucerne and the PBS control values. There was no significant effect on L3 migration for brambles.The response was significantly dose-dependent for oak and hazel tree (P<0.02), and close to significance for sainfoin (P<0.07).

Fig.1B

Concerning the intestinal species *T.colubriformis* (Fig.1C), the PBS controls showed a rate of migration close to 70%. With levamisole at 0.5%, only 7% of the larvae migrated. Compared to the PBS control values, the migration was affected after incubation with several plant extracts. Sainfoin (P<0.05) and lucerne (P<0.05) induced a significant decrease in migration. For brambles, the results were close to significance (P<0.07), but not with oak or hazel tree extracts. A significant dose-response was evident for brambles (P<0.03), but not for sainfoin and lucerne.

Fig.1C

Effects of plant extracts at 1200 µg/ml with or without PEG

PEG had no significant effect on the migration of larvae for the three species (Table 1), when added to PBS, in absence of plant extracts (78% to 93% of migration with or without PEG for the PBS controls). In addition, PEG did not modulate the migration of

larvae when added to levamisole (2.5% to 4.66% of migration with or without PEG for the anthelmintic controls).

Table 1

The rye-grass extracts did not show any inhibitory effect on the migration of the three species of nematodes compared to the values of the PBS controls. The addition of PEG did not modify the migration rate significantly.

Whatever the nematode species, a significant decrease in larval migration was found with the three plant extracts at the concentration of 1200 µg/ml. In contrast, the addition of PEG restored the percentage of migration to values which did not differ significantly to those found with PBS plus PEG.

Effects on adult worms

For the sake of clarity, the figures shown to illustrate the effects of the different plant extracts on the three species of nematodes, correspond only to the observations obtained at 48h.

Effects of plant extracts

For *T.circumcincta*, at 6 and 24h, all the worms were mobile, except those corresponding to the anthelmintic controls (the percentage of immobility ranged from 76% to 87%). The results acquired after 48h are represented on Fig.2A. After the 72-hour observations, statistical analysis showed a significant effect only for oak extracts ($P<0.02$) and close to significance for sainfoin ($P<0.07$).

Fig.2A

For *H.contortus*, all the worms were mobile after 6h (data not shown), except those in contact with the different concentrations of anthelmintic. The results acquired with this species are shown on Fig.2B. Over the 48 hours of observations, the statistical analysis showed a significant effect for sainfoin ($P<0.001$). Concerning the woody plants, oak ($P<0.01$) and hazel tree ($P<0.001$) extracts also showed a significant effect on the mobility of the adult worms. The results acquired with bramble extracts were close to significance ($P<0.07$).

Fig.2B

For *T.colubriformis*, at 6h (data not shown), all the worms were mobile except those in contact with the different concentrations of anthelmintic. The results acquired with this intestinal species after 48h are presented on Fig.2C. Sainfoin and lucerne extracts have no effect on *T.colubriformis*. Over the 48-hour observations, the

Fig.2C

statistical analysis showed a significant effect for oak and hazel tree extracts ($P<0.001$).

Effects of plant extracts at 1200 µg/ml with or without PEG

PEG had no significant effect on the motility of the adult worms for the three species (Table 2), when added to PBS, in absence of plant extracts. In addition, PEG did not modulate the effect of levamisole on the worms.

The rye-grass extracts did not show any effect on the motility of the three species of nematodes compared to the values of the PBS controls. The addition of PEG to rye-grass extracts did not modify the motility of the adult worms.

Table 2

Sainfoin and the woody plant extracts had a significant effect on the motility of the three adult worms species (Table 3). After addition of PEG , the effects of sainfoin were non significant for the three species of nematodes. The effects of oak with PEG were non significant for the abomasal species and significant for *T.colubriformis*. For hazel tree extracts with PEG, the effects were non significant for *T.circumcincta* and significant for *T.colubriformis* and *H.contortus* (Table 3).

Table 3

DISCUSSION

Different *in vitro* studies have been previously performed to assess the effects of tanniferous plants on gastrointestinal parasites. Molan *et al.* (2000a) have tested seven different forages on *T.colubriformis* larvae. Effects from sulla extracts (*Hedysarum coronarium*) were also measured on three species of nematodes (Molan *et al.* 2000b). However, only extracts of legume forages were examined and no information is currently available on the possible effects of woody plant extracts, despite their palatability for some ruminants, particularly goats (Silanikove, 2000) and deers (Kumar & Vaithiyanathan, 1990) under rangeland conditions. In addition, the effects of extracts were examined on eggs or third stage larvae, but *in vitro* data are still missing on adult worms.

