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Original article

In vitro acaricidal effect of *Syzygium aromaticum* and *Cymbopogon citratus* essential oil on engorged female of cattle tick *Rhipicephalus microplus* in Benin

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ABSTRACT

Ticks are external parasites of livestock and vector of several pathogens in infected animals. They lower production performances, which result in severe economic losses for breeders. So far a number of synthetic acaricide are used for the control of *Rhipicephalus microplus.* However, as a consequence of their extensive use, this tick specie has developed a resistance to the major classes of acaricide. In order to limit resistance and reduce the invasion of this tick, we have evaluated the potential acaricide action of *Syzygium aromaticum* of *Cymbopogon citratus* essential oils and their mixture of engorged females *Rhipicephalus microplus*. The Adults Immersion Test carried out showed sensitivity of ticks to the extract of *Syzygium aromaticum* and *Cymbopogon citratus* essential oil: 100% mortality of engorged female was obtained at the highest concentration, 10mg/ml, for the two essential oils. The mixture of *Syzygium aromaticum* and *Cymbopogon citratus* oil induced 100% death at a concentration of 1.25mg / ml. The lethal doses 50, 90 and 99% of individuals was calculated. The highest oviposition inhibition rate

(90.3% for *Syzygium aromaticum* and 60.7% for *Cymbopogon citratus)* was obtained at a concentration of 10mg/ml. However, no oviposition was seen from a concentration of 1.25mg /ml of the oil mixture. The results relevant to hatching reduction rate were similar. Hence, *Syzygium aromaticum* and *Cymbopogon citratus* essential oil mixture could be used as an alternative solution in the fight against ticks.

1. Introduction

Benin is a West African country located in the tropics with a climate and vegetation that are suitable for breeding. The national cattle herd is estimated to over 2.116,000 heads in 2013 (FAO, 2013) and plays an undeniable socio-cultural role. Unfortunately, the productivity of these animals is still low as a consequence of the existence of too much pathology. These pathologies include parasitic aggressions caused by ticks that induce huge losses every year (Rodriguez-Vivas et al., 2012). As reported previously, ticks represent the most important livestock ectoparasites, about 80% of worlds' cattle is infested by ticks (Laamri et al., 2012). Ticks are a constraint to the development of cattle breeding in several countries and a source of poverty for small holder (Bisusa et al., 2014). Taking into account their number, ticks are one of the most important vectors of animals' diseases (Farougou et al., 2012; Farougou et al., 2013).

Currently, synthesis acaricide are the only effective means of ticks'control. However, this method is limited as it leads to the development of resistance (Kearney, 2013; Lovis et al., 2012), meanwhile pharmaceutical companies have difficulty to find new molecule for rational control of these parasites. Previous studies reported that ticks are a major threat for animal and human health and the extensive use of acaricide may cause environment degradation. Rhipicephalus microplus has a great ecological adaptation capacity and is gradually competing with the native species of tick in livestock farms (Madder et al., 2012). It's one of the most resistant ticks to the commonly used acaricide (Adakal *et al*., 2012). To face this threat, new methods of fighting are explored including the use of plant extracts that own acaricide virtue (Chagas et al., 2014; Attia et al., 2011). Many studies were carried out to evaluate the activity of non-volatile extracts and essential oils of seeds and plants on *Rhipicephalus microplus.* The aim was to provide for farmer use tickicide that are more efficient, less expensive and less harmful for the environment (Borges et al., 2011). This study consisted in assessing the action of two novel plant (*Syzygium aromaticum* and *Cymbopogon citratus)* essential oils and their mixture on engorged females of *Rhipicephalus microplus*.

2. Materials and methods

2.1. Collection of ticks

Engorged female of *Rhipicephalus microplus* was harvested from the farm of Kpinnou. Ticks were transported to the laboratory in perforated bottles (about 1 mm of diameter for each whole) to allow better ventilation. The harvested ticks were used to complete the Adults Immersion Test.

2.2. Essential oils

The oils used here were previously extracted by steam distillation from *Syzygium aromaticum* flower buds and *Cymbopogon citratus* fresh leaves and analyzed by gas chromatography and gas chromatography coupled with mass spectrometry by Sessou et al. (2012, 2013).

