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EVALUACIÓN *in vitro* E *in vivo* DE PLANTAS BIOACTIVAS SOBRE EL CONTROL DE *Cooperia punctata* EN BOVINOS.

T E S I S

QUE PARA OPTAR POR EL GRADO DE:

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Für meine Eltern und Geschwister

… meine Familie, das größte Lebensglück !

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RESUMEN.

Las nematodosis gastrointestinales (NGI) representan uno de los principales problemas de salud dentro de las unidades de producción bovina en el mundo. El uso de plantas ricas en metabolitos secundarios se ha propuesto como una alternativa para el control químico de NGI en rumiantes. En la primera fase experimental de este estudio, se evaluó el potencial antihelmíntico *in vitro* de cinco plantas bioactivas (*Leucaena leucocephala*, *Gliricidia sepium*, *Cratylia argentea*, *Guazuma ulmifolia* y *Azadirachta indica*) sobre la inhibición del desenvaine larvario y sobre la inhibición de la eclosión de huevos de Cooperia *punctata*. Todas las plantas evaluadas interfirieron en el desenvaine larvario con un efecto dosis-dependiente ($P < 0.05$). Sin embargo, el efecto antihelmíntico sobre la eclosión de huevos fue menor. Los compuestos polifenólicos fueron el grupo de metabolitos secundarios involucrados en la actividad antihelmíntica *in vitro*. En una segunda fase experimental, se realizó el fraccionamiento biodirigido de *L. leucocephala* y *G. sepium* (plantas que mostraron mayor actividad ovicida) con la finalidad de aislar e identificar las moléculas químicas involucradas en el efecto AH sobre *C. punctata*, así como su posible mecanismo de acción. Para *G. sepium* se identificó una cumarina con potente actividad ovicida; y para *L. leucocephala* se observó la interacción de dos moléculas en el efecto antihelmíntico, la quercetina y el ac. caféico. Las moléculas aisladas de ambas plantas alteraron el grosor de la tri-capa de los huevos y generaron una pérdida en la continuidad de membranas. En la tercera fase experimental, se realizó la validación *in vivo* del potencial antihelmíntico *G. sepium* sobre el establecimiento larvario de *C. punctata* en becerros. El consumo de hojas frescas de *G. sepium* redujo 76.9 % el establecimiento parasitario de *C. punctata* ($P < 0.05$). Se concluye que las plantas bioactivas evaluadas podrían ser una alternativa dentro de un programa estratégico de control de NGI en bovinos.

Palabras clave: Cooperia punctata, plantas bioactivas, fraccionamiento biodirigido, establecimiento larvario.

ABSTRACT.

Gastrointestinal nematodes (GIN) represent one of the major constrains in cattle production units worldwide. The use of plants rich in secondary metabolites has been proposed as an alternative to chemoprofilaxis for GIN control in ruminants. The first stage of this investigation five bioactive plants (*Leucaena leucocephala*, *Gliricidia sepium*, *Cratylia argentea*, *Guazuma ulmifolia* y *Azadirachta indica*) were assesed *in vitro* for anti-exsheathment and ovicidal activity against *C. punctata*. All extracts showed a dose-dependent anti-exsheathment activity ($P < 0.05$). However, the ovicidal activity observed was much lower. Polyphenolic compounds were identified as prime effectors of the anthelmintic (AH)-like acctivity in both *in vitro* assays. For the second stage, *L. leucocephala* and *G. sepium* (plats which showed a higher ovicidal activity) were chosen for bioguided fractionation, sor the isolation and identification of the phytochemicals involved in the AH activity over *C. punctata*. For *G. sepium* a coumarin was identidied as a molecule with potent ovicidal activity; as for *L. leucocephala* an interaction between quecetin and caffeic acid was observed. The molecules isolated from both plants affected the thickness of the eggshell and caused discontinuity of the membranes. Finally the *in vivo* validation of *G. sepium* AH-like activity was demonstrated with a reduction of 76.9 % of *C. punctata* larvae establishment in calves ($P < 0.05$). The results obtained through this investigation confirms that the use of the bioactive plants used through this investigation represent a reliable alternative to consider when implementing an integrated GIN control management system.

Key words: *Cooperia punctata*, bioactive plants, bioguided fractionation, larval establishment

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EVENTO	LUGAR Y FECHA	TÍTULO	
1. Novel Approaches for Helminth Control.	Toulouse, Francia, Abril del 2013.	**Isolation, morphometric and scanning electron microscopy identification of Mecistocirrus digitatus in a cow from a Mexican herd in the tropics: first case report**	*POSTER*
2. V Congreso Internacional Biológico Agropecuario	Tuxpan, Veracruz, México, septiembre de 2013.	"**Reaparición de Mecistocirrus digitatus en un hato bovinio del trópico mexicano: identificación molecular y por microscopía electrónica (SEM)**"	*ORAL*
3. Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria (CENID-PAVET-INIFAP)	Jiutepec, Morelos, México, abril del 2014.	"**Fraccionamiento Bio-dirigido de *Gliricidia sepium*, con base en su efecto antihelmíntico contra *Cooperia* spp**".	ORAL
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7. 25th International Conference of the World Association for the Advancement of Veterinary Parasitology.	Liverpool, Reino Unido, Agosto 2015.	**Elucidation of *Leucaena leucocephala* anthelmintic-like phytochemicals, and the ultrastructural damage generated over *Cooperia* spp., eggs**	ORAL + POSTER
8. 25th International Conference of the World Association for the Advancement of Veterinary Parasitology.	Liverpool, Reino Unido, Agosto 2015.	***2H*-chromen-2-one an anthelmintic-like phytochemical isolated from *Gliricidia sepium* leaves**	ORAL
9. 25th International Conference of the World Association for the Advancement of Veterinary Parasitology.	Liverpool, Reino Unido, Agosto 2015.	**Elucidation of *Leucaena leucocephala* anthelmintic-like phytochemicals, and the ultrastructural damage generated over *Cooperia* spp., eggs**	ORAL

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ABREVIATURAS

NGI	Nematodos gastrointestinales
UPB	Unidad de producción bovina
LM	Lactonas macrocíclicas
L_1	Larva 1
L_2	Larva 2
L_3	Larva infectante
PI	Post-infección
Hpg	Huevos por gramo de heces
RA	Resistencia antihelmíntica
PM	Proteína metabolizable
EM	Energía metabolizable
CPF	Compuestos polifenólicos
EHA	Bioensayo de la inhibición de la eclosión de huevos
LEIA	Bioensayo de inhibición del desenvaine larvario artificial
MEB	Microscopía electrónica de barrido
MET	Microscopía electrónica de transmisión

I. INTRODUCCIÓN.

Los nematodos gastrointestinales (NGI) son el principal problema de salud endémico de las unidades de producción bovina en pastoreo (Fitzpatrick, 2013). Las infecciones por NGI en condiciones naturales se presentan de forma múltiple: *Haemonchus* spp., *Mecistocirrus digitatus*, *Ostertagia ostertagi*, *Cooperia* spp., *Trichostrogylus* spp., *Nematodirus* spp., y *Oesophagostommum* spp. (Quiroz, 1999); de los cuales *Cooperia* spp,. se ha posicionado como uno de los NGI de mayor prevalencia en ganado bovino en pastoreo (Fiel *et al*., 2012). Las infecciones por *Cooperia* spp., tienen un impacto económico negativo dentro de las unidades de producción bovina (UPB) debido a pérdida de la ganancia diaria de peso, disminución del consumo voluntario, disminución de los parámetros reproductivos por retraso en el crecimiento, disminución en la producción láctea, así como incremento de costos por tratamiento y por manejo (Li and Gasbarre, 2009; Li et al., 2009; Stromberg et al., 2012).

Desde el descubrimiento de los antihelmínticos hace más de 55 años, el control de las nematodosis se ha basado en la quimioprofilaxis (Benzimidazoles, Imidazotiazoles y Lactonas macrocíclicas); y durante las últimas dos décadas, las lactonas macrocíclicas (LM) ha sido la familia de antihelmínticos más empleada por ganaderos debido a las ventajas que ofrece para mejorar los parámetros productivos y por su amplio espectro (Jackson and Miller, 2006). Sin embargo, la sustentabilidad a largo plazo de dichos beneficios se ha puesto en duda, ya que su mala y excesiva utilización ha generado la emergencia de cepas de NGI resistentes y multi-resistentes a los antihelmínticos (Kaplan, 2004; Soutello et al., 2007; Suarez and Cristel, 2007; Suarez and Silvina, 2005). Además, el uso de antihelmínticos se ha cuestionado debido a problemas de ecotoxicidad y a la presencia de residuos químicos en los productos de origen animal (Waller *et al*., 2001). Otro aspecto, que ha sido poco señalado es que el uso excesivo de los antihelmínticos influye directamente sobre la epidemiología de los NGI en bovinos. Por ejemplo, *Cooperia* spp., es un nematodo que de forma innata posee mayor tolerancia a las LM en comparación con otros géneros, y esa característica puede influir en el incremento de su la prevalencia. Es posible, que debido a una fuerte presión con antihelmínticos, la presencia de algunos otros parásitos poco reportados sea más frecuente. Ante dicha problemática, múltiples investigaciones a nivel mundial se han enfocado en la búsqueda de alternativas de control.

El uso de plantas con compuestos secundarios como los taninos es una de las alternativas de control más estudiadas debido al efecto antihelmíntico directo e

indirecto que tienen sobre los NGI de pequeños rumiantes (Hoste *et al*., 2006), sin embargo, existen pocos estudios del efecto de plantas sobre NGI de bovinos. Estudios *in vitro* e *in vivo* han demostrado que la incubación de NGI de pequeños rumiantes en extractos de taninos y/o compuestos polifenólicos pueden reducir la eclosión, el desarrollo, la viabilidad, la motilidad, el establecimiento, la fertilidad y fecundidad de los mismos (Alonso-Diaz *et al*., 2008; Athanasiadou *et al*., 2001; Molan *et al*., 2002). Otros estudios indican que compuestos polifenólicos como los flavonoides también pueden jugar un papel importante en la actividad antihelmíntica (Ademola *et al*., 2005; Barrau *et al*., 2005; von Son-de Fernex *et al*., 2012). Se han reportado que existen más de 10,000 compuestos polifenólicos (CPF) presentes en plantas que pueden tener una amplia gama de actividad biológica (antiinflamatorio, antioxidante, antimicrobiano, antihelmíntico y con potencial anti-carcinogénico). No obstante, existen pocos trabajos de investigación que hayan logrado el aislamiento, la purificación y la identificación de las moléculas con actividad antihelmíntica, mediante el fraccionamiento biodirigido de los extractos (Barrau *et al*., 2005). La purificación e identificación de las moléculas químicas con efecto antihelmíntico sobre NGI de bovinos puede ayudar a explorar el mecanismo de acción, a estudiar y a comprender las posibles interacciones entre fitoquímicos, y finalmente ayudar con el desarrollo de nuevos productos con potencial antihelmíntico.

El presente trabajo de investigación se dividió en tres fases: i) en evaluar *in vitro* cinco leguminosas tropicales (*Leucaena leucocephala*, *Gliricidia sepium*, *Cratylia argentea*, *Guazuma ulmifolia* y *Azadirachta indica*) sobre la inhibición del desenvaine larvario y de la eclosión de huevos de *C. punctata*, ii) en realizar el fraccionamiento biodirigido de las leguminosas tropicales que mostraron mayor actividad antihelmíntica *in vitro* y iii) en evaluar el efecto *in vivo* del consumo de la leguminosa con mayor actividad antihelmíntica *in vitro* sobre el establecimiento parasitario de *C. punctata* en becerros F1 (Ho X Cebú).

II. REVISIÓN DE LITERATURA

2.1. Nematodos gastrointestinales y su impacto en la ganadería bovina

2.1.1. Producción bovina en México

La ganadería bovina en México representa una de las principales actividades del sector agropecuario del país, ya que aporta el 43% del valor de la producción pecuaria nacional (SHCP, 2014). Más del 50% del territorio se destina para el sector primario, donde se ubican 1.1 millones de unidades de producción bovina (SHCP, 2014). En México, la ganadería de doble propósito se desarrolla en más de 48 millones de hectáreas y posee 45% del inventario bovino nacional. De acuerdo con la FAO (2009), el consumo anual per cápita en México para el 2014 se estima en 16.9 kg de carne y 112.6 kg de leche (SIAP, 2014); sin embargo, la producción nacional cubre únicamente 31.4% y 67% del consumo total de carne y leche, respectivamente (García-Winder, 2011). En el 2012, la producción promedio de carne y de leche de bovino fue de 1,820.5 toneladas y 11.15 mil millones de litros. Este déficit en la producción, aunado a una mayor demanda debido al constante incremento demográfico, obliga a la estructuración de nuevas estrategias zootécnicas que permitan fortalecer la producción nacional, considerando tanto las necesidades de la industria como las de los consumidores.

En México, la ganadería bovina se desarrolla bajo un panorama extremo donde existen desde sistemas productivos altamente tecnificados (en menor proporción) hasta sistemas de producción extensivos y de doble propósito. En los sistemas donde el forraje se aprovecha como el principal recurso nutricional, las parasitosis son el problema sanitario más importante. Por lo tanto, si se establece un sistema de control parasitario eficiente, los estándares nacionales de producción podrían elevarse considerablemente.

2.1.2. Ciclo biológico de los nematodos gastrointestinales.

Los nematodos gastrointestinales son los helmintos de mayor importancia en el ganado bovino. Pertenecen al *phylum* Nematoda y existen más de 20,000 especies descritas. Los NGI de mayor importancia en rumiantes son: *Haemonchus* spp., *Mecistocirrus digitatus*, *Ostertagia ostertagi*, *Cooperia* spp., *Trichostrogylus* spp., *Nematodirus* spp., y *Oesophagostommum* spp. (Vázquez-Prats *et al*., 2004). Los NGI se caracterizan por presentar una morfología de tipo cilíndrica, cavidad pseudocelómica con simetría bilateral, sistema nervioso (4 troncos nerviosos longitudinales), aparato digestivo completo (boca, intestino, cloaca), sistema excretor (túbulos colectores que desembocan en un poro excretor) y aparato reproductor (dioicos) y son de tamaño variable según el género (Bird and Bird, 1991). Presentan un ciclo biológico directo dividido en una fase pre-parasítica (fases en vida libre) y otra parasítica (fases establecidas dentro del hospedero). Las fases de vida libre inicia con la eliminación de huevos del hospedero e incluye de la eclosión de huevos al desarrollo de larva 1 (L_1), larva 2 (L_2) y larva infectante (L_3). La duración de esta fase es variable y depende tanto de las condiciones climáticas como del género parasitario (Cuadro 1).

Cuadro 1. Duración de las fases del ciclo biológico de los nematodos gastrointestinales.

Fase		Localización	Tiempo de desarrollo	Referencia
Pre-parasitica	Huevo	Heces	Eliminado con las heces	
	Larva 1	Heces	24-30 horas post eliminación	Quiroz-Romero, 2011
	Larva 2	Heces	2-3 días	
Transición	Larva 3	Pastura	4-7 días	Quiroz-Romero, 2011
Parasítica	Larva 4	Tracto gastrointestinal	7 días post-infección	Cordero-de-campillo y Rojo-Vázquez, 1999
	Juvenil	Tracto gastrointestinal	8-15 días post infección	
	Adulto	Tracto gastrointestinal	15-21 días post-infección	

❖ El tiempo de duración entre cada fase dependerá del género parasitario.

La fase pre-parasítica inicia con la eliminación de los huevos junto con las heces, y este estadio se considera la fase biológica de los NGI con mayor resistencia no solo al estrés medioambiental, sino también a los productos antihelmínticos (Bird, 1971). Los huevos están constituidos por una triple capa externa (vitelina, quitinosa y lipídica) que le confiere la característica de resistencia (Bird and Bird, 1991). El desarrollo embrionario se desencadena tres horas post-eliminación de los huevos y se encuentra mediada por la acción de la hormona juvenil (Rogers, 1980). El proceso de eclosión se lleva a cabo durante las primeras 24 a 30 horas post-eliminación. En los trichostrongylidos la eclosión se divide en dos fases, ambas mediadas por el embrión. La primera, consiste en la producción del fluido de eclosión, el cual se encuentra constituido por diversas enzimas donde destacan la leucina aminopeptidasa (LAP) y lipasa (LIP) que son las encargadas de alterar la permeabilidad de las membranas, y de ocasionar la ruptura en el huevo. Y la segunda, consiste en la fractura mecánica, debido a los movimientos de la larva L_1 dentro del huevo, los cuales provocan la emulsión de la capa lipídica y consecuentemente la fractura del huevo (Rogers and Brooks, 1976). Otras enzimas que se han asociado al desarrollo y a la eclosión de huevos son la anhidrasa carbónica y la hormona juvenil (Rogers, 1980), y que al igual que las enzimas LIP y/o LAP, una falta o disminución de su producción bajo condiciones naturales produce una inhibición en la eclosión (Rogers y Brooks., 1976; Rogers, 1980) generando la muerte larvaria y con ello la interrupción del ciclo biológico de los NGI (von Son-de Fernex *et al*., 2015).

La larva infectante (L_3) representa la etapa de transición entre la fase de vida libre y la fase parasitaria. Debido a que ésta larva se localiza en los pastos para ser ingerida por el hospedero definitivo, la L_3 posee características específicas de resistencia medioambiental y de comportamiento que incrementan la probabilidad de la continuidad de su ciclo biológico. Estas características consisten en: i) la retención cuticular de su fase anterior (vaina) que sirve de protección del estrés medioambiental (temperatura y humedad), ii) el fototropismo positivo a la luz tenue, iii) el hidrotropismo positivo, y iv) la capacidad de ejercer una migración

vertical por los pastos para situarse en la sección media-alta de la hoja, para ser ingerida por los rumiantes (Niezen *et al*., 1998).

El establecimiento parasitario inicia con el desenvaine larvario, y ocurre pocas horas post-ingestión en el órgano precedente al sitio de establecimiento del parásito adulto (Lesage y Mallet., 1987). Si no ocurre el desenvaine, la L_3 no logra infectar al hospedero y muere (Hoste *et al*., 2012). El desenvaine larvario se desencadena al exponerse al microambiente ruminal, lo cual estimula la secreción del fluido de desenvaine a través del espacio intracuticular (Ozerol and Silverman, 1972). Este fluido es un líquido conformado por proteasas de diversos pesos moleculares que al entrar en contacto directo con la cutícula inducen la formación de un anillo refráctil aproximadamente 20 µm por debajo de la región cefálica de la vaina de la L_3, que tras ser digerido por las proteasas (ej. leucina aminopeptidasa), permitirá a la larva liberarse de la vaina y e iniciar su proceso de establecimiento (Hoste *et al*., 2012; Lesage y Mallet, 1987). Una vez desenvainada, la L_3 migra por el tracto gastrointestinal (TGI) donde dependiendo del género parasitario, penetrará o iniciará su establecimiento en el lumen de diversas zonas de la mucosa digestiva dando continuidad a su ciclo (Cuadro 2). Aproximadamente, el séptimo día post-infección (PI) se desarrolla en una L_4, que continúa su desarrollo hasta alcanzar la fase juvenil o pre-adulto (Cordero-de-campillo y Rojo-Vazquez, 1999). Tras un periodo prepatente de 15 a 21 días, dependiendo del género, el pre-adulto alcanza su madurez sexual culminando así su establecimiento, para posteriormente llevar a cabo la cópula e iniciar con la producción de huevos entre los días 16 y 23 PI (Cordero-de-campillo y Rojo-Vazquez, 1999).

Cuadro 2. Procesos del establecimiento parasitario de los nematodos gastrointestinales de mayor prevalencia en bovinos y su sitio de ocurrencia dentro del hospedero.

Género	Desenvaine L_3	Penetración de L_3	Establecimiento
O. ostertagi	Tránsito rumeno-omasal	Glándulas gástricas de la región antropilórica	Abomaso
H. placei		Mucosa fúndica del abomaso	
M. digitatus			
T. colubriformis	Tránsito omaso-abomasal	Primer tercio del intestino delgado, entre el epitelio y la membrana basal de la mucosa	Intestino delgado
C. oncophora		No penetra	
C. punctata		Mucosa del intestino delgado	
N. battus		No penetra	
O. radiatum	Intestino delgado	Intestino grueso	Intestino grueso

2.1.3. *Cooperia punctata*

Las especies de *Cooperia* que afectan al ganado son *C. oncophora* (Raillet, 1898), *C. punctata* (von Linstow, 1907), *C. pectinata* (Ransom, 1907), *C. surnabada* (Anitpin, 1931) y *C. spatulata* (Baylis, 1938). Los adultos de estas especies se localizan en los primeros seis metros del intestino delgado y su patogenicidad varía según la especie, siendo *C. punctata* la más patógena (Stromberg *et al*., 2012). El ciclo biológico de *C. punctata* es similar al de otros géneros de trichostrongylidos; sin embargo, diversos autores difieren sobre el sitio de establecimiento del adulto, ya que mencionan que tiene la capacidad de establecerse tanto en abomaso como en intestino delgado (Cholleta *et al*., 2000; Stromberg *et al*., 2012). Una vez que la L_3 ha sido ingerida por su hospedero, ésta desenvaina dentro de las primeras 13 horas post-ingestión. La L_3 de *Cooperia punctata,* a diferencia de las otras especies, tiene la capacidad de penetrar la mucosa del intestino delgado para emerger cuatro días post-infección como L_4, y convertirse en adulto en un periodo

aproximado de 9 a 11 días (Stromberg *et al*., 2012). El periodo prepatente de la especie es de 13 días y la patencia tiene una duración de 9 a 15 meses (Rickard, 1990).

El potencial biótico del género *Cooperia* es bajo comparado con otros nematodos como *H. placei* o *M. digitatus*. Lo anterior implica que animales infectados con *Cooperia*, eliminen pocos huevos en heces y son diagnosticados libres de NGI (animales falsos negativos). Las infecciones por *Cooperia punctata* representan una amenaza para las unidades de producción debido a su patogenicidad. Estudios recientes reportaron el impacto negativo sobre el desempeño y la salud animal en ganado infectado mono-específicamente con larvas de *C. punctata*. Se observaron alteraciones en el metabolismo de la albumina y en el balance/retención de nitrógeno, disminuyó el consumo voluntario hasta 680 gr/animal/día, y retraso en el crecimiento de hasta un 13.5% comparado con animales libres de infección (Coop et al., 1979; Li and Gasbarre, 2009; Stromberg et al., 2012). También existen reportes de muerte de becerros de 5 meses de edad producida directamente por una primo infección con 130,000 L_3 de *C. punctata* (Yatsuda and Vieira-Bressan, 2000).

2.1.4. Impacto de las nematodosis en la producción bovina.

Las nematodosis gastrointestinales se consideran el principal problema endémico de salud animal dentro de las unidades de producción bovina bajo sistemas de alimentación extensivos y semi-extensivos (Fitzpatrick, 2013; Vázquez-Prats *et al*., 2004). Las infecciones por NGI bajo condiciones naturales suelen ser de origen multi-etiológico (más de 20 especies) (Charlier *et al*., 2015). Los NGI con mayor grado de patogenicidad en bovinos son los Trichostrongylidos abomasales y del intestino delgado (Encalada-Mena *et al*., 2009), entre los que destacan *Haemonchus spp.*, *Mecistocirrus* sp., *Cooperia* spp., *Trichostrongylus* spp., y *Ostertagia* sp., (Quiroz *et al*., 2009). Las infecciones por NGI en bovinos suelen ser subestimadas y normalmente pasan desapercibidas debido a su naturaleza subclínica, ya que la

semiología y lesiones ocasionadas en el hospedero definitivo, varían con los géneros de NGI involucrados en la infección y la inmunocompetencia del mismo (Encalada-Mena *et al*., 2009). Es importante recalcar que todo proceso infeccioso se refleja con una afección directa sobre la salud general del hato. En bovinos, la edad de mayor susceptibilidad a las nematodosis gastrointestinales es entre los 4 a 12 meses (Encalada-Mena *et al*., 2009), pero se ha reportado que en el ganado adulto las infecciones por NGI afectan su desempeño productivo (Charlier *et al*., 2014).

En un estudio que se realizó en el trópico húmedo de México, se encontró una prevalencia del 98.8 % de *M. digitatus* en el abomaso de 68 animales adultos con un peso promedio de 384.7 ± 14.83 kg, y las lesiones observadas (Figura 1) (von Son de Fernex *et al*., 2014) son similares a las ocasionadas por las haemonchosis; las cuales se asocian a anemia, anorexia, y a alteraciones en el metabolismo de la proteína y de la energía (Garcia-Coiradas *et al*., 2010). En la misma región, también se encontró que animales adultos infectados con paratuberculosis, presentaron altas cargas parasitarias de *M. digitatus*. Estos hallazgos en animales adultos, indican que tanto los animales jóvenes como los adultos son susceptibles a infecciones de NGI, y se ha demostrado que causan fuertes pérdidas económicas dentro de las unidades de producción(Charlier *et al*., 2014).

Las acciones patógenas de mayor relevancia tanto médica como productiva en animales jóvenes y adultos (mecánica, traumática e irritativa), ocurren por el proceso de penetración en la mucosa gástrica o entérica y por la permanencia de los parásitos adultos sobre la mucosa abomasal o entre las vellosidades intestinales (Quiroz, 1999). Durante el proceso de establecimiento parasitario, se liberan sustancias de secreción y excreción que estimulan una reacción antigénica en el hospedero, debido a las reacciones inmunológicas humorales y celulares que se desencadenan tanto a nivel sistémico como local, y se traduce en un costo metabólico por pérdida de proteínas plasmáticas (Romero y Boero, 2001). Las manifestaciones clínicas que caracterizan a las parasitosis gastrointestinales son emaciación progresiva, anemia, diarrea, disminución del consumo voluntario y de

la tasa de conversión alimenticia (Li and Gasbarre, 2009). A mediano plazo, los animales infectados con NGI merman su producción láctea, cárnica y disminuye la tasa de fertilidad por retraso a la pubertad (Perri *et al*., 2011).

El costo directo que ocasiona el control de las parasitosis también se ha calculado. Nielsen (1997) reportó un gasto anual aproximado a los 14 millones de dólares por concepto de compra de antihelmínticos. En Australia, se estimaron pérdidas productivas de 235 millones de dólares y costos para su control de aproximadamente 122 millones de dólares australianos. En otros trabajos, la medición del impacto económico de las parasitosis en bovinos se ha estimado midiendo la asociación entre el nivel de infección y los parámetros productivos. Estudios con ganado lechero en Bélgica, reportan que el costo anual de una vaca infectada con NGI varía entre los 4 y 113 euros (Charlier *et al*., 2015). En México, se estimó un impacto económico anual de las nematodosis gastrointestinales en bovinos de 445.10 millones de dólares americanos, considerando únicamente las perdidas en la producción de leche y en la ganancia de peso de animales no tratados (Rodriguez-Vivas *et al*., 2015). Se han reportado reducciones en la producción láctea de 1.2 kg de leche por vaca/día (Barger y Gibbs, 1981; Charlier *et al*., 2005). Perri *et al*. (2011) reportan que la disminución en la producción láctea causada por infecciones de NGI, está mediada por la acción e inhibición de diversas hormonas metabólicas y galactopoyéticas como: la hormona del crecimiento, factor de crecimiento insulínico tipo 1 (IGF-I) y prolactina.

En animales en desarrollo y crecimiento, las parasitosis gastrointestinales están directamente relacionadas a las pérdidas de peso en los animales. Se ha demostrado que la pérdida de peso vivo en la primer etapa del pastoreo de los becerros es de tipo permanente (Charlier *et al*., 2014) y repercute en pérdidas productivas por retraso a la pubertad, retraso a la primer lactancia y la disminución en el desarrollo de la región pélvica en las hembras (Perri *et al*., 2011). Otros estudios realizados con infecciones artificiales de trichostrongylidos reportan una disminución en el consumo voluntario que varía del 15 al 50%, como consecuencia de la acción parasitaria e inmunológica dentro del hospedero (Basabe *et al*., 2009;

Fox, 1997). Una deficiente conversión alimenticia y digestibilidad de nutrientes, menor retención de nitrógeno, deficiencia en el desempeño metabólico y mala utilización tanto de proteínas, como de minerales y energía (Rossangio *et al*., 1992; Mutturi *et al*., 2005).

