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Effect of season and sampling location on acaricidal activity of *Petiveria alliacea* on larvae of *Rhipicephalus microplus* resistant to acaricides.

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Abstract

To determine the effect of season and sampling location on the acaricidal activity of *Petiveria alliacea* against *acaricides* resistant *Rhipicephalus microplus tick*, methanol extracts of leaves and stems collected during the dry and rainy season at three sites in Yucatan, Mexico were used. Adult specimens of *R. microplus* ticks were collected to rear larvae and perform larval bioassays. For testing the acaricidal activity of plant extracts, larval immersion technique using different concentrations, namely: 20, 10, 5 and 2.5% was used. Extracts collected during the dry season exhibited the following 50% lethal concentrations (LC50): 4.4% and 1.7% for leaves and stems (respectively) from site 1, 8.2% and 2.2% for samples from site 2, and 18.3% and 22.4% for site 3. Extracts from samples collected during the rainy season showed the following LC50 values: 7.2% and 6.6% for leaves and stems (respectively) from site 1, 15.8% and 7.5% for samples from site 2, and 10.6% and 38.7% for site 3. In addition, gas chromatography-mass spectrometry run for stem samples showed that benzyldisulfide (BDS) and benzyltrisulfide (BTS) were present at higher concentrations in stems of dry season from sites 1 and 2. We conclude that the acaricidal activity of leaf and stem extracts from *P. alliacea* on resistant larvae of *R. microplus* is contingent upon the season and site of sampling. Extracts collected on dry season showed the highest acaricidal activity against *R. microplus* larvae. Site 1 showed the highest acaricidal activity across both seasons. The stem of *P. alliacea* performs better to control *R. microplus*, and higher concentration of BDS was observed in stem extracts collected on dry season in most locations. Further studies are needed to address the influence of edaphic and climatic factors on the phytochemical composition of plants.

Keywords: Acaricidal activity, *Petiveria alliacea, Rhiphicephalus microplus*, Season, Benzyldisulfide, Benzyltrisulfide.

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Introduction

Ticks and the diseases they transmit generate substantial constraints on the production of cattle worldwide [1]. The most common method to control ticks is the use of chemical drugs. However, the indiscriminate use of these products results in the emergence of strains that are resistant to these drugs [2]. As a result, in recent years work has focused on developing alternative methods of tick control [3-5].

The use of plant extracts to control ticks, particularly in the case of Rhipicephalus microplus, the cattle tick, appears to be a potentially viable alternative to drug use given the large number of plants that produce chemical compounds with acaricidal activity which have been identified thus far [6-8]. These plant-based products can be of use not only for organic cattle farming but could also represent an alternative method for controlling resistant tick strains [7].

Dominguez, Garcia and Kobayashi make [9-11] the point that environmental factors such as soil conditions, climatic conditions, as well as the presence or abundance of associated flora may affect the production and concentration of secondary metabolites in plants. Accordingly, the insecticidal and acaricidal activity of the compounds produced by focal plant species may be contingent upon such factors. Petiveria alliacea L. (Phytolaccaceae) is an example of a plant species that has been recently investigated in Mexico due to its acaricidal effects on R. microplus ticks [7]. In Latin America this plant is used in traditional medicine due to its analgesic, anti-inflammatory, antimicrobial, antispasmodic, diuretic, antipyretic, as well as acaricidal properties [12,13].

Studies conducted thus far in Yucatan, Mexico, reported that extracts of leaves and stems of P. alliacea exhibit high acaricidal activity (>85% mortality) against larvae and adults of R. microplus resistant to acaricides. Benzyldisulfide (BDS) and benzyltrisulfide (BTS) were identified as the main constituents and were proposed to be responsible for such effects [7,14,15]. However, it is currently unknown whether aspects of plant sampling such as season of the year and location influence the acaricidal activity of P. alliacea extracts as well as the concentration of BDS and BTS in tissues of this species. The aim of this study was to determine the effect of season and

sampling location on the acaricidal activity of P. alliacea against R. microplus larvae resistant to acaricides.

Materials and Methods

Sampling sites

This study was carried out in Yucatan, Mexico (19° 30´and 21° 35´N, 90° 24´W). Climate is warm-humid (Aw) with an annual mean temperature of 26°C (mean monthly range: 16.2°C-35.6°C), and a mean annual rainfall that ranges from <500 mm in the northwest end of the State up to 1,200 mm in the southeastern end [16].

