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Abstract: Nanotechnology has the potential to overcome the challenges of sustainable agriculture, and nanopesticides can control agricultural pests and increase farm productivity with little environmental impact. However, it is important to evaluate their toxicity on non-target organisms, such as honeybees (Apis mellifera) that forage on crops. The aims of this study were to develop a nanopesticide that was based on solid lipid nanoparticles (SLNs) loaded with pyrethrum extract (PYR) and evaluate its physicochemical properties and short-term toxicity on a non-target organism (honeybee). SLN+PYR was physicochemically stable after 120 days. SLN+PYR had a final diameter of 260.8 ± 3.7 nm and a polydispersion index of 0.15 ± 0.02 nm, in comparison with SLN alone that had a diameter of 406.7 ± 6.7 nm and a polydispersion index of 0.39 ± 0.12 nm. SLN+PYR had an encapsulation efficiency of 99%. The survival analysis of honeybees indicated that PYR10ng presented shorter longevity than those in the control group ($P \leq 0.01$). Empty nanoparticles and PYR10ng caused morphological alterations in the bees' midguts, whereas pyrethrum-loaded nanoparticles had no significant effect on digestive cells, so are considered safer, at least in the short term, for honeybees. These results are important in understanding the effects of nanopesticides on beneficial insects and may decrease the environmental impacts of pesticides.

Can a nanopesticide based on solid lipid nanoparticles loaded with the botanical insecticide pyrethrum be toxic to honeybees?

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01th February 2019

COVER LETTER

Dear Editor of Chemosphere,

I am submitting the original article "Can a nanopesticide based on solid lipid nanoparticles loaded with the botanical insecticide pyrethrum be toxic to honeybees?" (Cristiane R. OLIVEIRA et al.) for the refereeing process, in order to publish it in the Chemosphere. Aiming to minimize the effects of pesticides on non-target beneficial insects, nanoparticles that act as carrier systems for agrochemicals are being developed by means of nanotechnology. The solid lipid nanoparticles encapsulated pyrethrum biocide releases small quantities over time and thereby reduces the amount of chemical compound bioavailable in the environment. Nevertheless, it is necessary to assess the adverse effects of nanopesticides in the terrestrial environment. In this sense, our study is pioneer in evaluating the toxicity of this system on a non-target pollinator insect, the honeybees.

We tried to follow precisely the journal's author guidelines, with the title page article, Introduction, Materials and Methods, Results and Discussion and Acknowledgment. *Additional Information* - Total number of words of the textual elements: 6117; Total number of Tables: 1; Total number of Figures: 4.

Sincerely,

Elaine C. M. Silva Zacarin

Dra. Elaine C. M. Silva-Zacarin

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Leonardo Fernandes Fraceto

Dr. Leonardo Fernandes Fraceto

Corresponding Author

Sorocaba, June 30th 2019.

Dear Prof. Willie J. G. M. Peijnenburg
Editor Chemosphere,

Ref. Chem60158

RESPONSE TO EDITOR IN CHIEF AND REVIEWER

Reviewer comment: I thank you very much for submitting your revised manuscript. Having evaluated the responses to the comments made by the reviewers, there is one issue that I do not agree on and that is on the issue of the definition of nanoparticle. 100 nm is considered the upper limit of size in one dimension to allow a particle to be termed a nanoparticle. In your case, the particles are of a size of 260 nm and they should therefore not be termed 'nanoparticle' but they are 'submicron particles'. Throughout the manuscript, the term 'nano' therefore needs to be replaced by 'submicron', including in the title of the manuscript. This is depite the arguments raised in Nature Nanotechnology*.*

Answer: The authors are very thankful to the Reviewer for his(her) valuable comment regarding the nano definition. We really respect his(her) point of view, however, we disagree to change the term nanoparticles as well as nanopesticides in the manuscript to submicron particles. Our arguments are:

i) *We can not use only size range to define a nanoparticle. In this way, the properties that we got with solid lipid nanoparticles in the range of size that we have in this study is totally different from the properties with bulk material. To support this statement, please look at A.D. Maynard, Don't define nanomaterials, Nature, 2011, 475, 31–31.*

ii) *It is clear in literature that nanoparticles prepared with polymeric and lipid materials showed a size distribution in the same range of the particles from our study and these particles are considered nanoparticles due its properties reached in the size range. Easily it is possible to find thousands of published papers in many different areas such as: cosmetics, food, medicine, pharmacy, agriculture that use particles with the same characteristics (lipid particles) and are considered by the scientific community as nanoparticles.*

iii) *It is stated by the editorial from Nature Nanotechnology that in the case of nanopesticides authors showed that the size range threshold is higher for this kind of systems.*

iv) *European Food Safety Authority, a regulatory agency, described in recent guidance that nanomaterials definitions should be reconsidered for food and agriculture since they described that particles larger than 100 nm but retain properties typical of nanoparticles.*

v) *Food and Drug Administration – USA – definition (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/considering-whether-fda-regulated-product-involves-application-nanotechnology#_ftn6):*

"At this time, when considering whether an FDA-regulated product involves the application of nanotechnology, FDA will ask:

1. *Whether a material or end product is engineered to have at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm);*

In addition, as we explain in more detail below, because materials or end products can also exhibit related properties or phenomena attributable to a dimension(s) outside the nanoscale range of approximately 1 nm to 100 nm that are relevant to evaluations of safety, effectiveness, performance, quality, public health impact, or regulatory status of products, we will also ask:

2. ***Whether a material or end product is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1,000 nm).****"*

vi) *Recently Nature Nanotechnology has published a series of papers that were written by worldwide specialists about the nanotechnology in agriculture (see below) and in all these papers there are a lot of citations of papers that showed size higher than 100 nm and they were considered nanomaterials/nanoparticles/nanopesticides/nanofertilizers.*

- https://www.nature.com/articles/s41565-019-0464-4

- https://www.nature.com/articles/s41565-019-0468-0

- https://www.nature.com/articles/s41565-019-0461-7

- https://www.nature.com/articles/s41565-019-0460-8

- https://www.nature.com/articles/s41565-019-0439-5

vii) *If you look at the EU homepage below it is possible to find the definition:*

"Upper size limit

Although 999 nm is still formally on the nanoscale, a very commonly used upper limit for nanomaterial size is 100 nm. This covers most nanomaterials, but there are exceptions. ***Nanomaterials clumped together can have outside dimensions larger than 100 nm, as can those which have been modified by adding a coating or an unusually large chemical group such as a long-chain organic molecule. Such materials include liposomes – small fatty globules – which can be loaded with nanoparticles for drug delivery or use in cosmetic products****."*

https://ec.europa.eu/health/scientific_committees/opinions_layman/nanomaterials2012/en/l-2/3.htm

In this way, as our system is a solid lipid nanoparticles, this mean a structure formed by lipid covered by a surfactant it is like a liposomes, fatty globules and as mentioned below, in the area of cosmetics this is considered as nanoparticles.

vii) The Chemosphere Journal has published papers aiming pest control with particles with mean size distributions higher than 400 nm and they accepted the use of the term nanoparticles. Just as example, look at: https://doi.org/10.1016/j.chemosphere.2013.11.056

Also, based on all arguments above, we do not agree to change the term nanoparticles to submicron particles. We would like thank you so much the reviewer for this discussion, but from our point of view is really more than a question of size limit (100 nm) and by properties of the material. In addition, the application of polymeric and lipid materials in agriculture are well known nowadays and the community that develop systems for this kind of application really considered sizes in the range from the particles of our study as nanoparticles.

Again, thank you for your comment that we really appreciate, but in this case, we can't agree with your suggestion to change the term in the manuscript since nowadays the scientific community has been accepted other definitions than a cut-off 100nm.

Sincerely yours

Dr. Leonardo Fraceto

Corresponding author

On-behalf of all authors.

Nanopesticide based on botanical insecticide pyrethrum and its potential effects on honeybees

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ABSTRACT

Nanotechnology has the potential to overcome the challenges of sustainable agriculture, and nanopesticides can control agricultural pests and increase farm productivity with little environmental impact. However, it is important to evaluate their toxicity on non-target organisms, such as honeybees (*Apis mellifera*) that forage on crops. The aims of this study were to develop a nanopesticide that was based on solid lipid nanoparticles (SLNs) loaded with pyrethrum extract (PYR) and evaluate its physicochemical properties and short-term toxicity on a non-target organism (honeybee). SLN+PYR was physicochemically stable after 120 days. SLN+PYR had a final diameter of 260.8 ± 3.7 nm and a polydispersion index of 0.15 ± 0.02 nm, in comparison with SLN alone that had a diameter of 406.7 ± 6.7 nm and a polydispersion index of 0.39 ± 0.12 nm. SLN+PYR had an encapsulation efficiency of 99%. The survival analysis of honeybees indicated that PYR_{10ng} presented shorter longevity than those in the control group ($P \leq 0.01$). Empty nanoparticles and PYR_{10ng} caused morphological alterations in the bees' midguts, whereas pyrethrum-loaded nanoparticles had no significant effect on digestive cells, so are considered safer, at least in the short term, for honeybees. These results are important in understanding the effects of nanopesticides on beneficial insects and may decrease the environmental impacts of pesticides.

KEYWORD: Nanopesticide; Biocide; Sustainable agriculture, Solid lipid nanoparticles; Bees.

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1. INTRODUCTION

Agri-food production and population growth are amongst the greatest challenges facing humanity. Agriculture is one of the primary drivers of the economy by providing food to the population and benefiting producing countries, but increased population growth has significantly increased humanity's global ecological footprint, surpassing the biocapacity of the Earth (SEKHON, 2014). Human populations increase exponentially over time, whereas food production increases in a linear manner. Conventional agricultural practices generally have negative impacts on the environment and biodiversity, as they require many resources such as energy, water, and soil, and large amounts of agrochemicals and fertilizers are used to improve productivity.

The U.S. Department of Agriculture's (USDA) National Institute of Food and Agriculture (NIFA, 2018) aims to find innovative solutions to issues related to agriculture, food, the environment, and communities. NIFA's priorities include global food security and hunger, food safety, plant health and production, and animal health and production (NANO, 2018). Many of these issues may be resolved using nanotechnology, which has demonstrated great potential in providing novel solutions to agricultural problems (SCOTT and CHEN, 2012; MUKHOPADHYAY, 2014). In the last few decades, nanoscience and nanotechnology have been at the forefront of the development of several nanomaterials for different medical and industrial purposes. Nanoparticles have been developed for a wide variety of applications in the biomedical and electronic fields, while research on nanoparticles as carriers of pesticides has only been conducted in the last decade, and there are still many variables to be investigated before their use on crops (LIU et al., 2008; ANJALI et al., 2010; GOPAL et al., 2012; KAH et al., 2014; SARLAK et al., 2014; MISHRA et al., 2017; KIM et al., 2018).

Nanotechnology can deliver agricultural substances such as nanopesticides and nanofertilizers that increase farm productivity, decrease the environmental impact and the amount of resources used, improve pest control, and support sustainable agriculture, particularly in developing countries. Furthermore, nanocarriers of pesticides and fertilizers have economic advantages for agriculture, because their stability and controlled-release mechanism increase efficiency and reduce the amount of chemicals required on crops (PEREZ-DE-LUQUE and RUBIALES; 2009; CHEN and YADA, 2011; GRILLO et al., 2016; PRASAD et al., 2017; WALKER et al., 2017).

However, the effects of nanoparticles should be fully evaluated before they are incorporated into sustainable agriculture. The U.S. National Science Foundation (NSF) and Environmental Protection Agency (EPA) encourage the investigation of various aspects of nanomaterials, such as their toxicity to non-target organisms, their destination, transportation, and safety in the environment, and their status in terms of food legislation, and support the creation of a nanomaterial database and the maintenance of food regulations (SCOTT and CHEN, 2012).

Pyrethrum extract is a natural botanical insecticide that is extracted from chrysanthemum (*Chrysanthemum cinerariaefolium* and *Chrysanthemum cineum*) flowers, is composed of pyrethrin types I and II and jasmolin, and can be used on crops to control pest insects (PEAY et al., 2006). Natural pyrethrum (a.i.) is highly lipophilic, photodegradable, has low water solubility (<10 $mg.L^{-1}$), does not exhibit biomagnification (SCHLEIER and PETERSON, 2011), and leaves no toxic residues in plants. However, it is more expensive than synthetic pyrethroids (PEAY et al., 2006) and is highly toxic to insects, aquatic invertebrates, and fish (USEPA, 2006). Pyrethroids are insecticides that were developed to improve the photodegradation of natural pyrethrin, and thus be used as an insecticide in the

field (SANTOS et al., 2007), and have great stability and target selectivity. Examples of pyrethroids include deltamethrin, permethrin, and cypermethrin (MONTANHA and PIMPÃO, 2012).

However, for the use of pyrethrum extract in the field it is necessary, at first, to load it into solid lipid nanoparticles (SLNs) to prevent its fast degradation, improving its stability and efficiency to allow its application on crops. Many benefits can be obtained by using SLNs, such as lower large-scale production costs, greater physicochemical stability, the possibility of hydrophilic and hydrophobic drug encapsulation, and the use of natural products in the formulation preparation (MULLER et al., 2000; MULLER et al., 2011; NASERI et al., 2015; SARANGI and PADHI et al., 2016).

Interactions between biological systems and nanomaterials are complex, so it is important to evaluate their toxicity to non-target organisms (JACQUES et al., 2017), particularly to beneficial insects such as honeybees (*Apis mellifera*), which play an important role in pollinating agricultural crops (GIANNINI et al., 2015). Honeybee populations are declining worldwide, and although multiple factors contribute to this decline (GOULSON et al., 2015), it is mainly caused by agrochemicals sprayed on crops visited by bees (POTTS et al., 2010). In this context, the physicochemical characterization of nanopesticides can enable their future use in organic farming and contribute to sustainable agriculture, because these carriers may have little effect on the environment and biodiversity (GRILLO et al., 2016; PRASAD et al., 2017). However, this carrier system must have low toxicity to honeybees and other beneficial insects.

The objectives of this study were to develop a nanopesticide that was based on SLNs loaded with pyrethrum extract biocide (nanobiocide), characterize its physicochemical properties, and evaluate its toxicity to honeybees (Africanized *A. mellifera*). We evaluated

sublethal effects on the histopathology of the bee midgut, an organ that plays a central role in food digestion and nutrient absorption. It is important to emphasize the fact that there are gaps of information in the literature regarding the toxicity of nanopesticides to non-target organisms, such as pollinator insects including honeybees. Our results can be applied in the field, can contribute to nanopesticide regulation, and can improve both environmental and food security.

2. MATERIALS AND METHODS

2.1. Chemicals

The pyrethrum extract Pestanal® (biocide, CAS 8003-34-7, analytical standard), polyvinyl alcohol (PVA, 30–70 kDa, CAS 9002-89-5, hydrolyzed >99%), and glyceryl tripalmitate (tripalmitin, CAS 555-44-2, purity ≥99%) were purchased from Sigma-Aldrich. Chloroform ($CHCl_3$, CAS 67-66-3, purity ≥99%) was purchased from a local supplier. All these products were used for the preparation of the nanoparticles. Acetone (CAS 67-64-1, purity = 100%) was used as a solvent in the preparation of the pyrethrum solution.

2.1.1. Solid lipid nanoparticles

SLNs containing pyrethrum were prepared by the method of emulsification/solvent evaporation with some modifications (VITORINO et al., 2011; de MELO et al., 2018). Initially, 30 mL of an aqueous phase containing 1.25% PVA and distilled water was prepared and magnetically stirred (100 rpm). An organic phase with 250 mg of glyceryl tripalmitate and 5 mg of pyrethrum (active ingredient – a.i.) was then prepared, which was dissolved in 5 mL of chloroform. The organic phase was added to the aqueous phase, and this mixture was sonicated at 40 W for 5 min producing an emulsion. The emulsion was placed in an

143 ULTRA-TURRAX™ homogenizer at 14,000 rpm for 7 min. The organic solvent was then
144 removed using a rotating evaporator in order to create a concentrated emulsion with 10 mL
145 of nanoparticles. The final concentration of biocide was 0.05 $mg.mL^{-1}$. SLNs without
146 pyrethrum extract (control) were also prepared.
147
148 *2.2. Nanoparticles*
149 The purpose of the formulations was to achieve greater physicochemical stability and
150 better efficiency of pyrethrum encapsulation in the nanoparticles. In order to evaluate the
151 physicochemical stability as a function of time were used the maintenance of colloidal
152 parameters in formulation. The colloidal parameters were the mean diameter, polydispersity
153 index, zeta potential, besides the nanoparticle concentration and encapsulation efficiency of
154 the pyrethrum extract. All analyses were conducted for 120 days and the results were
155 expressed (mean ± SEM).
156
157 *2.2.1. Nanoparticle characterization*
158 The mean diameter and polydispersion index were determined by dynamic light
159 scattering (DLS). Nanoparticle samples were diluted (10 µL:1 mL) in purified water and
160 analyzed using a Zetasizer Nano ZS90 analyzer (Malvern Panalytical, UK). Zeta potential
161 values (in mV) were also determined using the ZS90 analyzer, with the same dilution process.
162 The pH of the nanoparticles was determined using a pH meter (Tecnal®, Brazil). Further
163 details could be obtained in literature (VENKATRAMAN et al., 2005; de MELO et al., 2012;
164 OLIVEIRA et al., 2015).
165
166 *2.2.2. Nanoparticle concentration*

SLN size distributions and concentrations were analyzed using a nanoparticle tracking analysis (NTA) instrument (NanoSight LM10). Nanoparticle samples were diluted 10,000 times and analyzed by injecting 1 mL of the sample into the cell (more details in section 1.1 - Supplementary Material).

2.2.3. Differential Scanning Calorimetry (DSC)

A thermal analysis was performed to demonstrate that the pyrethrum was encapsulated in the nanocarriers using a DSC Q20 differential scanning calorimeter (TA Instruments). The samples of pyrethrum extract, lipid, SLNs, and SLNs loaded with pyrethrum were analyzed (Section 1.2 - Supplementary Material).

2.2.4. Fourier-transform infrared spectroscopy (FTIR)

FTIR was performed to investigate interactions between the biocide and the SLNs using an infrared spectrophotometer (Agilent). The pyrethrum extract, lipid, surfactant (PVA), physical mixture, SLNs, and SLNs loaded with pyrethrum were analyzed using an attenuated total reflectance accessory (POLLETO et al., 2007; WANG et al., 2010) (Section 1.3 - Supplementary Material).

2.3. Determination of encapsulation efficiency and quantification of pyrethrum by high-performance liquid chromatography (HPLC)

The total amount of pyrethrum extract present in the nanoparticle suspension was determined by the ultrafiltration/centrifugation method. After the suspension had been diluted with acetonitrile, it was filtered through a 0.22 μm Millipore™ membrane filter and quantified by HPLC (Varian ProStar). The pyrethrum extract association rate was calculated

as the difference between the non-associated fraction of biocide and the total amount initially added to the nanoparticles (GAMISANS et al., 1999; SCHAFFAZICK et al., 2003; KILIC et al., 2005) (Table 1S- Supplementary Material).

2.4. Toxicological bioassay

Operculated brood combs were collected from three healthy colonies of Africanized *Apis mellifera* located in apiaries at Sao Paulo State, Brazil. The emergence of worker bees was monitored in laboratory. Following emergence, the bees were transferred to plastic pots lined with filter paper and fed *ad libitum* sugar-aqueous solution (50%:50% water:inverted sugar, v:v) to acclimatize for 24 h.

Subsequently, the 1-day-old bees were divided into the following experimental groups in triplicate (each colony representing a replicate): I) Control (CTL) - sugar-aqueous solution (syrup); II) Sublethal dose (1 ng.μL^{-1}) of pyrethrum extract (PYR_{1ng}); III) Sublethal dose (10 ng.μL^{-1}) pyrethrum extract (PYR_{10ng}); IV) 1 ng.μL^{-1} of pyrethrum loaded in SLNs ($SLNP_{1ng}$); V) 10 ng.μL^{-1} of pyrethrum loaded in SLNs ($SLNP_{10ng}$); IV) Empty SLNs; V) Polyvinyl alcohol - surfactant control (PVA); VI) Acetone control (ACN) - vehicle/solvent control. The dose used per bee was based on the $LD50_{48h}$ of pyrethrum for honeybees, i.e., 22 ng.bee^{-1} (USEPA, 1991).

Acute exposure was performed individually by oral administration, i.e., the corresponding solution of the experimental group was administrated to the bees (1 µL) using a micropipette (*per os* administration). Two sublethal doses of 10 ng or 1 ng of biocide per bee were given of the pyrethrum extract (PYR) and pyrethrum loaded in nanoparticles (SLNs). The half the $LD50_{48h}$ value corresponded to a 1/2 dilution ($LD50_{/2}$ = 10 ng.μL^{-1} = 10 ppm), and the other dose corresponded to a 1:20 dilution of the $LD50_{48h}$ value ($LD50_{/20}$ = 1

215 ng.µL^{-1} = 1 ppm), both being sublethal concentrations for honeybees. Concentrations of the
216 solutions, which were used for getting the sublethal doses offered to bees, were obtained by
217 serial dilution of stock solution.

218 After individually acute exposure, the bees were kept in plastic pots (cages), being
219 fed with 50% (w/w) sucrose aqueous solution, in an incubator at a relative humidity of 70%
220 ± 5 and temperature of 32 ± 2ºC, under dark conditions. Two bioassays were performed,
221 being the first one for survival analysis (N = 12 bees per pot in triplicate, per experimental
222 group, totalizing 36 individuals) and another one for histology analyzes (N = 15 bees per pot
223 in triplicate per experimental group, totalizing 45 individuals).

224 In the first bioassay (survival analysis), the bees were monitored daily until the last
225 bee has died. Specifically for survival bioassay, the deltamethrin (DLT, 10 ng.µL^{-1})
226 experimental group was added as positive control. In the second bioassay, the bees were
227 collected 48 h after the acute exposure (N = 6 per group) and dissected for midguts' removal,
228 which were processed for resin embedding and histological analysis (section 2.4.1).

229

230 *2.4.1. Histology procedure*

231 The bee midguts were fixed in 4% buffered paraformaldehyde solution for 24 h and
232 immersed in phosphate-buffered saline (0.1 mol.L^{-1} phosphate buffer, pH 7.4). After, the
233 material was dehydrated in an increasing ethanol series according to Silva-Zacarin et al.
234 (2012). Subsequently, the material was embedded in historesin, and submitted to microtomy.
235 Slides containing 3-µm thick histological sections were stained with hematoxylin-eosin.
236 Posteriorly, the material was photodocumentated and both qualitative and semi-quantitative
237 histopathological analyses were performed using Leica Application Suite V3.8 coupled to
238 the light field photomicroscope (DM1000, Leica). For each bee from each experimental

group (N = 6), two slides were analyzed per individual and three non-sequential histological sections were analyzed for each slide.

Other slides containing 3-µm thick histological sections were submitted to histochemical analysis for detection of proteins, lipids and neutral glycoconjugates (SILVA-ZACARIN et al., 2012) (Section 1.4 - Supplementary Material and Figure 4S).