Todos los efectos negativos mencionados anteriormente han sido registrados bajo cifras de eliminación menores a los 300 huevos por gramo de heces (Rossangio *et al.*, 1988; Coop y Kyriazakis, 1999; Knox *et al.*, 2006; Basabe *et al*., 2009). Es importante considerar que en los animales parasitados resulta imperativo establecer un estado de homeostasis que les permita sobrevivir, y esto representa un elevado costo metabólico (tanto proteico como energético). Para lograrlo, los animales deben: i) incrementar la síntesis de proteínas plasmáticas, ii) producir moco para favorecer la expulsión parasitaria, iii) realizar la reparación del tracto gastrointestinal (TGI), y iv) alcanzar un estado de inmunocompetencia. Cuando el costo metabólico coincide con un bajo aporte nutricional y/o disminución del consumo de alimento, se suspenden las funciones fisiológicas secundarias como el crecimiento desarrollo óseo y muscular, deposición de grasa, reproducción y producción láctea (Coop y Kyriazakis, 1999; Mutturi *et al*., 2005). De esta forma, todas las modificaciones de los parámetros nutricionales y fisiológicos, en rumiantes con infecciones por NGI, ponen en continuo riesgo la rentabilidad de las unidades de producción en todo el mundo (Corwin, 1997).

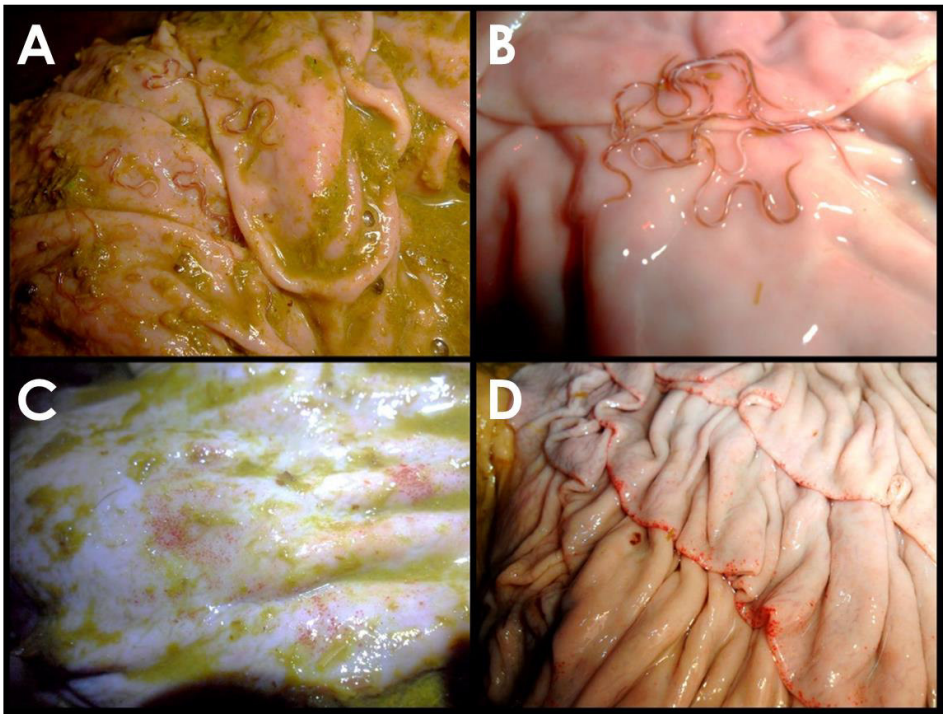

Figura 1. Lesiones del abomaso ocasionadas por *M. digitatus* en animales de rastro. A-B) Nematodos del género *M. digitatus* en mucosa abomasal de bovino, C-D) Lesiones ocasionadas en mucosa abomasal de bovino, asociado a la presencia de *M. digitatus*.

2.2. Estrategias de control de nematodos gastrointestinales

En la actualidad, un programa de control de NGI se debe diseñar de forma individual para cada UPB, considerando prolongar la vida útil y la efectividad de los antihelmínticos químicos y mejorar el desempeño animal (Kaplan, 2004). De acuerdo con Jackson y Miller (2006), un programa de control de NGI debe combinar diferentes estrategias de control de NGI, y simultáneamente potencializar la producción animal (Jackson and Miller, 2006). Para desarrollar dicho programa, es necesario partir de información epidemiológica considerando las variantes siguientes: i) las características del hospedero (estado nutricional, edad e inmunocompetencia), ii) las condiciones del NGI (género, especie y estatus de

resistencia antihelmíntica), iii) las condiciones medioambientales, iv) el sistema de producción (intensiva, extensiva, semi-extensiva y familiar o de traspatio), y v) el manejo zootécnico y médico que se realiza en la UPB. Después de esto, y con base en los resultados del análisis epidemiológico, se deben elegir las mejores opciones entre las alternativas de control existentes: químicas, inmunológicas, de manejo, y biológicos (Jackson y Miller, 2006).

2.2.1. Control químico.

El manejo de las NGI se ha basado en el uso de antihelmínticos de las familias de los benzimidazoles, imidazotiazoles y lactonas macrocíclicas (ver Cuadro 3). Desde su aparición en los 90´s, la ivermectina ha sido el antihelmíntico de elección para el control de las parasitosis debido a su fácil aplicación, a su acción endectocida, y a su capacidad de maximizar la productividad y la eficiencia alimenticia del ganado (Jackson y Miller, 2006). En general, la acción de las diferentes moléculas de antihelmínticos puede resumirse en dos mecanismos:

i) Alteraciones neuromusculares, como los imidazotiazoles (ej. levamisol) y lactonas macrocíclicas (ej. ivermectina) que producen una alteración directa sobre el sistema neuromuscular de los NGI provocando una parálisis irreversible y muerte.
ii) Alteraciones del metabolismo energético, como los BZDs (ej. albendazol) que inhiben la polimerización de los micro túbulos celulares que son los organelos encargados de la secreción de diversas enzimas digestivas de los nematodos, y que al ser inhibidas provocan la muerte parasitaria por inanición.

En el 2009, se liberó al mercado una nueva clase de AH químico derivados amino-acetonitrílicos (por sus siglas en inglés AAD), que posee un nuevo mecanismo de acción para evitar la aparición de resistencia cruzada (Ducray *et al*., 2008). Los AAD actúan a nivel de los receptores nicotínicos de acetilcolina (Hco-MPTL-1), que están

presentes únicamente en los nematodos, y son responsables de la transmisión de las señales nerviosas. Su capacidad para bloquear dichos receptores produce una parálisis en los NGI y con ellos su muerte y/o expulsión del hospedero. La primer molécula comercial perteneciente a dicha clase de antihelmínticos es el monepantel que mostró una eficacia del 100% contra cepas de NGI multirresistentes a los diferentes antihelmínticos (Kaminsky *et al*., 2008; Steffan *et al*., 2011). No obstante, y tras cuatro años de su lanzamiento al mercado ya se han publicado los primeros reportes de resistencia antihelmíntica (Leathwick, 2013). En principio, y con los beneficios obtenidos en la salud y en la producción animal, se consideró que los antihelmínticos químicos serían la solución a largo plazo para la problemática de parásitos gastrointestinales en el ganado; sin embargo, debido a la rápida aparición de la resistencia antihelmíntica se ha demostrado que depender de un método de control, no es sustentable (Nari *et al.*, 1996)

Cuadro 3. Principales antihelmínticos y su mecanismo de acción (Jabbar *et al*., 2006).

CLASE	AH	MECANISMO DE ACCIÓN
BENZIMIDAZOLES	Tiabendazole Albendazole Mebendazole Oxibendazole Oxfendazole	Se une a la β-tubulina, inhibiendo la polimerización de los microtúbulos
IMIDAZOTIAZOLES	Levamisol Tetramisol	Agonistas colinérgicos
LACTONAS MACROCÍCLICAS	Ivermectina Abamectina Doramectina Selamectina Milbemicina (Moxidectina)	Mantienen abiertos los canales de cloro
DERIVADOS AMINO-ACETONITRÍLICOS	Monepantel	Inhibe los receptores nicotínicos de acetilcolina (Hco-MPTL-1)

2.2.2. Resistencia antihelmíntica.

La resistencia antihelmíntica se define como la capacidad heredable de una población de NGI para tolerar dosis tóxicas de principios activos que normalmente son letales para una población susceptible (Torres-Vásquez *et al*., 2007). El desarrollo de la resistencia antihelmíntica se ha relacionado con la elevada tasa de evolución que presentan los NGI a nivel de secuencias de nucleótidos y a su elevado potencial biótico, característica que les confiere una amplia diversidad genética (Kaplan, 2004). El problema de resistencia antihelmíntica se ha documentado ampliamente en NGI de pequeños rumiantes (Kaplan, 2004); pero los reportes de la presencia de cepas multirresistentes de NGI en bovinos va en aumento (Becerra-Nava et al., 2014; Sutherland and Leathwick, 2011). Debido a la constante amenaza que representa la resistencia antihelmíntica en las UPB, es imperativo modificar el manejo de los antihelmínticos dentro de un modelo integral de alternativas de control que permitan optimizar la salud y la producción animal. Leathwick *et al*. (2015), mencionaron que con un manejo adecuado en las unidades de producción ovina, es posible revertir la resistencia antihelmíntica mediante el manejo siguiente:

1. EL uso adecuado de antihelmínticos y la evaluación anual de su efectividad en campo
2. El tratamiento selectivo de animales clínicamente enfermos
3. No administrar productos antihelmínticos en intervalos menores a 28 días
4. Minimizar o eliminar el uso de antihelmínticos con actividad persistente
5. Realizar una desparasitación única al final del verano con una molécula antihelmínticos nueva
6. Preservar el refugio parasitario
7. Maximizar zonas de pastoreo integrado por diferentes especies de rumiantes
8. Asegurar que las desparasitaciones no coincidan con el cambio de potrero
9. Realizar cuarentena de ganado de nuevo ingreso a la UPA, para evitar la diseminación de cepas resistentes

La investigación sobre alternativas para el control de NGI en rumiantes, puede ayudar a mejorar las condiciones de salud y de bienestar animal así como a aumentar la vida útil de los antihelmínticos disponibles para el control de NGI, antes de que éstos se vuelvan obsoletos para la ganadería.

2.3. Métodos alternativos de control contra nematodos gastrointestinales.

Los métodos alternativos de control representan todas aquellas herramientas ajenas a la quimioprofilaxis que permiten controlar las poblaciones de NGI tanto dentro como fuera del hospedero; y han sido clasificadas en: (Jackson y Miller, 2006):

i) Estrategias para el control de supra-poblaciones (fases en vida libre) que incluyen el manejo de pastoreo y el control biológico.
ii) Estrategias para el control de infra-poblaciones (dentro del hospedero) que incluye el manejo inmunológico del hospedero (inmunonutrición, selección genética y vacunación), agujas de óxido de cobre y el uso de plantas bioactivas.

Debido a la relación con el tema central de este estudio, se realizará una mayor descripción de los métodos alternativos de control de infra-poblaciones, en particular lo relacionado con el uso de plantas para el control de NGI.

2.3.1. Control de infra-poblaciones

2.3.1.1. Inmunonutrición

En las regiones tropicales donde se practica la ganadería bovina existen dos limitantes principales que impactan negativamente el desempeño animal, la alimentación y las parasitosis. Hay una estrecha relación entre la nutrición y los NGI. La implementación de un sistema nutricional óptimo mejora la resistencia y/o la resiliencia del hospedero para mitigar el impacto negativo de los NGI, y viceversa (Houdijk, 2012; Viney, 2002). La primer respuesta inmunológica que se desencadena en el hospedero es una reacción antigénica de tipo humoral o innata a las sustancias de secreción y excreción parasitaria (Schallig *et al*., 1996), mediante la intervención de las inmunoglobulinas IgA, IgG e IgM (Yatsuda *et al*., 2000). Posteriormente, se desarrolla una respuesta de tipo celular o adquirida mediada por células como las citokinas y eosinófilos. Las células de la inmunidad humoral y celular son de origen proteico y cuando no se cuenta con el aporte nutricional adecuado, el organismo animal opta por suspender funciones secundarias como el desarrollo muscular y la reproducción. Sykes (2010) resume que el costo nutricional generado por la reacción inmunológica en el hospedero se debe a los factores siguientes:

1. A un aumento en la actividad metabólica durante la activación de las células de la inmunidad, al reclutamiento y activación de leucocitos, y al aumento por duplicado o triplicado en el consumo de oxígeno, glucosa y glutamina (Colditz, 2008).
2. A una menor disponibilidad de nutrientes debido a la anorexia y a la mala absorción.
3. A una alteración en las prioridades de la utilización de nutrientes durante la activación inmunológica (impide su utilización por tejidos no inmunológicos); aumento del catabolismo proteico en musculo esquelético, y anabólico en el hígado y TGI.
4. A la rápida producción de células de la inmunidad de origen proteico.

5. Y a la reparación del daño ocasionado en el tejido gastrointestinal del hospedero.

Estudios que evaluaron el efecto de los niveles de proteína metabolizable (PM) y energía metabolizable (EM) sobre las poblaciones parasitarias en ovinos, encontraron que únicamente el incremento de proteína cruda tiene la capacidad de reducir las poblaciones parasitarias establecidas; y reportaron una correlación positiva con el incremento de mastocitos en la mucosa de los animales infectados (Jones *et al*., 2011). Dichos hallazgos son consistentes con otros autores que reportan la necesidad de una suplementación con 17 gramos extras de PM sobre el requerimiento fisiológico, a modo de compensar las pérdidas proteicas debido a los daños provocados por los NGI en ovinos (Knox *et al*., 2006). Gennari *et al*. (1995), reportaron que en becerros con mayor porcentaje proteico en su dieta, se disminuye la tasa de establecimiento parasitario, la presentación de semiología clínica y menor porcentaje de alteraciones bioquímicas y hematológicas. Estos reportes permiten concluir que la suplementación proteica de animales parasitados con NGI permite mejorar la resistencia y resiliencia de los mismos (Hoste *et al*., 2008; Houdijk, 2012; Jones *et al*., 2011). Se ha reportado que la EM tanto en rumiantes como en monogástricos, no es un factor determinante para mejorar la resistencia del hospedero contra las NGI, lo cual es asociado al bajo requerimiento energético que demanda el sistema inmunológico para cubrir sus funciones (Houdijk, 2012). No obstante, los autores recomiendan una suplementación equilibrada que aporte tanto PM como EM (Houdijk, 2012).

2.3.1.2. Medicina etnoveterinaria y el uso de plantas con efecto antihelmíntico

La ganadería mundial se enfrenta a la necesidad de incrementar los parámetros productivos para satisfacer la demanda de alimentar tres billones de persona más en los próximos 50 años, sin incrementar las extensiones de tierra destinadas para ello (Charlier *et al*., 2015). Este incremento en la producción se debe realizar

considerando las demandas sociales que exigen un menor impacto ambiental, un menor uso de productos químicos, y una mayor seguridad alimentaria (Lans, 2011). La investigación en medicina ethnoveterinaria se refiere a la validación de la seguridad y la eficacia de la medicina tradicional, como es el uso de plantas para tratar diversas enfermedades en los animales (Barrau *et al*., 2005). Debido a que la nutrición y las nematodosis gastrointestinales son de los principales problemas que afectan el desempeño productivo en las regiones tropicales (Fitzpatrick, 2013), durante la última década se ha evaluado el uso de plantas nutraceuticas con efecto antihelmíntico dentro de las unidades de producción animal. Esta búsqueda de plantas nutraceuticas con efecto antihelmíntico se ha enfocado en aquellas familias o especies que poseen mayor concentraciones de compuestos bioactivos o metabolitos secundarios, además de su aporte nutricional, de producción y de adaptación (Vercoe *et al*., 2010). De esta forma, se han desarrollado estudios *in vivo* donde mediante el consumo de plantas nutraceuticas, se ha corroborado el efecto antihelmíntico en parásitos de pequeños rumiantes (Martinez-Ortiz-de-Montellanos *et al*., 2010; Villalba *et al*., 2010).

2.3.1.2.1. Leguminosas forrajeras tropicales

El trópico mexicano se extiende en el 33 % del territorio nacional, concentra el mayor porcentaje del hato ganadero del país (64 %) y aporta 25 % y 35 % de la producción nacional de leche y carne, respectivamente (FUNPROVER, 2010). La mayoría de los sistemas de producción en el trópico se basan en el uso del forraje, mediante el pastoreo, como el principal recurso nutricional. Por lo tanto, uno de los principales problemas que enfrenta la ganadería en estas regiones, es la producción estacional del forraje muy marcada, que limita la alimentación y la nutrición del ganado en épocas de variación climática extrema (nortes y/o sequía) (González-Arcia *et al*., 2012). A parte de la escasez de forraje, la producción se ve limitada debido a la mala calidad de los pastos (Lascano and Avila, 1991). Una de las estrategias para resolver la falta de forraje y la calidad nutricional del mismo, es

la complementación alimenticia con leguminosas tropicales (González-Arcia *et al*., 2012). Las leguminosas, desde el punto de vista forrajero/nutricional, tienen como atributo principal, un alto contenido de proteína cruda (14-28%) y un contenido de fibra menor al 40%, lo cual favorece un mayor consumo voluntario y digestibilidad. Estudios enfocados a la evaluación del potencial productivo de las leguminosas reportan un incremento en los rendimientos productivos de carne y leche de hasta un 50% (Lascano y Ávila, 1991). Dentro de las ventajas que ofrece su implementación están: i) mayor producción de biomasa, ii) tolerancia a sequias prolongadas, iii) capacidad de rebrote en sequía, iv) mejor calidad del forraje; y de modo alternativo como v) barreras naturales, vi) división de potreros y vii) controlar la erosión del terreno (Argel and Lascano, 1998). Así mismo, se ha sugerido que el contenido moderado de CPF y/o taninos que poseen algunas de las leguminosas permiten proteger la proteína cruda de la degradación ruminal, aumentando la disponibilidad de proteína de sobrepaso para su absorción intestinal. También se ha reportado que el consumo de algunas leguminosas tropicales tiende a ser mayor que el de los pastos y cereales, debido a que poseen una mayor palatabilidad (D'Mello, 1992).

Una de las principales desventajas del uso de algunas leguminosas es la presencia de múltiples metabolitos secundarios (aminoácidos no proteicos, glucósidos, fitohemaglutininas, compuestos polifenólicos, alcaloides, tritrepenos y oxalatos) que son considerados factores anti-nutricionales que afectan el consumo voluntario y la digestibilidad en los rumiantes (Kumar, 1992). No obstante, estudios recientes reportan que los efectos anti-nutricionales varían dependiendo de factores como: i) la concentración y naturaleza de los metabolitos secundarios, ii) la especie que los consume, iii) la adaptación fisiológica del animal, iv) su estado fisiológico y v) la composición general de la dieta (Makkar, 2003).

Actualmente, el contenido de metabolitos secundarios en las leguminosas se ha asociado con múltiples efectos benéficos sobre la nutrición y sobre la salud de los consumidores. *Gliricidia sepium* y *Leucaena leucocephala* son dos de las leguminosas que más se utilizan en la alimentación del ganado en México (Olivares-Pérez *et al*., 2011), ambas con moderadas a altas concentraciones de MS

(Rojas *et al*., 2006). Estudios *in vitro* e *in vivo* con estas mismas leguminosas han reportado un efecto AH contra los NGI de pequeños rumiantes (Alonso-Diaz *et al*., 2008; von Son-de Fernex *et al*., 2012).

2.4. Metabolitos secundarios de las plantas

Los metabolitos secundarios de las plantas son compuestos que no están directamente relacionados al crecimiento, al desarrollo o a la reproducción de las mismas, sino que forman parte de la defensa química o de la interacción medioambiental (Sepúlveda-Jiménez *et al*., 2003). Los metabolitos secundarios de las plantas se sintetizan y/o se acumulan en la planta ante la presencia de daños o lesiones ocasionados por: i) agentes biológicos (bacterias, virus, parásitos u hongos), ii) agentes químicos (bactericidas, fungicidas, insecticidas, nematocidas), iii) situaciones de estrés climático (radiación solar) y/o herbivoria (artrópodos y vertebrados), iv) funciones orientadas a la adaptación medioambiental (atracción de polinizadores y dispersores) y, v) reparación celular y tisular.

Existen alrededor de 20, 000 estructuras de metabolitos secundarios, los cuales se encuentran clasificados en dos grandes grupos: compuestos nitrogenados (alcaloides, aminoácidos no proteicos, aminas, glucósidos cianogénicos y glucosinolatos) y compuestos no nitrogenados (terpenoides, poliacetilenos, policetidos y fenilpropanoides) (Sepúlveda-Jiménez *et al*., 2003). Los metabolitos secundarios de las plantas han mostrado poseer diversas actividades biológicas, por lo cual han sido ampliamente utilizados en la industria cosmética, alimentaria y farmacéutica (Ávalos-García and Pérez-Urria, 2009).

Dentro de los grupos de metabolitos secundarios de las plantas más empleados o estudiados en dichas industrias encontramos a cuatro grupos principales:

1. Terpenoides. Los terpenos se derivan de la fusión de cinco carbonos, unidad funcional conocida como isopreno. Se clasifican de acuerdo a la cantidad de unidades de isopreno que le conforman como monoterpenos, diterpenos, triterpenos, tetraterpenos y quinonas, etc. Existen más de 40,000

moléculas dentro de las cuales encontramos hormonas (giberelinas, ácido abscisico y citoquininas), pigmentos (clorofilas, carotenoides), esteroles y aceites esenciales (Ávalos-García y Pérez-Urria, 2009). Dentro de la amplia gama de actividad biológica que muestran, se ha reportado el efecto antihelmíntico de algunos fitoquímicos pertenecientes a éste grupo como las piretrinas, monoterpenos que afectan el sistema nervioso de insectos; y la cucubitacina, triterpenoide que posee acción nematocida (Sepúlveda-Jiménez *et al*., 2003).

2. Compuestos fenólicos. Los fenilpropanoides son moléculas que poseen en su estructura un anillo aromático con uno o más grupos hidroxilo. Se clasifican de acuerdo a la cantidad de anillos, grupos hidroxilo y a la posición de los anillos fenólicos que poseen. Dentro de éste grupo se encuentran como principales compuestos bioactivos a las cumarinas, al ácido caféico, a los flavonoides y a los taninos (Sepúlveda-Jiménez *et al*., 2004). Los compuestos polifenólicos han mostrado tener una amplia gama de compuestos con actividad AH como los taninos y flavonoides (Alonso-Diaz *et al*., 2008; Barrau *et al*., 2005; Hoste *et al*., 2012; von Son-de Fernex *et al*., 2012). Sin embargo, es necesario aislar e identificar los compuestos con mayor potencial antihelmíntico contra nematodos gastrointestinales de rumiantes.

3. Glicósidos. Son metabolitos constituidos a partir de una molécula de azúcar condensada con otra molécula que contiene un grupo hidroxilo. Dentro de ésta clasificación encontramos a las saponinas, glicósidos cardiacos, glicósidos cianogénicos y glucosinolatos. Este grupo se caracteriza por poseer propiedades detergentes.

4. Alcaloides. Estos compuestos heterocíclicos se sintetizan a partir de aminoácidos cuya toxicidad ha sido directamente asociada a su capacidad para bloquear neuroreceptores, debido a la similitud que

poseen con diversos neurotransmisores. Existen más de 15,000 moléculas reportadas con actividad biológica; dentro de las cuales algunas poseen actividad AH: i) la anarginina y cistina con actividad nematocida, y ii) la matrina y esparteína, que tienen la capacidad de afectan la motilidad de los helmintos (Sepúlveda-Jiménez *et al*., 2003).

2.4.1. Compuestos polifenólicos (CPF).

Los CPF contienen alrededor de 8,000 moléculas que se agrupan en tres categorías: ácidos fenólicos, flavonoides y taninos (Isaza, 2007; Robbins, 2003). Los compuestos polifenólicos se producen durante la síntesis de aminoácidos aromáticos a partir del ácido sjhkimico. La mayoría se constituyen por una estructura de 3 anillos, dos aromáticos (anillos A y B) y uno heterocíclico oxigenado (anillo C); y cuya distribución dentro de la molécula determinará su función, naturaleza, estabilidad y actividad biológica (Waghorn, 2008; Wojdylo *et al*., 2007). Pueden agruparse con base en la complejidad molecular como fenoles simples o polifenoles (Robbins, 2003). Los CPF se caracterizan por su amplia actividad biológica que incluye la precipitación de proteínas y la formación de complejos con polisacáridos, ácidos nucleicos, esteroides, alcaloides y saponinas (Lascano *et al*., 2003). Dichas propiedades biológicas generan un gran interés médico (antiinflamatorio, antioxidante, antimicrobiano, potencial anticarcinogénico) e industrial (Cushnie and Lamb, 2011; Handique and Baruah, 2002; Wojdylo et al., 2007). En los últimos 20 años los CPF han cobrado gran interés en la medicina veterinaria ya que se ha demostrado que tienen la capacidad de ejercer un efecto antihelmíntico contra los principales NGI del ganado doméstico (Alonso-Díaz *et al*., 2008; Novobilsky *et al*., 2011; von Son-de Fernex *et al*., 2012).

Dentro de los grupos de CPF con mayor actividad antihelmíntica encontramos a los siguientes:

1. Ácidos fenólicos. Los ácidos fenólicos son fenoles que poseen una unidad funcional de ácido carboxílico; no obstante, al referirse a los metabolitos secundarios de las plantas un ácido fenólico se considera cuando la molécula tienen adheridas una estructura hidroxicinámicas y otra hidroxibenzoica (Robins, 2003). Su función en la planta se asocia al aprovechamiento de nutrientes, actividad enzimática, fotosíntesis y como componentes estructurales (Robins, 2003). Se ha reportado que los ácidos fenólicos poseen propiedades antioxidantes, y que tienen la capacidad de inhibir enfermedades ocasionadas principalmente por daños oxidativos como enfermedades coronarias, infartos y cáncer (Block *et al*., 1992; Jacob y Burri, 1996). Así mismo, Ndhlala *et al*. (2015) reporta que los ácidos fenólicos como el ácido gálico, isoferúlico, rutina, clorogénico, ácido caféico y ferúlico entre otros, pueden tener propiedades antimicrobianas y antihelmínticas. Existe poca información sobre la participación de estas estructuras químicas aisladas de leguminosas tropicales, sobre el efecto antihelmíntico de NGI en rumiantes.

2. Flavonoides. Los flavonoides son un amplio grupo de compuestos que cuenta con aproximadamente 4000 moléculas conformadas por una benzo-y-pirona, y se subcategorizados en ocho clases diferentes. Su estructura química está conformada por un número variable de moléculas de carbono, hidrógeno y oxígeno (Grupos Hidroxilo + Hidrocarburo aromático), que dependiendo la cantidad y su localización dentro de la molécula dan origen a múltiples compuestos con denominación y función diferente. Estos metabolitos se sintetizan en la planta en respuesta a la invasión bacteriana. Su actividad biológica depende directamente de su estructura molecular (grado de hidroxilación, patrones de sustitución, conjugación y grado de polimerización). La actividad biológica más importante o estudiada dentro de éste grupo es la antioxidante. Existen pocos reportes sobre la actividad antihelmíntica de éstos compuestos, se ha reportado el efecto directo de algunos flavonoides como el nicotiflorin, narcisina, luteolina, quercetina, sobre la motilidad de larvas infectantes de NGI de

importancia económica y fitonematodos (Adekunle and Aderogba, 2008; Barrau et al., 2005; Kozan et al., 2013).

