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Use of encapsulated carvacrol with yeast cell walls to control resistant strains of *Rhipicephalus microplus* (Acari: Ixodidae)

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ABSTRACT

The tick *Rhipicephalus microplus* is typically controlled with synthetic acaricides, but indiscriminate use has selected for ticks that are resistant. The phenol compound carvacrol can serve as an alternative method for controlling *R. microplus*; however, environmental factors can increase its volatility. A microencapsulation technique using yeast cell walls can prolong the acaricide action of carvacrol, acting as a physical barrier against such environmental action. The aim of the present study was to investigate the encapsulation of carvacrol with yeast cell walls and its activity against resistant *R. microplus* larvae. Carvacrol was encapsulated with *Saccharomyces cerevisiae* cell walls; acaricide activity and volatility were measured using a larval immersion test with resistant strains of *R. microplus*. The efficacy of the encapsulation was confirmed by Fourier transform infrared spectroscopy and scanning electron microscopy. Fourier transform spectroscopy revealed similar vibrational peaks between the analyzed samples, supporting the scanning electron microscopy results that encapsulation occurred. The size difference between the yeast cell walls (diameter 2.5 ± 0.2 μm) and the encapsulated carvacrol (diameter de 4.5 ± 0.5 μm) was statistically significant ($P > 0.001$). The encapsulated carvacrol showed the highest larvicidal activity against *R. microplus*, exhibiting a lethal concentration 50 (LC_{50}) of 0.71 mg/mL; the LC_{50} of carvacrol alone was 1.82 mg/mL. The yeast cell walls promoted low volatility of carvacrol, maintaining high acaricidal activity for up to 60 h, and the reduced efficiency of carvacrol (18%) during 10 h following the test was significantly different ($P > 0.001$). The high acaricidal activity and lower volatilization of carvacrol encapsulated with yeast cell walls show that this technique is appropriate for the development of a delivery system and for protecting an active compound to control *R. microplus*.

1. Introduction

The tick *Rhipicephalus microplus* affects the health of cattle and, thus, cattle production in tropical and subtropical regions (Bendele et al., 2015; Guerreiro et al., 2016). Synthetic acaricides are most frequently used for controlling *R. microplus* worldwide (George et al., 2004; Rodriguez-Vivas et al., 2014; Castro-Janer et al., 2015). However, as the exhaustive use of this control method has selected for resistant tick populations (Singh et al., 2015), the search for alternatives to synthetic acaricides has accelerated in an effort to combat increasing tick populations (Lem et al., 2014).

Carvacrol, a volatile monoterpene found in several essential oils (Cacciatore et al., 2015), is a compound that is safe for oral intake and is being approved for use by the Food and Drug Administration (FDA) (Chalier et al., 2009; Yu et al., 2012; Suntres et al., 2015). Carvacrol has been used in antimicrobial, anticancer, insecticide and acaricidal applications (Jayakumar et al., 2012 Concepción et al., 2013; Miladi et al., 2016; Tunç et al., 2016). For example, it has demonstrated acaricidal activity against *Dermanyssus gallinae*, *Tetranychus urticae* and the ticks *Ixodes scapularis*, *Amblyomma americanum*, *Rhipicephalus sanguineus* and *R. microplus* (Cavalcanti et al., 2010; Jordan et al., 2011; Cruz et al., 2013; Tabari et al., 2015; Araújo et al., 2016), including resistant populations (Costa-Júnior et al., 2016). However, few formulations with carvacrol have been developed, and bioactive tests involving ticks have not been performed. Because of its high volatility, carvacrol requires a formulation that decreases volatilization and increases the residual

Abbreviations: AChE, acetylcholinesterase; CE, initial mass of yeast cell walls; CM, final mass of carvacrol encapsulated in yeast cell walls; EY, encapsulation efficiency; FTIR, fourier transform infrared spectroscopy; GABA, gamma-amino butyric acid; GPI, glycosylphosphatidylinositol; LC_{50}, lethal concentration 50; RH, relative humidity; RPMs, revolutions per minute; SEM, scanning electron microscopy

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period and, consequently, efficiency.

Microencapsulation has emerged as a pharmaceutical approach that promotes the stability of a compound by providing as a physical barrier to prevent the action of environmental factors on the encapsulated molecule (Madene et al., 2006; Pham-Hoang et al., 2013). It is a system characterized by microcomponent particles that comprise a coating capsule, resulting in a micro- or nanoscale formulation (Paramera et al., 2011).

Saccharomyces cerevisiae yeast cell walls are used as fermentation components in the food and alcohol industries (Sundh and Melin, 2011). Indeed, *S. cerevisiae* cell walls are by-products of the beverage and alcohol industry, with a high production of approximately 75,000 tons/year in Brazil (Martins, 2009), and they have been used for encapsulating hydrophobic compounds, including essential oils and monoterpenes (Pannell 1990; Shi et al., 2008; Paramera et al., 2011). The efficiency of encapsulation of monoterpenes using *S. cerevisiae* has been reported (Normand et al., 2005). In addition, encapsulation of carvacrol with β-cyclodextrin was effectively used to control the parasitic mite *Varroa destructor* (Leblanc et al., 2008), and a carvacrol microemulsion exhibited larvicidal action against *Aedes albopictus* (Seo et al., 2015).

As encapsulation of carvacrol with *S. cerevisiae* yeast cell walls can decrease its volatility and increase its acaricidal efficiency, the aim of this study was to assess the acaricidal activity of encapsulated carvacrol against resistant *R. microplus*.