Three study sites were selected, which exhibit markedly different environmental conditions:

Site 1, Yaxcaba: Located in the south-central portion of the State (between 20° 19' and 20° 49' N and 80° 36' and 88° 56' W), with a mean altitude of 29 meters above sea level. Climate is warm and humid with summer rains and lacks a defined winter thermal change. The mean annual temperature is 25.9°C; the highest mean monthly temperature is in May and the lowest in January. Mean annual rainfall is 1,111 mm, with a mean annual relative humidity of 89% [16,17]. The types of soil characteristic of this locality are Cambisols (CM), Calcisols (CL), and to a lesser extent Luvisols (LV) [18]. The vegetation is sub-deciduous tropical forest with different stages of succession [19].

Site 2, Hunucma: Located in the northwest portion of Yucatan (between 20° 55' and 21° 14' N, and 89° 48' and 90° 12' W), with a mean altitude above sea level of 10 m. The mean annual temperature is 26°C, the mean annual rainfall is 1,200 mm, and the mean relative humidity is 71% [16,17]. The predominant type of soil has a stony texture ("T'zekel"), which is fertile but retains very little moisture. In general, the soils in this locality are poorly developed and include a combination of Leptosols (LP) and CM [18]. The vegetation is represented by deciduous forest with different stages of secondary succession, abandoned henequen plantations, pasture lands for extensive livestock [19].

Site 3, Colonia Yucatan: Located at the eastern end of the State (between 21° 12'45"N and 87° 43'33"W), with a mean altitude above sea level of 17 m. The mean annual rainfall is 1,084 mm, with a relative humidity of 77% [16,17]. The soils in this locality are LP and CM, and the former are rich in organic matter and exhibit a high percentage of stoniness [18]. The vegetation at this locality is represented by sub-deciduous tropical forest [19].

Plant materials and extractions

At each study, location, leaf and stem samples of P. alliacea were collected once during the dry season (February-April 2013) and again during the rainy season (July-September 2013), for a total of 12 samples. Voucher specimens were authenticated and deposited at the herbarium of the Centro de Investigación Científica de Yucatán (CICY). Each plant sample (leaf and stem) was dried at 40°C for 2-3 days, shaked every 24 h during this drying period, and then ground with an electric grinder with a 5 mm diameter mesh. The material was macerated and compounds were extracted with MeOH (30 ml per 25 g of ground material) for 24 h. We performed two extractions for each plant sample [7,20].

The extracted methanol was filtered and evaporated at 45°C with a vacuum rotary evaporator. The crude extracts were then transferred to glass vials and kept at 4°C [21], until bioassays were performed to evaluate acaricidal activity on R. microplus larvae.

Tick collection and larval rearing

To determine the acaricidal of P. alliacea extracts against R. microplus, adult female ticks previously identified as resistant to amidines, organophosphates, synthetic pyrethroids and ivermectin were collected at a ranch in Yucatan [1,2,22-24]. From 200 to 300 engorged adult females from at least 20 cattle were collected. Ticks were placed in small plastic boxes with air holes and transported to the Parasitology Laboratory at the College of Veterinary Sciences (FMVZ-UADY). Females were rinsed with water and a sodium hypochlorite solution 0.05%, and then placed in Petri dishes (20 engorged females per dish) where oviposition took place. The Petri dishes were stored in a humid chamber (80-90% humidity and controlled temperature of 27 ± 2°C). Females were allowed to oviposit throughout a 15 day-period. The eggs were mixed and then transferred to five 3-mL glass vials with a cotton cap and placed in the incubator. Larvae emerged approximately 30 days after collection of engorged females, and we used 7-14 day-old larvae for the bioassays [7,23].

Larval immersion technique

Larval immersion test modified by Rosado-Aguilar et al. [7] was used for bioassays. Briefly, R. microplus larvae are immersed for 10 min in solutions with different concentrations of the leaf and stem extracts. Tween-20 at 2% was used to dilute and prepare four extract concentrations, namely: 20, 10, 5, and 2.5% [3,25]. The solutions were poured into Petri dishes with filter paper on the bottom and at least 500 larvae covered with another paper filter. After 10 min of exposure, we proceeded to fill packets, starting with the controls, and then following from the lowest concentration to the highest. A brush was used to place 80 to 120 larvae inside the packet. After filling the packets, we placed them in a clean tray inside an incubator (27 ± 2°C, 80-90% RH) for 48 h. The packets were opened 48 h post-treatment and we used a stereoscope to record the number of live larvae, dead larvae, as well as any toxicological effects. Larvae that were unable to walk forward were considered dead. We breathed on the packet while performing counts to stimulate live but inactive larvae. Three replicates of controls and of each extract concentration were performed [7].

