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Evaluation of antimicrobial activity of glycerol monolaurate nanocapsules against American foulbrood disease agent and toxicity on bees

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ABSTRACT

The American Foulbrood Disease (AFB) is a fatal larval bee infection. The etiologic agent is the bacterium *Paenibacillus larvae*. The treatment involves incineration of all contaminated materials, leading to high losses. The Glycerol Monolaurate (GML) is a known antimicrobial potential compound, however its use is reduced due to its low solubility in water and high melting point. The nanoencapsulation of some drugs offers several advantages like improved stability and solubility in water. The present study aimed to evaluate the antimicrobial activity against *P. larvae* and the toxicity in bees of GML nanoparticles. The nanocapsules were produced and presented mean diameter of 210 nm, polydispersity index of 0.044, and zeta potential of −23.4 mV demonstrating the acceptable values to predict a stable system. The microdilution assay showed that it is necessary 142 and 285 μg/mL of GML nanocapsules to obtain a bacteriostatic and bactericidal effect respectively. The time-kill curve showed the controlled release of compound, exterminating the microorganism after 24 h. The GML nanocapsules were able to kill the spore form of *Paenibacillus larvae* while the GML do not cause any effect. The assay in bees showed that the GML has a high toxicity while the GML nanoparticles showed a decrease on toxic effects. Concluding, the formulation shows positive results in the action to combat AFB besides not causing damage to bees.

1. Introduction

Infection diseases must be critically controlled, especially in dense environment populations. A typical example of this environment involves bee colonies, which are frequently attacked by important pathogens, leading in the most cases to the dead of insects and colonies devastation [1]. One of the most dangerous disease in these environments is the American foulbrood (AFB) which is a fatal disease that threatens apiculture, since it is highly contagious and can eliminate all hive causing losses to the productive sector of honey and derivatives [2]. The etiological agent is the bacillus Gram positive spore-forming *Paenibacillus larvae*, considered a fatal epizootic globally scattered, even killing only larvae of bees [3]. Actually, the treatments to control the AFB are extremely problematic and costly. For these reasons, the search for alternatives to control the AFB with high antimicrobial activity and low toxic effects is important [4].

The glycerol monolaurate (GML) is a natural compound recognized as safe by *The Food and Drug Administration* (FDA). The antimicrobial potential of GML against many Gram Positive coccus in addition to *Bacillus anthracis* [5], inhibits microorganisms related to candidiasis and bacterial vaginosis [6] is related. The use of GML is not expanded due the low solubility in water, leading to low bioavailability. In this context, the nanotechnology has shown great growth, with promising results in pharmaceutical industry, due to the fact that the nanoencapsulation of some compounds represents an increase of solubility, potential antimicrobial and consequently a decrease of toxicity [7]. In view of the lack of options to combat AFB without losses, the following study aimed to evaluate for the first time the antimicrobial activity of GML Nanocapsules against *Paenibacillus* species and the toxicity in honey bees.

2. Materials and methods

2.1. GML nanocapsules

The GML Nanocapsules were produced according to the method described previously [8] with modifications. The nanocapsules were characterized as size distribution and polydispersity index (PDI) by dynamic light scattering (DLS), zeta potential by electrophoresis in a Zetasizer Nano-ZS (Malvern Instruments, United Kingdom). The pH was evaluated using potentiometer (Digimed®). Each parameter was evaluated in triplicated (n = 3) and results were expressed by means ± standard deviation.

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2.2. Microorganisms

Six isolates of *Paenibacillus* species from the collection of the Ministry of Agriculture (LANAGRO/RS) Brazil were used in this study. The test organisms included isolates of *Paenibacillus alginolyticus*, *Paenibacillus azotofixans*, *Paenibacillus borealis*, *Paenibacillus gluconolyticus*, *Paenibacillus validus* and *Paenibacillus larvae* (ATCC 9545) were used, as well as three strains of *P. larvae* well characterized isolated from different regions of Argentina: Cobo (37°40′S-57°19′W), Miramar (38°13′ S-57°52′W), Chapadmalal (38°03′S-57°42′W). These strains were gently donated by Research Center in Social Bees, Faculdad de Ciências Exactas y Naturales, Universidad Nacional de Mar del Plata (Mar del Plata, Buenos Aires, Argentina). All strains were maintained in −80 °C on Brain Heart Infusion (BHI) broth with glycerol and unfrozen 2 days before the experiments.

