https://doi.org/10.22319/rmcp.v15i2.6348

Article

A novel effect of aqueous extract of *Pimpinella anisum* seeds on ticks of domestic dogs (*Canis lupus familiaris*)

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Abstract:

Synthetic pesticides used to combat ticks lose their effectiveness against certain species and can affect human health. The present study evaluated *in vitro* and *in vivo* the effect of the aqueous extract of *Pimpinella anisum* (*P. anisum*) seeds against *Rhipicephalus sanguineus* and *Ixodes affinis*, domestic dog ticks. In the *in vitro* evaluations, concentrations of 1.25, 2.5, 5, 10, 25, 50, 75 and 100 % of the aqueous extract of *P. anisum* were applied directly to ticks. The concentrations that had the highest effectiveness in immobilization were 50 %, 75 % and 100 %, but the latter caused immobilization for a longer time (55.89 ± 0.16 min). In the *in vivo* evaluation, the concentrated aqueous extract was applied to ticks attached to the skin of domestic dogs. Amitraz, a commercial tickicide, was used as a positive control. Both the concentrated aqueous extract and Amitraz caused 100 % of tick detaching. Nonetheless, concentrated aqueous extract of *P. anisum* seeds was more effective in reducing the average time of tick detaching (60.81 ± 3.17 min) compared to the commercial tickicide Amitraz (145.12 ± 15.97 min). This research suggests that the p-anisaldehyde identified in the aqueous extract could be linked to the immobilization and detaching of *R. sanguineus* and *I. affinis* from domestic dogs, suggesting that this extract could be used as a biopesticide to control ticks in domestic dogs.

Keywords: Biopesticide, Ticks, Immobilization, Domestic dogs, *In vitro*, *In vivo.*

Received: 31/10/2022

Accepted: 26/12/2023

Introduction

Ticks of the family Ixodidae are hematophagous arthropods of worldwide distribution, which parasitize various species of mammals, birds, and reptiles. When these ectoparasites feed on their host (various domestic and wild animals, including humans), they can transmit pathogenic microorganisms such as bacteria, viruses, protozoa, and helminths[1,2].

Ticks are pests that are considered economically harmful in livestock and other animal species as they can cause severe anemia and weight loss[3]. Dogs that inhabit rural and urban areas are hosts of the species *R. sanguineus*. These ticks can be found in tropical and subtropical regions, adapting to indoor conditions[4]. *Ixodes affinis* is a species that is also common in dogs and cats worldwide[5].

The use of synthetic pesticides causes damage to the environment and is a health hazard[6,7]; however, in recent years, alternatives have been sought to control pests without polluting the environment, one of them is the use of plants with insect or mite control capabilities.

The use of medicinal plants is a practice that has carried out since ancient times and has contributed to the origin of modern medicine[8]. Numerous medicinal plants contain secondary metabolites and pigments, among other components that are toxic against various microorganisms. It has been reported that phytochemicals isolated from medicinal plants are key to the generation of biopesticides; they are also considered less toxic and easily degraded[9,10]. In recent years, the applications of extracts and essential oils from plant species have become new alternatives as environmentally friendly pesticides, with the purpose of limiting the use of synthetic pesticides in the agricultural sector[7,11].