2.3. The adults immersion test

The Adult Immersion Test (AIT) (Drummond et al., 1973) was used to assess the action of volatile extracts and their mixture on engorged females of *Rhipicephalus microplus*. The experiment was conducted in two phases. Essential oils were tested separately and after mixing was performed at equal concentrations (50% *Cymbopogon* and 50% *citratus Syzygium aromaticum*). In prelude to the test, engorged females which weight approximately 0.2 g were selected and placed by group of 10 in perforated Petri dishes(whole were large of approximately nine (9) centimeters in diameter). Control ticks were dipped into solution made of distilled water and 2% of Tween-20 for thirty seconds. This control solution was used to prepare a series of essential oils solution at different concentration as follow: 0.312 mg/ml, 0.625 mg /ml, 1.25 mg /ml, 2.5 mg /ml, 5 mg /ml and 10mg/ml. The test was repeated three times as recommended previously (Rosado-Aguilar et al., 2010). After soaking, ticks are replaced in their respective Petri dish. During the following seven days, the number of alive and dead female was recorded using a stereo microscope. Eggs from each group were weighed using an analytical scale and oviposition inhibition rates were calculated (Drummond et al., 1973). Harvested eggs were placed in bottles whose caps were perforated and, incubated (27 ± 1 ° C and 85-90% humidity) in an oven (IBELLI, 2012). Hatching rate was estimated twenty-one days after egg incubation (Chagas et al., 2014). Adult mortality was corrected by Abbott's formula (Abbott, 1925), in case the mortality in the control group was greater than or equal to 5%.

$$OR = \frac{\text{Mean weight of eggs in controls (g)} - \text{mean weight of eggs in treated group (g)}}{\text{Mean weight of eggs in controls (g)} \times 100} \times 100$$

OR= reduction of oviposition

$$HR = \frac{\text{Hatching rate in controls} - \text{hatching rate in treated group}}{\text{Hatching rate in controls}} \times 100$$

HR = reduction of hatching

2.4. Data analysis

Data related to mortality, oviposition and hatching rate were subjected to variance analysis (ANOVA) followed by Tukey's test at 5%. The lethal concentrations (LC) which kill 50%, 90% and 99% of adults and their confidence intervals (CI) at 95% were also calculated by probit analysis (Polo more software version 1).

3. Results and discussion

3.1. *Rhipicephalus microplus* engorged female mortality rate

The results of tests performed on engorged female of *Rhipicephalus microplus* indicate a correlation between tick mortality percentages and the different concentration of essential oils applied (Table 1). The concentration needed to obtain 100% mortality, seven days after application, was estimated at 10mg /ml for both *Cymbopogon citratus and Syzygium aromaticum* taking separately and 1,25mg/ml for the mixture (*Cymbopogon citratus + Syzygium aromaticum*). Significant difference of the mortality rate was observed between different dosage of mixture application (P <0.0001). Our results showed that the efficiency of the essentials oil increased gradually as its concentration increased. But the plant mixture appears to have a higher efficiency compare to the essential oils tested individually. The toxic effect of the mixture of volatile extracts (*Cymbopogon citratus + Syzygium aromaticum*) was also studied (Table 2). The values of LC50 (IC), CL90 (IC) and CL99 (IC) were respectively 0.257 (0.211 - 0.296) 0.667 (0.593 to 0.779) and 1.450 (1.157 to 2.035). These were very low compared to the values of the oils tested separately. The lethal concentration 50 (LC50) of *Cymbopogon citratus* is lower than that of *Syzygium aromaticum*. In general, he three essential oils exhibit adulcide activity on the engorged females of *Rhipicephalus microplus* but the mixture remains the most effective, with its CL values 3-18 times lower than that of the two individual oil.

3.2. Effect of essential oil of *Syzygium aromaticum*, *Cymbopogon citratus* and their mixture on *Rhipicephalus microplus* female oviposition and hatching.