Larval mortality was estimated using Abott´s formula [26], recommended by FAO [27]:

Corrected mortality=(% treated mortality-% controls mortality/ 100-% controls mortality) × 100

% mortality mean=(mortality replicate 1+mortality 2+mortality 3)/3

Detection of benzyldisulfide and benzyltrisulfide in P. alliacea extracts

The gas chromatography-mass spectrometry (GC-MS) analysis of P. alliaceae extracts was carried out with an Agilent 6890N gas chromatography equipment coupled with a mass selective detector 5975B using the following chromatographic conditions: split injection of 1 µl of a 1% concentration sample in CHCl3 analytical grade (Baker), and a flow rate of 1.0 ml/min using helium as carrier gas. Separation was performed using a 100% dimethylpolisiloxane capillary column (25 m × 0.2 mm i.d. × 0.25 µm film thickness). Samples were analyzed with the column held initially at 100oC for 4 min after injection, and then increased to 200°C with a heating program using a gradient of 10°C/min. All injections were done in split mode (split ratio 50:1). The interphase and injector temperature were set at 250°C. The mass spectrometer was operated under electron impact ionization (70 eV). All the detected peaks were identified by comparing the mass spectra with those available in the NIST mass spectral library. Benzyldisulfide (retention time 18.44 min) and benzyltrisulfide (retention time 5.38 min) were the most abundant components in the samples analyzed. The measurement of areas of all the detected peaks permitted a preliminary determination of the yield percentage of each metabolite present in the crude extract.

Statistical analyses

The mortality results of the four concentrations from each extract were analyzed by Probit methodology using Polo-Plus program in order to obtain the lethal concentrations at 50% (LC50) [28]. LC50 and 95% confidence intervals (CI) were calculated for leaf and stem extracts from each sampling season, at each site. If the CIs overlapped between a given pair of treatment levels, it's considered as a non-significant difference [28].

Results

Our estimates of LC50 for leaf and stem extracts of P. alliacea indicated that samples from the dry season were more effective against R. microplus larvae at most of the studied sites (Tables 1 and 2). No mortality was observed in the control group.

Leaf	20%	10%	5%	2.5%	LC50 (CI)
Dry season					
Site 1	96.0 ± 3.4	80.2 ± 5.7	48.99 ± 8.9	48.82 ± 17.4	4.4 (3.1-5.5)[a]
Site 2	83.8 ± 9.7	42.2 ± 12.8	36.5 ± 11.6	24.7 ± 2.3	8.2 (5.8-11.8)[b]
Site 2	56.8 ± 3.4	25.03 ± 5.1	14.54 ± 1.5	9.13 ± 1.0	18.3 (14.9-24.1)[c]
Rainy season					
Site 1	93.5 ± 0.7	64 ± 4.3	26.7 ± 2.6	12.7 ± 0.5	7.21 (6.5-7.9)[db]
Site 2	58.6 ± 7.1	35.1 ± 3.0	3.96 ± 1.4	3.0 ± 3.0	15.8 (13.3-20.1)[ec]
Site 3	72.0 ± 12.1	55.2 ± 1.4	20.71 ± 8.3	3.8 ± 3.5	10.6 (9.1-12.7)fb

LC_{50}: Lethal Concentration at 50%; CI: Confidence Intervals. ± Standard deviation. Different letters indicate significant differences ($p<0.05$).

Table 1 shows that the leaf extracts collected at site 1 in the dry season had the highest mortality of 96.0%, 80.2%, 48.99%, and 48.82% at concentrations of 20%, 10%, 5%, and 2.5%, respectively. This situation was corroborated with the lowest lethal concentration to kill 50% of larvae population (LC50: 4.4, CI: 3.1-5.5, $p<0.05$) of this leaf extract (Table 1). Similar results were found when the stem extract of site 1 in the dry season was evaluated (mortality of 100%, 92.0%, 88.1%, and 0.6% at concentrations of 20%, 10%, 5%, and 2.5%, respectively). The LC_{50} with stem of site 1 in the dry season was 1.7 (CI: 1.1-2.2, $p<0.05$) (Table 2). However when it was compared with the stem extract from site 2 collected during the dry season, these did not differ significantly ($p>0.05$) (Table 2).