Among the plant species used to combat pests is *Pimpinella anisum*[12], commonly known as anise, green anise or badian[13], it belongs to the family Umbelliferae, currently called Apiaceae, and has been used in traditional medicine as a carminative, aromatic, disinfectant and galactagogue. *P. anisum* seeds have antimicrobial, antifungal, antiviral, antioxidant and anticonvulsant activities and muscle relaxing, analgesic, and hypoglycemic effects[14], and have also been reported to have insecticidal activity[15].The essential oil of the seeds of this plant species has been shown to have a lethal effect against *Tribolium castaneum*[12], repellent activity against adults of *Culex pipiens*[16] and is toxic against *Daphnia magna*[17]. Recent studies have shown that *P. anisum* seed oil has acaricidal activity against *Tetranychus urticae*[18], while aqueous and methanolic extracts have antimicrobial activity against *Candida albicans*[19] and *Escherichia coli*[20], respectively. Compounds such as *trans*-anethole, methyl chavicol, anisaldehyde, estragole, and γ-hymachalen[14] have been identified in the seeds of *P. anisum*, and p-anisaldehyde[14] has been identified in the essential oil, which is a compound that causes immobilization, repellency, and mortality effects in insects such as *Haematobia irritans irritans* (L.) and *Musca domestica* L., and the response of its application depends on the stage of development in these insects[21,22,23]. According to the background and effects caused by the species *P. anisum* against other insects, this study aimed to evaluate the effect of the aqueous extract of *P. anisum* seeds against ticks that commonly attack domestic dogs, in order to reduce the use of synthetic insecticides that are not friendly to the environment.

Material and methods

Biological material

The seeds of *P. anisum* were obtained from the commercial house Granos y semillas Yael, located at Calle 52 No. 540 and 67, Centro, Mérida, Yucatan, which were dehydrated.

Preparation of aqueous extract of *P. anisum* seeds

The concentrated aqueous extract of *P. anisum* seeds was obtained from 50 g of seeds, which were crushed in a manual mill (Del Rey brand) to obtain particles of 1.5 mm in diameter on average. The seeds were decocted using 12.5 g in 1 L of purified H_2O (Bonafont® brand) at 90 °C. Cooking time was 20 min. Finally, the aqueous extract obtained was kept in amber bottles and preserved under refrigerated conditions at 4 °C until use. Subsequently, the concentrated aqueous extract was diluted with purified H_2O (Bonafont®) to prepare concentrations of 1.25, 2.5, 5, 10, 25, 50, 75 % and the 100 % concentrated aqueous extract. Purified H_2O was used as a negative control and the commercial compound Combatick® (12.5 % Amitraz), an insecticidal and acaricide solution, was used as positive control, which was prepared and applied according to the indications on the product label (2 ml of the solution per 1 L of H_2O).

Toxicity bioassay

To determine the toxicity of the aqueous extract of *P. anisum* seeds, a toxicity test was performed on *Artemia salina* (White Mountain, Great Salt Lake, Utah, USA); the cysts were incubated in filtered seawater for 24 h, at a temperature of 29 ± 4 °C, with constant aeration[24].

Bioassays were performed on 24-well plates. Four concentrations (0.0005, 0.05, 5, 500 mg/ml) of the aqueous extract of *P. anisum* seeds were prepared by serial dilution. Ten nauplii of *A. salina* were placed in each well. Filtered sea H_2O without extract was used as a negative control. All treatments were analyzed fivefold. The plates were incubated at 29 ± 4 °C for 24 h; after this time, they were observed under a stereo microscope (SMZ800, Nikon) and the number of live nauplii was counted. Mortality was considered when no movement

was observed after 10 sec. Mortality percentage and median lethal dose (LC_{50}) were calculated. To consider whether the plant extract is toxic, the toxicity criteria proposed by Clarkson *et al*[25] were followed: non-toxic when LC_{50} >1,000 μg/mL, low toxicity 500 < LC_{50} <1,000 μg/mL, moderate toxicity 100 < LC_{50} <500 μg/mL, and highly toxic 0 < LC_{50} <100 μg/mL.

Tick collection on domestic dogs

A total of 270 adult ticks were collected from 10 naturally infested domestic dogs of different breeds, ages, and sexes from the municipalities of Ticul (20°23′43″N, 89°32′02″W) and Oxkutzcab (20°18′10″N 89°25′06″W), Yucatan, Mexico; these animals received no previous treatment. The ticks were placed in glass bottles with perforated lids and stored in the laboratory at 29 ± 4 °C for 24 h.

