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Research paper

# Effect of *Gliricidia sepium* leaves intake on larval establishment of *Cooperia punctata* in calves and bio-guided fractionation of bioactive molecules

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ABSTRACT

The aims of this study were: 1) to assess the anthelmintic effect of *Gliricidia sepium* on the establishment of *C. punctata* third-stage larvae ($L_3$) in calves, and 2) to isolate and to elucidate an anti-exsheathment phytochemical from the plant offered during the trial. Twelve ¾ Holstein × Zebu calves were divided in two experimental groups: control (T1) and treatment (T2) (n = 6). After adaptation, each calf was infected with an oral dose of 400 *C. punctata* $L_3$/Kg LW. Basal diet consisted of *Digitaria decumbens* hay (6.27% CP) and commercial concentrate (12% CP). In addition, during the experimental period T2 received fresh *G. sepium* leaves (26.88% CP) *ad libitum*. On day 9 post-infection, three calves per treatment were randomly selected for slaughter, and worm counts were performed. Larval establishment rates obtained were 13.44 ± 0.13% and 3.1 ± 1.42% for T1 and T2, respectively ($P < .05$). The reduction of larval establishment was 76.9%. The total length of worms recovered from the animals was also affected by the intake of *G. sepium* ($P < .05$). Phytochemicals present in *G. sepium* leaves offered to calves were isolated through silica gel columns and elucidated through Magnetic Nuclear Resonance (1H and 13C). Bio-guided isolation procedures lead to the elucidation of Oxytroside (Kaempferol 3-*O*-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside-7-O-rhamnopyranoside), which fully inhibited the *C. punctata* exsheathment process (2400 μg $mL^{-1}$). *Gliricidia sepium* represents an alternative to prevent severe *C. punctata* infections by reducing larval establishment in cattle.

## 1. Introduction

The resistance of gastrointestinal nematodes (GIN) to anthelmintics represents a continuous threat to cattle production units worldwide. Despite of the social and economic importance of the cattle industry, there are few reports regarding the possible benefits of implementing the use of bioactive plants for GIN control. *Cooperia* spp. represents one of the GIN with highest prevalence and resistant features in cattle (Becerra-Nava et al., 2014; Bartley et al., 2012; Stromberg et al., 2012). *Cooperia punctata* has been found to induce important production losses associated to a significant decrease of: i) dry matter intake, ii) nutrient uptake and iii) weight gain (Stromberg et al., 2012; Li and Gasbarre, 2009), highlighting the urge of novel control strategies to be both developed and implemented.

Recent *in vitro* studies have demonstrated the anthelmintic effect of bioactive plants against the cattle nematodes *Cooperia oncophora* and *Ostertagia ostertagi* (Novobilsky et al., 2011). *Gliricidia sepium* is a tropical legume with a worldwide distribution, which is widely used in cattle production units as a natural defense and nutritional resource during dry seasons (Wood et al., 1998). Previous studies have reported an anthelmintic-like effect of *G. sepium* leaves extracts affecting the egg hatching, the exsheathment process and the motility of either *H. contortus* or *C. punctata* (von Son-de Fernex et al., 2016, 2012). The *in vitro* assessment of anti-exsheathment activity of *G. sepium* against *Cooperia* spp. has reported mean effective concentrations ($EC_{50}$) of 250.5 μg $mL^{-1}$ and 270.7 μg $mL^{-1}$ of aqueous and acetone:water extracts, respectively (von Son-de Fernex et al., 2014). Therefore, *G. sepium* has been considered as an adequate prototype for evaluating its potential in the reduction of *C. punctata* larval establishment in calves. The goals of this study were: 1) to assess the anthelmintic effect of *G.*

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*sepium* on the establishment of *C. punctata* third-stage larvae in calves, and 2) to isolate and elucidate an anti-exsheathment phytochemical from the plant offered during the trial.

## 2. Materials and methods

### 2.1. Plant material

Fresh daily harvested leaves of *G. sepium* were used in the experiment. The material was harvested a daily basis during the trial, from an experimental area located at the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical (Centre for Research, Teaching and Extension in Tropical Livestock) of the Facultad de Medicina Veterinaria y Zootecnia (Faculty of Veterinary Medicine and Animal Science) of the Universidad Nacional Autónoma de México (National Autonomus Univesity of México), in Martinez de la Torre (20°03´ N y 93°03´ O; 151 msnm), Veracruz, Mexico.

### 2.2. Infective larvae

*Cooperia punctata* third-stage larvae ($L_3$) were obtained after culturing feces from donor calves with a mono-specific infection (isolate *C. p*. CEIEGT-FMVZ-UNAM strain, Mexico). Calves were housed indoors on a concrete floor, provided with hay, commercial concentrate and free access to water (complying with the Internal Committee for Care and Use of Animals of the National Autonomous University of México [CICUA-UNAM] regulations, described below). Feces were collected daily and cultured for seven days, after which larvae were recovered with the Corticelli-Lai methodology (Corticelli and Lai, 1963); then, larvae were stored in distillate water at 4 °C until use (two months old).