Two main objectives were therefore assigned to the current study. Firstly, we aimed at assessing the effects of three woody plant extracts, which are usually browsed by goats. For comparison, extracts of one forage, sainfoin, were also included in the study. Secondly, in *in vivo* controlled conditions, some differences on the effects of quebracho on two parasitic stages have been described in goats. On established

populations of adult worms, quebracho induced a significant reduction in egg output but without any change in worm numbers. In contrast, the main consequence of quebracho on incoming larvae of *T.colubriformis* and *T.circumcincta* was a significant reduction in the worm number (Paolini *et al*. 2003c). Since the results differed according to the parasitic stages and the nematode species, the effects of plant extracts were tested concomitantly on both L3 and adult worms. The larval migration inhibition bioassay is a method recognized to measure the inhibitory effects of anthelmintics on nematodes (Rabel, Mc Gregor & Douch, 1994). In contrast, no standardised *in vitro* method has been previously described to assess the anthelmintic properties of various substances on adult worms. In the current study, we therefore used a motility index to assess the effects of plant extracts on adult worms. The results on *T.circumcincta* and *T.colubriformis* appeared coherent. Those for *H.contortus* were more difficult to interpret because of lower index of motility in the controls. Despite these limits, the method allowed to assess statistical effects on adult worms of various species. These effects did not always correspond with those obtained by the LMI bioassay but *in vivo* studies suggested that tannins could act differently on incoming larvae and adult populations (Paolini *et al*. 2003c).

In vitro tests are useful in order to screen the effects of different plants on the L3 and on adult worms and also to investigate the possible direct effects of tannins on the worms. In some instances, a good relationship has been found in results acquired from both *in vitro* and *in vivo* studies. For example, Molan *et al*. (2000a, b) showed that purified condensed tannins from several herbages reduced the motility and migration ability of the third-stage larvae of nematodes and these results agreed with those observed *in vivo* in ruminants (Athanasiadou *et al.* 2000a, b; Paolini *et al*. 2003a,c). However, cautions have to be taken before transposing *in vitro* results to the *in vivo* conditions. Firstly, the chemical components of the plants could be modified after passage in the gastrointestinal tract. In particular, the ruminal conditions can affect tannins because of bacteria degradation (Makkar, 2003) and/or formation of complexes with proteins. Secondly, the question of the physiological significance of concentrations of tannins tested *in vitro* is also relevant. In sheep, the range of total condensed tannin concentrations in the abomasal digesta of animals fed diets containing tannins was estimated to 1100 to 2800µg/ml whilst the extractable condensed tannins ranged between 350 to 900 µg/ml (Terrill *et al*. 1994;

Molan *et al*. 2000a). The concentrations applied in the present study on both larvae and adult worms were thus within the range of the physiological concentrations measured in sheep digesta. Last, the toxicity of tannins for the ruminants can not be tested *in vitro*. It is well known that the hydrolysable tannins of acorn (Jean-Blain, 1998) and oak leaves (Garg *et al*., 1992) are dangerous for ruminants and specially for cattle. High concentrations and astringent condensed tannins reduce live-weight gain and productivity and can cause death (Waghorn & Mc Nabb, 2003). However, the tannin excess in the diet of the ruminants is mainly worrying in tropical conditions (Jean-Blain, 1998; Waghorn & Mc Nabb, 2003) and their toxicity is linked to their nature and their concentrations.

Two main conclusions can be drawn from our study. Firstly, the different plant extracts showed an effect on the third-stage larvae and adult worms. In particular, the present study is the first record on *in vitro* effects of woody plants on parasitic nematodes. However, some modulations appeared according to the nematode species and the plant sources. Overall, the two abomasal species were found more susceptible to the plant extracts than *T.colubriformis*. For example, the three woody plants were effective on *H.contortus* and *T.circumcincta*, but less on *T.colubriformis*. Secondly, the effects of plant extracts varied also according to the parasitic stages. For example, the effects of sainfoin, oak and hazel tree extracts on *T.colubriformis* differed according to the parasitic stages. On the two abomasal species, the results observed with oak and hazel tree extracts were similar on adult worms and L3, but the results differed between L3 and adult parasites with the bramble extracts for *H.contortus* and with sainfoin for *T.circumcincta*. These *in vitro* results tend thus to confirm previous *in vivo* data obtained with quebracho tannins on the two parasitic stages of *T.circumcinta* and *T.colubriformis* (Paolini *et al*. 2003c).