Ours results showed that the oil mixture has the best inhibition rate (Table3), which is 100% at a concentration of 1.25 mg/ml. Data were significantly different between treatments (P<0.001). The highest inhibition rate of *Cymbopogon citratus* was 60.7% and that of *Syzygium aromaticum* was 90.3%. Both were reached at a concentration of 10mg/ml. The essential oil of *Syzygium aromaticum* has higher inhibitory potency than *Cymbopogon citratus*. The mixture induced inhibition rate higher than 50% after one week of laying (51.7% to 100%). Thus, analysis of variance p<0.05 for treatments confirmed the positive development of well marked inhibition rate with increasing concentration. The mixture is effective against *Rhipicephalus microplus* engorged females since its effect is fast and is expressed at a low concentration on reducing the rate of lay. With concentrations of 5 and 10 mg/ml the reduction in hatchability (Table 4) was 94.5% and 100% respectively for *Syzygium aromaticum*. Also a very significant difference is noted between the average volatile extracts hatchability *Syzygium aromaticum* of *Cymbopogon citratus*. At the level of oil mixtures, reducing the hatching rate is higher at all concentrations and presents a very significant different (P <0.001). The mixture of oil gave a strong inhibition rate and oviposition reduction *Rhipicephalus microplus* engorged females over two other oils.

Table 1

Mean and standard deviation of the mortality rate of engorged females.

Concentration (mg/ml)	*S. aromaticum*	*C. citratus*	*S. aromaticum + C. citratus*
0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
0.312	0.0 ± 0.0^{d}	33.3 ± 5.774^{c}	63.3 ± 5.7^{c}
0.625	20.0 ± 0.0^{cd}	50.0 ± 10.0^{c}	83.3 ± 5.7^{b}
1.25	33.3 ± 5.7^{c}	73.3 ± 5.7^{b}	100.0 ± 0.0^{a}
2.5	63.3 ± 20.8^{b}	80.0 ± 10.0^{b}	100.0 ± 0.0^{a}
5	96.6 ± 5.7^{a}	83.3 ± 5.7^{ab}	100.0 ± 0.0^{a}
10	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
ANOVA	***	***	***

Different letters in the same column represent a significant difference at 5% within the same parameter, *** P <0.001.

Table 2

Lethal concentration of essential oils on the engorged females.

Variable	Con. létale 50 (IC)	Con. létale 90 (IC)	Con. létale 99 (IC)
C. citratus	0.605 (0.427- 0.790)	4.735 (3.361-7.817)	25.342(13.651-67.042)
S. aromaticum	1.590 (1.333-1.897)	4.288 (3.379-5.996)	9.629 (6.738-16.396)
C. citratus + *S. aromaticum*	0.257 (0.211- 0.296)	0.667 (0.593-0.779)	1.450 (1.157-2.035)

Con, Lethal: Lethal concentration; CI: confidence interval of 95%.

Table 3

Oviposition rate of engorged females.

Concentration(mg/ml)	*S. aromaticum*	*C. citratus*	*C. citratus + S. aromaticum*
0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
0.312	5.2 ± 4.6^{d}	8.2 ± 4.7^{cd}	51.7 ± 3.5^{c}
0.625	12.8 ± 1.1^{cd}	11.0 ± 4.6^{cd}	86.8 ± 3.4^{b}
1.25	19.6 ± 1.7^{c}	18.1 ± 6.4^{bcd}	100.0 ± 0.0^{a}
2.5	41.4 ± 6.9^{b}	20.4 ± 6.2^{bc}	100.0 ± 0.0^{a}
5	79.6 ± 2.6^{a}	33.9 ± 2.9^{b}	100.0 ± 0.0^{a}
10	90.3 ± 8.8^{a}	60.7 ± 0.0^{a}	100.0 ± 0.0^{a}
ANOVA	***	***	***

Different letters in the same column represent a significant difference at 5% within the same parameter, *** P <0.001.

Table 4
Reduction of the hatching rate of the engorged females.

Concentration (mg/ml)	*S. aromaticum*	*C. citratus*	*S. aromaticum + C. limonum*
0	0.0 ± 0.0[d]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
0.312	21.0 ± 8.0[c]	8.0 ± 3.8[de]	48.6 ± 14.6[c]
0.625	32.8 ± 4.1[c]	18.0 ± 0.6[cd]	75.4 ± 13.2[b]
1.25	58.4 ± 14.6[b]	29.9 ± 5.1[c]	100.0 ± 0.0[a]
2.5	81.1 ± 6.9[a]	50.0 ± 8.8[b]	100.0 ± 0.0[a]
5	94.5 ± 5.2[a]	73.9 ± 4.4[a]	100.0 ± 0.0[a]
10	100.0 ± 0.0[a]	85.9 ± 4.0[a]	100.0 ± 0.0[a]
ANOVA	***	***	***

Different letters in the same column represent a significant difference at 5% within the same parameter, *** P <0.001.