2.3. Bacterial suspension

After unfrozen, the strains were seeded in MYPG agar (Mueller Hinton, Yeast Extract, Potassium Phosphate Dibasic and Glucose) and the plates were incubated at 37 °C for 24 h [9]. After incubation, the colonies were suspended in sterile saline to make the bacterial suspension. The absorbance was adjusted in spectrophotometer to obtain 0.5 in McFarland scale (Optic density in 600 nm = 0.8 to 1.0).

2.4. Determination of minimal inhibitory and bactericidal concentration

The minimal inhibitory concentration (MIC) was performed by macrodilution method in tubes with Mueller Hinton Broth with Thiamine (MHBT) [10]. The GML and GML Nanocapsules were sequentially diluted in the concentration range of 500–3.90 μg/mL in MHBT. A group of only MHBT was considered such Negative Control and MHBT with microorganism such Positive Control. This assay was performed in triplicated. All tubes were incubated at 37 °C for 48 h. After incubation, the substance (2,3,5 Triphenyltetrazolium chloride) was used to visualize the MIC and was considered the lowest concentration which the microorganism does not demonstrate visible growth. To evaluate the minimal bactericidal concentration (MBC), samples of each tube were seeded in MYPG agar and the lowest samples concentration which no show colonies growth was defined as the MBC.

2.5. Time-kill curves

This assay was performed to determine the necessary time for the GML and GML nanocapsules, eliminate the microorganism. The same experiment design used in the previous item was applied for this study. The tubes containing MHBT with GML or GML Nanocapsules on MBC concentration were incubated at 37 °C and in times of 0, 6, 12 and 24 h, a sample was seemed in plate with MYPG agar. The plate was maintained at 37 °C for 48 h and the colonies were counted. A tube with only MHBT was considered Negative Control and the Positive Control was considered a tube with MHBT and microorganism. The assay was performed with 3 replicates.

2.6. Spore suspension

The *Paenibacillus larvae* (ATCC 9545) was incubated on Mueller Hinton agar with Thiamine (MHAT) at 37 °C for 1 week. The vegetative cells with endospores were suspended in cold deionized water and then sonicated for 10 min to destroy vegetative cells. Spores were collected by centrifugation (2000 × g for 15 min) with Histopaque® and subsequently washed by repeated centrifugation and suspension in sterile distilled water at 4 °C. After two washes, each spore pellet was suspended in sterile 0.85% NaCl solution. The condition of the spores was examined using malachite green stain [11]. The spore suspension was stored at −70 °C.

2.7. Sporicidal activity

The sporicidal activity was measured by colony counting on plate with MHAT. Were added 100 μl of spore suspension in 100 μl of GML or GML Nanocapsules. Only spore suspension was considered positive control. The tubes were incubated in shaker by 4 h. After incubation an aliquot of the tubes was seeded on MHAT and incubated for 24 h at 37 °C and them, the colonies were counted.

2.8. Toxicity assay in bees

The toxicity of GML and GML Nanocapsules was verified against adult honey bees (*Apis mellifera*). The test was performed by pulverization described previously [12] adapted by Santos et al. [13]. The assay was developed with seven groups in triplicate, each group with six bees. It were evaluated 1× and 2× MIC of GML and GML Nanocapsules. Saline was considered Positive Control and the insecticide Deltametrin ® (DTT) was used such negative control. The samples were pulverized during 5 days, once a day, and the number of bees was evaluated all days during the experiment.

2.9. Statistical analysis

For the toxicity assay, differences in survival after 120 h of observation were assessed by Two-Way analysis of variance (ANOVA) followed by Bonferroni's test. A p-value < 0.05 and < 0.001 was considered statistically significant. Two-Way analysis of variance (ANOVA) followed by Bonferroni's test was used to determine the significant differences between treatment groups in the cell viability assay of *P. larvae*. These tests were chosen were selected because the existence of more than one interferer, in this case, besides the treatment time also influences. The statistical analyzes were performed with the software package GraphPad Prism 5.00 for windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. GML nanocapsules

The physicochemical characterization of the nanocapsules showed mean diameter of 209.3 ± 1.5, polydispersion index of 0.044 ± 0.02, zeta potential of - 23.2 ± 3 and pH values of 6.19 ± 0.21. The results demonstrated acceptable values to predict de stable system showing the success on development of nanocapsules.

3.2. Determination of minimal inhibitory and bactericidal concentration

Using the macro dilution method, it can be seen the effect of GML and GML nanocapsules (Table 1). The MICs ranged from 7.8 to 62.8 μg/mL (GML) and 35.7–142.8 μg/mL (GML Nanocapsules). The MBCs ranged from 35.7 to 142.8 μg/mL (GML) and

Table 1
The MICs and MBCs values of GML and GML nanocapsules against different *Paenibacillus* species. The dada are showed in μg/ml.

