Development of an environmentally friendly formulation of silk fibroin associated with fatty acid from Astrocaryum murumuru Mart. fat, effective against larvae of the Aedes aegypti vector

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Abstract

The *Aedes aegypti* mosquito is a vector of several diseases, such as dengue, malaria and the Zika virus. Synthetic insecticides such as chlorpyrifos and chlorothalonil have been used for plague control, despite causing damage to the environment and to humans. It is therefore important to study natural active compounds with a low environmental impact. The present paper developed an environmentally friendly formulation of silk fibroin (SF) associated with fatty acid esters [ethyl (FAEE-SF), propyl (FAPE-SF) and butyl (FABE-SF)] from *Astrocaryum murumuru* Mart. fat, which was effective against *Aedes aegypti* 3th instar larvae. The FABE-SF nanoemulsion induced a higher mortality rate in the larvae of the *A. aegypti* after 48 h (LC_{50} = 18.92 µg / mL). The stabilities of the nanoemulsions were monitored for 21 days, and FABE-SF exhibited greater stability throughout the monitored period, with average particle, zeta and PDI values of around 217 ± 0.85 nm, -25.6 ± 3.24 mV and 0.338 ± 0.01, respectively. This paper reported the first effective action of fatty acid esters from *A. murumuru* Mart. associated with silk fibroin against *A. aegypti*. The FABE-SF solution also had a low hemolytic index, suggesting that treatment may be safe for animal and human use.

1. Introduction

The effective and sustainable control of mosquito populations and vectors of diseases is currently a challenge (Baldacchino et al. 2015; Benelli and Mehlhorn 2016). For many years, the control of mosquito populations has involved the preferential use of various synthetic insecticides, such as organochlorines, organophosphates and pyrethroids (Rodrigues et al. 2019). However, the indiscriminate and frequent use of such substances has caused selected populations of mosquitoes to become more resistant, as well as resulting in environmental pollution caused by their non-selective inhibitory enzymatic action (Gilliom and Hamilton 2006), that sets humans and other non-target organisms at risk (Sarwar et al. 2009). An alternative is the search for an effective low-cost insecticide, with lower environmental risk (Baldacchino et al. 2015; Benelli and Mehlhorn 2016).

In this context, there is growing demand for the use of natural products such as bioinsecticides, as plant extracts or fractions thereof contain a diversity of bioactive compounds, such as triglycerides, free fatty acids, fatty esters, alkanolamides, terpenes, and flavonoids, which in some cases act as acetylcholinesterase inhibitors (Sharma et al. 2009). Thus, the fat extracted from the fruits of *Astrocaryum murumuru* Mart represent a favorable alternative for hypothetically larvicidal biocomposites. *A. murumuru* fat is easily obtained, as the species of plant is widely distributed around the Amazon estuary (Bezerra 2012). Its natural oil is widely used as a raw material for the manufacture of cosmetics and in the food industry, such as in the production of margarine (Bezerra 2012), due to its favorable composition of saturated fatty acids such as palmitic and stearic acid (Erdemoglu and Kusmenoglu 2003; Korul'kina et al. 2004).

The larvicidal, insecticidal, and repellent action of fatty acid against mosquitoes has been widely reported in literature (Suarez et al. 2007), (Bosch et al. 2000; Perumalsamy et al. 2015), although there are few

studies about the effects of natural products on insects, when used as larvicidal agents (Sharma et al. 2011).

Emulsification methods using co-polymers and proteins have become a viable alternative for enhancing the dispersion of essential or fixed oils in aqueous vehicles, as mosquitoes and other disease vectors need an aqueous environment to develop their life cycle (Echeverría and Albuquerque, 2019). Emulsions are formed and stabilized by surfactants or by amphiphilic polymers, which aim to reduce interfacial tension when adsorbed at the oil/water interface (Kaci et al. 2017; Sarquis et al. 2020). In comparison, silk fibroin is as an alternative to the various types of surfactants used in the formulation of emulsions, due to its low production cost, low cellular and environmental toxicity and high biodegradability, as well as being a biomaterial based on proteins (Ferreira et al. 2017a).

During the last decade, nanotechnology has presented a wide scope of applications in different areas such as medicine, electronics, catalysis and agriculture (Pavoni et al. 2019). With its advent, nanopesticides or nanoformulations that are thin films or encapsulation of the active ingredient of the pesticide in nanostructures have emerged, the main advantage of these nanostructures is the slow and controlled release of their active principles (Bilia et al. 2014). These make them environmentally safe and non-hazardous compared to chemical pesticides (Mehlhorn 2016).