In vivo studies showing an effect of tanniferous plants on the gastrointestinal nematodes are now numerous in sheep, goats, and deers. The tannins have generally been proposed as the secondary plant compounds responsible for these effects. In some cases, this hypothesis was partly supported by the use of polyethylene glycol, which has the property to inactivate the tannins (Jones & Mangan, 1977). *In vitro* results showed that the addition of 2 µg PEG/ µg to condensed tannins of different legume forages eliminated 81-93% of the inhibitory

effects on *T.colubriformis* (Molan *et al*. 2000a). In the current study, after addition of PEG, the plant effects on the parasites were reduced, and usually did not differ statistically from the PBS values. These data were coherent for the three species, and the two parasitic stages. These results confirmed that tannins were the source of inhibition of motility of the third-stage larvae and adult worms. However, the restoration of activity, related to PEG, was only partial for adult parasites. One possible explanation for this partial effect is that other substances could be involved. For example, Guarrera (1999) showed that other non tannic secondary compounds, from garlic and a naphtoquinone contained in walnut were active components towards nematodes. Moreover, Molan *et al*. (2003a) observed that crude sesquiterpene lactones, extracted from chicory, had a profound effect on the larval motility.

Condensed tannins from different plant species differ in their molecular weight and in chemical composition (Foo *et al*. 1982, 1996, 1997; Singh *et al*. 1997; De Bruyne *et al*. 1999). It could be hypothesized that this variability in quality and chemical composition of tannins could explain the variation in effects on species and/or parasitic stages. Supporting this hypothesis, it has recently been shown that the inhibitory activities of condensed tannins might be influenced by the prodelphinidin:procyanidin ratios (Molan *et al*. 2003b).

The efficiency of woody plant extracts on nematodes raises the question of the role of plants in the regulation of host-parasite relationships in browsing animals but these *in vitro* results clearly need further *in vivo* confirmation. The variability of results obtained depending on the parasite species, stages and the plant species underlined the complexity of the tannin/parasite interactions. Because in naturally infected ruminants, multispecific infections are the rule, it is important to understand the causes of this variability and the mechanisms of action of tannins. Nevertheless, our results confirmed that the use of condensed tannins of several sources against gastrointestinal parasites could represent an attractive approach to infrapopulation regulation and could provide a valuable alternative to chemotherapy.

ACKNOWLEDGMENTS

The authors would like to thank the financial support from the European Union through the Contract WORMCOPS (number QLK5-CT 2001-01843) which is part of collaboration between Denmark, U.K., the Netherlands, Sweden, Spain and France.

REFERENCES

ATHANASIADOU, S., KYRIAZAKIS, I., JACKSON, F. & COOP, R.L. (2000a). Effects of short term exposure to condensed tannins on adult *T. colubriformis*. *Veterinary Record* **146** ,728-732.

ATHANASIADOU, S., KYRIAZAKIS, I., JACKSON, F. & COOP, R.L. (2000b). Consequences of long-term feeding with condensed tannins on sheep parasited with *T. colubriformis*. *International Journal of Parasitology* **30**, 1025-1033.

ATHANASIADOU, S., KYRIAZAKIS, I., JACKSON, F. & COOP, R.L. (2001). Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep *in vitro* and *in vivo* studies. *Veterinary Parasitology* **99**, 205-219.

DE BRUYNE, T., PIETERS, L., DEELSTRA, H. & VLIETINCK, A. (1999). Condensed vegetable tannins: Biodiversity in structure and biological activities. *Biochemical Systematics and Ecology* **27**, 445-459.

FOO, L. Y., JONES, W.T., PORTER, L. J. & WILLIAMS, V.M. (1982). Proanthocyanidin polymers of fodder legumes. *Phytochemistry* **21**, 933-935.

FOO, L. Y., LU, Y., MCNABB, W. C., WAGHORN, G. & ULYATT, M. J. (1997). Proanthocyanidins from *Lotus pedunculatus*. *Phytochemistry* **45**, 1689-1696.

FOO, L. Y., NEWMAN, R., WAGHORN, G., MCNABB, W. C. & ULYATT , M. J. (1996). Proanthocyanidins from *Lotus corniculatus*. *Phytochemistry* **41**, 617-624.

GARG, S.K., MAKKAR, H.P.S., NAGAL, K.B., SHARMA, S.K., WADHWA, D.R. & SINGH, B. (1992). Toxicological investigations into oak (*Quercus incana*) leaf poisoning in cattle. *Veterinary Human Toxicology* **34**, 161-164.

GORDON, I. J. (2003). Browsing and grazing ruminants: are they different beasts?. *Forest Ecology and Management* **181**, 13-21.

GUARRERA, P.M. (1999). Traditional anthelmintic, antiparasitic and repellent uses of plants in Central Italy. *Journal of Ethnopharmacology* **68**, 183-192.

