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Ruta chalepensis full extract and organic phases exhibit nematocidal activity against *Haemonchus contortus* eggs and infective larvae (L_3)

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Summary

The ovicidal and larvicidal effect of a full aqueous extract (FE) and two phases: an aqueous (Aq-Ph) and an ethyl acetate (EtOAc-Ph) from *Ruta chalepensis* (*Rc*) stems and leaves against *Haemonchus contortus* (*Hc*) were assessed. The egg hatching inhibition (EHI) assay and larval mortality (LM) test were performed by triplicate in 96-well micro-titration plates (n=4 wells). The FE against *Hc* eggs and larvae was assessed at 1.25, 2.5, 5, 10 and 20 mg/mL; and 30, 60, 90, 120,150 and 200 mg/mL, respectively. The ovicidal effect of Aq-F and EtOAc-F was assessed at 1.25, 2.5, 5, 10 and 20 mg/mL. Plates were incubated at 28 °C for 48 (ovicidal assay) and 72 h (larvicidal assay). The EHI results were considered based on the mean number of eggs hatching failure after 48 h exposure. The LM was recorded after 72 h exposure to the *Rc* phases and expressed as mortality percentage. The *Rc* FE caused 96 and 100% EHI at 10 and 20 mg/mL, respectively; meanwhile, 74% LM was recorded at 200 mg/mL ($p<0.05$). The Aq-Ph showed 78.5% EHI at 2.5 mg/mL. Likewise, the EtOAc-Ph caused 100% EHI in almost all concentrations. Eighteen compounds including alkaloids, cumarins, triterpens, flavonoids, tannins, saponins and sterols were identified by GC-MS analysis. The results indicate that the aqueous extract from *Rc* possesses bioactive compounds with *in vitro* nematocidal activity against *Hc*; mainly in the EtOAc-Ph. Further studies should be performed to elucidate those compounds searching for alternative methods of control of the sheep haemonchosis.

Keywords: plant extracts; *Ruta chalepensis*; Ruda; nematodes; egg hatch inhibition; larval mortality

Introduction

Sheep and goat gastrointestinal parasitic nematodiasis (GIPN) are one of the most concerning diseases diminishing the zootechnical productivity of small ruminants mainly in tropical and sub-tropical areas of the world (Abebe *et al.*, 2010; Roeber *et al.*, 2013). The blood-feeding nematode *Haemonchus contortus* is considered as the most pathogenic parasite affecting sheep and goats all over the world (Saminathan *et al.*, 2015; Selemon, 2018). Chemical deworming of flocks is the most common practice of control of these parasites; however, the incorrect use of anthelmintic drugs triggers an anthelmintic resistance in the parasites (Ramos *et al.*, 2018; Wakayo & Dewo, 2015). Possible public health risk for residues drugs remaining in meat, milk or sub-products for human con-

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sumption (Imperiale *et al.*, 2004; Macedo *et al.*, 2015) is imminent. Further, there is potential damage to beneficial organisms in soil or aquifers due to the elimination of bio-active molecules derived from the chemical anthelmintic drugs in urine or faeces of treated animals (Beynon, 2012; Baydan *et al.*, 2015).

The use of plants with medicinal properties has been used by millenarian cultures as curative remedies of a number of diseases including parasitosis both in human beings and in animals (Bauer-Petrovska, 2012). *Ruta chalepensis* is an herbaceous plant belonging to the Rutaceae family and it has been widely used in the traditional medicine because of possesses a number of medicinal properties including: analgesic and antipyretic (Martínez-Pérez *et al.*, 2017), antimicrobial and antioxidant (Jaradat *et al.*, 2017) and anticancer (Terkmane *et al.*, 2017). Recent studies have assessed the *in vitro* anthelmintic effect of methanolic extracts obtained from *R. chalepensis* and other plants and has showed anthelmintic activity against gastrointestinal parasitic nematodes (Ortu *et al.*, 2016; Jasso-Díaz *et al.*, 2017). The objective of this study was to assess the *in vitro* ovicidal and larvicidal activity of a *R. chalepensis* full extract (FE) and two phases of this extract: an aqueous (Aq-Ph) and an Ethyl-acetate (EtOAc-Ph) against the sheep parasitic nematode *H. contortus*.