In this context, the researchers have been developing different types of nanopesticides, such as nanoencapsulated formulations, nanoemulsions, nanogels, nanospheres, among others (Kah and Hofmann, 2014). In this way, nanoparticles can help in the production of new pesticides, insecticides and repellents and nanoemulsions can guarantee an efficient and controlled release of the active ingredients (Athanassiou et al. 2018). With this, the nanotechnology can revolutionize insect management, as it can provide innovative tools for the controlled and safe delivery of pesticides (Rai and Ingle, 2012).

Silk fibroin is a natural polymer produced by various insects, and is therefore subject to great diversity in its sequence, structure, and properties (Zhang et al. 2007). The study of silks has been carried out from the cocoon of the domesticated silkworm *Bombyx mori* and the *Nephila clavipes* spider (Noufi 1995). Silk fibroin is a natural amphiphilic block of hydrophobic and hydrophilic co-polymers that collectively mix, giving silk fibroin flexibility and robustness (Tanaka et al. 1999; Zhang et al. 2007). Due to its excellent mechanical properties, high biocompatibility, biodegradability, and low cost (Pham and Tiyaboonchai 2020), silk fibroin is used in tissue engineering, and in the delivery system of bioactive compounds, such as drugs, peptides and proteins, among others (Kundu et al. 2010; Koh et al. 2015), in addition to a variety of biomaterials, such as nanoparticles, nanospheres, hydrogels and films (Cheng et al. 2017).

Our research group has been investigating the action of emulsions prepared with silk protein and larvicidal actives. Recently, our Group has developing biopesticides based on the use of silk fibroin as a vehicle co-polymeric. Araújo (Araújo et al. 2020) evaluated the larvicidal action of the hydroethanolic, methanolic, and hexane extracts of *Acmella oleracea* leaves solubilized in silk fibroin (2%), and Sarquis (Sarquis et al. 2020), that reported the use of free fatty acids from *Carapa guianensis* Aubl oil associated

with silk fibroin, and its activity against *A. aegpyti* larvae, and found excellent LC_{50} results after 48 h of 16.7 µg.mL^{-1}.

Therefore, continuing the study of silk protein as a vehicle for active larvicides, the present study aimed to evaluate the preparation of potentially sustainable, stable, and low-cost larvicidal emulsions containing fatty esters from *A. murumuru* fat, associated with silk fibroin.

2. Material And Methods

2.1 Reagents and solvents

The fat of *Astrocaryum murumuru* Mart. was purchased from Aspacs (Amazonas, Brazil). Ethanol (99%) was purchased from Solven (São Paulo, Brazil), isopropanol and butanol were purchased from Quimex. The solvents *n*-hexane (98.5%) and ethyl acetate (98%), used in the purification of the esters by column chromatography, were obtained from Synth (São Paulo, Brazil) and Amano AK Lipase from *Pseudomonas fluorescens* (20.000 U/g) was purchased from Sigma-Aldrich (São Paulo, Brazil).

2.2 Synthesis of ethyl, isopropyl and butyl esters of *A. murumuru* Mart. fat catalyzed by Amano AK lipase from *Pseudomonas fluorescens*

The enzymatic transesterification of fat was performed separately for ethyl, isopropyl and *n*-butyl alcohols to generate the respective esters (FAEE, FAPE and FABE), as described by Ferreira with some modifications (Ferreira et al. 2017b).

Transesterification was performed in a 50 mL reaction flask containing 1.0 g of fat, 3 mL of respective alcohol and 0.1 g (10%) of Lipase from *Pseudomonas fluorescens*. The reaction mixtures were magnetically stirred for 24 h at room temperature. Afterwards, the reaction solutions were filtered, the organic phases were dried with anhydrous sodium sulfate and filtered, and the solvents were removed by vacuum under reduced pressure. The products were purified by silica gel column chromatography, with the mobile phase being a mixture of *n*-hexane and ethyl acetate (9:1). The isolated products were characterized by their spectroscopic data (GC-MS and FTIR).