2.4.2. Semi-quantitative analysis of midguts

Parameters for semi-quantitative analysis were defined according to the Bernet et al. (1999) protocol, and histological alterations (lesions) in midgut of bees were based on Soares-Lima et al. (2018) protocol. To determine alterations in the bee midguts, the lesion index and the organ index, were calculated using two parameters: the importance factor and the score value (BERNET et al., 1999). Alterations were classified from 0 to 3, depending on their degree and extent: 0- no alteration, 1- slight alteration, 2- moderate alteration, and 3- severe alteration. The importance factor was established for each lesion observed (cells eliminated from the epithelium, increased apocrine secretions from the digestive cells, cellular vacuolization, changes in regenerative cells' nests, and the presence of pyknotic nuclei in cells of the epithelium) by a qualitative analysis based on pathological severity. This factor was categorized as (1) minimal pathological importance (repairable damage), (2) moderate pathological importance (damage was repairable in most cases), or (3) severe pathological importance (irreparable damage) (Table 2S and section 1.4 - Supplementary Material).

2.5. Statistical analysis

All data were previously subjected to homogeneity of variance (Bartlett's) and normality (Shapiro-Wilk and Kolmogorov-Smirnov) tests. The physicochemical characterization data were subjected to a Student's t-test followed by a Mann Whitney test. A semi-quantitative analysis of the bee midguts was performed using a Kruskal-Wallis test followed by Dunn's multiple comparison test. The significance level was set at $\alpha = 0.05$. GraphPad Prism v.5.0 was used for these statistical analyses.

The survival curve of honeybees per each experimental group was analyzed by the Log-Rank test (Kaplan-Meier method), and comparison between survival time of the groups was performed by the Holm-Sidak test. The significance level was set at $\alpha = 0.05$. SigmaPlot 13 software was used these analyze.

3. RESULTS AND DISCUSSION

3.1. Nanoparticle characterization

The SLNs were prepared using approved components that are generally recognized as safe (GRAS). Tripalmitin (glyceryl tripalmitate) was used as a solid lipid and PVA was used as a surfactant. Physicochemical stability of the empty and encapsulated biocide in SLNs were evaluated from maintenance measurements of the colloidal parameters (mean diameter, polydispersity and zeta potential), besides the concentration of nanoparticles and pyrethrum encapsulation efficiency, over time (0 to 120 days). Colloidal parameter values and other parameters are shown in Table 1.

The initial and final hydrodynamic diameters (mean ± SEM) of the empty solid lipid nanoparticles (SLN) were 290.0 ± 5.0 and 406.7 ± 6.7 nm, respectively. For the SLNs loaded with pyrethrum (SLN+PYR) the initial and final hydrodynamic diameters were 264.9 ± 2.8 and 260.8 ± 3.7 nm, respectively. There was a significant difference between the empty

nanoparticles and those loaded with pyrethrum in the initial ($P \leq 0.0001$ and $T = 18.18$) and final ($P \leq 0.0001$ and $T = 48.51$) analyses. The hydrodynamic diameter values of empty SLNs increased after 60 days of storage with significant differences between the timepoints ($P \leq 0.0001$ and $T = 54.60$), while these values remained stable for SLN+PYR over the experimental period (120 days) (Figure 1SA- Supplementary Material). The empty SLNs had a larger mean diameter and less physicochemical stability than SLN+PYR, indicating that active ingredient of pyrethrum can stabilize nanoparticle formulation and decrease aggregate formation.

The polydispersion index at 0 and 120 days was 0.12 ± 0.01 and 0.39 ± 0.12 nm, respectively, in empty SLNs, and 0.12 ± 0.01 and 0.15 ± 0.02 nm, respectively, in SLN+PYR (Table 1), and values below 0.2 nm in the initial analysis were considered indicative of good stability and a small distribution of particle diameters. The low values indicate that the nanoparticles were of similar size and without aggregates (MASARUDIN et al., 2015). Similar results were obtained by de Melo et al. (2016) in a 120-day experiment with 15d-PGJ2-loaded SLNs, and by González et al. (2015) at the beginning of their experiment with poly (ethylene glycol)-nanoparticles containing geranium (an essential oil). However, the time-based analysis revealed that the SLN polydispersion index had increased after 60 days of storage (0.3 and 0.39 nm; Figure 1SB - Supplementary Material), with significant differences between the timepoints ($P \leq 0.005$ and $T = 0.0$) and significant differences between SLN_{120} and $SLN+PYR_{120}$ ($P \leq 0.005$ and $T = 0.0$). These data indicate that there was a heterogeneous distribution of particle diameters, i.e., there was a greater aggregation of particles in the empty system (SLN). Particle aggregation and degradation occur in SLN formulations that increase and decrease particle size, respectively, due to the loss of a surfactant coating that protects the material (MULLER et al., 1996).

Both nanosystems had a negative zeta potential, with initial and final values of -13 ± 0.4 and -14 ± 0.3 mV, respectively, for empty SLNs and -9.7 ± 0.2 and -18.2 ± 0.3 mV, respectively, for SLN+PYR. There was a significant difference between the empty nanoparticles and those loaded with pyrethrum ($P \leq 0.0001$, $T_{0d} = 8.989$, and $T_{120d} = 24.50$; Table 1). After decreasing on the 30th day (-5.48 ± 0.13 mV), the zeta potential of SLN+PYR increased to -12.2 ± 0.18 and -18.2 ± 0.35 mV after 90 and 120 days, respectively (Figure 1SD- Supplementary Material). Similarly, the empty SLN zeta potential decreased after 15 (-4.85 ± 0.19 mV) and 30 (-6.27 ± 0.18 mV) days, but increased on the 60th day (-15.43 ± 0.23 mV), indicating good stability until the end of the analysis time (Figure 1SD- Supplementary Material). Zeta potential values greater than 30 mV indicate excellent electrostatic stabilization (60 mV is the ideal value), while values lower 15 mV may result in partial flocculation (SCHWARZ et al., 1994). Low zeta potentials were observed, but the nanoparticle formulations were stable over time due to steric stabilization provided by the PVA (LOURENÇO et al., 1996). Stabilizers can be used in nanoparticle formulations to prevent particle aggregation (ABDELWAHED et al., 2006). In the present study, the nonionic surfactant PVA was used to prepare the SLNs, which is absorbed onto surface nanoparticles and promotes steric stabilization (ADITYA et al., 2013; OLIVEIRA et al., 2015). Therefore, unlike in previous studies, it was not superficial electrostatic repulsion that provided stability to the system (PASQUOTO-STIGLIANI et al., 2017). Particles in suspension are more stable if the zeta potential is greater than 20 mV, and 40 mV indicates excellent stability (ADITYA et al., 2013). Similar results were obtained by Oliveira et al. (2018) in zein nanoparticles loaded with the essential oil citronella (geraniol and R-citronellal), and by Kah et al. (2014) in a polymer-based nanoformulation of atrazine.

Table 1: Characterization of empty SLN and SLN loaded with pyrethrum extract over a period from 0 to 120 days.

PARAMETERS	*SLN_0*	*SLN_{120}*	*$SLN{+}PYR_0$*	*$SLN{+}PYR_{120}$*
MD_{DLS} (NM)	290.0 ± 5.0	406.7 ± 6.7[a,c]	264.9 ± 2.8	260.8 ± 3.7
MD_{NTA} (NM)	185.9 ± 4.6[c]	263.8 ± 18.5[a,c]	161.5 ± 2.7	227.0 ± 12.3[b]
PDI	0.12 ± 0.01	0.39 ± 0.12[a,c]	0.12 ± 0.01	0.15 ± 0.02
ZP (-mV)	13 ± 0.4[c]	14 ± 0.3[c]	9.7 ± 0.2	18.2 ± 0.3[b]
CT (10^{13} particles/mL)	2.7 ± 0.5	3.8 ± 0.2	5.9 ± 0.5	2.0 ± 0.1
pH	4.9 ± 0.04	5,7 ± 0.04[a,c]	5.0 ± 0.02	7.1 ± 0.02[b]
EE (%)	-	-	> 99%	> 99%

Legend - Mean diameter (MD) using dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) techniques; polydispersion index (PDI); zeta potential (ZP), concentration of nanoparticles(CT); hydrogenionic potential (pH) and encapsulation efficiency (EE). The values are expressed as the mean ± standard error of six measurements. [a] Significant difference between SLN group and times; [b] Significant difference between SLN+PYR group and times; [c] Significant difference between SLN and SLN+PYR group. Paired T Test for parametric test, and Mann Whitney U test for nonparametric test.

SLNs showed good stability for the encapsulated a.i, evidencing that physicochemical properties not changed over time. According to Naseri et al. (2015), SLNs are good nanocarriers and can be used to deliver drugs and agrochemicals. Their properties include great physicochemical stability during production and storage, a good release profile, the ability to solubilize lipophilic actives, and low toxicity (NASERI et al., 2015).

There was a significant difference in the pH of the empty SLN suspension between 0 and 120 days (4.9 ± 0.04 and 5.7 ± 0.04, respectively; $P \leq 0.0001$ and $T = 16.08$), and of SLN+PYR (5.0 ± 0.02 and 7.1 ± 0.02, respectively; $P \leq 0.0001$ and $T = 10.04$; Table 1). Only at 120 days was there a significant difference in pH between the treatment groups ($P \leq 0.0001$ and $T = 107.9$) with SLN+PYR having a pH of 7.16 ± 0.02 (Figure 1SC - Supplementary Material), indicating that hydrolytic processes occurred during this period. Similar results were obtained by Oliveira et al. (2015).

354 The NTA revealed that the empty SLNs contained 2.7 ± 0.5 x 10^{13} particles per mL
355 with an initial size of 185.9 ± 4.6 nm, and SLN+PYR contained 5.9 ± 0.5 x 10^{13} particles per
356 mL with an initial size of 161.5 ± 2.7 nm. Table 1 shows that there was a significant
357 difference among timepoints for empty SLNs ($P \leq 0.02$ and $T = 3.65$) and SLN+PYR ($P \leq$
358 0.007 and $T = 4.92$), as well as between empty SLNs and SLN+PYR at 0 ($P \leq 0.0004$ and T
359 = 10.68) and 120 ($P \leq 0.007$ and $T = 5.23$) days. NTA counts the number of particles per mL
360 and is a complementary technique in the analysis of hydrodynamic diameters, and DLS and
361 NTA did not provide similar diameter values and particle concentrations. This difference
362 may have been caused by sample dilution during the NTA, which could have caused some
363 aggregates to rupture in suspension and result in smaller particles than the DLS
364 (MARUYAMA et al., 2016).

365 The encapsulation efficiency of pyrethrum into the SLNs was evaluated using an
366 analytical curve of pyrethrum determined by HPLC (Peak area (a.u.) = 4.69442 +
367 1952.15769* [pyrethrum concentration], r = 0.99341). The encapsulation efficiency was as
368 high as 99%, suggesting that the pyrethrum extract was efficiently encapsulated in this carrier
369 system. Nevertheless, is important verify the release profile of pyrethrum in field conditions
370 and it is expected that due the high encapsulation efficiency that the particles protect the a.i.
371 in order to increase its shelf life in field conditions. A high encapsulation efficiency has also
372 been reported in polymeric nanocapsules and SLNs loaded with carbendazim and
373 tebuconazole (CAMPOS et al., 2015), in chitosan nanoparticles carrying the herbicides
374 imazapic and imazapyr (MARUYAMA et al., 2016), and in microcapsules containing
375 dementholized peppermint oil (ZHAO et al., 2016). The high encapsulation value indicates
376 the affinity of the biocide to the lipid matrix (de MELO et al., 2016) due to its low solubility
377 in water (<10 $mg.L^{-1}$) and high solubility in organic solvents (USEPA, 2006).

3.2. Differential scanning calorimetry (DSC)

DSC thermograms for SLN+PYR, empty SLNs, tripalmitin, and pyrethrum extract are presented in Figure 1. The DSC analyzes in this study were carried out with the objective of demonstrating that the pyrethrum interacts with nanocarriers components. There were no endothermic peaks for the pyrethrum extract. Tripalmitin's lowest peak was observed at 61ºC, which agrees with the melting point described in the literature (CHEN et al., 2006). Analysis of the empty SLNs and SLN+PYR revealed that the melting points for tripalmitin were 65 and 64°C, respectively, indicating that tripalmitin in the SLNs was solid, and that the pyrethrum did not change the lipid core organization of the SLNs. Similar results were obtained by Oliveira et al. (2015), who found that the herbicides simazine and atrazine were dispersed on a nanoparticle matrix; as well as, Nasseri et al. (2016), verified that SLNs containing *Zataria multiflora* essential oil (ZEO) not showed DSC pick of *Zanataria multiflora*, and authors suggested that essential oil was incorporated and dissolved in the lipid matrix. Analysis of the empty and encapsulated SLNs revealed two peaks, one indicating a tripalmitin peak and the other possibly indicating PVA. Thermal studies of PVA have reported an 88.1°C peak, probably due to moisture evaporation (GUIRGUIS; MOSELHEY, 2012).

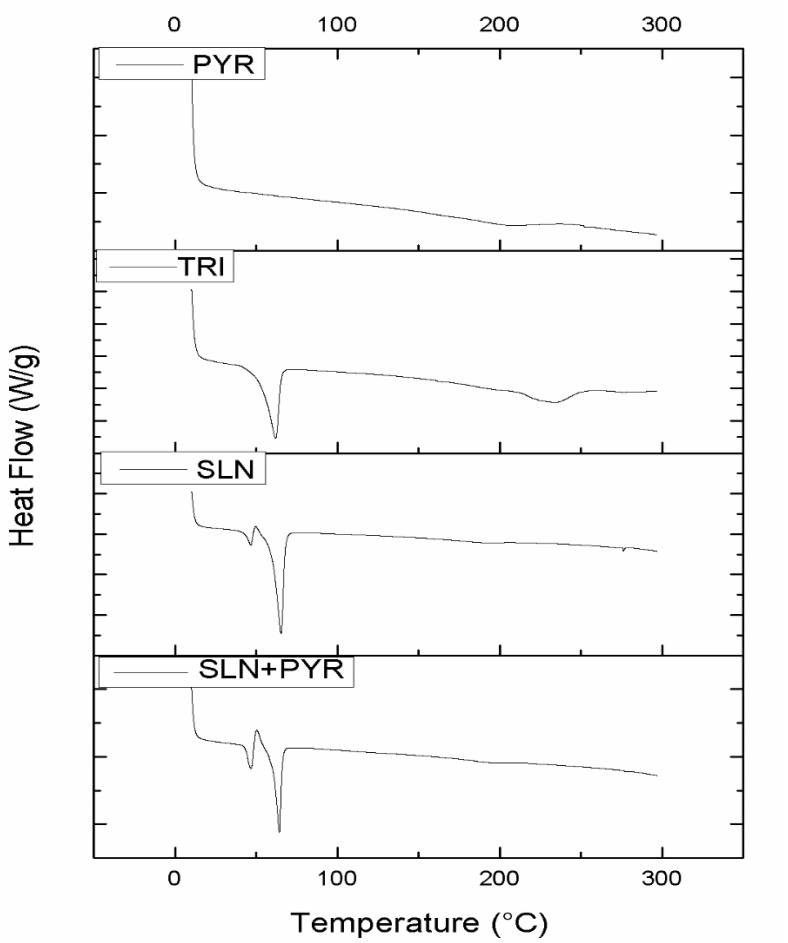

Figure 1 - Differential scanning calorimetry evaluation of interaction between pyrethrum extract and components of the SLN formulation: Thermograms for (PYR) Pyrethrum extract, (TRI) Tripalmitin, (SLN) Solid lipid nanoparticles, (SLN+PYR) Pyrethrum loaded in solid lipid nanoparticles. Conditions: N_2 flow - 50 mL/minute, heating ramp of 10 to 300°C at a rate of 10°C per minute.

3.3. Fourier Transform Infrared Spectroscopy (FTIR)

The physical mixture had three specific bands at 2914, 2368, and 1654 cm^{-1} (Figure 2), which corresponded with tripalmitin (2914 cm^{-1}); and pyrethrum extract bands at 2368 and 1654 cm^{-1}, corresponding with peak CO_2 (OLIVEIRA and PASSOS, 2013) and a stretching of the –C=C group, respectively. The infrared spectra of PVA, empty SLNs, and SLN+PYR exhibited similar specific bands at 3335 cm^{-1} (Figure 2), which suggests the presence of an –O-H group in the formulations. These groups were probably derived from the water and PVA used in the preparation of the nanoparticles (ZAIN et al., 2011). The

411 specific bands at 2914 and 2848 cm^{-1} that were observed in the nanoparticles indicates a
412 stretching of the –C-H group (Figure 2), corresponding to tripalmitin (CAMPOS et al., 2015).
413 It was also possible to observe bands at 1735 cm^{-1}, corresponding to a stretching of the –
414 C=O group, at 1470 cm^{-1}, corresponding to a bending of the $–C-H_2$ group, and at 1178 cm^{-1},
415 corresponding to a stretching of the –C-O group.

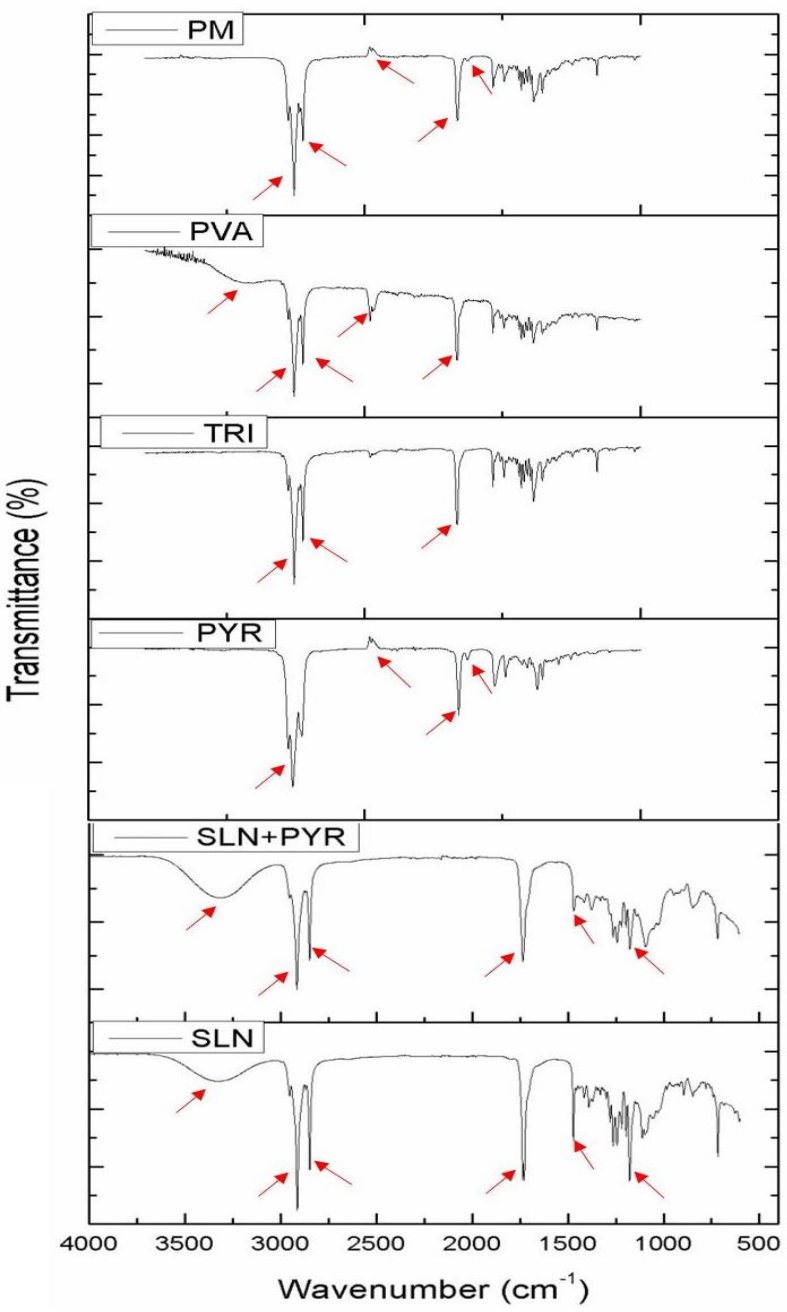

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Figure 2 - Infrared spectroscopic evaluation of interaction between pyrethrum extract and components of the SLN formulation: FTIR spectra for (PM) Physical mixture (PVA) Surfactant - polyvinyl alcohol; (TRI) Tripalmitin; (PYR) Pyrethrum extract; (SLN+PYR) Pyrethrum loaded in solid lipid nanoparticles; (SLN) Solid lipid nanoparticles. Arrows indicate the main characteristic absorption bands in each spectrum. Conditions: infrared spectrophotometer with a range of 400 to 4000 cm^{-1}, 128 scans per sample and 2 cm^{-1} resolutions.

3.4. Toxicological bioassay

Exposure to deltamethrin or pyrethrum extract (10 $ng.\mu L^{-1}$) affected the longevity of bees, reducing their life span. Bees exposed to pyrethrum extract ($P < 0.01$; 141.18 ± 21.3 hours) and pyrethroid ($P < 0.001$; 25.33 ± 0.93 h) presented shorter longevity than those in the control group (257.83 ± 21.79 h). There is not significant difference between control and other experimental groups (ACN; PVA; SLN; $SLNP_{1ng}$; $SLNP_{10ng}$ and PYR_{1ng}; $P > 0.05$). The ACN (252.7 ± 25.03 h) data was similar to control group, as well as $SLNP_{1ng}$ (256.24 ± 21.00 h) and $SLNP_{10ng}$ (241.33 ± 18.81 h). The mean survival time of PVA (171.16 ± 18.09 h), SLN (196.54 ±11.38 h) and PYR_{1ng} (175.33 ± 28.12 h) groups was lower than the control group, but not significant ($P > 0.05$). The data of survival analysis were showed in Supplementary Material (Figure 2S).

Pyrethroids can be dangerous to honeybees (JOHNSON et al., 2010), for example, they interfere in the behavior (PALMQUIST et al., 2012), learning and memory performance (LIAO et al., 2018). In addition, exposure to Lambda-Cyhalothrin negatively affects the life span (LIAO et al., 2018; DOLEZAL et al., 2016). In line with these data, the pyrethrum extract and deltamethrin also reduced survival of Africanized *Apis mellifera*.

The sublethal doses of 1 $ng.\mu L^{-1}$ (1 ppm) and 10 $ng.\mu L^{-1}$ (10 ppm) of biocide free or encapsulated that were administered to the bees, induced short-term responses, at morphological level, in the midguts of newly emerged workers.

444 The bee midgut is mainly responsible for food digestion and nutrient absorption, and
445 is composed of three cell types: digestive, endocrine, and regenerative cells. Digestive cells
446 are responsible for the production of digestive enzymes and nutrient absorption, endocrine
447 cells produce hormones, and regenerative cells, which are within nests, are responsible for
448 cell renewal of the epithelium (MARTINS et al., 2006).

449 Histological analysis of the bee midguts revealed morphological alterations in the
450 epithelium (Figure 3), specifically in the digestive cells, whereas the regenerative cell nests
451 maintained their normal morphological pattern. An increase in the elimination of digestive
452 cells to the intestinal lumen was observed in some treatment groups (empty SLNs, $SLNP_{1ng}$,
453 and PYR_{10ng}; Figure 3D, 3E, and 3H) in comparison to the control groups (CTL, ACN, and
454 PVA), which was significant in the empty SLN group (Figure 4A and Table 3S -
455 Supplementary Material).