3. Taninos. Pertenecen al grupo de los flavonoides, son considerados taninos cuando poseen tres o más subunidades de fenol en su estructura (Robins, 2003). La palabra tanino tiene su origen de la palabra germana *tanna*, que se significa roble (fuente original de los taninos). Los taninos se han clasificado según su complejidad molecular en tres diferentes grupos: hidrolizables, condensados y derivados del ácido elágico. En general, los taninos se encuentran conformados por unidades de flavonoides enlazadas mediante ligaduras de carbono (C-C) (Schofield and Pell, 2001) y su longitud de cadena es variable llegando a poseer en su estructura hasta más de 20 unidades de flavonoides (Waghorn, 2008). Los taninos condensados se caracterizan por su capacidad de formar complejos con proteínas, polisacáridos, ácidos nucleicos, esteroides, alcaloides y saponinas; no obstante, la formación de dichos complejos será dependiente de múltiples factores como el tamaño molecular del tanino, la composición de aminoácidos, el peso molecular de la proteína y el pH del medio de incubación (Haslam, 1989; Hagerman, 1989). La estabilidad del complejo dependerá del tipo de interacción molecular: i) cuando se realiza mediante enlaces hidrofóbicos y de hidrógeno, la unión tendrá una naturaleza reversible y ii) cuando es mediante oxidación, su unión es irreversible denominándosele como una quinona. Uno de los factores más importantes en la formación y disociación de complejos tanino-proteína es el pH, presentándose la conjugación molecular cuando el pH es cercano a neutro y desasociándose cuando el pH es menor o igual a tres. El complejo tanino-proteína se forma a partir de enlaces de hidrógeno entre los grupos fenólicos de los taninos y el grupo cetoamida de las proteínas, éste proceso de formación es normalmente reversible y tanto las proteínas como los taninos pueden recuperarse intactos (Hagermann, 1989). Esta característica ha captado la atención de diversos investigadores, quienes mediante diversos estudios *in vitro* e *in vivo* han reportado la actividad antihelmíntica de los taninos condensados

contra los principales NGI en pequeños rumiantes (Alonso-Díaz *et al*., 2010; Katiki *et al*., 2013; Macedo *et al*., 2012; Saric *et al*., 2015; Vargas-Magana *et al*., 2014).

2.4.2. Efecto antihelmíntico de los CPF/taninos. ¿Directo o Indirecto?

La hipótesis del posible efecto antihelmíntico de los CPF surge tras observar que pequeños rumiantes que pastorearon en praderas con leguminosas de clima templado, presentaban una menor eliminación de huevos por gramo de heces (hpg) (Hoskins *et al.*, 2000; Niezen *et al*., 1995). A partir de este reporte, la evaluación de plantas bioactivas se han enfocado a la utilización de plantas que poseen elevadas concentraciones de taninos o CPF. En primer instancia, se planteó que el efecto antihelmíntico de los taninos era de modo indirecto, debido a su habilidad de proteger la proteína de la digestión ruminal y favorecer un incremento de la proteína de sobrepaso para la absorción a nivel intestinal; lo cual repercute en una mejora de la resistencia/resiliencia del hospedero contra las infecciones por NGI (Coop y Kyriazakis, 1999) (Ver sección 2.3.1.1.).

También se planteó la hipótesis de un efecto antihelmíntico de los taninos, debido a la unión con proteínas de diferentes estructuras de los parásitos gastrointestinales (Hoste *et al*., 2006). Existen estudios que han demostrado el efecto antihelmíntico directo de los taninos condensados, y se relacionan a la formación de complejos tanino-proteína. Diversas estructuras parasitarias como la cutícula, la cavidad bucal, el esófago, la cloaca y la vulva, son ricas en prolina, un aminoácido con afinidad para formar complejos estables con los taninos (Hoste *et al*., 2006). Hay evidencias de un efecto antihelmíntico directo ejercido por plantas ricas en taninos sobre la cutícula, la motilidad y el desenvaine larvario, así como en la reducción de hpg y establecimiento de parásitos adultos en el hospedero (Alonso-Diaz et al., 2008; Athanasiadou et al., 2001; Brunet et al., 2008; Heckendorn et al., 2007; Hoste et al., 2006; Paolini et al., 2003; Rojas et al., 2006). La mayoría de los estudios han sido realizados utilizando pequeños rumiantes como modelo experimental, por lo

que es necesario evaluar el efecto de compuestos polifenólicos extraídos y purificada de leguminosas tropicales sobre NGI que afectan a los bovinos.

2.4.3. Evaluaciones *in vitro* de la actividad antihelmíntica de metabolitos secundarios

Las evaluaciones del efecto antihelmíntico *in vitro* de los metabolitos secundarios han sido adaptadas de las metodologías existentes para la evaluación de la eficacia de moléculas antihelmínticas y/o de la resistencia antihelmíntica (Athanasiadou and Kyriazakis, 2004). Las evaluaciones del efecto antihelmíntico *in vitro* de plantas bioactivas son una metodología rápida y de bajo costo. Esta herramienta también permite realizar el monitoreo de varias plantas contra varios géneros de NGI. La mayoría de las evaluaciones *in vitro* se realizan con las fases parasitarias de vida libre (huevo, L_1, L_2 y L_3) debido a la facilidad en la obtención y en el mantenimiento del material biológico bajo condiciones de laboratorio. Los bioensayos estandarizados para dichas evaluaciones son: i) la inhibición de la eclosión de huevos (EHA), ii) la inhibición de la alimentación larvaria, iii) la inhibición del desarrollo larvario, iv) la inhibición de la migración larvaria, y v) la inhibición del desenvaine larvario artificial (LEIA) (Hoste *et al*., 2012). También se han logrado adaptar bioensayos con parásitos adultos (micromotilidad); sin embargo, el tiempo que se logra mantener con vida a los adultos fuera del hospedero (24 - 48 horas) es una de las principales limitantes para su evaluación (Athanasiadou y Kyriazakis, 2004).

En cuanto al tipo de extracción de los metabolitos secundarios de las plantas bioactivas, posiblemente el método más popular es el sistema mixto de acetona:agua (70:30) debido a que ofrece un mayor rendimiento de compuestos polifenólicos (Hoste *et al*., 2015). No obstante, existe evidencia de la presencia de otros metabolitos secundarios con actividad antihelmíntica (Molan *et al*., 2003; Barrau *et al*., 2005; Kozan *et al*., 2013), que podría ser recuperados y aislados de las plantas mediante otros métodos de extracción. Barrau *et al*. (2005), reportó que los

flavonoides como rutina, narcissina y quercetina, de origen sintético, también ejercen un efecto antihelmíntico directo sobre diferentes fases biológicas de los NGI.

Para realizar un mejor perfil fitoquímico de plantas bioactivas, se sugiere utilizar métodos de extracción que combinen diferentes polaridades así como realizar el fraccionamiento biodirigido de los extractos de plantas con actividad antihelmíntica. Lo anterior permite elucidar las moléculas químicas involucradas en la bioactividad (Adekunle y Aderogba, 2008). Sin embargo, existen pocos estudios sobre el aislamiento y la identificación de fitoquímicos con actividad antihelmíntica presentes en los extractos de las plantas bioactivas, debido a la complejidad del procedimiento. Esto puede ser aún más complejo con leguminosas tropicales debido a que poseen una composición bioquímica más diversa (Alonso-Díaz *et al*., 2010). En el cuadro 4, se presenta un resumen de los fitoquímicos aislados de plantas bioactivas con actividad antihelmíntica.

Cuadro 4. Metabolitos secundarios de plantas, reportados con actividad antiparasitaria.

METABOLITO	GRUPO	PARASITO	REFERENCIA
ALFA-HEDERINA	Saponinas	*Fasciola hepática* *Dicrocelium* spp	Julien *et al*. (1985)
EMETINA **QUELERITRINA** **BERBERINA**	Alcaloides	*Toxocara canis*	Satou *et al*. (2000)
TANINOS **LACTONAS SESQUITERPENICAS**	Polifenoles Terpenos	*Dictyocaulus viviparus*	Molan *et al*. (2003)
RUTINA **NICOTIFLORINA** **NARCISINA**	Polifenoles	*Haemonchus contortus*	Barrau *et al*. (2005)
QUERCETINA	Polifenoles	*C. elegans*	Adekunle y Aderogba, (2008)
TANINOS	Polifenoles	*H. contortus* *T. colubriformis*	Alonso-Díaz *et al*. (2008 a,b)
QUERCETINA **LITEOLINA**	Polifenoles	*Trichostrongylus* sp.	Kozan *et al*. (2013)

Los estudios *in vitro* pueden ser potencializados mediante la combinación de otras metodologías como el fraccionamiento biodirigido, el cual permite conocer las moléculas químicas involucradas con el efecto antihelmíntico así como el o los posibles mecanismos de acción.

2.4.3.1. Fraccionamiento biodirigido

El fraccionamiento biodirigido de las plantas es una metodología que permite el aislamiento, la purificación y la caracterización de compuestos con una bioactividad específica. Para realizar el fraccionamiento, se sugiere la metodología siguiente (Tsuda, 2004):

1. Extracción primaria. El método de extracción dependerá de la naturaleza del compuesto bioactivo. En caso de desconocerse, se sugiere realizar una extracción con 100 g de material vegetal seco y molido; (1) 200 ml hexano (3 veces), (2) 200 ml acetato de etilo (2 veces) y (3) 200 ml metanol (2 veces) por un periodo de 3 - 4 horas.
2. Realizar una evaluación cualitativa de las tres fracciones mediante la utilización de Cromatografía en capa fina.
3. Realizar el bioensayo para determinar la actividad biológica de cada una de las fracciones.
4. Colocar la fracción de mayor actividad biológica en una columna cromatográfica. El sistema de solventes a utilizar en la columna depende de la naturaleza y de la polaridad de los compuestos observados en la cromatografía en capa fina.
5. La molécula purificada deberá enviarse a resonancia magnética nuclear para realizar la elucidación molecular.

Para el diseño y/o selección del bioensayo que permita aislar el fitoquímico bioactivo, es importante tomar en consideración cuatro características esenciales que son: i) la simplicidad (que sea fácil, rápido de elaborar y que requiera de bajas concentraciones o dosis), ii) la rapidez (los resultados deberán obtenerse en un

lapso de dos días y una semana como máximo), iii) la repetitividad (para considerar resultados confiables, al ser realizado por otra persona o en otro momento, los resultados deben ser similares) y iv) la comparabilidad (los resultados obtenidos deben ser de naturaleza cuantitativa).

El aislamiento y purificación de fitoquímicos con actividad antihelmíntica permite no solo la identificación de nuevas moléculas con potencial antihelmíntico, sino que en conjunto con otros métodos como la microscopía electrónica de barrido (MEB) y de transmisión (MET) podría ayudar a definir con mayor claridad el mecanismo de acción de los fitoquímicos (von Son-de Fernex *et al*., 2015).

2.4.3.2. Microscopía electrónica de barrido (MEB)

La microscopía electrónica de barrido emite información topográfica y tridimensional de la superficie de los ejemplares analizados, después de un proceso de fijación que permite preservar la estructura celular evitando cambios significativos en su tamaño, forma y en la relación espacial de los componentes celulares (Dykstra and Reuss, 2003). En la parasitología, ésta técnica se utiliza con fines de caracterización y de diferenciación morfológica de especies; así como para la observación de características ultraestructurales que no es posible identificar mediante microscopía óptica (Lichtenfels, 1990; von Son-de Fernex *et al*., 2014). Recientemente, la MEB se ha empleado para determinar los posibles mecanismos de acción de los metabolitos secundarios con actividad antihelmíntica (von Son-de Fernex *et al*., 2015). Estudios recientes demostraron que adultos de *H. contortus* incubados con taninos condensados presentaban adherencias de materia orgánica en la región cefálica y en la vulva de las hembras parásitas, además de daño cuticular (Hoste *et al*., 2012).

2.4.3.3. Microscopía electrónica de transmisión (MET)

La microscopía electrónica de transmisión es una técnica que permite la observación de la estructura y conformación interna del objeto de estudio, y se ha convertido en una herramienta primaria dentro de las ciencias biológicas (Stach, 2008). La MET permite realizar observaciones de tipo anatomo-patológicas de las muestras biológicas. La microscopía de transmisión aplicada a la parasitología se ha utilizado como una herramienta de identificación de cambios ultraestructurales en los diversos nematodos, producidos por sustancias (Bleve-Zacheo *et al*., 1993). De esta forma, Mansfield *et al*. (1992), reportaron no solo la composición estructural de la tri-capa del huevo de *H. contortus*, sino también el proceso de eclosión larvaria, así como las alteraciones y daños que sufren las diferentes capas del huevo tras su incubación en proteasas, cloroformo y biotina. La MET también se utilizó para esclarecer la composición de las paredes de los oocistos de coccidias (Mai et al., 2009). El primer reporte de su uso en el ramo de la ethnoveterinaria fue por Brunet *et al*. (2011), quienes observaron cambios patológicos a nivel cuticular y de hipodermis así como en células somáticas musculares e intestinales en larvas infectantes de *H. contortus* y *T. colubriformis* incubadas con Sanfoin (1200 µg/ml). Este estudio también les permitió corroborar la hipótesis del efecto antihelmíntico directo que pueden ejercer los extractos de plantas bioactivas y/o los taninos condensados (Hoste *et al*., 2012).

2.4.4. Evaluaciones *in vivo* del efecto antihelmíntico de los metabolitos secundarios

La evaluación *in vivo* es indispensable para validar el efecto antihelmíntico *in vitro* de una planta o de sus compuestos. La actividad biológica puede ser evaluada con base en su capacidad para inhibir/reducir el establecimiento parasitario y su efecto sobre poblaciones de parásitos ya establecidas (fertilidad y como desparasitante). No obstante, también existen controversias con respecto al efecto antihelmíntico generado tras su administración directa con los hospederos (efecto

directo o indirecto; ver secc. 2.4.2.). Actualmente existen dos metodologías aceptadas para la evaluación de plantas con potencial antihelmíntico *in vivo*.

1. Suplementación de la planta bajo un ambiente parcialmente controlado y mediante corte y acarreo de la planta en cuestión
2. Pastoreo directo con plantas ricas en metabolitos secundarios

Cada una de las metodologías mencionadas tiene ventajas y desventajas y su elección deberá realizarse con base en el objetivo deseado.

La suplementación con plantas bajo ambientes parcialmente controlados (consumo voluntario, calidad de la dieta, grado de infección o infección artificial) se considera una de las mejores opciones para determinar no solo el efecto de la planta si no también su impacto productivo y de salud en los animales (Hoste *et al*., 2015). La mayoría de las evaluaciones *in vivo* se han realizado con pequeños rumiantes, donde se han reportado reducciones hasta del 80 % de la población adulta de NGI dentro del hospedero. Estas plantas también pueden ayudar al control de los NGI en ganado bovino y en otras especies productivas (Sandoval-Castro *et al*., 2012).

Con relación a la segunda metodología de pastoreo directo, se han obtenido resultados alentadores; no obstante, algunas inconsistencias ponen en duda el efecto de las mismas. En experimentos donde se utilizó la misma planta bioactiva, se reportaron resultados diferentes asociado a la naturaleza y concentración de metabolitos secundarios de las plantas (Athanasiadou y Kyriazakis, 2004). El contenido de metabolitos secundarios de las plantas se modifican de acuerdo a las condiciones geográficas, climáticas y medio ambientales (Hoste *et al*., 2015). Por lo tanto, uno de los mayores retos bajo un sistema de pastoreo es controlar el consumo individual de los animales, lo cual trae consigo una elevada variabilidad en los resultados y con ello un mayor margen de error para sobre-estimar o sub-estimar las propiedades antihelmínticas de una planta. A pesar de esto, los estudios han demostrado que la utilización de plantas bioactivas son una alternativa viable para el control parasitario en rumiantes (Hoste *et al*., 2015).

Las inconsistencias entre el efecto antihelmíntico *in vitro* e *in vivo* que han sido reportadas, pueden verse asociadas a múltiples factores dentro de los cuales encontramos (Athanasiadou y Kyriazakis, 2004):

1. Diferencias en las concentraciones de los metabolitos secundarios de las plantas
2. Tiempo de incubación con los compuestos bioactivos
3. Falta de contacto directo de los metabolitos secundarios de las plantas y los parásitos
4. Microambiente de incubación

La mayoría de los factores antes mencionados son controlados bajo condiciones de laboratorio y se ven totalmente modificadas en condiciones naturales dentro del hospedero. Motivo por el cual, tras la obtención de un efecto antihelmíntico *in vitro* es indispensable su evaluación *in vivo* antes de poder realizar alguna conclusión.

2.4.5. Efecto de los CPF en la nutrición de los rumiantes.

Existen controversias sobre el efecto de los CPF en la nutrición de rumiantes, ya que se ha reportado efectos tanto benéficos (mayor disponibilidad de proteína de sobrepaso, prevención de timpanismo, efecto antihelmíntico preventivo y curativo) como detrimentales (disminución del consumo voluntario, sensación de saciedad, reducción de las poblaciones microbianas en rumen, mala digestión de fibra y proteína). Se ha sugerido que el efecto ejercido sobre los animales dependerá tanto de la naturaleza y concentración de los CPF como de la especie animal que les ingiere (Waghorn, 2008). Dentro de los efectos detrimentales con mayor impacto económico negativo está la disminución del consumo voluntario. La disminución del consumo voluntario ha sido asociado en primer instancia a una disminución en la digestibilidad de la materia seca en rumen, y con que al reaccionar con la mucosa del TGI interfieren directamente con la absorción de los nutrientes

generando una señal de distención física en los animales. En segunda instancia, la disminución del consumo voluntario también se asocia a la palatabilidad y/o a la astringencia provocada por a la capacidad que poseen los CPF para formar complejos con las proteínas salivales (Mera, 2004).

También se ha reportado que la inclusión en la dieta de 6.1 % - 8.3 %, los CPF tienen la capacidad proteger parte del nitrógeno de la degradación ruminal (Werne *et al*., 2013). El complejo entre los CPF y las proteínas se caracteriza por mantener su estabilidad bajo condiciones de pH entre 4 - 7 y por disociarse fuera de dicho rango. Esto explica la hipótesis de que el complejo CPF-Proteína se forma o pasa a través del rumen, y se disocia en abomaso debido a la presencia de los jugos gástrico (pH 2.5- 3.5) y/o en el duodeno por la acción del ácido pancreático (pH 8-9), permitiendo la absorción de proteínas de mayor calidad nutricional en el intestino (Bernal-Bechara, 2007). Se ha reportado que la inclusión de CPF en la dieta de rumiantes tiene la capacidad de mejorar los parámetros productivos (reproducción, producción láctea y prevención de timpanismo) (Min *et al*., 2003). Aunado a las ventajas productivas que ofrece la inclusión de CPF, está la disminución de la carga parasitaria en pequeños rumiantes (disminuciones en el establecimiento parasitario, reducción de la población adulta, y disminución de la fertilidad de las hembras parásitas) (Hoste *et al*., 2015).

2.4.6. Automedicación en rumiantes

La preferencia de los animales por algún alimento se encuentra regulado por dos factores principales: la palatabilidad y su efecto post-ingesta. Ambos factores son cualidades de los alimentos que están directamente relacionados a su composición química (Provenza, 1996). Se ha reportado que los rumiantes tienen la capacidad de seleccionar sus alimentos con base en sus propiedades nutricionales, e inclusive de seleccionar forrajes con propiedades medicinales que les permitan reducir el índice de enfermedades (Janzen, 1978). El comportamiento

de la automedicación en animales silvestres fue propuesto en los años 60´s cuando se observó que los chimpancés ingerían plantas, carentes de valor nutricional, sin masticarlas (Shurkin, 2014), con la finalidad de auto desparasitarse (Fruth *et al*. 2014). La auto-desparasitación en rumiantes se ha propuesto como un comportamiento de regulación fisiológica, y se ha corroborado en pequeños rumiantes que al tener altas cargas parasitarias muestran una preferencia en el consumo de plantas con elevadas concentraciones de metabolitos secundarios de las plantas, obteniendo como resultado una disminución en la eliminación de hpg (Martinez-Ortiz-de-Montellano et al., 2010; Villalba and Landau, 2012; Villalba et al., 2010). De igual manera, González-Arcia *et al*. (2014), reportó que bovinos infectados con NGI también tienden a modificar su patrón de consumo al ofrecer en la dieta leguminosas con potencial antihelmíntico. El mismo autor reportó que al comparar el consumo entre animales libres de infección por NGI con el de bovinos que presentaban una eliminación promedio de 80 hpgh, los animales infectados incrementaron el consumo de *Cratylia argentea* mediante un comportamiento de sustitución de componentes en la dieta. Por lo anterior, diversos investigadores sugieren que el acceso de los animales a leguminosas o plantas bioactivas con moderadas concentraciones de metabolitos secundarios pudiese mitigar el impacto de las parasitosis, disminuir la utilización de medicamentos y dependiendo de la calidad, mejorar la nutrición de los mismos.

III. HIPÓTESIS.

Los extractos (acuosos, acetona:agua y acetónicos) de las plantas: *Leucaena leucocephala, Cratylia argentea, Gliricidia sepium, Guazuma ulmifolia* y *Azadirachta indica* inhibirán el desenvaine larvario y la eclosión de huevos de *Cooperia punctata*.

La complementación de la dieta de bovinos con hojas frescas de la leguminosa que haya mostrado mayor efecto antihelmíntico *in vitro,* reducirá el establecimiento de *C. punctata in vivo*.

IV. OBJETIVO GENERAL

Evaluar el efecto antihelmíntico *in vitro* e *in vivo* de plantas bioactivas sobre el control de *Cooperia punctata* en bovinos.

4.1. OBJETIVOS ESPECÍFICOS

4.1.1. Realizar tres métodos de extracción [acuosos, acetona:agua (70:30) y acetónicos] a cinco plantas bioactivas: *Leucaena leucocephala, Cratylia argentea, Gliricidia sepium, Guazuma ulmifolia* y *Azadirachta indica*; y determinar las principales clases de fitoquímicos presentes en cada extracto.

4.1.2. Evaluar el efecto antihelmíntico *in vitro* de los 15 extractos de plantas bioactivas sobre el desenvaine larvario artificial y la eclosión de huevos de *Cooperia punctata*; y determinar la participación de los compuestos polifenólicos en la actividad anntihelmíntica.

4.1.3. Aislar y purificar los fitoquímicos de la planta bioactiva que haya mostrado un mayor efecto antihelmíntico *in vitro*, mediante fraccionamiento biodirigido.

4.1.4. Evaluar el efecto antihelmíntico *in vivo* de la planta bioactiva que muestre el mayor efecto antihelmíntico *in vitro*, sobre el establecimiento parasitario de *Cooperia punctata*, en becerros ¾ Ho X Cebú infectados artificialmente.

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6. ARTÍCULO 1

In vitro anthelmintic effect of fifteen organic extracts on the exsheathment process of the cattle nematode *Cooperia punctata*.

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ABSTRACT

Larval exsheathment process of gastrointestinal nematodes is vital and necessary for the successful establishment in the host. The objectives of this study were: (1) to assess the anthelmintic (AH) potential of *Leucaena leucocephala, Gliricidia sepium, Guazuma ulmifolia*, *Cratylia argentea* and *Azadirachta indica* on impairing larval exsheathment of *Cooperia* spp., (2) to assess the role of polyphenols in AH activity, (3) to evaluate the best plant extraction procedure (Aqueous [AQ], Acetone:Water [AW] and Acetonic [AC]) against the exsheathment process. Infective larvae of *C. punctata* were exposed to increasing concentrations of each plant extract (150, 300, 600, 1200 and 2400 μg mL^{-1}). A general linear model test was used to assess dose-dependent behavior. A Kruskal-Wallis test was used to evaluate the AH effect among extraction procedures and the addition polyethylene glycol. All fifteen extracts showed a significant dose-dependent response ($P<0.05$). At the lowest concentration, *Leucaena leucocephala* inhibited 100% of larval exsheathment. Fourteen extracts fully inhibited the exsheathment process at the highest concentration, except for *A. indica*-AQ which inhibited 96.63 ± 1.2 %. At the highest concentration, no significant differences were found among plants and extraction procedures ($P > 0.05$). The best-fit LC_{50} values were: 86.77 ± 48.12 (*A. indica*-AC), 225.27 ± 57.5 (*C. argentea*-AC), and 250.48 ± 11.45 μg mL^{-1} (*G. sepium*-AQ). Polyethylene glycol addition revealed polyphenols as the main bioactive compounds. Results suggest that all three extraction procedures have similar bioactivity; thus, the AW extraction offers both AH-like activity and higher yield. The five plants could be considered for further *in vivo* investigations to corroborate their capacity to reduce larval establishment within the host.

Keywords: Cattle, *C. punctata*, exsheathment, plant extracts

INTRODUCTION

Over recent decades, special emphasis has been placed on cattle production unit management due to current concerns on food safety (Fitzpatrick, 2013). Implementation of legumes in livestock nutrition in the tropics has been proposed as an alternative to counteract the low quality of most native forages available for grazing cattle (González Arcia ***et al.***, 2012). Furthermore, the use of legumes has been proposed as an alternative for helminth control in small ruminants (Alonso-Díaz ***et al.***, 2010). Plants rich in secondary metabolites (PSM) have been shown to act against different life stages of gastrointestinal nematodes (GINs) (Bizimenyera ***et al.***, 2006; Alonso-Diaz ***et al.***, 2008b; von Son-de Fernex ***et al.***, 2012). Although several ***in vitro*** and ***in vivo*** studies have proved the AH efficacy of bioactive plants (BP) against GINs in small ruminants, the information on cattle nematodes is scarce (Novobilsky ***et al.***, 2011).

Infective larvae (L_3) are the transitional stage between the pre-parasitic and parasitic life cycles of nematodes, representing a critical time-framing step of nematode biology (Hoste ***et al.***, 2012). The larva exsheathment inhibition assay (LEIA) is an ***in vitro*** test which looks for compounds capable of impairing or delaying that biological process in L_3, with the final objective of reducing larval establishment within the host (Hoste ***et al.***, 2012). ***Cooperia*** spp. is a highly resistant cattle nematode, which causes direct losses to the cattle industry (Stromberg ***et al.***, 2012). The continuous reports of ***Cooperia*** resistant strains to the anthelmintics available in the market for their control (Sutherland and Leathwick, 2011; Arnaud-Ochoa and Alonso Díaz, 2012; Becerra-Nava ***et al.***, 2014) increases worm-burden regulation inside the host; thus, new strategies are required.

Although the ovicidal effect of tropical plants against ***C. punctata*** has been reported (von Son-de Fernex ***et al.***, 2016), other biological stages such as the exsheathment process should also be targeted to propose an alternative focus of control, which includes more than one development stage of GIN. The objectives of this study were: (1) to assess the potential of ***Leucaena leucocephala, Gliricidia sepium, Guazuma ulmifolia***, ***Cratylia argentea*** and ***Azadirachta indica*** at impairing larval exsheathment of ***C. punctata*** infective larvae, (2) to state the role of polyphenols in AH activity, and (3) to evaluate the best plant extraction procedure against the larval exsheathment process.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Leucaena leucocephala* (LL), *Cratylia argentea* (CA), *Gliricidia sepium* (GS), *Guazuma ulmifolia* (GU) and *Azadirachta indica* (AI) were harvested in March 2013 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 Zootechnia) of the Universidad Nacional Autónoma de México (National Autonomus Univesity of México), located in Martinez de la Torre (20°03´ N y 93°03´ O; 151 msnm), Veracruz, Mexico.

Extraction procedure

For each plant species, 1±0.15 kilograms of fresh leaves were air-dried at 60 °C for 72 h and placed in a grinder to obtain particles of 1 mm. Air-dried and powdered material was used for different polarity extraction procedures. First, plant material was placed in a glass beaker (2L) containing acetone:water (70:30) with a magnetic stirrer. The mixture was then sonicated for 4 h in a water bath (Branson Sonicator 2510®). The second extraction was performed by placing 500±36.97g of dried-ground material of each plant species in an individual glass beaker with acetone and then kept at room temperature (24 °C) for 24 h. Finally, an aqueous extract was performed, using the same ground material used for the acetonic extraction, which after dried, was placed in distilled water previously heated to 58 °C for two hours. For all three extraction procedures, the extract was obtained from the filtered material using filter paper (Whatman® qualitative filter paper, Grade 1). Solvents were evaporated from the extracts at 58 °C using a low pressure distillation procedure in a rotovapor machine (Rotovapor® R-3). Extracts were washed four times with 500 ml of n-hexane to remove chlorophyll and lipids, and a separation funnel was used to discard the n-hexane fraction. Finally, extracts were frozen and lyophilized to obtain the dry ground extracts.

The percentage yield from fresh young leaves of each plant species was obtained through three extraction procedures (aqueous, acetone:water, acetonic) are presented in Table 1.

Thin layer chromatography (TLC) of the fifteen extracts

Qualitative phytochemical screening of the fifteen plant extracts was performed using silica gel 60 F_{254}- pre-coated TLC plates with capillary tubes, and developed in a TLC chamber using suitable mobile phase for terpenoids and flavonoids. The developed TLC plates were air dried and observed under ultra-violet light at both 254 nm and 365 nm. They were then sprayed with the Natural products-polyethylene glycol reagent (NEU-reagent) and Anisaldehyde-sulphuric acid reagent (AS), for flavonoids and terpenoids, respectively (Wagner and Bladt, 1996). Finally, plates were placed in a hot air oven for 1 min for the development of color in separated bands. The qualitative determination of compounds was performed according to colorimetric standards (Wagner and Bladt, 1996). The phytochemicals identified in the fifteen organic extracts are presented in Table 2.