Table 2. *Percent mortality and LC_{50} (and CI) values for Rhipicephalus microplus larvae exposed to Petiveria alliacea stem extracts collected at different sites and during different seasons.*

Stem	20.0%	10.0%	5.0%	2.5%	LC50 (CI)
Dry season					
Site 1	100 ± 0	92.0 ± 3.7	88.1 ± 4.5	0.6 ± 0.1	1.7 (1.1-2.2)a
Site 2	99.3 ± 1.2	94.1 ± 5.7	74.6 ± 9.8	56.2 ± 5.1	2.2 (1.6-2.8)ba
Site 3	54.9 ± 10.4	35.6 ± 37.2	6.6 ± 1.4	6.8 ± 2.0	22.4 (16.6- 37.1)c
Rainy season					

Site 1	88.3 ± 6.8	67.3 ± 8.6	31.4 ± 2.3	20.6 ± 2.4	6.6 (5.7-7.6)d
Site 2	91.9 ± 1.7	65.4 ± 10.3	29.3 ± 2.6	6.5 ± 1.6	7.5 (6.9-8.2)ed
Site 3	33.0 ± 10.6	22.1 ± 5.0	4.7 ± 2.2	6.4 ± 3.5	38.7 (24.3-110.8)fc

LC_{50}: Lethal Concentration at 50%; CI: Confidence Intervals. ± Standard deviation. Different letters indicate significant differences ($p<0.05$).

Results for stem extracts collected from sites 1 and 2 during the dry and rainy season exhibited the highest mortality values: 88.3-100%, 65.4-94.1%, 29.3-88.1%, and 6.5-56.2% at concentrations of 20%, 10%, 5%, and 2.5%, respectively. Stem extracts were generally more effective against R. microplus than leaf extracts ($p<0.05$), except at site 3 (Tables 1 and 2), where the leaf extract collected during rainy season had the highest acaricidal activity. In addition, the LC50 of the stem extract collected at site 3 did not differ significantly between seasons (Table 2).

The stem sample from site 1 collected during the dry season had a higher concentration of both BDS and BTS than that collected at this site during the rainy season. Likewise, the concentration of BDS in stems collected from site 2 was higher for samples from the dry season than for the wet season; however, BTS was not detected in samples from this site. In contrast, for site 3 we found that both compounds were present at a higher concentration in samples collected during the rainy season (0.738 and 2.390% for each compound, respectively) compared with the dry season (0.594% and 0.681%, respectively) (Table 3).

Table 3. *LC50 values for Rhipicephalus microplus larvae and concentrations of benzyldisulfide and benzyltrisulfide for stem methanolic extracts of Petiveria alliacea samples collected at different locations and during different seasons*.

Stem	LC50 (CI)	Benzyldisulfide (%)	Benzyltrisulfide (%)
Dry season			
Site 1	1.7 (1.1-2.2)a	0.641	0.860
Site 2	2.2 (1.6-2.8)ba	0.666	0.0
Site 3	22.4 (16.6- 37.1)c	0.594	0.681
Rainy season			
Site 1	6.6 (5.7 - 7.6)d	0.493	0.490
Site 2	7.5 (6.9 - 8.2)ed	0.492	0.0
Site 3	38.7 (24.3 - 110.8)fc	0.738	2.390

LC_{50}: Lethal Concentration at 50%; CI: Confidence Intervals. Different letters indicate significant differences ($p<0.05$).

Discussion

There is increasing evidence for the acaricidal activity of plant secondary metabolites, based on which the use of plant extracts could represents a viable strategy for controlling pesticide-resistant tick species, as in the case of R. microplus [3-5]. The use of plant extracts for tick control provides several advantages over conventional chemical drug use. For example, extracts can be used in organic cattle farming and even replace synthetic acaricides, they are associated with lower environmental and food contamination, result in slower development of arthropod resistance, and are of lower toxicity to animals and humans [7,8].

Previous work by Rosado-Aguilar et al. [7] showed that P. alliacea exhibited acaricidal activity against R. microplus resistant to acaricides; however, the efficacy of such effects is likely to be contingent upon the season and location where the plant was grown. Accordingly, previous work has pointed out that the concentration and nature of secondary metabolites in plants can be affected by intrinsic and extrinsic factors, which can ultimately alter the biological activity of these compounds against arthropod pests [20].

The present study showed that the acaricidal activity of leaf and stem extracts of P. alliacea on larvae of R. microplus were influenced by the time of year during which samples were collected. Higher activity against R. microplus was observed for extracts collected during the dry season at sites 1 and 2. Lower water availability during the dry season could play an important role in determining the concentration of plant secondary metabolites. For instance, previous work has shown that plants produce a higher or lower concentration of metabolites depending on conditions of abiotic stress, and this may produce concomitant variation in the acaricidal activity of plant extracts [29,30]. Likewise, other abiotic conditions such as temperature and amount of solar radiation may vary across study sites and could also play an important role in shaping plant, flowering, phenology, growth, and defense investment [31].

