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Assessment and determination of LC_{50} of carvacrol and salicylic acid analogues with acaricide activity in larvae and adult ticks of *Rhipicephalus* (*Boophilus*) *microplus*

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ABSTRACT

Rhipicephalus (*Boophilus*) *microplus* is a tick that causes huge economic losses in cattle. The indiscriminate use of acaricides has generated resistance to most compounds present on the market. Carvacrol and salicylic acid have been widely studied for their biological activities and have been evaluated in different strains of *Rhipicephalus*. In this research the analogues carvacrol and salicylic acid were evaluated in larvae of *R.* (*B.*) *microplus* with data obtained in larval packet test (LPT) and larval immersion test (LIT). A lethal concentration 50 (LC_{50}) was assessed. The most potent compounds were evaluated in the adult ticks since there are no reports of evaluation in the life state of the parasite. From all the tested compounds, the ethyl 2-methoxybenzoate (91.82 ± 1.66%, 0.91 μmol/mL) and ethyl 2,5-dihydroxybenzoate (89.14 ± 1.61%, 2.04 μmol/mL) showed the highest percentage of mortality and the lowest LC_{50}. They were found to be the best candidates for a study in vivo.

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1. Introduction

R. (*B.*) *microplus* is one of the main ectoparasites of cattle in the tropical and subtropical regions in the world (Borges et al., 2011). The export of cattle to the United States is an economically important activity for Mexican livestock since it generates foreign exchange for about $ 700 million USD annually (González and Hernández, 2012). In Mexico, this tick causes huge economic losses in the livestock sector of approximately 48 million dollars because of direct damage such as damage to the skin by bites, blood loss, as well as indirect damage (transmission of the etiological agent), thus decreasing production and causing cost parameters for the control of the parasites such as: *Anaplasma marginale*, *Anaplasma centrale*, *Babesia bigemina*, *Babesia bovis*, *Borrelia theileri.* (Alonso et al., 2006; Rosario et al., 2011).

Since the indiscriminate use of acaricides has caused *R.* (*B.*) *microplus* to develop resistance to most acaricides available, it is necessary to find new compounds with acaricide activity. Carvacrol (Du et al., 2008; Di Pasqua et al., 2007; Cristani et al., 2007; Yin et al., 2012; Jayakumar et al., 2012; Ultee and Smid, 2001) and salicylic acid (Rangel et al., 2010; Mackowiak, 2000) have been studied extensively for their biological activities and have been evaluated in different strains of *Rhipicephalus*

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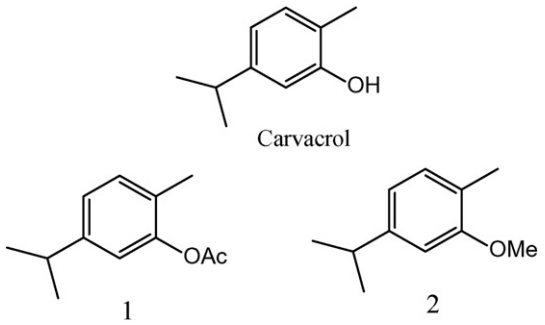

Fig. 1. Analogues of Carvacrol: carvacrol acetate (**1**) and carvacrol methyl ether (**2**).

(Coskun et al., 2008; Silveira et al., 2007). In this study the acaricidal activities of carvacrol analogues (carvacrol acetate (1) and carvacrol methyl ether (2) (Fig. 1) and salicylic acid analogues (2,5-dihydroxybenzoic acid (3), methyl 2,5-dihydroxybenzoate (4), ethyl 2,5-dihydroxybenzoate (5), 2-(α, α'-dimethoxymethyl)phenol (6), ethyl 2-methoxybenzoate (7), 2-hydroxybenzyl alcohol (8), methyl *p*-hydroxybenzoate (9) and propyl *p*-hydroxybenzoate (10) (Fig. 2), were measured by calculating the larval mortality percentage in a strain of *R.* (*B.*) *microplus*. The most potent compounds were selected according to the lethal concentration 50 (LC_{50}) and selected compounds were tested on the adult tick of this strain.

2. Materials and methods

Carvacrol, salicylic acid, 2,5-dihydroxybenzoic acid (**3**), methyl *p*-hydroxybenzoate (**9**) and propyl *p*-hydroxybenzoate (**10**) were purchased commercially. Carvacrol and salicylic acid analogues (carvacrol acetate (**1**), carvacrol methyl ether (**2**), methyl 2,5-dihydroxybenzoate (**4**), ethyl 2,5-dihydroxybenzoate (**5**), 2-(α,α'-dimethoxymethyl) phenol (**6**), ethyl 2-methoxybenzoate (**7**)) were prepared as described in the literature (Briard et al., 2008; Pilyugin et al., 2004; Yan et al., 2010; Liable et al., 2001; Ben Arfa et al., 2006). The 2-hydroxybenzyl alcohol (**8**) was provided by Dr. Héctor J. Salgado Z. obtained by a *Novo* technique.