JACKSON, F.S., BARRY, T.N., LASCANO., C. & PALMER, B. (1996). The extractable and bound condensed tannin content of leaves from tropical tree, shrub and forage legumes. *Journal of Sciences, Food and Agriculture* **71**, 103-110.

JACKSON, F. & COOP, R.L. (2000). The development of anthelmintic resistance in sheep nematodes. *Parasitology* **120** , 95-107.

JEAN-BLAIN, C. (1998). Aspects nutritionnels et toxicologiques des tanins. *Revue de Médecine vétérinaire* **149**, 911-920.

JONES, W.T. & MANGAN, J.L. (1977). Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* scop.) with fraction 1 leaf protein and with submaxillary

mucoprotein and their reversal by polyethylene glycol and pH. *Journal of Sciences, Food and Agriculture* **28**, 126-136.

KABASA, J.D., OPUDA-ASIBO, J. & TER MEULEN, U. (2000). The effect of oral administration of polyethylene glycol on faecal helminth egg counts in pregnant goats grazed on browse containing condensed tannins. *Tropical Animal Health and Production* **32**, 73-86.

KAHIYA, C., MUKARATIRWA, S. & THAMSBORG, S. M. (2003). Effects of *Acacia nilotica* and *Acacia karoo* diets on *Haemonchus contortus* infection in goats. *Veterinary Parasitology* **29**, 265-274.

KAHN, L.P. & DIAZ-HERNANDEZ, A. (2000). Tannins with anthelmintic properties. *Tannins in Livestock and Human nutrition: Proceedings of an International workshop, Adelaide, Australia. ACLAR proceedings* **92**, 130-138.

KUMAR, R. & VAITHIYANATHAN, S. (1990). Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. *Animal Feed Science and Technology* **30**, 21-38.

LANDAU, S., PEREVOLOTSKY, A., BONFIL, D., BARKAI, D. & SILANIKOVE, N. (2000). Utilization of low quality resources by small ruminants in Mediterranean agro-pastoral systems: the case of browse and aftermath cereal stubble. *Livestock Production Science* **64**, 39-49.

MAKKAR, H. P. S. (2003). Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* **49**, 241-256.

MIN, B. R., POMROY, W. E., HART, S. P. & SAHLU, T. (2003). The effect of short-term consumption of a forage containing condensed tannins on gastro-intestinal nematode parasite infections in grazing wether goats. *Small Ruminant Research* **In Press**.

MOLAN, A.L., ALEXANDER, R.A., BROOKES, I.M. & MCNABB, W.C. (2000a). Effect of an extract from Sulla (*Hedysarum coronarium*) containing condensed tannins on the migration of three sheep gastrointestinal nematodes *in vitro*. *Proceedings of New Zealand Society for Animal Production* **60**, 21-25.

MOLAN, A., DUNCAN, A., BARRY, T. & MCNABB, W. (2003a). Effects of condensed tannins and crude sesquiterpene lactones extracted from chicory on the motility of larvae of deer lungworm and gastrointestinal nematodes. *Parasitology International* **52**, 209-218.

MOLAN, A. L., MEAGHER, L. P., SPENCER, P. A. & SIVAKUMARAN, S. (2003b). Effect of flavan-3-ols on in vitro egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis*. *International Journal for Parasitology* **33,** 1691-1698.

MOLAN, A.L., WAGHORN, G.C., MIN, B.R. & MCNABB, W.C. (2000b). The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration *in vitro*. *Folia Parasitologica* **47**, 39-44.

MOLLE, G., DECANDIA, M., FOIS, N., LIGIOS, S., CABIDDU, A. & SITZIA, M. (2003). The performance of Mediterranean dairy sheep given access to sulla (*Hedysarum coronarium* L.) and annual ryegrass (*Lolium rigidum Gaudin*) pastures in different time proportions. *Small Ruminant Research* **49**, 319-328.

NIEZEN, J.H., CHARLESTON, W.A.G., ROBERTSON, H.A., SHELTON, D., WHAGHORN, G.C. & GREEN, R. (2002). The effect of feeding sulla (*Hedysarum coronarium*) or lucerne (*Medicago sativa*) on lamb parasite burdens and development of immunity to gastrointestinal nematodes. *Veterinary Parasitology* **105**, 229-245.

NIEZEN, J.H., ROBERTSON, H.A., WAGHORN, G.C. & CHARLESTON, W.A.G.(1998a) .Production, faecal egg counts and worm burdens of ewe lambs which grazed six contrasting forages. *Veterinary Parasitology* **80**, 15-27.

