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Original article

In vitro evaluation of the acaricidal effect of vegetal oils extracted from the kernel of *Thevetia peruviana* and *Annona muricata* on the *Rhipicephalus* (*Boophilus*) *microplus* larvae

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ABSTRACT

The objective of this work was to study the acaricide activity of oils extracted from kernels of *T. peruviana* and *A. muricata* with the hexane at different concentrations on *Rhipicephalus (Boophilus) microplus* larvae aged from 14 to 21 days. For this purpose, the Larva Immersion Test was performed. Twice dilutions of both oils were tested from a starting dilution of 40% to 2.5%. There were three repetitions for each concentration and for each oil. The control solution in which these larvae were treated consisted of Tween-20, diluted at 2% in distilled water. The experimental groups were stored in an incubator at 27 ± 1 °C and at 85-90% of relative humidity for 24 hours. The larvae mortality was greater than 55% in all tested groups and reached 100% at the 40% concentration for *A. muricata*, while the control group had a 0% mortality. The LC_{50} and CL_{90} obtained were respectively 4.0331% and 10.7594% for *A. muricata* and 7.0942% and 41.4247% respectively for *T. peruviana*.

These results clearly indicate that these oils all have acaricidal effects on *Rhipicephalus (Boophilus) microplus* larvae. However, *A. muricata* oil is the most toxic. It can therefore be used as an effective alternative to control the *Rhipicephalus (Boophilus) microplus* tick and there is a high probability that it can be used for other ticks affecting cattle and even other ectoparasites in Benin and worldwide, thereby reducing the use of synthetic acaricides which are toxic to the environment and ineffective against this tick.

1. Introduction

In Benin, cattle account for the majority (56.68%) of national meat production and total milk production (Countrystat, 2015). Improving domestic production of meat and especially milk will necessarily appeal to improving the performance of this species through research works on animal health (Farougou et al., 2013). In parallel with those researches, development researches aiming at improving domestic milk and meat production were carried out on cattle through projects. One of the improvement strategies developed by these projects is the introduction of new breeds. For example, in 2004, the Livestock Development Project Phase III imported cattle of the Girolando breed from Brazil. As a result, the arrival of these Girolando cattle at the Kpinnou farm in Benin led to the introduction of the invasive tick *Rhipicephalus (Boophilus) microplus* in Benin. Of all the ticks in the world, *Rhipicephalus (Boophilus) microplus* is a problematic species for livestock in the tropics. It acts as an important vector for three mains parasites, which cause tick fever: These include protozoa such as Babesia bovis, Babesia bigemina and marginal Anaplasma (Jonsson et al., 2008). The economic losses generated by this tick include the direct costs of ingested blood and indirect costs related to the transmission of pathogens (Barros-Battesti et al., 2006).

Therefore, the use of synthetic acaricides is the only one means to control the tick. However, the cost of acaricidal treatments and labor for their application, in addition to commercial restrictions by contamination of meat, milk and the environment because of the use of these chemicals slow down the profitability of the production (Benavides *et* Romero, 2002; FAO, 2003). Moreover, these chemical acaricides do not reach 100% efficiency: 28.2% for pyrethroids (Pereira, 2006), 98.6% for cypermethrin chlorpyrifos (Furlong et al., 2007) and 34.6% For amitraz (Davey et al., 2008). The use of synthetic acaricides is therefore limited and leads to the development of resistance (Yessinou et al., 2016b; Kearney, 2013; Lovis et al., 2012). Thus, the need to develop alternative methods of control from plant extracts. For this purpose, several research works have been carried out with different plant extracts on *Rhipicephalus (Boophilus) microplus* in Benin (Adinci et al., 2015; Yessinou et al., 2016a).

Those researches proved that plant extracts are a promising means of controlling *Rhipicephalus (Boophilus) microplus*. Furthermore, according to (Roel, 2001), plants having acaricidal activity has many advantages compared to synthetic products. They are rapidly degradable, the development of the resistance of these compounds is a slow process. However, apart from the researches of (Chougourou, 2012) on oils extracted from seed kernels in order to develop alternatives to environmental toxic chemical control of houseflies, no other information is known on oils extracted from seed kernels in order to develop alternative control methods against *Rhipicephalus (Boophilus) microplus* in Benin. The above reason justifies the current research whose purpose is to determine the larvicide effect of oil extracted from the kernel of the seeds of *T. peruviana* and *A. muricata* on *Rhipicephalus (Boophilus) microplus.*

2. Materials and methods

2.1. Vegetal material

The vegetal material consists of the kernel of *T. peruviana* from the family *Apocyanaceae* and *A. muricata* belonging to the *Annonaceae* family harvested in different ares of the district of Abomey-Calavi in Benin.