2.1. Obtaining larvae of Rhipicephalus (B.) microplus

Adult ticks from cattle previously infected with a susceptible strain of *R.* (*B.*) *microplus* were donated by the Centro Nacional de Servicios de Constatación en Salud Animal (CENAPA) located in Jiutepec, Morelos, México; They were incubated during a two-week period at 28 °C and 80% relative humidity; the eggs were then collected in vials and incubated under the same conditions. After 14 days the hatched larvae were suitable for the bioassay (Bravo et al., 2008).

2.2. Acaricidal activity evaluation in vitro larvae R. (B.) microplus

There are two techniques for in vitro evaluation of the larval stage: LTP (Larval Packet Test; lipo-soluble compounds), LIT (larval immersion test; water-soluble compounds). The type of procedure depends on the solubility of the compound being evaluated.

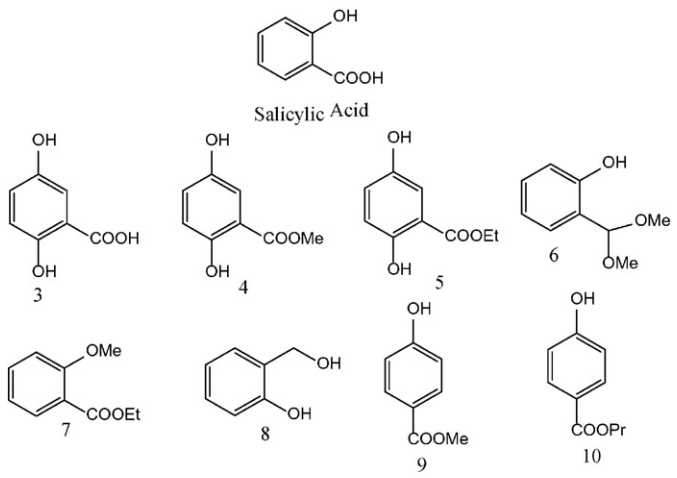

Fig. 2. Analogues of Salicylic acid: 2,5-dihydroxybenzoic acid (**3**), methyl 2,5-dihydroxybenzoate (**4**), ethyl 2,5-dihydroxybenzoate (**5**), 2-(α,α'-dimethoxymethyl)phenol (**6**), ethyl 2-methoxybenzoate (**7**), alcohol 2-hydroxybenzyl (**8**), methyl *p*-hydroxybenzoate (**9**) and propyl *p*-hydroxybenzoate (**10**).

2.3. Bioassay (Larval Packet Test, LPT)

Dilutions of each compound were prepared at concentrations of 1.0, 0.5, 0.25, 0.125 and 0.0625% (*w*/*v*) using trichloroethylene and extra virgin olive oil (2:1). Afterwards 670 μL for each concentration were applied to filter paper (Whattman No. 1, 10 × 12 cm) and left to dry for 30 min at room temperature. With the impregnated papers, envelopes were made in which approximately 100 larvae of *R.* (*B.*) *microplus* were placed and these packages were incubated for 24 h at 28 °C and 80% relative humidity. From each solution six repetitions were carried out and a negative control was included in every experiment (Ducornez et al., 2005).

After incubation, the percentage of mortality was estimated with a stereoscopic microscope and a manual laboratory counter; the percentage of mortality was corrected according to the formula applied by Abbot (1925) (Bravo et al., 2008).

$$\%\,\text{Mortality} = \left(\frac{(\%\ \text{treated mortality} - \%\ \text{control mortality})}{100 - \%\ \text{control mortality}}\right) x\ 100$$

2.4. Bioassay (Larval immersion test, LIT)

A standard solution was prepared by dissolving 84.2 mg of the compound in 2 mL of ethanol; it was subsequently brought to a volume of 25 mL by adding distilled water. Serial dilutions were prepared at 1.0, 0.5, 0.25, 0.125 and 0.065% (*w*/*v*) of the standard solution, using distilled water as the main solvent. Solutions were applied on filter paper (Whattman No. 1, 10 × 12 cm); the paper was allowed to dry for a period of 30 min at room temperature; afterwards, impregnated papers were used to form envelopes where 100 larvae of *R.* (*B.*) *microplus* were placed inside the envelope with a brush; then the larval packages were incubated for 24 h at 28 °C and 80% relative humidity. The experiment was carried out with six replicates for each dilution and a negative control was used. After 24 h, dead larvae were quantified and the mortality rate was calculated with the aid of a stereoscopic microscope and a laboratory manual counter. Later on the mortality rate was corrected according to the formula applied by Abbot (1925) (Bravo et al., 2008; Sabatini et al., 2001).

