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RESEARCH ARTICLE

OLEOVET FORMULATIONS AS LARVICIDAL ON *RHIPICEPHALUS SANGUINEUS* TICKS

FORMULACIONES DE OLEOVET COMO LARVICIDA CONTRA GARRAPATAS RHIPICEPHALUS SANGUINEUS

Roxana Gómez Zaldivar [a,*] (0000-0002-8388-4571)
Zullyt Zamora Rodríguez,[a] (0000-0002-9387-3761)
Gabriela Guerra Collazo [a](0000-0003-3435-3763)
Lidia Asela Fernández García[a](0000-0002-1043-5505)
Oscar Ernesto Ledea Lozano[a] (0000-0001-9106-9502)
Yamil Bello Soto[b] (0000-0003-0394-8561)
Alina Rodríguez Mallon[b] (0000-0002-6950-6793)

[a] National Center for Scientific Research.
[b] Center for Genetic Engineering and Biotechnology

a,* roxana.gomez@cnic.cu

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ABSTRACT

The dog tick (*Rhipicephalus sanguineus s.l*) is a vector of hemaparasitic diseases. Ozonated sunflower oil (OSO) called OleoVET has shown an acaricidal effect against *Demodex canis* and *Psoroptes cuniculi*. The objective of this research was to evaluate OleoVET and its formulations on *R. sanguineus* larvae. Three experiments were conducted using the larval immersion method. In the first, larvae were distributed in four groups: control group: sunflower oil (SO) and three groups of OSO with different peroxide index (PI). In the second, they were distributed in seven groups: SO, three formulations of OleoVET (45, 60 and 75 % PI) and others with Amitraz 12.5 %, Bayticol® and Asuntol®. In the third, four groups were treated: negative control with SO, Asuntol® as a positive control and two others exposed to OleoVET formulations (45 and 60 %). Mortality percentages at 72 h were determined. The results showed that larvae exposed to OSO, OleoVET and high PI showed mortalities of 99.34%, 100% and 100%, respectively, while formulations with 45 and 60 % PI caused 93.41 and 98.28 % mortality, with no significant statistical differences between them. In conclusion, the *in vitro* acaricidal efficacy of the ozonized substances, OleoVET and its formulations, was demonstrated.

Keywords: *Rhipicephalus sanguineus*, larvae, ozonized sunflower oil, formulations, OleoVET-95.

RESUMEN

La garrapata del perro (*Rhipicephalus sanguineus*), es un vector de enfermedades hematológicas. El uso inadecuado de los acaricidas, conducen al desarrollo de resistencia por parte de los ácaros. El Aceite de Girasol Ozonizado (AGO) identificado como OleoVET, posee efecto acaricida frente a *Demodex canis* y *Psoroptes cuniculi*, por lo que se tiene como objetivo, evaluar la acción del OleoVET 95 y sus formulaciones frente al cultivo de larvas *R. sanguineus*. Mediante el método de inmersión de larvas, se realizaron dos experimentos, en el primero, las larvas se dividieron en cuatro grupos: un grupo control tratado con Aceite de Girasol (AG), y tres grupos AGO; OleoVET-95 y HPI (Alto Índice de Peróxidos por en sus siglas en inglés), siendo los valores de IP (mmol-equiv. / kg) 9,91, 312, 569, 1184, respectivamente. En el segundo experimento, se conformaron siete grupos: el placebo, cuatro tratados con Amitraz 12,5%, Bayticol®, Asuntol® y Fipronil y dos con formulaciones de OleoVET-95 (F1 y F2). El porcentaje de mortalidad se determinó mediante conteo en estereoscopio después de 72 h de exposición. La mortalidad de las larvas expuestas a AGO, OleoVET-95 y HPI, incrementó significativamente en 99, 100 y 100 % respectivamente, con respecto al control (4,12%). La efectividad del Amitraz, Bayticol, Asuntol y Fipronil, fue 100; 90,9; 99,7 y 100 %, respectivamente, mientras que la efectividad de la F1 fue 93,41% y F2 98,28 %, evidenciando la superioridad de F2, sin diferencias con los productos de referencias. Concluyendo que F2 es un producto efectivo para el control de larvas de garrapatas.