NIEZEN, J.H., WHAGHORN, G.C. & CHARLESTON, W.A.G.(1998b). Establishment and fecundity of *Ostertagia circumcincta* and *Trichostrongylus colubriformis* in lambs fed Lotus (*L.pedunculatus*) or perennial ryegrass (*Lolium perenne*). *Veterinary Parasitology* **78**, 13-21.

PAOLINI, V., BERGEAUD, JP., GRISEZ, C., PREVOT, F., DORCHIES, Ph. & HOSTE, H. (2003a). Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. *Veterinary Parasitology* **113**, 253-261.

PAOLINI, V., DORCHIES, Ph. & HOSTE, H. (2003b). Effects of sainfoin hay on gastrointestinal nematode infections in goats. *Veterinary Record* **152**, 600-601.

PAOLINI, V., FRAYSSINES, A., DE LA FARGE, F., DORCHIES, Ph. & HOSTE, H. (2003c). Efficacy of condensed tannins on established populations and on incoming larvae of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in goats. *Veterinary Research* **34**, 331-339.

RABEL, B., MC GREGOR, R. & DOUCH, P.G.C. (1994). Improved bioassay for estimation of inhibitory effects of ovine gastrointestinal mucus and anthelmintics on nematode larval migration. *International Journal for Parasitology* **24**, 671-676.

SILANIKOVE, N. (2000). The physiological basis of adaptation in goats to harsh environments. *Small Ruminant Research* **35**, 181-193.

SILANIKOVE, N., GILBOA, N., PEREVOLOTSKY, A. & NITSAN Z. (1996). Goats fed tannin-containing leaves do not exhibit toxic syndromes. *Small Ruminant Research* **21**, 195-201.

SINGH, S., MCCALLUM, J., GRUBER, M. Y., TOWERS, G. H. N., MUIR, A. D., BOHM, B.A., KOUPAI-ABYAZANI, M. R. & GLASS, A. D. M. (1997). Biosynthesis of flavan-3-ols by leaf extracts of *Onobrychis viciifolia. Phytochemistry* **44**, 425-432.

TERRILL, T.H., WAGHORN, G.C., WOOLLEY, D.J., MCNABB, W.C. & BARRY, T.N. (1994). Assay and digestion of 14 C-labelled condensed tannins in the gastrointestinal tract of sheep. *British Journal of Nutrition* **72**, 467-477.

WAGHORN, G.C. & McNABB, W.C. (2003). Consequences of plant phenolic compounds for productivity and health for ruminants. *Proceedings of the Nutrition Society* **62**, 383-392.

WAGLAND, B.M., JONES, W.O., HRIBAR, L., BENDIXSEN, T. & EMERY D.L. (1992). A new simplified assay for larval migration inhibition. *International Journal for Parasitology* **22**, 1183-1185.

Table 1 . Percentages of migration of infective larvae of *T.circumcincta*, *H.contortus* or *T.colubriformis* after incubation with extracts at 1200µg/ml (± standar error) of rye-grass, hazel tree, oak, or sainfoin with or without PEG. ** indicates a significant result.

	T.circumcinta	***H.contortus***	***T.colubriformis***
PBS	82(±5,5)	92,5(±4,9)	93(±0,7)
PEG	78(±1,5)	90,5(±2,1)	91,25(±3,6)
Rye-grass	86 (±2,5)	85,66(±1,5)	86,25(±7,9)
PEG	77,3 (±5)	85(±1)	85,3(±3,8)
Hazel tree**	49(±6,5)	44,25(±1,9)	45,5(±2,4)
PEG	72,3(±2,5)	68,5(±8,9)	84,25(±1,8)
Oak**	29,6(±4,5)	38,25(±10)	33,5(±4,7)
PEG	69(±3,6)	67,25(±12,9)	79,25(±3,6)
Sainfoin**	46(±7,9)	38,24(±5,6)	58(±1,8)
PEG	68(±7,2)	66,5(±14,5)	81,5(±7,2)
AH**	4(±2,6)	3(±2,5)	3,60(±1,2)
PEG**	4,66(±2,5)	2,5(±1,5)	4(±3,8)

Table 2. Percentage of mortality of adult worms of *T.circumcincta*, *H.contortus* or *T.colubriformis* after 6, 24 or 48 hours (and 72 hours for *T.circumcincta*) of incubation with extracts at 1200µg/ml of rye-grass, hazel tree, oak, or sainfoin with or without PEG.