2.3 Gas chromatography-mass espectrometry (GC-MS)

The samples (FAEE, FAPE and FABE) were analyzed by gas chromatography mass spectrometry. The gas chromatography-mass spectrometry (GC−MS) analyses were performed in a Shimadzu GC2010 with a mass selective detector (Shimadzu MS2010plus) in electron ionization mode (EI, 70 eV). The GC−MS (equipped with a 30m×0.25mm×0.25µm RTX-5MS column) conditions were: the oven temperature started at 130°C and was kept for 2 min, increased to 290°C at 5°C min^{-1}, maintained for 2 min. The total analysis time was 36 min. The injector and detector temperature was maintained at 210°C; 1 µL was

injected with 1:15 split and helium was used as the carrier gas at a 1.0 mL min^{-1} flow rate. The ions were monitored from 3 to 36 min in the m/z 40–500. The components present in the samples were identified through comparison of spectral data with those in the Wiley library.

2.4 Infrared spectroscopy analysis

A Fourier transform infrared spectrophotometer (Shimadzu FTIR IRTracer-100) recorded the spectra of FAT (*A. murumuru*), FAEE, FAPE and FABE using a potassium bromide beam splitter. The KBr pellet method was employed, and the background spectrum was collected. The range was set from 400 to 4000 cm^{-1} with 16-cm^{-1} resolution.

2.5 Preparation of the silk fibroin solution

The silk fibroin solution was prepared based on the method developed by Ferreira (Ferreira et al., 2014). Silkworm cocoon (3.0 g, from Bratac, Brazil) were degummed in boiling a (2%, w/v) Na_2CO_3 solution for 30 min. The resultant fibers were filtrated and washed with distilled water (3 x 500 mL). Subsequently, silk fiber were dissolved in a ternary solution (50 mL) of H_2O:EtOH:$CaCl_2$ (8:2:1 molar proportions) at 30ºC for 4 h. This mixture was then dialyzed (cellulose tube with an exclusion limit of 16 kDa, from Viskase, Brazil) for 3 days at room temperature, and water changed every 24 hours. The fibroin solution was centrifuged (6000 rpm for 10 min) to remove impurities and larger particles. The concentration of the silk fibroin solution was adjusted to 2% (w/w).

2.6 Preparation of ester/silk fibroin emulsions

The emulsions were produced using a spontaneous emulsification process that occurs when an aqueous phase and an organic phase are mixed. In summary, deionized water was added to a mixture of esters (FAEE, FAPE or FABE) and the silk fibroin solution (2%). The 10 mL solution contained 94% silk fibroin solution (2%), 1% active compounds and 5% of a mixture of ethanol and isopropanol (1:1). The emulsions were prepared according to Sarquis et al. (2020), with slight modifications.

Initially, a mixture of ethanol and isopropanol was added to the esters (FAEE, FAPE or FABE) under constant magnetic agitation (300 rpm) for 30 min. Next, the aqueous phase containing silk fibroin solution was added with continuous agitation for 5 min in vortex.

Emulsions were stored under 4 ºC and evaluated from 1 to 21 days following preparation. The droplet size, polydispersity index and zeta potential of the emulsions were determined using a ZS zetasizer (Malvern, United Kingdom). Each sample was diluted with distilled water (1:10) for analysis. Measurements were made in triplicate. The average droplet size was expressed as mean diameter. All analyzes were performed at 25 ºC.

2.7 Larvicidal activity

Esters combined with silk fibroin emulsions (FAEE-SF, FAPE-SF and FABE-SF) were prepared in different concentrations (7.5, 15, 25, 50 and 75 µg.mL^{-1}) for the larvicidal tests in *A. aegypti.* Five replicates with

10 larvae each were performed. The negative control consisted of the silk fibroin solution without the asset, and dichlorvos solution (6.25 ng.mL^{-1}) was used as the positive control. The larval mortality rate was determined after 24 h and 48 h of incubation at a temperature of 25 ºC and a humidity of 75%. The larvae were considered dead when they did not respond to any stimulus or did not move on the surface of the solution, in contrast to those observed in the control. The bioassay was conducted according to WHO guidelines (2005).

2.8 Morphological analysis on larvae

After treatment, larvae were fixed in 10% formalin. Their external morphology was then analyzed under an optical microscope (with 6 V DC and 20 W output) and was photographed using a digital camera (MDCE - SC USB 2.0) with the Scopelm age software 9.0 package.