In vitro evaluation in ticks

For the *in vitro* toxicity evaluation, was used the concentrated aqueous extract of *P. anisum* seeds and seven dilutions of this same extract (1.25, 2.5, 5, 10, 25, 50, 75 %) and purified H_2O as a negative control. For each of the treatments, 30 ticks and 0.5 ml of the solution were used; the solution was applied by spraying it on the ticks. After 30 min, the percentage of immobilized ticks (% I) was calculated using the formula:

$$\% I = (N_i/N_T) \times 100$$

Where: % I= percentage of immobilized ticks; N_i= number of ticks immobilized; N_T= total number of ticks treated.

In vivo evaluation in domestic dogs

The concentrated extract was used to evaluate the effect of the aqueous extract of *P. anisum* seeds on ticks in domestic dogs. For this test, the positive control was Amitraz, which is a commercial miticide and insecticide, and purified water of the Bonafont® brand was used as a negative control. For the evaluation, 12 domestic dogs of different breeds, sexes and ages that presented problems with the presence of ticks on their body were included and they were divided into three groups of four canine specimens each for the application of the product to

be evaluated and the count of the number of ticks present in each individual (Tables 1, 2 and 3). For each dog, a volume of 0.5 ml of concentrated extract of *P. anisum* was used and applied to the left ear, tail, armpits and on the back of the animal where the ticks were. Amitraz was applied in accordance with the commercial producer's instructions. To determine the time it took for each treatment to detach the ticks, we waited until the last tick became detached by application area.

Table 1: Breeds of dogs infested with ticks in different areas, treated with the negative control (purified H_2O)

	Number of ticks by area				
Breed	**Left ear**	**Tail**	**Armpits**	**Back**	**Total number of ticks**
Chihuahua	6	12	5	12	35
Maltese dog	5	15	11	11	42
German shepherd	5	15	11	11	42
Mixed breed	13	7	12	3	35

Table 2: Breeds of dogs infested with ticks in different areas, treated with concentrated aqueous extract of *P. anisum*

	Number of ticks by area				
Breed	**Left ear**	**Tail**	**Armpits**	**Back**	**Number of ticks by area**
Chihuahua	7	6	11	7	31
Maltese dog	15	15	12	7	49
German shepherd	7	7	8	9	31
Mixed breed	5	16	15	5	41

Table 3: Breeds of dogs infested with ticks in different areas, treated with the positive control (Amitraz)

	Number of ticks by area				
Breed	**Left ear**	**Tail**	**Armpits**	**Back**	**Number of ticks by area**
Chihuahua	10	6	7	8	31
Maltese dog	13	5	14	11	43
German shepherd	7	7	8	9	31
Mixed breed	5	16	15	5	41

Identification of tick species in domestic dogs

The identification of the 100 randomly selected ticks, treated in the laboratory, as well as those that became detached from domestic dogs after the application of the aqueous extract and Amitraz, was by morphological characteristics, the shape of the hypostome, capitulum, and pedipalp of each of the ectoparasites. The ticks were placed in a 70 % ethanol solution for 8 min, during which time the ticks remained motionless. They were then observed with a stereo microscope (Stemi 305, Zeiss). The identification of the species was carried out using the images of ticks published by Lord CC[26] and Solís Hernández[27] as a reference.

Identification of p-anisaldehyde in the aqueous extract of *P. anisum*

To determine the presence of p-anisaldehyde, 5 mL of the concentrated extract was used, which was filtered through 0.22 µM nylon membranes (Thermo Scientific Cat. No. 726-2520). Subsequently, the solution was frozen in a deep freezer for 3 h (Thermo Fisher Scientific Inc., Model-TSX400D, No. 144DT0B01A) and lyophilized (LABCONCO-No. cat 77540-00) for 24 h. The lyophilized product was resuspended in 5 mL MeOH (TEDIA-MS1922-001), stirred (Vortex-genie-Serial No G-560) and centrifuged again for 5 min (Galaxy mini centrifuge – Serial No. 1204), obtaining the supernatant. For sample analysis, 2 µL of the supernatant was injected into a gas chromatograph coupled to mass spectrometry (Agilent 7890A, Wilmington, Delaware USA), equipped with a hydrogen flame ionization detector for compound identification. Compound separation was performed with an HP5MS column (Agilent Technologies, 30 m × 0.250 mm, 0.25 µm, Cat. No. 190915-435, USA). The injector temperature was set to 250 °C and the initial oven temperature was 70 °C for 3 min, increasing by 5 °C/min to 250 °C.