2.5. Bioassay (Adult immersion test, AIT)

The following protocol was modified from an earlier test described by Drummond et al. (1973) (Sabatini et al., 2001; White et al., 2004; Cutullé et al., 2013).

Serial dilutions were prepared at 250, 200, 150, 100 and 50 ppm, using 1 mL of DMSO (dimethyl sulfoxide) as a co-solvent; subsequently it was brought to a volume of 25 mL with distilled water.

A group of 10 engorged females were weighed and immersed for 30 min in the dilution. Ticks were recovered from the solutions, dried and stuck dorsal surface down on double sided tape on the perforated lid of a plastic petri dish (5.5 cm diameter, 1.5 cm high). The eggs fell in a petri dish, thus facilitating the collection. The holes in the lid provided air circulation and the petri dishes were incubated at 27–28 °C, 85–95% relative humidity to complete the life cycle. At the end of oviposition (14 days), the eggs were weighed and transferred to adapted plastic syringes, identified and sealed with cotton. The eggs were put back into the incubator under the same conditions for larval hatching. The hatching rate was read after 14 days and the data obtained were used to determine the percent reduction of oviposition (**% OR**) and hatching (**% HR**) as described by Domingues et al. (2013), as well as the egg production rate (**EPR**), to calculate the treatment efficacy (**E**), according to Domingues et al. (2013).

$$OR = \frac{mean\ weight\ of\ eggs\ in\ controls\ (g) - mean\ weight\ of\ eggs\ in\ treated\ group\ (g)}{mean\ weight\ of\ eggs\ in\ controls\ (g)} x100$$

$$HR = \frac{hatching\ rate\ in\ controls - hatching\ rate\ in\ treated\ group}{hatching\ rate\ in\ controls} x100$$

$$EPR = \frac{weight\ of\ eggs\ (g)}{weight\ of\ females\ (g)} x100$$

$$E = \frac{EPR\ controls - EPR\ treated}{EPR\ controls} x100$$

2.6. Statistical analysis

The mean of corrected mortality percentage using Abbot's formula and its standard deviation was calculated. Lethal concentration 50 (LC_{50}) was calculated by using a Probit regression. Regarding AIT, all tests were performed in triplicate; a one-way ANOVA was performed and in case significant differences were detected a Dunnett test was performed.

Table 1
Percentage mortality of analogues on *R.* (*B.*) *microplus* larvae *in vitro* by LPT.

Conc. %	Carvacrol[b]	carvacrol acetate (1)[d]	carvacrol methyl ether (2)[d]	methyl 2,5-dihydroxybenzoate (4)[c]	ethyl 2,5-dihydroxybenzoate (5)[e]	2 - (α, α'-dimethoxymethyl) phenol (6)[a]	ethyl 2-methoxybenzoate (7)[e]
1	35.85 ± 3.18	67.83 ± 2.07	71.69 ± 01.57	46.49 ± 0.78	89.14 ± 1.61	26.29 ± 2.46	91.82 ± 1.66
0.5	31.51 ± 3.30	46. 77 ± 16.65	59.41 ± 01.64	43.90 ± 0.61	73.94 ± 4.82	21.92 ± 2.66	80.67 ± 0.46
0.25	27.75 ± 3.95	45.42 ± 00.26	57.06 ± 18.82	38.46 ± 0.59	66.30 ± 1.63	16.38 ± 1.40	83.81 ± 159
0.125	20. 20 ± 3.95	46.38 ± 00.32	31.20 ± 02.61	25.26 ± 03. 48	44.69 ± 0.36	13.12 ± 1.03	73.37 ± 3.65
0.0625	14.52 ± 3.28	36.48 ± 01.90	17.56 ± 03.18	21.61 ± 05.09	44.94 ± 1.13	10.02 ± 0.13	46. 98 ± 10.69

Different letters indicate significant difference ($p < 0.05$).

Table 2
Percentage mortality of analogues on *R.* (*B.*) *microplus* larvae *in vitro* by LIT.