Materials and Methods

Location

This study was performed at the Laboratory of the Research Unit in Helminthology from Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, (CENID-SAI), belonging to the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), situated in Progreso, Jiutepec Municipality, Morelos State, Mexico. The plant extraction and the chromatographic techniques for partitions were performed at Centro de Investigación Biomédica del Sur (CIBIS-IMSS) in Xochitepec Municipality, Morelos State, Mexico.

Plant material

Five kg of the plant *R. chalepensis* (in fresh) was collected from a particular garden at La Milpa, locality, Villa García Municipality, Zacatecas State, Mexico. The plant material was deposited at the ethnobotanical garden of Cuernavaca city, State of Morelos, Mexico and the taxonomic identification of the plant, was performed by the Biologist Margarita Avilés, from the Autonomous University of the State of Morelos, Mexico.

Extraction procedure

The plant material (*R. chalepensis* aerial parts) was washed with tap water and was dried into a conventional stove at 45 °C for 3 days. Dried plant material (1.1 Kg) was ground to obtain small particles (1 mm) and transferred to a 4 L Erlenmeyer flask and distilled water was added to afore up to 4 L. The material remained under water maceration for 24 h. After this period, material was filtered through a Whatman paper (Nº 4). The extract was concentrated into a rotary evaporator (Heidolph Laborota 4000, Germany) at 50 °C with 90 rpm and at continuous vacuum. The whole solvent was eliminated until obtain a dense mixture that was frozen at -80 °C. Later on, the extract was lyophilized using a Heto spywinner lyophilizer to obtain a brown powder (308 g) which was finally maintained under darkness at -20 °C until use.

Aqueous extract fractionation

The *R. chalepensis* full aqueous extract (FE, 100 g) was subjected to a liquid-liquid bipartition with Ethyl acetate (EtOAc), what is a water immiscible solvent (used in 1:1 proportion). The liquid separation of the whole extract was performed as follows: One hundred grams of the extract were eventually resuspended into 1000 ml of distilled water. The liquid extract was processed for a bi-partition with Ethyl Acetate to obtain one aqueous phase and an EtOAc phase. The mixture was put into a crystal separation funnel (Pixer® 2000 mL N° 6400, pear-shape) and remained for 5 min to allow the phases separation. Then, the funnel key was opened to recover the individual phases. The denser phase was the aqueous phase (Aq-Ph) and this was the first collected phase and the less dense phase (EtOAc-Ph) was the last recovered phase. This method was repeated several times until the whole aqueous extract was processed. Both phases were concentrated using the previously described rotary evaporator. The aqueous phase was concentrated at 50 °C with 90-rpm rotations and at continuous vacuum. The solvent was eliminated until obtaining a dense mixture that it was frozen at -80 °C. The phase EtOAc-Ph was concentrated at 46 °C with 90-rpm rotations and also at a continuous vacuum until eliminating the whole solvent. Both phases were eventually lyophilized using the previously described lyophilizer and were maintained at -20 °C under dark conditions until use (García, 2015).

Thin-layer chromatography (TLC)

A phytochemical screening of the EtOAc phase of the *R. chalepensis* aqueous extract was performed in silica gel 60 F254 pre-coated plates. Samples were seeded using capillary tubes. Plates were developed into a crystal chamber with a low polarity system (95:5 dichloromethane:methanol) to allow the visualization of the highest amount of compounds present in the analysed sample. After developing, plates were dried though a soft agitation. Once the plates were dried these were observed under ultraviolet light at 245 nm and 365 nm wavelengths searching for compounds. Plates were developed using reagents specific for flavonoids, 2-aminoethyl diphenylborinate, Cerium (IV) sulphate solution and 4-dimethylamino benzaldehyde. After developing plates were dried on a hot stirrer until, visualize the coloured bands indicating the presence of compounds Delgado-Núñez *et al.* (2020). The bands corresponding to compounds of interest were identified by comparison with pure compounds either isolated or commercial that showed both a similar colorimetric pattern and similar development (von Son-de Fernex *et al.*, 2015).