Statistical analysis

The data obtained in the *in vitro* and *in vivo* treatments were analyzed with one-way ANOVA with the Holm-Sidak test using the Sigma Plot version 12 program.

Results

Toxicity bioassay

The toxicity results of the aqueous extract of *P. anisum* seeds showed that at a concentration of 500 mg/mL, the highest mortality percentage (14.3 %) was obtained, while with the other concentrations no mortality was observed (Table 4). The median lethal dose (LC_{50}) was 4645 µg/mL. With the toxicity and LC_{50} results obtained and, according to the toxicity criteria proposed by Clarkson *et al*[25], the aqueous extract of *P. anisum* seeds is considered to be non-toxic for use in canines.

Table 4: Percentage of mortality of *Artemia salina* in the presence of the aqueous extract of *P. anisum* seeds

Concentration (mg/mL)	Mortality (%)
Control	0.0
0.0005	2.0
0.05	0.0
5	0.0
500	14.3

Immobilizing effect of aqueous extract of *P. anisum* seeds *in vitro*

In the *in vitro* treatment, the aqueous extract of *P. anisum* seeds was sprayed on the ticks, and after 30 min it was observed that the concentration of 1.25 % of the aqueous extract had no effect on tick mobility. At the concentration of 2.5 %, an immobilization percentage of 16.7 ± 5.8 was observed. In the higher concentrations, the percentage of immobilization increased significantly; with 25 %, more than 96 % of the total number of ticks evaluated were immobilized, and with concentrations of 50, 75 and 100 % of extract, 100 % of the treated ticks were immobilized, with no significant difference observed in the last four treatments (Table 5).

The average time of tick immobilization differed depending on each concentration of the aqueous extract of *P. anisum* seeds. It was observed that the 2.5 % aqueous extract caused the ticks to remain motionless for 3.00 ± 0.04 min; with 10 % of the aqueous extract the time was 14.9 ± 0.21 min; with 50 %, the immobilization time was 45.14 ± 0.07 min. The 100 %

concentrated extract caused the ticks to remain motionless for more than 50 min (55.89 ± 0.16 min). Immobilization time was statistically different between each treatment (Table 5).

Table 5: Percentage of immobilized ticks and immobilization time caused by the effect of the aqueous extract of *P. anisum* seeds under *in vitro* conditions

Aqueous extract concentration (%)	Immobilized ticks (%)	Immobilization time (min)
0	0^{a}	0^{a}
1.25	0^{a}	0^{a}
2.5	16.7 ± 5.8^{b}	3 ± 0.04^{b}
5	43.3 ± 5.8^{c}	10.07 ± 0.13^{c}
10	60 ± 00^{d}	14.9 ± 0.21^{d}
25	96.7 ± 5.8^{e}	28.07 ± 0.07^{e}
50	100 ± 00^{e}	45.14 ± 0.07^{f}
75	100 ± 00^{e}	50.14 ± 0.07^{g}
100	100 ± 00^{e}	55.89 ± 0.16^{h}

^{abcd} Different letters, placed as a superscript, indicate significant differences. One-way ANOVA ($P<0.005$).

In vivo effect of aqueous extract of *P. anisum* seeds on ticks attached to domestic dogs

In order to demonstrate the effect of *P. anisum* seeds on ticks attached to domestic dogs, the concentrated aqueous extract was evaluated, which, under *in vitro* conditions, caused ticks to be immobilized for longer than dilutions (1.25, 2.5, 5, 10, 25, 50, and 75 %).