2.9 Hemolysis assay

Potential membrane injuries were evaluated according to the hemolysis assay protocol (Kang et al. 2009). Mouse blood was collected and centrifuged (3000 rpm/5 min), and erythrocytes were washed and suspended at 2% with 0.85% saline solutions. The FAEB-SF and negative control was used at final concentrations of 5, 10, 25, 50 and 75 µg.mL^{-1}, and the cells were incubated for 1h at room temperature. 0.1% Triton X-100 was used as the positive control. Samples were centrifuged (3000 rpm/5 min), and the hemoglobin released was measured at 450 nm. The results were expressed in % of hemolysis compared to the positive control.

2.10 Statistical analysis

Probit analysis was performed with a 95% confidence interval to determine the Lethal concentrations (LC$_{50}$ and LC$_{90}$) and Chi-square using software Statgraphics Centurion XV version 15.2.11 (Statpoint Technologies, Inc., Warrenton, VA). If the control mortality of the treated groups was between 5% and 20%, the analysis was corrected in accordance with the WHO guidelines (2005) formula *mortality (%) = X − Y / X × 100*, where X = percentage survival in the untreated control group and Y = percentage survival in the treated sample. Results of hemolysis test were expressed as mean ± standard deviation (SD) using GraphPad Prism 8.0 software. Normality and homogeneity of variances were verified with Kolmogorov− Smirnov and Levene's tests, respectively. Comparisons among the Triton X-100, Murumuru fat and FABE-SF groups results were performed using one-way Analysis of variance (ANOVA) and a Tukey post-test. The minimum significance level was set at p < 0.05 in all cases.

3. Results And Discussion

The transesterification reaction of *A. murumuru* with ethanol, isopropanol and *n*-butanol using Amano AK lipase from *P. fluorescens* provided a fatty acid ethyl ester (FAEE) percentage of 78.4%, a fatty acid propyl ester (FAPE) percentage of 75.1% and a fatty acid butyl ester (FABE) percentage of 63.5%, after purification in a chromatographic column with silica gel.

The gas chromatographic analysis of FAEE from *A. murumuru* showed a composition formed of saturated, monounsaturated and polyunsaturated fatty acids (Table 1). However, *A. murumuru* fat from the present study identified a highest saturated fatty acid percentage of 95.5%, with the predominant presence of lauric acid (C12:0) and myristic acid (C14:00), at 53.5% and 25.8% respectively. Other saturated fatty acids, such as palmitic (C16:0), stearic (C18:0), capric (C10:0) and caprylic (C14:00) acids, were also present in *A. murumuru* fat, in smaller proportions (Table 1). From the unsaturated fatty acid, oleic (C18:1) and linoleic (C18:2) acid were identified, at 3.8% and 0.7%, respectively. Due to a high content of short chain fatty acid, these fats are used in the chemical industry for the manufacture of detergents and soaps (Kostik et al. 2013). The chromatographic profile of the compounds found in the samples were compared with the spectral data of those presented in the Wiley library, and exhibited high levels of similarity.

The polyunsaturated/saturated fatty acid ratio (P/S) of the *A. murumuru* fat used was 0.007, while several studies indicate that a higher P/S index means a reduced deposition of lipids in the body (Kang et al. 2004). According to the investigation of (Pereira et al. 2019), the lauric acid (C12:0) content was found in major proportions (47.6%), and the P/S index was 0.04. In another species, *Astrocaryum aculeatum* G. Mey (Pereira Lima et al. 2017), lauric acid also represented the majority of fatty acids.

The FTIR spectra (Fig. 2) for the three esters obtained from *A. murumuru* revealed evidence of the formation of esters, including C-H stretching vibration in the $2950 - 2853$ cm^{-1} region for the presence of unsaturated and saturated carbons. Stretching bands C = O at 1740 cm^{-1} are related to carbonyl group vibration, and bands characteristic of unsaturated esters were observed, such as C − C − O at 1164 cm^{-1} (Enumo et al. 2020). Stretching vibration C = C in the region of $1655–1686$ cm^{-1} provided evidence of the presence of unsaturated carbons. CH asymmetric angular stretching bands were observed at $1466 - 1371$ cm^{-1} and O-C-C stretching bands in the region of $1113 - 1111$ cm^{-1}.