Palabras clave: *Rhipicephalus sanguineus*, larvas, aceite de girasol ozonizado, formulaciones, OleoVET.

INTRODUCTION

Ticks that parasitize canines, commonly called, dog ticks, or brown ticks (*Rhipicephalus sanguineus s.l*), are hematophagous parasitic arthropods of the Ixodidae family. This tick species preferentially parasitizes dogs (Cruz & García, 1999) although it can also parasitize other domestic and wild animals and occasionally humans (Otranto & Dantas, 2010). The health harm caused by ticks extends beyond the discomfort produced on animal's skin due to bites; they also transmit various hematoparasites causing diseases, such as, Ehrlichiosis (*Ehrlichia canis)* Babesiosis (*Babesia canis)* and Hepatozoonosis (*Hepatozoon canis)*. These diseases transmitted by ticks have an impact on human health, as they are zoonotic; therefore, the control of these ectoparasites is of utmost importance (Gaínza *et al.,* 2014; León *et al.,* 2008). As a consequence, there are many scientists studying ticks and the control of their reproduction, including new developments of safe and effective acaricide products (Pérez *et al.,* 2002). The climatic conditions in Cuba are favorable to tick proliferation (Bermúdez *et al*., 2012). Rodríguez *et al.,* 2021 reported, the only presence of *Rhipicephalus sanguineus* s.l. tick species in dogs studied, with a higher prevalence, mostly in wet than dry period (León *et al*., 2008; González *et al*., 2016; da Silva *et al*., 2016). There are many other Latin-American countries, such as Panama, México and Brazil in which, there are also favorable climatic conditions for tick proliferation (Pérez *et al*., 2014; Pessoa *et al*., 2006; Braz *et al*., 2007; Silveira *et al.,* 2009; Ramírez *et al*., 2008). There is a wide range of products used for the control of tick populations. Most commonly used products include Asuntol®, which contains Coumofos as active ingredient, pyrethroids, including permethrin, cypermethrin, tetramethrin, and flumethrin, that is the active component in Bayticol®, amidines, such as Amitraz, (Bovitraz®), which inhibits monoamine oxidases enzymes and Fipronil among others (Romero, 2012). Fipronil is a phenylpyrazole that interferes with the central nervous system in arthropods through the blockage of GABA-gated and glutamate-gated chloride channels (Raymond *et al.,* 2005). Fipronil is one of the most widely used insecticides/acaricides to control fleas and ticks on both dogs and cats (Kuzner *et al.,* 2013). The indiscriminated use of these products has led to the appearance of tick resistant populations to these active ingredients (Romero, 2012).

Ozonized sunflower oil (OSO), commercially named as OleoVET 95 for veterinary use, consists of a mixture of active ingredients including hydroperoxides, peroxides, aldehydes, ozonides, among others obtained from the ozone oxidation reaction with unsaturated triglycerides (Lozano *et al.,* 2005). All these ingredients are responsible for the main pharmacological actions of OSO as a broad-spectrum germicide (Lezcano *et al*., 1996, 2000; Curtiellas *et al*., 2005, 2008 and Lozano *et al*., 2010); its demonstrated anti-inflammatory activity (Zamora *et al*., 2006; Zamora *et al*, 2018) and its action as a healing stimulator (Stable *et al*., 2021). The antifungal activity of OSO, was also evidenced in rabbits (Zamora *et al.,* 2007) and calves with dematophytosis (Romulo *et al.,* 2022), and in dogs with skin infection by malassezia (Delgado *et al.,* 2015). The *in vitro* OSO acaricidal action was firstly demonstrated on mites isolated from rabbit ears infested with psoroptic mange (Camp *et al.,* 2012). The *in vivo* effectiveness against this parasitosis was confirmed by the same study. Other studies, have supported the OSO effectiveness as a treatment for sarcoptic mange in pigs (Camps *et al.,* 2013), in the control of demodectic mange in sheep (Camps *et al.,* 2016) and in dogs (Rodriguez *et al*., 2021). The OSO safety was demonstrated by toxicological studies of dermal and ophthalmic irritability in rabbits, the phototoxic effect in puppies (Díaz *et al*., 2006, Rodríguez *et al*., 2019) and a single-dose toxicity study in rats and rabbits (Martínez *et al*., 1999). Mutagenicity, teratogenicity and genotoxicity studies similarly, showed the product safety, even when administered orally (Rodríguez *et al*., 1990; Remigio *et al*., 1998). Considering the previous acaricidal effect