In the comparison of the effects of the negative control of purified H_20 (Bonafont®), the aqueous extract of *P. anisum* seeds and Amitraz as a positive control, it was shown that the purified water did not detach ticks from the skin of domestic dogs; in contrast, the aqueous extract of *P. anisum* seeds caused all ticks to detach in an average time of 60.81 ± 3.17 min, while Amitraz required 145.12 ± 15.97 min, observing that the average time of tick immobilization of these treatments was statistically different (Table 6). After the detachment of the ticks from the domestic dogs, the concentrated aqueous extract maintained the effect of immobilizing the ticks in the ground for 14.625 ± 1.36 min. On the other hand, Amitraz did so for 44.93 ± 2.38 min (Table 7), with a significant difference in the time of immobilization of ticks on the ground.

Table 6: Effect of aqueous extract of *P. anisum* seeds on the detaching time of ticks attached to domestic dogs

Treatments	Detached ticks (%)	Total time of tick detaching (min)
Purified water	0	0
Aqueous extract (100%)	100+00	60.813 ± 3.17^{a}
Amitraz	100±00	145.125 ± 15.97^{b}

[ab] Different letters, placed as a superscript, indicate significant differences. One-way ANOVA (P<0.005).

Table 7: Immobilization time of ticks after detachment in domestic dogs, due to the effect of the aqueous extract of *P. anisum* seeds

Treatments	Average immobilization time of the total number of ticks (min)
Purified water	0
Aqueous extract (100%)	14.625 ± 1.36^{a}
Amitraz	44.938 ± 2.38^{b}

[ab] Different letters, placed as a superscript, indicate significant differences. One-way ANOVA (P<0.005).

Morphology of ticks evaluated

Morphological analyses suggest that ticks that were immobilized *in vitro*, as well as those that became detached from domestic dogs when the aqueous extract of *P. anisum* and Amitraz were applied, vary in shape and size of the hypostome, pedipalp, capitulum shape, and color. In all the ticks that were evaluated, four pairs of legs were counted, observing 80 % *R. sanguineus* and 20 % *I. affinis*.

Identification of compounds of the aqueous extract of *P. anisum* seeds

Nine compounds were identified in the aqueous extract of *P. anisum* seeds: p-anisaldehyde (4-methoxybenzaldehyde), butanoic acid, benzyl alcohol, and falcarinol (compounds with antimicrobial activity), phenol (antioxidant property), 2-myristynoyl pantetheine (aromatic compound), paromomycin (antileishmaniasis property), 10-heptadecen-8-ynoic acid, methyl ester, (E)- and *d*-mannose (anti-inflammatory activity) (Table 8).

Table 8: Compounds identified in the aqueous extract of *P. anisum* seeds by gas chromatography coupled to mass spectrometry

N	Compound name	Formula	Area (%)	Reported biological activity
1	4-methoxybenzaldehyde	$C_8H_8O_2$	0.55	Immobilizing and repellent effect[21,22,23] Antifungal activity[28]
2	Butanoic acid	$C_4H_8O_2$	6.31	Antibacterial activity[29]
3	Phenol	C_6H_6O	0.96	Antioxidant property[30]
4	Paromomycin	$C_{23}H_{45}N_5O_{14}$	0.04	*Leishmania amazonensis* treatment[31]
5	2-myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	0.02	Sensory property[32]
6	Benzyl alcohol	$C_8H_{10}O_2$	2.62	It inhibits the reproduction of β-hemolytic *Streptococcus* and *Proteus* spp[33]
7	Falcarinol	$C_{17}H_{24}O$	0.49	Antimycobacterial activity[34]
8	10-heptadecen-8-ynoic acid, methyl ester, (E)-	$C_{18}H_{30}O_2$	0.03	Anti-inflammatory[35]
9	*d*-Mannose	$C_6H_{12}O_6$	0.02	Anti-inflammatory[36]

Discussion

In the evaluation of the aqueous extract of *P. anisum* seeds under *in vitro* conditions, the main effect was observed to be the immobilization of ticks in domestic dogs. The percentage of ticks that were immobilized and the duration of the effect depended on the increase in the concentrations of the aqueous extract of *P. anisum* seeds. The effect of tick detachment and immobilization when *P. anisum* extract concentrate is applied could be due to the compound identified as p-anisaldehyde (4-methoxybenzaldehyde).