Microorganisms	MIC (μg/ml)		MBC (μg/ml)	
	GML	GML nanocapsules	GML	GML nanocapsules
P. larvae (ATCC 9545)	62.2	142.8	142.8	285.7
P. larvae (Cobo)	62.2	142.8	142.8	285.7
P. larvae (Miramar)	62.2	142.8	142.8	285.7
P. larvae (Chapadmalal)	62.2	142.8	142.8	285.7
P. borealis	31.2	71.4	71.4	142.8
P. gluconolyticus	31.2	71.4	71.4	142.8
P. alginolyticus	7.8	35.7	35.7	71.4
P. pabuli	31.0	71.4	71.4	142.8
P. thiaminolyticus	31.2	71.4	71.4	142.8
P. azotofixans	7.8	35.7	35.7	71.4

71.4–285.7 μg/mL (GML Nanocapsules). The result can be visualized in Table 1.

3.3. Time-kill curves

The concentration used in this test was taken by MBC. After incubation the colonies were counted and the results (Fig. 1). While the GML killed the microorganism after hours, to the GML Nanocapsules, the total elimination of microorganism was verified in 24 h. This result demonstrates the controlled release of GML from nanocapsules.

3.4. Sporicidal activity

The result (Fig. 2) showed an efficacy action of GML nanocapsules comparing the positive control with statistically significance for $p < 0.001$. The GML don't showed significant effect against *Paenibacillus larvae* spores. The use of GML becomes impracticable if only kill the microorganism on bacillus form.

3.5. Toxicity assay

The toxicity assay in adult honey bees was addressed to verify the toxic effects of GML and GML nanocapsules. The results (Fig. 3) showed that GML cause an important toxic effect on bees comparing with Positive Control ($p < 0.001$) invalidating the use. On the other hand, the GML Nanocapsules showed a significant decrease on toxic effects comparing with Positive Control ($p < 0.05$) and it can be used to combat the AFB.

4. Discussion

The GML is a compound with antimicrobial activity showed against many microbes such *Staphylococcus aureus*, *Streptococcus* species [14,15], inhibited the virulence factors such such as β-lactamase, α-hemolysin, and toxic shock syndrome toxin-1 [16,17]. A study performed by Ref. [18] showed the ability to inhibit the development of detectable *S. aureus* and *Enterococcus faecalis* biofilm. However, physical properties of GML such high melting point and poor solubility in water lead to difficulties in its use as an antimicrobial [19].

Different approaches have been tried to improve the solubility of GML, for possible cosmetic and food application, and to increase its antimicrobial activity [20,21]. Studies have been demonstrated the enhanced solubility such as oral administration using microemulsion system [22,23]. In our study we used a nanoformulation with GML.