Conc. %	Salicylic Acid calculated [e]	Salicylic Acid reported [a]	2, 5-dihydroxybenzoic acid (3)[c]	2-hydroxybenzyl alcohol (8)[b]	methyl *p*-hydroxybenzoate(9) [d]	propyl *p*-hydroxybenzoate (10)[c]
1	92.62 ± 6.17	0	27.62 ± 5.42	15.96 ± 2.98	79.31 ± 7.52	34.14 ± 4.33
0.5	12.88 ± 1.32	1.08 ± 1.55	13.68 ± 3.06	1.89 ± 0.78	49.47 ± 7.34	10.71 ± 1.28
0.25	0	0.64 ± 1.05	2.18 ± 0.84	0	15.50 ± 2.97	0
0.125	0	0	0	0	4.21 ± 1.22	0
0.0625	0	0	0	0	1.19 ± 0.49	0

* Results reported by Silveira et al., 2007.
Different letters indicate significant difference ($p < 0.05$).

3. Results and discussion

The following compounds were evaluated by LPT: carvacrol acetate (**1**), carvacrol methyl ether (**2**), methyl 2,5-dihydroxybenzoate (**4**), ethyl 2,5-dihydroxybenzoate (**5**), 2- (α,α'-dimethoxymethyl)phenol (**6**), ethyl 2-methoxybenzoate (**7**) and carvacrol (Table 1). The compounds evaluated by LIT were: 2,5-dihydroxybenzoic acid (**3**), 2-hydroxybenzyl alcohol (**8**), methyl *p*-hydroxybenzoate (**9**) and propyl *p*-hydroxybenzoate (**10**) (Table 2). Salicylic acid activity was also evaluated by Silveira et al. (2007), against *R* (*B*) *microplus* on a farm in the town of Coronel Pacheco in the State of Minas Gerais in Brazil.

All tested compounds showed ixodicide activity against *R.* (*B.*) *microplus*, where 2,5-dihydroxybenzoic acid (**3**), methyl 2,5-dihydroxybenzoate (**4**), 2-(α,α'-dimethoxymethyl)phenol (**6**), 2-hydroxybenzyl alcohol (**8**) and propyl *p*-hydroxybenzoate (**10**) presented a mortality below 50% at concentrations of 1.0%. A significant difference was found ($p < 0.05$) among compounds that showed a mortality percentage above 50% at concentrations of 1.0%, being these carvacrol acetate (**1**), carvacrol methyl ether (**2**), ethyl 2,5-dihydroxybenzoate (**5**), ethyl 2-methoxybenzoate (**7**), methyl *p*-hydroxybenzoate (**9**) and salicylic acid. The results obtained in this research with salicylic acid are different to those reported by Silveira et al. (2007) perhaps because the strain of the town of Coronel Pacheco in the State of Minas Gerais in Brazil is a strain that already presents resistance.

With the results obtained, the LC_{50} was calculated with a probit regression of all compounds tested in larvae of *R.* (*B.*) *microplus* (Table 3). Compounds that showed an LC_{50} lower than 10 μmol/mL were considered to be evaluated in the adult tick (AIT) since, according to the literature, compounds **1**, **2**, **5** and **7** can be considered for *in vivo* studies. Carvacrol (Ramírez et al., 2013) and salicylic acid (Silveira et al., 2007) have already been evaluated on *R.* (*B.*) *microplus* larvae; however, there are no reports of evaluation on the adult tick in this species; therefore, acaricide activity on this stage of life cycle of *R.* (*B.*) *microplus* was evaluated.

Table 3
LC_{50} of each compound evaluated in larvae *R.* (*B.*) *microplus in vitro*.

Compound	LC_{50} (μmol/mL)
Carvacrol	59.72
carvacrol methyl ether (1)	6.00
carvacrol acetate (2)	5.01
Salicylic acid	**
2,5-dihydroxybenzoic acid (3)	35.69
methyl 2,5-dihydroxybenzoate (4)	2.04
ethyl 2,5-dihydroxybenzoate (5)	30.32
2 - (α, α'-dimethoxymethyl) phenol (6)	333.13
ethyl 2-methoxybenzoate (7)	0.91
2-hydroxybenzyl alcohol (8)	**
propyl *p*-hydroxybenzoate (9)	**
methyl *p*-hydroxybenzoate (10)	11.61

** Cannot compute (insufficient data).

Table 4
Mean results for ovoposition reduction (OR), of carvacrol, salicylic acid and analogues.

