Efficacy of 11 Brazilian essential oils on lethality of the cattle tick *Rhipicephalus (Boophilus) microplus*

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**A B S T R A C T**

Herbal extracts have been investigated as an alternative for parasite control, aiming to slow the development of resistance and to obtain low-cost biodegradable parasiticides. The goal of this study was to evaluate the efficacy, *in vitro*, of 11 essential oils from Brazil on reproductive efficiency and lethality of the cattle tick *Rhipicephalus (Boophilus) microplus*. The effects of oils extracted from *Cupuama longa*, *Zingiber officinale*, *Lippia alba*, *Lippia gracilis*, *Lippia origanoides*, *Lippia sidoides*, *Mentha arvensis*, *Mentha piperita*, *Croton cajucara* (white and red), and *Croton sacquaquina* on ticks were investigated by the Immersion Test with Engorged Females (ITEF) and the modified Larval Packet Test (LPT). Distilled water and 2% Tween 80 were used as control treatments. Chemical analysis of the oils was done with gas chromatography coupled with mass spectrometry. Analysis of the *in vitro* tests using Probit (SAS program) allowed the calculation of lethal concentrations (LCs). Lower reproductive efficiency indexes and higher efficacy percentages in the ITEF were obtained with the oils extracted from *C. longa* (24 and 71%, respectively) and *M. arvensis* oils (27 and 73%, respectively). Lower LC50 was reached with *C. longa* (10.24 mg/mL), *L. alba* (10.78 mg/mL), *M. arvensis* (22.31 mg/mL), *L. sidoides* (27.67 mg/mL), and *C. sacquaquina* (29.88 mg/mL) oils. In the LPT, species from Zingiberaceae and Verbenaceae families caused 100% lethality at 25 mg/mL except for *L. sidoides*. The most effective oils were from *C. longa*, *L. gracilis*, *L. origanoides*, *L. alba*, and *Z. officinalis*. The LC50 and LC90 were, respectively: 0.54 and 1.80 mg/mL, 3.21 and 7.03 mg/mL, 3.10 and 8.44 mg/mL, 5.85 and 11.14 mg/mL, and 7.75 and 13.62 mg/mL. The efficacy was directly related to the major components in each essential oil, and the oils derived from *Croton* genus presented the worst performance, suggesting the absence of synergistic effect among its compounds. Since *C. longa*, containing 62% turmerone, was the one most efficient against ticks, this compound may be potentially used for tick control, but further research is needed, especially to assess toxicity of these compounds to the host. These new studies, together with the results presented here, may provide a strong rationale for designing pre-clinical and clinical studies with these agents.

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1. Introduction

Brazil is the world’s largest beef producer and exporter. In 2013, Brazil had approximately 212 million bovine heads and from July to September of 2014, almost 8.5 million bovines were slaughtered (IBGE, 2014). Due to its territorial size, the country still has enormous potential to expand its animal production, but parasites represent a substantial constraint in tropical areas. Economic losses related to *Rhipicephalus (Boophilus) microplus* are due to a decrease in the production of meat and milk, hide damage, costs for tick control (labor, equipment, facilities, acaricides) and loss or treatment of animals with tick-borne diseases caused by *Babesia bigemina*, *Babesia bovis*, and *Anaplasma marginale* (Leal et al., 2003). Recent estimates of annual losses caused by the cattle tick, also known as southern cattle tick, reach around $3.2 billion. The annual potential economic losses due to the five major ectoparasites (*R. (B.) microplus*, *Haematobia irritans*, *Dermatobia hominis*, *Cochliomyia*

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amitraz, with volatile resistance populations of increasing oils. Recently, that cost of chemical products in developing countries, there is an emerging interest in alternative approaches to manage parasite populations (Lem et al., 2014). From the 1960s to the 1990s, resistance of the southern cattle tick to commercial acaricides such as organochlorates, organophosphates, synthetic pyrethroids, amitraz, and macrocyclic lactones was observed (Castro-Janer et al., 2015). Although fipronil and fluazuron were not commonly adopted for tick control, reduction in their costs and tick resistance to other molecules increased their use at the beginning of this century and currently, fluazuron is the only molecule for which no reports have described the presence of resistance in Uruguay. Recently, however, resistance to fluazuron, and cross-resistance between fipronil and lindane in R. (B.) microplus have been reported in Brazil (Reck et al., 2014; Castro-Janer et al., 2015). The emergence of highly resistant organisms has created a worrisome scenario that has influenced the course of scientific research in the field of healthcare. Therefore, the search for alternatives to minimize this problem has been proposed, such as the use of botanical insecticides based on plant extracts.

Essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal, and cosmetic applications, especially in the pharmaceutical, sanitary, cosmetic, agricultural, and food industries (Bakkali et al., 2008). Some of these oils constitute effective alternatives with reduced secondary effects, which can be used as single agents or together with synthetic chemical compounds (Carson and Riley, 2003). Extracts from some botanical families have been recognized as presenting biological activities against different organisms. In addition, there are an increasing number of studies concerning the use of essential oils in the veterinary field, particularly as control agents against ectoparasites like lice, mites, and ticks. A possible advantage of essential oils over conventional ectoparasite treatments may be associated with their reported ovicidal efficacy, repellent action due to their volatile components, and effect on tick fecundity when females are exposed to sub-lethal doses of essential oils (Ellse and Wall, 2014).

In the present study, essential oils extracted from plant species of the following families were evaluated: Zingiberaceae (Curcuma longa and Zingiber officinale), Verbenaceae (Lippia alba, Lippia gracilis, Lippia origanoides, and Lippia sidoides), Lamiaceae (Mentha arvensis and Mentha piperita), and Euphorbiaceae (Croton cajucara (red and white morphotypes) and Croton saccaquinha). The aim of this study was to investigate the efficacy of these 11 essential oils on the reproductive efficiency of engorged females, as well as whether any of them can be lethal to engorged females and larval of R. (B.) microplus.

## 2. Materials and methods

### 2.1. Plant material

The plant species used in the present study (Fig. 1; Table 1) were grown in 2014 at Embrapa Amazônia Ocidental, located in the city of Manaus, Amazonas State (AM), Brazil. Botanical samples of each species were deposited at the EAFM Herbarium of the Instituto Federal do Amazonas, located in Manaus. The cultivation occurred in field plots with soil classified as Dystrophic Yellow Latosol and which were composted using chicken manure (1.0 kg m⁻²) incorporated before cultivation. The field plots were located at the geographic coordinates 03° 06′ 23.04″S and 60° 01′ 35.14″W, having average altitude of 50 m, average temperature of 25.6 °C, and average annual rainfall of 2200 mm. The equatorial climate is characterized as Af, according to the Köppen classification (Köppen and Geiger, 1928).

### 2.2. Oil extraction

After harvest in July 2014, the parts of the plants (Table 1) were separated and put to dry in a covered shed for a week at an average temperature of 27 °C. The essential oils from each species and plant part were extracted by hydrodistillation in a modified Clevenger apparatus for 4 h. After separation was complete, oils were dried over anhydrous sodium sulfate and kept refrigerated (8 °C) in closed vials until used.

### 2.3. Chemical analysis

Chemical analysis of the oils was done with gas chromatography coupled with mass spectrometry (GC–MS) in an Agilent 5973N system (Agilent Technologies, DE, USA) equipped with an HP-5MS capillary column (5%-diphenyl, 95%-dimethylsilicone, 30 m × 0.25 mm; film thickness 0.25 μm). Helium was used as the carrier gas (1.0 mL min⁻¹). A total volume of 1.0 μL of a 1% solution of the oil in dichloromethane was injected into an injector heated to 250 ºC, operating in split mode (split ratio 1:100). Oven temperature was raised from 60 to 240 ºC at 3 ºC min⁻¹. The mass detector was operated in electronic ionization mode (70 eV) with the mass analyzer maintained at 150 ºC, the ionization source at 220 ºC, and the transfer line at 260 ºC. Linear retention indices were calculated by injection of a series of n-alkanes (C₇-C₉₆) in the same column and conditions as above. Identification of the essential oil components was done by comparison of both mass spectra and retention indices with the Wiley sixth edition spectral database and other data from the literature.