demonstrated by OleoVET, the objective of this study was the evaluation of its effect as an active ingredient in different formulations, on *Rhipicephalus sanguineus* ticks by a larval immersion test.

MATERIALS AND METHODS

Ticks

Tick larvae were obtained from the *R. sanguineus* colony established at the Center for Genetic Enginering and Biotechnology (CIGB) by Encinosa *et al*., 2016.

Larval immersion method

Whatman No. 1 filter paper was placed in a 7 cm petri dish and 1.7 ml of each substance to be evaluated was poured into by triplicate. Subsequently, approximately 100 *R. sanguineus* larvae were placed in each plate and allowed to be in contact with the substances during 10 min. After that, 2.2 mL of each test substance was added to the larvae and a second filter paper was placed on top. Then again 1.7 mL of substance test was added. After 10 minutes, the filter papers containing the larvae were placed on sterile paper towels until all the contained product was absorbed. Once the filters paper was dried, the larvae were passed into a glass jar and incubated under controlled conditions at 28ºC and 80 % of relative humidity, during 72 hours. Thereafter, the alive and dead larvae were counted (White *et al*., 2004). The larvae mortality percentage was calculated using the following formula:

% mortality = (dead larvae) (100) / total larvae

Experimental Designs

First experiment

Larvae of *R. sanguineus* were divided into four experimental groups of 100 larvae each which were placed into the Petri dish of 7 cm diameter. The first group was treated with Sunflower oil (SO) with peroxide index (PI) of 9.9 mmol-equiv/Kg as negative control. The second group was treated with Ozonized sunflower oil (OSO) with a PI of 313 mmol-equiv/kg. The third group was treated with OleoVET with a corresponding PI of 569 mmol-equiv/Kg and the last group of larvae was treated with a high PI (HPI) of 1 185 mmol-equiv/Kg.

All these products, OSO, OleoVET, HPI and their formulations were prepared at the laboratory of Chemical formulations from the research group of Ozonized Substances belonging to the Directorate of Research, Development and Innovation at the National Center for Scientific Research in Havana, Cuba.

Second experiment

Larvae were divided in seven groups. The first group (negative control) was treated with SO: The second, third and fourth groups were treated with OleoVET in milk formulations at 45, 60, and 75 %, with PI of 214.78, 401.98, and 561.27 mmol-equiv/kg, respectively. The fifth, sixth and seventh groups were treated with the commercial acaricides Amitraz at 12.5%, prepared in /water in a proportion of 8mL/50 mL (v/v); Bayticol ® prepared as 0.025mL/50mL (v/v) and Asuntol ® prepared 0.25mL/50mL, respectively. (v/v). All substances were assayed in triplicate.