Stem extracts from samples collected during the dry season at sites 1 and 2 also had higher concentrations of BDS and BTS compared with stems collected during the rainy season. BTS has been isolated previously from root tissues of P. alliacea and shows an efficacy of 50% at 0.001 mg/ml against R. microplus adults [14]. This result also agrees with findings by Rosado-Aguilar et al. [7] who assessed the effects of P. alliacea stem extracts on R. microplus resistant to acaricides, and found that BTS and BDS were the biologically active compounds present at the highest concentration. Based on this, the authors proposed that these compounds were responsible for the observed acaricidal activity against R. microplus [15]. These results are also in agreement with a study conducted by Chaves et al. [29], who showed that the concentration of total phenolic (compounds with insecticidal activity) from Cistus landanifer was influenced by abiotic factors such as radiation, UV, and water stress, and reported that these compounds were present to the highest concentration during the spring.

From a physiological standpoint, lack of water in the dry season causes plants close their stomata and restricts photosynthesis process [32]. In such conditions, nonstructural carbohydrates tend to be accumulated and could explain the increase in synthesis of carbon-based defenses substances belonging to secondary metabolism. Confirmation of this balance carbon/nutrient has been found in species that grow in media with low availability of nutrients or water, in which occurs an increase in the concentration of compounds such as condensed tannins, lignin, total phenol and/or glycosides phenols [33,34]. Such findings could explain why the acaricidal activity of P. alliacea extracts against R. microplus was stronger during the dry season.

An unexpected result was found for samples from site 3, where stem extracts collected during the rainy season exhibited a higher concentration of BTS and BDS. Nonetheless, the acaricidal activity of extracts from this site was similar between seasons ($p>0.05$). This suggests that other compounds are involved in the acaricidal effects of P. alliacea on R. microplus. In agreement with this interpretation, we found strong acaricidal effects of stem extracts from site 2, but did not detect BTS in stem samples from this site. Accordingly, previous work has reported that P. alliacea contains numerous biologically active compounds such as terpenoids, saponins, polyphenols, tannins, alkaloids, stilbenes and acids which could have driven the observed patterns and may vary in concentration among plant tissues [7,35].

In general terms, plant extracts from site 1 showed a higher acaricidal activity (i.e. lower LC50) across both seasons compared with extracts from sites 2 and 3. Accordingly, previous work has shown that the chemical composition of P. alliacea extracts varies with the site of collection and soil type where plants were grown [8]. The higher acaricidal activity of site 1 extracts could be associated with the type of soil in the area, which is Calcisol (not present in sites 2 and 3). This type of soil is characterized by an accumulation of secondary carbonates and cemented carbonate [18]. This means that plants growing at this site have a higher availability of carbon in the soil and this could be influencing investment in carbon-based secondary metabolites under conditions of abiotic stress, and ultimately result in stronger acaricidal effects against R. microplus [34].

Overall, across all sites and seasons, stem extracts of P. alliacea showed a higher acaricidal activity against R. microplus larvae (lower LC50) relative to leaf extracts. This finding is consistent with a previous study by Rosado-Aguilar et al. [7] who also reported that P. alliacea stem extracts had stronger acaricidal effects on R. micoplus than leaf extracts.

Plant extracts can exert multiple effects on arthropods, including repellence and deterrence [36,37] on ingesta and oviposition, as well as toxicity when fumigated, topically applied, or ingested [38]. Toxicity is mediated by effects on cuticle disruption and inhibition of molting and respiration, and results in lower growth and fecundity [39]. In our case, P. alliacea contains sulfur compounds (e.g. BTS, BDS), stilbenes, and secondary metabolites [7,14,15] which may also have acaricidal activity; however, these metabolites have not been investigated yet and deserve further attention in order to elucidate the mechanisms and identify the compounds responsible for the observed effects of this plant species on R. microplus. Further studies should also address the influence of soil conditions, abiotic factors, and growing conditions which favor the production of target secondary compounds and thus maximize the efficacy of plant extracts used against ticks.

Conclusion

The acaricidal activity of leaf and stem extracts from P. alliacea on resistant larvae of R. microplus is contingent upon the season and site of sampling. Extracts collected on dry season showed the highest acaricidal activity against R. microplus larvae. Site 1 showed the highest acaricidal activity across both seasons. The stem of P. alliacea performs better to control R. microplus, and higher concentration of BDS was observed in stem extracts collected on dry season in most locations. Further studies are needed to address the influence of edaphic and climatic factors on the phytochemical composition of plants.

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