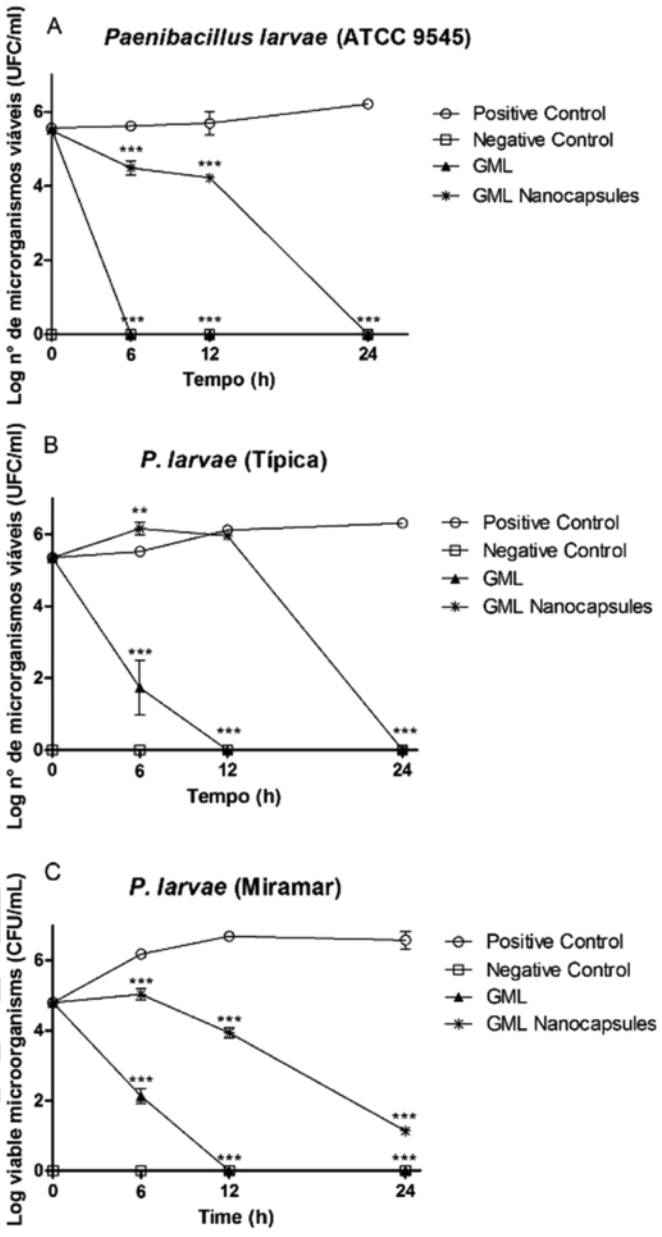

Fig. 1. Time-kill curve of GML and GML Nanocapsules against (A) *P. larvae* (ATCC 9545), (B) *P. larvae* (Chapadmalal), (C) *P. larvae* (Miramar). Data showed on Average ± Standard Deviation. Analysis of variance (ANOVA) followed by Bonferroni's test considering statistically significant to $p < 0.01$ (**) and $p < 0.001$ (***). The study was performed in triplicate.

After the antimicrobial tests, was possible observe that the GML in free form eliminated the microbial population in 6 h, while the GML Nanocapsules took 24 h for the total elimination of microorganism. The positive control (containing only microorganism) did not present fall in the number of colonies forming units (CFU) while the negative control presented no microbial growth. The assay against Argentinean isolates showed that the GML eliminated the microbial population in 12 h and the GML nanocapsules in 24 h, except the strain from Miramar region. The experiment showed the controlled release of the compound, characteristic of nanostructured system [24,25]. It is important to note that this is the first study that shows the antimicrobial activity of nanoparticles with GML.

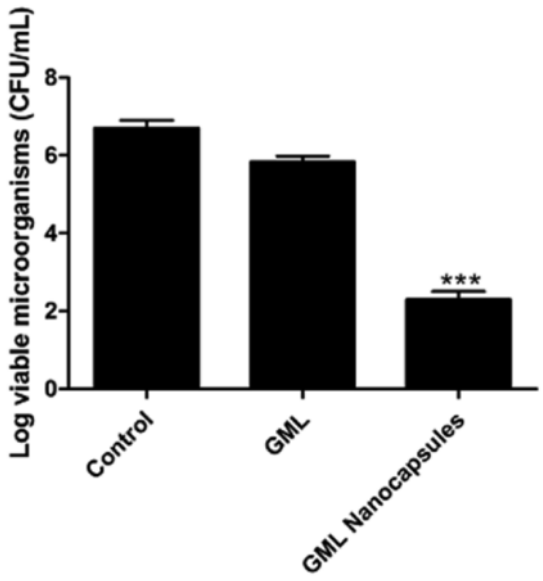

Fig. 2. Sporicidal activity of GML and GML nanocapsules against *Paenibacillus larvae* spores. Data showed on Average ± Standard Deviation. Analysis of variance (ANOVA) has been applied followed by Tukey's test considering values $p < 0.001$ statistically significant (***). The study was performed in triplicate.

In the microdilution assay the results is according to previous studies with nanoparticles against *Paenibacillus* species. Studies with essential oils such *Copaifera Officinalis* and *Carapa guaianensis* show the potent antimicrobial activity against *Paenibacillus* species. A study performed with Andiroba and Copaiba oil nanostructured showed antimicrobial activity against many *Paenibacillus* species. The nanostructuration of *Melaleuca alternifolia* showed an increase antimicrobial activity gainst AFB agent. The use of *Paenibacillus* than *Paenibacillus larvae* help us such screening test [13,26,27].

The spores may show resistant to heat and various chemicals also stay dormancy for many periods of time [28]. The present study showed the sporicidal potential of GML nanocapsules against *Paenibacillus larvae* spores. Previous In previous studies, microparticles killed *Bacillus subtilis* spores [29].

