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Seasonal variation in the effects of Mediterranean plant extracts on the exsheathment kinetics of goat gastrointestinal nematode larvae

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ABSTRACT

The use of chemical drugs for the control of Gastrointestinal nematodes (GINs) causes rapid development of resistance to anthelmintics in worm populations. The possible use of bioactive ingredients from plants has been identified as a valuable solution to modulate the biology of parasitic nematodes and consequently to counteract the negative effects in the hosts. However, the concentration of these bio-actives in anthelmintic plants can be seasonal and we hypothesized that this may cause different anthelmintic bio-activity. Using a two-species but steady population of parasitic nematodes (ca. 20% *Teladorsagia circumcinta* and 80% *Trichostrongylus colubriformis*), we tested this hypothesis by using the larval exsheathment inhibition assay (LEIA). We examined effect on L3 larval exsheathment kinetics and the polyphenol content of ethanol 70% extracts of *Pistacia lentiscus*, *Phillyrea latifolia*, and *Inula viscosa* clipped on December (winter), May (spring), June (summer), and September (fall). Extract concentrations in assays were 600, 900, 1200, and 2400 ppm. Extracts obtained from *P. latifolia* showed similar inhibition of larval exsheathment throughout the year; in contrast, the inhibition by both extracts of *P. lentiscus* and *I. viscosa* was affected by season, ranking fall > summer > spring = winter and spring > summer = fall > winter, respectively. The total polyphenol content in foliage of the three plant species was highest in the fall for *P. lentiscus* and in spring for *I. viscosa*, but did not vary significantly for *P. latifolia*. Differences in larval exsheathment inhibition did not completely fit differences in phenolics contents. Seasonal variation in anthelmintic activity should be taken into account where plants are integrated into anthelmintic strategies.

1. Introduction

Gastrointestinal nematode (GIN) parasitism in grazing ruminants is a severe problem worldwide. Over the past five decades, the control of these parasites has been achieved mainly through intensive chemoprophylaxis, based on the repeated use of anthelmintic drugs. However, this quasi exclusive reliance on synthetic molecules is not sustainable and is nowadays facing several limits. The first one is the heightened concern of consumers that chemicals administered to farm animals leave residues in food products or have negative environmental consequences (McKellar, 1997). However, the main threat to the use of chemical drugs for the control of GINs comes from the worms themselves: rapid development of resistance to anthelmintics in worm populations after commercialisation of chemical drugs (Jackson and Coop, 2000; Waller, 2006a,b), worldwide diffusion of resistance to anthelmintics within worm populations, and the occurrence, in some regions, of multi-resistant strains (Kaplan, 2004): in the Southern US, resistance to all classes of anthelmintics was detected on 48% of farms (Howell et al., 2008). Taken together these reasons explain the strong impetus given to current research on alternative approaches that could complement or replace chemical drugs. Literature on the value of plants as anthelmintics in the diet of grazing livestock is accumulating (Athanasiadou and Kyriazakis, 2004; Hoste et al., 2006, 2010; Athanasiadou et al., 2001, 2007). Phytotherapy could be part of an integrated control system (Thamsborg et al., 1999).

The eggs of parasitic strongyles are excreted in the animal feces, hatch under suitable environmental conditions, producing two non-parasitical larval stages followed by a third-stage infective larvae (L3) that is ensheathed, i.e., retaining the shed cuticle

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from the previous molt for protection. L3 exsheathment is therefore a critical process in the life cycle, being a transitionary step from the free-living to the parasitic stages (Hertzberg et al., 2002). Studies of the kinetics of larval exsheathment have revealed that the process must occur within a narrow time frame and that it is sensitive to disturbing factors (DeRosa et al., 2005).

The larval exsheathment inhibition assay (LEIA; see, for example, Bahuaud et al., 2006) is a well-standardized procedure that have served to evaluate the effects on the kinetics of exsheathment of a wide array of plant extracts: chestnut nut tegument (*Castanea sativa*), pine tree (*Pinus sylvestris*) leaves (Bahuaud et al., 2006), sainfoin hay (Brunet et al., 2007), *Acacia pennatula*, *Lysiloma latisiliquum*, *Piscidia piscipula*, *Leucaena leucocephala* (Alonso-Díaz et al., 2008), *Pistacia lentiscus*, and *Phillyrea latifolia* foliage (Azaizeh et al., 2013).

Inula viscosa (L.) Aiton (*Compositae*) (common local name: Taioon) is a perennial plant of the Compositae family common in different regions of the Mediterranean Basin (Celik and Aslanturk, 2010; Said et al., 2002). In traditional medicine, *I. viscosa* has many uses (Said et al., 2002) and the plant kills soil nematodes (Oka et al., 2001). As phenolic components, such as chlorogenic acid, caffeic acid, rutin, myricetin, quercetin, luteolin and kaempferol were quantified by HPLC-DAD in the methanol extracts of the *Inula* species collected in Turkey (Gökbulut et al., 2013) are also found in strongly anthelmintic *P. lentiscus* and mildly anthelmintic *P. latifolia* (Azaizeh et al., 2013), we hypothesized that *I. viscosa* could also be a likely candidate for anthelmintic activity.