Identification by Gas Chromatography-Mass Spectrometry (GC-MS)

The identification of molecules using Gas chromatography/mass spectrometry analysis was performed based on the protocol described by Pineda-Alegría *et al.* (2017). The chemical composition of EtOAc phase was analysed on a Gas Chromatograph-Mass Spectrometry (GC-MS) equipped with a quadruple mass detector in electron impact mode at 70 eV. Volatile compounds were separated onto a HP 5MS capillary column (25 m long, 0.2 mm i.d., with 0.3-µm film thickness). Oven temperature was set at 40 °C for 2 min, then programmed from 40 – 260 °C at 10 °C/min and maintained for 20 min at 260 °C. Mass detector conditions were as follows: interphase temperature 200 °C, and mass acquisition range, 20 – 550. Injector and detector temperatures were set at 250 and 280 °C, respectively. Splitless injection mode was carried out with 1 µL of each fraction (3 mg/mL solution). The carrier gas was helium at a flow rate of 1 mL/min. Identification of the compounds was performed with the comparison of mass spectra with those of the National Institute of Standards and Technology (NIST, 1.7 Library) and with data from the literature, which identified eighteen compounds (1 – 18).

Haemonchus contortus egg and larvae obtaining

One 10 months, Pelibuey male sheep previously infected with 350 *H. contortus* infective larvae (L3) per kg of body weight (BW) (per os) was used as an egg donor of the parasite. Faeces from this animal were processed to recover the eggs (in fresh) following the technique descried by Coles *et al.* (1992). The faeces were suspended in water and were passed through different sized meshes to separate detritus and residues of large size and eggs were eventually retained in a 37-µm-size mesh. Eggs were recovered into a precipitate glass and then the egg suspension was centrifuged at 300 rpm for 1 min with a separation gradient using saturated saline solution. After spinning down eggs floated up forming a white ring in the upper part of the centrifuge tube. Eggs were recovered using a crystal Pasteur pipette and re-suspended in water. Then, several washes with water were necessary to obtain perfectly clean egg suspension (Jasso-Díaz *et al.*, 2014). Likewise, faeces put into a plastic bowl for the elaboration of coprocultures. Faeces were crushed using a wooden spoon and tap water and small particles of rubber foam were added and mixed to create an adequate water and oxygen micro-environment for promoting the best nematode egg hatching (MAFF, 1986). Coprocultures were maintained under room temperature (18 – 25 ºC) for 5 days to allow the highest larval rate recovering. Infective larvae were obtained through the Baermann funnel technique for 12 h (Mesquita *et al.*, 2018).

Egg hatching inhibition assay using the whole extract, the Aq-Ph and the EtOAc-Ph of Ruta chalepensis

The egg hatching inhibition assay was performed on 96-well micro-titration plates. Five treatments were settle down as follows: 1) the whole extract, 2) aqueous (Aq-Ph) and 3) ethyl acetate (EtOAc-Ph) phases at 1.25, 2.5, 5, 10 and 20 mg/mL. 4) distilled water (a negative control, water was used to dissolve the whole extract and Aq-Ph) and 4) 4 % methanol (another negative control, methanol was used to dissolve the EtOAc-Ph). Fifty microliters of an aqueous suspension containing 100±10 *H. contortus* eggs were deposited in every well (n=4) and additionally 50 µL of the corresponding to extract, phases and controls were added in each well obtaining a 100 µL final volume. The plates were maintained at 28 °C for 48 h into a humidity chamber. After incubation 5-µL of lugol were deposited on each well in order to stop the egg hatching. Both first stage larvae (L1) and eggs of the parasite into each well were counted. The results were considered using an egg hatching inhibition rate; where the number of recovered larvae was considered as 100 % hatching eggs. The results were eventually expressed as egg hatching inhibition percentage. This experiment was performed by triplicate.

In vitro lethal activity of R. chalepensis whole extract against Haemonchus contortus (L3) infective larvae

This assay was also performed in 96-well micro-titration plates considering four well per treatment (n=4). It is important to mention that in preliminary results the same doses used in the ovicidal assay did not show any important larvicidal effect. So, we decided to increase the concentrations to identify some potential important larvicidal effect. The treatments were the serial concentrations of whole extract (at 30, 60, 90, 120, 150 and 200 mg/mL). Likewise,

Table 1. *Haemonchus contortus* egg hatching inhibition values attributed to the effect of a *Ruta chalepensis* full extract at different concentrations.