Conc. (ppm/mL)	Carvacrol	Salicylic acid	carvacrol methyl ether (1)	carvacrol acetate (2)	ethyl 2,5-dihydroxybenzoate (5)	ethyl 2-methoxybenzoate (7)
250	15.71 ± 3.26	-1.26 ± 3.19	26.72 ± 1.06	25.80 ± 0.27	26.56 ± 1.07	27.00 ± 1.50
200	6.48 ± 4.26	-1.89 ± 0.98	23.59 ± 1.22	21.42 ± 3.12	21.45 ± 3.02	24.60 ± 2.64
150	0.02 ± 1.45	-11.05 ± 5.7	20.29 ± 1.99	11.30 ± 1.52	14.02 ± 2.398	22.33 ± 8.91
100	0.09 ± 2.36	-8.76 ± 2.93	16.03 ± 3.06	6.85 ± 0.49	7.20 ± 3.20	13.91 ± 2.43
50	-2.62 ± 1.21	-7.27 ± 7.39	11.13 ± 2.39	2.34 ± 1.57	6.20 ± 0.2	11.21 ± 0.68

Table 5
Mean results for hatching inhibition rate (HR), of carvacrol, salicylic acid and analogues.

Conc. (ppm/mL)	Carvacrol	Salicylic acid	carvacrol methyl ether (1)	carvacrol acetate (2)	ethyl 2,5-dihydroxybenzoate (5)	ethyl 2-methoxybenzoate (7)
250	7.95 ± 0.37	7.09 ± 1.71	12.90 ± 0.42	10.95 ± 0.47	15.59 ± 1.07	13.52 ± 0.75
200	8.17 ± 1.49	6.19 ± 2.39	11.65 ± 0.28	9.15 ± 0.51	11.22 ± 1.42	10.84 ± 0.89
150	6.93 ± 1.97	5.69 ± 2.49	10.29 ± 0.23	7.75 ± 0.63	8.81 ± 0.36	9.82 ± 0.73
100	5.26 ± 1.36	3.99 ± 1.31	9.03 ± 1.06	6.71 ± 0.81	7.01 ± 0.65	8.11 ± 0.50
50	0.26 ± 0.28	0.52 ± 0.21	8.30 ± 0.74	6.03 ± 0.84	6.43 ± 0.92	6.81 ± 0.40

Table 6
Mean results for acaricidal efficacy percentage (E), of carvacrol, salicylic acid and analogues.

Conc. (ppm/mL)	Carvacrol	Salicylic acid	carvacrol methyl ether (1)	carvacrol acetate (2)	ethyl 2,5-dihydroxybenzoate (5)	ethyl 2-methoxybenzoate (7)
250	16.76 ± 2.27	1.11 ± 2.22	26.76 ± 1.12	26.33 ± 0.19	27.29 ± 0.75	27.97 ± 1.05
200	9.36 ± 2.97	-1.31 ± 0.68	23.33 ± 0.85	22.50 ± 2.17	22.77 ± 2.10	24.21 ± 1.84
150	0.98 ± 1.01	-7.86 ± 3.97	21.88 ± 1.39	11.22 ± 1.05	15.36 ± 15.36	17.97 ± 6.20
100	1.26 ± 1.64	-6.47 ± 2.04	18.26 ± 2.13	7.45 ± 0.34	6.81 ± 2.23	15.77 ± 1.69
50	-2.26 ± 0.84	-8.12 ± 5.14	11.53 ± 1.66	2.98 ± 1.10	6.68 ± 0.14	11.87 ± 0.47

With the collected data of the selected compounds, parameters such as ovoposition reduction (OR, Table 4), hatching inhibition rate (HR, Table 5) and efficacy (E, Table 6) were determined.

All selected compounds showed an **OR** from 27.00% to 2.34%, **HR** values of 15.59% to 6.03% and **E** of 27.97% to 2.98% (at 250 ppm/mL and 50 ppm/mL respectively), no significant difference was found between analogues. **OR**, **HR** and **E** values of carvacrol and salicylic acid were lower than their analogues.

4. Conclusion

Carvacrol acetate (**1**), carvacrol methyl ether (**2**), ethyl 2,5-dihydroxybenzoate (**5**), ethyl 2-methoxybenzoate (**7**) and methyl *p*-hydroxybenzoate (**9**) presented the highest mortality percent in larvae stage, where compounds **1**, **2**, **5** and **7** presented a LC_{50} below 10 μmol/mL. Based on the latter, compounds were evaluated in adult tick presenting similar **OR**, **HR** and **E** percentages proportional to dilutions used. In both stages salicylic acid and carvacrol were less effective than their analogues; thus indicating a significant increase in their acaracide activity of the analogues compared to the parent molecule.

All compounds tested showed greater acaracide activity in the larval stage compared to the adult stage of *R.* (*B.*) *microplus*. Ethyl 2,5-dihydroxybenzoate (**5**) and ethyl 2-methoxybenzoate (**7**), gave the best results of acaracide activity and can be considered candidates for more in depth studies *in vivo*.

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