Bioassays

Larval recovery

Cooperia punctata third stage larvae (L_3) were obtained from donor calves with a mono-specific infection (CEIEGT-FMVZ-UNAM strain, Mexico). Calves were housed indoors on concrete floors, provided with hay, commercial concentrate and free access to water. Feces were collected daily and cultured for seven days, after which larvae were Baermanized and filtered. Finally, larvae were stored at 4 °C for 2 months before use. Final concentration of 1000 L_3 mL^{-1} was achieved either by concentrating the larval inocula through centrifugation, or by diluting with distilled water (DW).

Larval exsheathment assay

Four thousand ensheathed *C. punctata* were separately incubated for each of the three different polarity extracts at increasing concentrations of 150, 300, 600, 1200 and 2400 µg mL^{-1} of distilled water (DW), for 3 h at 21 °C. For negative control treatments, larvae were incubated either in DW (AQ and AW extracts) or Tween 80 (TW) at 2% (AC extracts) (Katiki *et al* 2011). After incubation, L_3 were washed and centrifuged (2500 rpm) three times in DW (pH 7.2). Larvae were then subjected to an artificial exsheathment process by contact with a sodium hypochlorite solution (75 µL domestic bleach with 112 µL of 6% sodium hypochlorite, diluted in 15.813 mL of distilled water) modified from Katiki *et al*. (2011). This was performed to obtain 100% larval exsheathment after 60 min in control groups. The kinetics of the larval exsheathment process was monitored by microscopic observation (40x). Exsheathed and unsheathed larvae were counted at 0, 10, 20, 30, 40, 50 and 60 min. At each time interval, L_3 were killed and examined immediately. Six replicates were run for each dose, extract and control, to look for possible changes in the proportion of exsheathed larvae over time. To confirm the role of polyphenolic compounds in the AH effect, another series of incubations were made. This included three treatments: i) the negative control (distilled water or Tween 80 at 2%), ii) the maximum dose of the extract to be tested (2400 µg of extract/ml) with PEG (4800 µg/ml; after a pre-incubation period of 3 h to bind tannins), and iii) the maximum dose, but without PEG (Makkar *et al*., 1995).

Statistical analysis

General linear model (GLM) tests were used to assess a dose-dependent behavior for each plant extract (STATGRAPHICS, Centurion XVI v. 16.1.18, USA). Kruskal-Wallis tests were used to evaluate differences in exsheathment rate with and without PEG addition. Statistical differences were considered significant at $P<0.05$. The lethal concentration to inhibit 50% of L_3 exsheathment (LC_{50}) was calculated for each extract using a Probit Analysis Program (Minitab® 17.1.0, Minitab Inc. USA).

RESULTS

Larval exsheathment inhibition assay (LEIA)

The exsheathment kinetics of *C. punctata* (L_3) was similar in all control groups (DW and TW) with 100% of exsheathment obtained after a 60 min exposure to the artificial exsheathment fluid. However, after 3 h incubation period of L_3 to the highest concentration (2,400 µg mL^{-1}), the exsheathment process of *C. punctata* was fully inhibited with all fifteen extracts (Figs. 1-5), showing dose-dependent behaviors ($P < 0.05$).

Best impairment of exsheathment was achieved with *L. leucocephala*-AQ and AW extracts, where the lowest concentration (150 µg mL^{-1}) induced exsheathment inhibition by 100%

(Fig. 1 a, b). Lethal concentrations required for 50% exsheathment inhibition are presented in Table 3. The best fit LC_{50} were: 86.77 ± 48.12, 225.27 ± 57.50, 250.48 ± 11.45, and 270.73 ± 22.98 µg mL^{-1} from ***A. indica*** (AC), ***C. argentea*** (AC), ***G. sepium*** (AQ) and ***G. sepium*** (AW), respectively. For ***L. leucocephala*** extracts, the LC_{50} was not possible to calculate due to the high inhibitory effect.

When assessing AH performance among extraction procedures, lethal concentrations (LC_{50}) were taken into consideration (Mean ± SE): 519.11 ± 176.29, 142.94 ± 104.04 and 479.43 ± 167.04 µg mL^{-1} for AQ, AW and AC extractions, respectively. Best mean values were obtained with the AW extracts.

Role of polyphenols in the anthelmintic activity of bioactive plant extracts

Polyethylene glycol addition evinced the role of polyphenolic compounds in the AH-similar activity of most plants, restoring the exsheathment values similar to control groups (distilled water or TW 80 at 2%) (Table 4). However, non-reestablishment was achieved with ***L. leucocephala***-AC and ***G. sepium***-AC, discarding polyphenols as the bioactive compounds present in both acetonic extracts (Table 4).

DISCUSSION

Cooperia punctata infective larvae (L_3) were chosen for AH evaluations because their prevalence in grazing cattle is one of the highest worldwide (Hoglund ***et al***., 2013), and reports of their resistance to anthelmintic drugs are increasing (Sutherland and Leathwick 2011; Arnaud-Ochoa and Alonso Díaz 2012; Becerra-Nava ***et al***., 2014; Gasbarre 2014). One objective of this study was to assess the potential of ***L. leucocephala, G. sepium, G. ulmifolia***, ***C. argentea*** and ***A. indica*** at impairing the larval exsheathment of ***C. punctata*** infective larvae. The exsheathment process of ***C. punctata*** was fully inhibited with all fifteen extracts used. There are scarce reports where extracts from plants with potential use for cattle nutrition also had an AH effect against the cattle nematode ***C. punctata***. Previous studies reported that ***L. leucocephala***, ***G. sepium*** and ***C. argentea*** acetone:water (70:30) extracts inhibited or delayed the ***H. contortus*** L_3 exsheathment process at 1200 µg mL^{-1} (Alonso-Diaz ***et al***., 2008a; von Son-de Fernex ***et al***., 2012). This concurs with our findings, where at the highest concentrations the exsheathment process of ***C. punctata*** was inhibited. According to Hertzberg ***et al***. (2002), larvae are not able to infect their host when the exsheathment process is blocked. The latter might explain why the consumption of bioactive plants by small ruminants negatively affects the larval establishment of ***T. colubriformis*** or ***H. contortus*** (Brunet ***et al***., 2008). It is necessary to conduct ***in vivo*** trials to corroborate the AH effect against cattle nematodes with bioactive plants when are voluntary consumed by cattle. This information might help to build strategic/integrated mechanisms to control GINs in grazing cattle.

In this study, the best inhibitory values were observed with ***L. leucocephala*** (AQ and AW extracts), which inhibited the exsheathment of ***C. punctata*** at 150 µg mL^{-1}. Indeed, the best-fit LC_{50} were: 86.77 ± 48.12, 225.27 ± 57.50, 250.48 ± 11.45, and 270.73 ± 22.98 µg mL^{-1} from ***A. indica***-AC, ***C. argentea***-AC, ***G. sepium***-AQ and ***G. sepium***-AW, respectively. Novobilsky ***et al***. (2011) reported significant delays, but no full exsheathment inhibition by bioactive plants against ***Cooperia oncophora*** using similar concentrations, but different plant species. Differences could be associated to the phytochemical constituents in each extract and their affinity with certain structural molecules of GIN morphology (Martins ***et al***., 2011;

Hoste *et al*., 2012). Previous reports have emphasized tannins or polyphenolic compounds as responsible for the AH effect of some plant extracts. Thus, the second objective of this study was to state the role of tannins/flavonoids in plant extracts on the AH activity against *C. punctata*. Although phytochemical evaluations in the present investigation allowed for the identification of different phytochemical classes in plant extracts, the use of polyethylene glycol addition evinced the role of polyphenolic compounds in the AH-like activity of most plants, confirming that PEG is a specific tannin/flavonoid neutralizer (Makkar *et al*., 1995; Barrau *et al*., 2005). Nevertheless, it should also considered that for *G. sepium* and *L. leucocephala* acetonic extracts, PEG failed to restore inhibition values, suggesting the participation or co-participation of other phytochemical classes in the AH effect. Thin layer chromatography represents a low cost analytical tool for the qualitative phytochemical screening of plant extracts, which facilitates the separation and visualization of organic substances (Nicoletti, 2013). The qualitative phytochemical analysis allowed for the visualization of flavonoids as the most active phytochemicals inhibiting the *C. punctata* exsheathment process. Thus, further fractionation is suggested as it could provide important information for the elucidation of novel anthelmintic molecules.

When comparing extraction procedures to determine the best method for anti-exsheathment activity screening, the mean LC_{50} values (±SE) obtained were: 519.11 ± 176.29, 142.94 ± 104.04 and 479.43 ± 167.04 µg mL^{-1} for AQ, AW and AC extractions, respectively. For extraction methods, acetone:Water was the best procedure when screening for AH-like molecules. The results are consistent with von Son-de Fernex *et al*. (unpublished data) who reported the AW extraction procedure to be the best methodology when screening for ovicidal activity against *C. punctata*.

CONCLUSIONS

It is concluded that: i) the five tropical plants assessed against *C. punctata* have anti-exsheathment activity, ii) flavonoids are potent phytochemicals to inhibit *Cooperia*'s exsheathment process, and iii) acetone:water is the best extraction procedure for anti-exsheathment activity and to obtain higher yield percentages. Nevertheless, *Leucaena leucocephala* proved to be the forage with stronger anthelmintic-like flavonoids. Therefore, after *in vivo* confirmation, this legume could be considered a good alternative for inclusion into an integrated control strategy in tropical regions to reduce *C. punctata* establishment within hosts.

CONFLICT OF INTEREST STATEMENT

The authors of this manuscript have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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AUTHOR CONTRIBUTIONS

Elke von Son de Fernex conceived the study, performed all the plant extractions and assays, and wrote the manuscript.

Miguel Ángel Alonso Díaz performed the experimental design and helped with the manuscript redaction and revision.

Pedro Mendoza de Gives helped to isolate *C. punctata* isolation and revised the manuscript.

Braulio Valles de la Mora performed the statistical analysis of the experiments and revised the manuscript.

Alejandro Zamilpa designed the plant extraction and phytochemical evaluation methods and revised the manuscript.

Manases González Cortazar interpreted the results of the phytochemical screening and revised the manuscript.

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356 **TABLES**

357

358 Table 1. Yield percentage from of each plant species obtained through three extraction
359 procedures (aqueous, acetone:water, acetonic).

Plant material	Extraction Yield (%)		
	Aqueous	Acetone:water	Acetonic
L. leucocephala	6.408	7.312	1.335
C. argentea	6.444	9.791	2.673
G. sepium	5.742	9.533	1.013
G. ulmifolia	5.780	10.624	1.001
A. indica	6.548	12.947	1.491
mean±SE	6.18±0.17 [a]	10.04±0.91[b]	1.50±0.31[c]

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363 Table 2. Qualitative identification of phytochemicals present in the fifteen extracts.

Plant	Extract	Flavonoids	Coumarins	Hydroxycinnamic acids	Terpenoids	Un-identified
LL	Aqueous	+++		+++	+	
	70:30	+++		+++	+++	
	Acetonic	+++			++	
CA	Aqueous	+		+++	+	+
	70:30	+		++	++	++
	Acetonic			+	++	+
GS	Aqueous	++		+++	+	+
	70:30	++		+	+++	++
	Acetonic	++		+	++	+++
GU	Aqueous	+	+	++	+	
	70:30	++	+++	+	+	
	Acetonic	+	+++	+	+	+
AI	Aqueous	+++	+++	+++	++	
	70:30	++	+++	+	+	
	Acetonic	++	+++	+	+	

364 +++: visible under UV-254 or 365 nm, for terpenoids and flavonoids respectively; ++: viewed after reagent application; +: slightly seen
365 after reagent application. Unidentified compounds were not reactive to the reagents employed.
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Table 3. Lethal concentrations required to inhibit 50% of *C. punctata* exsheathment, after a 3 h incubation period with the bioactive extracts (µg/ml).

Plant species	Extraction		Probit analysis for *C. punctata* exsheathment inhibition (95.0% Normal CI) Mean	Std. Error	Limits	
					Lower	Upper
L. leucocephala	Aqueous	a	ND	ND	ND	ND
	70:30	a	ND	ND	ND	ND
	Acetonic	b	698.27	11082.6	-21023.3	22419.8
C. argentea	Aqueous	a	994.17	45.91	904.18	1084.17
	70:30	b	ND	ND	ND	ND
	Acetonic	c	225.27	57.50	112.57	337.98
G. sepium	Aqueous	a	250.48	11.45	228.04	272.92
	70:30	a	270.73	22.98	225.68	315.78
	Acetonic	b	377.37	4411.15	-8268.33	9023.08
G. ulmifolia	Aqueous	a	623.76	31.19	197.54	322.58
	70:30	b	309.47	544.92	-758.56	1377.5
	Acetonic	c	1009.46	40.89	929.32	1089.60
A. Indica	Aqueous	a	727.16	52.05	625.15	829.17
	70:30	b	332.34	40.20	253.55	411.14
	Acetonic	c	86.77	48.12	-7.54	181.08

* For each plant species, different letters in the same column represent statistically significant differences for the extraction procedures with the same plant. * ND: Not determined due to an abrupt exsheathment of larvae, except for LL.

Table 4. *Cooperia punctata* larval exsheathment (%) obtained with the highest concentration tested (2,400 µg/ml) among plant species and extraction procedures, with or without PEG addition.

Plant species	*Cooperia* spp., larval exsheathment (%)		
	Extraction	Exsheathment (%)	PEG (%)
L. leucocephala	Aqueous	0.00 ± 0.00 [a]	82.66 ± 1.03 [b]
	70:30	0.00 ± 0.00 [a]	79.24 ± 1.50 [b]
	Acetonic	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
C. argentea	Aqueous	0.00 ± 0.00 [a]	100.00 ± 0.00 [b]
	70:30	0.00 ± 0.00 [a]	100.00 ± 0.00 [b]
	Acetonic	0.00 ± 0.00 [a]	88.03 ± 1.60 [b]
G. sepium	Aqueous	0.00 ± 0.00 [a]	98.15 ± 1.04 [b]
	70:30	0.00 ± 0.00 [a]	89.03 ± 0.21 [b]
	Acetonic	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
G. ulmifolia	Aqueous	0.00 ± 0.00 [a]	100.00 ± 0.00 [b]
	70:30	0.00 ± 0.00 [a]	100.00 ± 0.00 [b]
	Acetonic	0.00 ± 0.00 [a]	98.48 ± 0.85 [b]
A. indica	Aqueous	3.37±1.20 [a]	98.48±0.85 [b]
	70:30	0.00±0.00 [a]	100.00±0.00 [b]
	Acetonic	0.00±0.00 [a]	100.00±0.00 [b]

* Larvae in the control group exsheathed at 100% for all plant species and extract procedures. * Different letters in the same row represent statistically significant differences ($P<0.05$).

FIGURES

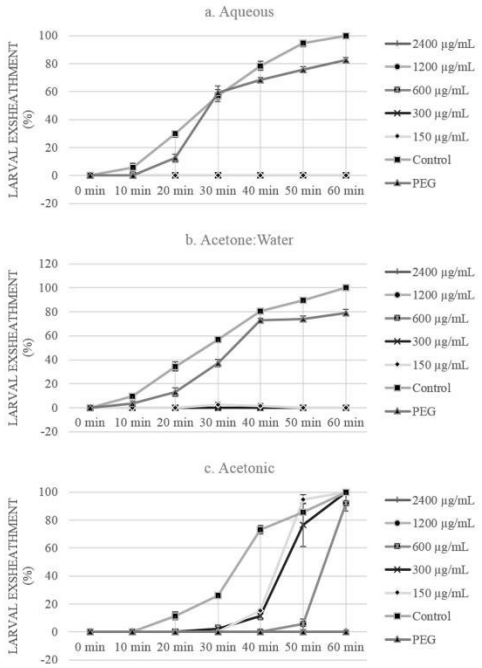


Figure 1 (a-c). Effect of different concentrations of three extracts of *Leucaena leucocephala* on the *Cooperia punctata* exsheathment process.

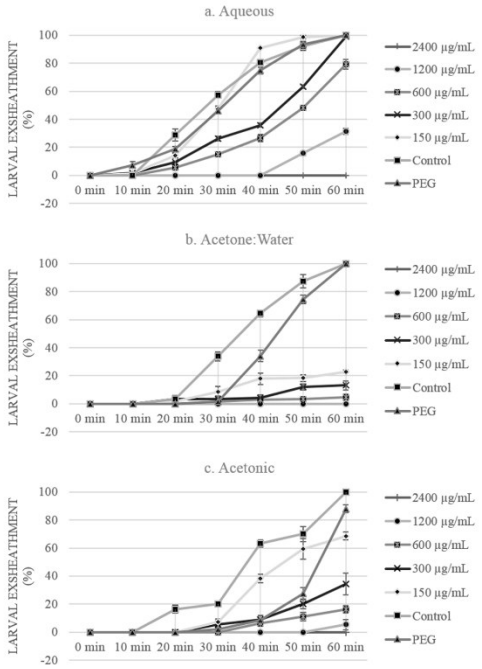


Figure 2 (a-c). Effect of different concentrations of three extracts from *Cratylia argentea* on the *Cooperia punctata* exsheathment process.

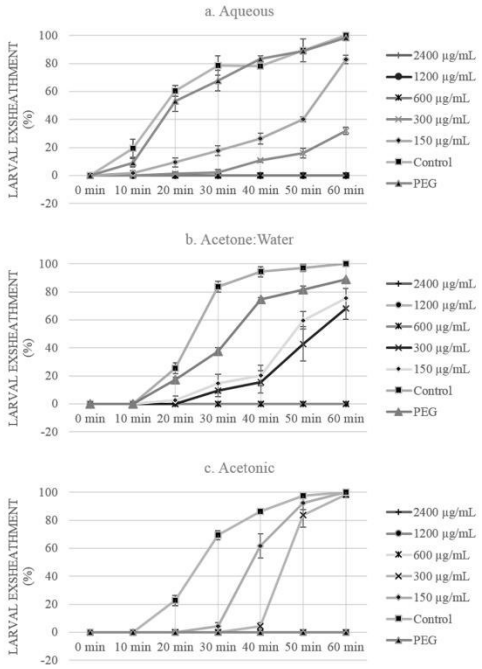


Figure 3 (a-c). Effect of different concentrations of three extracts of *Gliricidia sepium* on the *Cooperia punctata* exsheathment process.

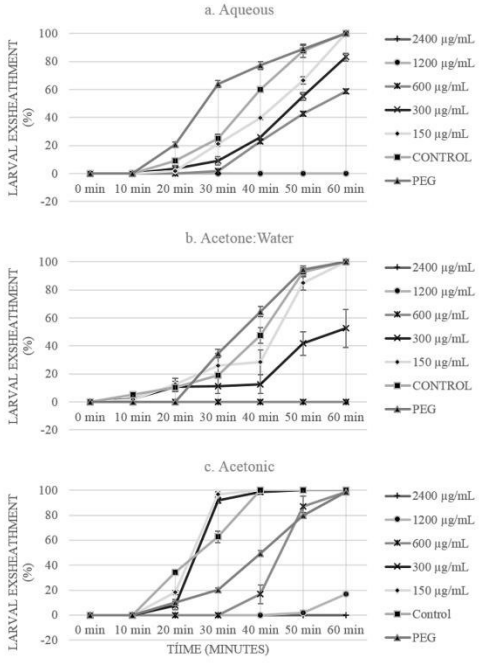
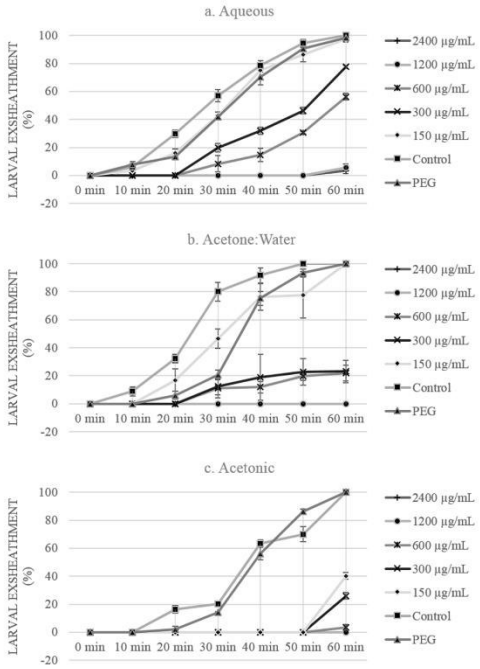


Figure 4 (a-c). Effect of different concentrations of three extracts from *Guazuma ulmifolia* on the *Cooperia punctata* exsheathment process.



401

402 **Figure 5 (a-c)**. Effect of different concentrations of three extracts of *Azadirachta indica* on
403 the *Cooperia punctata* exsheathment process.

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7. ARTÍCULO 2

Ovicidal activity of extracts from four plant species against the cattle nematode *Cooperia punctata*.

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Ovicidal activity of extracts from four plant species against the cattle nematode *Cooperia punctata*

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Additional information and declarations can be found on page 7

Abstract

Bioactive plants might represent an alternative for *Cooperia punctata* control in grazing cattle. The objectives of this study were (1) to assess the ovicidal activity of extracts from 4 plant species against *C. punctata*, (2) to determine the role of the polyphenols in the plants' anthelmintic (AH) activity, and (3) to evaluate the best plant extraction procedure when searching for ovicidal activity. The egg hatch assay was used with different extraction procedures, aqueous (AQ), acetone:water (AW) and acetonic (AC), to evaluate the ovicidal activity of *Leucaena leucocephala*, *Glinicidia sepium*, *Guazuma ulmifolia* and *Cratylia argentea*. Eggs of *C. punctata* were exposed in quadruplicate to 0.6, 1.2, 2.4, 4.8 and 9.6 mg mL^{-1} of each plant extract. The roles of the polyphenols were assessed using polyethylene glycol (PEG). The 12 plant extracts inhibited egg hatching in a dose-dependent manner. Best-fit LC_{50} values were 1.03 ± 0.17 and 7.90 ± 1.19 mg mL^{-1} for *G. sepium*-AC and *L. leucocephala*-AQ, respectively. Differences in AH activity were found among the extraction procedures ($P < 0.05$). At the highest concentration, *L. leucocephala*-AQ inhibited more than 50% of *C. punctata* hatching. The *G. sepium*-AC extract fully inhibited egg hatching. The addition of polyethylene glycol revealed the role of the polyphenols in the bioactivity of most plant extracts; however, for *G. sepium*-AC, the polyphenols were not the main bioactive compounds. Overall, acetone:water extraction represented the best extraction procedure to obtain both ovicidal activity and higher yield. The inhibition rates suggested that *L. leucocephala* and *G. sepium* should be evaluated as a means of reducing larval density in pastures.

Keywords: Cattle; Nematodes; *Cooperia punctata*; Ovicidal effect; Plant extracts.

Introduction

Gastrointestinal nematodosis has been ranked as the main endemic parasitic disease in cattle production units (Fitzpatrick, 2013), reducing the productivity and health of livestock (Charlier *et al.*, 2009; Perri *et al.*, 2011). Among the gastrointestinal nematodes (GIN) that affect cattle, *Cooperia* spp. have been highlighted as the nematodes with higher prevalence in grazing cattle around the world (Kenyon

and Jackson, 2012; Stromberg *et al.*, 2012). These parasites decrease the amount of dry feed consumed and nutrient uptake or utilization (Stromberg *et al.*, 2012). Broad-spectrum anthelmintics (AHs) are a suitable tool for the control of GIN, and the application of these anthelmintics enhances the productivity and performance of animals (Sutherland and Leathwick, 2011). Unfortunately, resistance has become an emerging problem among cattle nematodes (Gasbarre, 2014). Recent studies have reported the emergence of *Cooperia* spp. strains resistant to macrocyclic lactones (ML) (Bartley *et al.*, 2012), benzimidazoles (Arnaud-Ochoa and Alonso Díaz, 2012) and imidazothiazoles (Becerra-Nava *et al.*, 2014). Thus, novel approaches for helminth control in cattle are required before GIN becomes a major problem due to the spread of highly resistant and multi-resistant strains among farms.

One of the most studied novel approaches has been the use of bioactive plants having anthelmintic effects (Hoste *et al.*, 2012). *In vitro* and *in vivo* studies have shown the anthelmintic effect of some plants when using GIN from small ruminants as models (Alonso-Díaz *et al.*, 2008a; Alonso-Díaz *et al.*, 2008b; Alonso-Díaz *et al.*, 2010; Martínez-Ortíz-de-Montellano *et al.*, 2010; von Son-de Fernex *et al.*, 2012), whereas little research has been carried out with cattle nematodes.

Tropical browse legumes are one of the most studied forages due to their high content of plant secondary metabolites (PSMs), along with their benefits, which are obtained from their nutritional quality. Anthelmintic activity of PSMs has been mainly associated with the presence of tannins due to their capacity to interrupt specific nematode life-stages, such as inhibiting egg hatching, larval development, larval motility and larval exsheathment (Molan *et al.*, 2000; Athanasiadou *et al.*, 2001; Alonso-Díaz *et al.*, 2008b; von Son-de Fernex *et al.*, 2012). The AH effect from tannins is related to the ability of the tannins to create chemical bonds with structural proteins present in nematodes morphology (Hoste *et al.*, 2012). However, few reports have identified other bioactive molecules such as flavonol glycosides, flavones and sesquiterpene lactones as being involved in AH effects (Molan *et al.*, 2003; Barrau *et al.*, 2005; Kozan *et al.*, 2013).

Over the last decade, multiple extraction procedures that use different solvents and mixtures for phytochemical extractions have been standardized for *in vitro* AH evaluations. As condensed tannins (CTs) have been the most targeted compounds for investigation, the system proven most efficient for CT recovery is the mixture of acetone:water (Chavan *et al.*, 2001; Chavan and Amarowicz, 2013), although different extraction methods (oleaginous, ethyl acetate, aqueous and acetonic) have also shown bioactivity against different parasites (Katiki *et al.*, 2011; Botura *et al.*, 2013; Kozan *et al.*, 2013). The evaluation of different extraction procedures for tropical plants might help to standardize extracts with possible AH effects against cattle nematodes and to identify the phytochemical classes present. The objectives of this study were (1) to assess the ovicidal activity of extracts from four plant species against *C. punctata*, (2) to state the role of polyphenols in the plants' anthelmintic activity, and (3) to evaluate the best plant extraction procedure when searching for ovicidal activity.

Materials and methods

Plant material

Fresh leaves of *Leucaena leucocephala*, *Cratylia argentea*, *Gliricidia sepium* and *Guazuma ulmifolia* were harvested during March 2013 from an experimental area located at the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical (Center 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 University of Mexico), located in Martínez de la Torre (20°03' N y 97°03' O; 151 m above sea level), Veracruz, Mexico. These plants were chosen because they have high levels of secondary plant metabolites, and some of the plants have been reported to exhibit AH activity against GIN of small ruminants (**Alonso-Díaz *et al.*, 2008**; von Son-de Fernex *et al.*, 2012). Furthermore, these plants are predominant within the native vegetation of Veracruz and are also distributed in other tropical areas of the world (Flores-Guido, 2001). These fodder trees and shrubs are an important nutritional alternative for animal production.

Extraction procedure

For each plant species, 1 ± 0.15 kg of fresh leaves were air-dried at 60 °C for 72 h and then placed in a grinder to obtain particles of 1 mm in size. This material was then placed in a glass beaker (2 L) containing acetone:water (70:30) with a magnetic stirrer. The mixture was then sonicated for 4 h in a water bath (Branson Sonicator 2510MT®, Emerson Industrial Automation, Danbury, USA). The second extraction was performed by placing 500 ± 36.97 g of dried ground material from each plant species in a mixer with acetone and maintained at room temperature (24 °C) for 24 h. Finally, an aqueous extraction was performed, using the same ground material used for the acetonic extraction, which, after being dried, was placed in distilled water previously heated at 58 °C for 2 hours. For all extraction procedures, the extract was obtained from the filtered material using filter paper (Whatman® qualitative filter paper, Grade 1). Solvents were evaporated from the extracts at 58 °C using a low pressure distillation procedure in a rotovapor machine (Rotovapor® R-3, Büchi®, Switzerland). Extracts were washed 4 times with 500 mL of n-hexane to remove the chlorophyll and lipids, and a separation funnel was used for discarding the n-hexane fraction. Before the n-hexane fraction was discarded, a qualitative chromatographic profile was performed to confirm that only chlorophyll and lipids were removed. Finally, extracts were frozen and lyophilized to obtain the dry ground extracts.