Concentration mg/mL	Average of recovered eggs (± Standard deviation)	Average of recovered larvae (± Standard deviation)	Egg hatching inhibition (%)
1.25	6 ± 3	144 ± 18	1[a]
2.5	87 ± 86	103 ± 79	41[a]
5	122 ± 86	95 ± 96	57[a]
10	225 ± 27	9 ± 6	96[b]
20	221 ± 69	0 ± 0	100[b]
Distilled water	5 ± 4	195 ± 10	3[a]

Different letters indicate statistic differences ($p \leq 0.05$)

Table 2. *Haemonchus contortus* egg hatching inhibition values attributed to the effect of two phases (aqueous and organic) and a full organic extract of *Ruta chalepensis* at different concentrations.

Concentration mg/mL	Aqueous phase (Aq-Ph)			Organic phase (EtOAc-Ph)			Whole organic extract		
	Average of recovered eggs (±Standard deviation)	Average of recovered larvae (±Standard deviation)	Egg hatching inhibition (%)	Average of recovered eggs (±Standard deviation)	Average of recovered larvae (±Standard deviation)	Egg hatching inhibition (%)	Average of recovered eggs (±Standard deviation)	Average of recovered larvae (±Standard deviation)	Egg hatching inhibition (%)
1.25	75 ± 5	57 ± 10	57^{c}	108 ± 15	0	100^{b}	6 ± 3	144 ± 18	1^{a}
2.5	107 ± 10	30 ± 12	78^{b}	105 ± 16	0	100^{b}	87 ± 86	103 ± 79	41^{a}
5	65 ± 6	65 ± 3	50^{c}	112 ± 15	0	100^{b}	122 ± 86	95 ± 96	57^{a}
10	63 ± 9	70 ± 9	47^{c}	105 ± 16	0	100^{b}	225 ± 27	9 ± 6	96^{b}
20	34 ± 4	135 ± 15	20^{d}	86 ± 17	0	100^{b}	221 ± 69	0 ± 0	100^{b}
Controls									
Distilled water	4 ± 2	127 ± 8	3^{a}	--------	---------	--------	5 ± 4	195 ± 10	3^{a}
Methanol 4%	--------	-------	--------	72 ± 12	3 ± 1	3^{a}	NA	NA	NA

Different letters in same column and row indicate statistic differences ($p<0.05$).------ not tested

Table 3. Volatile compounds identified by Gas Chromatography-Mass Spectrometry (GC-MS) from *Ruta chalepensis* EtOAc-Ph.

Compound	Retention time (min)	Molecular weight (a.m.u.)	Identified compound	Type of compounds
1	14.92	208	Elemicin	Phenylpropene
2	15.53	192	Piperonyl acetone	Phenylbutanone
3	16.33	186	1,2,3,3a,4,9,10,10a-Octahydrobenzo[f] azulene	phenylcycloalkanes
4	18.02	220	1-piperonylpiperazine	phenylpiperazine
5	18.48	186	7H-Furo[3,2-g][1] benzopyran-7-one, 4-methoxy	Furocoumarins
6	18.60	270	Ethyl pentadecanoate	fatty acid ester
7	19.36	256	Hexadecanoid acid	saturated long-chain fatty acid
8	20.20	248	PTH-4-hydroxyproline	nonessential amino acid
9	20.43	216	Methoxsalen	Furocoumarins
10	20.49	252	1-Octadecanol	Stearyl alcohol
11	21.21	308	Ethyl linoleate	fatty acid ethyl ester
12	21.23	284	Octadecanoic acid	saturated fatty acid
13	21.56	230	Procerin	cyclic alkene
14	21.69	229	4,4'-Dimethoxydiphenylamine	aromatic amine
15	22.16	246	5,8-Dimethoxypsoralen	Furocoumarins
16	23.24	340	n-butyl stearate	Fatty acid ester
17	27.28	314	12-methoxy-5β-abieta-8,11,13-trien-7-one	Diterpenoids
18	29.09	356	17α-methoxyprogesterone	steroidal progestin

distilled water was used as control. Ten microliters of an aqueous suspension containing 200±15 *H. contortus* infective larvae (L3) were deposited in each well (n=4 wells). Additionally 90 µL of the corresponding extract at different concentrations were added to each well, giving 100 µL as a final volume. The plates were maintained at 28 °C for 72 h into a humidity chamber. After the confrontation period, live and dead larvae were counted in ten 5-µL aliquots and the means of dead or live larvae per treatment were recorded. This assay was performed by triplicate. Mortality rate was expressed as a percentage mortality and was calculated according to the following formula:

Mortality= [(dead larvae mean) / (live larvae mean + dead larvae mean)] × 100

Statistical analysis

Data were arcsin√Xij transformed; since the distribution was not normal. Transformed data were analysed with a fix effect model completely random with six treatments with the PROC GLM program using the statistical package SAS and a Tukey mean comparison prove (SAS, 1999). The lethal concentrations (LC 50) for egg hatching inhibition and larvae mortality were estimated by the Probit analysis.