456 Therefore, sublethal concentrations of pyrethrum extract in both non-encapsulated
457 and encapsulated form in nanoparticles, as well as in empty nanoparticles (SLN), caused
458 changes in digestive cells. Digestive cells have many microvilli close to the peritrophic
459 matrix in the lumen, and among these cells, nests of small regenerative cells are in the
460 intestinal epithelium (NEVES et al., 2002). These undifferentiated cells that remain in the
461 nest are a source for cell renewal in epithelium of bee midgut (CAVALCANTE and CRUZ-
462 LANDIM, 2004). Thus, regenerative cells replace dead digestive cells, which were released
463 into the lumen, for new epithelial digestive cells by differentiation process (CRUZ et al.,
464 2011). In this study, regenerative nests were observed in midgut epithelium, but histological
465 alterations indicative of cytotoxicity were not found in these cells, such as pyknotic nuclei.
466 If the regenerative cells from nests had presented nuclear pyknosis, which is an indicative of
467 cell death in undifferentiated cells, this alteration would have a "severe pathological

468 importance" because regenerative cells in adults does not suffer mitosis (CRUZ et al., 2011),
469 and consequently epithelial renewal of midgut would be compromised, resulting to partial or
470 total loss of the organ function.

471 Digestive cells are eliminated by cell degeneration under natural conditions,
472 meanwhile this process can be accelerated and/or intensified in response to xenobiotic
473 exposure (e.g., SLNs; Table 3S - Supplementary Material). Therefore, cell renewal is an
474 important process in maintaining the organ function, because the differentiation process from
475 regenerative cells can replace dead digestive cells and to renew the midgut epithelium.

476 There was less elimination of digestive cells to the intestinal lumen in bees exposed
477 to pyrethrum-loaded nanoparticles than in those exposed to empty nanoparticles (SLN).
478 Probably, the reduced cell-to-lumen liberation has been due to the interaction of the
479 pyrethrum with the active sites in the nanoparticle, providing greater stability of the colloidal
480 system over the time (0-120d) and high encapsulation efficiency (> 99% along 120d), as
481 evidenced in the physicochemical characterization data. On the contrary, empty SLNs are
482 more reactive and form aggregates more easily over time. Therefore, reactive empty SLNs
483 could interact with the epithelial cells of the midgut (oral exposure) and induce cytotoxicity
484 in digestive cells, which would trigger their elimination to the organ's lumen. The compounds
485 used in nanoparticle formulations, and the colloidal instability of the system, can affect
486 interactions with cell membranes and trigger cytotoxicity (NAFEE et al., 2009). Whereas
487 the worker honeybee has lifetime of 45 days, and considering the acute exposure to the
488 nanopesticide during its application, probably the whole SLNP will remain stable during its
489 life span. Associating this information with the survival analysis, it can be noted that
490 encapsulated pyrethrum kept the survival time (256.24 ± 21.00 h and 241.33 ± 18.81 h,
491 $SLNP_{1ng}$ and $SLNP_{1ng}$, respectively) of the bees similar to the control group (257.83 ± 21.79

492 h). Given that 10 ng of pyrethrum extract and pyrethroid (deltamethrin) reduced life span of
493 the bees, it may be noted that pyrethrum-loaded in nanoparticle is more safe for honeybees,
494 probably because of the stability of the encapsulated pyrethrum and its release as a function
495 of time.

496 Another important process that we observed was increased apocrine secretions from
497 the midgut epithelium onto the apical surfaces of midgut digestive cells (Figure 3SD and 3SE
498 - Supplementary Material). These epithelial cells secrete digestive enzymes and peritrophic
499 matrix substances normally by means of apocrine secretion. Therefore, an increase in
500 secretion may be a protective compensatory response to xenobiotic exposure. Increased
501 apocrine secretion occurred in both the empty nanoparticle-exposed and 1 $ng.\mu L^{-1}$ of
502 pyrethrum-loaded nanoparticle-exposed groups (SLN and $SLNP_{1ng}$; Table 3S and Figure
503 4B). A previous study reported an increase in apocrine secretion of midgut digestive cells in
504 bees exposed to sublethal doses of thiamethoxam insecticide (0.428 $ng.\mu L^{-1}$ and 0.0428
505 $ng.\mu L^{-1}$ per day for 18 days), as well as the increase in both cell vacuolization and cell
506 elimination from the epithelium to the midgut lumen over the exposure period (OLIVEIRA
507 et al., 2014).

508 Higher frequency of eliminated digestive cells and release of apocrine secretion
509 (Figure 4) were considered reversible alterations in the bee midgut and that did not affect
510 survival of bees in empty SLNs or encapsulated pyrethrum (SLNPs) groups. In normal
511 physiological situations, there is low frequency of senescent or dead cells eliminated to the
512 lumen (CAVALCANTE; CRUZ-LANDIM, 1999), and releasing of digestive enzymes from
513 cells to the peritrophic matrix in the lumen, usually by apocrine secretion (TERRA;
514 FERREIRA, 2012). Therefore, these alterations were classified as importance factor 1 in the
515 semi-quantitative analysis, because normally they are reversible, i.e., damage recovery in

516 epithelium occurs through the differentiation of regenerative cells from their nests in order
517 to have new digestive cells. Thus, there is a compensatory response to the potential
518 physiological stress triggered by agrochemicals or nanocarriers that can lead to the
519 elimination of cells and/or intensification of apocrine secretion. Soares et al. (2012) reported
520 an elimination of cells into the lumen, increased apocrine secretion, and pyknotic nuclei in
521 the epithelial cells of the *Scaptotrigona postica* midgut after applying sublethal doses of the
522 insecticide imidacloprid. Similarly, Rossi et al. (2011) exposed Africanized *A. mellifera* to
523 sublethal doses of imidacloprid and observed an increase in both cell elimination and
524 apocrine secretion in the midgut.

525 Aljedani (2017) evaluated the effects of acute exposure to deltamethrin on foraging
526 worker honeybees (*A. mellifera jemenatica*). The bees that were fed a sugary solution
527 containing 2.5 ppm of pyrethroid presented morphological changes in the midgut. In our
528 study, sublethal concentrations of pyrethrum extract (1 and 10 ng.µL^{-}) did not induce
529 histopathological effects on midguts' honeybees when the cell biomarkers were analyzed
530 separately, but the total organ index analysis showed alterations in 10 ng.µL^{-1} pyrethrum
531 extract that could potentially impair midgut function, since there was a decrease in the
532 longevity of the bees, demonstrating the relevance of evaluation of total organ index in bees
533 exposed to pesticides coupled to survival analysis.

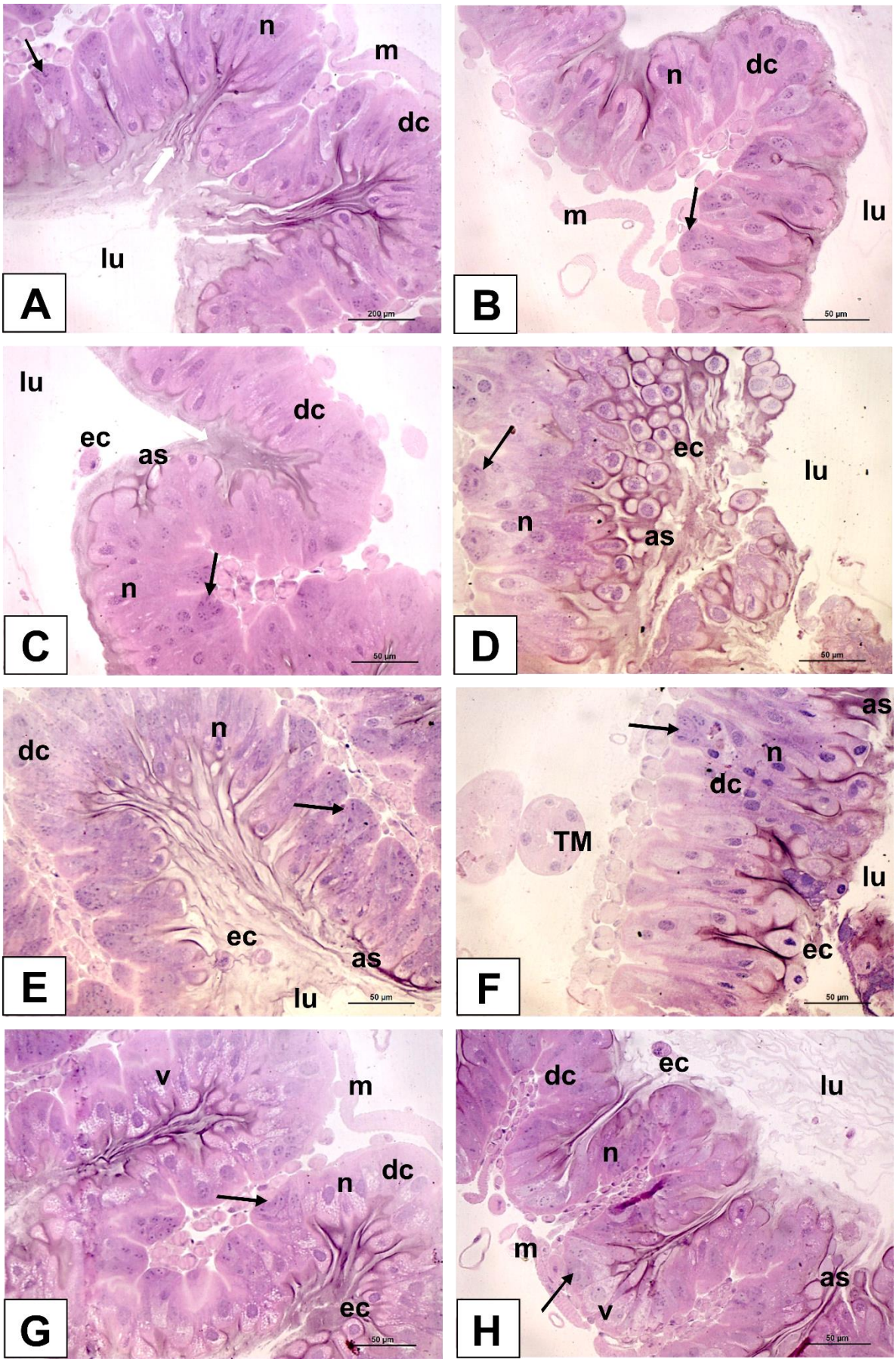
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Figure 3 – Honeybees (Africanized *A. mellifera*) midguts after 48 h of acute exposure. A) CTL - syrup control; B) ACN –acetone control; C) PVA - surfactant control; D) SLN – Solid lipid nanoparticles; E) $SLNP_{1ng}$– 1 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles; F) $SLNP_{10ng}$ – 10 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles G) PYR_{1ng} – 1 ng.µL^{-1} of pyrethrum extract; H) PYR_{10ng} – 10 ng.µL^{-1} of pyrethrum extract. Legend: dc = digestive cell; ec = eliminated cell in the lumen; lu = lumen; n = nucleus, v = vacuolization; as = apocrine secretion; Black arrow = Regenerative cell; TM = Malpighi's tubes; m = muscle. Staining: Hematoxylin-Eosin. Bars: 50 µm.

Although vacuolization can be present in bee midgut cells as a physiological process of autophagy for intracellular turnover, their increased level frequently has been associated to side-effects of xenobiotics, especially in bees exposed to pesticides. For example, Cruz et al. (2010) reported cytoplasmic vacuolization and cell elimination in *A. mellifera* larvae midguts exposed to fipronil (0.1 and 1 µg.g^{-1}) and boric acid (1.0, 2.5, and 7.5 mg.g^{-1}). Kakamand et al. (2008) observed an increase in the vacuolization of midgut cells in honeybees exposed to deltamethrin (1, 2.5, 5, and 10 mg.L^{-1}) and the degeneration of the midgut epithelium of bees exposed to the highest concentration of this compound.

Histochemical analysis of vacuolization areas in digestive cells (Figure 4S - Supplementary Material) showed that they are negative for proteins or neutral glycoconjugates, but had positive labelling for lipids that could indicate multivesicular bodies, because newly emerged honeybees have no spherocrystals yet. Multivesicular bodies are frequently found in midgut cells of insects (SERRAO; CRUZ-LANDIM, 1996), and are formed from early endosomes due to an inward budding of its membrane resulting in intralumenal vesicles whose main function is “collecting” plasma membrane receptors to be degraded into the lysosomes. Multivesicular bodies and autophagy are closely related (FADER; COLOMBO, 2009).

At the present study, intensification of cytoplasm vacuolization was considered a morphological alteration indicative of cytoplasmic loss, which is of greater pathological

563 importance than the other alterations analyzed because, especially in insects, autophagy may
564 act as a pro-death process at the cellular/organ level (MALAGOLI et al., 2010), although its
565 effects at the organismal level can still be considered as fundamental for survival.

566 Cell vacuolization increased in both groups exposed to pyrethrum extract (Figure 3G
567 and 3H, Figure 3SG and 3SH, and Table 3S), but there was no significant difference due to
568 the highly variable degree of vacuolization among individuals exposed to pyrethrum extract
569 (Figure 4C). However, when the organ index was calculated, vacuolization accounted for a
570 higher total index under 10 ng.μL^{-1} of pyrethrum extract (Figure 4D), as this alteration was
571 classified as importance factor 2 in the semi-quantitative analysis (Table 3S) because of the
572 loss of cytoplasmic material and the severity level.

573 In the total organ index analysis, the empty nanoparticles and 10 ng.μL^{-1} of pyrethrum
574 extract caused more significant changes than the other experimental groups (Table 3S). In
575 contrast, nanoparticles loaded with 1 ng.μL^{-1} pyrethrum extract did not increase cell
576 alterations more than the other groups (nanoparticles and pyrethrum extract). The SLNP
577 groups exhibited a decrease in short-term cell alterations, so in this respect was considered
578 safer for bees over short exposure times.

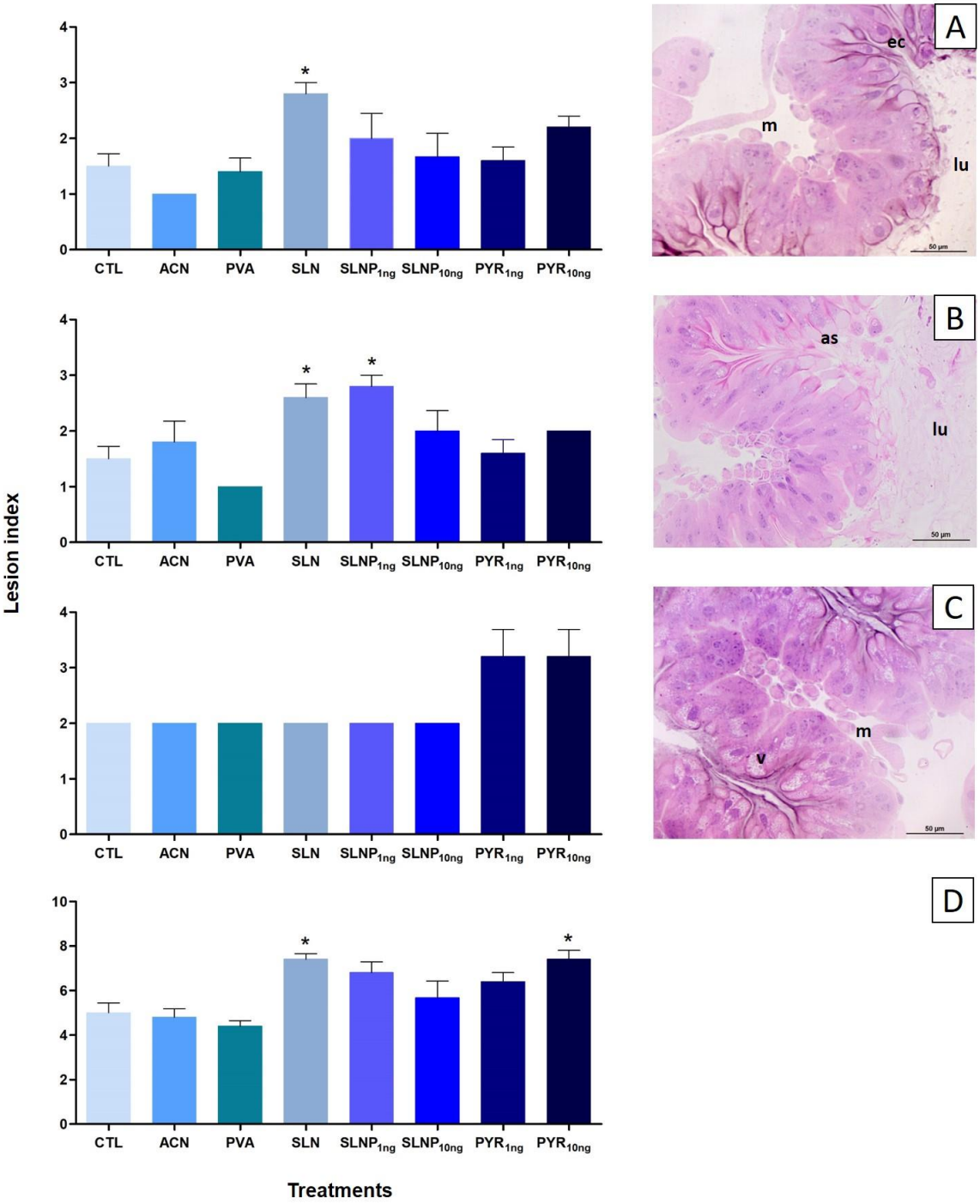

Figure 4 – Alterations and organ index in honeybee (Africanized *A. mellifera*) midguts. a) Eliminated cell index; b) Apocrine secretion index; c) Vacuolization index; d) Total organ index. Legend: CTL – syrup control; ACN – acetone control; PVA - surfactant control; SLN – Solid lipid nanoparticles; $SLNP_{1ng}$ – 1 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles; $SLNP_{10ng}$ – 10 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles PYR_{1ng} – 1 ng.µL^{-1} of pyrethrum extract; PYR_{10ng} – 10 ng.µL^{-1} of pyrethrum extract. Kruskal Wallis One-way ANOVA, followed by Dunn's multiple comparison test. *represent significant differences between groups.

588 At the lowest sublethal doses (1 ng.µL^{-1}), the biocide did not evidence significant
589 histopathological changes in the total lesion index, indicating that could be applied on crops.
590 A carrier system could be developed to improve pyrethrum extract stability, thus allowing its
591 use as nanopesticides. Besides, when the pyrethrum extract was encapsulated in nanocarriers
592 and demonstrated lower toxicity when compared with pyrethrum only. Therefore,
593 nanocarriers are an alternative to conventional pesticide applications. Nanotechnology
594 applied in the agricultural sector could increase agricultural production and crop protection,
595 contribute to sustainable agriculture and eco-friendly carrier systems, and reduce
596 environmental effects and toxicity to organisms (GRILLO et al., 2016). Oliveira et al. (2018)
597 found that zein nanoparticles loaded with citronella effectively controlled the pest species
598 *Tetranychus urticae* with low toxicity.
599 The empty SLNs showed effects onto honeybee, for example, in the total lesion index,
600 with the increase the eliminated cells and apocrine secretion. Therefore, nanocarrier system
601 itself may have reactive sites capable of changing their biological system because it has no
602 active ingredient encapsulated. These reactive sites could interact with organic molecules of
603 the organism, inducing negative effects that indirectly decreased the mean survival time of
604 the bees (196.54 ±11.38 h; $P > 0.05$). By the way, further studies need to be performed in
605 order to evaluate these hypotheses.
606 Nanopesticides can be able to increase the efficiency of agrochemicals and biocides,
607 because it is possible that in the field low doses of the active ingredients can be used.
608 However, in the case of pyrethrum and SLNs this fact will be confirmed with biological
609 assays in target organisms that will be run in the future. In addition, they increase production
610 and reduce damage to the environment (PRASAD et al., 2017). However, there are still many
611 gaps in information to be filled, normative instructions to be written, and legislation to be

made before they can be extensively and safely employed in agriculture (KAH; HOFMANN, 2014; KOOKANA et al., 2014). According Kah et al. (2018), further studies that investigate the efficacy of nanopesticides in crop farming are needed, in order to elucidate their effects on biodiversity and human health, and their benefits and costs compared with conventional formulations.

4. CONCLUSION

It is important to develop and analyze carrier systems as they have many potential benefits in comparison to synthetic and natural agrochemicals, such as reducing the amount of biocide in the environment and greater stability. However, nanotoxicological studies should be undertaken to evaluate the effects of nanoparticles on non-target organisms. In conclusion, this study demonstrates that nanoparticles loaded with pyrethrum extract at sublethal dose (1 or 10 ng.μL^{-1}) are relatively safe for honeybees, because they do not cause morphological changes in digestive cells. In contrast, empty nanoparticles and 10 ng.μL^{-1} of pyrethrum extract caused changes in digestive cells during acute exposure. The concentration of 1 ng.μL^{-1} of pyrethrum extract could be used for pest control. These data reflect the effects of a sublethal and acute exposure, and more studies are needed to check if a chronic exposure to these compounds would have different effects on bees. Our results added information for subsidizing future decision making, regulatory framework creation, risk assessments, and legislation development, and improve food security. In addition, based on the results we are planning to run biological assays in order to investigate the efficacy of the nanopesticide against target organisms.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interest in the present study.

REFERENCES

Abdelwahed, W.; Degobert, G.; Stainmesse, S.; Fessi, H. 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. *Adv. Drug Delivery Rev*. 58 (15), 1688-1713. https://doi.org/10.1016/j.addr.2006.09.017

About NIFA, 2018. National Institute of Food and Agriculture (NIFA) - United States Department of Agriculture (USDA). White House Office of Science and Technology Policy: Alexandria, VA, 2018. https://nifa.usda.gov/about-nifa (accessed Mar 04, 2018).

Aditya, N.P.; Shim, M.; Lee, I.; Lee, Y.; Im, M.H.; Ko, S. 2013. Curcumin and gnistein coloaded nanostructured lipid carriers: in vitro digestion and antiprostate cancer activity. *J. Agri. Food Chem*. 61 (8), 1878–1883. doi: 10.1021/jf305143k.

Aljedani, D.M. 2017. Effects of Abamectin and Deltamethrin to the foragers honeybee workers of *Apis mellifera jemenatica* (Hymenoptera: Apidae) under laboratory conditions. *Saudi J. Biol. Sci.* 24 (5), 1007-1015. doi: http://dx.doi.org/10.1016/j.sjbs.2016.12.007.

Anderson, W.; Kozak, D.; Coleman, V.A.; Jämting, Å.K.; Trau, M. 2013. A comparative study of submicron particle sizing platforms: Accuracy, precision and resolution analysis of polydisperse particle size distributions. *J. Colloid Interface Sci.* 405, 322–330. https://doi.org/10.1016/j.scienta.2007.11.013

Anjali, C.H.; Sudheer Khan, S.; Margulis-Goshen, K.; Magdassi, S.; Mukherjee, A.; Chandrasekaran, N. 2010. Formulation of water-dispersible nanopermethrin for larvicidal applications. *Ecotoxicol. Environ. Saf.* 73 (8), 1932-1936. doi: 10.1016/j.ecoenv.2010.08.039.