### 2.3. Experimental design and diets

Twelve nematode free calves (10.08 ± 0.08 month-old) were divided in two experimental groups (control and treatment group; n = 6), wich were balanced according to body weighed. Control group weighted 166.2 ± 5.1 kg and treated group weighted 167.3 ± 7.0 (Mean ± S.E.). All animals were treated with levamisole (8.5 mg/kg live weight, 14 days prior infection; Helmisol® 12% ADE-B-SE, ADLER FARMA, México, Reg. SAGARPA Q-0970-005), and were also drenched with oral Benzimidazole (15 mg/kg PV live weight; Alben min®, Mederilab, México, Reg. SAGARPA Q-1190-059) before being placed on individual concrete floor pens to avoid further natural nematode infection (10 days prior infection). Animal feces were analyzed daily with the McMaster technique (Raynaud, 1970) for 10 days prior to the artificial infection, in order to corroborate the free gastrointestinal nematodes (GIN) status.

Animals were fed daily with 500 grams/head of commercial concentrate (12% CP; sorghum grain, wheat bran, oleaginous paste) and had free access to water and *Digitaria decumbens* hay (6.27% CP). The trial lasted for 17 days and was divided in three successive periods: I) 7-day adaptation period to diet (D-7 to D-1), II) a 6-day experimental period (D0 to D5), and III) 4-day post treatment period (D6 to D9) where animals remained on the basal diet prior to being slaughtered. Day-0 corresponded to the day when the twelve calves were experimentally oral infected with 400 $L_3$/kg BW of *C. punctata*. In addition, during the experimental period (D0-D5) T2 received every morning and for six hours (08:00 to 14:00 h), fresh leaves of *Gliricidia sepium* (26.88% CP; *ad libitum*), while T1 remained on the basal diet with the amount of concentrate in individual troughs adjusted to obtain isoproteic amounts between the experimental diets. Animals were fed on individual concrete troughs. Both, feeds on offer and refusals were measured daily in order to estimate the intake of fresh leaves of *G. sepium* and *D. decumbens* hay.

Six calves (three calves per treatment) were humanely slaughtered 9 days after infection (D9) complying with the Internal Committee for the Care and Use of Animals of the National Autonomous University of Mexico (CICUA-UNAM) regulations. The first 6 m of the small intestine were immediately recovered. The intestines were opened and washed in order to recover the worms present in the luminal contents. Contents were washed with approximately 20 L of warm water through 1000, 149 µm and 74 µm mesh sieves, and stored in a 10% formalin solution until analyses. The total number of larvae present in the luminal contents was estimated using the 10% aliquot technique (Martínez-Ortiz-de-Montellano et al., 2007). For each animal 20 non-damaged worms were recovered from the intestinal washings and transferred to individual petri dishes with distilled water. Measurements were performed manually using a micrometer slide through optic microscopy.

### 2.4. Plant analysis

During the experimental period (D0 to D5), samples of *G. sepium* fresh leaves and *D. decumbens* hay were collected daily and were individually oven-dried at 50 °C for 72 h. Pool samples of either *G. sepium* or *D. decumbens* were obtained respectively by mixing the same proportion of the six daily dried samples. Samples were kept in airtight containers until analyses. Dry matter (DM), ash (AS), crude protein (CP) and total fiber (TF) were determined, according to the A.O.A.C. (1980).

### 2.5. Parasitological techniques

#### 2.5.1. Larval exsheathment inhibition assay (LEIA)

Infective larvae were obtained from a donor calf with a mono-specific infection (see sec 2.2). The LEIA was performed as described by Alonso-Díaz et al. (2010) with slight modifications: 1) a single concentration of 2400 µg $mL^{-1}$ of each plant fraction was used for $L_3$ incubation and 2) no counts of exsheathment rates were performed over time, a single measurement was performed 60 min after $L_3$ were exposed to the artificial exsheathment fluid (75 µL domestic bleach with 112 µL of 6% sodium hypochlorite, Sigma Aldrich®, diluted in 15.813 ml of distilled water). The LEIA was used to determine exsheathment inhibitions of 17 fractions isolated through extraction of *G. sepium* (described below), because the *in vivo* AH effect of *G. sepium* consumption is directly associated with the impairment of the exsheathment process of infective larvae.

### 2.6. Bio-guided fractionation of the leave's aqueous extract

#### 2.6.1. Extraction procedure

Leaves of *G. sepium* (773.86 g) obtained from a pool sample of the plant offered to animals were air-dried at 60 °C for 72 h and were subsequently milled (Pulvex-Plastic®) to smaller particles (4–6 mm). The ground *G. sepium* (180 g) was placed in a glass beaker with distilled water (previously heated to 58 °C) and a magnetic stir-bar, and were continuously sonicated for 4 h in a water bath (Branson Sonicator 2510®; 40KHz). The extract was separated from the solid material using filter paper (Whatman® qualitative filter paper, Grade 1), and the solvent was evaporated from the extract at 58 °C using low pressure distillation in a rotovapor machine (Rotovapor® R-3). The extract was washed four times with 500 mL of n-hexane in order to remove chlorophyll and lipids, and a separation funnel was used for discarding the n-hexane fraction. Finally, the extract was frozen and lyophilized to obtain the dry ground extract.

#### 2.6.2. Chromatographic purification of the bioactive fraction

The bio-guided fractionation procedure was performed as reported by von Son-de Fernex et al. (2015) using the larval exsheathment inhibition assay. Prior to all AH evaluations performed during the experiment, fractions were concentrated by distillation under reduced pressure in a rotary evaporator (58 °C, R-3 Heidolph ®, Germany), and were lyophilized (Heto Drywinner DW3®, USA). The obtained aqueous extract was re-suspended with 0.8 L of n-hexane to eliminate waxes and