	T.circumcinta				***H.contortus***			***T.colubriformis***		
	6 h	**24 h**	**48 h**	**72 h**	**6 h**	**24 h**	**48 h**	**6 h**	**24 h**	**48 h**
PBS	0	0	0	0	0	8,125	52,5	0	0	0
PEG	0	0	0	0	0	2,5	55,75	0	0	0
Rye-grass	0	0	12,5	12,5	0	0	71,4	0	0	0
PEG	0	0	0	0	0	8	100	0	0	0
Hazel tree	0	0	50	50	0	41,66	100	40	70	100
PEG	0	0	25	25	0	9	81	11	32	32
Oak	0	0	8	66	0	27	91	4	34,5	73
PEG	0	0	9	27	0	0	54,5	0	15	45
Sainfoin	0	0	6,25	25	0	33	88	0	0	35
PEG	0	0	0	7,7	0	0	46,15	0	0	9
AH	76	85	91	100	91,5	91,5	100	68	72,3	85,5
PEG	70	83	83	100	83	91,5	100	57,5	78,5	84

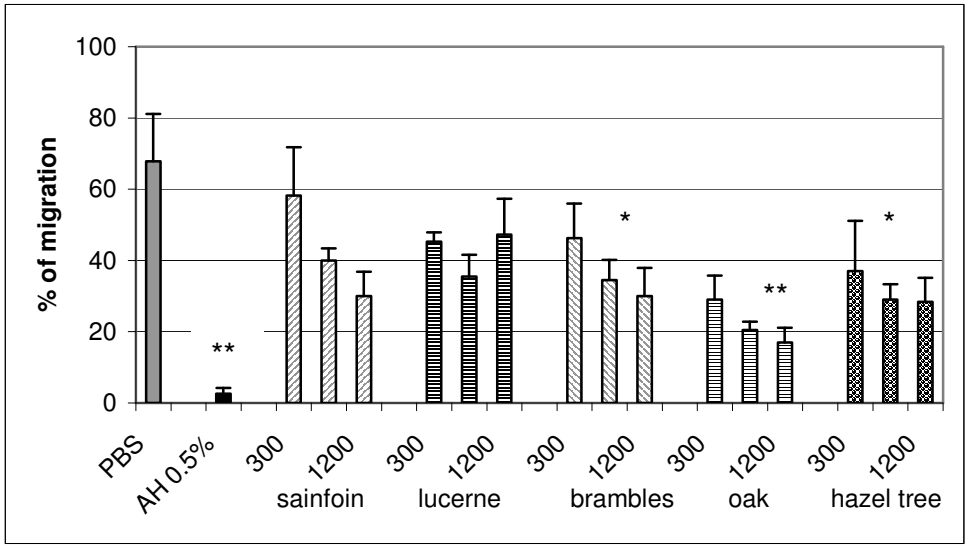
Table 3 . Statistical results on the motility of adult worms after incubation with extracts at 1200µg/ml of rye-grass, sainfoin, hazel tree or oak, with or without PEG, compared to values obtained with PBS or PBS+PEG. (NS: non significant result)

	T.circumcincta	*H.contortus*	*T.colubriformis*
Rye-grass	NS	NS	NS
PEG	NS	NS	NS
Sainfoin	P<0,07	P<0,03	P<0,01
PEG	NS	NS	NS
Hazel tree	P<0,01	P<0,01	P<0,01
PEG	P<0,07	P<0,04	P<0,04
Oak	P<0,01	P<0,03	P<0,01
PEG	NS	NS	P<0,01

Figure 1. Effects of sainfoin, lucerne, bramble, oak, hazel tree extracts at concentrations 300, 600 and 1200 µg/ml on the migration of infective larvae of *T.circumcincta* (figure 1A)*, or H.contortus* (figure 1B), or *T.colubriformis* (figure 1C). Results are shown as means (± standar error) of triplicates. * indicates a result close to significance. ** indicates a significant result.

Figure 2. Effects at 48h of sainfoin, lucerne, bramble (rubus), oak and hazel tree extracts at five concentrations (75, 150, 300, 600 and 1200 µg/ml) on the plot viability of the adult worms *T.circumcincta* (figure 2A)*,* or *H.contortus* (figure 2B), or *T.colubriformis* (figure 2C). The concentrations of the anthelmintic are 0.125%, 0.25%, 0.5% and 1%.