Bernet, D.; Schmidt, H.; Meier, W.; Burkhardt-Holm, P.; Wahli, T. 1999. Histopathology in fish: Proposal for a protocol to assess aquatic pollution. *J. Fish Dis.*, 22 (1), 25-34. https://doi.org/10.1046/j.1365-2761.1999.00134.x

Campos, E.V.R.; de Oliveira, J.L.; da Silva, C.M.; Pascoli, M.; Pasquoto, T.; Lima, R.; Abhilash, P.C.; Fraceto, L.F. 2015. Polymeric and Solid Lipid Nanoparticles for Sustained Release of Carbendazim and Tebuconazole in Agricultural Applications. *Sci Rep.* 5, 13809. doi: 10.1038/srep13809

Cavalcante, V.M.; C. Cruz-Landim. 1999. Types of cells present in the midgut of the insects: A review. *Naturalia* (Rio Claro) 24, 19-40.

Cavalcante, V.M.; Cruz-Landim, C. 2004. Padrão eletroforético de proteínas e atividade de fosfatase ácida em extratos do intestino médio de *Apis mellifera L*. durante a metamorfose. *Neotrop. Entomol.* 33 (2), 169-172. http://dx.doi.org/10.1590/S1519-566X2004000200007.

Chen, H; Chang, X.; Du, D.; Liu, W.; Liu, J.; Weng, T.; Yang, Y.; Xu, H.; Yang, X. 2006. Podophyllotoxin-loaded solid lipid nanoparticles for epidermal targeting. *J. Controlled Release* 110 (2), 296 – 306. doi: 10.1016/j.jconrel.2005.09.052

Chen, H., Yada, R. 2011. Nanotechnologies in agriculture: new tools for sustainable development. *Trends Food Sci. Technol* 22, 585–594. https://doi.org/10.1016/j.tifs.2011.09.004

Cruz, A.S.; da Silva-Zacarin, E.C.; Bueno, O.C.; Malaspina, O. 2010. Morphological alterations induced by boric acid and fipronil in the midgut of worker honeybee (*Apis mellifera L.*) larvae. *Cell Biol. Toxicol.* 26 (2), 165–176. doi: 10.1007/s10565-009-9126-x.

Cruz, L.C.; Araújo, V.A.; Dolder, H.; Araújo, A.P.; Serrão, J.E.; Neves, C.A. 2011. Morphometry of the midgut of *Melipona quadrifasciata anthidioides* (Lepeletier) (Hymenoptera: Apidae) during metamorphosis. *Neotrop. Entomol.* 40 (6), 677-681. http://dx.doi.org/10.1590/S1519-566X2011000600007.

de Melo, N.F.S.; Araújo, D.R.; Grillo, R.; Moraes, C.M.; Matos, A.P.; Paula, E.; Rosa, A.H.; Fraceto, L.F. 2012. Benzocaine-loaded polymeric nanocapsules: Study of the anesthetic activities. *J. Pharm. Sci.* 101, 1157-1165.

de Melo, N.F.; de Macedo, C.G.; Bonfante, R.; Abdalla, H.B.; da Silva, C.M.; Pasquoto, T.; de Lima, R.; Fraceto, L.F.; Clemente-Napimoga, J.T.; Napimoga, M.H. 2016. 15d-PGJ_2-Loaded Solid Lipid Nanoparticles: Physicochemical Characterization and Evaluation of Pharmacological Effects on Inflammation. *PLoS One* 11 (8), e0161796. https://doi.org/10.1371/journal.pone.0161796

Dolezal, A.G.; Carrillo-Tripp, J.; Miller, W.A.; Bonning, B.C.; Toth, A.L. 2016. Pollen Contaminated with Field-Relevant levels of Cyhalothrin affects Honey Bee Survival, Nutritional Physiology, and Pollen Consumption Behavior. J. Econ, Entomol. 109 (1), 41-8. https://doi.org/10.1093/jee/tov301.

724 Fader, C.M.; Colombo, M.I. 2009. Autophagy and multivesicular bodies: two closely related
725 partners. Cell Death Differ. 16 (1), p. 70–78. doi: 10.1038/cdd.2008.168.
726
727 Gamisans, F.; Lacoulonche, F.; Chauvet, A.; Espina, M.; García, M.L.; Egea, M.A. 1999.
728 Flurbiprofen-loaded nanospheres: analysis of the matrix structure by thermal methods. *Int. J.*
729 *Pharm.* 179 (1), 37-48. https://doi.org/10.1016/S0378-5173(98)00381-0
730
731 Giannini, T.C.; Cordeiro, G.D.; Freitas, B.M.; Saraiva, A.M. Imperatriz-Fonseca, V.L. 2015.
732 The dependence of crops for pollinators and the economic value of pollination in Brazil. *J.*
733 *Econ. Entomol*. 108, 1-9. doi: 10.1093/jee/tov093.
734
735 Gopal, M.; Kumar, R.; Goswami, A. 2012. Nano-pesticides - A recent approach for pest
736 control. *The J. Plant Protec. Sci.* 4 (2), 1-7.
737
738 Goulson, D.; Nicholls, E.; Botías, C.; Rotheray, E.L. 2015.Bee declines driven by combined
739 stress from parasites, pesticides, and lack of flowers. *Science.* 347 (6229), 1255957. Doi:
740 10.1126/science.1255957
741
742 Grillo, R.; Abhilash, P.C.; Fraceto, L.F. 2016. Nanotechnology applied to Bio-encapsulation
743 of pesticides. *J. Nanosci. Nanotechnol.* 16, 1231-1234.
744 https://doi.org/10.1166/jnn.2016.12332
745
746 Guirguis, O.W.; Moselhey, M.T.H. 2012. Thermal and structural studies of poly(vinyl
747 alcohol) and hydroxypropyl cellulose blends. *Natural Sci.* 4 (1), 57-67.
748 http://dx.doi.org/10.4236/ns.2012.41009
749
750 Jacques, M.T.; Oliveira, J.L.; Campos, E.V.; Fraceto, L.F.; Ávila, D.S. 2017. Safety
751 assessment of nanopesticides using the roundworm *Caenorhabditis elegans*. *Ecotoxicol.*
752 *Environ. Saf.* 139, 245–253. doi: 10.1016/j.ecoenv.2017.01.045
753
754 Johnson, R.M.; Ellis, M. D.; Mullin, C. A.; Frazier, M. 2010. Pesticides and honey bee
755 toxicity – USA. *Apidologie* 41, 312–331. doi: 10.1051/apido/2010018
756
757 Kah, M.; Machinski, P.; Koerner, P.; Tiede, K.; Grillo, R.; Fraceto, L.F. 2014. Hofmann
758 T.Analysing the fate of nanopesticides in soil and the applicability of regulatory protocols
759 using a polymer-based nanoformulation of atrazine. *Environ. Sci. Pollut. Res.* 21 (20), 11699-
760 707. doi: 10.1007/s11356-014-2523-6.
761
762 Kah, M.; Hofmann, T. 2014. Nanopesticide research: Current trends and future priorities.
763 *Environ. Int.* 63, 224-235. doi: 10.1016/j.envint.2013.11.015.
764
765 Kah, M.; Kookana, R.S.; Gogos, A.; Bucheli, T.D. 2018. A critical evaluation of
766 nanopesticides and nanofertilizers against their conventional analogues. *Nat. Nanotechnol.*
767 13, 677–684. doi: 10.1038/s41565-018-0131-1.
768

769 Kakamand, F.A.K.; Mahmoud, T. T.; Amin, A.M. 2008. The role of three insecticides in
770 disturbance the midgut tissue in honey bee *Apis mellifera L*. Workers. *J. Dohuk Univ*. 11 (1),
771 144-151.
772
773 Kearns, C.A.; Inouye, D. W. 1997. Pollinators, Flowering Plants, and Conservation Biology.
774 *BioScience* 47 (5), 297-307.
775
776 Kilic, A. C.; Capan, Y.; Vural, I.; Gursoy, R.N.; Dalkara, T.; Cuine, A.; Hincal, A.A. 2005.
777 Preparation and characterization of PLGA nanospheres for the targeted delivery of NR2B-
778 specific antisense oligonucleotides to the NMDA receptors in the brain. *J.*
779 *Microencapsulation* 22, 633–641.
780
781 Kim, D.Y.; Kadam, A.; Shinde, S.; Saratale, R.G.; Patra, J.; Ghodake, G. 2018. Recent
782 developments in nanotechnology transforming the agricultural sector: a transition replete
783 with opportunities. *J. Sci. Food Agric.* 98 (3), 849-864. doi: 10.1002/jsfa.8749.
784
785 Kookana, R.S.; Boxall, A.B.; Reeves, P.T.; Ashauer, R.; Beulke, S.; Chaudhry, Q.; Cornelis,
786 G.; Fernandes, T.F.; Gan, J.; Kah, M.; Lynch, I.; Ranville, J.; Sinclair, C.; Spurgeon, D.;
787 Tiede, K.; Van den Brink, P.J. 2014. Nanopesticides: guiding principles for regulatory
788 evaluation of environmental risks. *J. Agric. Food. Chem*. 62 (19), 4227-4240.
789 doi: 10.1021/jf500232f
790
791 Liao, C.H.; He, X.J.; Wang, Z.L.; Barron, A.B.; Zhang, B.; Zeng, Z.J.; Wu, X.B. 2018. Short-
792 Term Exposure to Lambda-Cyhalothrin Negatively Affects the Survival and Memory-
793 Related Characteristics of Worker Bees *Apis mellifera*. Arch. Environ. Contam. Toxicol. 75
794 (1), 59-65. doi: 10.1007/s00244-018-0514-1.
795
796 Liu, Y.; Tong, Z.; Prud'homme, R.K. 2008. Stabilized polymeric nanoparticles for controlled
797 and efficient release of bifenthrin. *Pest. Manag. Sci*. 64 (8), 808-812. doi: 10.1002/ps.1566.
798
799 Lourenço, C.; Teixeira, M.; Simões, S.; Gaspar, R. 1996. Steric stabilization of nanoparticles:
800 Size and surface properties. *Int. J. Pharm*. 138 (1), 1-12. https://doi.org/10.1016/0378-
801 5173(96)04486-9
802
803 Malagoli, D.; Abdalla, F.C.; Cao, Y.; Feng, Q.; Fujisaki, K.; Gregorc, A.; Matsuo, T.; Nezis,
804 I.P.; Papassideri, I.S.; Sass, M.; Silva-Zacarin, E.C.; Tettamanti, G.; Umemiya-Shirafuji, R.
805 2010. Autophagy and its physiological relevance in arthropods: current knowledge and
806 perspectives. Autophagy. 6 (5), 575-88. doi: 10.4161/auto.6.5.11962.
807
808 Martins, G.F.; Neves, C.A.; Campos, L.A.; Serrão, J.E. 2006. The regenerative cells during
809 the metamorphosis in the midgut of bees. *Micron* 37 (2), 161-168.
810 https://doi.org/10.1016/j.micron.2005.07.003
811
812 Maruyama, C.R.; Guilger, M.1.; Pascoli, M.; Bileshy-José, N.; Abhilash, P.C.; Fraceto, L.F.;
813 de Lima, R. 2016. Nanoparticles Based on Chitosan as Carriers for the Combined Herbicides
814 Imazapic and Imazapyr. *Sci. Rep*. 6 (19768). Doi: 10.1038/srep19768
815

816 Masarudin, M.J.; Cutts, S.M.; Evison, B.J.; Phillips, D.R.; Pigram, P. J. 2015. Factors
817 determining the stability, size distribution, and cellular accumulation of small, monodisperse
818 chitosan nanoparticles as candidate vectors for anticancer drug delivery: application to the
819 passive encapsulation of [^{14}C]-doxorubicin. *Nanotechnol. Sci. Appl.* 8, 67–80.
820 http://doi.org/10.2147/NSA.S91785
821
822 Mishra, S.; Keswani, C.; Abhilash, P.C.; Fraceto, L.F.; Singh, H.B. 2017. Integrated
823 Approach of Agri-nanotechnology: Challenges and Future Trends. *Front. Plant. Sci.* 8, 1-12.
824
825 Montanha, F.P.; Pimpão, C.T. 2012. Efeitos toxicológicos de piretróides (cipermetrina e
826 deltametrina) em peixes. *Rev. Cient. Elet. Med. Vet.* 9 (18), 1-58.
827
828 Mukhopadhyay, S.S. 2014. Nanotechnology in agriculture: prospects and constraints.
829 *Nanotechnol., Sci. Appl.* 7, 63–71. doi: 10.2147/NSA.S39409
830
831 Muller, R. H.; Rühl, D.; Runge, S.A. 1996. RungeBiodegradation of solid lipid nanoparticles
832 as a function of lipase incubation time. *Int. J. Pharm.* 144 (1), 115-121.
833
834 Muller, H.R.; Mäder, K.; Gohla, S. 2000. Solid lipid nanoparticles (SLN) for controlled drug
835 delivery: a review of the state of the art. *Eur. J. Pharm. Biopharm.* 50 (1), 161-177.
836 https://doi.org/10.1016/S0939-6411(00)00087-4
837
838 Muller, H.; Shegokar, R.; Keck, C.M. 2011. 20 Years of Lipid Nanoparticles (SLN & NLC):
839 Present State of Development & Industrial Applications. *Curr. Drug. Discov. Technol*. 8,
840 207-227. https://doi.org/10.2174/157016311796799062
841
842 Nafee, N.; Schneider, M.; Schaefer, U.F.; Lehr, C.M. 2009. Relevance of the colloidal
843 stability of chitosan/PLGA nanoparticles on their cytotoxicity profile. *Int. J. Pharm.* 381
844 (2, 3), 130-139. https://doi.org/10.1016/j.ijpharm.2009.04.049
845
846 Naseri, N.; Valizadeh, H.; Zakeri-Milani, P. 2015. Solid Lipid Nanoparticles and
847 Nanostructured Lipid Carriers: Structure, Preparation and Application. *Adv. Pharm. Bull.* 5
848 (3), 305–313. http://doi.org/10.15171/apb.2015.043
849
850 Nasseri, M.; Golmohammadzadeh, S.; Arouiee, H.; Jaafari, M.R.; Neamati, H. 2016.
851 Antifungal activity of *Zataria multiflora* essential oil-loaded solid lipid nanoparticles in-vitro
852 condition. *Iran. J. Basic Med. Sci.* 19 (11), 1231-1237. Doi: 10.22038/ijbms.2016.7824
853
854 Neves, C.A.; Bhering, L.L.; Serrão, J.E.; Gitirana, L.B. 2002. FMRFamide-like midgut
855 endocrine cells during the metamorphosis in *Melipona quadrifasciata anthidioides*
856 (Hymenoptera Apidae). *Micron* 33 (5), 453-460. https://doi.org/10.1016/S0968-
857 4328(01)00043-9
858
859 Oliveira, J.L.; Campos, E.V.; Gonçalves da Silva, C.M.; Pasquoto, T.; Lima, R.; Fraceto,
860 L.F. 2015. Solid Lipid Nanoparticles Co-loaded with Simazine and Atrazine: Preparation,
861 Characterization, and Evaluation of Herbicidal Activity. *J. Agric. Food Chem.* 63, 422-432.
862 Doi: 10.1021/jf5059045

Oliveira, J.L.; Campos, E.V.R.; Pereira, A.E.S.; Pasquoto, T.; Lima, R.; Grillo, R.; Andrade, D.J.; Santos, F.A.D.; Fraceto, L.F. 2018. Zein Nanoparticles as Eco-Friendly Carrier Systems for Botanical Repellents Aiming Sustainable Agriculture. *J. Agric. Food Chem.* 66, 1330-1340. doi: 10.1021/acs.jafc.7b05552.

Oliveira, R.A.; Roat, T.C.; Carvalho, S.M.; Malaspina, O. 2014. Side-effects of thiamethoxam on the brain and midgut of the africanized honeybee *Apis mellifera* (Hymenopptera: Apidae). *Environ. Toxicol*. 29 (10), 1122-33. doi: 10.1002/tox.21842.

Oliveira, R.L.; Passos, F.B. 2013. Estudo da oxidação parcial do etanol em catalisadores de Rh por DRIFTS. *Quim. Nova* 36 (3), 375-381.

Palmquist, K.; Salatas, J.; Fairbrother, A. 2012. Pyrethroid insecticides: use, environmental fate, and ecotoxicology, in: Perveen, F. (Ed.), *Insecticides: Advances in Integrated Pest Management*, InTech, Rijeka: Croatia, cap. 11, pp. 251-278.

Pasquoto-Stigliani, T.; Campos, E.V.R.; Oliveira, J.L.; Silva, C.M.G.; Bilesky-José, N.; Guilger, M.; Troost, J.; Oliveira, H.C.; Stolf-Moreira, R.; Fraceto, L.F.; de Lima, R. 2017. Nanocapsules Containing Neem (*Azadirachta Indica*) Oil: Development, Characterization, And Toxicity Evaluation. *Sci. Rep.* 7 (5929). doi:10.1038/s41598-017-06092-4

Peay, S.; Hiley, P.D.; Collen, P.; Martin, I. 2006. Biocide treatment of ponds in Scotland to eradicate signal crayfish. *Bull. Fr. Pêche Piscic.* 380–381, 1363–1379. https://doi.org/10.1051/kmae:2006041

Perez-de-Luque, A.; Rubiales, D. 2009. Nanotechnology for parasitic plant control. *Pest Manage. Sci.* 65 (5), 540-545. https://doi.org/10.1002/ps.1732

Polleto, F.S.; Jäger, E.; Ré, M.I.; Guterres, S.S.; Pohlmann, A.R. 2007. Rate modulating PHBHV/PCL microparticles containing weak acid model drugs. *Int. J. Pharm.* 345, 70-80. https://doi.org/10.1016/j.ijpharm.2007.05.040

Potts, S.G.; Biesmeijer, J.C.; Kremen, C.; Neumann, P.; Schweiger, O.; Kunin, W.E. 2010. Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* 25 (6), 345-353. https://doi.org/10.1016/j.tree.2010.01.007

Prasad, R., Bhattacharyya, A.; Nguyen, Q.D. 2017. Nanotechnology in Sustainable Agriculture: Recent Developments, Challenges, and Perspectives. *Front. Microbiol*. 20; 8:1014. https://doi.org/10.3389/fmicb.2017.01014

Rossi, C.A., Roat, T.C.; Tavares, D.A.; Cintra-Socolowski, P.; Malaspina, O. 2011. Effects of sublethal doses of imidacloprid in malpighian tubules of africanized *Apis mellifera* (Hymenoptera, Apidae). *Microsc. Res. Tech.* 76 (5), 552-558. https://doi.org/10.1002/jemt.22199

Santos, M.A.T.; Areas, M.A.; Reyes, F.G. 2007. Piretróides – uma visão geral. *Alim. Nutr.* 18 (3), 339-349.

Sarangi, M.J.; Padhi, S. Solid lipid nanoparticles – A Review. *J. Crit. Rev*. 2016, *3* (3), 5-12.

Sarlak, N.; Taherifar, A.; Salehi, F. 2014. Synthesis of Nanopesticides by Encapsulating Pesticide Nanoparticles Using Functionalized Carbon Nanotubes and Application of New Nanocomposite for Plant Disease Treatment. *J. Agric. Food Chem* 62 (21), 4833–483. doi.10.1021/jf404720d

Schaffazick, S.R.; Guterres, S.S.; Freitas, L.L.; Pohlmann, A.R. 2003. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. *Quím. Nova* 26 (5), 726-737. http://dx.doi.org/10.1590/S0100-40422003000500017.

Schleier, J.J.; Peterson, R.K.D. 2011. Pyrethrins and Pyrethroid Insecticides, in: López, O.; Fernández-Bolaños, J.G. (Eds.), *Green Trend. Insect Cont*, J. Publishing. London: Burlington House, cap. 3, pp. 94-131.

Schwarz, C.; Mehnert, W.; Lucks, J.S.; Müller, R.H. 1994. Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterization and sterilization. *J. Controlled Release.* 30 (1), 83-96. https://doi.org/10.1016/0168-3659(94)90047-7

Serrão, J. E., C. Cruz-Landim. 1996. Ultrastructure of digestive cells in stingless bees of various ages (Hymenoptera, Apidae, Meliponinae). *Cytobios* 88, 161-171.

Scott, N.; Chen, H. 2012. Nanoscale Science and Engineering for Agriculture and Food Systems. *Ind. Biotechnol.* 9 (1), 17-18. https://doi.org/10.1089/ind.2013.1555

Sekhon, B.S. 2014. Nanotechnology in agri-food production: an overview. *Nanotechnol., Sci. Appl*. 7, 31–53. http://doi.org/10.2147/NSA.S39406

Silva-Zacarin, E.C.M.; Chauzat, M.P.; Zeggane, S.; Drajnudel, P.; Schurr, F.; Faucon, J.P.; Malaspina, O.; Engler, J.A. 2012. Protocol for optimization of histological, histochemical and immunohistochemical analyses of larval tissues: application in histopathology of honey bee. In: Méndez-Vilas, A. (Ed.), *Current microscopy contributions to advances in science and technology*, Formatex Research Center: Badajoz, 5. ed., v. 1, pp. 696-703.

Silva, M. dos S. Cocenza, D. S. Grillo, R. de Melo, N. F. S. Tonello, P. S. Oliveira, L. C. de Cassimiro, D. L. Rosa, A. H. Fraceto, L. F. 2011. Paraquat-loaded alginate/chitosan nanoparticles: preparation, characterization and soil sorption studies. *J. Hazard. Mater*. 190 (1-3), 366-374.

Soares, H.M. *Avaliação dos efeitos do inseticida imidacloprido para abelhas sem ferrão Scaptotrigona postica Latreille, 1807 (Hymenoptera, Apidae, Meliponini).* Dissertação de Mestrado, Universidade Estadual Paulista - campus Rio Claro/SP, 2012.