2.2. Extraction of oils

The extraction of vegetable oils was made from the shelled seeds of both plant species. The kernel obtained from the different seeds were crushed with a moulinex. After grinding and weighing, the oil was extracted from the ground material using hexane Soxhlet. The residue of hexane in the oil was evaporated under reduced pressure consisting of a water bath and a rotavapor. The oil obtained was stored in shaded glass bottles.

2.3. Cultivation of larvae

The cultivation of *Rhipicephalus (Boophilus) microplus* larvae was carried out in the Acarology laboratory of the Biotechnology Unit of Animal Production and Animal Health (URBPSA) of the Polytechnic School of Abomey-Calavi in Benin. The gorged females of this tick, taken from the cattle of the Kpinnou state farm in the Mono department, were fixed on cardboard paper and left in the open air in the laboratory for egg production. These eggs were transferred into flasks of about nine centimeters of diameter at a rate of 5g of egg per vial and the lid is a thin net that can prevent larvae from emerging after hatching. These flasks loaded with the eggs were placed in the oven under the conditions described by (Ibelli et al., 2012) (27-28 °C, 85-90% of relative humidity). Regular monitoring using an information sheet helped to identify the duration of the various phases of the evolutionary cycle leading to the obtaining of the larvae. These larvae were used for this test at their maturity (14-21 days after hatching).

2.4. Biological tests

The larvae immersion test was carried out to determine the efficacy of the extracts of both targeted kernelson the larvae of *Rhipicephalus (Boophilus) microplus* (Soberanes et al., 2002). The Tween-20, an emulsifier, was diluted in distilled water to serve as a control solution. Plant extracts were used to prepare a series of five solutions of 2.5%, 5%, 10%, 20% and 40% concentration for each extract. Each of the prepared solutions contains 2% of Tween-20. Three milliliters of each extract concentration were transferred into the 150 mm diameter Petri dishes prepared for the immersion tests. Using a N°4 brush, approximately one hundred (100) larvae were gently transferred into an envelope made from Whatman N°1 filter papers previously immersed in 3 ml of the corresponding solution and dried in the dark and dust free place for two hours (Rosado-Aguilar et al., 2010).

These envelopes containing the larvae were closed with plastic clasps and marked with a pencil (date of immersion, tested extract, concentration). Then, they were placed in an incubator at a temperature of 27 ± 1 °C and at 85-90% relative humidity for 24 hours. Finally, they were opened and the larvae, they contained were observed separately with a stereoscope. The number of living and dead larvae was recorded. Only larvae that can no longer move at all were considered dead.

2.5. Statistical analysis of data

The mean mortality rates of ticks and their standard errors were calculated for the different concentrations of the two oils tested and graphs were made to see their evolution as a function of the concentrations. To determine CL_{50} and CL_{90} lethal concentrations, regression models of the binomial family were fitted to the data for each of the oils using the *logit, probit* and *cloglog* link functions. The link function selected is the one with the smallest AIC (Akaike's Information Criterion). The CL_{50} and CL_{90} concentrations were then determined on the basis of the coefficients of the retained models and the predicted values. Moreover, in order to test any significant difference between the mortality rates induced by the different concentrations of the oils, Tukey multiple comparison tests were carried out on the previously retained models. All analyzes were made with the software R (R Core Team, 2015). Finally, a regression model of the binomial family was also adjusted to the data by taking into account the effect of the oil species to test whether the mortality induced by the different concentrations differs from one species to another.

3. Results and discussion

3.1. Duration of the phases of the evolutionary cycle leading to the larvae

From the sampling of engorged females to larval hatching, the average length of a part of the evolutionary cycle, namely: The pre-oviposition, the oviposition and the incubation of *Boophilus microplus* tick bred in the laboratory was determined. Thus, we obtained:

- An average of 5 days for pre-oviposition.
- An average of 17 days for oviposition.
- An average of 26 days for incubation.