Likewise, it was shown that the correlation between the aqueous extract and its effect in this study agrees with published results, Showler and Harlien[21], where they evaluated the activity of p-anisaldehyde powder at 98 % purity of the sigma brand, observing that by increasing the concentration of this product from 0.125 to 2.5 %, the number of immobilized adults of *Haematobia irritans irritans* (L) increased. Showler and Harlien[22,23] reported that p-anisaldehyde has lethal and repellent effects on *Musca domestica*. It has also been shown that the increase in the concentration of p-anisaldehyde powder causes greater mortality of

Amblyomma americanum larvae; nevertheless, the effect it generated was in accordance with the application technique[37]. Considering this background, the present study suggests that the effect of tick immobilization could be due to the presence of p-anisaldehyde in the aqueous extract of *P. anisum* seeds, which the less concentrated it is, the shorter the duration of its immobilization effect.

When the aqueous extract was applied to ticks attached to domestic dogs, it caused them to detach, suggesting that the aqueous extract of *P. anisum* seeds generates immobilization; this effect causes ticks to detach from the skin of domestic dogs, as does Amitraz (positive control in this study). Amitraz is a widely used product for the treatment of ticks in domestic animals; unfortunately, the extensive use of this product has caused certain species of ticks such as *Rhipicephalus microplus* to become resistant[38]; it is also a product that can cause poisoning by inhalation and dermal contact[39].

In this research, it was found that, under the applied conditions, the aqueous extract of *P. anisum* seeds detaches adult ticks from the skin of domestic dogs by 100 %, having similar effects to the commercial product Amitraz. This means that the effect generated by this extract could be related to the concentration used, or the application technique.

Likewise, it was found that the aqueous extract of *P. anisum* seeds formulated with 50 g of seeds per liter of purified water is not toxic to the person who applies it, according to the studies carried out with *A. salina* and with the criteria of Clarkson *et al*[25]; in addition, the aqueous extract of seeds did not cause irritation or redness of the skin of domestic dogs.

According to morphological analyses, ticks with an elongated, brown body, with short hypostome and pedipalp, and a hexagonal shape of their capitulum belong to the species of *R. sanguineus*, which are characteristics that coincide with data published by Lord CC[26]. On the other hand, ticks with a round body, with long hypostome and pedipalp, and a triangular shape of their capitulum and dorsal shield indicate that they belong to the genus *Ixodes* or to the species of *I. affinis*, characteristic data that coincide with specimens of *I. affinis* published by Solís-Hernández *et al*[27].

Although the oily extract has been used in some publications, this does not limit the evaluation of the use of the aqueous extract of *P. anisum* seeds as a sustainable and economical alternative. The purpose of this research was to demonstrate that the aqueous extract of *P. anisum* seeds can also work for the treatment of ticks, in addition to being prepared in a simple manner and at a lower cost than extracting the essential oil from the seeds.

In this work, it was observed that the aqueous extract of *P. anisum* seeds has potential as a commercial use for tick control and is also affordable for domestic consumers. This study is

one of the first to be published in which the aqueous extract of *Pimpinella anisum* seeds is evaluated.

Conclusions and implications

Aqueous extract of *P. anisum* seeds can be a sustainable alternative for the treatment of ticks in domestic dogs; this extract has been shown to have a more effective detaching time than Amitraz, in addition to having an effect of 100 % ectoparasite detaching from the skin of domestic dogs and keeping them immobilized for a certain amount of time, although shorter than Amitraz. It was shown that the number of seeds used per liter of water for the production of the aqueous extract makes it non-toxic. In addition, this extract did not cause irritation in the area of application. It is expected that the results of this research will provide the basis for future research on the aqueous extract made from the seeds of *Pimpinella anisum* and that it will be applied to other pests that afflict living beings.

Acknowledgements

To the Higher Technological Institute of the South of the State of Yucatan for allowing the use of materials and facilities to carry out this research, and the support provided by the parents who allowed their canines to be evaluated.

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