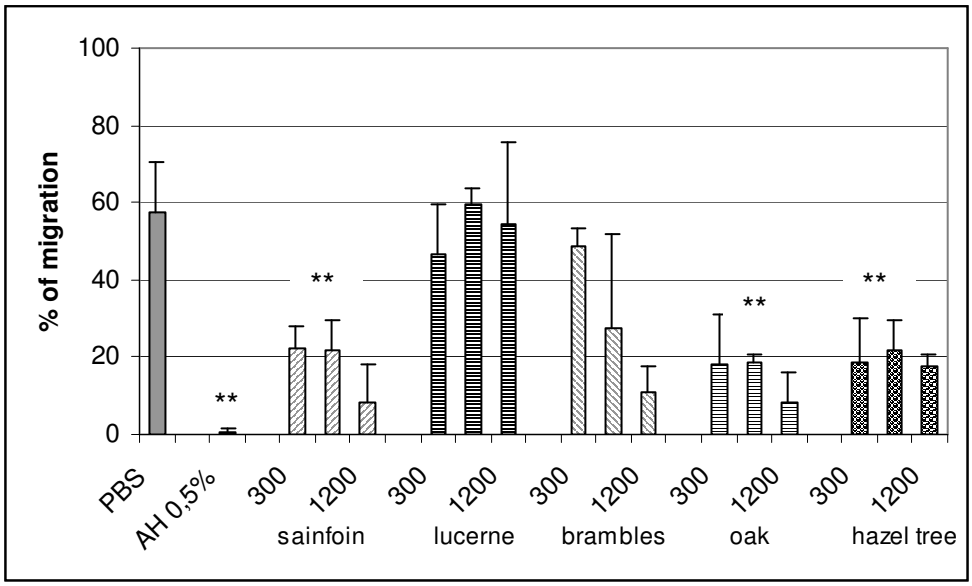
Figure 1A



V. PAOLINI , I. FOURASTE , H. HOSTE .

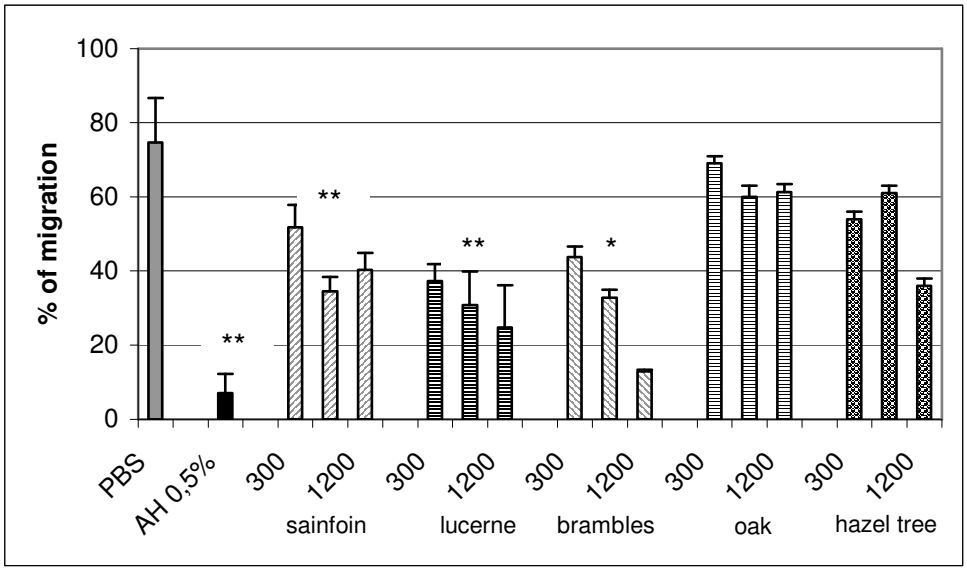
In vitro effects of tanniferous plants on nematodes

Figure 1B



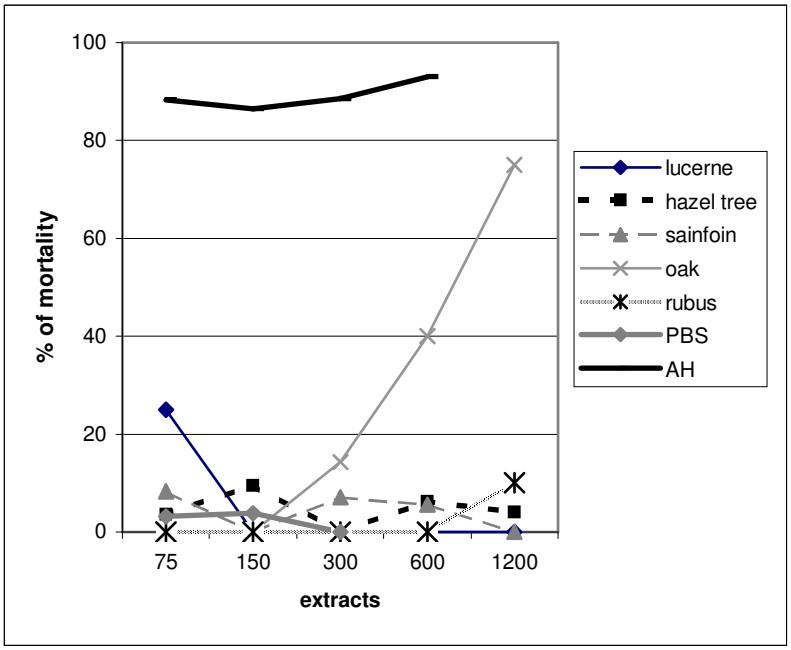
V. PAOLINI , I. FOURASTE , H. HOSTE .
In vitro effects of tanniferous plants on nematodes