Plant secondary compounds – of which some exhibit anthelmintic properties – are synthesized by plants as a result of stress conditions: in Germany, the content of phenolic glycosides in willow bark decreased abruptly from March to July (Förster et al., 2008) and in Spain, the concentration in *Quercus ilex* foliage of flavonols was higher in winter than in summer (Brossa et al., 2009). Flavonoids accumulate in leaves of *P. latifolia* when exposed to excess sun radiation (Tattini et al., 2000), implying a possible seasonal effect on their anthelmintic value. As the survival of L3 larvae is dictated by temperature and humidity (O'Connor et al., 2006), if plants are intended to be used to combat GIN infections, their contents of anthelmintic must be high at the periods of potential infection. We are not aware of studies of seasonality in the anthelmintic value of plant extracts.

The main objectives of the current work are to verify if the anthelmintic value of plants is seasonal, using *P. lentiscus* (*Anacardiaceae*); *P. latifolia* (*Oleaceae*), and *Inula viscosa* (*Compositae*), plants growing naturally in the Eastern Mediterranean basin and the larval exsheathment inhibition assay to estimate anthelmintic value.

2. Materials and methods

2.1. Preparation of plant extracts for exsheathment tests

The plant extracts were prepared from *P. lentiscus* L., *P. latifolia* L., and *I. viscosa* leaves collected from the Ramat Hanadiv Nature Park (Carmel heights, 32°33′N, 34°56′E) and Shefa Amr (Lower Galilee, 32°79′N, 35°17′E), each time randomly from 15 to 20 bushes on different slopes and exposure, merged into one composite sample within each species. This was done for four periods including 12/2012 (winter), 5/2013 (spring), 6/2013 (early summer) and 9/2013 (fall). Samples were dried at 50 °C for 24 h. Based on our previous results (Azaizeh et al., 2013), the 70% ethanol was selected as the main extraction solution, because in addition to being an acceptable solvent to be used for extraction bioactive compounds it can be used in food and feed animal additives. Ten grams of dried leaves (randomly collected from at least 10 bushes) were ground and incubated with 100 ml of 7:3 ethanol:water (v:v) (70% ethanol for 24 h). The mixture was filtered and evaporated under vacuum (Rotorvapor Hie-VAP; Hiedolph, Germany) at 35 °C to remove the ethanol and water to dryness. Extraction yield was calculated as g extract per g dried matter (DM) of plant leaves. Total polyphenols in plant extracts were determined in three replicates according to the Folin–Ciocalteu method (Zhishen et al., 1999), as described previously (Azaizeh et al., 2013). The extract was kept at −20 °C until analysis.

2.2. Larval exsheathment inhibition assays (LEIA)

We compared the effects of pre-incubation with plant extracts at different concentrations of 600, 900, 1200 and 2400 ppm plant extracts with two-species L3 larvae produced by culturing the feces of a donor goat for 7 days at 26 °C. Larval suspensions were obtained and harvested by the Baermann procedure. The L3 larvae (mixture of the three species) were washed three times with a phosphate-buffered saline solution (PBS; 0.01 M phosphate, 0.05 M NaCl, pH 7.2) and kept at 4 °C until the assay. Larvae were used for assays within 1 month of refrigeration. We used one donor goat that was parasitized by two nematode species, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Species was identified as described by Van Wyk et al. (2004): their proportions in the suspension were steady throughout the year: 25, 75; 25, 75; 20, 80; and 18, 82%; in winter, spring, summer, and fall, respectively.

The LEIA procedure was according to Bahuaud et al. (2006) in triplicates and the percentage of exsheathed larvae were averaged for each time point. Approximately 200 L3 larvae were incubated with each plant extract at a concentration with PBS solution for 3 h at 20 °C. Each tube contained 30 μl of PBS, 150 μl of larvae solution, and 150 μl of diluted extract. Larvae were next washed and centrifuged three times in PBS before they were subjected to an artificial process of exsheathment which was induced by exposure to a solution of sodium hypochlorite (2% w/v) and sodium chloride (16.5% w/v) diluted 1:300 in PBS. The kinetics of exsheathment was determined by removing an aliquot containing somewhat less than one-fifth of the larvae at time intervals of 0, 15, 30, 45, and 60 min from exposure, and counting the number of exsheathed and non-exsheathed individuals under a microscope (×400). Counts were averaged within each time point.

2.3. Statistical analyses

Values of semi-log slopes for the percentages of exsheathed larvae at each of the five time intervals were calculated and subjected, as well as final exsheathment percentages, to analysis of variance using the General Linear Model procedure. Area under curve (AUC) was also estimated. For LEIA, the model included 10 treatments (three extracts of *P. lentiscus*, three extracts of *P. latifolia*, three extracts of *I. viscosa* and a PBS control). As the effects of concentration × plant, concentration × season, and concentration × plant × season interactions were significant, analysis was carried out with the true replicate assay nested within plant and season. Therefore the effects of plant and season were tested against the variance among assays within plant and season. Then, means on sampling days were separated by contrast *t*-tests using the Bonferroni correction for multiple testing.

3. Results

3.1. Polyphenol content of plant extracts

Extraction yields ranged between 11 and 23% depending on the plant species and seasonal collection time. Highest extraction yields were found in spring for all plants with *P. lentiscus* showing the highest value (Table 1). Total phenol content, expressed as tannic,