3.2. Effect of oils extracted from seed kernels on the larvae of *Boophilus microplus*

The average mortality rate of larvae grows exponentially for oil concentrations from 2.5% to 20% after passing through an inflection point between 2.5% and 5% concentrations. This rate varies from 28.85% (*Annona muricata*) and 14.35% (*Thevetia peruviana*) then begins to stabilize after the concentration of 20% and then reaches for the last concentration applied (40%) the mortality rate of 100% and 80% respectively for *Annona muricata* oil and *Thevetia peruviana* oil (Fig. 1). Tukey's comparison tests showed that the average mortality rates observed for the different concentrations were significantly different from each other for to the two oils except for the 2.5% concentration which was not significantly different from the witness.

In addition, larvae mortality induced by *T. peruviana* oil was significantly different ($P<0.001$) from the one observed for *A. muricata*. The CL_{50} and CL_{90} values were 4.0331%; 10.7594% and 7.0942%; 41.4247% respectively for *A. muricata* and *T. peruviana* (Fig. 2).

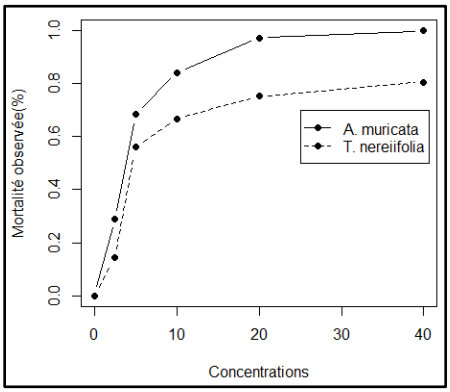

Fig. 1. Evolution of the average mortality rates based on oils concentrations.

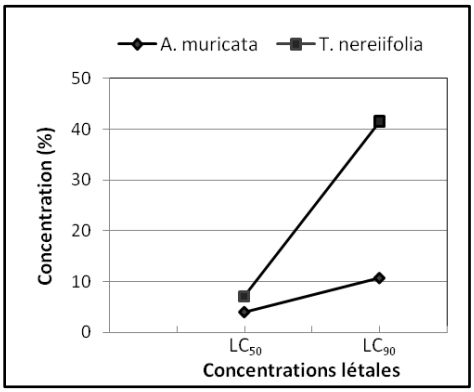

Fig. 2. Evolution of concentrations as a function of lethal concentrations (CL_{50} and CL_{90}).

3.3. Duration of the phases of the evolutionary cycle leading to obtaining the larvae

All along our experiment, the average durations of pre-oviposition, oviposition and incubation observed during the cultivation of *Rhipicephalus (Boophilus) microplus* ticks in the first part of their evolutionary cycle at a temperature of 27 ± 1 °C with 85 -90% relative humidity are respectively 5, 17 and 26 days. These durations are justified by the respect of the optimal cultivation conditions reported with respect to the *Rhipicephalus (Boophilus) microplus* tick. They correspond more or less to those reported in the literature and observed in other laboratory experiments on different species of ticks. Indeed, (Dantas-Torres, 2010) reports that pre-oviposition lasts between three days and a few weeks for *Rhipicephalus sanguineus.* (Koch, 1982) finds out that the duration of oviposition for *Rhipicephalus sanguineus* in the laboratory with variable conditions (Temperature: 10-35 °C, moisture: 15-95%) can last up to several weeks. For this same species, hatching takes place after an incubation period which varies between six days and a few weeks (Thirteen days in average) (Dantas-Torres, 2008b). Moreover, at a temperature between 19-23 °C, for the *Hyalomma* species, (Atte, 1992) obtained longer durations than those obtained in the present study. (Karimou, 1990) also reported higher durations with *Hyalomma impeltatum* and *Amblyomma variegatum* using a temperature of 25-27 °C and moisture maintained by cotton slightly moistened with water. In contrast, with the species *Hyalomma. marginatum rufipes* and *Boophilus geigyi*, this author obtained results close to ours. And our results are similar to those obtained by (Miko, 1985). For this author, with a temperature of 25 °C at night and 32 °C during the day, the duration of the pre-oviposition varies from 8 to 11 days, the oviposition is 6 to 11 days and the incubation is 21 to 26 days. It is then obvious that the duration of the phases of the evolutionary cycle of *Ixodidae* is influenced by environmental conditions (Temperature and relative humidity), the genus and the species.