957 Soares-Lima, H.M.; Silva-Zacarin, E.C.M.; Camargo, I; Nocelli, R.C.F.; Malaspina, O.
958 Histopathological alterations on honeybees midgut infected by *Nosema ceranae* and exposed
959 to imidacloprid. In: Society of Environmental Toxicology and Chemistry Asia-Pacific
960 Conference, Daegu, Korea, Sep 16-19, 2018; SETAC AP, Eds.; SETAC: Korea, 2018.
961
962 Terra, W.R.; Ferreira, C. 2012. Biochemistry and molecular biology of digestion. In, Gilbert,
963 L.I. (Ed.), *Insect Molecular Biology and Biochemistry*, Academic Press: San Diego, pp. 365–
964 418. https://doi.org/10.1016/C2009-0-62118-8
965
966 *U.S. Department of Agriculture, National Institute of Food and Agriculture (USDA/NIFA)*
967 *2018;* National Nanotechnology Initiative (NANO). White House Office of Science and
968 Technology Policy: Alexandria, VA, 2018. https://www.nano.gov/node/137 (accessed Mar
969 04, 2018).
970
971 *Data Evaluation Record - Pyrethrum extract 1991;* U.S. Environmental Protection Agency
972 (USEPA). White House Office of Science and Technology Policy: Philadelphia, PA, 1991.
973 https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-069001_3-Sep-
974 91_a.pdf (accessed Mar 14, 2018).
975
976 *Reregistration Eligibility Decision for Pyrethrins 2006;* U.S. Environmental Protection
977 Agency (USEPA). White House Office of Science and Technology Policy: Philadelphia, PA,
978 2006. http://www.epa.gov/oppsrrd1/REDs/pyrethrins_red.pdf (accessed Mar 14, 2018).
979
980 Venkatraman, S. S. 2005. Micelle-like nanoparticles of PLA–PEG–PLA triblock copolymer
981 as chemotherapeutic carrier. *Int. J. Pharm.* 298, 219–232.
982 https://doi.org/10.1016/j.ijpharm.2005.03.023
983
984 Vitorino, C.; Carvalho, F.A.; Almeida, A.J.; Sousa, J.J.; Pais, A.A. 2011. The size of solid
985 lipid nanoparticles: An interpretation from experimental design. *Colloids Surf., B.* 84 (1),
986 117-130. https://doi.org/10.1016/j.colsurfb.2010.12.024
987
988 Walker, G.W.; Kookana, R.S.; Smith, N.E.; Kah, M.; Doolette, C.L.; Reeves, P.T.; Lovell,
989 W.; Anderson, D.J.; Turney, T.W.; Navarro, D.A. 2017. Ecological risk assessment of nano-
990 enabled pesticides: A perspective on problem formulation. *J. Agric. Food Chem*. 66 (26),
991 6480-6486. https://doi: 10.1021/acs.jafc.7b02373
992
993 Wang, Q.; Guan, Y.X.; Yao, S.J.; Zhu, Z.Q. 2010. Microparticle formation of sodium
994 cellulose sulfate using supercritical fluid assisted atomization introduced by hydrodynamic
995 cavitation mixer. *Chem. Eng. J.* 159(1-3), 220-229. https://doi.org/10.1016/j.cej.2010.02.004
996
997 Zain, N.A.M.; Suhaimi, M.S.; Idris, A. 2011. Development and modification of PVA–
998 alginate as a suitable immobilization matrix. *Process Biochem.* 46 (11), 2122-2129.
999 https://doi.org/10.1016/j.procbio.2011.08.010
1000
1001 Zhao, D.; Jiao, X.; Zhang, M.; Ye, K.; Shi, X.; Xihua, Lu. Qiu, G.; Sheac, K.J. 2016.
1002 Preparation of high encapsulation efficiency fragrance microcapsules and their application
1003 in textiles. *RSC Adv.* 84, 80924-80933. doi: 10.1039/C6RA16030A

Zhou, T.; Zhou, W.; Wanga, Q.; Dai, P.L.; Feng, L.; Zhang, Y.L; Sun, J.H. 2011. Effects of pyrethroids on neuronal excitability of adult honeybees *Apis mellifera*. *Pestic. Biochem. Physiol.* 100 (1), 35-40. https://doi.org/10.1016/j.pestbp.2011.02.001

***Highlights (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

HIGHLIGHTS

- Nanoparticles showed good properties to be used as pyrethrum carrier system
- Pyrethrum extract in nanocarrier and sublethal concentrations is safer for honeybees
- Pyrethrum and nanotechnology showed promising results aiming agriculture applications

Nanopesticide based on botanical insecticide pyrethrum and its potential effects on honeybees

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ABSTRACT

Nanotechnology has the potential to overcome the challenges of sustainable agriculture, and nanopesticides can control agricultural pests and increase farm productivity with little environmental impact. However, it is important to evaluate their toxicity on non-target organisms, such as honeybees (*Apis mellifera*) that forage on crops. The aims of this study were to develop a nanopesticide that was based on solid lipid nanoparticles (SLNs) loaded with pyrethrum extract (PYR) and evaluate its physicochemical properties and short-term toxicity on a non-target organism (honeybee). SLN+PYR was physicochemically stable after 120 days. SLN+PYR had a final diameter of 260.8 ± 3.7 nm and a polydispersion index of 0.15 ± 0.02 nm, in comparison with SLN alone that had a diameter of 406.7 ± 6.7 nm and a polydispersion index of 0.39 ± 0.12 nm. SLN+PYR had an encapsulation efficiency of 99%. The survival analysis of honeybees indicated that PYR_{10ng} presented shorter longevity than those in the control group ($P \leq 0.01$). Empty nanoparticles and PYR_{10ng} caused morphological alterations in the bees' midguts, whereas pyrethrum-loaded nanoparticles had no significant effect on digestive cells, so are considered safer, at least in the short term, for honeybees. These results are important in understanding the effects of nanopesticides on beneficial insects and may decrease the environmental impacts of pesticides.

KEYWORD: Nanopesticide; Biocide; Sustainable agriculture, Solid lipid nanoparticles; Bees.

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1. INTRODUCTION

Agri-food production and population growth are amongst the greatest challenges facing humanity. Agriculture is one of the primary drivers of the economy by providing food to the population and benefiting producing countries, but increased population growth has significantly increased humanity's global ecological footprint, surpassing the biocapacity of the Earth (SEKHON, 2014). Human populations increase exponentially over time, whereas food production increases in a linear manner. Conventional agricultural practices generally have negative impacts on the environment and biodiversity, as they require many resources such as energy, water, and soil, and large amounts of agrochemicals and fertilizers are used to improve productivity.

The U.S. Department of Agriculture's (USDA) National Institute of Food and Agriculture (NIFA, 2018) aims to find innovative solutions to issues related to agriculture, food, the environment, and communities. NIFA's priorities include global food security and hunger, food safety, plant health and production, and animal health and production (NANO, 2018). Many of these issues may be resolved using nanotechnology, which has demonstrated great potential in providing novel solutions to agricultural problems (SCOTT and CHEN, 2012; MUKHOPADHYAY, 2014). In the last few decades, nanoscience and nanotechnology have been at the forefront of the development of several nanomaterials for different medical and industrial purposes. Nanoparticles have been developed for a wide variety of applications in the biomedical and electronic fields, while research on nanoparticles as carriers of pesticides has only been conducted in the last decade, and there are still many variables to be investigated before their use on crops (LIU et al., 2008; ANJALI et al., 2010; GOPAL et al., 2012; KAH et al., 2014; SARLAK et al., 2014; MISHRA et al., 2017; KIM et al., 2018).

Nanotechnology can deliver agricultural substances such as nanopesticides and nanofertilizers that increase farm productivity, decrease the environmental impact and the amount of resources used, improve pest control, and support sustainable agriculture, particularly in developing countries. Furthermore, nanocarriers of pesticides and fertilizers have economic advantages for agriculture, because their stability and controlled-release mechanism increase efficiency and reduce the amount of chemicals required on crops (PEREZ-DE-LUQUE and RUBIALES; 2009; CHEN and YADA, 2011; GRILLO et al., 2016; PRASAD et al., 2017; WALKER et al., 2017).

However, the effects of nanoparticles should be fully evaluated before they are incorporated into sustainable agriculture. The U.S. National Science Foundation (NSF) and Environmental Protection Agency (EPA) encourage the investigation of various aspects of nanomaterials, such as their toxicity to non-target organisms, their destination, transportation, and safety in the environment, and their status in terms of food legislation, and support the creation of a nanomaterial database and the maintenance of food regulations (SCOTT and CHEN, 2012).

Pyrethrum extract is a natural botanical insecticide that is extracted from chrysanthemum (*Chrysanthemum cinerariaefolium* and *Chrysanthemum cineum*) flowers, is composed of pyrethrin types I and II and jasmolin, and can be used on crops to control pest insects (PEAY et al., 2006). Natural pyrethrum (a.i.) is highly lipophilic, photodegradable, has low water solubility (<10 $mg.L^{-1}$), does not exhibit biomagnification (SCHLEIER and PETERSON, 2011), and leaves no toxic residues in plants. However, it is more expensive than synthetic pyrethroids (PEAY et al., 2006) and is highly toxic to insects, aquatic invertebrates, and fish (USEPA, 2006). Pyrethroids are insecticides that were developed to improve the photodegradation of natural pyrethrin, and thus be used as an insecticide in the

field (SANTOS et al., 2007), and have great stability and target selectivity. Examples of pyrethroids include deltamethrin, permethrin, and cypermethrin (MONTANHA and PIMPÃO, 2012).

However, for the use of pyrethrum extract in the field it is necessary, at first, to load it into solid lipid nanoparticles (SLNs) to prevent its fast degradation, improving its stability and efficiency to allow its application on crops. Many benefits can be obtained by using SLNs, such as lower large-scale production costs, greater physicochemical stability, the possibility of hydrophilic and hydrophobic drug encapsulation, and the use of natural products in the formulation preparation (MULLER et al., 2000; MULLER et al., 2011; NASERI et al., 2015; SARANGI and PADHI et al., 2016).

Interactions between biological systems and nanomaterials are complex, so it is important to evaluate their toxicity to non-target organisms (JACQUES et al., 2017), particularly to beneficial insects such as honeybees (*Apis mellifera*), which play an important role in pollinating agricultural crops (GIANNINI et al., 2015). Honeybee populations are declining worldwide, and although multiple factors contribute to this decline (GOULSON et al., 2015), it is mainly caused by agrochemicals sprayed on crops visited by bees (POTTS et al., 2010). In this context, the physicochemical characterization of nanopesticides can enable their future use in organic farming and contribute to sustainable agriculture, because these carriers may have little effect on the environment and biodiversity (GRILLO et al., 2016; PRASAD et al., 2017). However, this carrier system must have low toxicity to honeybees and other beneficial insects.

The objectives of this study were to develop a nanopesticide that was based on SLNs loaded with pyrethrum extract biocide (nanobiocide), characterize its physicochemical properties, and evaluate its toxicity to honeybees (Africanized *A. mellifera*). We evaluated

sublethal effects on the histopathology of the bee midgut, an organ that plays a central role in food digestion and nutrient absorption. It is important to emphasize the fact that there are gaps of information in the literature regarding the toxicity of nanopesticides to non-target organisms, such as pollinator insects including honeybees. Our results can be applied in the field, can contribute to nanopesticide regulation, and can improve both environmental and food security.

2. MATERIALS AND METHODS

2.1. Chemicals

The pyrethrum extract Pestanal® (biocide, CAS 8003-34-7, analytical standard), polyvinyl alcohol (PVA, 30–70 kDa, CAS 9002-89-5, hydrolyzed >99%), and glyceryl tripalmitate (tripalmitin, CAS 555-44-2, purity ≥99%) were purchased from Sigma-Aldrich. Chloroform ($CHCl_3$, CAS 67-66-3, purity ≥99%) was purchased from a local supplier. All these products were used for the preparation of the nanoparticles. Acetone (CAS 67-64-1, purity = 100%) was used as a solvent in the preparation of the pyrethrum solution.

2.1.1. Solid lipid nanoparticles

SLNs containing pyrethrum were prepared by the method of emulsification/solvent evaporation with some modifications (VITORINO et al., 2011; de MELO et al., 2018). Initially, 30 mL of an aqueous phase containing 1.25% PVA and distilled water was prepared and magnetically stirred (100 rpm). An organic phase with 250 mg of glyceryl tripalmitate and 5 mg of pyrethrum (active ingredient – a.i.) was then prepared, which was dissolved in 5 mL of chloroform. The organic phase was added to the aqueous phase, and this mixture was sonicated at 40 W for 5 min producing an emulsion. The emulsion was placed in an

143 ULTRA-TURRAX™ homogenizer at 14,000 rpm for 7 min. The organic solvent was then
144 removed using a rotating evaporator in order to create a concentrated emulsion with 10 mL
145 of nanoparticles. The final concentration of biocide was 0.05 $mg.mL^{-1}$. SLNs without
146 pyrethrum extract (control) were also prepared.
147
148 *2.2. Nanoparticles*
149 The purpose of the formulations was to achieve greater physicochemical stability and
150 better efficiency of pyrethrum encapsulation in the nanoparticles. In order to evaluate the
151 physicochemical stability as a function of time were used the maintenance of colloidal
152 parameters in formulation. The colloidal parameters were the mean diameter, polydispersity
153 index, zeta potential, besides the nanoparticle concentration and encapsulation efficiency of
154 the pyrethrum extract. All analyses were conducted for 120 days and the results were
155 expressed (mean ± SEM).
156
157 *2.2.1. Nanoparticle characterization*
158 The mean diameter and polydispersion index were determined by dynamic light
159 scattering (DLS). Nanoparticle samples were diluted (10 µL:1 mL) in purified water and
160 analyzed using a Zetasizer Nano ZS90 analyzer (Malvern Panalytical, UK). Zeta potential
161 values (in mV) were also determined using the ZS90 analyzer, with the same dilution process.
162 The pH of the nanoparticles was determined using a pH meter (Tecnal®, Brazil). Further
163 details could be obtained in literature (VENKATRAMAN et al., 2005; de MELO et al., 2012;
164 OLIVEIRA et al., 2015).
165
166 *2.2.2. Nanoparticle concentration*

SLN size distributions and concentrations were analyzed using a nanoparticle tracking analysis (NTA) instrument (NanoSight LM10). Nanoparticle samples were diluted 10,000 times and analyzed by injecting 1 mL of the sample into the cell (more details in section 1.1 - Supplementary Material).

2.2.3. Differential Scanning Calorimetry (DSC)

A thermal analysis was performed to demonstrate that the pyrethrum was encapsulated in the nanocarriers using a DSC Q20 differential scanning calorimeter (TA Instruments). The samples of pyrethrum extract, lipid, SLNs, and SLNs loaded with pyrethrum were analyzed (Section 1.2 - Supplementary Material).

2.2.4. Fourier-transform infrared spectroscopy (FTIR)

FTIR was performed to investigate interactions between the biocide and the SLNs using an infrared spectrophotometer (Agilent). The pyrethrum extract, lipid, surfactant (PVA), physical mixture, SLNs, and SLNs loaded with pyrethrum were analyzed using an attenuated total reflectance accessory (POLLETO et al., 2007; WANG et al., 2010) (Section 1.3 - Supplementary Material).

2.3. Determination of encapsulation efficiency and quantification of pyrethrum by high-performance liquid chromatography (HPLC)

The total amount of pyrethrum extract present in the nanoparticle suspension was determined by the ultrafiltration/centrifugation method. After the suspension had been diluted with acetonitrile, it was filtered through a 0.22 μm Millipore™ membrane filter and quantified by HPLC (Varian ProStar). The pyrethrum extract association rate was calculated

as the difference between the non-associated fraction of biocide and the total amount initially added to the nanoparticles (GAMISANS et al., 1999; SCHAFFAZICK et al., 2003; KILIC et al., 2005) (Table 1S- Supplementary Material).

2.4. Toxicological bioassay

Operculated brood combs were collected from three healthy colonies of Africanized *Apis mellifera* located in apiaries at Sao Paulo State, Brazil. The emergence of worker bees was monitored in laboratory. Following emergence, the bees were transferred to plastic pots lined with filter paper and fed *ad libitum* sugar-aqueous solution (50%:50% water:inverted sugar, v:v) to acclimatize for 24 h.

Subsequently, the 1-day-old bees were divided into the following experimental groups in triplicate (each colony representing a replicate): I) Control (CTL) - sugar-aqueous solution (syrup); II) Sublethal dose (1 ng.μL^{-1}) of pyrethrum extract (PYR_{1ng}); III) Sublethal dose (10 ng.μL^{-1}) pyrethrum extract (PYR_{10ng}); IV) 1 ng.μL^{-1} of pyrethrum loaded in SLNs ($SLNP_{1ng}$); V) 10 ng.μL^{-1} of pyrethrum loaded in SLNs ($SLNP_{10ng}$); IV) Empty SLNs; V) Polyvinyl alcohol - surfactant control (PVA); VI) Acetone control (ACN) - vehicle/solvent control. The dose used per bee was based on the $LD50_{48h}$ of pyrethrum for honeybees, i.e., 22 ng.bee^{-1} (USEPA, 1991).

Acute exposure was performed individually by oral administration, i.e., the corresponding solution of the experimental group was administrated to the bees (1 µL) using a micropipette (*per os* administration). Two sublethal doses of 10 ng or 1 ng of biocide per bee were given of the pyrethrum extract (PYR) and pyrethrum loaded in nanoparticles (SLNs). The half the $LD50_{48h}$ value corresponded to a 1/2 dilution ($LD50_{/2}$ = 10 ng.μL^{-1} = 10 ppm), and the other dose corresponded to a 1:20 dilution of the $LD50_{48h}$ value ($LD50_{/20}$ = 1

215 ng.µL^{-1} = 1 ppm), both being sublethal concentrations for honeybees. Concentrations of the
216 solutions, which were used for getting the sublethal doses offered to bees, were obtained by
217 serial dilution of stock solution.

218 After individually acute exposure, the bees were kept in plastic pots (cages), being
219 fed with 50% (w/w) sucrose aqueous solution, in an incubator at a relative humidity of 70%
220 ± 5 and temperature of 32 ± 2ºC, under dark conditions. Two bioassays were performed,
221 being the first one for survival analysis (N = 12 bees per pot in triplicate, per experimental
222 group, totalizing 36 individuals) and another one for histology analyzes (N = 15 bees per pot
223 in triplicate per experimental group, totalizing 45 individuals).

224 In the first bioassay (survival analysis), the bees were monitored daily until the last
225 bee has died. Specifically for survival bioassay, the deltamethrin (DLT, 10 ng.µL^{-1})
226 experimental group was added as positive control. In the second bioassay, the bees were
227 collected 48 h after the acute exposure (N = 6 per group) and dissected for midguts' removal,
228 which were processed for resin embedding and histological analysis (section 2.4.1).

229

230 *2.4.1. Histology procedure*

231 The bee midguts were fixed in 4% buffered paraformaldehyde solution for 24 h and
232 immersed in phosphate-buffered saline (0.1 mol.L^{-1} phosphate buffer, pH 7.4). After, the
233 material was dehydrated in an increasing ethanol series according to Silva-Zacarin et al.
234 (2012). Subsequently, the material was embedded in historesin, and submitted to microtomy.
235 Slides containing 3-µm thick histological sections were stained with hematoxylin-eosin.
236 Posteriorly, the material was photodocumentated and both qualitative and semi-quantitative
237 histopathological analyses were performed using Leica Application Suite V3.8 coupled to
238 the light field photomicroscope (DM1000, Leica). For each bee from each experimental

group (N = 6), two slides were analyzed per individual and three non-sequential histological sections were analyzed for each slide.

Other slides containing 3-µm thick histological sections were submitted to histochemical analysis for detection of proteins, lipids and neutral glycoconjugates (SILVA-ZACARIN et al., 2012) (Section 1.4 - Supplementary Material and Figure 4S).

2.4.2. Semi-quantitative analysis of midguts

Parameters for semi-quantitative analysis were defined according to the Bernet et al. (1999) protocol, and histological alterations (lesions) in midgut of bees were based on Soares-Lima et al. (2018) protocol. To determine alterations in the bee midguts, the lesion index and the organ index, were calculated using two parameters: the importance factor and the score value (BERNET et al., 1999). Alterations were classified from 0 to 3, depending on their degree and extent: 0- no alteration, 1- slight alteration, 2- moderate alteration, and 3- severe alteration. The importance factor was established for each lesion observed (cells eliminated from the epithelium, increased apocrine secretions from the digestive cells, cellular vacuolization, changes in regenerative cells' nests, and the presence of pyknotic nuclei in cells of the epithelium) by a qualitative analysis based on pathological severity. This factor was categorized as (1) minimal pathological importance (repairable damage), (2) moderate pathological importance (damage was repairable in most cases), or (3) severe pathological importance (irreparable damage) (Table 2S and section 1.4 - Supplementary Material).

2.5. Statistical analysis

All data were previously subjected to homogeneity of variance (Bartlett's) and normality (Shapiro-Wilk and Kolmogorov-Smirnov) tests. The physicochemical characterization data were subjected to a Student's t-test followed by a Mann Whitney test. A semi-quantitative analysis of the bee midguts was performed using a Kruskal-Wallis test followed by Dunn's multiple comparison test. The significance level was set at $\alpha = 0.05$. GraphPad Prism v.5.0 was used for these statistical analyses.

The survival curve of honeybees per each experimental group was analyzed by the Log-Rank test (Kaplan-Meier method), and comparison between survival time of the groups was performed by the Holm-Sidak test. The significance level was set at $\alpha = 0.05$. SigmaPlot 13 software was used these analyze.

3. RESULTS AND DISCUSSION

3.1. Nanoparticle characterization

The SLNs were prepared using approved components that are generally recognized as safe (GRAS). Tripalmitin (glyceryl tripalmitate) was used as a solid lipid and PVA was used as a surfactant. Physicochemical stability of the empty and encapsulated biocide in SLNs were evaluated from maintenance measurements of the colloidal parameters (mean diameter, polydispersity and zeta potential), besides the concentration of nanoparticles and pyrethrum encapsulation efficiency, over time (0 to 120 days). Colloidal parameter values and other parameters are shown in Table 1.

The initial and final hydrodynamic diameters (mean ± SEM) of the empty solid lipid nanoparticles (SLN) were 290.0 ± 5.0 and 406.7 ± 6.7 nm, respectively. For the SLNs loaded with pyrethrum (SLN+PYR) the initial and final hydrodynamic diameters were 264.9 ± 2.8 and 260.8 ± 3.7 nm, respectively. There was a significant difference between the empty

nanoparticles and those loaded with pyrethrum in the initial ($P \leq 0.0001$ and $T = 18.18$) and final ($P \leq 0.0001$ and $T = 48.51$) analyses. The hydrodynamic diameter values of empty SLNs increased after 60 days of storage with significant differences between the timepoints ($P \leq 0.0001$ and $T = 54.60$), while these values remained stable for SLN+PYR over the experimental period (120 days) (Figure 1SA- Supplementary Material). The empty SLNs had a larger mean diameter and less physicochemical stability than SLN+PYR, indicating that active ingredient of pyrethrum can stabilize nanoparticle formulation and decrease aggregate formation.

The polydispersion index at 0 and 120 days was 0.12 ± 0.01 and 0.39 ± 0.12 nm, respectively, in empty SLNs, and 0.12 ± 0.01 and 0.15 ± 0.02 nm, respectively, in SLN+PYR (Table 1), and values below 0.2 nm in the initial analysis were considered indicative of good stability and a small distribution of particle diameters. The low values indicate that the nanoparticles were of similar size and without aggregates (MASARUDIN et al., 2015). Similar results were obtained by de Melo et al. (2016) in a 120-day experiment with 15d-PGJ2-loaded SLNs, and by González et al. (2015) at the beginning of their experiment with poly (ethylene glycol)-nanoparticles containing geranium (an essential oil). However, the time-based analysis revealed that the SLN polydispersion index had increased after 60 days of storage (0.3 and 0.39 nm; Figure 1SB - Supplementary Material), with significant differences between the timepoints ($P \leq 0.005$ and $T = 0.0$) and significant differences between SLN_{120} and $SLN+PYR_{120}$ ($P \leq 0.005$ and $T = 0.0$). These data indicate that there was a heterogeneous distribution of particle diameters, i.e., there was a greater aggregation of particles in the empty system (SLN). Particle aggregation and degradation occur in SLN formulations that increase and decrease particle size, respectively, due to the loss of a surfactant coating that protects the material (MULLER et al., 1996).

