The latex of *Euphorbia tirucalli* L. (LET) has great etnopharmacological relevance for several traditional communities. In this study, the *in vitro* and *in vivo* (using *Tenebrio molitor* larvae) antimicrobial effects of LET were evaluated. LET did not inhibit the growth of *S. aureus*, however a reduction on staphyloxanthin production (an important virulence factor of *S. aureus*) was observed. LET (at 10 μL/kg) was also able to enhance the survival of larvae infected with a lethal dose of *S. aureus*, an effect associated with reduction in the haemocytes. These results encourage us to carry out new studies to better characterize the anti-infective effects of this latex in order to guide the development of new therapies for the treatment of infections caused by *S. aureus*.

**Keywords**: virulence factors, bacterial infections, haemocytes.

1. **Experimental section**

   **1.1. Obtaining of *Euphorbia tirucalli* latex**

The latex of *Euphorbia tirucalli* (LET) was collected in the urban area of *São Luís* (Brazil; latitude 2°3 0’09 "S and longitude 1 44° 7’ 07 "W), between the months of March and April 2017. LET was obtained by incisions in the stem of the plant, still planted, where there was extravasation of the same that was stored in a sterile container.
After, the latex was diluted in sterile distilled water in order to obtain a concentration of 100 μL/L. The aqueous solution was kept refrigerated.

1.2. Minimum inhibitory concentration (MIC)
The antimicrobial activity of the extracts was evaluated by the determination of the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* O42. Briefly, LET was serially diluted in Mueller Hinton broth and 10 μL of bacterial suspension (1.5 x 10^8 CFU/mL). The plates were incubated at 37°C and after 24 h the bacterial growth were evaluated using 30 μL of 0.03% resazurin solution (da Silva et al. 2016).

1.3. Qualitative and quantitative Staphyloxanthin assays
Overnight culture of *S. aureus* ATCC 6538 were diluted in Mueller Hinton broth (1:100) and cultivated at 37°C with and without different concentrations of LET. After 24 h, the bacteria were harvested by centrifugation and washed twice with sterile saline, and at this time the cells were photographed to compare the production of staphyloxanthin. Following, cell pellets were resuspended in 0.2 mL of methanol and heated at 55°C for 30 min. The supernatant was collected and the entire process was repeated 2 times to maximize the extraction of staphyloxanthin. Finally, the pigment production was measured at 465 nm using a spectrophotometer (Silva et al. 2017).

1.4. Infection model using Tenebrio molitor

1.4.1. Larval survival test
Larvae of *Tenebrio molitor* with approximately 100 mg were randomized distributed into groups of 10 subjects. After anaesthesia and disinfection, microbial suspension (10 μL) was injected in the ventral membrane between the second and third abdominal segments from the tail to the head (Czarniewska et al. 2018). Each group were
placed in Petri dishes and incubated at 37°C. After 2 hours, three groups received LET at different doses (5 μL/kg, 10 μL/kg or 15 μL/kg). Infected-animals treated with PBS (vehicle) were used as control; while uninfected-larvae treated with PBS or LET at different doses (5 μL/kg, 10 μL/kg or 15 μL/kg) were also used as controls. The larvae viability was evaluated daily for lack of movement.

1.4.2. Total haemocytes count
For total haemocytes count, the larvae (n=15/group) were cut with a scalpel in the ventral part and squeezed to remove the hemolymph, which was collected in an ice cold microtube. After centrifugation (500 g for 10 min at 4°C), the pellet was resuspended in 1 mL of ice-cold insect physiological saline (IPS) (pH= 6.9; containing 150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris-HCl and 10 mM EDTA and 30 mM sodium citrate as anticoagulants) (Bergin et al. 2005). Following, the cell suspension was centrifuged again and resuspended in 1 mL of IPS. Cell viability was assessed in a Neubauer chamber by the exclusion test using tripan blue.

1.4.3. Measurement of Lysosomal Membrane Stability
In order to determine the effect of LET on lysosomal membrane stability, the larvae (n=15/group) were treated and the haemocytes were obtained and treated as described above. The cells were washed twice with IPS and resuspended in an acridine orange solution (1 μg/mL in IPS). The cells were incubated for 20 min (protected from light), and then they were washed three times with IPS. The haemocytes were resuspended in IPS and analysed by flow cytometry (BD AccuriTM, United States; FL3 channel). A total of 10,000 events were analysed for each sample. Haemocytes from untreated larvae were used as positive control, while
cells submitted for a cycle of thermal shock (5 min at 100 °C, followed for 5 min at -20 °C).

1.5. Statistical Analysis

Data were presented as means ± standard variation (SD) or percentages. Statistical analyses were performed using the software GraphPad Prism version 5.0. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey test. A p-value less than or equal 0.05 was considered as statistically significant. The larvae survival assays were analyzed using the Kaplan–Meier method to calculate survival fractions and log-rank test was used to compare survival curves.
References


Supplementary Figures captions

**Figure S1:** Effects of *Euphorbia tirucalli* latex on the production of staphyloxanthin by *S. aureus* ATCC 6538. (*) Statistical differences in relation to untreated bacterial cells (p < 0.05).

**Figure S2:** Effects of *Euphorbia tirucalli* latex on *Tenebrio molitor* larvae infected by *S. aureus*. (A-C) Survival of larvae infected with *S. aureus* ATCC 6538 and treated with LET at 5 μL/kg (A), 10 μL/kg (B) and 15 μL/kg (C). (D) Survival of larvae infected with *S. aureus* ATCC 25923 and treated with LET at 10 μL/kg. Sa= *S. aureus*; (E) Effects of LET (10 μL/kg) on haemocytes amounts in *Tenebrio molitor* larvae infected or not with *S. aureus* ATCC 6538. (F) Effects of LET (10 μL/kg) on the stability of lysosomal membrane of haemocytes from *Tenebrio molitor* larvae. (*) Statistical differences with PBS-treated larvae (p <0.05).
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