Ethical Approval and/of Informed Consent

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. The animal used as a donnor of *H. contortus* eggs was housed following the ethical, care/welfare and non-unnecessary suffering standard regulations of the Mexican Official Rule NOM-051-ZOO-1995.

Results

The percentages of the hatching inhibition (EHI) due to the egg exposure to *R. chalepensis* whole extract are shown in the Table 1. The EHI assay (EHI) revealed 41 and 57 % activity at 2.5 and 5 mg/mL concentration, respectively. Meanwhile, 96 and 100 % EHI were recorded at 10 and 20 mg/mL, respectively.

On the other hand, the *H. contortus* larval mortality attributed to the effect of *R. chalepensis* stems and leaves of the organic phases and the complete extract are shown in Table 2. The aqueous extract showed a low nematicidal activity at 30 and 120 mg ($p<0.05$); however, an increasing effect in the larval mortality proportional to the extract concentration increasing was observed at 150 and 200 mg/mL, reaching mortality values close to 60 to 75 % respectively.

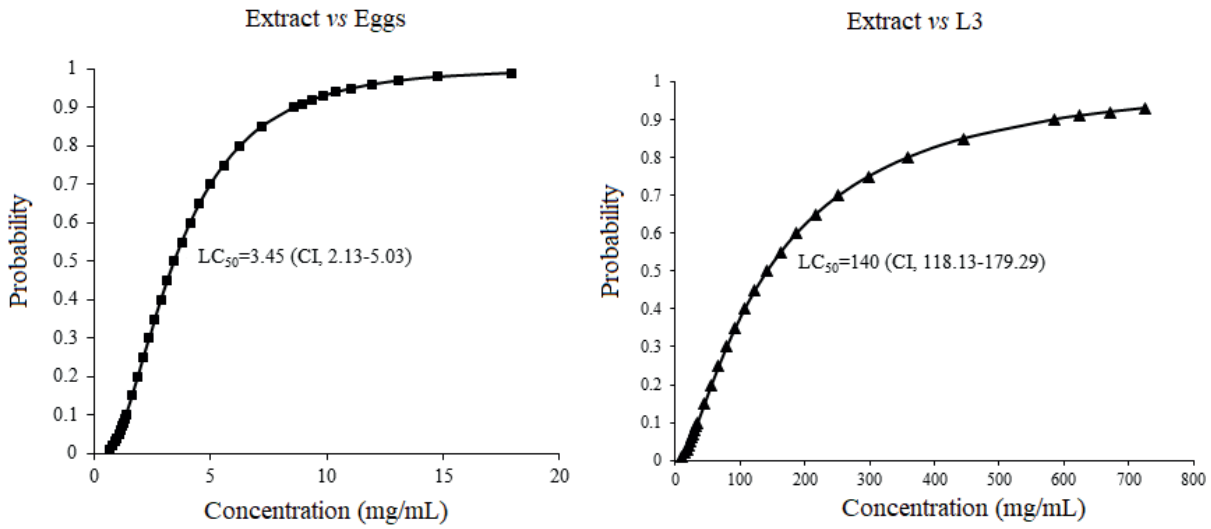

Fig. 1. Lethal concentrations (LC50) required to inhibit 50% of *Haemonchus contortus* eggs hatching after 48 h and larval mortality after 72 h exposure with a *Ruta chalepensis* full extract. CI= confidence interval (95%)

The results with the Aq-Ph were unexpected; since although, 57 % and 78 % egg-hatching inhibition were obtained using 1.26 mg/mL and with 2.5 mg/mL; respectively the subsequent concentrations (5, 10 and 20 mg/mL) showed a decreasing activity when the concentration was increased until reach 20 % inhibition with 20 mg/mL. In contrast, the organic phase (EtOAc-Ph) caused 100 % EHI at all the assessed concentrations.

The lethal concentration (LC50) caused by *R. chalepensis* FE both for eggs and infective larvae are shown on Figure 1. The analysis of AcOEt-Ph by GC-MS allowed the identification of eighteen compounds, into a molecular weight range between 192 and 356 a.m.u. The HPLC chromatograms resulted from *R. chalepensis* FE are shown in Figure 2.