Generally, the fatty acid esters acted as an emulsion stabilizer, with different degrees of stability improvement (Park and Walsh 2019). Size distribution for DLS analysis of the emulsion (FAEE-SF, FAPE-SF and FABE-SF) from the fatty acid ester of *A. murumuru* associated with silk protein were recorded at the beginning of the experiment and every 7 days during storage (Table 2). The size of all the emulsions prepared was satisfactory (Fig. 3), and no phase separation was observed in the formulation during storage at -4 ºC for 21 days, showing that silk protein could play an important role in the stability of the lipid particles from the conjunct of fatty acid esters. Watanabe (Watanabe et al. 2018) showed that sucrose fatty acid esters with HLB = $5–7$ were suitable for obtaining O/W emulsions. In addition, the creaming phenomenon was inhibited for 30 days or more when fatty acids with a linear saturated alkyl chain with 14 or more carbon atoms were added. These findings are useful for designing stable O/W emulsions for food and cosmetic products.

Silk fibroin/oil emulsion stability is based on a number of factors, as follows: 1) Silk fibroin is an amphiphilic polymer with large hydrophobic domains. These hydrophobic regions are interrupted by small hydrophilic spacers, and the N and C-termini of the chains are also highly hydrophilic (Lu et al.

2015). Therefore, the amphiphilicity of the chain organization likely plays a significant in the stability of the emulsions; 2) The viscosity of the aqueous phase increases with the addition of the silk fibroin solution, which may have inhibited droplet aggregation and gravitational separation (Feng et al. 2018). This favors Brownian motion and inhibits flocculating and coalescing (Khuwijitjaru et al. 2004; Al-Sabagh et al. 2012).

However, the results of the present study revealed that the stability of the mixture depended on the fatty acid ester type present in the sample. For FABE-SF, the particle diameter was smaller (215.5 ± 1.57 nm and 217.5 ± 0.85 at 0 days and the 21st day, respectively) than the particle size of emulsions formed from fatty acid esters with propanol and ethanol [274.06 ± 2.41 nm (0 days) and 702.5 ± 92.43 nm (0 days), respectively] (Table 2). Alcohols, like ethanol, induce fibroin to transform into a Silk II crystalline (Tsukada et al. 1995). However, a decay in the particle size was observed, significantly for the FAEE-SF emulsion, starting at 702.5 (± 92.43) nm (0 day) and reducing to 229.4 (± 8.31) nm (21st day). It is therefore believed that the relationship between the ester structures can influence the particle diameter in the case of an emulsion formed by ethanol, propanol, and butanol fatty acid esters from *A. murumuru* fat (fatty acid mixture).

The present study showed that silk fibroin which could play an important role in the stability of the lipid particles from the conjunct of the fatty acid esters. The polydispersity index (PDI) indicates the homogeneity and stability of the size of the droplets distributed in the emulsions. According to (Lemarchand et al. 2003), PDI values of around 0.3 or below indicate the more homogeneous size distribution of the particles dispersed in the suspension. FABE-SF exhibited an excellent PDI, ranging from 0.320 (± 0.03) to 0.338 (± 0.01), while FAPE-SF had a PDI ranging from 0.452 (± 0.02) to 0.415 (± 0.04), and FAEE-SF a PDI of 0.709 (± 0.08) to 0.396 (± 0.03). One interesting finding was the reduction in the PDI values of the FAEE-SF and FAPE-SF nanoemulsions on the 21st day, to 0.396 and 0.415, respectively (Table 2). The results of this work indicate that the elongation of the ester carbon chain (-Ethyl; -Isopropyl and -*n*-Buthyl) influenced the thermodynamic balance of the phases. Factors such as the spontaneous diffusion and evaporation of volatile material from the internal phase have been observed as a mechanism for reducing droplet size (Silva et al. 2011).

Zeta potential is the electrostatic potential at the slipping plane a few molecules away from the surface (Dalgleish 1997). For the nanoemulsion to be considered stable by electrostatic repulsion alone, a zeta potential value ± 20 is required (Honary and Zahir 2013). All nanoemulsions associated with silk protein exhibited zeta values ranging from − 53.9 (± 4.50) to -25.6 (± 3.24) mV, during the 21 days of monitoring. It is noteworthy that FABE-SF and FAPE-SF showed greater stability throughout the entire monitoring period (Table 2). The stability of a polymer is important for the storage of a drug delivery device. The stability of these emulsions may be related to the improved balance between the dissociation of the propyl and butyl esters with the carboxylic and amino groups present in silk fibroin, resulting in a slight variation in the zeta potential for these emulsions, making them more stable. Electrophoretic mobility experiments have demonstrated that SF was positively charged below pH 3.9 and negatively charged above pH 3.9 (Malay et al. 2008), which is its pI (isoelectric point = 3.9).