Third experiment

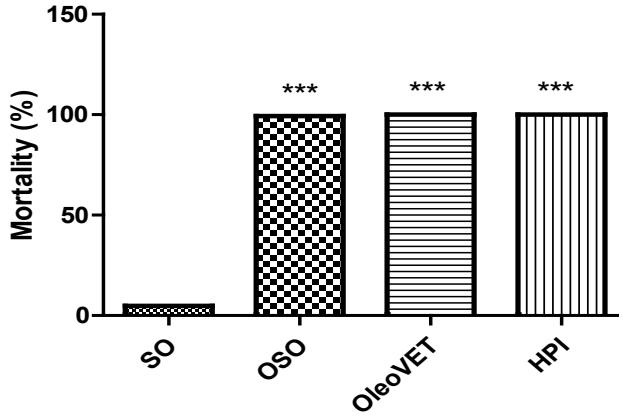
Four groups of larvae were defined. The first group was treated with SO as negative control. The second group was treated with Asuntol ® as positive group and the others two groups were treated with formulations of OleoVET prepared at 45% and 60 % in milk formulations with PI ranging between 230-270 and 350-470 mmol-equiv/kg, respectively.

Statistical analysis

The mortality percentages obtained for each experimental group were calculated and analyzed using Graph Pad Prism program version 0.5 as statistical package. The data were transformed and compared using an ANOVA of repeated measurements followed by the a Bonferroni test for multiple comparisons. Linear correlation analysis was performed to analyze the dose-response curves, according to concentration of the formulations.

RESULTS

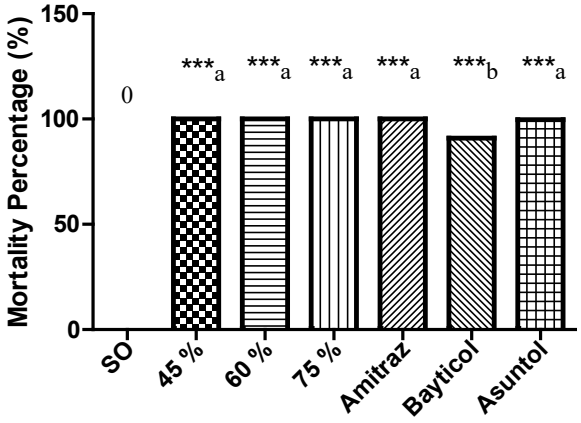
The mortality percentages of *R. sanguineus* larvae exposed to SO, OSO, OleoVET and HPI are shown in the **Figure 1**. The group of larvae exposed to the placebo substance (SO) had a mortality rate of 4.12 %. The group treated with OSO (PI of 312 mmol-equiv/kg), showed a statistically significant increase in the mortality percentage of larvae corresponding to 99.34 %, compared to the control group (SO). Similarly, both groups treated with OleoVET (569 PI) and HPI (1 184.92 PI) showed 100 % mortality of tick larvae (*R. sanguineus*), which was statistically significant different compared to the mortality in the control group. In the curve response analysis, there was not significant value $R^2 = 0.52$.



*** p< 0,001. Differences vs control.

Fig. 1. *Mortality percentage of R. sanguineus tick larvae, exposed to SO, OSO, OleoVET and HPI substances.*

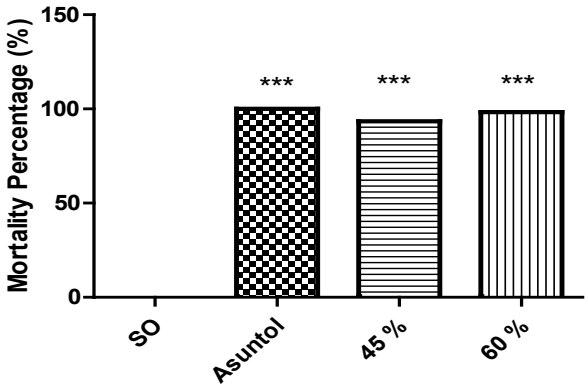
The mortality percentages of *R. sanguineus* tick larvae treated with OleoVET at different concentrations (45, 60 and 75%) in milk formulations and Amitraz, Bayticol and Asuntol as reference products, are shown in the **Figure 2**. The three OleoVET milk formulations significantly increased the mortality percentage of treated larvae (100 %), respect to the control group (SO). Similarly, reference acaricide products (Amitraz, Bayticol ® and Asuntol ®) reached mortality percentages of 100; 90.95 and 99.71 %, respectively, with significant differences respect to the control group (SO). There were not statistical differences among the OleoVET formulations and the commercial acaricides used as reference.