In Table 3, these compounds are presented and listed in order of elution.

Discussion

During the last decades a number of studies focused to assess plant extracts from a diversity of worldwide flora against endo and ecto parasites of livestock have been carried out with encouraging results (Molento *et al*., 2020). This study shows evidence about a high anthelmintic activity of a whole extract and two phases an organic and an aqueous from *R. chalepensis*. This is the first time, to our knowledge, that the presence of the compounds (1 – 18) are report in aerial parts of *R. chalepensis*, as well as the anthelmintic activity. It is important to remark that from this list some compounds ie., the Ethyl linolenate obtained from other plants like *Ophiorrhiza rugosa* var. *prostrata* have been associated with anthelmintic properties (Adnan *et al*., 2019). Ethyl linoleate derives from a linoleic acid, produced by other organisms ie., nematophagous fungi and it has identified with anthelmintic properties (Stadler *et al*., 1993). Likewise, n-hexadecanoic acid and 1-octadecanoic acid presents also in other plants ie., *Corallocarpus epigaeus* have been also related to an anthelmintic activity against *Pheretima posthuma* (earthworms) (Ishnava & Konar, 2020).

On the other hand, the whole extract showed a dose/dependence effect in the egg hatching inhibition activity; where the highest activity (100 %) was recorded at 20 mg/mL concentration (Figure 1). It is interesting to mention that the phases obtained by bi-partition of the whole extract shown a much higher ovicidal activity than the whole extract; being the Aq-Ph and the EtOAc-Ph eight and sixteen times higher than the whole extract.

On the other hand, the aqueous phase of the extract, showed a different behaviour; since the peak of the highest activity was observed using 2.5 mg/mL. This was contrarious to what we expected; since no increasing in the ovicidal effect was observed and in contrast higher than 2.5 mg/mL concentrations provoked a proportional diminishing in the egg hatching inhibition effect where only 20 % activity was recorded at 20 mg/mL. In contrast, the organic phase from the bi-partition liquid: liquid with ethyl acetate inhibited 100 % the *in vitro* egg hatching at all assessed concentrations (1.25, 2.5, 5, 10 and 20 mg/mL). We have no categorical explanation about this finding observed with *R. chalepensis* aqueous phase; however, the results of the present research confirm a theory that other authors have suggested with respect to the non-polar solvents ie., ethyl acetate that concentrate a large number of compounds with anthelmintic activity. In this study the organic phase showed a lethal effect against nematode eggs at all assessed concentrations. Similarly, Akkari *et al.* (2015) assessed the *R. chalepensis* flowers