Bioassays

Egg recovery

Eggs were obtained from a donor calf infected with the *C. punctata* strain multi-resistant towards macrocyclic lactones, benzimidazoles and imidazothiazoles (CEI-EGT-FMVZ-UNAM strain, Mexico). Adult males of *C. punctata* were identified using taxonomic keys (Gibbons, 1981) and molecular techniques (**von Son de Fernex, unpublished data**). Calves were housed indoors on a concrete floor, provided with hay and commercial concentrate and allowed free access to water. Feces were

collected daily using harnesses and polyurethane collection bags; samples were stored and processed at temperature of 23.37 ± 0.21 °C (mean ± SE). Tap water (1 L) was added to 200 g of feces with a fecal egg count (FEC) of 150 eggs per gram of feces (EPGF) and mixed to produce a relatively liquid suspension. Liquid feces were filtered through a household sieve with a 400-µm mesh size to remove coarse plant debris. Then, the suspension was serially filtered through sieves with pore sizes of 1000, 149 and 74 µm, with the eggs finally being trapped on a 24 µm mesh. The material on the 24-µm mesh was washed into 50-mL centrifuge tubes, which were filled with a saturated NaCl solution and centrifuged at 3000 rpm for 15 min. The supernatant was washed in tap water through a 24-µm mesh sieve, on which the eggs were collected. Clean eggs were concentrated and placed in 15-mL centrifuge tubes for counting. The egg concentration was estimated by counting the number of eggs in aliquots of 10 % of the suspension on a microscope slide. A final concentration of 500 eggs/mL was achieved either by concentrating the egg suspension through centrifugation or by diluting it with distilled water. The egg recovery process was standardized for completion in 1.25 ± 0.08 hours (mean ± SE).

Egg hatching assay (EHA)

Approximately 100 eggs/200 µL of egg suspension were pipetted into each well of a 24 well culture plate, and 200 µL of increasing concentrations (1.2, 2.4, 4.8, 9.6 and 19.2 mg mL^{-1}) of the corresponding plant species extract were placed in each test well. Thus, we obtained final concentrations of 0.6, 1.2, 2.4, 4.8 and 9.6 mg mL^{-1}. Levamisole was used as a positive control at a concentration of 10 % to equal the highest plant extract concentration (**Dobson *et al.*, 1986**). Distilled water was employed as a negative control for the 70:30 and aqueous extracts; whereas 2.5 % dimethyl sulfoxide (DMSO) was used for acetonic plant extracts (because it was employed as a low/non-polar compound solvent for the bioassays). Control wells also contained 200 µL of the egg suspension. Four replicates were run for each dose, extract and control. The plates were incubated at 27.7 ± 0.1 °C (mean ± SE) for 48 h. A drop of Lugol's iodine solution was added to each well to stop further hatching, and all the unhatched eggs and larvae (dead or alive) in each well were counted (Coles *et al.*, 1992). To confirm the role of polyphenolic compounds in an AH effect, another series of incubations was performed for 3 treatments: i) negative control (distilled water or DMSO 2.5 %), ii) the maximum dose of the extract to be tested (9.6 mg of extract/mL) with PEG (19.2 mg mL^{-1}) and a pre-incubation period of 3 h to bind the polyphenolic compounds (before egg exposure), and iii) the maximum dose of incubation without PEG (Makkar *et al.*, 1995).

Statistical analysis

A General Lineal Model (GLM) was used to assess a dose-dependent behaviour within each plant species extract ($Yij = \mu + Tj + Eij$), where the dependant variable was the egg hatching (Yij), which represents the *i*th observation taken under the *j*th treatment; the independent variable was the increasing concentration of each plant extract (Tj); μ represents the general mean; and Eij represents the residual variation or experimental error. Treatment means comparisons were performed with a Least Significant Difference (LSD) test, and the probability value indicative of statistical

significance was $P < 0.05$ (F-test). No transformation was required because the data had normal distribution and homoscedasticity (STATGRAPHICS, Centurion XVI version 16.1.18, USA). A Kruskal-Wallis test was used to i) compare the egg hatching rates obtained for each plant species extract with and without PEG addition, ii) compare the egg hatching rate among extraction procedures within each plant species, and iii) evaluate extract yields among extraction procedures. Kruskal-Wallis test was employed when assumptions of ANOVA analysis did not met. The probability value indicative of statistical significance was $P < 0.05$ (H-test).

The percentage of egg hatching inhibition (EHI) was calculated using the following formula: Inhibition (%) = 100 $(1 - P_t / P_c)$,where 1 represents the total number of eggs, P_t is the number of eggs hatched in a treatment group, and P_c is the respective number in water or DMSO control groups (Bizimenyera *et al.*, 2006). The lethal concentration to inhibit 50 % of egg hatching (LC_{50}) was calculated for each extract using a Probit Analysis Program (Minitab® 17.1.0, Minitab Inc., USA).

Results and discussion

Egg hatching assay (EHA)

The mean egg hatching (± SE) of *C. punctata* in negative and positive control groups ranged from 92.48 ± 1.97 % to 95.29 ± 0.76 % and 31.17 ± 4.69 % to 35.49 ± 4.37 %, respectively. Egg hatching showed a dose-dependent behaviour when exposed to each of the 12 extracts ($P < 0.01$) (Figures 1 to 3). *Leucaena leucocephala*-AQ inhibited more than 50 % of the *C. punctata* egg hatching ($P < 0.05$; $r^2 = 69.51$ %) (Figure 1). For *G. ulmifolia* and *C. argentea*, the highest inhibition rate was obtained with the AW extraction procedure: 45.42 ± 2.3% ($P < 0.01$; $r^2 = 95.71$ %) and 35.07 ± 1.40 % ($P < 0.01$; $r^2 = 97.46$ %), respectively (Figure 2). At the highest concentration, the *G. sepium*-AC fully inhibited hatching of the *C. punctata* eggs ($P < 0.01$; $r^2 = 94.42$ %) (Figure 3). *Cooperia* spp. is responsible for one of the GINs with higher prevalence in grazing cattle. Increasing reports of nematode resistance to chemotherapy notes the need to develop effective and secure strategies of control (Bartley *et al.*, 2012; Demeler *et al.*, 2013). This work provides evidence of the ovicidal effect of bioactive plant extracts against the egg and free-living stages of *C. punctata*. Reports on the novel technologies available to control free-living stages of cattle nematodes are scarce (Novobilsky *et al.*, 2011). Previous *in vitro* assessments have shown temperate legumes to be active against the infective larvae of *C. oncophora* (Novobilsky *et al.*, 2011), but few reports exist on the novel technologies available for other free-living stages such as egg hatching. The nematode egg is a GIN biological stage with a relatively thick tri-layered shell (Mansfield *et al.*, 1992), which provides resistance to adverse environmental conditions (temperature, moisture, UV radiation and trampling). These characteristics complicate the development of effective control strategies.

The lethal concentrations required for 50 % of hatching inhibition calculated for all 12 extracts are presented in Table1. Best-fit LC_{50} values were 1.03 ± 0.17 and 7.9 ± 1.19 mg mL^{-1} for *G. sepium*-AC and *L. leucocephala*-AQ, respectively. For the AW extracts, the LC_{50} ranged from 8.84 to 15.12 mg mL^{-1}. The significant dose-dependent effect observed for most of the plant extracts indicates a toxicological response of the phytochemicals present in the 4 plant species evaluated (Hoste *et*

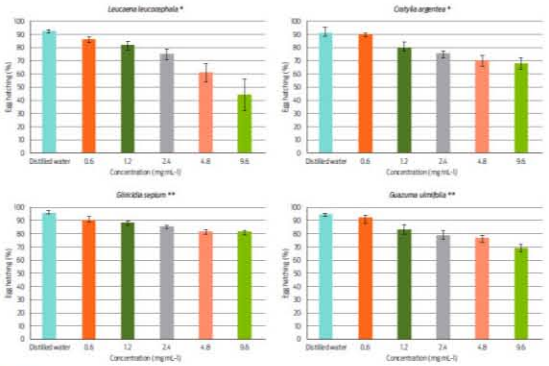

Figure 1. *Cooperia punctata* egg hatching after incubation in aqueous plant extracts (* P < 0.05; ** P< 0.01).

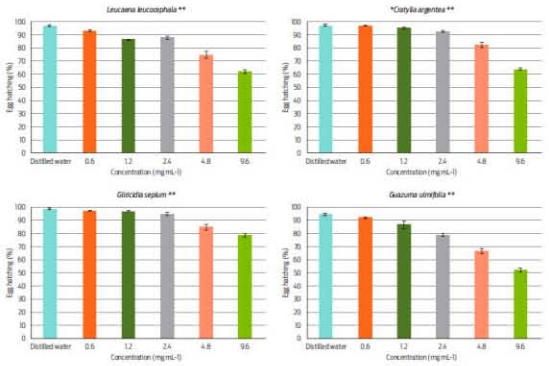

Figure 2. *Cooperia punctata* egg hatching after incubation in acetone:water plant extracts (* P < 0.05; ** P< 0.01).

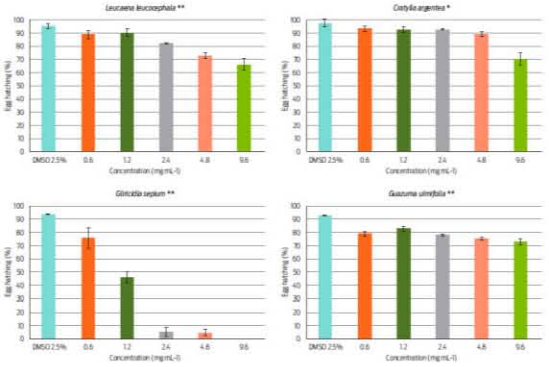

Figure 3. *Cooperia punctata* egg hatching after incubation in acetonic plant extracts (* P < 0.05; ** P< 0.01).

Table 1. Lethal concentrations required to inhibit 50% of *Cooperia punctata* egg hatching (LC_{50}), after a 48-h incubation period with bioactive extracts (mg mL^{-1}).

Probit analysis (LC_{50}) for *C. punctata* egg hatching inhibition (95% Normal CI)					
Plant material	Extraction	Mean	Std. Error	Limits	
				Lower	Upper
L. leucocephala	Aqueous	7.93	1.19	5.59	10.27
	70:30	11.77	1.19	9.42	14.12
	Acetonic	13.84	1.19	11.51	16.16
C. argentea	Aqueous	15.05	4.44	6.34	23.75
	70:30	11.31	0.79	9.78	12.86
	Acetonic	14.79	2.51	9.88	19.71
G. sepium	Aqueous	32.63	16.41	0.47	64.79
	70:30	15.12	1.86	11.47	18.76
	Acetonic	1.03	0.17	0.69	1.37
G. ulmifolia	Aqueous	16.41	5.35	5.92	26.89
	70:30	8.84	0.89	7.09	10.59
	Acetonic	29.08	9.31	10.83	47.34

al., 2012). However, further studies are necessary for the identification and isolation of the AH-like molecules present in the most active extracts. Phytochemical identification could help to understand the ongoing mechanisms involved in their activity.

Role of polyphenols in the ovicidal activity of bioactive plant extracts

The addition of polyethylene glycol revealed the role of polyphenolic compounds in the ovicidal activity of most plant extracts, restoring egg hatching to values similar to those for control groups (distilled water or DMSO 2.5 %) (Table 2). However, almost no reestablishment was achieved with *G. sepium*-AC (EHI of 79.85 ± 1.2 %), discarding polyphenols as the main bioactive compound present in those extracts (Table 2). The hydrophilic polymer PEG was utilized to test for the role of polyphenols in the extracts regarding bioactivity (Makkar, 2003). When PEG was added, the inhibition values of most extracts were restored to values similar to those obtained with negative controls indicating the predominate role of polyphenols (Table 2). Nevertheless, inhibition values of 79.85 ± 1.2 % were obtained after PEG addition for *G. sepium*-AC, which suggests the possible involvement of other phytochemicals in the ovicidal activity of the acetonic extracts. Thin layer chromatography analysis is one of the most frequently used techniques when evaluating herbal medicines for the identification and differentiation of phytochemical classes (Rafi *et al.*, 2011). The chromatographs obtained for each plant extract, as well as the use of PEG, suggest that medium-polar flavonoids have ovicidal activity against *C. punctata*. This is in agreement with previous authors who have reported flavonols, such as quercectin, rutin and kempherol, as having AH properties (Barrau *et al.*, 2005). On the other hand, fingerprint analysis of *G. sepium*-AC extract show the predominant constituent to be a low polar phytochemical visible under UV

Table 2. Egg hatching inhibition (EHI) obtained with the highest concentration tested (9.6 mg mL^{-1}) with or without addition of polyethylene glycol (PEG), among plant species and extraction procedures. Different small letters in the same row and different capital letters amongst extraction procedures of the same plant, represent statistically significant differences ($P < 0.05$).

Plant material	Extraction	Cooperia punctata egg hatching inhibition (%)	
		EHI (%)	PEG EHI (%)
L. leucocephala	Aqueous	52.01 ± 12.4 ^{a}	12.59 ± 2.5 ^{b}
	70:30	35.75 ± 1.53 ^{a}	0.82 ± 0.90 ^{b}
	Acetonic	29.80 ± 1.50 ^{a}	2.76 ± 1.50 ^{b}
C. argentea	Aqueous	25.73 ± 4.10 ^{a}	5.86 ± 3.40 ^{b}
	70:30	35.07 ± 1.40 ^{a}	0.16 ± 0.20 ^{b}
	Acetonic	26.76 ± 4.90 ^{a}	0.67 ± 1.60 ^{b}
G. sepium	Aqueous A	15.40 ± 1.40 ^{a}	1.78 ± 0.90 ^{b}
	70:30 A	19.59 ± 1.70 ^{a}	2.40 ± 0.50 ^{b}
	Acetonic B	100.0 ± 0.00 ^{a}	79.85 ± 1.2 ^{b}
G. ulmifolia	Aqueous A	27.26 ± 3.40 ^{a}	2.48 ± 3.10 ^{b}
	70:30 B	45.42 ± 2.30 ^{a}	0.95 ± 1.20 ^{b}
	Acetonic A	21.46 ± 3.00 ^{a}	1.59 ± 1.50 ^{b}

short wave (254 nm) but non-reactive when sprayed with AS and NEU reagents. Additionally, PEG failed to restore egg hatching inhibition to control values (79.85 ± 1.2 %), thus supporting the suggestion of a non-flavonoid phytochemical with AH-like activity but disagreeing with Wabo Poné *et al.* (2011), who linked the role of CT in *G. sepium*-AC extracts to *H. contortus* egg hatching inhibition. It was not possible to elucidate the phytochemical involved in the AH effect in the present study; however, this information could be helpful to a better understanding of the possible mechanisms of action on *C. punctata*.

Plant extract yields

Phytochemical extraction showed yield differences among the extraction procedures ($P < 0.05$). The AW extraction provided a yield of 10.04 ± 0.91 % (Mean ± SE). Individual yield percentages are shown in Table 3. In this study, extraction procedures were also compared based on their ovicidal activity. Best inhibitory values were observed using uni-solvent extractions (*G. sepium*-AC and *L. leucocephala*-AQ), leading to the perception that compounds with similar polarity features could enhance bioactivity. Yet the overall performances of each extraction procedure, assessed through LC_{50}, were 16.64 ± 4.27 (Mean ± SE), 12.41 ± 1.19 and 13.49 ± 4.59 mg mL^{-1} for AQ, AW and AC, respectively (Table 3). Furthermore, among the extraction procedures, AW showed the highest yield percentage ($P < 0.05$).

Analyses in this investigation allowed for the determination of both i) flavonoids having ovicidal activity and ii) flavonoids predominant in the AW extractions. The latter is consistent with previous studies that report the acetone:water extraction as the most efficient system for phenol recovery (Chavan *et al.*, 2001; Chavan and Amarowicz, 2013). Previous phytochemical studies have reported a synergistic/antagonistic effect among components from the same extract (Biavatti, 2009),

Table 3. Phytochemical yield of 4 plant materials using 3 extraction procedures (aqueous, acetone:water, and acetonic). Different letters in the means of each extraction procedure represent statistically significant differences ($P < 0.05$).

Plant material	Extraction yield (%)		
	Aqueous	Acetone:water	Acetonic
L. leucocephala	6.40	7.31	1.33
C. argentea	6.44	9.79	2.67
G. sepium	5.74	9.53	1.01
G. ulmifolia	5.78	10.62	1.00
Mean ± SE	6.09 ± 0.17 [a]	9.31 ± 0.91 [b]	1.50 ± 0.31 [c]

which could explain how the *G. sepium*-AC extract showed an exceptional ovicidal performance, but when the extractions were performed with acetone:water, the bioactivity was barely noticeable. Thus, such data trends were only observed in one of the four plants analyzed, *G. sepium*, and overall, the AW extraction showed equal or improved AH activity. However, further bio-guided phytochemical fractionation is suggested for the determination of the active molecules present in each plant extract and their interactions.

Conclusions

The present investigation corroborated the ovicidal potential of acetone:water plant extracts against *C. punctata*. The use of PEG indicated that polyphenolic compounds were the main phytochemical class involved in the AH activity. *Leucaena leucocephala* and *Glinicidia sepium* were the forages with the strongest anthelmintic-like phytochemicals, and they could be considered for further *in vivo* evaluations.

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Conflicts of interest

Miguel Ángel Alonso Díaz is the director of Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical from Facultad de Medicina Veterinaria y Zootecnia. The rest of the authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Author contributions

Elke von Son de Fernex conceived the study, performed all the plant extractions and assays, and wrote the manuscript.

Miguel Ángel Alonso Díaz performed the experimental design and helped with the manuscript redaction and revision.

Pedro Mendoza de Gives helped isolate *C. punctata* and revised the manuscript.

Braulio Valles de la Mora performed the statistical analysis of the experiments and revised the manuscript.

Alejandro Zamilpa designed the plant extraction and phytochemical evaluation methods and revised the manuscript.

Manases González Cortazar interpreted the results of the phytochemical screening and revised the manuscript.

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8. ARTÍCULO 3

Veterinary Parasitology 214 (2015) 89–95

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Elucidation of *Leucaena leucocephala* anthelmintic-like phytochemicals and the ultrastructural damage generated to eggs of *Cooperia* spp.

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ABSTRACT

Leucaena leucocephala is a tropical forage legume suggested as an alternative method to control gastrointestinal parasitism in ruminants. This study: (1) performed a bio-guided fractionation of an aqueous extract of *L. leucocephala* using the egg hatch assay (EHA) to identify the anthelmintic (AH)-like phytochemicals present in fresh leaves, and (2) assessed the ultrastructural damage to eggs of *Cooperia* spp. after incubation with the final fraction. Phytochemicals were isolated using silica gel columns and identified using high performance liquid chromatography and standards for comparison. The final fraction was evaluated using EHA at 0.06, 0.125, 0.250, 0.500 and 1.1 mg ml $^{-1}$. The lethal concentration to inhibit 50% of *Cooperia* spp. egg hatching (LC_{50}) was calculated using a Probit analysis. Scanning and transmission electron microscopy revealed the ultrastructural changes present in *Cooperia* spp. eggs. Bio-guided isolation procedures led to the recognition of an active fraction (LIC1F3) mainly composed of quercetin (82.21%) and caffeic acid (13.42%) which inhibited 90.49 ± 2.8% of *Cooperia* spp. egg hatching ($P < 0.05$), and an LC_{50} of 0.06 ± 0.14 mg ml $^{-1}$. Scanning electron microscopy (SEM) showed eggs exposed to the active fraction had an irregular external layer with small projections and ruptures of lateral eggshell walls. Transmission electron microscopy (TEM) showed changes to *Cooperia* spp. eggs in electro-density, including the thickness of the eggshell layers and fractures after incubation with the final fraction (LIC1F3).

Changes in bioactivity after purification suggest synergistic interactions between quercetin and caffeic acid.

1. Introduction

Cooperia spp. is an endemic genus of gastrointestinal nematodes (GINs) of grazing cattle in tropical and subtropical environments. These parasites have recently been reported as one of the most prevalent group of GINs in grazing cattle worldwide (Fiel et al., 2012; Stromberg et al., 2012; Vlaminck et al., 2015). The negative impact of *Cooperia* spp. is due to the high costs of control schemes and the decreased productivity of cattle (Stromberg et al., 2012). Broad-spectrum anthelmintics (AHs) are a suitable tool for the control of GINs, which also enhance the productivity and performance of other animals (Sutherland and Leathwick, 2011). However, the increasing presence of *Cooperia* spp. strains resistant to broad-spectrum AHs on cattle farms (Bartley et al., 2012; Becerra-Nava et al., 2014; Njue and Prichard, 2004) supports the development of novel treatments to control GIN populations inside and outside hosts. The use of bioactive plants as a source of secondary metabo-

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lites with AH-like properties represents a viable alternative (Hoste et al., 2006).

The tropical forage *Leucaena leucocephala* (Lam.) de Wit is a member of the family Fabaceae. It is a perennial browse legume native to Central America and southern Mexico, and has been introduced into most tropical and subtropical areas of the world (Nehdi et al., 2014). *L. leucocephala* preparations have been used in traditional medicine mainly for its anti-inflammatory and anti-diabetic activities (Souza Pinto et al., 1995; Syamsudin et al., 2010). Furthermore, *L. leucocephala* has been reported to have AH properties against some of the most important GINs of small ruminants, involving tannins/polyphenols as the phytochemical group responsible for the AH effect (Ademola et al., 2005; Alonso-Díaz et al., 2008). Similar results were found against cattle nematodes where *L. leucocephala* aqueous extracts inhibited both egg hatching and the exsheathment process of *Cooperia* spp. (von Son-de Fernex et al., unpublished data), and flavonoids/polyphenols were identified as the most important compounds involved in the AH effect. Nevertheless, in the latter study, the participation or co-participation of other phytochemical classes in the active extract, especially on the exsheatment process of *Cooperia*, were suggested.

There are limited reports identifying the AH-like phytochemicals present in *L. leucocephala*, but to our knowledge there are no reports targeting cattle nematodes. Therefore, bio-guided fractionation of *L. leucocephala* leaves due to its ovicidal activity against *Cooperia* spp. might help to elucidate and evaluate the phytochemical involved in the AH effect to better understand the possible mechanisms of action on *Cooperia* spp. It has been suggested that the AH effect of phytochemicals such as tannins, might be linked to direct interactions between polyphenols and the structural proteins in the nematode cuticle (Hoste et al., 2006; Brunet et al., 2011). However, there are no studies assessing the ultrastructural damage created by *L. leucocephala* phytochemicals on the free living stages of *Cooperia* spp.

This study: (1) performed a bio-guided fractionation of an aqueous extract from *L. leucocephala* using the EHA to identify the AH-like phytochemicals present in fresh leaves, and (2) assessed the ultrastructural damage to *Cooperia* spp. eggs after incubation with the final fraction.

2. Materials and methods

2.1. Plant material

Fresh leaves of *L. leucocephala* (1865 g) were harvested during February 2014 from an experimental area located at the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical, de la Facultad de Medicina Veterinaria y Zootecnia, de la Universidad Nacional Autónoma de México (CEIEGT-FMVZ-UNAM) (Centre for Research, Teaching and Extension in Tropical Livestock, of the Faculty of Veterinary Medicine, of the National Autonomous University of Mexico), located in Martínez de la Torre (20°03′ N and 93°03′ W; 151 masl), Veracruz, Mexico.

2.2. Extraction procedure

Fresh leaves were air-dried at 60 °C for 72 h and placed in a grinding mill to obtain smaller particles (1 mm). Air-dried powdered material (404 g) was placed in a glass beaker with distilled water (previously heated to 58 °C) and a magnetic stir-bar, and was continuously sonicated for 4 h in a water bath (Branson Sonicator 2510®; 40 KHz). The extract was separated from the solid material using filter paper (Whatman® qualitative filter paper, Grade 1), and the solvent was evaporated from the extracts at 58 °C using low pressure distillation in a rotovapor machine (Rotovapor® R-3). Extracts were washed four times with 500 ml of *n*-hexane to remove chlorophyll and lipids, and a separation funnel was used for discarding the *n*-hexane fraction. Finally, extracts were frozen and lyophilized to obtain the dry ground extracts.

2.3. Chemical fractionation of the aqueous extract using the egg hatch assay

The aqueous extract (2.77 g) was fractioned through a bipartition process using ethyl acetate and water, obtaining a low-polarity fraction (LIC'-AcoET, organic) and a polar fraction (LIA'-Aq, aqueous). The fraction LIC'-AcoET (1.6 g) was subjected to further fractionation using a silica gel column, eluted with dichloromethane and methanol with an ascending polarity of 5% resulting in five main fractions (LIC1F1, 35.3 mg; LIC1F2, 48.9 mg; LIC1F3, 500.0 mg; LIC1F4, 409.9 mg and, LIC1F5, 42.0 mg). The best AH value was obtained with LIC1F3, which was further analyzed using high performance liquid chromatography (HPLC) (Section 2.4.2). Due to the identification of a compound mixture, LIC1F3 was suspended in dichloromethane resulting in two final fractions (soluble fraction LIC1F3A and precipitate fraction LIC1F3B) for which AH evaluations also were performed and were analyzed using HPLC. Prior to all AH evaluations performed during the experiment, fractions were concentrated to dryness using low-pressure distillation in a rotovapor machine (Rotovapor® R-3), and then were lyophilized. The EHA was used to perform the bio-guided fractionation of *L. leucocephala* because in previous studies the exsheathment process showed higher sensitivity to plant extracts.

2.4. Phytochemical identification

2.4.1. Thin layer chromatography analysis of fractions obtained through isolation procedures

Qualitative phytochemical screening of *L. leucocephala* fractions was performed in silica gel 60 F_{254}-pre-coated thin layer chromatography (TLC) plates using capillary tubes, and developed in a TLC chamber using suitable mobile phases for either terpenoids or flavonoids. The developed TLC plates were air-dried and observed under ultraviolet light at both 254 nm and 365 nm. They were later sprayed with natural products-polyethylene glycol reagent and anisaldehyde–sulphuric acid reagent, for flavonoids and terpenoids, respectively (Wagner and Bladt, 1996). Finally, the plates were placed in a hot air oven for 1 min for the development of color in separated bands. Qualitative determination of compounds was performed according to the colorimetric standards established by Wagner and Bladt (1996). Retention factors (Rf) of phytochemicals were obtained using the formula: Rf = MDP/MDS, where MDP and MDS represent the migration distance of the phytochemical and of the solvent, respectively. Qualitative TLC analysis was employed starting with the aqueous crude extract, leading up to the final fractions in order to perform an accurate fractionation procedure.

2.4.2. High performance liquid chromatography analysis of fractions obtained through isolation procedures

High performance liquid chromatography (HPLC) was used for the final fractions analysis, using a Waters® 2695 separation-module HPLC system equipped with a Waters® 996 photodiode array detector and Empower Pro software (Waters Corporation®, USA). Compounds were separated on a supersphere 100 RP-18 column (4 × 125 mm, 5 μm) (Merck, Darmstadt, Germany). The mobile phase consisted of water (5% TFA, solvent A) and acetonitrile (solvent B). The gradient system was: 0–1 min, 0% B; 2–4 min, 10% B; 5–7 min, 20% B; 8–14 min, 30% B; 15–18 min, 40% B; 19–22 min, 80% B; 23–26 min, 100% B, and 27–28 min, 0% B. Flow rate was maintained at 1 ml min^{-1} and the injection volume was 20 μL. Absorbance was measured at 360 nm. Flavonoid peaks

were identified by comparison of retention times (RT) and ultraviolet spectra with those of commercial reference compounds. The RT for quercetin was 11.3 min (λ = 205, 250 and 360 nm), caffeic acid 8.25 min (λ = 205 nm) and scopoletin 5.278 min (λ = 325 nm). Percentages of phytochemicals present in the mixture were estimated by interpolation of peak areas. Results were expressed as percentages.