For quantification, the essential oils were analyzed in an Agilent 7890A chromatograph equipped with a flame ionization detector (FID) kept at 280 ºC and fitted with an HP-5MS capillary column.
(5% diphenyl–95% dimethyl silicone; 30 m × 0.25 mm; film thickness 0.25 μm). The same injection and chromatographic conditions described above were applied, but hydrogen was used as the carrier gas at 1.5 mL min⁻¹. The results are expressed as relative area (percent area).

### 2.4. In vitro tests

#### 2.4.1. Immersion Test of Enorganged Females (ITEF)

In vitro tests were performed at the Animal Health Laboratory of the Embrapa Southeast Livestock Unit (Embrapa Pecuária Sudeste – CPPSE), São Paulo, Brazil. *R. (B.) microplus* females were collected from naturally infested cattle at the CPPSE farm. In the laboratory, they were washed, dried, and selected according to integrity, motility, and maximum engorgement. They were divided into three groups of 10, to establish uniformity among the different experimental population, and placed in disposable cups to proceed with the immersion (Drummond et al., 1973). For each oil, seven dilutions were tested in triplicate: 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/mL. Distilled water and 2% Tween 80 were used as control treatments, also in triplicate.

The females were immersed in 5 mL of the respective treatment for 5 min. Next, they were dried and placed in Petri dishes, then labeled and kept in an incubator (±27 °C and RH > 80%) for oviposition. After 18 days, the eggs were weighed and transferred to adapted plastic syringes, identified, and sealed with cotton. They were then placed back in the incubator under the same conditions for larval hatching, which was read visually after 16 days of incubation. With these data, the reproductive efficiency index (REI) and the efficacy of the extracted product (EP) were calculated according to the formulas proposed by Drummond et al. (1973).

\[
\text{REI} = \frac{\text{egg mass weight} \times \% \text{egg hatching/engorged female weight}}{20,000^*}
\]

where * shows the constant that indicates the number of eggs present in 1 g.

\[
\text{EP} = \frac{(\text{REI control} - \text{REI treated})}{\text{REI control}} \times 100
\]

#### 2.4.2. Sensitivities of larvae on impregnated paper (Larval Packet Test – LPT)

For this test, 14- to 21-day-old larvae derived from the colony of *R. (B.) microplus* at CPPSE were used. Approximately 100 larvae were placed between 2 cm × 2 cm filter papers impregnated with the oils. The larvae were placed inside a filter paper envelope and sealed. The envelopes were placed in the incubator (±27 °C and RH > 80%) and readings were performed after 24 h, counting the dead and live larvae using a vacuum pump with an adapted pipette tip. Each of the extracts was dissolved in 2% Tween 80. The two controls consisted of distilled water and 2% Tween 80 plus distilled water. All tests were conducted in triplicate (Chagas et al., 2002). The essential oils were tested at the same concentrations of the ITEF.

#### 2.5. Data analyses

Calculation of each oil’s lethal concentration (LC) was performed by fitting the Probit linear regression using the normal distribution, and the generalized linear model was used for binary data (logistic regression), with estimates of the parameters of these equations by maximum likelihood. The procedure used was the SAS Probit to estimate the LC₅₀ and LC₉₀ (concentration necessary for 50% and 90% lethality, respectively) with the independent variable (dose) transformed by natural logarithm (log dose). The goodness-of-fit was assessed using Pearson’s chi-square test and maximum likelihood ratio.

### 3. Results

Each of the essential oils from the four plant families had its efficacy investigated against the tick *R. (B.) microplus* through two in vitro tests. To better understand the efficacy observed, we performed chemical analysis of the essential oils to identify their components. The main ones (higher than 10%) can be seen in Table 1. Among the plant species tested, the largest amount of compounds was detected in the essential oil extracted from *C. sacquaquinha* (57 different compounds), while the smallest amount was present in the *L. sidoides* essential oil (13 different compounds). Table 1 shows that *L. alba* and *M. piperita* had the lowest compound yield, while *L. sidoides*, *M. arvensis*, *L. origanoides*, and *L. gracilis* had the highest yield.