The choice of solvents of the formulation is essential for the larvicidal activity on an aqueous medium. Initially, we evaluated the effect of the fatty esters solubilized in DMSO (5%), and the emulsions of the fatty esters associated with the silk fibroin solution, at concentrations of 25 and 75 µg.mL^{-1} (Table 3). The results were then obtained after 24h against the *A. aegypti* larva of the III instar larvae.

For at concentrations of 25 and 75 µg.mL^{-1} tested, the results showed that all fatty acid esters (FAEE, FAPE and FABE) from *A. murumuru* fat associated with silk fibroin nanoemulsion have greater larvicidal activity against the mosquito than normal fatty acid esters in DMSO (Table 3). The FAPE-SF and FABE-SF emulsions, for example, exhibited 92% mortality at a concentration of 75 µg.mL^{-1}, while the FAEE-SF emulsion yielded a mortality rate of 68% at the same concentration. This result highlights the carrying capacity of the emulsion from fibroin and its positive impact on biological activity. It is likely that nanoemulsions associated with silk fibroin solution interact inside the larva cells, leading to a more rapid death than with the DMSO solution, due to changes in the active release kinetics (Table 4). Additionally, the hydrophobic interactions showed to be the main cause of interactions between SF and the fatty acid esters from *A. murumuru* fat. Similarly, previous reports by Sarquis (Sarquis et al. 2020) proposed that the nanoemulsion containing 75% silk fibroin solution (2%), 5% fatty acid from *C. guianensis* Aubl. and 24% ethanol, shown be effective against *A. aegypti* (III instar larvae) with an LC$_{50}$ 94.45 µg.mL^{-1} at 24 h and 16.79 µg.mL^{-1} at 48 h.

One of the great advantages of silk fibroin in the emulsion system is the biodegradation process, which occurs via enzymatic degradation, the production of non-toxic by-products, and the controllable degradation rate, setting it apart from other synthetic or natural polymers (Nguyen et al. 2019).

Table 4 Here

In view of the preliminary results, other concentrations were tested to determine LC$_{50}$ and LC$_{90}$ at 24 and 48 h of nanoemulsion larvae *A. aegypti* larvae. Among the nanoemulsions in fibroin solution, FABE-SF exhibited the highest mortality percentage. After 24 h, the mortality rate was 94% at 75 µg.mL^{-1} and 4% at 7.5 µg.mL^{-1}, the lowest concentration tested. At 48 h, the mortality rate was 100% at 75 and 20% at 7.5 µg.mL^{-1} (Fig. 4A and B). In contrast, a negative control consisting of fibroin solution only exhibited no larvicidal action. This result demonstrates the lack of toxicity of the fibroin solution towards *A. aegypti*.

From these results, it was also possible to establish values for the lethal concentrations LC$_{50}$ and LC$_{90}$ (Table 4) using Probit analysis. The fatty acid ester emulsions from murumuru oil in SF tested here exhibited good efficacy on III instar *A. aegypti* larvae, with LC$_{50}$ values ranging from 29.68 to 44.56 µg.mL^{-1} at 24 h, and from 18.92 to 30.29 µg.mL^{-1} at 48 h (Table 4). The FAEE-SF exhibited a LC$_{50}$ of 44.56 µg.mL^{-1}, while the FAPE-SF and the FABE-SF had LC$_{50}$ of 40.02 µg.mL^{-1} and 29.68 µg.mL^{-1}, respectively, at 24 h. When the larvae of *Ae. aegypti* were exposed to the FAEB-SF nanoemulsion for 48 h, the LC$_{50}$ was 18.92 µg.mL^{-1} and the LC$_{90}$ was 48.51 µg.mL^{-1}.

The present study found that around 78% of mortality was observed at a concentration lower than 75 $\mu g.mL^{-1}$ for the FAEE-SF and FAPE-SF nanoemulsions, after 24 h of exposure of the larvae to nanoemulsions. The FABE-SF nanoemulsion at 24 h exhibited mortality of 92% at a concentration of 75 $\mu g.mL^{-1}$, and 100% mortality after 48 h. These results make it clear that emulsions of different esters with silk fibroin represent promising larvicidal agents against *Ae. aegypti.*

Although the toxicity mechanism of these nanoemulsions is not very well known, we suggest that the larvicidal properties of nanoemulsions, especially FABE-SF, come from its hydrophobic character/interaction with the silk protein, enhanced by the increase in the alkyl grease chain.