2.5. Parasitological techniques

2.5.1. Egg recovery

Cooperia spp. eggs were obtained from two donor calves with a mono-specific infection (CEIEGT-FMVZ-UNAM strain, Mexico), which were housed indoors on a concrete floor, fed hay and commercial concentrate, and had free access to water, practices complying with regulations of the Comité Institucional para Cuidado y Uso de los Animales de Experimentación de la Universidad Nacional Autónoma de México [CICUAE-UNAM] (Internal Committee for Care and Use of Experimental Animals of the National Autonomous University of México). Feces were collected daily using harnesses and polyurethane collection bags, and samples were stored and processed at an environmental temperature of 22.30 ± 0.09 °C (Mean ± SE). To process the fecal samples, tap water (1 l) was added to 200 g of feces, which were then homogenized to make a relatively liquid suspension. The liquid feces were then filtered through a household sieve having 400 mm mesh, and the suspension then serially filtered through sieves having pore sizes from 1000, 149, 74 μm, with eggs finally being trapped on a 24 μm mesh. The material retained on the 24 μm mesh was washed into 15 ml centrifuge tubes, which were filled with saturated salt solution (specific gravity 1.2) and centrifuged at 806 × *g* for 5 min. Then, the supernatant was washed in tap water through a 24 μm mesh sieve, and the eggs were collected. Finally, clean eggs were concentrated and placed in 15 ml centrifuge tubes for counting. Egg concentration was estimated by counting the number of eggs in aliquots of 10% of the suspension on a microscope slide. Final concentration of 500 eggs ml^{-1} was achieved either by concentrating the egg suspension through centrifugation, or by diluting with distilled water. The egg recovery process was standardized for completion in 1.45 ± 0.06 h (Mean ± SE).

2.5.2. Egg hatch assay

The EHA was performed according to Bizimenyera et al. (2006), using a 24-well culture plate. The initial concentration of *L. leucocephala* fraction was 9.6 mg ml^{-1}, which was lowered through the fractionation process to 1.1 mg ml^{-1}. Distilled water and 2.5% dimethyl sulfoxide (DMSO) were used as negative controls (for the LIA-IAq and the rest of the fractions, respectively). Levamisole at 1% was used as a positive control (Olmedo-Juárez et al., 2014). Four replicates were run for each concentration, fraction and control. The plates were incubated at 28 °C for 48 h. To stop further hatching, a drop of Lugol's iodine solution was added as described by Bizimenyera et al. (2006). The number of unhatched eggs and larvae (dead or alive) per well were then counted (Coles et al., 1992). Finally, the percentage of egg hatching inhibition was calculated (Bizimenyera et al., 2006). For the final fraction obtained through isolation procedures (Section 2.3), the same methodology was performed, although the concentrations employed were: 1.1, 0.50, 0.250, 0.125 and 0.06 mg ml^{-1} to obtain an LC_{50}.

2.6. Scanning electron microscopy

A new set of EHAs was performed for SEM analysis according to the technique previously described (Section 2.5.2). Two hundred *Cooperia* eggs were exposed to fraction LIC1F3 at 1.1 mg ml^{-1}. Control eggs were incubated in 2.5% DMSO for 2 h in order to avoid embrionation or hatching. All samples were fixed in a 25% glutaraldehyde solution and in a 0.1 M sodium cacodylate buffer, and post-fixed in a 2.5% glutaraldehyde solution and 0.1 M Sorensen's phosphate buffer solution (pH 7.2) for 24 h (Bozzola and Russell, 1992). After three consecutive washes with Sorensen's phosphate buffer solution (0.1 M), the samples were dehydrated in a graded ethanol series, dried in a critical-point dryer (SANDRI-780A), and coated with gold for 5 min in an ionizer (Ion Sputter JFC-1100, Jeol, Fine Coat). Eggs were then observed with a scanning electron microscope (TESCAN MIRA3 LM) at an accelerating voltage of 10 kV. Four micrographs were obtained for each treatment and control, respectively.

2.7. Transmission electron microscopy

A new set of EHAs was performed for TEM analysis according to the technique previously described (Section 2.5.2). Exposed and control eggs were incubated as mentioned above (Section 2.6). Samples were fixed in a mixture of glutaraldehyde and paraformaldehyde at 2.5% and 4%, respectively, and then diluted in a 0.1 M sodium cacodylate buffer. Afterwards, samples were washed three times in a cacodylate buffer solution for 15 min, and post-fixed with 1% osmium tetroxide for one hour. Samples were then dehydrated on increasing concentrations of acetone, and preserved in polymerized epoxy resin at 60 °C for 24 h. Finally, four ultrathin slices (150 nm) were placed on a microscope slide and stained with toluidine blue and the eggs observed with a transmission electron microscope.

2.8. Statistical analysis

Egg hatching inhibition percentage (%) was calculated as: Inhibition (%) = $100(1 - P_I/P_C)$, where P_I is the number of eggs hatched in a treatment group, and P_C is the number of eggs hatched in the control group (Bizimenyera et al., 2006). The egg hatching rates obtained with each dose, and eggshell thickness were compared using a Kruskal–Wallis test, and significant statistical differences were considered when $P < 0.05$ (STATGRAPHICS® Centurion XVI version 16.1.18). Lethal concentrations that inhibited 50% of *Cooperia* spp. egg hatching (LC_{50}) were calculated using a Probit analysis (Minitab® 17.1.0, Minitab Inc. USA).

3. Results

3.1. Identification of anthelmintic-like phytochemicals present in the final fractions

The full bio-guided fractionation procedure is presented in Table 1. Bio-guided fractionation produced three final active fractions: LIC1F3, LIC1F3A and LIC1F3B, which inhibited 90.49 ± 2.85%, 25.73 ± 4.99% and 37.78 ± 9.60% of *Cooperia* spp. egg hatching, respectively (Table 1). High performance liquid chromatography of the most bioactive fraction, LIC1F3, identified the presence of quercetin (RT: 11.233 min), caffeic acid (RT: 8.254 min) and scopoletin (RT: 5.274 min) in proportions of 82.21%, 13.42% and 4.37%, respectively. After final purification, the same phytochemicals were found in each fraction (LIC1F3A and LIC1F3B), but their concentrations were highly modified (fraction composition is presented in Table 2).

3.2. Mean lethal concentrations of the final fractions

Mean lethal concentrations (LC_{50}) were obtained for the three final fractions with AH-like activity. A best-fit LC_{50} was obtained with LIC1F3 (0.06 ± 0.14 mg ml^{-1}), followed by LIC1F3B and

Table 1
Egg hatching inhibition values obtained from bio-guided fractionation (Mean ± SE).

Fractionation steps	Fraction tested	Egg hatching inhibition (%)
1	*Leucaena leucocephala* aqueous extract	52.02 ± 12.39 [a]
Bipartition (9.6 mg ml^{-1})		
2	LIA'-Aq, aqueous	36.34 ± 3.77 [a]
	LIC'-AcnET, organic	76.53 ± 5.02 [b]
First chromatographic column (1.1 mg ml^{-1})		
3	LIC1F1	10.72 ± 4.61 [a]
	LIC1F2	78.49 ± 3.54 [b]
	LIC1F3	90.49 ± 2.85 [b]
	LIC1F4	11.12 ± 3.12 [a]
	LIC1F5	30.78 ± 9.27 [c]
	Levamisole at 1%	56.47 ± 3.83 [d]
Second chromatographic column and bipartition (1.1 mg ml^{-1})		
4	LIC1F3A	25.73 ± 4.99 [a]
	LIC1F3B	37.78 ± 9.60 [a,b]
	Levamisole at 1%	48.41 ± 1.60 [b]

Different letters within each fractionation step represent significant differences $P < 0.05$.

Table 2
Identification and concentration (%) of phytochemicals present in the main active fractions.

Fraction	Phytochemical concentrations within the mixture (%)		
	Quercetin	Caffeic acid	Scopoletin
LLC1F3	82.21	13.42	4.37
LLC1F3A	11.99	84.44	3.57
LLC1F3B	97.32	2.31	1.37

LIC1F3A (1.47 ± 0.31 mg ml^{-1} and 1.82 ± 0.44 mg ml^{-1}, respectively (Table 3).

3.3. Scanning electron microscopy

Ultrastructural features of *Cooperia* eggs exposed and not exposed to LIC1F3 are shown in Fig. 1. Control eggs showed well-defined external layers with no apparent alterations (Fig. 1A); while eggs exposed to LIC1F3 had irregular external layers with small projections (Fig. 1B) and ruptures of lateral eggshell walls (Fig. 1C).

3.4. Transmission electron microscopy

Control eggs showed a well-defined tri-layered eggshell structure (Fig. 2A). Ultrathin slices of *Cooperia* eggs exposed to LIC1F3 revealed changes in electro-density and thickness of the eggshell layers (Fig. 3); yet when compared to the control group, no statistically significant difference was found ($P > 0.05$). Fractures and material infiltration through the eggshell layers were also evident (Fig. 3B).

Table 3
Lethal concentrations required to inhibit 50% of *Cooperia* spp. egg hatching (LC_{50}) after a 48 h incubation period with the most active fractions (mg ml^{-1}; CI = confidence interval).

Plant material	Fraction	LC_{50}		95% CI limits	
		Mean	Std. error	Lower	Upper
Leucaena	LI-AQ	7.93 [a]	1.19	5.50	10.27
leu-	LIC1F3	0.06 [b]	0.14	-0.22	0.34
co-	LIC1F3A	1.82 [c]	0.44	0.95	2.69
cephala	LIC1F3B	1.47 [c]	0.31	0.86	2.27

Different letters in the same column represent statistically significant differences $P < 0.05$.

4. Discussion

The bio-guided fractionation procedure has been described as a key method for the successful purification of phytochemicals with specific bioactive features that could further help to the understanding of the ongoing mechanism of their activity. In this study, bio-guided isolation procedures led to the recognition of a final active fraction (LIC1F3) composed of quercetin (82.21%), caffeic acid (13.42%) and scopoletin (4.37%), which inhibited 90.49 ± 2.85% of *Cooperia* spp. egg hatching. Quercetin, the main compound found in the *L. leucocephala* final fraction, is a flavonol occurring in many plants, and has been described as the most potent antioxidant among polyphenols. Furthermore, quercetin has been reported to have antiviral and antibacterial properties (Materska, 2008). Adekunle and Aderogba (2008) reported quercetin as an antinematocidal compound present in *L. leucocephala* leaves, which affected egg hatching and larval viability of the phytonematode *Meloidogyne incognita*. Furthermore, the derivative quercetin-3-*O*-β-glucopyranoside has been described as having AH activity affecting the motility and viability of *Trichostrongylus* spp. third-stage larvae (Kozan et al., 2013). Previous studies reported that *L. leucocephala* has AH properties against some of the most important GINs of small ruminants, involving tannins/polyphenols as the phytochemical group responsible for the AH effect (Alonso-Díaz et al., 2008; Ademola et al., 2005). Similar results were found against cattle nematodes, with *L. leucocephala* aqueous extracts inhibiting both the egg hatching and exsheathment processes of *Cooperia* spp. (von Son-de Fernex et al., unpublished data), and with flavonoids/polyphenols as important compounds involved in

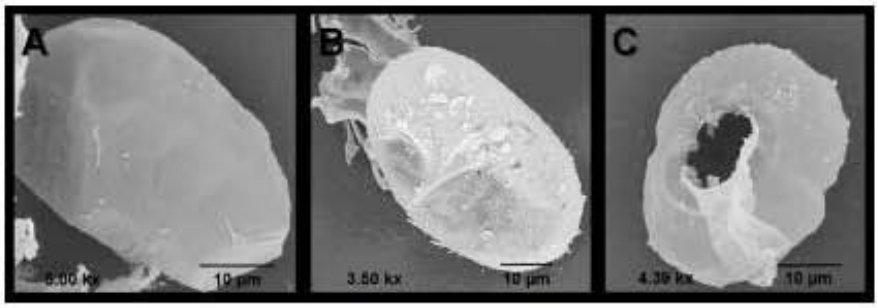

Fig. 1. *Cooperia* spp. eggs observed using scanning electron microscopy, before and after 48 h of incubation with *Leucaena leucocephala* active fraction LIC1 F3.
(A) External layer of untreated egg; (B) Egg structure slightly collapsed and with debris aggregation on the surface; (C) Crater shaped rupture in a lateral eggshell wall.

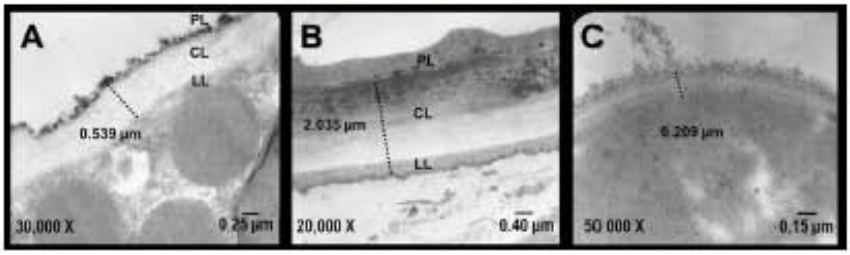

Fig. 2. *Cooperia* spp. eggs observed using transmission electron microscopy, before and after 48 h of incubation with *Leucaena leucocephala* active fraction LIC1F3. (A) Eggshell of the control group. (B,C) *Cooperia* spp. eggs incubated in active fraction LIC1F3 with evident changes in the tri-layer electro-density, thickness and material infiltration through the eggshell layers.

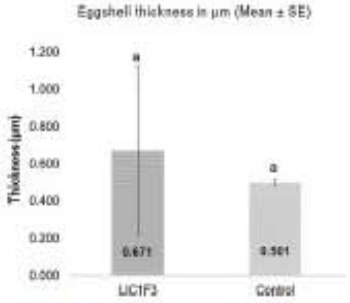

Fig. 3. *Cooperia* spp. eggshell thickness analysis before and after incubation with *Leucaena leucocephala* active fraction LIC1F3.

the AH effect. The AH effect from tannins is related to their ability to create chemical bonds with structural proteins present in GIN morphology (Hoste et al., 2012), which has also been described for flavonol glycosides against the GINs in ruminants (Brunet et al., 2011; Kozan et al., 2013). Such results suggest that due to the similarity of biochemical structure between tannins and flavonol glycosides (Kozan et al., 2013), a similar or close mechanism of action for both types of compounds could occur. The present research also contributes to this topic.

On the other hand, little is known about the AH effect of caffeic acid and its mechanism of action against *Cooperia* spp. In nature, caffeic acid has various derivatives such as caffeic acid phenethyl ester (CAPE), a bioactive polyphenol (Murtaza et al., 2014). It possesses several biological and pharmacological activities, such as antioxidant, anti-cancer and anti-inflammatory (Murtaza et al., 2014; Ulrih, 2015). Therefore, the application of CAPE in the pharmaceutical industry is increasing due to its potential health benefits. The AH properties of caffeic acid against GINs of ruminants have been scarcely studied. Vargas-Magaña et al. (2014) reported that *Coffea arabica* extract did not show any AH effect against *Haemonchus contortus* egg hatching; yet in the latter study chemical compounds were not isolated. To our knowledge, this is the first report involving caffeic acid as a potential AH against *Cooperia* spp., and below we discuss the possible co-participation/synergism of this chemical compound on the egg hatching inhibition of *Cooperia* spp. However, further investigation on the AH effect of caffeic acid against free-living stages of *Cooperia* spp. is suggested.

In tropical regions, plant biochemical composition is usually much more complex than that of temperate legume forages (Alonso-Díaz et al., 2010). A combination of condensed tannins, hydrolysable tannins, polyphenols, and other plant secondary metabolites such as alkaloids or saponins can be found in such plants, making the use of these resources less straightforward. In the AH effect of these plants it is possible to find participation or co-participation of other phytochemical classes present in the active fractions. In fact, unfavorable interactions between tannins/polyphenols and other unidentified plant secondary metabolites on the AH effect (measured as inhibition of egg hatching of *H. contortus*) of plants has been proposed (Vargas-Magaña et al., 2014). An important finding of this study was the possible synergistic interaction observed between quercetin and caffeic acid from *L. leucocephala* on the egg hatching of *Cooperia* spp. Due to the slight change of scopoletin concentration in the mixture after the last purification process and the evident decrease in AH activity, quercetin and caffeic acid were considered the primary AH-like phytochemicals. When quercetin and caffeic acid were 82.21% and 13.42% in the final fraction, the egg hatching inhibition was higher than 90% and the LC_{50} was 0.06 mg ml^{-1}. Yet, when the proportion of quercetin and caffeic acid changed to 97% and 2.3%, respectively, the AH effect decreased to nearly 40% and the LC_{50} was 1.82 mg ml^{-1}. The synergistic interaction observed between compounds could be associated in the first instance to quercetin traits of P-glycoprotein (Pgp) efflux mediator (Mandery et al., 2010). P-glycoproteins are ABC transporters participating in cell membranes as barriers against drug penetration (Xu et al., 2005). Although, the mechanism of plant extracts in the inhibition of GIN egg hatching has not been elucidated, the presence of Pgp on the surface of nematode eggs has been corroborated and strongly related to GIN anthelmintic resistance (De Graef et al., 2013). Furthermore, recent investigations have reported quercetin as an enhancer of ivermectin effectivity when counteracting *Haemonchus placei* resistant strains, and it has been associated with the capacity to inhibit specific drug evasion mechanisms (Heckler et al., 2014). Secondly, caffeic acid has been reported to inhibit proteolysis, lipolysis, and to suppress the activation of matrix metallo-proteinases (Raghavendra et al., 2007; Qiang, 2011; Sullivan and Zeller, 2013); pathways and enzymes known to be either released or activated by the nematode embryo prior to hatching (Kovaleva et al., 2004; Rogers and Brooks, 1976). Throughout this investigation, the embrionation process was not inhibited, as 90.20 ± 2.45% of the eggs failing to hatch contained fully developed larvae (Fig. 4). This suggests that the main AH effect of the isolated phytochemicals consisted of blocking egg hatching, possibly by thickening the eggshell and suppressing matrix metallo-proteinases and other hatching-related enzymes (Molan et al., 2002). Yet, embryo mortality within the eggs might also have been the result of asphyxia and/or cellular toxicity associated with the accumulation of metabolic products (Brunet et al., 2011; Weston et al., 1984). Knowledge of specific interactions between quercetin and caffeic acid (in different proportions) on the AH effect

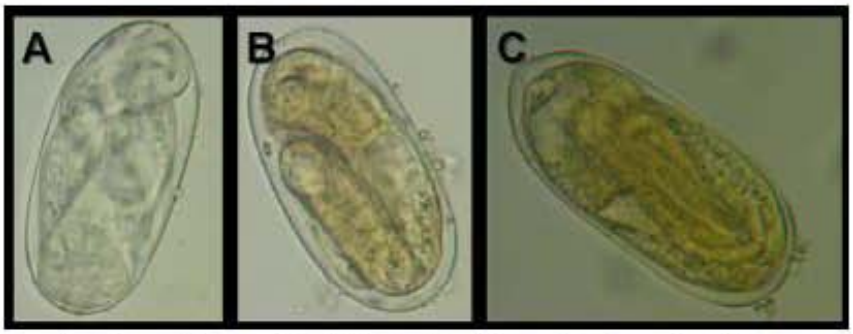

Fig. 4. *Cooperia* spp. eggs observed using optical microscopy (40×) after 48 h of incubation with *Leucaena leucocephala* active fraction LlCF3. (A) Embryonated egg with a living larva failing to hatch. (B,C) Embryonated *Cooperia* spp. egg with a dead larva inside.

against egg hatching inhibition of *Cooperia* spp. is required in order to know more about their possible interaction.

In this study, the ultrastructural damage to *Cooperia* spp. eggs after incubation with the final fraction is presented. Scanning electron microscopy revealed severe structural damage to the external surface of eggs incubated with the final fraction. According to Brunet et al. (2011), this kind of structural damage might lead to the death of nematodes. Furthermore, TEM micrographs of *Cooperia* spp. eggs revealed either thickening or thinning of the tri-layered eggshell, although non-significant values were obtained when calculated together. Fractures and both material aggregation and infiltration, which might be the result of changes in membrane permeability, were also evident. To our knowledge, there are no reports of direct AH effects of quercetin and caffeic acids on free-living stages of *Cooperia* spp. In a study using *H. contortus* and *Trichostrongylus colubriformis* third-stage larvae as models, Brunet et al. (2011) reported similar ultrastructural damage to parasites caused by sainfoin (*Onobrychis viciifolia* Scop.) tannins. Thus, we can suggest that the lesions caused by quercetin and caffeic acids might be involved in the possible AH mechanism of action from *L. leucocephala* extracts on the cattle nematode *Cooperia* spp.

5. Conclusions

The mixture of quercetin and caffeic acids isolated from *L. leucocephala* leaves has synergistic interactions, which enhance AH activity against *Cooperia* spp. egg hatching. Elucidation of bioactive phytochemicals will help to develop novel AHs targeted to reduce larval density in pastures.

Conflict of interest

The authors of this manuscript have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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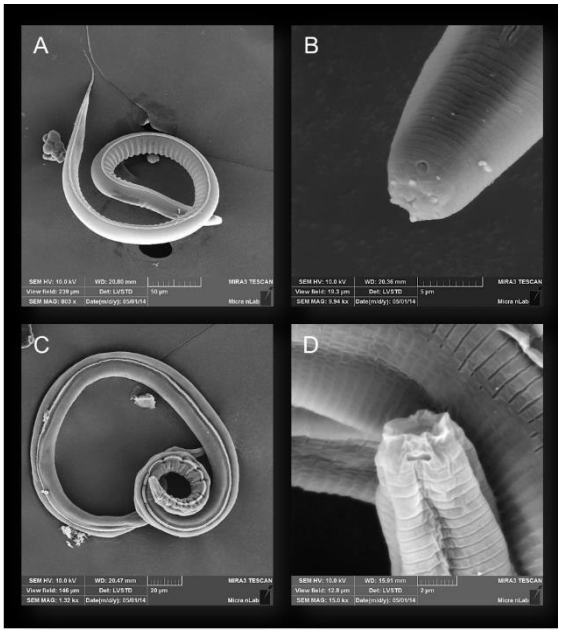
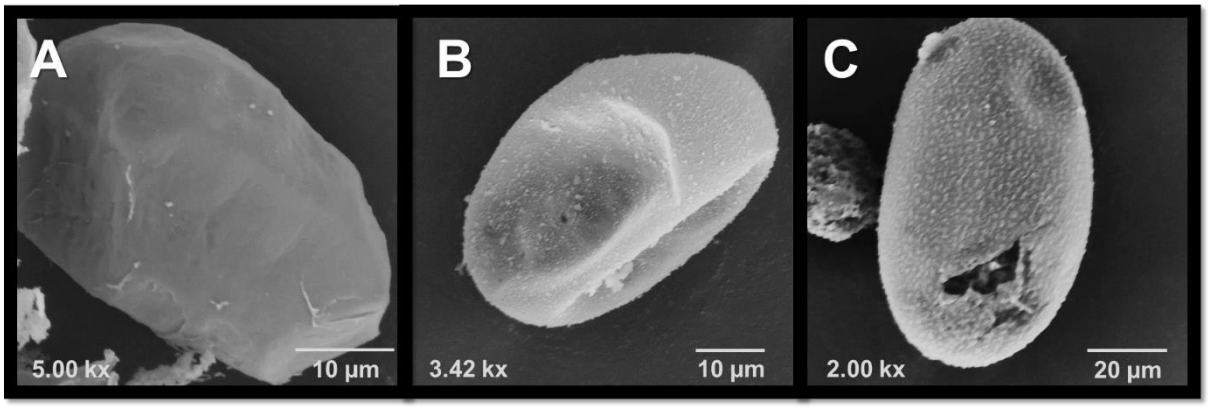
9. ARTÍCULO 4

Anthelmintic effect of 2H-chromen-2-one isolated from *Gliricidia sepium* leaves against *Cooperia punctata*.

von Son-de Fernex, Elke [a*]; Alonso-Díaz, Miguel Ángel [a*]; Valles-de la Mora, Braulio [a]; Mendoza-de Gives, Pedro [b]; González-Cortazar, Manases [c]; Zamilpa, Alejandro [c].





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INTRODUCTION

Gastrointestinal nematodes (GIN) represent one of the mayor constraints for animal welfare and performance, causing important economic losses in the cattle industry (Stromberg et al., 2012). For the past few years, GIN control has become a major problem because anthelmintic resistance rapidly increases among herds worldwide (Becerra-Nava et al., 2014; Bartley et al., 2012; Njue and Prichard, 2004). Cooperia species have been reported as one of the most prevalent species worldwide (Fiel et al., 2012; Vlaminck et al., 2015); being ***C***. ***punctata*** the most pathogenic species of the genus, affecting cattle performance (Stromberg et al., 2012). In recent years, both academic and commercial institutions have joined efforts to form collaborations focused on anthelmintic drug discovery (Wolstenholme and Martin, 2014).

The use of plants as medicinal sources has been reported since Claudius Galënus (AD 130-200) developed the ”galenical” drugs from vegetables (Waller et al., 2001). Currently, more than 50,000 plants have been described possessing useful biological activity (Cordell, 2014). Moreover, it has been estimated that one-fourth of all prescribed drugs contain compounds which are directly or indirectly derived from plants (Zhong, 2011); thus, phytochemistry represents a suitable alternative for AH-like molecules screening.

Gliricidia sepium is tropical forage legume widely used in cattle production units as natural fences and nutritional resource during dry seasons (Wood et al., 1998). Previous ***in vitro*** evaluations have reported anthelmintic activity of ***G***. ***sepium*** against GIN nematodes associating the presence of tannins, flavonoids and lectins to its bioactivity (Kabore et al., 2012; Rios-de Alvarez et al., 2012; von Son-de Fernex et al., 2012). A recent study demonstrated that ***G***. ***sepium*** crude extracts blocked the egg hatching of ***C***. ***punctata***, however, polyphenols/tannins were not the main bioactive compounds suggesting the possible involvement of other phytochemicals in the ovicidal activity (von Son de Fernex et al., 2016). The proper identification of the bioactive(s) molecule(s) has not been completed and it might help to understand the ongoing AH mechanism. The objectives of this investigation were: (1) to perform a bio-guided fractionation of an acetonic extract of ***G***. ***sepium*** leaves using the egg hatch assay (EHA), (2) to elucidate the anthelmintic (AH)-like phytochemical using nuclear magnetic resonance (NMR), and (3) assess the ultrastructural damage of ***Cooperia punctata*** eggs exposed to the AH-like phytochemical.

2. MATERIALS AND METHODS

2.1. Plant material

Fresh leaves of ***G***. ***sepium*** (1695 g) were harvested during February 2014 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 Zootechnia) of the Universidad Nacional Autónoma de México (National Autonomus University of México), located in Martinez de la Torre (20°03´ N y 93°03´ O; 151 masl), Veracruz, Mexico.

2.2. Extraction procedure

Leaves of ***G***. ***sepium*** were air-dried at 60° C for 72 h and were subsequently milled (Pulvex-Plastic®) for smaller particles (4 - 6 mm). The powder (240 g) was placed in a flask and was added 2 L of acetone (GR, Merck) to be macerated for 24 h at room temperature (23 °C). The extract was filtered with filter paper (No. 4 Whatman), and concentrated by distillation under reduced pressure in a rotary evaporator (58 ° C, R-3 Heidolph®, Germany). The acetone extract obtained was re-suspended with 0.8 L of n-hexane to eliminate waxes and chlorophyll. The precipitate (Fraction soluble in acetone, Fr-Acet) was lyophilized (Heto Drywinner DW3®, USA) and stored at 5 ° C for later use.

2.3. Chromatographic purification of bioactive fraction

Fraction Fr-Acet (2.10 g) was subjected to a liquid-liquid fractionation using ethyl acetate (1000 mL) and water (1000 mL) to obtain an organic fraction (GSB-EA) and aqueous fraction (GS-Aq). Both fractions were analyzed on the pharmacological model described in section 2.5.2. Fraction GSB-EA (1.80 g) was subjected to a silica gel 60 open chromatographic column (5.4 g, 00-00 mesh, Merck) using as mobile phase a mixture of dichloromethane/methanol gradient system. Samples of 10 mL were collected and grouped according to their chemical similarity obtaining five final fractions: GSB1 (100:0, 320.4 mg); GSB2 (90:10, 152 mg); GSB3 (80:20, 99.8 mg); GSB4 (50:50, 35.7 mg), and GSB5 (0:100, 77.7 mg). These fractions were biologically evaluated to choice the most active fraction. As proposed by von Son-de Fernex et al. (2015), the EHA was used to perform the bio-guided fractionation of ***G***. ***sepium***.