2. Materials and methods

2.1. Carvacrol encapsulation in the Saccharomyces cerevisiae cell wall

The process of carvacrol encapsulation in the cell wall of *S. cerevisiae* was performed according to Paramera et al. (2011), with modifications. Briefly, 3 g of yeast cell walls (Biorigin, São Paulo, Brazil) was washed in distilled water. After centrifugation at 3000 rpm for 10 min, the supernatant was discarded, and the washed material was used for the encapsulation process. Carvacrol (3 g, Sigma Aldrich, Saint Louis, MO, USA) was added to the washed yeast cell walls, and the mixture was stirred at 45 ± 1 °C for 4 h. The encapsulated sample was washed with distilled water and stirred at 3000 rpm for 10 min twice to remove non-encapsulated carvacrol. The encapsulated sample was maintained at −20 °C and subsequently freeze-dried. The same washing procedure and freeze-drying was performed on yeast cell walls in the absence of carvacrol. The encapsulation efficiency (% EY) was calculated in mg of carvacrol encapsulated in yeast cell walls according to Paramera et al. (2011):

$$EY\ (\%) = (CE/CM) * 100$$

where, CE refers to the initial mass of the yeast cell walls and CM refers to the final mass of carvacrol encapsulated in the yeast cell wall.

2.2. Chemical and morphological analysis

Encapsulated carvacrol, isolated carvacrol, and yeast cell walls were analyzed by Fourier transformed infrared spectroscopy (FTIR) (IRPrestige-21, Shimadzu, Kyoto, Japan) using KBr pellets. FTIR spectra were recorded for vibrations ranging from 500 to 4000 cm^{-1}.

Encapsulated carvacrol was placed on carbon-coated tape and examined using scanning electron microscopy (Phenom ProX, Phenom-World, Eindhoven, Netherlands) with an acceleration of 15 kV. The yeast cell wall particles and encapsulated carvacrol were measured from scanning electron microscopy images using the counting methods described by Maciel et al. (2003). Sixty particles of each sample were measured and statistically analyzed using the program Origin 8.6 (OriginLab, Northampton, Massachusetts, USA) with a descriptive statistical analysis in columns and GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA); a *t*-test with respective confidence intervals of 95% (95% CIs) was also applied. Statistical significance was set at $P < 0.05$.

2.3. Acaricidal tests

2.3.1. Tick maintenance

Engorged *R. microplus* females resistant to all known synthetic chemical acaricides (Reck et al., 2014) were collected from calves experimentally infested and without recent contact with chemical acaricides. The engorged females collected were washed in water and maintained in the laboratory at 27 °C and ≥80% relative humidity (RH) until oviposition was completed. Larvae aged 14–21 days were used in larval immersion tests.

2.3.2. Larval immersion test

The larval immersion test was performed according to Klafke et al. (2006). The encapsulated formulation was diluted in distilled water at concentrations ranging from 2.0 to 0.41 mg/mL. A 1-mL aliquot of each concentration was transferred to 1.5-mL tubes, and approximately 500 tick larvae were placed in each tube. The controls used were distilled water, non-encapsulated carvacrol (concentrations of 2.0–0.41 mg/mL) and yeast cell walls without carvacrol (concentrations of 2.0–0.41 mg/mL). Immediately after addition of the larvae, the tube was closed, and the mixture was vigorously agitated. After 10 min, the larvae were transferred to a filter paper to dry. After drying, approximately 100 larvae were transferred to a clean dry filter paper (8.5 × 7.5 cm) that was folded and closed with clips. The packets were incubated at 27 ± 1 °C and a relative humidity (RH) of ≥80% for 24 h. The dead and live larvae were counted. Each test concentration was included four replicates. The lethal concentration (LC_{50}) of carvacrol and encapsulated carvacrol was calculated using GraphPad Prism 6.0 (GraphPad Software), with respective 95% CIs. The encapsulated carvacrol was considered to be significantly ($P < 0.05$) more (or less) efficient than the yeast cell walls when there was no overlap between the 95% CIs of the LC_{50} values (Roditakis et al., 2005).

2.3.3. Volatility test

For the volatility test, 1 mL of either the encapsulated carvacrol solution, carvacrol or yeast cell walls (2 mg/mL) was transferred to 1-mL microtubes. The tubes remained open during incubation at 27 °C and RH ≥ 40% and were then closed at intervals of 5 h up to 60 h. The larval immersion test was performed as described above with all samples to verify the volatility time. The negative control was distilled water and yeast cell walls. Statistical analysis was performed using two-way analysis of variance (ANOVA; GraphPad Software) with 95% CIs; significance was considered at $P < 0.05$.

3. Results and discussion

Yeast cell wall encapsulation has emerged as a promising system for novel drug formulations (Blanquet et al., 2005). This encapsulation technique with yeast cell walls has been used for hydrophobic molecules, such as essential oils and monoterpenes (Bishop et al., 1998; Shi et al., 2008). The encapsulation yield for carvacrol with yeast cell walls showed an efficiency of 60%. FTIR spectra demonstrated the encapsulation of carvacrol with yeast cell walls (Fig. 1). Stretching vibrations of 3500–3000 cm^{-1} correspond to −OH bands, a characteristic of carbohydrates (Laroche and Gervais, 2003). Peaks at 1300–1000 cm^{-1} are attributed to C−O vibrations, corresponding to phenols group (Pavia et al., 2008). This peak intensity can be attributed to the encapsulation of carvacrol. Stretching vibrations less than 860 cm^{-1} correspond to benzene (aromatic ring) (Fig. 1) (Pavia et al., 2008). The spectrum profile confirms the encapsulation of carvacrol by the yeast cell walls. Morphological analysis by scanning electron microscopy revealed size differences between the yeast cell walls