Both nanosystems had a negative zeta potential, with initial and final values of -13 ± 0.4 and -14 ± 0.3 mV, respectively, for empty SLNs and -9.7 ± 0.2 and -18.2 ± 0.3 mV, respectively, for SLN+PYR. There was a significant difference between the empty nanoparticles and those loaded with pyrethrum ($P \leq 0.0001$, $T_{0d} = 8.989$, and $T_{120d} = 24.50$; Table 1). After decreasing on the 30th day (-5.48 ± 0.13 mV), the zeta potential of SLN+PYR increased to -12.2 ± 0.18 and -18.2 ± 0.35 mV after 90 and 120 days, respectively (Figure 1SD- Supplementary Material). Similarly, the empty SLN zeta potential decreased after 15 (-4.85 ± 0.19 mV) and 30 (-6.27 ± 0.18 mV) days, but increased on the 60th day (-15.43 ± 0.23 mV), indicating good stability until the end of the analysis time (Figure 1SD- Supplementary Material). Zeta potential values greater than 30 mV indicate excellent electrostatic stabilization (60 mV is the ideal value), while values lower 15 mV may result in partial flocculation (SCHWARZ et al., 1994). Low zeta potentials were observed, but the nanoparticle formulations were stable over time due to steric stabilization provided by the PVA (LOURENÇO et al., 1996). Stabilizers can be used in nanoparticle formulations to prevent particle aggregation (ABDELWAHED et al., 2006). In the present study, the nonionic surfactant PVA was used to prepare the SLNs, which is absorbed onto surface nanoparticles and promotes steric stabilization (ADITYA et al., 2013; OLIVEIRA et al., 2015). Therefore, unlike in previous studies, it was not superficial electrostatic repulsion that provided stability to the system (PASQUOTO-STIGLIANI et al., 2017). Particles in suspension are more stable if the zeta potential is greater than 20 mV, and 40 mV indicates excellent stability (ADITYA et al., 2013). Similar results were obtained by Oliveira et al. (2018) in zein nanoparticles loaded with the essential oil citronella (geraniol and R-citronellal), and by Kah et al. (2014) in a polymer-based nanoformulation of atrazine.

Table 1: Characterization of empty SLN and SLN loaded with pyrethrum extract over a period from 0 to 120 days.

PARAMETERS	***SLN_0***	***SLN_{120}***	***$SLN{+}PYR_0$***	***$SLN{+}PYR_{120}$***
MD_{DLS} (NM)	290.0 ± 5.0	406.7 ± 6.7[a,c]	264.9 ± 2.8	260.8 ± 3.7
MD_{NTA} (NM)	185.9 ± 4.6[c]	263.8 ± 18.5[a,c]	161.5 ± 2.7	227.0 ± 12.3[b]
PDI	0.12 ± 0.01	0.39 ± 0.12[a,c]	0.12 ± 0.01	0.15 ± 0.02
ZP (-mV)	13 ± 0.4[c]	14 ± 0.3[c]	9.7 ± 0.2	18.2 ± 0.3[b]
CT (10^{13} particles/mL)	2.7 ± 0.5	3.8 ± 0.2	5.9 ± 0.5	2.0 ± 0.1
pH	4.9 ± 0.04	5,7 ± 0.04[a,c]	5.0 ± 0.02	7.1 ± 0.02[b]
EE (%)	-	-	> 99%	> 99%

Legend - Mean diameter (MD) using dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) techniques; polydispersion index (PDI); zeta potential (ZP), concentration of nanoparticles(CT); hydrogenionic potential (pH) and encapsulation efficiency (EE). The values are expressed as the mean ± standard error of six measurements. [a] Significant difference between SLN group and times; [b] Significant difference between SLN+PYR group and times; [c] Significant difference between SLN and SLN+PYR group. Paired T Test for parametric test, and Mann Whitney U test for nonparametric test.

SLNs showed good stability for the encapsulated a.i, evidencing that physicochemical properties not changed over time. According to Naseri et al. (2015), SLNs are good nanocarriers and can be used to deliver drugs and agrochemicals. Their properties include great physicochemical stability during production and storage, a good release profile, the ability to solubilize lipophilic actives, and low toxicity (NASERI et al., 2015).

There was a significant difference in the pH of the empty SLN suspension between 0 and 120 days (4.9 ± 0.04 and 5.7 ± 0.04, respectively; $P \leq 0.0001$ and $T = 16.08$), and of SLN+PYR (5.0 ± 0.02 and 7.1 ± 0.02, respectively; $P \leq 0.0001$ and $T = 10.04$; Table 1). Only at 120 days was there a significant difference in pH between the treatment groups ($P \leq 0.0001$ and $T = 107.9$) with SLN+PYR having a pH of 7.16 ± 0.02 (Figure 1SC - Supplementary Material), indicating that hydrolytic processes occurred during this period. Similar results were obtained by Oliveira et al. (2015).

354 The NTA revealed that the empty SLNs contained 2.7 ± 0.5 x 10^{13} particles per mL
355 with an initial size of 185.9 ± 4.6 nm, and SLN+PYR contained 5.9 ± 0.5 x 10^{13} particles per
356 mL with an initial size of 161.5 ± 2.7 nm. Table 1 shows that there was a significant
357 difference among timepoints for empty SLNs ($P \leq 0.02$ and $T = 3.65$) and SLN+PYR ($P \leq$
358 0.007 and $T = 4.92$), as well as between empty SLNs and SLN+PYR at 0 ($P \leq 0.0004$ and T
359 $= 10.68$) and 120 ($P \leq 0.007$ and $T = 5.23$) days. NTA counts the number of particles per mL
360 and is a complementary technique in the analysis of hydrodynamic diameters, and DLS and
361 NTA did not provide similar diameter values and particle concentrations. This difference
362 may have been caused by sample dilution during the NTA, which could have caused some
363 aggregates to rupture in suspension and result in smaller particles than the DLS
364 (MARUYAMA et al., 2016).

365 The encapsulation efficiency of pyrethrum into the SLNs was evaluated using an
366 analytical curve of pyrethrum determined by HPLC (Peak area (a.u.) = 4.69442 +
367 1952.15769* [pyrethrum concentration], r = 0.99341). The encapsulation efficiency was as
368 high as 99%, suggesting that the pyrethrum extract was efficiently encapsulated in this carrier
369 system. Nevertheless, is important verify the release profile of pyrethrum in field conditions
370 and it is expected that due the high encapsulation efficiency that the particles protect the a.i.
371 in order to increase its shelf life in field conditions. A high encapsulation efficiency has also
372 been reported in polymeric nanocapsules and SLNs loaded with carbendazim and
373 tebuconazole (CAMPOS et al., 2015), in chitosan nanoparticles carrying the herbicides
374 imazapic and imazapyr (MARUYAMA et al., 2016), and in microcapsules containing
375 dementholized peppermint oil (ZHAO et al., 2016). The high encapsulation value indicates
376 the affinity of the biocide to the lipid matrix (de MELO et al., 2016) due to its low solubility
377 in water (<10 $mg.L^{-1}$) and high solubility in organic solvents (USEPA, 2006).

3.2. Differential scanning calorimetry (DSC)

DSC thermograms for SLN+PYR, empty SLNs, tripalmitin, and pyrethrum extract are presented in Figure 1. The DSC analyzes in this study were carried out with the objective of demonstrating that the pyrethrum interacts with nanocarriers components. There were no endothermic peaks for the pyrethrum extract. Tripalmitin's lowest peak was observed at 61ºC, which agrees with the melting point described in the literature (CHEN et al., 2006). Analysis of the empty SLNs and SLN+PYR revealed that the melting points for tripalmitin were 65 and 64°C, respectively, indicating that tripalmitin in the SLNs was solid, and that the pyrethrum did not change the lipid core organization of the SLNs. Similar results were obtained by Oliveira et al. (2015), who found that the herbicides simazine and atrazine were dispersed on a nanoparticle matrix; as well as, Nasseri et al. (2016), verified that SLNs containing *Zataria multiflora* essential oil (ZEO) not showed DSC pick of *Zanataria multiflora,* and authors suggested that essential oil was incorporated and dissolved in the lipid matrix. Analysis of the empty and encapsulated SLNs revealed two peaks, one indicating a tripalmitin peak and the other possibly indicating PVA. Thermal studies of PVA have reported an 88.1°C peak, probably due to moisture evaporation (GUIRGUIS; MOSELHEY, 2012).

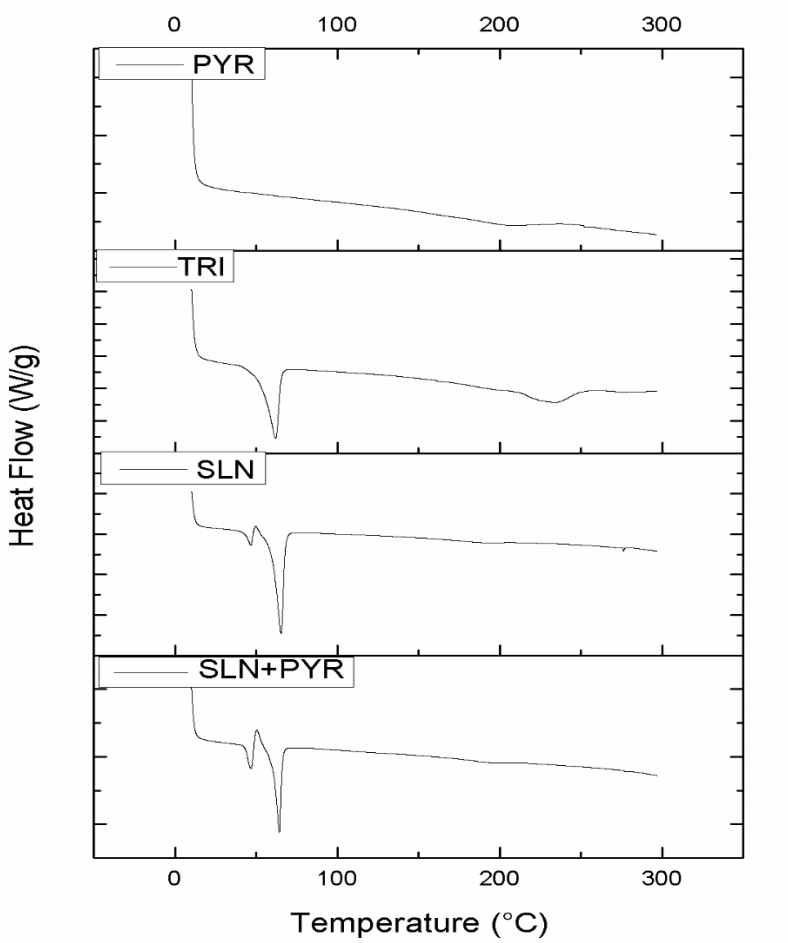

Figure 1 - Differential scanning calorimetry evaluation of interaction between pyrethrum extract and components of the SLN formulation: Thermograms for (PYR) Pyrethrum extract, (TRI) Tripalmitin, (SLN) Solid lipid nanoparticles, (SLN+PYR) Pyrethrum loaded in solid lipid nanoparticles. Conditions: N_2 flow - 50 mL/minute, heating ramp of 10 to 300°C at a rate of 10°C per minute.

3.3. Fourier Transform Infrared Spectroscopy (FTIR)

The physical mixture had three specific bands at 2914, 2368, and 1654 cm^{-1} (Figure 2), which corresponded with tripalmitin (2914 cm^{-1}); and pyrethrum extract bands at 2368 and 1654 cm^{-1}, corresponding with peak CO_2 (OLIVEIRA and PASSOS, 2013) and a stretching of the –C=C group, respectively. The infrared spectra of PVA, empty SLNs, and SLN+PYR exhibited similar specific bands at 3335 cm^{-1} (Figure 2), which suggests the presence of an –O-H group in the formulations. These groups were probably derived from the water and PVA used in the preparation of the nanoparticles (ZAIN et al., 2011). The

411 specific bands at 2914 and 2848 cm^{-1} that were observed in the nanoparticles indicates a
412 stretching of the –C-H group (Figure 2), corresponding to tripalmitin (CAMPOS et al., 2015).
413 It was also possible to observe bands at 1735 cm^{-1}, corresponding to a stretching of the –
414 C=O group, at 1470 cm^{-1}, corresponding to a bending of the $–C-H_2$ group, and at 1178 cm^{-1},
415 corresponding to a stretching of the –C-O group.

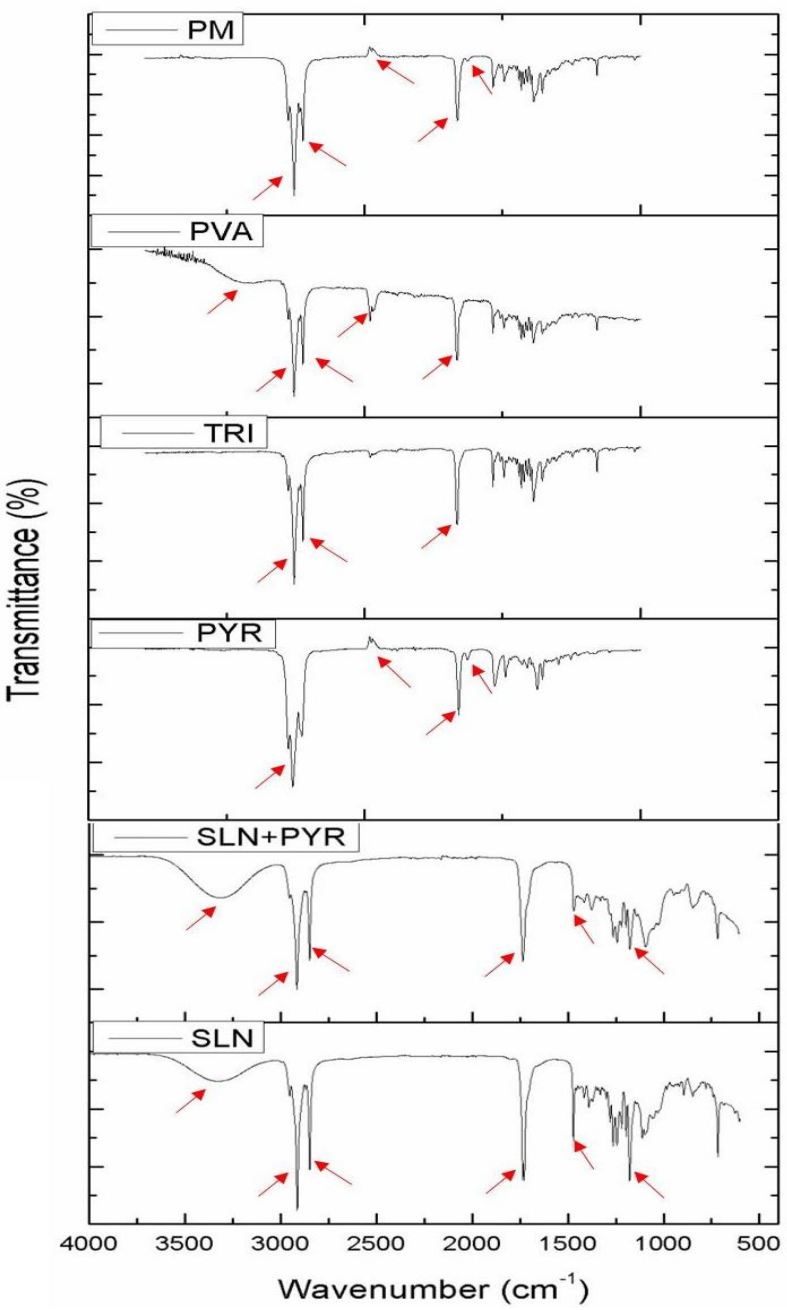

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Figure 2 - Infrared spectroscopic evaluation of interaction between pyrethrum extract and components of the SLN formulation: FTIR spectra for (PM) Physical mixture (PVA) Surfactant - polyvinyl alcohol; (TRI) Tripalmitin; (PYR) Pyrethrum extract; (SLN+PYR) Pyrethrum loaded in solid lipid nanoparticles; (SLN) Solid lipid nanoparticles. Arrows indicate the main characteristic absorption bands in each spectrum. Conditions: infrared spectrophotometer with a range of 400 to 4000 cm^{-1}, 128 scans per sample and 2 cm^{-1} resolutions.

3.4. Toxicological bioassay

Exposure to deltamethrin or pyrethrum extract (10 $ng.\mu L^{-1}$) affected the longevity of bees, reducing their life span. Bees exposed to pyrethrum extract ($P < 0.01$; 141.18 ± 21.3 hours) and pyrethroid ($P < 0.001$; 25.33 ± 0.93 h) presented shorter longevity than those in the control group (257.83 ± 21.79 h). There is not significant difference between control and other experimental groups (ACN; PVA; SLN; $SLNP_{1ng}$; $SLNP_{10ng}$ and PYR_{1ng}; $P > 0.05$). The ACN (252.7 ± 25.03 h) data was similar to control group, as well as $SLNP_{1ng}$ (256.24 ± 21.00 h) and $SLNP_{10ng}$ (241.33 ± 18.81 h). The mean survival time of PVA (171.16 ± 18.09 h), SLN (196.54 ±11.38 h) and PYR_{1ng} (175.33 ± 28.12 h) groups was lower than the control group, but not significant ($P > 0.05$). The data of survival analysis were showed in Supplementary Material (Figure 2S).

Pyrethroids can be dangerous to honeybees (JOHNSON et al., 2010), for example, they interfere in the behavior (PALMQUIST et al., 2012), learning and memory performance (LIAO et al., 2018). In addition, exposure to Lambda-Cyhalothrin negatively affects the life span (LIAO et al., 2018; DOLEZAL et al., 2016). In line with these data, the pyrethrum extract and deltamethrin also reduced survival of Africanized *Apis mellifera*.

The sublethal doses of 1 $ng.\mu L^{-1}$ (1 ppm) and 10 $ng.\mu L^{-1}$ (10 ppm) of biocide free or encapsulated that were administered to the bees, induced short-term responses, at morphological level, in the midguts of newly emerged workers.

444 The bee midgut is mainly responsible for food digestion and nutrient absorption, and
445 is composed of three cell types: digestive, endocrine, and regenerative cells. Digestive cells
446 are responsible for the production of digestive enzymes and nutrient absorption, endocrine
447 cells produce hormones, and regenerative cells, which are within nests, are responsible for
448 cell renewal of the epithelium (MARTINS et al., 2006).

449 Histological analysis of the bee midguts revealed morphological alterations in the
450 epithelium (Figure 3), specifically in the digestive cells, whereas the regenerative cell nests
451 maintained their normal morphological pattern. An increase in the elimination of digestive
452 cells to the intestinal lumen was observed in some treatment groups (empty SLNs, $SLNP_{1ng}$,
453 and PYR_{10ng}; Figure 3D, 3E, and 3H) in comparison to the control groups (CTL, ACN, and
454 PVA), which was significant in the empty SLN group (Figure 4A and Table 3S -
455 Supplementary Material).

456 Therefore, sublethal concentrations of pyrethrum extract in both non-encapsulated
457 and encapsulated form in nanoparticles, as well as in empty nanoparticles (SLN), caused
458 changes in digestive cells. Digestive cells have many microvilli close to the peritrophic
459 matrix in the lumen, and among these cells, nests of small regenerative cells are in the
460 intestinal epithelium (NEVES et al., 2002). These undifferentiated cells that remain in the
461 nest are a source for cell renewal in epithelium of bee midgut (CAVALCANTE and CRUZ-
462 LANDIM, 2004). Thus, regenerative cells replace dead digestive cells, which were released
463 into the lumen, for new epithelial digestive cells by differentiation process (CRUZ et al.,
464 2011). In this study, regenerative nests were observed in midgut epithelium, but histological
465 alterations indicative of cytotoxicity were not found in these cells, such as pyknotic nuclei.
466 If the regenerative cells from nests had presented nuclear pyknosis, which is an indicative of
467 cell death in undifferentiated cells, this alteration would have a "severe pathological

468 importance" because regenerative cells in adults does not suffer mitosis (CRUZ et al., 2011),
469 and consequently epithelial renewal of midgut would be compromised, resulting to partial or
470 total loss of the organ function.

471 Digestive cells are eliminated by cell degeneration under natural conditions,
472 meanwhile this process can be accelerated and/or intensified in response to xenobiotic
473 exposure (e.g., SLNs; Table 3S - Supplementary Material). Therefore, cell renewal is an
474 important process in maintaining the organ function, because the differentiation process from
475 regenerative cells can replace dead digestive cells and to renew the midgut epithelium.

476 There was less elimination of digestive cells to the intestinal lumen in bees exposed
477 to pyrethrum-loaded nanoparticles than in those exposed to empty nanoparticles (SLN).
478 Probably, the reduced cell-to-lumen liberation has been due to the interaction of the
479 pyrethrum with the active sites in the nanoparticle, providing greater stability of the colloidal
480 system over the time (0-120d) and high encapsulation efficiency (> 99% along 120d), as
481 evidenced in the physicochemical characterization data. On the contrary, empty SLNs are
482 more reactive and form aggregates more easily over time. Therefore, reactive empty SLNs
483 could interact with the epithelial cells of the midgut (oral exposure) and induce cytotoxicity
484 in digestive cells, which would trigger their elimination to the organ's lumen. The compounds
485 used in nanoparticle formulations, and the colloidal instability of the system, can affect
486 interactions with cell membranes and trigger cytotoxicity (NAFEE et al., 2009). Whereas
487 the worker honeybee has lifetime of 45 days, and considering the acute exposure to the
488 nanopesticide during its application, probably the whole SLNP will remain stable during its
489 life span. Associating this information with the survival analysis, it can be noted that
490 encapsulated pyrethrum kept the survival time (256.24 ± 21.00 h and 241.33 ± 18.81 h,
491 $SLNP_{1ng}$ and $SLNP_{1ng}$, respectively) of the bees similar to the control group (257.83 ± 21.79

492 h). Given that 10 ng of pyrethrum extract and pyrethroid (deltamethrin) reduced life span of
493 the bees, it may be noted that pyrethrum-loaded in nanoparticle is more safe for honeybees,
494 probably because of the stability of the encapsulated pyrethrum and its release as a function
495 of time.

496 Another important process that we observed was increased apocrine secretions from
497 the midgut epithelium onto the apical surfaces of midgut digestive cells (Figure 3SD and 3SE
498 - Supplementary Material). These epithelial cells secrete digestive enzymes and peritrophic
499 matrix substances normally by means of apocrine secretion. Therefore, an increase in
500 secretion may be a protective compensatory response to xenobiotic exposure. Increased
501 apocrine secretion occurred in both the empty nanoparticle-exposed and 1 $ng.\mu L^{-1}$ of
502 pyrethrum-loaded nanoparticle-exposed groups (SLN and $SLNP_{1ng}$; Table 3S and Figure
503 4B). A previous study reported an increase in apocrine secretion of midgut digestive cells in
504 bees exposed to sublethal doses of thiamethoxam insecticide (0.428 $ng.\mu L^{-1}$ and 0.0428
505 $ng.\mu L^{-1}$ per day for 18 days), as well as the increase in both cell vacuolization and cell
506 elimination from the epithelium to the midgut lumen over the exposure period (OLIVEIRA
507 et al., 2014).