The most active fraction (GSB2, 152 mg) was rechromatographed in a silica gel open column (452 mg, 70-230 mesh, MERCK). The mobile phase consisted on an n-hexane/ethyl acetate gradient system (samples of 50 mL). This process was followed by thin layer chromatography and fraction with similar chemical constituents were grouped in three final fractions (GBS2A, 90:10 system, 25.9 mg; GBS2B, 80:20 system, 83.8 mg; GBS2C, 70:30 system, 32.6 mg). The fraction with highest AH-like activity (GSB2b) was analyzed by ^{1}H and ^{13}C NMR showing the same spectra data the previously reported 2H-chromen-2-one.

2.5. Parasitological techniques.

2.5.1. Egg recovery.

Cooperia punctata eggs were obtained from a donor calve with a mono-specific infection (isolate C. p. CEIEGT-FMVZ-UNAM strain, Mexico), which was housed indoors on concrete floor, fed hay and commercial concentrate, and had free access to water (complying with the Internal Committee for Care and Use of Experimental Animals of the National Autonomous University of México [CICUAE-UNAM] regulations). Feces were collected daily using harnesses and polyurethane collection bags, and samples were kept and processed at an environmental temperature of 22.30 ± 0.09 °C (Mean ± SE). Egg recovery technique was performed as described by von Son-de Fernex et al. (2015).

2.5.2. Egg hatch assay (EHA)

The EHA was performed according to Bizimenyera et al. (2006), using a 24-well culture plate. Initial concentration of ***G***. ***sepium*** Fr-Acet was 9.6 mg mL^{-1}, which was lowered through fractionation to 1.1 mg mL^{-1}. For the final fraction obtained through isolation procedures (see section 2.3), the same methodology was performed, although the concentrations employed were: 1.1, 5 x 10^{-1}, 2.5 x 10^{-1}, 1.25 x 10^{-1}, 6 x 10^{-2}, 1 x 10^{-3}, 1 x 10^{-5} mg mL^{-1} to obtain EC_{50}.

Dimethyl sulfoxide (DMSO) 2.5% was employed as a negative control. Four replicates were run for each concentration, fraction and control. The plates were incubated at 28 °C for 48 h. A drop of Lugol's iodine solution was added to each well to stop further hatching, and all the unhatched eggs and larvae (death or alive) per well were counted (Coles et al., 1992). Finally, the percentage of egg hatching inhibition was calculated.

2.6. Scanning Electron Microscopy (SEM)

A new set of EHA was performed for SEM analysis (sec 2.5.2). Two hundred ***C. punctata*** eggs exposed to the fraction GSB2b at 1.1 mg mL^{-1}. Control eggs were incubated in DMSO 2.5 % during 2 hours in order to avoid embrionation or hatching. All samples were fixed in a 25 % glutaraldehyde solution and in a 0.1 M sodium cacodylate buffer, and post-fixed in a 2.5 % glutaraldehyde solution and 0.1 M Sorensen´s phosphate buffer solution (pH 7.2) for 24 h (Bozzola and Russell, 1992). After three consecutive washes with the Sorensen´s phosphate buffer solution (0.1M), the samples were dehydrated in a graded ethanol series, dried in a critical-point dryer (SANDRI-780A), and coated with gold for 5 min in an ionizer (Ion Sputter JFC-1100, Jeol, Fine Coat). Eggs were then observed with a scanning electron microscope (TESCAN MIRA3 LM) at an accelerating voltage of 10 kV. Four micrographs were obtained for each treatment and control, respectively.

2.7. Transmission Electron Microscopy (TEM)

For TEM analysis a new set of EHA were performed according to the technique previously described (sec 2.5.2). Treated and control eggs were incubated as mentioned above (sec 2.6). Samples were fixed in a mixture of glutaraldehyde and paraformaldehyde at 2.5 % and 4%, respectively, and then diluted in a 0.1 M sodium cacodylate buffer. Afterwards, samples were washed three times in a cacodylate buffer solution for 15 min, and post-fixed with osmium tetroxide at 1% for one h. Samples were then dehydrated on increasing concentrations of acetone, and preserved in polymerized epoxy resin at 60 °C for 24 h. Finally, four ultrathin slices (150 nm) were placed on a microscope slide and stained with toluidine blue and the eggs observed with a transmission electron microscope.

2.8. Statistical analysis

The percentage (%) of egg hatching inhibition was calculated using the formula: Inhibition (%) =100 (1 - P_t / P_c). Where P_t is the number of eggs hatched in treatment group, and P_c is the respective numbers in DMSO control group (Bizimenyera et al., 2006). The egg hatching rates obtained for each fraction were compared using a Kruskal-Wallis statistical test and eggshell thickness was analyzed using an ANOVA test (STATGRAPHICS ® Centurion XVI vs 16.1.18) Significant statistical differences were considered when $P < 0.05$.

To fit the dose-response data by non-linear regression, a four-parameter logistic equation with a variable slope was used using the computer program GraphPad Prism ® V. 6.1. All analyses were performed after transforming the data into their logarithms (X=l ogX) and constraining the bottom value to 0%. The EC_{50} values, the 95% confidence intervals and R^2 values were calculated.

III. RESULTS

3.1. Bio-guided fractionation of the acetonic extract of G. sepium leaves

The Bio-guided fractionation process was carried out in four steps, where 10 different fractions were analyzed (Table 1). Through the third step of fractionation, the fractions GSB2 and GSB4 showed potent AH-like activity (100.00 ± 0.00 and 94.24 ± 3.04, respectively); however, fraction GSB2 was chosen for further purification as it had the capacity to fully inhibit the embrionation within the egg, while GSB4 acted only as a blocking agent of ***C. punctata*** hatching process.

3.1. Elucidation of the AH-like phytochemical present in the final fraction

Nuclear Magnetic Resonance (1H and C13) provided two chromatograms (Fig. 1 a,b) with number and distance of both Hydrogen and Carbon atoms present in the bioactive molecule ($C_{10}H_6$). Atoms position and coupling constants are shown in Table 2. The anthelmintic-like phytochemical isolated from ***G. sepium***, was identified as 2H-chromen-2-one.

3.2. Half maximal effective concentration (EC50) of 2H-chromen-2-one

At 1.1 mg mL-1 egg hatching was fully inhibited (Table 3). Furthermore, embrionation of ***C. punctata*** eggs was also fully inhibited with the isolated Coumarin (Fig. 2). Half maximal effective concentration (EC50) obtained for 2H-chromen-2-one was 0.024 ± 0.074 mg mL-1 (Table 3). Dose-response curve is presented in figure 3.

3.4 Scanning Electron Microscopy (SEM)

Ultrastructure features of ***C. punctata*** eggs exposed and not exposed to 2H-chromen-2-one were assessed through SEM (Fig. 4). Control eggs show a well-defined external layer with slight content of debris attached, which is consistent with the external vitelline layer sticky nature (Fig. 4 A). Eggs exposed to the 2H-chromen-2-one showed less debris attachment, collapsed eggshell structure and multiple external fractures (Fig. 4 B-C).

3.5 Transmission Electron Microscopy (TEM)

Ultrathin cuts of ***C. punctata*** eggs showed multiple eggshell fractures involving the proteic and chitin layers (Fig. 5). Furthermore, changes in the electrodensity and thickness of C. punctata eggshell layers were also noticeable (Fig. 5 B). Statistical significant difference of the eggshell thickness was observed between groups (Fig. 6; $P < 0.05$).

DISCUSSION

Gliricidia sepium is a tropical forage legume widely used as a cattle-feeding resource and for medicinal purposes (Rastrelli et al., 1999). Medicinal appliances of the legume have been reporting bioactivities such as antioxidant, antimicrobial, anthelmintic, antifungal, and insecticidal properties (Nazli et al., 2011; Sinha, 2013). Bio-guided fractionation performed through this investigation identified the molecule 2H-chromen-2-one (chromone or coumarin) as a potent dose-dependent inhibitor of ***C***. ***punctata*** embrionation and egg hatching. The presence of coumarin in multiple aerial parts of ***G***. ***sepium*** has been reported since 1946, when its rodenticide activity was firstly described (Standley and Steyermark, 1964). Further studies have stated the presence of at least four different coumarin derivatives to be present in ***G***. ***sepium*** leaves (Wood et al., 1998). Coumarin is a secondary heterocyclic metabolite composed of fused benzene and pyrone rings, found in a wide range of plant families and microorganisms (fungi and bacteria). In fact, the bioactivity of various coumarin derivatives depends on the presence and position of hydroxyl groups. The bioactive phytochemical isolated from ***G***. ***sepium*** through this investigation represents the simplest coumarin (2H-chromen-2-one); of which, to our knowledge, AH bioactivity has not been reported. Coumarins described with AH activity are mainly Acylhydrazones and 3-carbamoyl-4-hydroxycoumarins (Pubchem, NCBI).

Through this investigation ***C***. ***punctata*** eggs incubated in ***G***. ***sepium*** 2H-chromen-2-one, were not just inhibited for hatching, but mainly for embryo development (Fig. 2). Such findings are consistent with previous statements of both coumarin and coumarin derivatives to be potential inhibitors of cellular proliferation (Lacy and O'Kennedy, 2004), and to deregulate mitochondrial respiration by uncoupling oxidative phosphorylation and inhibiting succinate oxidase (Du et al., 2011). Even more, Coumarins have been proved as potent inhibitors of carbonic anhydrases (Balboni et al., 2012) which are metalloenzimes present in nematode species including cattle trichostrongylids (DeRosa et al., 2008; Emameh et al., 2014; Güzel et al., 2009), and are associated with membrane proteins of eggshells and to interact with the juvenile hormone; which is responsible for nematodes early stages development (Rogers, 1980). Furthermore, carbonic anhydrase inhibitors, have been reported to alter cell membrane permeability and to affect the control of embryonic development (Rogers, 1980). In addition, coumarin and its derivatives have been characterized as Cytochrome P450 (CYP) inhibitors (Kleiner et al., 2003; Laing, 2010), which is also present in multiple nematodes genera such as: Caenorhabditis elegans, Haemonchus contortus and Cooperia oncophora (Benenati et al., 2009; Laing, 2010; van der Veer et al., 2003). Cytochrome P450 has been related with eggshell lipid production, meiosis and embryo polarization of C. elegans; and its depletion leads to embryonic lethality associated to: i) osmotic imbalance, ii) incorrect execution of meiosis and iii) impaired establishment of polarity (Benenati et al., 2009).

Although more studies are required to fully understand the ongoing ovicidal mechanism of 2H-chromen-2-one, optic microscopy allowed to observe that ***C***. ***punctata*** larvae development within the egg was fully inhibited. Benzimidazoles (BZD) have been described as the anthelmintic group inhibiting trichostrongylid embrionation, and many hypothesis have been proposed for its mechanism of action, including: i) inhibition of protein and nucleotide synthesis, ii) inhibition of fumarate reductase, iii) inhibition of glucose uptake and iv) inhibition of tubulin polymerization to form microtubules (Lacey et al., 1987). The molecular size has also been associated to the mechanism of action, as ovicidal compounds such as BZD should not be bigger than 400-500 Da to exert their activity inside the egg (Vargas-Magana et al., 2014). On this matter, the 2H-chromen-2-one isolated throughout this investigation has a molecular weight of 146.14 Da, which is very similar to BZDs 118.14

Da (Pubchem-NCBI CID:323 and CID:5798, respectively). Moreover, trichostrongylid eggs incubated in BZD have been reported with atypical blastomers formation, resulting in abnormal cellular division and also a hatching-blockage associated to the transport inhibition of substances crucial for the hatching process of nematodes (Lacey et al., 1987). Kirsch and Schleich (1982) observed and characterized some morphological alteration of eggs exposed to BZD such as Bubble-like/ or crater-like structures and knot-like shape of blastomers and granular appearance; most of which are consistent with damages observed through this investigation (Fig. 2). On the other hand, SEM micrograph set revealed both collapsed and multi-fractured eggshells; which might be consistent with alterations of membrane permeability. Finally, TEM analysis allowed the corroboration of most of the previous observations. Furthermore, comparison of exposed and unexposed eggs revealed a significant thickening of the eggshell tri-layer complex, with multiple fractures involving the external and mid-layer. Damage observed is consistent with recent studies using SEM and TEM, where when analyzing Cooperia spp., eggs exposed to a mixture of flavonoids isolated from Leucaena leucocephala, direct alterations of eggshell permeability and thickness were reported; however, embrionation was not affected (von Son-de Fernex et al., 2015). Suggesting that depending on the nature of phytochemicals, those may act either as hatch-blocking agents or ovicidal molecules. However, further in vitro assays targeting other development stages of C. punctata and toxicity analysis are suggested for the better understanding of 2H-chromen-2-one ongoing AH-like mechanism; and to determine the safety of the isolated phytochemical prior to ***in vivo*** evaluations.

CONCLUSIONS

The coumarin (2H-chromen-2-one) isolated from ***G***. ***sepium*** leaves has potent ovicidal activity against ***C***. ***punctata***. After toxicity evaluations, 2H-chromen-2-one could be suggested as a feasible novel AH-like phytochemical targeting GINs supra populations in the field.

Conflict of interest statement

The authors of this manuscript have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Figures

Figure 1 a-b. Proton and Cabron chromatograms.

Figure 2. ***Cooperia punctata*** eggs observed using optical microscopy (40X) after 48 h of incubation with ***2H***-chromen-***2***-one.

A. Apparent disruption of eggshell continuity and lack of embrionation; B. Blastomers with Bubble / crater-like structures; C. knot-like shape of blastomers.

Figure 3. Dose-response curve of 2***H***-chromen-2-one against ***C***. ***punctata*** egg hatching.

Figure 4. ***Cooperia punctata*** eggs observed using scanning electron microscopy, before and after 48 h of incubation with 2***H***-chromen-2-one.

A. External layer of unexposed egg; B. Collapsed egg structure; C. Eggshell fractures.

Figure 5. ***Cooperia punctata*** eggs observed using transmission electron microscopy, before and after 48 h of incubation with 2***H***-chromen-2-one.

A. Eggshell tri-layer complex of unexposed egg; B. ***Cooperia punctata*** eggs incubated in ***2H***-chromen-***2***-one with evident changes in the tri-layer electro-density; C. Disruption of eggshell continuity involving proteic and chitin layers.

Figure 6. ***Cooperia punctata*** eggshell thickness analysis before and after incubation with 2***H***-chromen-2-one.

Tables

Table 1. Egg hatching inhibition values obtained from bio-guided fractioning (Mean ± SE).

Table 2. ^{1}H and ^{13}C NMR data (300 and 75 MHz) of 2***H***-chromen-2-one in $CDCl_3$.

Table 3. Half maximal effective concentration (EC_{50}), 95 % confidence intervals (CI) and correlation coefficients (R-square) obtained after 48h incubation of ***C***. ***punctata*** eggs in 2***H***-chromen-2-one (mg mL^{-1}).

Figure 1 a-b. Proton and Cabron chromatograms.

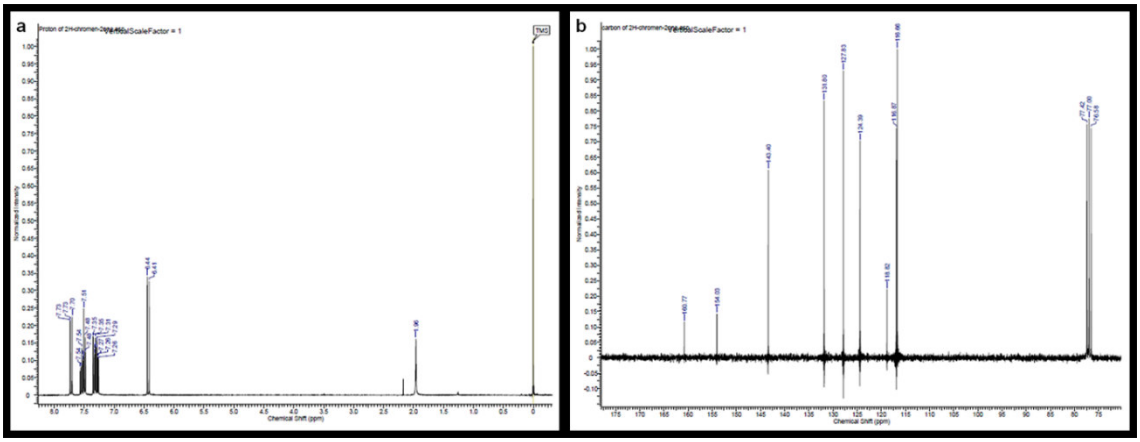
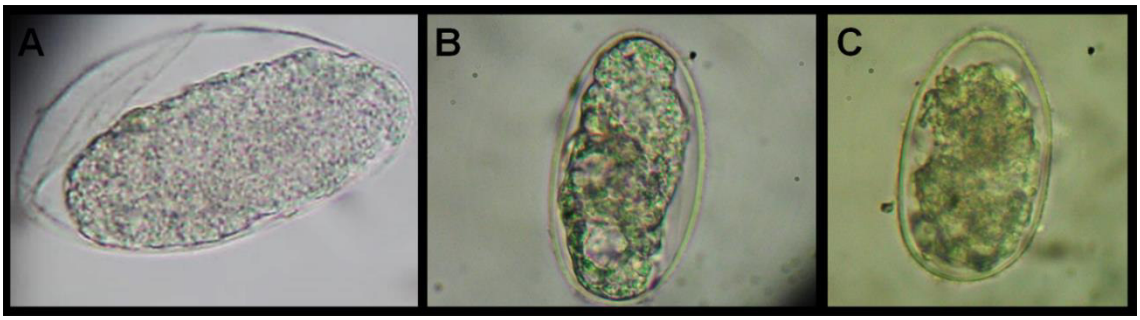


Figure 2. ***Cooperia punctata*** eggs observed using optical microscopy (40X) after 48 h of incubation with ***2H***-chromen-***2***-one.



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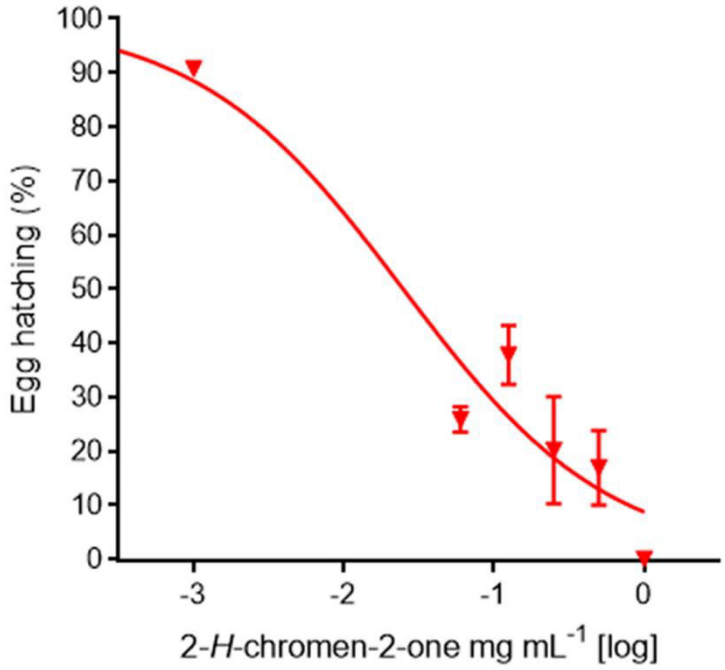

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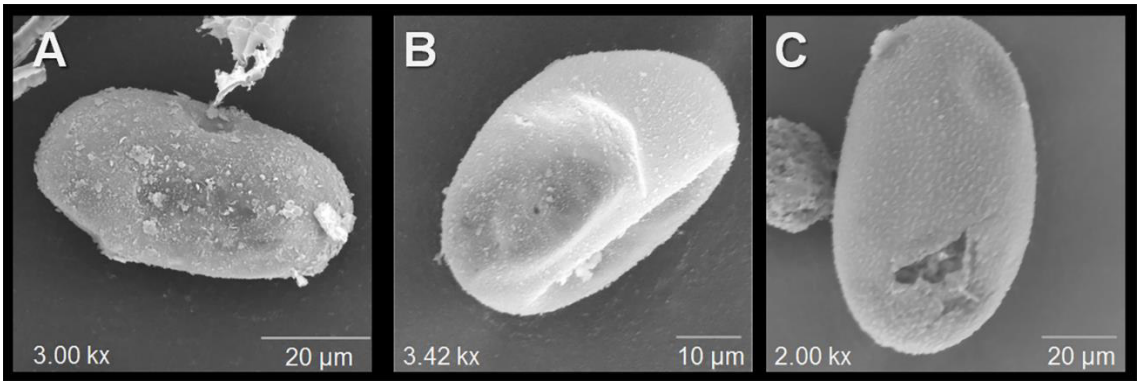
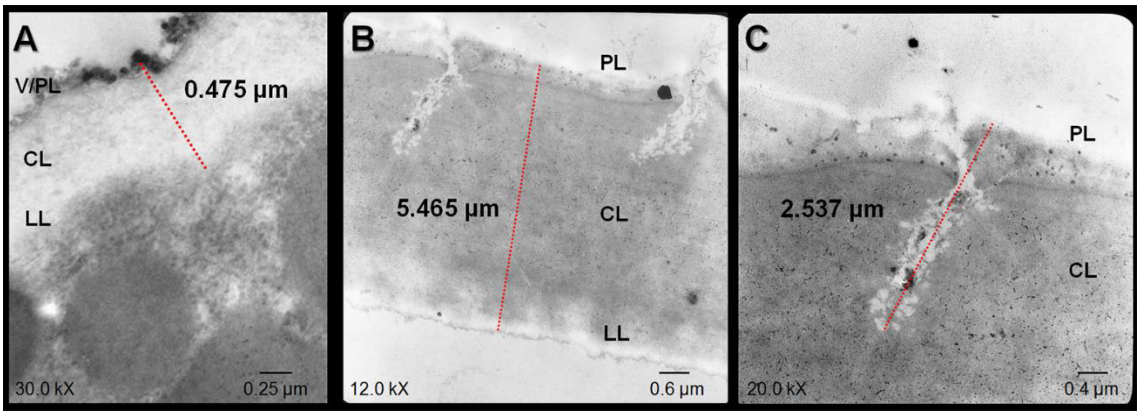


B. External layer of unexposed egg; B. Collapsed egg structure; C. Eggshell fractures.

Figure 5. *Cooperia punctata* eggs observed using transmission electron microscopy, before and after 48 h of incubation with 2***H***-chromen-2-one.



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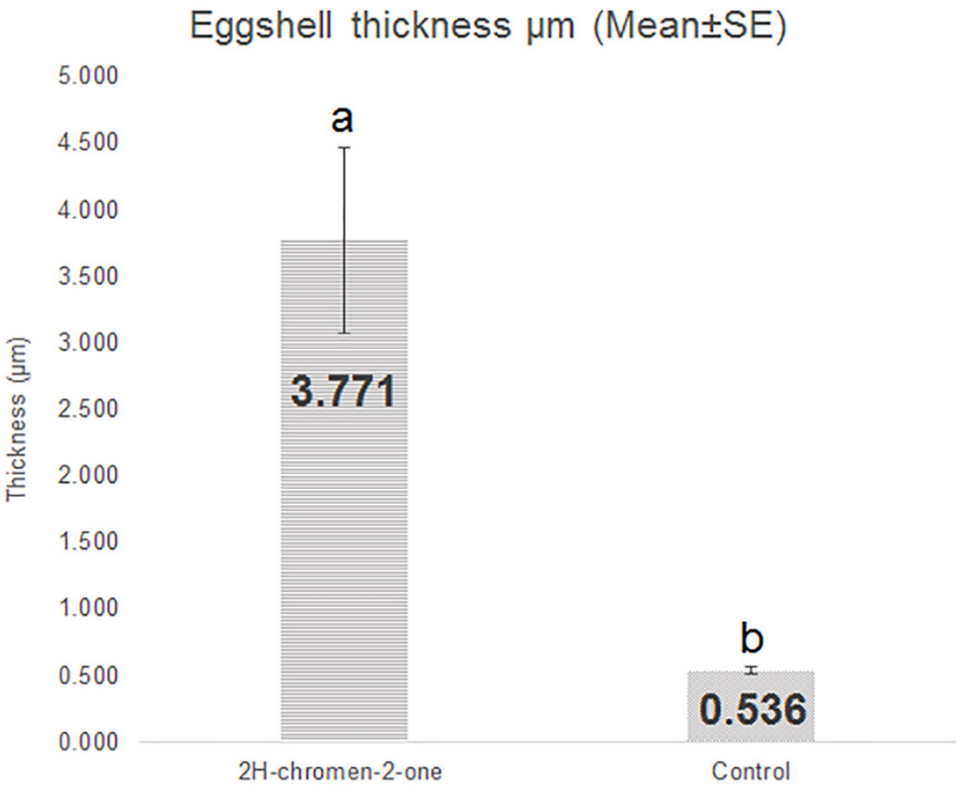

478 **Table 1.** Egg hatching inhibition values obtained from bio-guided fractioning (Mean ± SE).

Fractionation steps	Fraction tested	Egg hatching inhibition (%)
1	***G. sepium*** acetonic extract	100.00 ± 0.00 [a]
	Bipartition (9.6 mg mL^{-1})	
2	GSA'-Aq, aqueous	5.98 ± 3.53 [a]
	GSB'-AcoET, organic	100.00 ± 0.00 [b]
	First chromatographic column (1.1 mg mL^{-1})	
3	GSB1	3.57 ± 2.96 [a]
	GSB2	100.00 ± 0.00 [b]
	GSB3	59.84 ± 2.13 [c]
	GSB4	94.24 ± 3.04 [b]
	GSB5	-1.11 ± 0.45 [a]
	Second chromatographic column (1.1 mg mL^{-1})	
4	GSB2a	36.44 ± 2.79 [a]
	GSB2b	100.00 ± 0.00 [b]
	GSB2c	17.35 ± 2.69 [c]

479 Different letters within each fractionation step represent statistically significant differences $P < 0.05$.

480

481 **Table 2.** ^{1}H and ^{13}C NMR data (300 and 75 MHz) of 2***H***-chromen-2-one in $CDCl_3$.

Position	δ_C	δ_H
2	160.7	--
3	116.6	6.42 (d, J= 9.6 Hz)
4	143.4	7.72 (d, J= 9.6 Hz)
5	127.8	7.55 (dd, J= 1.5, 8.6 Hz)
6	124.4	7.31 (ddd, J= 1.1, 8.5, 8.5 Hz)
7	131.8	7.51 (ddd, J= 1.3, 8.6, 8,6 Hz)
8	116.8	7.29 (dd, J= 1.1, 8.8 Hz)
9	154.04	--
10	118.8	--

482

483

484 **Table 3.** Half maximal effective concentration (EC_{50}), 95 % confidence intervals (CI) and
485 correlation coefficients (R-square) of ***2H***-chromen-***2***-one against ***C***. ***punctata*** egg hatching
486 (mg mL^{-1}).

Molecule	EC_{50}	Std. Error	95 % CI Limits		R square
			Lower	Upper	
***2H*-chromen-*2*-one**	0.0247	0.074	0.01522	0.04021	0.9466

487

10. ARTÍCULO 5

Effect of *Gliricidia sepium* leaves intake on larval establishment of *Cooperia punctata* in calves.

von Son-de Fernex, Elke*[a]; Alonso-Díaz, Miguel Ángel[a]; Valles-de la Mora, Braulio[a]; Mendoza-de Gives, Pedro[b]; Castillo-Gallegos, Epigmenio[a]; González-Cortazar, Manases[c]; Zamilpa, Alejandro[c].

Veterinary Parasitology

(Artículo por enviar)

Effect of *Gliricidia sepium* leaves intake on larval establishment of *Cooperia punctata* in calves.

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ABSTRACT

The use of bioactive plants has been suggested as an alternative for the control of gastrointestinal nematodes. Recent *in vitro* studies have demonstrated the anthelmintic (AH) effect of *Gliricidia sepium* extracts against cattle nematodes. This study aimed to: 1) assess the anthelmintic effect of *G. sepium* on the establishment of *Cooperia punctata* third-stage larvae in calves, 2) assess morphological features of established worms. Twelve ¾ Holstein x Zebu calves were divided in two experimental groups: control and treatment (T1 and T2; n=6). The 17-day trial was divided in three periods: i) adaptation (7-d), ii) experimental (6-d), and iii) post-treatment (4-d). After adaptation, each calf was infected with an oral dose of 400 *C. punctata* L_3 / Kg LW. During the trial, the 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*, while T1 remained on the basal diet with the amount of concentrate adjusted to obtain isoproteic diets. Six calves were randomly selected for slaughter 9-d post-infection, and worm counts and measurements were performed. Larval establishment rates were: 13.44 ± 0.13% and 3.1 ± 1.42% for T1 and T2, respectively ($P < 0.05$). The reduction of larval establishment was 76.9%. Differences were found for dry matter intake and worm length ($P < 0.05$). Results confirm that the ingestion of *G. sepium* leaves reduced larval establishment of *C. punctata* in cattle.