The reproductive efficiency index of the engorged female submitted to different essential oils was lower with *C. longa* and *M. arvensis* and therefore presented the better efficacy (Table 2). The ITEF determined the LC₅₀ of the oils extracted from *C. longa* (10.24 mg/mL), *L. alba* (10.78 mg/mL), *M. arvensis* (22.31 mg/mL), *L. sidoides* (27.67 mg/mL), and *C. sacquaquinha* (29.88 mg/mL). The lethality observed for the remaining essential oils against the engorged females was not sufficient to determine LC₅₀ values. In the LPT, species from Zingiberaceae and Verbenaceae families caused 100% lethality at 25 mg/mL, except for *L. sidoides*. *C. longa*
presented notable results, causing 100% lethality up to 6.25 mg/mL. The most effective oils against the tick larvae were from *C. longa*, *L. gracilis*, *L. origanoides*, *L. alba*, and *Z. officinalis*. The LC<sub>50</sub> and LC<sub>90</sub> were, respectively: 0.54 and 1.79 mg/mL, 3.21 and 7.03 mg/mL, 3.08 and 8.44 mg/mL, 5.84 and 11.14 mg/mL, and 7.74 and 13.62 mg/mL. *L. sidioides*, *M. arvensis*, and *M. piperita* oils revealed intermediate efficacy, while *C. cajucara* (red and white morphotypes) and *C. sacquaquina* oils had the worst performance. It was not possible to calculate the LC<sub>90</sub>, since these three oils reached a maximum efficacy of 39.9%, 56.9%, and 53.8%, respectively. All the results can be seen in Table 2.

4. Discussion

Extensive phytochemical analyses have led to the characterization of various essential oils and identification of compounds that are of wide scientific interest. Among them, terpenoids and phenylpropanoids are responsible for most of the biological properties of the essential oils. Many plant molecules are effective against drug-sensitive as well as drug-resistant strains of organisms (Raut and Karuppai, 2014). Thus, this study screened 11 essential oils for their biological effect on different developmental stages of the cattle tick *R. (B.) microplus*, which is strongly resistant to today’s commercial acaricides.

In the case of the species of the Zingiberaceae family, *C. longa* and *Z. officinalis*, the major components differed completely, except for 1,8-cineole, which was present in similar concentrations in both species. *C. longa* oil had the best performance in the tick tests (LC<sub>50</sub> of 10.24 and 0.54 mg/mL against females and larvae, respectively), presenting efficacy of 71% in engorged females and 100% in larvae from 6.25 to 25 mg/mL. According to its essential oil compounds, the presence of the sesquiterpenes α, β, and α-turmerone (62% in total) can be related to the effect of *C. longa*. The antimicrobial activity of extracts from *C. longa* obtained through hydrodistillation and extraction with hexane was evaluated by Nagethini (2006). Antifungal activity of both samples was observed against *Alternaria brassicicola* and *Aspergillus flavipes*. Higher antimicrobial activity was provided by the hexanic extract compared to the essential oil, and both ar-turmerone and α-turmerone were the prevalent compounds (Nagethini, 2006). Ar-turmerone is a compound with effective cytotoxic potential for tumor cells and fungi. Also, biochemical and histopathological analyses revealed the safety of using ar-turmerone for 14 days by oral gavage, as they did not reveal any changes (David, 2013).

A previous study evaluated the larvicidal activity of *Z. officinalis* essential oil against the filarial mosquito *Culex quinquefasciatus*. The late third instar larvae showed the LC<sub>50</sub> value of 50.78 ppm after 24 h of treatment (Pushpanathan et al., 2008). According to Maede et al. (2012), some monoterpenes are common constituents of a number of essential oils described in the literature as presenting mosquito repellent activity. In the study by Kumari et al. (2014), ginger essential oil contained 27.45% zingiberene, 13.82% copaene, 11.10% camphene, and 10.98% germacrene as the main components. In the present study, *Z. officinalis* oil presented 22.2% germacrene, 16.7% neral, 15.8% 1,8-cineole, and 11.3% camphene, among other minor components. Thus, although some differences are observed across oil composition, most of these components have been recognized as presenting biological activities against diverse organisms and as being responsible for the efficacy observed in our results, mainly those in the larvae stage (100% lethality at 25 mg/mL).