508 Higher frequency of eliminated digestive cells and release of apocrine secretion
509 (Figure 4) were considered reversible alterations in the bee midgut and that did not affect
510 survival of bees in empty SLNs or encapsulated pyrethrum (SLNPs) groups. In normal
511 physiological situations, there is low frequency of senescent or dead cells eliminated to the
512 lumen (CAVALCANTE; CRUZ-LANDIM, 1999), and releasing of digestive enzymes from
513 cells to the peritrophic matrix in the lumen, usually by apocrine secretion (TERRA;
514 FERREIRA, 2012). Therefore, these alterations were classified as importance factor 1 in the
515 semi-quantitative analysis, because normally they are reversible, i.e., damage recovery in

516 epithelium occurs through the differentiation of regenerative cells from their nests in order
517 to have new digestive cells. Thus, there is a compensatory response to the potential
518 physiological stress triggered by agrochemicals or nanocarriers that can lead to the
519 elimination of cells and/or intensification of apocrine secretion. Soares et al. (2012) reported
520 an elimination of cells into the lumen, increased apocrine secretion, and pyknotic nuclei in
521 the epithelial cells of the *Scaptotrigona postica* midgut after applying sublethal doses of the
522 insecticide imidacloprid. Similarly, Rossi et al. (2011) exposed Africanized *A. mellifera* to
523 sublethal doses of imidacloprid and observed an increase in both cell elimination and
524 apocrine secretion in the midgut.

525 Aljedani (2017) evaluated the effects of acute exposure to deltamethrin on foraging
526 worker honeybees (*A. mellifera jemenatica*). The bees that were fed a sugary solution
527 containing 2.5 ppm of pyrethroid presented morphological changes in the midgut. In our
528 study, sublethal concentrations of pyrethrum extract (1 and 10 ng.µL^{-}) did not induce
529 histopathological effects on midguts' honeybees when the cell biomarkers were analyzed
530 separately, but the total organ index analysis showed alterations in 10 ng.µL^{-1} pyrethrum
531 extract that could potentially impair midgut function, since there was a decrease in the
532 longevity of the bees, demonstrating the relevance of evaluation of total organ index in bees
533 exposed to pesticides coupled to survival analysis.

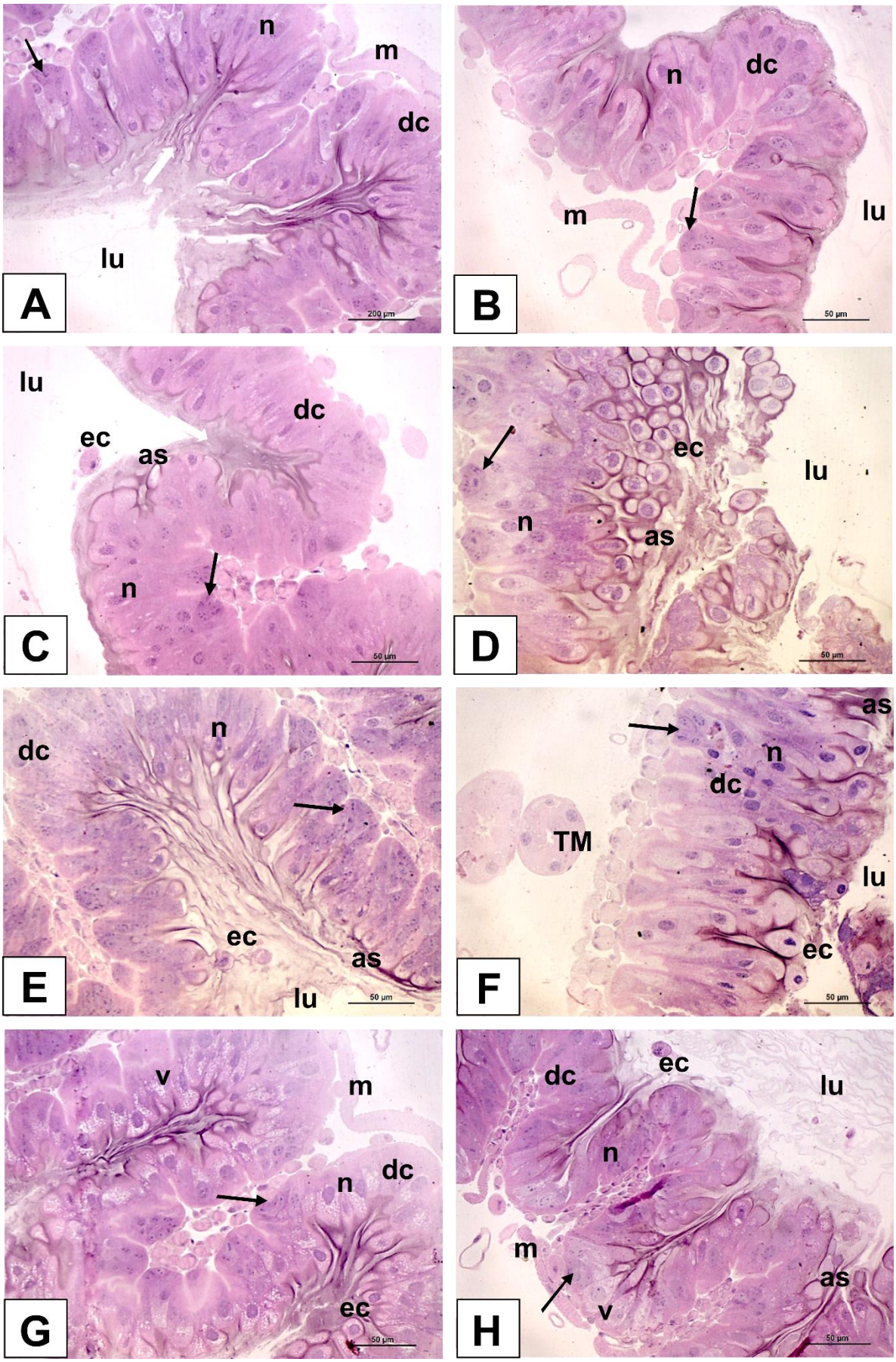
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Figure 3 – Honeybees (Africanized *A. mellifera*) midguts after 48 h of acute exposure. A) CTL - syrup control; B) ACN –acetone control; C) PVA - surfactant control; D) SLN – Solid lipid nanoparticles; E) $SLNP_{1ng}$– 1 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles; F) $SLNP_{10ng}$ – 10 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles G) PYR_{1ng} – 1 ng.µL^{-1} of pyrethrum extract; H) PYR_{10ng} – 10 ng.µL^{-1} of pyrethrum extract. Legend: dc = digestive cell; ec = eliminated cell in the lumen; lu = lumen; n = nucleus, v = vacuolization; as = apocrine secretion; Black arrow = Regenerative cell; TM = Malpighi's tubes; m = muscle. Staining: Hematoxylin-Eosin. Bars: 50 µm.

Although vacuolization can be present in bee midgut cells as a physiological process of autophagy for intracellular turnover, their increased level frequently has been associated to side-effects of xenobiotics, especially in bees exposed to pesticides. For example, Cruz et al. (2010) reported cytoplasmic vacuolization and cell elimination in *A. mellifera* larvae midguts exposed to fipronil (0.1 and 1 µg.g^{-1}) and boric acid (1.0, 2.5, and 7.5 mg.g^{-1}). Kakamand et al. (2008) observed an increase in the vacuolization of midgut cells in honeybees exposed to deltamethrin (1, 2.5, 5, and 10 mg.L^{-1}) and the degeneration of the midgut epithelium of bees exposed to the highest concentration of this compound.

Histochemical analysis of vacuolization areas in digestive cells (Figure 4S - Supplementary Material) showed that they are negative for proteins or neutral glycoconjugates, but had positive labelling for lipids that could indicate multivesicular bodies, because newly emerged honeybees have no spherocrystals yet. Multivesicular bodies are frequently found in midgut cells of insects (SERRAO; CRUZ-LANDIM, 1996), and are formed from early endosomes due to an inward budding of its membrane resulting in intralumenal vesicles whose main function is “collecting” plasma membrane receptors to be degraded into the lysosomes. Multivesicular bodies and autophagy are closely related (FADER; COLOMBO, 2009).

At the present study, intensification of cytoplasm vacuolization was considered a morphological alteration indicative of cytoplasmic loss, which is of greater pathological

563 importance than the other alterations analyzed because, especially in insects, autophagy may
564 act as a pro-death process at the cellular/organ level (MALAGOLI et al., 2010), although its
565 effects at the organismal level can still be considered as fundamental for survival.

566 Cell vacuolization increased in both groups exposed to pyrethrum extract (Figure 3G
567 and 3H, Figure 3SG and 3SH, and Table 3S), but there was no significant difference due to
568 the highly variable degree of vacuolization among individuals exposed to pyrethrum extract
569 (Figure 4C). However, when the organ index was calculated, vacuolization accounted for a
570 higher total index under 10 $ng.\mu L^{-1}$ of pyrethrum extract (Figure 4D), as this alteration was
571 classified as importance factor 2 in the semi-quantitative analysis (Table 3S) because of the
572 loss of cytoplasmic material and the severity level.

573 In the total organ index analysis, the empty nanoparticles and 10 $ng.\mu L^{-1}$ of pyrethrum
574 extract caused more significant changes than the other experimental groups (Table 3S). In
575 contrast, nanoparticles loaded with 1 $ng.\mu L^{-1}$ pyrethrum extract did not increase cell
576 alterations more than the other groups (nanoparticles and pyrethrum extract). The SLNP
577 groups exhibited a decrease in short-term cell alterations, so in this respect was considered
578 safer for bees over short exposure times.

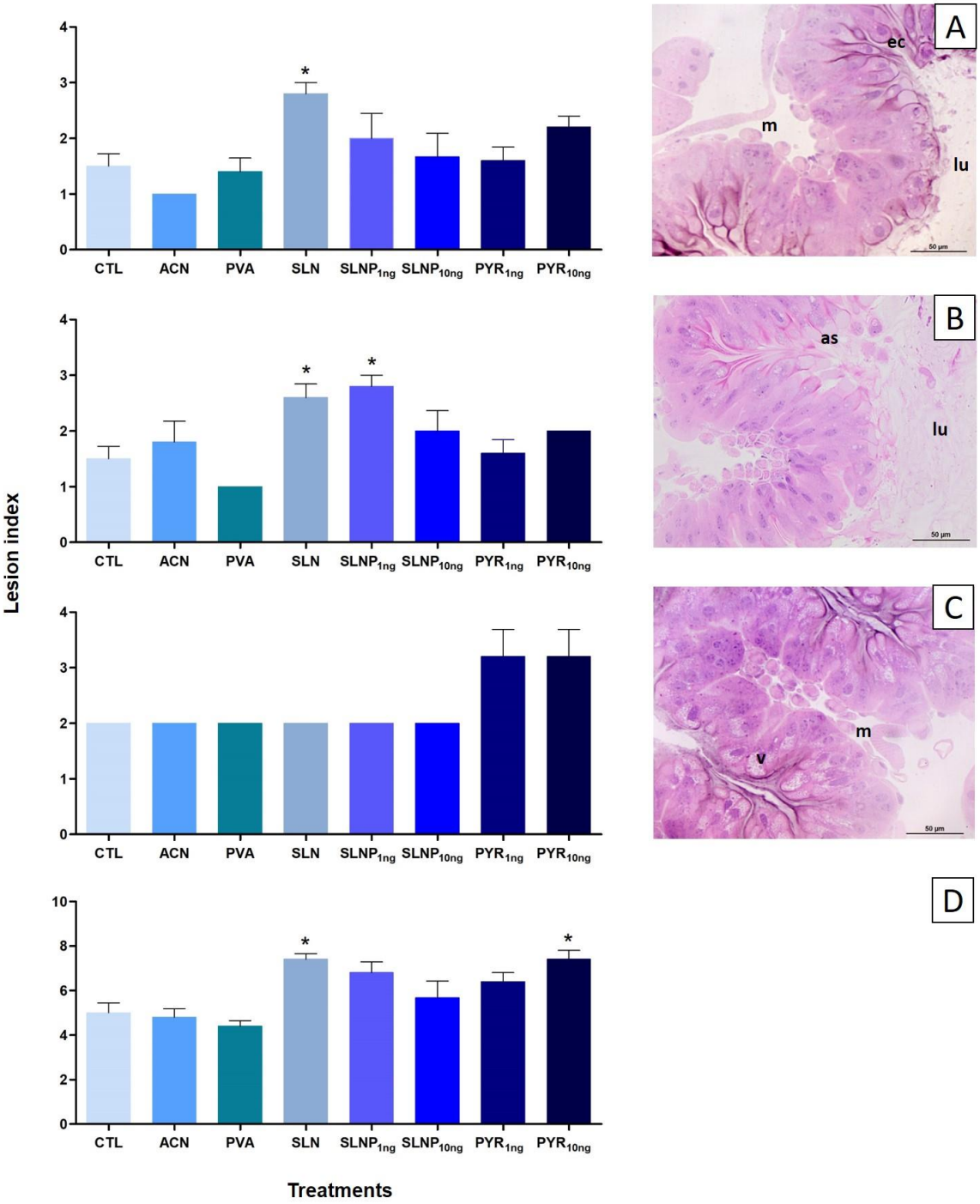

Figure 4 – Alterations and organ index in honeybee (Africanized *A. mellifera*) midguts. a) Eliminated cell index; b) Apocrine secretion index; c) Vacuolization index; d) Total organ index. Legend: CTL – syrup control; ACN – acetone control; PVA - surfactant control; SLN – Solid lipid nanoparticles; $SLNP_{1ng}$ – 1 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles; $SLNP_{10ng}$ – 10 ng.µL^{-1} of pyrethrum loaded in solid lipid nanoparticles PYR_{1ng} – 1 ng.µL^{-1} of pyrethrum extract; PYR_{10ng} – 10 ng.µL^{-1} of pyrethrum extract. Kruskal Wallis One-way ANOVA, followed by Dunn's multiple comparison test. *represent significant differences between groups.

588 At the lowest sublethal doses (1 ng.µL^{-1}), the biocide did not evidence significant
589 histopathological changes in the total lesion index, indicating that could be applied on crops.
590 A carrier system could be developed to improve pyrethrum extract stability, thus allowing its
591 use as nanopesticides. Besides, when the pyrethrum extract was encapsulated in nanocarriers
592 and demonstrated lower toxicity when compared with pyrethrum only. Therefore,
593 nanocarriers are an alternative to conventional pesticide applications. Nanotechnology
594 applied in the agricultural sector could increase agricultural production and crop protection,
595 contribute to sustainable agriculture and eco-friendly carrier systems, and reduce
596 environmental effects and toxicity to organisms (GRILLO et al., 2016). Oliveira et al. (2018)
597 found that zein nanoparticles loaded with citronella effectively controlled the pest species
598 *Tetranychus urticae* with low toxicity.

599 The empty SLNs showed effects onto honeybee, for example, in the total lesion index,
600 with the increase the eliminated cells and apocrine secretion. Therefore, nanocarrier system
601 itself may have reactive sites capable of changing their biological system because it has no
602 active ingredient encapsulated. These reactive sites could interact with organic molecules of
603 the organism, inducing negative effects that indirectly decreased the mean survival time of
604 the bees (196.54 ±11.38 h; $P > 0.05$). By the way, further studies need to be performed in
605 order to evaluate these hypotheses.

606 Nanopesticides can be able to increase the efficiency of agrochemicals and biocides,
607 because it is possible that in the field low doses of the active ingredients can be used.
608 However, in the case of pyrethrum and SLNs this fact will be confirmed with biological
609 assays in target organisms that will be run in the future. In addition, they increase production
610 and reduce damage to the environment (PRASAD et al., 2017). However, there are still many
611 gaps in information to be filled, normative instructions to be written, and legislation to be

made before they can be extensively and safely employed in agriculture (KAH; HOFMANN, 2014; KOOKANA et al., 2014). According Kah et al. (2018), further studies that investigate the efficacy of nanopesticides in crop farming are needed, in order to elucidate their effects on biodiversity and human health, and their benefits and costs compared with conventional formulations.

4. CONCLUSION

It is important to develop and analyze carrier systems as they have many potential benefits in comparison to synthetic and natural agrochemicals, such as reducing the amount of biocide in the environment and greater stability. However, nanotoxicological studies should be undertaken to evaluate the effects of nanoparticles on non-target organisms. In conclusion, this study demonstrates that nanoparticles loaded with pyrethrum extract at sublethal dose (1 or 10 $ng.\mu L^{-1}$) are relatively safe for honeybees, because they do not cause morphological changes in digestive cells. In contrast, empty nanoparticles and 10 $ng.\mu L^{-1}$ of pyrethrum extract caused changes in digestive cells during acute exposure. The concentration of 1 $ng.\mu L^{-1}$ of pyrethrum extract could be used for pest control. These data reflect the effects of a sublethal and acute exposure, and more studies are needed to check if a chronic exposure to these compounds would have different effects on bees. Our results added information for subsidizing future decision making, regulatory framework creation, risk assessments, and legislation development, and improve food security. In addition, based on the results we are planning to run biological assays in order to investigate the efficacy of the nanopesticide against target organisms.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interest in the present study.

REFERENCES

Abdelwahed, W.; Degobert, G.; Stainmesse, S.; Fessi, H. 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. *Adv. Drug Delivery Rev*. 58 (15), 1688-1713. https://doi.org/10.1016/j.addr.2006.09.017

About NIFA, 2018. National Institute of Food and Agriculture (NIFA) - United States Department of Agriculture (USDA). White House Office of Science and Technology Policy: Alexandria, VA, 2018. https://nifa.usda.gov/about-nifa (accessed Mar 04, 2018).

Aditya, N.P.; Shim, M.; Lee, I.; Lee, Y.; Im, M.H.; Ko, S. 2013. Curcumin and gnistein coloaded nanostructured lipid carriers: in vitro digestion and antiprostate cancer activity. *J. Agri. Food Chem*. 61 (8), 1878–1883. doi: 10.1021/jf305143k.

Aljedani, D.M. 2017. Effects of Abamectin and Deltamethrin to the foragers honeybee workers of *Apis mellifera jemenatica* (Hymenoptera: Apidae) under laboratory conditions. *Saudi J. Biol. Sci.* 24 (5), 1007-1015. doi: http://dx.doi.org/10.1016/j.sjbs.2016.12.007.

Anderson, W.; Kozak, D.; Coleman, V.A.; Jämting, Å.K.; Trau, M. 2013. A comparative study of submicron particle sizing platforms: Accuracy, precision and resolution analysis of polydisperse particle size distributions. *J. Colloid Interface Sci.* 405, 322–330. https://doi.org/10.1016/j.scienta.2007.11.013

Anjali, C.H.; Sudheer Khan, S.; Margulis-Goshen, K.; Magdassi, S.; Mukherjee, A.; Chandrasekaran, N. 2010. Formulation of water-dispersible nanopermethrin for larvicidal applications. *Ecotoxicol. Environ. Saf.* 73 (8), 1932-1936. doi: 10.1016/j.ecoenv.2010.08.039.

Bernet, D.; Schmidt, H.; Meier, W.; Burkhardt-Holm, P.; Wahli, T. 1999. Histopathology in fish: Proposal for a protocol to assess aquatic pollution. *J. Fish Dis.*, 22 (1), 25-34. https://doi.org/10.1046/j.1365-2761.1999.00134.x

Campos, E.V.R.; de Oliveira, J.L.; da Silva, C.M.; Pascoli, M.; Pasquoto, T.; Lima, R.; Abhilash, P.C.; Fraceto, L.F. 2015. Polymeric and Solid Lipid Nanoparticles for Sustained Release of Carbendazim and Tebuconazole in Agricultural Applications. *Sci Rep.* 5, 13809. doi: 10.1038/srep13809

Cavalcante, V.M.; C. Cruz-Landim. 1999. Types of cells present in the midgut of the insects: A review. *Naturalia* (Rio Claro) 24, 19-40.

Cavalcante, V.M.; Cruz-Landim, C. 2004. Padrão eletroforético de proteínas e atividade de fosfatase ácida em extratos do intestino médio de *Apis mellifera L*. durante a metamorfose. *Neotrop. Entomol.* 33 (2), 169-172. http://dx.doi.org/10.1590/S1519-566X2004000200007.

Chen, H; Chang, X.; Du, D.; Liu, W.; Liu, J.; Weng, T.; Yang, Y.; Xu, H.; Yang, X. 2006. Podophyllotoxin-loaded solid lipid nanoparticles for epidermal targeting. *J. Controlled Release* 110 (2), 296 – 306. doi: 10.1016/j.jconrel.2005.09.052

Chen, H., Yada, R. 2011. Nanotechnologies in agriculture: new tools for sustainable development. *Trends Food Sci. Technol* 22, 585–594. https://doi.org/10.1016/j.tifs.2011.09.004

Cruz, A.S.; da Silva-Zacarin, E.C.; Bueno, O.C.; Malaspina, O. 2010. Morphological alterations induced by boric acid and fipronil in the midgut of worker honeybee (*Apis mellifera L.*) larvae. *Cell Biol. Toxicol.* 26 (2), 165–176. doi: 10.1007/s10565-009-9126-x.

Cruz, L.C.; Araújo, V.A.; Dolder, H.; Araújo, A.P.; Serrão, J.E.; Neves, C.A. 2011. Morphometry of the midgut of *Melipona quadrifasciata anthidioides* (Lepeletier) (Hymenoptera: Apidae) during metamorphosis. *Neotrop. Entomol.* 40 (6), 677-681. http://dx.doi.org/10.1590/S1519-566X2011000600007.

de Melo, N.F.S.; Araújo, D.R.; Grillo, R.; Moraes, C.M.; Matos, A.P.; Paula, E.; Rosa, A.H.; Fraceto, L.F. 2012. Benzocaine-loaded polymeric nanocapsules: Study of the anesthetic activities. *J. Pharm. Sci.* 101, 1157-1165.

de Melo, N.F.; de Macedo, C.G.; Bonfante, R.; Abdalla, H.B.; da Silva, C.M.; Pasquoto, T.; de Lima, R.; Fraceto, L.F.; Clemente-Napimoga, J.T.; Napimoga, M.H. 2016. 15d-PGJ_2-Loaded Solid Lipid Nanoparticles: Physicochemical Characterization and Evaluation of Pharmacological Effects on Inflammation. *PLoS One* 11 (8), e0161796. https://doi.org/10.1371/journal.pone.0161796

Dolezal, A.G.; Carrillo-Tripp, J.; Miller, W.A.; Bonning, B.C.; Toth, A.L. 2016. Pollen Contaminated with Field-Relevant levels of Cyhalothrin affects Honey Bee Survival, Nutritional Physiology, and Pollen Consumption Behavior. J. Econ, Entomol. 109 (1), 41-8. https://doi.org/10.1093/jee/tov301.