*** p< 0,001. Differences vs control. Different letters, differences between groups.

Fig. 2. *Mortality percentages of R. sanguineus tick larvae treated with OleoVET in milk formulations and reference acaricide products.*

The **Figure 3** shows the comparison among the mortality percentage of *R. sanguineus* tick larvae induced by exposure to Asuntol as positive control group and the mortalities obtained by exposure to the 45 and 60 % OleoVET milk formulations. As expected, the larva treatment with SO did not cause any mortality (0.01 %). On the other hand, the larva treatment with Asuntol as positive control, showed a mortality percentage of 100 %. Meanwhile, 45 and 60 % of OleoVET in milk formulations caused an increase in the mortality percentage of 93.41 and 98.28 %, respectively, compared to the negative control group. No significant differences were found between the mortality percentages achieved by the OleoVET formulations (45 % and 60 %), compared to the positive control group treated with Asuntol.



*** p< 0,001. Differences vs control.

Fig. 3. *Comparison of mortality percentage of R. sanguineus tick larvae treated with Asuntol and 45 and 60 % of OleoVET in milk formulations.*

DISCUSSION

These results demonstrated for the first time, that ozonized oil, has an *in vitro* acaricidal effect on the *R. sanguineus* tick larvae. All ozonized products with a PI ranging between 300 and 1200 mmol/equv/kg, produced between 99 and 100 % of mortality in larvae with 10 min of a contact time period as maximum. Figure 1. This high mortality caused by the larva exposition to Ozonized oil, compared to the mortality of larvae treated with SO, suggests that the active compounds of ozonized substances such as hydroperoxides, peroxides, aldehydes, ozonides, among others could be the responsible for the larva toxicity. In this point, it is interesting noting that the acaricidal action of these substances does not depend to the PI. Pharmaceutical forms of OleoVET obtained by formulating it at 45, 60 and 75 % in milk demonstrated also an acaricidal effect by the same larva immersion test Figure 2. The mortality percentages of *R. sanguineous* larvae reached for the OleoVET formulations were similar to those achieved by larva treatment with OSO, OleoVET and HPI.

In the same way, the mortality percentages achieved by the larva treatment with the OleoVET formulations did not differ significantly from those obtained with treatments using commercial acaricides such as Amitraz, Bayticol ® and Asuntol ®.

The effectiveness of OSO (Ozone & live, Brazil) was reported in a clinical case of tick-infected dogs (de Oliveira *et al*., 2014). The authors reported that OSO applications reduced the erythematous damage caused by tick bites. Now, our results have demonstrated the acaricidal effectiveness of OSO against *R. sanguineus* ticks.

According to the results of this study and the FAO classification (1993) of acaricides as products that reach at least 60 % of ectoparasite mortality, it is suggested that the products OleoVET, HPI, OSO and the OleoVET formulations could be considered as acaricides.

The acaricidal mechanism of OSO has not been studied in depth, however, if it is known that its broad-spectrum germicidal power is given by oxidizing action of its peroxidic compounds on microorganism's structures (Lozano *et al*., 2010) and that there are previous evidences that the exposure of *R. sanguineous* ticks to gaseous ozone caused a destruction of their cuticle structures and their respiratory spiracles causing a 100% of mortality (Moreira *et al*., 2018). Obviously, it could be suggested that the larvicidal effect of OSO against *R. sanguineous* larvae is due to the oxidative mechanisms that could damage structures and vital functions of the larvae. In order to corroborate the mechanisms involved in the acaricide effects shown here, further studies will be required.

A recent report has shown that *R. sanguineous* ticks exposed during 5 min to ozonated water caused an irreversible damage to tegument tissue, interfering in attachment processes, engorgement, and reproduction in females; and causing the death of epithelial cells and compromising the epithelium physiological function (Abreu *et al*., 2020).