The main constituents in the essential oils extracted from species of the genus *Lippia* (Verbenaceae family) varied. In the case of *L. sidioides* oil, the main component detected was thymol, while for *L. alba* it was camvone, and for both oils extracted from *L. origanoides* and *L. gracilis*, carvacrol accounted for almost half of the components. Regarding lethality assessed by IETF, *L. alba* and *L. sidioides* essential oils showed the best efficacy in the genus (10.78 and 27.67 mg/mL, respectively), highlighting the better impact of *L. alba* on REI (33). In the LPT, the oils extracted from *L. gracilis* and *L. origanoides* caused 100% lethality in larvae at 25 mg/mL and had similar CL<sub>50</sub> of 3.21 and 3.08 mg/mL, respectively (they also have similar quantities of carvacrol and p-cymene). The next most effective oils in the LPT came from *L. alba* and *L. sidioides* (CL<sub>50</sub> of 5.84 and 9.89 mg/mL, respectively), although the latter had the worst performance among the Verbenaceae family for larvae lethality (84%). Cruz et al. (2013) evaluated the efficacy of *L. gracilis* essential oil and its major components, carvacrol and thymol, against *R. (B.) microplus*. The LC<sub>50</sub> obtained for the essential oil was 1.31 mg/mL on larvae for the genotype LGRA-201 and 4.66 mg/mL on engorged females for LGRA-106. This last genotype presented 59.26% thymol and for the other genotypes evaluated, the major component was carvacrol. Carvacrol (LC<sub>50</sub> of 0.22 and 4.46 mg/mL) was more efficient than thymol (LC<sub>50</sub> of 3.86 and 5.50 mg/mL, for larvae and engorged females, respectively). As reported by Cruz et al. (2013), other studies also detected acaricidal effects in vitro for these two isolated substances against *Amblyomma*, *Rhipicephalus*, and *Ixodes* tick genus (Panella et al., 2005; Novelino et al., 2007; Daemon et al., 2009; Mendes et al., 2011; Scolarick et al., 2012).

Species of the genus *Mentha* (Lamiaceae family) have menthol in their oil composition. Carvalho et al. (2012) evaluated the chemical composition of *M. piperita* essential oil and found quantities of menthol, menthyl acetate, pulegone, and menthone similar to what was observed in the present study, besides detecting 22.5% menthofuran. In this study, the oils derived from *M. arvensis* presented more than twice the amount of this substance as compared to *M. piperita* oil, which probably explains the better performance of *M. arvensis* in the IETF (LC<sub>50</sub> of 22.31 mg/mL). Although *M. arvensis*

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Table 2

<table>
<thead>
<tr>
<th>Plant species</th>
<th>IETF</th>
<th>LPT</th>
<th>LCP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Curcuma longa</em></td>
<td>2471</td>
<td>10.24 (4.44–84.37)</td>
<td>100</td>
</tr>
<tr>
<td><em>Zingiber officinalis</em></td>
<td>6227</td>
<td>10.78 (6.01–30.26)</td>
<td>100</td>
</tr>
<tr>
<td><em>Lippia alba</em></td>
<td>3362</td>
<td>7.57 (6.01–30.26)</td>
<td>100</td>
</tr>
<tr>
<td><em>Lippia gracilis</em></td>
<td>5532</td>
<td>10.78 (6.01–30.26)</td>
<td>100</td>
</tr>
<tr>
<td><em>Lippia origanoides</em></td>
<td>4939</td>
<td>7.57 (6.01–30.26)</td>
<td>100</td>
</tr>
<tr>
<td><em>Lippia sidoides</em></td>
<td>5148</td>
<td>27.67 (13.64–35.97)</td>
<td>84</td>
</tr>
<tr>
<td><em>Mentha arvensis</em></td>
<td>2773</td>
<td>22.31 (10.84–38.44)</td>
<td>86</td>
</tr>
<tr>
<td><em>Mentha piperita</em></td>
<td>5543</td>
<td>10.78 (6.01–30.26)</td>
<td>40</td>
</tr>
<tr>
<td><em>Crotan cajucara (red)</em></td>
<td>960</td>
<td>5.84 (4.59–7.26)</td>
<td>57</td>
</tr>
<tr>
<td><em>Crotan cajucara (white)</em></td>
<td>930</td>
<td>5.84 (4.59–7.26)</td>
<td>54</td>
</tr>
</tbody>
</table>