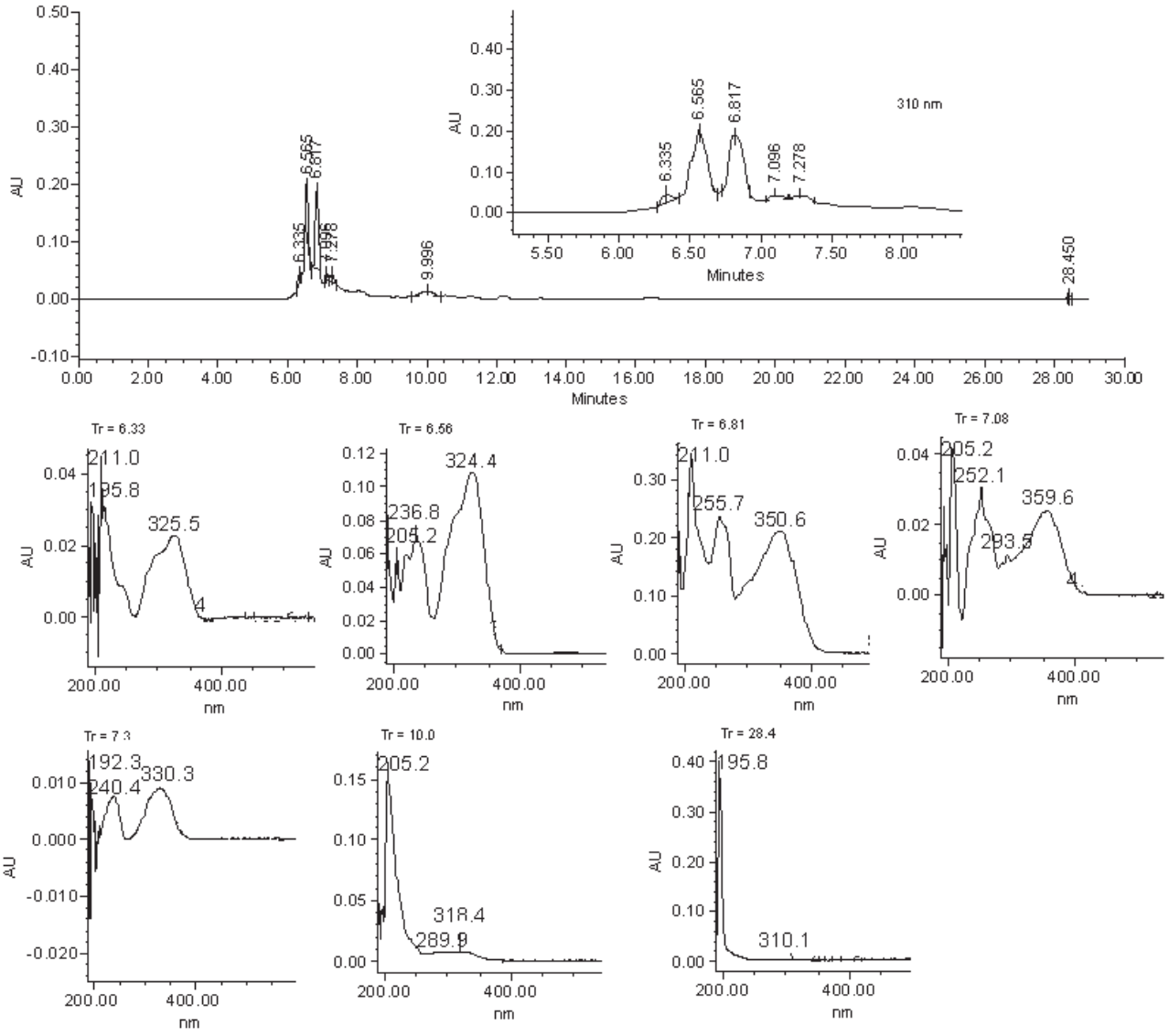

Fig. 2. Diagrams generated from HPLC chromatography showing spectra about phenolic compounds obtained from *Ruta chalepensis* FE under different retention times.

and leaves essential oil and they reported 100 % egg hatching inhibition of *H. contortus* using 1 mg/mL concentration. This is similar to the observed in the present study using an organic phase. It is interesting that a dose/dependent effect was recorded. The study shows evidence about both stems and leaves of *R. chalepensis* possess *in vitro* bioactive compounds against *H. contortus.* Similar results about the nematicidal activity of this kind of extracts were reported by Sánchez (2002), who observed that the aqueous extract of the upper aerial parts of other species of the genus *Ruta* (*R. graveolens*) caused 87 % mortality of *Radopholus similis*, a parasitic nematode of banana. Likewise, Sasanely (1992) reported a lethal effect of a *R. graveolens* aqueous extract dependent of the dose and confrontation times against *Xiphinema index*; causing 100 % mortality at 12 h exposure.

The mortalities recorded in the present study with the aqueous extract at 150 and 200 mg/mL are very important; mainly if we consider that this is only a complete extract and this can contain a large amount of compounds with no activity that can be discarded through chromatographic purification processes and on this way the most bio-active compounds can be selected and theoretically they could be very active even with much lower concentrations than the ones we used in this study (Jasso-Díaz *et al.*, 2017). In the present study, the egg hatching inhibition assay, recorded a response dependent of concentration and an absolute egg hatching

inhibition was recorded at 20 mg/mL. This fact is interesting; since some of the identified groups of compounds belong to alkaloids, coumarin, triterpenes, flavonoids, tannins, saponins and sterols that are been associated with either anthelmintic (Nayak & Manjari, 2011; Castillo-Mitre *et al.*, 2017) or entomopathogenic activity (Cárdenas *et al*., 2010; Barboza *et al*., 2010). The thin layer chromatography of the organic phase revealed the presence of similar chemical groups like coumarins, this compound could be responsible of the ovicidal activity recorded in the present study; although, more studies will be necessary to demonstrate this hypothesis.

Conflict of interest

Authors state no conflict of interest.

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