724 Fader, C.M.; Colombo, M.I. 2009. Autophagy and multivesicular bodies: two closely related
725 partners. Cell Death Differ. 16 (1), p. 70–78. doi: 10.1038/cdd.2008.168.
726
727 Gamisans, F.; Lacoulonche, F.; Chauvet, A.; Espina, M.; García, M.L.; Egea, M.A. 1999.
728 Flurbiprofen-loaded nanospheres: analysis of the matrix structure by thermal methods. *Int. J.*
729 *Pharm.* 179 (1), 37-48. https://doi.org/10.1016/S0378-5173(98)00381-0
730
731 Giannini, T.C.; Cordeiro, G.D.; Freitas, B.M.; Saraiva, A.M. Imperatriz-Fonseca, V.L. 2015.
732 The dependence of crops for pollinators and the economic value of pollination in Brazil. *J.*
733 *Econ. Entomol*. 108, 1-9. doi: 10.1093/jee/tov093.
734
735 Gopal, M.; Kumar, R.; Goswami, A. 2012. Nano-pesticides - A recent approach for pest
736 control. *The J. Plant Protec. Sci.* 4 (2), 1-7.
737
738 Goulson, D.; Nicholls, E.; Botías, C.; Rotheray, E.L. 2015.Bee declines driven by combined
739 stress from parasites, pesticides, and lack of flowers. *Science.* 347 (6229), 1255957. Doi:
740 10.1126/science.1255957
741
742 Grillo, R.; Abhilash, P.C.; Fraceto, L.F. 2016. Nanotechnology applied to Bio-encapsulation
743 of pesticides. *J. Nanosci. Nanotechnol.* 16, 1231-1234.
744 https://doi.org/10.1166/jnn.2016.12332
745
746 Guirguis, O.W.; Moselhey, M.T.H. 2012. Thermal and structural studies of poly(vinyl
747 alcohol) and hydroxypropyl cellulose blends. *Natural Sci.* 4 (1), 57-67.
748 http://dx.doi.org/10.4236/ns.2012.41009
749
750 Jacques, M.T.; Oliveira, J.L.; Campos, E.V.; Fraceto, L.F.; Ávila, D.S. 2017. Safety
751 assessment of nanopesticides using the roundworm *Caenorhabditis elegans*. *Ecotoxicol.*
752 *Environ. Saf.* 139, 245–253. doi: 10.1016/j.ecoenv.2017.01.045
753
754 Johnson, R.M.; Ellis, M. D.; Mullin, C. A.; Frazier, M. 2010. Pesticides and honey bee
755 toxicity – USA. *Apidologie* 41, 312–331. doi: 10.1051/apido/2010018
756
757 Kah, M.; Machinski, P.; Koerner, P.; Tiede, K.; Grillo, R.; Fraceto, L.F. 2014. Hofmann
758 T.Analysing the fate of nanopesticides in soil and the applicability of regulatory protocols
759 using a polymer-based nanoformulation of atrazine. *Environ. Sci. Pollut. Res.* 21 (20), 11699-
760 707. doi: 10.1007/s11356-014-2523-6.
761
762 Kah, M.; Hofmann, T. 2014. Nanopesticide research: Current trends and future priorities.
763 *Environ. Int.* 63, 224-235. doi: 10.1016/j.envint.2013.11.015.
764
765 Kah, M.; Kookana, R.S.; Gogos, A.; Bucheli, T.D. 2018. A critical evaluation of
766 nanopesticides and nanofertilizers against their conventional analogues. *Nat. Nanotechnol.*
767 13, 677–684. doi: 10.1038/s41565-018-0131-1.
768

769 Kakamand, F.A.K.; Mahmoud, T. T.; Amin, A.M. 2008. The role of three insecticides in
770 disturbance the midgut tissue in honey bee *Apis mellifera L*. Workers. *J. Dohuk Univ*. 11 (1),
771 144-151.
772
773 Kearns, C.A.; Inouye, D. W. 1997. Pollinators, Flowering Plants, and Conservation Biology.
774 *BioScience* 47 (5), 297-307.
775
776 Kilic, A. C.; Capan, Y.; Vural, I.; Gursoy, R.N.; Dalkara, T.; Cuine, A.; Hincal, A.A. 2005.
777 Preparation and characterization of PLGA nanospheres for the targeted delivery of NR2B-
778 specific antisense oligonucleotides to the NMDA receptors in the brain. *J.*
779 *Microencapsulation* 22, 633–641.
780
781 Kim, D.Y.; Kadam, A.; Shinde, S.; Saratale, R.G.; Patra, J.; Ghodake, G. 2018. Recent
782 developments in nanotechnology transforming the agricultural sector: a transition replete
783 with opportunities. *J. Sci. Food Agric.* 98 (3), 849-864. doi: 10.1002/jsfa.8749.
784
785 Kookana, R.S.; Boxall, A.B.; Reeves, P.T.; Ashauer, R.; Beulke, S.; Chaudhry, Q.; Cornelis,
786 G.; Fernandes, T.F.; Gan, J.; Kah, M.; Lynch, I.; Ranville, J.; Sinclair, C.; Spurgeon, D.;
787 Tiede, K.; Van den Brink, P.J. 2014. Nanopesticides: guiding principles for regulatory
788 evaluation of environmental risks. *J. Agric. Food. Chem*. 62 (19), 4227-4240.
789 doi: 10.1021/jf500232f
790
791 Liao, C.H.; He, X.J.; Wang, Z.L.; Barron, A.B.; Zhang, B.; Zeng, Z.J.; Wu, X.B. 2018. Short-
792 Term Exposure to Lambda-Cyhalothrin Negatively Affects the Survival and Memory-
793 Related Characteristics of Worker Bees *Apis mellifera*. Arch. Environ. Contam. Toxicol. 75
794 (1), 59-65. doi: 10.1007/s00244-018-0514-1.
795
796 Liu, Y.; Tong, Z.; Prud'homme, R.K. 2008. Stabilized polymeric nanoparticles for controlled
797 and efficient release of bifenthrin. *Pest. Manag. Sci*. 64 (8), 808-812. doi: 10.1002/ps.1566.
798
799 Lourenço, C.; Teixeira, M.; Simões, S.; Gaspar, R. 1996. Steric stabilization of nanoparticles:
800 Size and surface properties. *Int. J. Pharm*. 138 (1), 1-12. https://doi.org/10.1016/0378-
801 5173(96)04486-9
802
803 Malagoli, D.; Abdalla, F.C.; Cao, Y.; Feng, Q.; Fujisaki, K.; Gregorc, A.; Matsuo, T.; Nezis,
804 I.P.; Papassideri, I.S.; Sass, M.; Silva-Zacarin, E.C.; Tettamanti, G.; Umemiya-Shirafuji, R.
805 2010. Autophagy and its physiological relevance in arthropods: current knowledge and
806 perspectives. Autophagy. 6 (5), 575-88. doi: 10.4161/auto.6.5.11962.
807
808 Martins, G.F.; Neves, C.A.; Campos, L.A.; Serrão, J.E. 2006. The regenerative cells during
809 the metamorphosis in the midgut of bees. *Micron* 37 (2), 161-168.
810 https://doi.org/10.1016/j.micron.2005.07.003
811
812 Maruyama, C.R.; Guilger, M.1.; Pascoli, M.; Bileshy-José, N.; Abhilash, P.C.; Fraceto, L.F.;
813 de Lima, R. 2016. Nanoparticles Based on Chitosan as Carriers for the Combined Herbicides
814 Imazapic and Imazapyr. *Sci. Rep*. 6 (19768). Doi: 10.1038/srep19768
815

Masarudin, M.J.; Cutts, S.M.; Evison, B.J.; Phillips, D.R.; Pigram, P. J. 2015. Factors determining the stability, size distribution, and cellular accumulation of small, monodisperse chitosan nanoparticles as candidate vectors for anticancer drug delivery: application to the passive encapsulation of [^{14}C]-doxorubicin. *Nanotechnol. Sci. Appl.* 8, 67–80. http://doi.org/10.2147/NSA.S91785

Mishra, S.; Keswani, C.; Abhilash, P.C.; Fraceto, L.F.; Singh, H.B. 2017. Integrated Approach of Agri-nanotechnology: Challenges and Future Trends. *Front. Plant. Sci.* 8, 1-12.

Montanha, F.P.; Pimpão, C.T. 2012. Efeitos toxicológicos de piretróides (cipermetrina e deltametrina) em peixes. *Rev. Cient. Elet. Med. Vet.* 9 (18), 1-58.

Mukhopadhyay, S.S. 2014. Nanotechnology in agriculture: prospects and constraints. *Nanotechnol., Sci. Appl.* 7, 63–71. doi: 10.2147/NSA.S39409

Muller, R. H.; Rühl, D.; Runge, S.A. 1996. RungeBiodegradation of solid lipid nanoparticles as a function of lipase incubation time. *Int. J. Pharm.* 144 (1), 115-121.

Muller, H.R.; Mäder, K.; Gohla, S. 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art. *Eur. J. Pharm. Biopharm.* 50 (1), 161-177. https://doi.org/10.1016/S0939-6411(00)00087-4

Muller, H.; Shegokar, R.; Keck, C.M. 2011. 20 Years of Lipid Nanoparticles (SLN & NLC): Present State of Development & Industrial Applications. *Curr. Drug. Discov. Technol*. 8, 207-227. https://doi.org/10.2174/157016311796799062

Nafee, N.; Schneider, M.; Schaefer, U.F.; Lehr, C.M. 2009. Relevance of the colloidal stability of chitosan/PLGA nanoparticles on their cytotoxicity profile. *Int. J. Pharm.* 381 (2, 3), 130-139. https://doi.org/10.1016/j.ijpharm.2009.04.049

Naseri, N.; Valizadeh, H.; Zakeri-Milani, P. 2015. Solid Lipid Nanoparticles and Nanostructured Lipid Carriers: Structure, Preparation and Application. *Adv. Pharm. Bull.* 5 (3), 305–313. http://doi.org/10.15171/apb.2015.043

Nasseri, M.; Golmohammadzadeh, S.; Arouiee, H.; Jaafari, M.R.; Neamati, H. 2016. Antifungal activity of *Zataria multiflora* essential oil-loaded solid lipid nanoparticles in-vitro condition. *Iran. J. Basic Med. Sci.* 19 (11), 1231-1237. Doi: 10.22038/ijbms.2016.7824

Neves, C.A.; Bhering, L.L.; Serrão, J.E.; Gitirana, L.B. 2002. FMRFamide-like midgut endocrine cells during the metamorphosis in *Melipona quadrifasciata anthidioides* (Hymenoptera Apidae). *Micron* 33 (5), 453-460. https://doi.org/10.1016/S0968-4328(01)00043-9

Oliveira, J.L.; Campos, E.V.; Gonçalves da Silva, C.M.; Pasquoto, T.; Lima, R.; Fraceto, L.F. 2015. Solid Lipid Nanoparticles Co-loaded with Simazine and Atrazine: Preparation, Characterization, and Evaluation of Herbicidal Activity. *J. Agric. Food Chem.* 63, 422-432. Doi: 10.1021/jf5059045

Oliveira, J.L.; Campos, E.V.R.; Pereira, A.E.S.; Pasquoto, T.; Lima, R.; Grillo, R.; Andrade, D.J.; Santos, F.A.D.; Fraceto, L.F. 2018. Zein Nanoparticles as Eco-Friendly Carrier Systems for Botanical Repellents Aiming Sustainable Agriculture. *J. Agric. Food Chem.* 66, 1330-1340. doi: 10.1021/acs.jafc.7b05552.

Oliveira, R.A.; Roat, T.C.; Carvalho, S.M.; Malaspina, O. 2014. Side-effects of thiamethoxam on the brain and midgut of the africanized honeybee *Apis mellifera* (Hymenopptera: Apidae). *Environ. Toxicol*. 29 (10), 1122-33. doi: 10.1002/tox.21842.

Oliveira, R.L.; Passos, F.B. 2013. Estudo da oxidação parcial do etanol em catalisadores de Rh por DRIFTS. *Quim. Nova* 36 (3), 375-381.

Palmquist, K.; Salatas, J.; Fairbrother, A. 2012. Pyrethroid insecticides: use, environmental fate, and ecotoxicology, in: Perveen, F. (Ed.), *Insecticides: Advances in Integrated Pest Management*, InTech, Rijeka: Croatia, cap. 11, pp. 251-278.

Pasquoto-Stigliani, T.; Campos, E.V.R.; Oliveira, J.L.; Silva, C.M.G.; Bilesky-José, N.; Guilger, M.; Troost, J.; Oliveira, H.C.; Stolf-Moreira, R.; Fraceto, L.F.; de Lima, R. 2017. Nanocapsules Containing Neem (*Azadirachta Indica*) Oil: Development, Characterization, And Toxicity Evaluation. *Sci. Rep.* 7 (5929). doi:10.1038/s41598-017-06092-4

Peay, S.; Hiley, P.D.; Collen, P.; Martin, I. 2006. Biocide treatment of ponds in Scotland to eradicate signal crayfish. *Bull. Fr. Pêche Piscic.* 380–381, 1363–1379. https://doi.org/10.1051/kmae:2006041

Perez-de-Luque, A.; Rubiales, D. 2009. Nanotechnology for parasitic plant control. *Pest Manage. Sci.* 65 (5), 540-545. https://doi.org/10.1002/ps.1732

Polleto, F.S.; Jäger, E.; Ré, M.I.; Guterres, S.S.; Pohlmann, A.R. 2007. Rate modulating PHBHV/PCL microparticles containing weak acid model drugs. *Int. J. Pharm.* 345, 70-80. https://doi.org/10.1016/j.ijpharm.2007.05.040

Potts, S.G.; Biesmeijer, J.C.; Kremen, C.; Neumann, P.; Schweiger, O.; Kunin, W.E. 2010. Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* 25 (6), 345-353. https://doi.org/10.1016/j.tree.2010.01.007

Prasad, R., Bhattacharyya, A.; Nguyen, Q.D. 2017. Nanotechnology in Sustainable Agriculture: Recent Developments, Challenges, and Perspectives. *Front. Microbiol*. 20; 8:1014. https://doi.org/10.3389/fmicb.2017.01014

Rossi, C.A., Roat, T.C.; Tavares, D.A.; Cintra-Socolowski, P.; Malaspina, O. 2011. Effects of sublethal doses of imidacloprid in malpighian tubules of africanized *Apis mellifera* (Hymenoptera, Apidae). *Microsc. Res. Tech.* 76 (5), 552-558. https://doi.org/10.1002/jemt.22199

Santos, M.A.T.; Areas, M.A.; Reyes, F.G. 2007. Piretróides – uma visão geral. *Alim. Nutr.* 18 (3), 339-349.

Sarangi, M.J.; Padhi, S. Solid lipid nanoparticles – A Review. *J. Crit. Rev*. 2016, *3* (3), 5-12.

Sarlak, N.; Taherifar, A.; Salehi, F. 2014. Synthesis of Nanopesticides by Encapsulating Pesticide Nanoparticles Using Functionalized Carbon Nanotubes and Application of New Nanocomposite for Plant Disease Treatment. *J. Agric. Food Chem* 62 (21), 4833–483. doi.10.1021/jf404720d

Schaffazick, S.R.; Guterres, S.S.; Freitas, L.L.; Pohlmann, A.R. 2003. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. *Quím. Nova* 26 (5), 726-737. http://dx.doi.org/10.1590/S0100-40422003000500017.

Schleier, J.J.; Peterson, R.K.D. 2011. Pyrethrins and Pyrethroid Insecticides, in: López, O.; Fernández-Bolaños, J.G. (Eds.), *Green Trend. Insect Cont*, J. Publishing. London: Burlington House, cap. 3, pp. 94-131.

Schwarz, C.; Mehnert, W.; Lucks, J.S.; Müller, R.H. 1994. Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterization and sterilization. *J. Controlled Release.* 30 (1), 83-96. https://doi.org/10.1016/0168-3659(94)90047-7

Serrão, J. E., C. Cruz-Landim. 1996. Ultrastructure of digestive cells in stingless bees of various ages (Hymenoptera, Apidae, Meliponinae). *Cytobios* 88, 161-171.

Scott, N.; Chen, H. 2012. Nanoscale Science and Engineering for Agriculture and Food Systems. *Ind. Biotechnol.* 9 (1), 17-18. https://doi.org/10.1089/ind.2013.1555

Sekhon, B.S. 2014. Nanotechnology in agri-food production: an overview. *Nanotechnol., Sci. Appl*. 7, 31–53. http://doi.org/10.2147/NSA.S39406

Silva-Zacarin, E.C.M.; Chauzat, M.P.; Zeggane, S.; Drajnudel, P.; Schurr, F.; Faucon, J.P.; Malaspina, O.; Engler, J.A. 2012. Protocol for optimization of histological, histochemical and immunohistochemical analyses of larval tissues: application in histopathology of honey bee. In: Méndez-Vilas, A. (Ed.), *Current microscopy contributions to advances in science and technology*, Formatex Research Center: Badajoz, 5. ed., v. 1, pp. 696-703.

Silva, M. dos S. Cocenza, D. S. Grillo, R. de Melo, N. F. S. Tonello, P. S. Oliveira, L. C. de Cassimiro, D. L. Rosa, A. H. Fraceto, L. F. 2011. Paraquat-loaded alginate/chitosan nanoparticles: preparation, characterization and soil sorption studies. *J. Hazard. Mater*. 190 (1-3), 366-374.

Soares, H.M. *Avaliação dos efeitos do inseticida imidacloprido para abelhas sem ferrão Scaptotrigona postica Latreille, 1807 (Hymenoptera, Apidae, Meliponini).* Dissertação de Mestrado, Universidade Estadual Paulista - campus Rio Claro/SP, 2012.

957 Soares-Lima, H.M.; Silva-Zacarin, E.C.M.; Camargo, I; Nocelli, R.C.F.; Malaspina, O.
958 Histopathological alterations on honeybees midgut infected by *Nosema ceranae* and exposed
959 to imidacloprid. In: Society of Environmental Toxicology and Chemistry Asia-Pacific
960 Conference, Daegu, Korea, Sep 16-19, 2018; SETAC AP, Eds.; SETAC: Korea, 2018.
961
962 Terra, W.R.; Ferreira, C. 2012. Biochemistry and molecular biology of digestion. In, Gilbert,
963 L.I. (Ed.), *Insect Molecular Biology and Biochemistry*, Academic Press: San Diego, pp. 365–
964 418. https://doi.org/10.1016/C2009-0-62118-8
965
966 *U.S. Department of Agriculture, National Institute of Food and Agriculture (USDA/NIFA)*
967 *2018;* National Nanotechnology Initiative (NANO). White House Office of Science and
968 Technology Policy: Alexandria, VA, 2018. https://www.nano.gov/node/137 (accessed Mar
969 04, 2018).
970
971 *Data Evaluation Record - Pyrethrum extract 1991;* U.S. Environmental Protection Agency
972 (USEPA). White House Office of Science and Technology Policy: Philadelphia, PA, 1991.
973 https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-069001_3-Sep-
974 91_a.pdf (accessed Mar 14, 2018).
975
976 *Reregistration Eligibility Decision for Pyrethrins 2006;* U.S. Environmental Protection
977 Agency (USEPA). White House Office of Science and Technology Policy: Philadelphia, PA,
978 2006. http://www.epa.gov/oppsrrd1/REDs/pyrethrins_red.pdf (accessed Mar 14, 2018).
979
980 Venkatraman, S. S. 2005. Micelle-like nanoparticles of PLA–PEG–PLA triblock copolymer
981 as chemotherapeutic carrier. *Int. J. Pharm.* 298, 219–232.
982 https://doi.org/10.1016/j.ijpharm.2005.03.023
983
984 Vitorino, C.; Carvalho, F.A.; Almeida, A.J.; Sousa, J.J.; Pais, A.A. 2011. The size of solid
985 lipid nanoparticles: An interpretation from experimental design. *Colloids Surf., B.* 84 (1),
986 117-130. https://doi.org/10.1016/j.colsurfb.2010.12.024
987
988 Walker, G.W.; Kookana, R.S.; Smith, N.E.; Kah, M.; Doolette, C.L.; Reeves, P.T.; Lovell,
989 W.; Anderson, D.J.; Turney, T.W.; Navarro, D.A. 2017. Ecological risk assessment of nano-
990 enabled pesticides: A perspective on problem formulation. *J. Agric. Food Chem*. 66 (26),
991 6480-6486. https://doi: 10.1021/acs.jafc.7b02373
992
993 Wang, Q.; Guan, Y.X.; Yao, S.J.; Zhu, Z.Q. 2010. Microparticle formation of sodium
994 cellulose sulfate using supercritical fluid assisted atomization introduced by hydrodynamic
995 cavitation mixer. *Chem. Eng. J.* 159(1-3), 220-229. https://doi.org/10.1016/j.cej.2010.02.004
996
997 Zain, N.A.M.; Suhaimi, M.S.; Idris, A. 2011. Development and modification of PVA–
998 alginate as a suitable immobilization matrix. *Process Biochem.* 46 (11), 2122-2129.
999 https://doi.org/10.1016/j.procbio.2011.08.010
1000
1001 Zhao, D.; Jiao, X.; Zhang, M.; Ye, K.; Shi, X.; Xihua, Lu. Qiu, G.; Sheac, K.J. 2016.
1002 Preparation of high encapsulation efficiency fragrance microcapsules and their application
1003 in textiles. *RSC Adv.* 84, 80924-80933. doi: 10.1039/C6RA16030A

Zhou, T.; Zhou, W.; Wanga, Q.; Dai, P.L.; Feng, L.; Zhang, Y.L; Sun, J.H. 2011. Effects of pyrethroids on neuronal excitability of adult honeybees *Apis mellifera*. *Pestic. Biochem. Physiol.* 100 (1), 35-40. https://doi.org/10.1016/j.pestbp.2011.02.001

Nanopesticide based on botanical insecticide pyrethrum and its potential effects on honeybees

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ABSTRACT

Nanotechnology has the potential to overcome the challenges of sustainable agriculture, and nanopesticides can control agricultural pests and increase farm productivity with little environmental impact. However, it is important to evaluate their toxicity on non-target organisms, such as honeybees (*Apis mellifera*) that forage on crops. The aims of this study were to develop a nanopesticide that was based on solid lipid nanoparticles (SLNs) loaded with pyrethrum extract (PYR) and evaluate its physicochemical properties and short-term toxicity on a non-target organism (honeybee). SLN+PYR was physicochemically stable after 120 days. SLN+PYR had a final diameter of 260.8 ± 3.7 nm and a polydispersion index of 0.15 ± 0.02 nm, in comparison with SLN alone that had a diameter of 406.7 ± 6.7 nm and a polydispersion index of 0.39 ± 0.12 nm. SLN+PYR had an encapsulation efficiency of 99%. The survival analysis of honeybees indicated that PYR_{10ng} presented shorter longevity than those in the control group ($P \leq 0.01$). Empty nanoparticles and PYR_{10ng} caused morphological alterations in the bees' midguts, whereas pyrethrum-loaded nanoparticles had no significant effect on digestive cells, so are considered safer, at least in the short term, for honeybees. These results are important in understanding the effects of nanopesticides on beneficial insects and may decrease the environmental impacts of pesticides.

KEYWORD: Nanopesticide; Biocide; Sustainable agriculture, Solid lipid nanoparticles; Bees.

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Can a nanopesticide based on solid lipid nanoparticles loaded with the botanical insecticide pyrethrum be toxic to honeybees ?

AUTHORS

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