* The lethality observed was not sufficient to determine LC<sub>50</sub> values. REI and E from the highest concentration evaluated (25 mg/mL).
presented greater impact on the REI (27) and higher efficacy (73) than L. alba (33 and 62%, respectively), the latter presented lower LC50 by achieving higher efficacy in lower concentrations (data not shown). M. arvensis, however, obtained greater efficacy (73) only at the highest concentration (25 mg/mL), which consequently increased its LC50. There is a linear correlation between the size and weight of tick females and their oviposition capacity (Bennett, 1974). When the essential oil presents a good effect on egg production or hatching, it reduces the REI and shows better efficacy when compared to the non-treated groups (control).

Finally, the essential oils extracted from the members of the genus Croton, C. cajucara (white and red), and C. sacaquinha (Euphorbiaceae family) did not demonstrate potential efficacy against the studied cattle tick for all parameters here evaluated. Interestingly, extracts from these species have many components but none can be considered as the major one. Instead, besides having similar quantities of germacrene, they have a large number of compounds (46, 50, and 57, respectively) detected at relatively lower concentrations but that together represent most of the essential oil composition. The lack of efficacy observed suggests that these compounds have no synergic effect.

The genus Croton comprises over 1200 species that have been widely used in traditional medicine in Africa, Asia, and South America. Previous studies have shown that compounds obtained from Croton aromaticus, C. cajucara, Croton californicus, and Croton linearis are efficient insecticides or growth inhibitors of Heliotris violacea (Lepidoptera) (Salatino et al., 2007). Additionally, there are a few studies reporting the effects of Croton against parasites. The dichloromethane extract of Croton sphaerogyne was evaluated against R. (B.) microplus larvae at 0.625%, 2.5%, 5.0%, 10.0%, and 20.0% (v/v) and yielded mortality rates of 2.25%, 8.26%, 8.81%, 24.80%, 83.66%, and 99.32%, respectively. In the present study, Croton species had no relevant effect on R. (B.) microplus larvae, reaching a maximum lethality of 57% at 25 mg/mL for C. cajucara (white). Relevant constituents already identified for this species are abietanes, podocarpenes, and clerodane-type furano-diterpenes (Righi et al., 2013). Differently from what is described in the literature, the essential oils from Croton species evaluated here did not significantly interrupt the tick life cycle (oviposition and larva hatching) as demonstrated by the highest REI indexes and zero efficacies observed for C. cajucara (red and white). In both situations, engorged females presented the best performance under good conditions of incubation, and the egg mass weight mainly depends on the female’s initial weight. According to Gaxiola-Camacho et al. (2009), the egg-laying potential of an engorged female is directly related to her capacity to feed; therefore heavier females show highest values in the number of eggs laid as well as the weight of the egg mass, and subsequently a higher hatch rate.

It is known that the biological activity of essential oils varies with parasite species, oil concentration, method of application, and exposure time. During the past three decades, scientists have looked for less persistent and biodegradable alternatives (Maedeh et al., 2012) for plague management. Screenings such as the one described here allow the discovery of potential candidates to be selected and studied more carefully in terms of safety and the development of formulations for parasite control.

5. Conclusions

In this study, we showed that several essential oils extracted from a variety of Brazilian plants have excellent biocide potential against R. (B.) microplus. Especially C. longa and L. alba showed remarkable efficacy and lower LCs for engorged females (LC50 of 10.24 and 10.78 mg/mL, respectively) and larvae (LC50 of 0.54 and 5.84 mg/mL, respectively), with both species causing 100% lethality in larvae at 25 mg/mL. Efficacy was directly related to the major components in each essential oil. Curiously, the essential oils from species of the Croton genus were not efficient against the ticks. The potential of turmerone isolates warrants further investigation, which should include assessment of toxicity to the host. This new information, when combined with our results, may provide a strong rationale to design pre-clinical and clinical assays to evaluate their potential use for parasite control.

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