Other studied oils, such as ozonized olive oil (OOO), garlic oil (*Allium sativum L.*), marjoram oil (*Origanum majorana L.*), compared to a permethrin treatment of infested cats with *O. cynotis* demonstrated a 100 % of effectiveness using OOO treatment, similar to that obtained with permethrin treatment. The treatments

with oils of *O. majorana L.* and *A. sativum L.*, reached effectiveness of 99.85 and 98.36 %, respectively, after 30 days of treatment (Yipel *et al.*, 2016). Some essential oils of *S. aromaticum* and Eugenol, were evaluated against *R. sanguineus*, using the larva immersion test. A mortality higher than 90 % was achieved in this experiment (Lambert *et al.*, 2021).

Natural products, such as the aqueous extract of Neem, produced 63 % of mortality in an *in vitro* assay using the same immersion method but with adult ticks of *Rhipicephalus (Boophilus) microplus*, while the reference product (Amitraz) used in this experiment induced 87 % of mortality (Pavón-Leyva, 2014). For this reason, the effectiveness of OleoVET formulations against tick adults of *R. sanguineus* should be assayed in subsequent studies.

The safety of OSO, OleoVET in milk formulations and HPI, at different PI, has been demonstrated through previous toxicological studies (Díaz *et al.*, 2006; Rodríguez *et al.*, 2019; Martínez *et al.*, 1999; Rodríguez *et al.*, 1990; Remigio *et al.*, 1998). The results of these studies and the low PI of these products, suggest them as products of choice for the control of *R sanguineous* tick infestations without any toxic effect on the host and the ecosystem (Polewczyk *et al.*, 2020; Pratiwi *et al.*, 2021) compared to the toxic effects reported for most of the reference acaricides, such as Amitraz, (Alemán Ortega, 2017). Asuntol and Bayticol (Junquera, 2021) for which a series of adverse effects have been described. Sometimes, these side effects limit their use, besides their possible ineffectiveness due to the development of ectoparasite resistance (Herrera *et al.*, 2016; Coles & Dryden, 2014). OleoVET and its formulations unlike the previously mentioned products, does not produce toxic effects applied topically, as has been tested by Díaz *et al.*, 2006; Rodríguez *et al.*, 2019.

It is important to note that reference acarides cause also the ecosystem contamination, however ozonized oils have been used for soil bio-remediation as part of treatment schemes that include also the presence of microorganisms (Polewczyk *et al.*, 2020). At the same time, a study demonstrated that ozonization of used cooking oil, reduced environmental pollution (Pratiwi *et al.*, 2021). Suggesting, to that ozonized sunflower oil does not represent a soil contaminant, on the contrary, its use implies the improvement of contaminated soils or the dumping of more oxygenated oils.

CONCLUSION

The larvicidal effect of ozonated sunflower oil at different peroxide index and its formulations was demonstrated, constituting a new product to be developed for the control of *R sanguineus* ticks.

ETHICS DECLARATION

This study was approved by the National Center for Scientific Research (CNIC) and the Center for Genetic Engineering and Biotechnology (CIGB).

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AUTHORIAL CONTRIBUTION

Roxana Gómez Zaldívar: Conceptualization, research, methodology, formal analysis and interpretation of results, drafting of the original manuscript, and editing

Zullyt Zamora Rodríguez: Conceptualization, research, methodology, formal analysis and interpretation of results, critical review and approval of the final version, drafting of the original manuscript, and editing

Gabriela Guerra Collazo: Conceptualization, research, methodology, validation

Lidia Asela Fernández García: Conceptualization, research, methodology, formal analysis and interpretation of results, critical review and approval of the final version, drafting of the original document, and editing

Oscar Ernesto Ledea Lozano: Conceptualization, research, methodology, and validation

Yamil Bello Soto: Conceptualization, research, methodology, formal analysis, and interpretation of results

Alina Rodríguez Mallon: Conceptualization, research, methodology, formal analysis, and interpretation of results; critical review and approval of the final version; drafting of the original document; and editing

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