3.4. Effect of oils extracted from seed kernels on the larvae of *Rhipicephalus (Boophilus) microplus*

Our results show that each of the oils extracted from the seed kernels of *A. muricata* and *T. nereiifolia* has a proven acaricidal effect on the larvae of *Rhipicephalus (Boophilus) microplus*. The acaricidal effect of these oils could be linked to the active substances contained in the vegetal material. In fact, the work of (Champy, 2004b) revealed that bioactive substances such as Thevetine and annonacin are respectively present in the seeds of *T. peruviana* and *A. muricata*. There was a significant difference (P<0.001) between average larval mortality rates obtained for the different concentrations for both oils. At a concentration of 40%, *A. muricata* oil induced 100% mortality after 24 hours, whereas at the same concentration *T. peruviana* oil induced only 80% mortality. It follows that the acaricidal effect of the *A. muricata* oil was higher than that of *T. peruviana*. Our results are better compared to those obtained by some authors such as (Shyma et al., 2014) in India with crude methanolic extract of *A. indica*, *C. procera*, *D. stramonium*, *A. sativum* and *C. papaya* on the larvae of *Rhipicephalus (Boophilus) microplus* which gave a significant mortality (P<0.001): 55.2, 63.2, 71.8, 69.0 and 82.2% respectively at the highest concentration of 100 mg / ml after 15 days of exposure; (Bisen et al., 2009) who conducted *in vitro* efficacy trials of neem seed oils and Karanj seeds against *Rhipicephalus (Boophilus) microplus* and found that Karanj seed oil was the most effective one (70%); (Duarte, 2014) who evaluated the acaricidal effect of ethanolic extract from the fruit of Capsicum L. (Solanaceae) and obtained a maximum level of 55% mortality at a concentration of 75 mg / ml.

Several authors evaluated the acaricidal effect of oils extracted from various seed kernels on *Rhipicephalus (Boophilus) microplus* and other ticks. In those studies, the mortality rates induced by these oils are very often high and similar to our results. Thus, (Maykelin Fuentes Zaldivar, 2014) obtained very significant larval mortality for *Rhipicephalus (Boophilus) microplus*, up to 98 and 99% with the oil from the almonds of *Jatropha curcas*. In the studies of (Farias et al., 2007, 2009), oil from *C. guianensis* seeds with concentrations ranging from 10% to 100% for the control of *R. (Boophilus) microplus* had given 100% mortality. The work of (Abdel-Shafy et al., 2002) on the larvae of *Hyalomma anatolicum excavatum* showed that *Azadirachta indica* oil caused 100% mortality after 15 days of exposure at a concentration between 1.6 and 12.8%.

Based on our results and those obtained by these authors, we can conclude that the acaricidal effect of a plant extract closely depends on the genus and species of tick, the nature of the extract, its concentration and the duration of the experiment. According to (Fourie et al., 2013), the efficiency can also vary considerably depending on the age of the plant and the harvest season of the vegetal material in each region. Moreover, based on the different concentrations and the duration of the experiment, our oils have a better efficiency compared to those obtained by certain authors. The maximum concentration in our study was 40% and the duration of the experiment was 24 hours. In addition, the CL_{50} and CL_{90} values of 4.0331%; 10.7594% and 7.0942%; 41.4247% respectively for *A. muricata* and *T. peruviana* will help to better fix the concentrations in order to obtain the same higher mortality rates (100%) but using less oil. This could lead to obtaining an effective and less expensive acaricidal product. For the oils tested, their uses as acaricide in a real environment must be preceded by the study of certain variables such as climatic effects, toxicology, stability, presence of residues in meat and milk, as well as tests on other tick species. Numerous studies have reported the results of in vitro efficiency of plant species on *Rhipicephalus (Boophilus) microplus* similar to the results of commercial acaricides (Silva et al., 2009). Similarly, many plant species have been recognized to have promising insecticidal effects in several tropical countries such as: *Sambucus nigra, Cumanenses Ambrosia, Ruta graveolens, Urtica dioica, Azadirachta indica* (Srivastava et al., 2008). Our results, then contribute to the objectives of the scientific community for the implementation of alternative vector control measures for long-term pest management without harming the environment and health (Saiyed et al., 2003).

4. Conclusion

This study is carried out with the purpose of implementing innovative initiatives that may lead in the future to the manufacture of acaricidal products for the control of *Bm*. Oil from fruit of *T. peruviana* and *A. muricata*. showed a remarkable efficiency in the mortality of the larvae of *Rhipicephalus (Boophilus) microplus.* The identification of the main chemical constituents using High Performance Liquid Chromatography (HPLC) and the active component as well as the prospects of future toxicological experiments can also be an important environmental and health indicator for the use of these oils in real environment by breeders.

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