Figure 1C



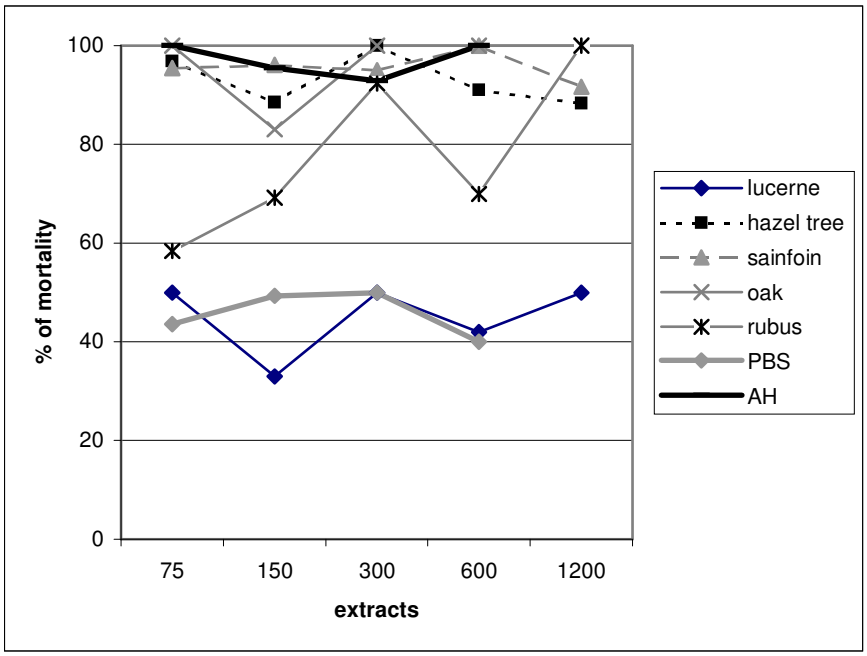
V. PAOLINI , I. FOURASTE , H. HOSTE .
In vitro effects of tanniferous plants on nematodes

Figure 2A



V. PAOLINI , I. FOURASTE , H. HOSTE .
In vitro effects of tanniferous plants on nematodes

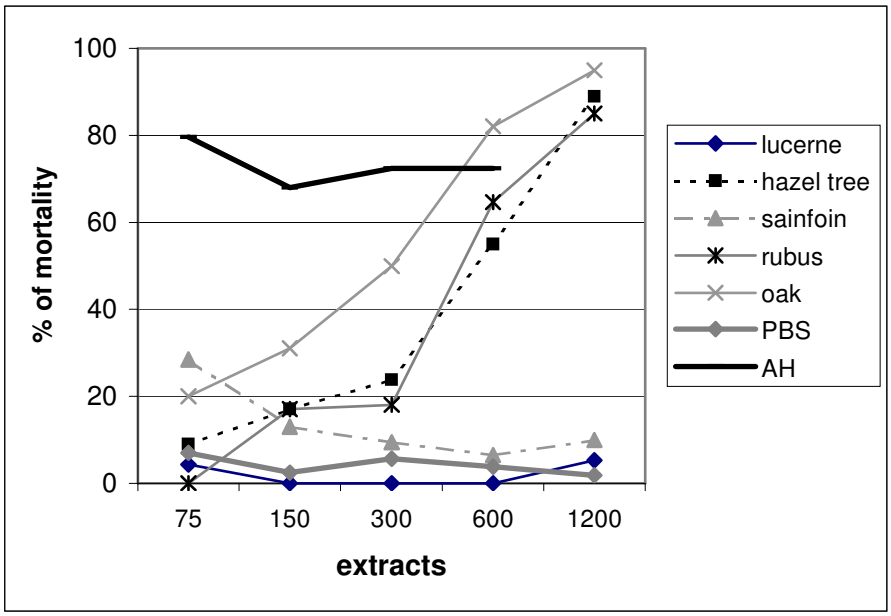
Figure 2B



V. PAOLINI , I. FOURASTE , H. HOSTE .

In vitro effects of tanniferous plants on nematodes

Figure 2C



V. PAOLINI , I. FOURASTE , H. HOSTE .
In vitro effects of tanniferous plants on nematodes