The toxicity assay demonstrated a high toxic effect of GML for the *A. mellifera*. Both concentrations used in the experiment decreased the number of survivor bees to 55%. However the formulation with nanocapsules of GML decreases the toxic effect, showing 95% of survivor bees. The obtained result shows the advantage in using a nanostructured system, in which the release is controlled and gradual. The controlled release of nanoparticles [30] can be the reason of nonexistence of toxic effects on bees. Studies corroborated the present work [26,27]. Both studies show the beneficial effects of nanoparticles in the toxicity on bees. In the first study, *Melaleuca alternifolia* oil caused 85% of bee mortality while the nanoencapsulated oil showed 0% of mortality. In the second study, the Andiroba oil showed a high

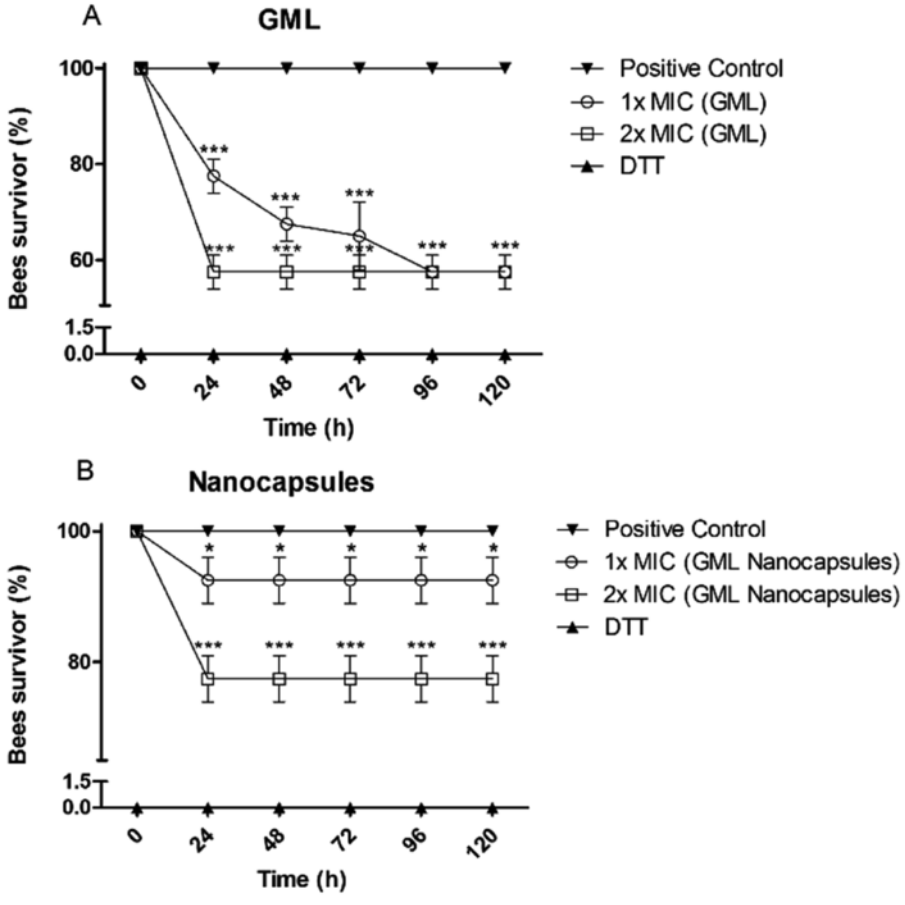

Fig. 3. Toxicity assay of GML and GML nanocapsules in *Apis mellifera*. The (A) GML test and (B) GML nanocapsules. Data showed on Average ± Standard Deviation. Was used analysis of variance (ANOVA) Two-Way followed by Bonferroni's test, considering values $p < 0.05$ (*) and $p < 0.0001$ (***) statistically significant. The study was performed in triplicate.

toxic effect while the nanostructured oil decreased significantly the toxic effect. A reason for this reduction in toxicity is the controlled release characteristics of nanoparticle, leading to increased time of action and a decreased concentration of the compound on the action site.

A study performed on soil with Zinc Oxide Nanoparticles does not cause effect on springtails at high concentrations [31]. A study performed by Sarma et al. [32] demonstrate that the toxicity, fate, and stability of silver nanoparticles in any medium is highly dependent on the type of surface-coated organic. Hydrophilic and hydrophobic monolayers-modified gold nanoparticles were studied for their uptake, distribution, and toxicity in media fish. It was found that hydrophilic particles were present in intestines of fish but no obvious health effects were observed [33]. There are fewer studies on ecotoxicology of organic nanoparticles than inorganic nanoparticles. With the great development of organic nanoparticles, the environment safety must be addressed urgently.

5. Conclusions

In conclusion, this study demonstrated for the first time the high antimicrobial potential of GML and GML nanocapsules against *P. larvae* species. Furthermore, the GML showed an important toxic effect. To exceed this effect, an alternative was nanostructured GML. The toxic effect caused by GML decreased significantly, increasing the bee survivor. Therefore, the formulation containing GML nanocapsules can be an alternative for the treatment or prevention of AFB without honey bee losses.

Ethical statement

This article does not contain any studies with animals that need approval of the ethics committee performed by any of the authors.

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