I. INTRODUCTION

The alarming emergence of anthelmintic resistance (AR) in gastrointestinal nematodes (GIN) represents a continuous threat to cattle production units worldwide. The use of bioactive plants (BP) has been suggested as an alternative to reduce the dependence of chemoprophylaxis in small ruminants (Martinez-Ortiz-de-Montellano et al., 2010) due to their potential to inhibit larvae establishment, development and to affect adult fertility of GIN (Brunet et al., 2008; Martinez-Ortiz-de-Montellano et al., 2010). Thus, and despite of the social and economic importance of the cattle industry, there are scarce reports of the possible benefits of implementing the use of bioactive plants for GIN control. Recent *in vitro* studies have demonstrated the anthelmintic effect of BP against the cattle nematodes *Cooperia oncophora* and *Ostertagia ostertagi* (Novobilsky et al., 2011). Furthermore, *Cooperia* spp., represents one of the gastrointestinal nematode with highest prevalence and resistant features in cattle (Bartley et al., 2012; Becerra-Nava et al., 2014; Stromberg et al., 2012). On the other hand, *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 (Li and Gasbarre, 2009; Stromberg et al., 2012); highlighting the urge of

novel control strategies to be both developed and implemented. The possible implementation of BP as an alternative to control gastrointestinal nematodes in ruminants might be restricted by the consumption of the feedstuff offered to animals. *Gliricidia sepium* is a tropical legume with a worldwide distribution; which is commonly used as natural fence or feedstuff for small ruminants. Furthermore, *in vitro* studies have reported an anthelmintic-like effect of leaves extracts; affecting the hatching, exsheathment and motility of either *H. contortus* or *C. punctata* (von Son-de Fernex et al., 2012). Therefore, *G. sepium* has been considered a suitable prototype to assess its potential to reduce larval establishment of *C. punctata* in calves. The objectives of this study were to: 1) assess the anthelmintic effect of *G. sepium* on the establishment of *C. punctata* third-stage larvae in calves, and 2) assess morphological features of established worms.

II. MATERIALS AND METHODS

2.1. Plants

Fresh daily harvested leaves of *G. sepium* were used in the experiment. The material was harvested on 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 Zootechnia) of the Universidad Nacional Autónoma de México (National Autonomus Univesity of México) located 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 a donor calve with a mono-specific infection (isolate *C. p.* Fernex-MEX), which was housed indoors on concrete floor, fed hay and commercial concentrate, and had free access to water (complying with the Internal Committee for Care and Use of Experimental Animals of the National Autonomous University of México [CICUAE-UNAM] regulations).

2.3. Experimental design and diets.

Twelve, 10.08 ± 0.08 month-old (Mean ± S.E.), nematode free calves were involved in the study. They were divided in two experimental groups (n=6), balanced according to body weight T1 (control): 166.17 ± 5.09 kg and T2 (treatment): 167.33 ± 7.03 (Mean ± S.E.). Animals were treated with levamisole (8.5 mg/kg live weight, 14 days prior infection); furthermore, animals were also drenched with oral Benzimidazole (15 mg/kg PV live weight) before

being placed on individual concrete floor pens to avoid further natural nematode infection (10 days prior infection). Animal feces were analyzed with McMaster technique (Reynaud, 1970) daily for 10 days prior to the artificial infection, to corroborate the free GIN status. The calves were fed daily with 500 grams of commercial concentrate (12% CP) 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 the 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). Day-0 corresponded to the day when the twelve calves were experimentally infected with 400 L_3/kg BW of *C. punctata*. Six calves were humanely slaughtered 9 days after infection (D9) complying with the Internal Committee for the Care and Use of Experimental Animals of the National Autonomous University of Mexico (CICUAE-UNAM) regulations. During the 17-day trial, the basal diet consisted of *Digitaria decumbens* hay (6.27 % CP; *ad libitum*) and commercial concentrate (12 % CP; 500 g). 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 individually adjusted to obtain isoproteic fares between the experimental diets.

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 AOAC procedures (1980).

2.6. Measurements of the plant consumption

Animals were fed on individual concrete troughs. Both, offer and refusal were measured daily in order to estimate the intake of fresh leaves of *G. sepium* and *D. decumbens* hay.

2.7. Parasitological techniques

2.7.1. Worm recovery and morphological analysis procedures

On D9, complying with the Internal Committee for the Care and Use of Experimental Animals of the National Autonomous University of Mexico (CICUAE-UNAM) regulations; six calves were humanely slaughtered and 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 pre-heated water through 1000 and 149 µm mesh sieves, and stored in a 10% formalin solution until analyses. The total number of larvae present in the luminal contents were estimated using the 10% aliquot technique (Martinez-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.

2.7. Statistical analysis

Daily consumption of dry matter and worm total length were analyzed through a one-way analysis of variance (STATGRAPHICS, Centurion XVI version 16.1.18). Differences in the worm counts between groups were obtained through a Kruskal-Wallis test (STATGRAPHICS, Centurion XVI version 16.1.18). The rates of larval establishment were calculated according to the following formula: $100*(1-L_r/L_i)$, where L_r represents the total number of worms recovered and L_i, represents the total number of L_3 used for infection. Reduction of larval establishment was calculated using the formula: $100*(1-E_t/E_C)$ where E_t represents the establishment obtained in the treatment group, and E_c represents the establishment obtained for the control group.

III. RESULTS

3.1. Plant analysis

The chemical composition of both *G. sepium* and *D. decumbens* are presented in Table 1.

3.2. *Gliricidia sepium* leaves and *D. decumbens* hay daily intake.

Dry matter (DM) intake between treatments was statistically significant through both adaptation and experimental periods ($P < 0.05$). Moreover, differences between the periods of T2 were also observed ($P < 0.05$), presenting a higher DM intake in percentage of body weight (DM % BW) throughout the adaptation period (data presented in Table 2). According to the chemical analyses and DM ingestion, the daily protein intake was of 415.59 ± 7.39 g/d and 481.9 ± 60.52 g/d, for T1 and T2 respectively ($P > 0.05$; Figure 1). The inclusion of *G. sepium* on regards of the total diet for T2 was of

26.64 ± 3.59 % (Mean ± S.E.), with an average voluntary intake of 0.62 ± 0.14 DM % BW.

3.3. Worm counts, larvae measurements and establishment rates

A significant decrease in *C. punctata* (L_3) establishment was observed between groups ($P < 0.05$; Table 3; Figure 2). The percentage of establishment reduction obtained through the trial was of 76.9 %. Total length of fourth stage recovered were 2.43 ± 0.1 mm and 1.66 ± 0.05 mm, for control and treatment respectively ($P < 0.05$); while young adults measured 6.58 ± 0.29 mm and 5.3 ± 0.26 mm for T1 and T2 respectively ($P < 0.05$; Figure 3).

4. DISCUSSION

The future implementation of bioactive plants as an alternative to control gastrointestinal nematodes in ruminants might be restricted by the consumption of the feedstuff offered to animals; as most bioactive plants have high contents of plant secondary metabolites which might restrain ingestion of cattle mainly due to astringency or rumen distention (Frutos et al., 2004). Condensed tannins are known for their anthelmintic activity in a threshold of 3-5 % in the diet (Hoste et al., 2012); though, recent reports state a concentration of 6.1 % - 8.3 % to achieve an anthelmintic activity towards GIN of small ruminants (Werne et al., 2013). However, concentrations higher than 5 % adversely affect both DM intake and digestibility, and might also be toxic (Hoste et al., 2006; Rogosic et al., 2008). Furthermore, opposite to small ruminants which have higher diet selectivity and other physiological mechanisms such as proline-rich proteins in their saliva (Alonso-Díaz et al., 2010), cattle cannot successfully withstand the anti-nutritional properties of CT; therefore, it could be a limiting issue for the use of bioactive plants with anthelmintic properties in large ruminants.

Previous trials have reported *G. sepium* as a nutritional alternative to compensate the deficit of both quantity and quality of native forages in the tropics (Tesorero and Combellas, 2003); and mean consumptions of 1.3 DM kg /day for crossbreed calves have been reported (Abdulrazak et al., 1997). Furthermore, *G. sepium* has been characterized as a tropical legume with traces of tannin contents (< 1 %) (Balogun et al., 1998), which might explain the high acceptance observed when offered to cattle in nutritional trials. Through the trial, differences in DM intake between groups were observed within both adaptation and the experimental period. However, the treated group revealed differences of DM intake between periods, with a significant decrease of DM intake in the experimental period ($P < 0.05$); which might be consistent with Smith and van Houtert (1987) who suggested that, when consumed, *G. sepium* distends the rumen, reducing intake of basal diet.

Nevertheless, and despite the decrease of DM intake, a high acceptance of *G. sepium* was observed throughout the six-day experimental period (1.04 ± 0.2 DM kg / day).

Throughout this investigation, the establishment rate obtained in the control group (13.44 ± 0.13 %) was similar to the establishment rates reported by Li and Gasbarre (2009), who recovered 10.67 % of *C. oncophora* 7 days after infection. Furthermore, Sauerman (2014) reported that the establishment rate of *C. oncophora* in animals with prior exposure to the nematode was less than 20%. Conversely, in animals fed with fresh leaves of *G. sepium* the mean establishment rate was 3.1 ± 1.42 %; which represents an establishment reduction of 76.9 %. To our knowledge, no studies are available for comparison; as most have been performed using small ruminants and were feed with tanniniferous plants. Brunet et al., (2008) reports that the use of tanniniferous plants led to a 70.99 % reduction in the establishment of *H. contortus* and *T. colubriformis* larvae in small ruminants; and the AH-like activity has been directly associated to the capacity of tannins to create bonds with some nematodes structural features (Hoste et al., 2012). To our knowledge, this is the first report of a non-tanniniferous plant reducing larval establishment of GIN in calves; suggesting the participation of other AH-like phytochemicals. Characteristics which might fulfill the goal of bioactive plants incorporation in grazing cattle, which is the prevention of severe GIN infections that might compromise animal performance and welfare; by lowering down both dynamics and rates of infection through their consumption.

The anthelmintic activity observed with *G. sepium* consumption through this investigation, might be directly related to its capacity to inhibit infective larvae exsheathment process (von Son-de Fernex et al., 2012); which represents the most critical time-framing process for the establishment of GIN within the host (Hertzberg et al., 2003). Furthermore, the total length of worms recovered were also different between groups ($p<0.05$), advising that the AH effect might not be only restricted to the exsheathment blocking, but could also be targeting larval development. Belief that might be consistent with prior *in vitro* and *in vivo* reports of plants rich in secondary metabolites simultaneously affecting multiple key biological stages of GIN such as: i) egg hatching, ii) development, iii) feeding, iv) migration, v) exsheathment, vi) adult motility, and vii) fecundity (Hoste et al., 2012).

CONCLUSIONS

The consumption of *Gliricidia sepium* fresh leaves reduces *C. punctata* establishment in calves.

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Statement of interest

None.

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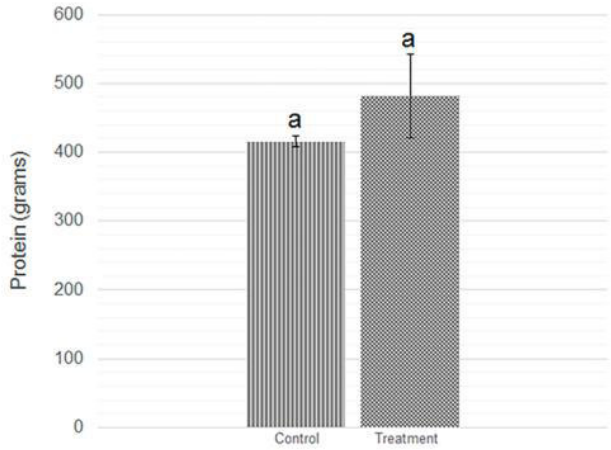
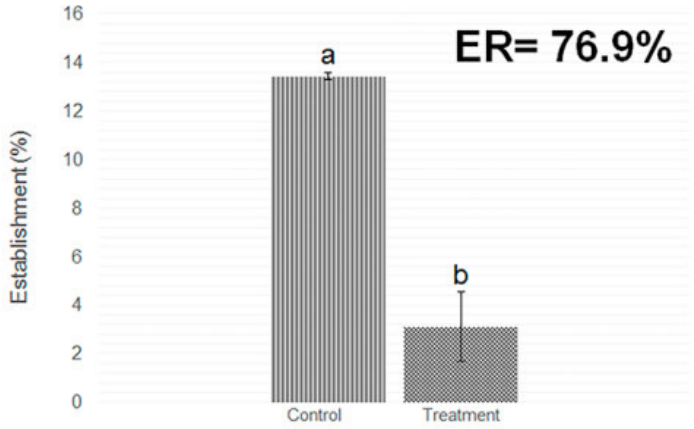
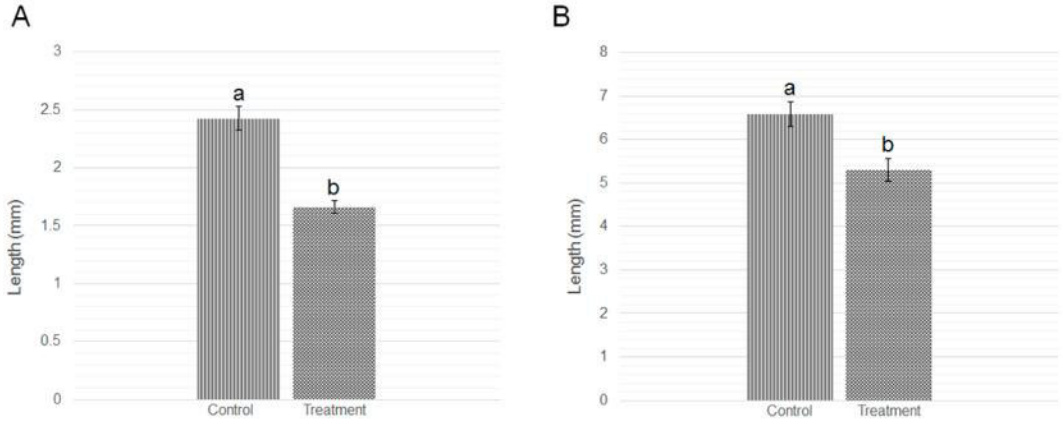


345
346 **Figure 1**. Daily protein intake during the 6-d experimental period, data presented as
347 grams per day (g / d; Mean ± SE).
348



349
350 **Figure 2**. *Cooperia punctata* larval establishment and reduction percentage
351 between experimental groups (Mean ± SE).



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353
354 **Figure 3**. Total length (mm) of worms recovered from intestinal washings (Mean ± SE).

Table 1. Chemical composition of the feedstuff (*G. sepium* and *D. decumbens*) offered to the calves during the trial (g/100g).

Diet	Material	O/R	DM	CP	EE	As	CF	NFE
Control (**T1**)	Dd	O	97.77	5.71	6.60	7.84	35.31	44.54
		R	98.43	6.00	5.23	11.94	35.62	41.21
Treatment (**T2**)	Dd	O	97.2	5.02	6.68	7.65	31.29	49.36
		R	98.06	5.57	4.56	12.02	38.17	39.68
	Gs	O	23.26	27.61	9.28	8.11	15.24	39.76
		R	35.12	24.52	9.46	8.24	15.98	41.80

*Dd: *Digitaria decumbens*; Gs: *Gliricidia sepium*; O: feedstuff offered; R: feedstuff rejected; DM: Dry matter; CP: Crude proten; EE: Ethereous extract; As: Ashes; CF: Crude fiber and NFE: nitrogen free elements.

Table 2. Dry Matter (DM) intake during the adaptation and experimental periods, presented as percentage of live weight (% LW; Mean ± SE).

	Dry matter intake (% LW)	
	Adaptation-period	Experimental-period
Control (**T1**)	2.78 ± 0.03 ^{Aa}	2.86 ± 0.11 ^{Aa}
Treatment (**T2**)	2.59 ± 0.05 ^{Ba}	2.24 ± 0.25 ^{Bb}

*Different capital letter in the same column represents statistically significant differences between treatments ($P < 0.05$).
*Different small letter in the same row represents statistically significant differences between periods ($P < 0.05$)

Table 3. *Cooperia punctata* third-stage infective larvae used for artificial infection (400 L_3 / kg LW) and fourth-stage / young adults recovered from intestinal contents.

Larval establishment of *Cooperia punctata*			
	Animal-ID	**Inocula**	**Recovered**
Control (T1)	A3	58,800	7,750
	A4	63,800	8,980
	A6	66,400	8,680
Treatment (T2)	B1	65,400	2,270
	B3	78,000	510
	B4	58, 200	3,550

Inocula were individually calculated according to live weight

11. DISCUSION GENERAL

La evaluación AH de plantas bioactivas sobre el control de NGI, surge como una alternativa para mitigar el impacto económico y de salud animal en las UPAs. Para implementar una estrategia eficiente de control de NGI, se necesita información epidemiológica de los NGI en la región de estudio (Hoste et al., 2015). Este trabajo se desarrolló tomando como base la información epidemiológica en animales sacrificados en rastro y en animales infectados artificialmente con NGI, en el trópico húmedo de Veracruz. En rastro, se encontró que el nematodo abomasal de mayor prevalencia fue *Mecistocirrus digitatus* (von Son-de Fernex et al., 2014) y en becerros menores a un año, infectados naturalmente con NGI fue el parásito intestinal *Cooperia punctata*. También se observó, que la prevalencia de *C. punctata* fue mayor a través del año en UPBs de la región. *Mecistocirrus digitatus* es un parásito abomasal que no había sido reportado desde hace 30 años en México; y los hallazgos en rastro a partir de éste trabajo de investigación demuestran que es un parásito hematófago que afecta al ganado adulto y cuyas lesiones al ser similares a las generadas por la hemonchosis pudiesen tener un impacto directo en el desempeño animal. La obtención de datos epidemiológicos sobre la prevalencia y distribución de los NGI en una región determinada permite el diseño e implementación delos métodos de control más apropiados. A diferencia de otros géneros de NGI, *M. digitatus* ha sido reportado en infecciones accidentales en humanos, lo cual pudiese representar un problema de salud pública (Tantaleán y Sánchez, 2007).

Se seleccionó a *C. punctata* como modelo de estudio debido a que fue el parásito que se pudo reproducir exitosamente en infecciones monoespecíficas. Además, *C. punctata* se ha reportado como un nematodo que causa importantes pérdidas a la ganadería bovina en el trópico y actualmente ha mostrado dificultades para su control mediante estrategias convencionales. Por otro lado, *M. digitatus* a diferencia de otros trichostrongylidos presenta un periodo de establecimiento que va de 28 a 45 días (Fernando, 1965), situación que dificulta la evaluación del efecto

antihelmíntico directo de plantas sobre el establecimiento parasitario en una infección combinada con *C. punctata*, ya que éste último se establece entre los días 7 y 12 post-infección (Stewart, 1954). Investigaciones previas recomiendan que para la validación del efecto antihelmíntico directo de plantas bioactivas, el diseño experimental debe considerar periodos cortos de evaluación para evitar la posible co-participación del sistema inmunológico en el efecto AH, y con ello la obtención de conclusiones erróneas (Hoste et al., 2015). Finalmente, y con base en las recomendaciones propuestas por Vercoe et al. (2010), la selección de las plantas utilizadas en las evaluaciones *in vitro* de éste trabajo de investigación, fueron consideradas con base en su potencial bioactivo, consumo voluntario y palatabilidad (Alonso-Diaz et al., 2008; González-Arcia et al., 2012; Iqbal et al., 2010; Juma et al., 2006; von Son-de Fernex et al., 2012).

Evaluación *in vitro* de cinco leguminosas tropicales (*Leucaena leucocephala*, *Gliricidia sepium*, *Cratylia argentea*, *Guazuma ulmifolia* y *Azadirachta indica*) sobre la inhibición del desenvaine larvario y la inhibición de la eclosión de huevos de *Cooperia punctata*.

La evaluación del efecto AH *in vitro* es una herramienta rápida y de bajo costo que permite valorar la actividad de múltiples extractos contra diferentes fases del ciclo biológico de uno o más NGI. Es este estudio se evaluó el efecto AH de extractos de plantas leguminosas que se utilizan para la nutrición y/o para el control de parásitos del ganado bovino en el trópico. Se seleccionaron bioensayos que permitieron evaluar el efecto AH sobre una fase de vida libre (huevos) y sobre la fase de transición parasitaria (larvas infectantes) de *Cooperia* spp. El huevo de los NGI se considera la fase biológica de mayor resistencia a los antihelmínticos (Bird and Bird, 1991), mientras que la larva infectante no solo representa a la fase biológica más longeva sino que al encontrarse expuesta por largos periodos de tiempo al medioambiente, también posee adaptaciones estructurales que les confiere un mayor grado de resistencia (Hoste et al., 2012). En ambos casos, las adaptaciones morfológicas y fisiológicas de resistencia que poseen representan un blanco

prometedor para el desarrollo de nuevas alternativas de control orientadas tanto a la reducción de la población larvaria en los potreros como a la disminución en el establecimiento parasitario dentro del hospedero (Hoste et al., 2012). Los resultados de este estudio permitieron determinar el potencial AH de las plantas, el método de extracción más eficiente para el análisis de la actividad AH, y el grupo de MS responsables del efecto AH contra *Cooperia* spp. Sin embargo, y tal como se programó en el protocolo de investigación, fue necesario profundizar en las plantas con mayor actividad AH, sobre el conocimiento de la(s) molécula(s) química(s) y/o sus posibles interacciones involucradas en el efecto AH así como el posible mecanismo de acción sobre los parásitos.

Fraccionamiento biodirigido de las leguminosas tropicales que mostraron mayor actividad antihelmíntica *in vitro*

En esta fase se realizó el fraccionamiento biodirigido de los extractos bioactivos, con el objetivo de aislar y determinar la(s) estructura(s) química(s) de los MS y/o sus interacciones con actividad AH. También se utilizó microscopía electrónica de barrido (SEM) y microscopía electrónica de transmisión (TEM) con la finalidad de conocer sobre los posibles mecanismos de acción de dichas moléculas. Se realizó el fraccionamiento biodirigido de los extractos *G. sepium*-AC y *L. leucocephala*-AQ. El efecto AH de los fitoquímicos aislados tanto de *G. sepium* como de *L. leucocephala* fueron ovicidas y de bloqueo de la eclosión, respectivamente. Estos mecanismos de acción se reportaron en un estudio previo donde se evaluó el efecto AH de leguminosas sobre la eclosión de huevos de *Haemonchus contortus* y las diferencias se asocian al tamaño molecular de los fitoquímicos y a su naturaleza (Vargas-Magana et al., 2014). Sin embargo, estos autores no determinaron las estructuras químicas de los compuestos responsables del efecto AH. En este estudio, para *G. sepium*, se identificó una cumarina (2H-chromen-2-one) como la responsable del mayor efecto ovicida (100 %) a una concentración de 1.1 mg / ml (CL_{50} de 0.024 ± 0.064 mg / ml). Mientras que para *L. leucocephala*, se observó la interacción de dos moléculas en el efecto AH, la quercetina y ac.

Caféico. Esta interacción inhibió el 90.49 ± 2.85 % de la eclosión de huevos a una concentración de 1.1 mg/ml (CL_{50} fue de 0.060 ± 0.14 mg / ml). Perry (2002) reporta que para que los MS ejerzan un efecto ovicida deberán pasar a través de las membranas del huevo, y para ello deberán tener una tamaño molecular no mayor a los 400-500 Da. Las moléculas que se aislaron es este estudio tienen un peso molecular de 146.14 Da para la 2H-chromen-2-one y de 302.235 Da y 180.1574 Da, para quercetina y ácido caféico, respectivamente (Pubchem-NCBI CID:323; CID: 5280343; CID: 689043). Lo anterior sugiere que a menor peso molecular, mayor potencial AH tanto para la inhibición de la eclosión como para ejercer un efecto ovicida. La microscopía electrónica de barrido (SEM) y de transmisión (TEM) permitió observar lesiones ultraestructurales que pueden estar relacionados con el mecanismo de acción de las moléculas aisladas e identificadas. Hubo alteraciones ultra-estructurales en el grosor y en la pérdida de continuidad de la tri-capa de los huevos de *Cooperia* spp. Hasta nuestro conocimiento, no existe ningún reporte del efecto AH directo de la cumarina, ni de la quercetina combinada con ácido caféico sobre huevos u alguna otra fase de vida libre de *Cooperia* spp. Brunet et al. (2011) reportaron daños similares en larvas infectantes de *H. contortus* y *T. colubriformis* incubadas en Sainfoin (*Onobrychis viciiifolia* Scop.), y que fueron asociadas con la muerte larvaria. Los diferentes análisis en ésta fase de investigación permitieron determinar que *G. sepium* y *L. leucocephala* tienen un potencial AH y que su validación *in vivo* es un paso necesario para poder utilizarse dentro de una estrategia de control de NGI en campo.

Evaluación del efecto *in vivo* del consumo de la leguminosa con mayor actividad AH *in vitro* sobre el establecimiento parasitario de *C. punctata* en becerros F1 (Ho X Cebú).

Para esta fase experimental, se seleccionó *G. sepium* debido a que en los estudios previos mostró el mayor efecto AH medido mediante la inhibición de la eclosión de huevos y de la inhibición del desenvaine larvario artificial contra *Cooperia* spp. Otro de los criterios de inclusión de esta planta fueron su disponibilidad como material

vegetativo en las UPB, sus características nutricionales así como los reportes previos de consumo voluntario (1.04 ± 0.2 MS kg / día) (Smith y van Houtert, 1987). Por lo tanto, *G. sepium* se utilizó para evaluar el efecto AH sobre el establecimiento larvario de *Cooperia punctata*. Se observó que el consumo de hojas frescas de *G. sepium* por becerros infectados redujo 76.9 % el establecimiento de *C. punctata*. Estos resultados son similares a lo reportado por Brunet et al. (2008) quienes al suplementar caprinos con plantas ricas en taninos se redujo 70.99 % el establecimiento de *H. contortus* y *T. colubriformis*. Hasta nuestro conocimiento éste es el primer reporte del efecto AH directo del consumo de plantas no taniniferas en bovinos. No obstante, los resultados obtenidos a lo largo de éste trabajo de tesis permiten confirmar mediante una validación integral, que el consumo de hojas frescas de *G. sepium* tienen un efecto AH de tipo preventivo; lo cual permitirá reducir las poblaciones parasitarias dentro del hospedero y posiblemente las densidades larvarias de *Cooperia punctata* en pastos debido al potente efecto ovicida que posee. No obstante, se recomienda la realización de otras evaluaciones *in vivo* que permitan determinar su potencial para ejercer un efecto AH de tipo curativo sobre las poblaciones adultas de *C. punctata* ya establecidas en el hospedero.

Finalmente, éste trabajo de investigación permite concluir que para la determinación de las propiedades nutraceuticas de una planta, es indispensable la utilización de diversas herramientas *in vitro* que deriven en resultados confiables y orientativos sobre su potencial AH. La integración de las diversas técnicas en éste trabajo de tesis nos permitió no solo elucidar nuevas moléculas con actividad AH que pudiesen ser utilizadas en la industria farmacéutica y su posible mecanismo de acción, sino que nos permitió orientar la evaluación *in vivo* a las características bioactivas propias de la planta; lo cual finalmente nos permite ofrecer una alternativa confiable para implementar dentro de un programa de estrategias de control contra NGI.

12. CONCLUSIONES GENERALES

Los extractos acuosos, acetona:agua (70:30) y acetónicos de las plantas bioactivas: *Leucaena leucocephala, Cratylia argentea, Gliricidia sepium, Guazuma ulmifolia* y *Azadirachta indica* inhibieron significativamente el desenvaine de larvas infectantes y la eclosión de huevos de *C. punctata in vitro*.

El fraccionamiento biodirigido de *L. leucocephala* y de *G. sepium* permitió el aislamiento de una combinación de moléculas (Quercetina y ácido caféico) y de la molécula pura 2H-chromen-2-one, respectivamente. Las cuales inhibieron la eclosión y/o el desarrollo embrionario de *C. punctata*.

La complementación de la dieta de bovinos con hojas frescas de *Gliricidia sepium*, redujo significativamente el establecimiento de *Cooperia punctata in vivo*.

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