Rhipicephalus microplus was accidentally introduced in Benin (Madder *et al*., 2012), and spread over the country very quickly (De Clercq et al., 2012). It serves as a vector for many cattle pathogen (Farougou et al., 2013). The emergence of this new specie is a bottleneck for farmers as it is resistance to a lot of miticides. In order to fight against the ticks and find an alternative to the acaricide resistance, we conducted a study on the volatile extracts and the mixtures of some endogenous plant essential oil. As shown by our results, the mortality rates after a seven-day exposure to essential oil concentration of 0.312 mg / ml 0.625 mg/ml, 1.5 mg/ml, 2.5 mg/ml, 5 mg/ml and 10mg/mg are respectively 0,0%; 20%; 33.3%; 63.3%; 96.6% and 100% for *Syzygium aromaticum* and 33.3%; 50%; 73.3%; 80%; 83.3% and 100% for *Cymbopogon citratus*. As for the mixture it induced 100% mortality at a concentration of 1.25 mg/ml. The mixture appeared to be more toxic on the ticks compare to oils tested separately. Regarding the egg hatching and reduction rate, oil of *Syzygium aromaticum* is more effective than that of *Cymbopogon citratus*. The mixture enhanced the oils action on the ticks and it inhibited totally the oviposition rate and hatching rate at a concentration of 1,25mg/ml. Previous studies have demonstrated that eugenol and trans-β-caryophyllene are toxic for arthropod (Barbosa et al., 2012; Jacques Huignard, 2012). Eugenol from flower buds of clove (*Syzygium aromaticum*, Myrtaceae) binds to octopamine receptors which is a specific invertebrate neurotransmitter (Barbosa et al., 2012). Thus nerve impulse is blocked, resulting in paralysis of the arthropod. The major compounds of the essential oil of *Syzygium aromaticum* are eugenol and trans-β-caryophyllene and may explain it action on the adult female of *Rhipicephalus microplus*. The essential oil of *Cymbopogon citratus* contains mainly geranial and myrcene mends that are repellent for arthropods (Weldon et al., 2011) and can explain the effect of the oil on ticks. The combined action of these various constituents reinforced the miticide effect of essential oils mixtures on the targeted engorged female of *Rhipicephalus microplus*. Several authors have reported the effectiveness of mixtures of oils on arthropods and bacteria (Traboulsi et al., 2002; Alçiçek et al., 2003; et Djenane , 2015). The combination of oils (*Cymbopogon citratus + Syzygium aromaticum*) has led to a reduction of the ticks' life expectancy, and a decrease of the number of eggs laid by ticks and the hatchability rate. The results of our studies are similar to those obtained by Giglioti et al., (2011) who reported that the essential oil of Azadirachta indica induced reduction of egg laying and hatching rate of *Rhipicephalus microplus* engorged females. Also Srivastava et al, (2008) claimed that extracts prepared from the seed of Azadirachta indica showed high toxicity against *Rhipicephalus microplus* (80%) after 5 h of treatment. Chiasson and Beloin (2007) hypothesized that the essential oils would act directly on the cuticle of mites and rapidly penetrated into their bodies. This could also explain the mortality rate recorded in this study with the essential oils of *Cymbopogon citratus* and *Syzygium aromaticum*. Concerning the mixture, the synergistic effect of the different additive components of the oils may be the determinant factor of the remarkable activity revealed by the mixture on the rate of egg laying and hatching. Velazquez *et al*. (2011) found that 1.25% of the mixture of essential oil of *Pimenta dioica* and *Ocimum basilicum*, have a larvicidal effect on *Rhipicephalus microplus*. The use of synthetic acaricide is the cause of resistance occurrence in a range of mites. In this context, the use of natural molecules, environment friendly and economic, that have acaricide properties, less toxicity for humans, is an alternative approach.

4. Conclusion

Cattle tick *Rhipicephalus microplus* tick is spreading very quickly over Benin. The control of tick invasion is increasingly ineffective due to it resistance against conventional miticides. Thus, *R. microplus* control through the use of plant extracts is a promising alternative since a very large number of plants having an activity against the tick has already been identified.

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