A set of methyl esters, from long saturated chains, extracted from the leaves of *Vitex trifolia*, exhibited good larvicidal activity against the *C. quinquefasciatus* mosquito, exhibiting LC_{50} and LC_{90} values of 9.26 and 21.28 $\mu g.mL^{-1}$, respectively, after 24 h treatment. It should be stated that this species had a high composition of lauric acid (Kannathasan et al. 2008).

De Melo (De Melo et al. 2018) evaluated the larvicidal action of oleic, linoleic, linolenic, palmitic and stearic acids, as well as their respective methyl esters, against the larvae of *C. quinquefasciatus*, and found that the oleic, linoleic and linolenic acid exhibited values of CL_{50} of 8.58, 10.04, and 19.78 $\mu g.mL^{-1}$, respectively, after 24 h.

Methyl esters from canola, corn, sunflower and soybean oils show LC_{50} values varying 42.32 to 196.27 $\mu g.mL^{-1}$, against the larvae of 3rd and 4th *C. quinquefasciatus* after 144 h of exposure. The lowest CL_{50} value was observed for the soybean oil methyl ester (42.32 $\mu g.mL^{-1}$) (Ribeiro-Neto et al. 2017).

However, it should be mentioned that most research with emulsions, nanoemulsions or solutions, even when obtained from plant extracts, use solvents or emulsifiers of significant toxicity, which can interfere with biological processes or cell maintenance.

Toxicity is therefore an important factor in the choice of surfactants, which are potentially irritating or poorly tolerated, as they have nonspecific effects on biological membranes. In general, cationic surfactants are more toxic than anionic surfactants, which in turn are more toxic than nonionic surfactants (Pouton and Porter 2008). In view of the above, silk protein represents a highly viable alternative in the use of these surfactants, as it is a biomaterial that does not exhibit cellular toxicity (Fig. 5).

The effects of FABE-SF and the solution of *A. murumuru* fat in silk fibroin were evaluated on erythrocytes isolated of the blood from rats in function of incubation time (1 h) at the following concentrations (5, 10, 25, 50 and 75 $\mu g.mL^{-1}$). The FABE-SF emulsion was chosen for the hemolysis test as it exhibited better larvicidal activity results.

The hemolysis percentage results are shown in Fig. 5. Both the emulsion and the fat solution of *A. murumuru* in silk fibroin exhibited a low capacity for hemolysis at the tested concentrations, of less than

6% even at the highest concentration (75 µg.mL^{-1}), compared with the 0,1% Triton X-100 solution (positive control). The silk fibroin solution used as a negative control did not exhibit erythrocyte lysis, suggesting excellent biocompatibility, behaving like a highly sustainable emulsion. Amino acid-based surfactants have the ability to interact with the lipid bilayer of cell membranes. The action of five amino acids derived from anionic lysine type N^α, N^ε- dioctanoyl lysine and three cationic surfactants derived from arginine (methyl ester of N^α- lauroyl-L- arginine, methyl ester of N^α- myristoyl -L- arginine and derivatives of methyl N^α-acyl- arginine), both surfactants, exhibited strong anti-hemolysis action (Sánchez et al. 2007).

The action of surfactants based on amino acids in plasma membranes is complex. A possible relationship between these surfactants, and their effects on the cell membrane may be due to the amphiphilic character of these surfactants, since they are interspersed in the lipid bilayer of the membrane. So the hydrophilic region is located at the hydrophilic/hydrophobic interface of the membrane and the hydrophobic region in the core of the bilayer (Zachowski and Durand 1988).

The results of the present study also suggest that there is no interaction between the FABE-SF nanoemulsion and erythrocytes, since the presence of silk fibroin in the negative control did not cause cell lysis. Silk fibroin, as well as surfactants based on amino acids, has an amphiphilic character; in addition, repulsion forces can occur between the negative surface charge of the silk fibroin and the negative charges present in the erythrocyte membranes.

Optical microscopy images of *A. aegypti* larvae after 48 h of exposure in the nanoemulsions (FAEE-SF, FAPE-SF and FABE-SF) revealed particles of the emulsions precipitated in the larvae, which causes lesions in the cuticles of the initial segment of the larvae, such as the head and thorax. In addition to adhering to its lateral bristles, while darkening and torsion in the larvae body was also observed (Fig. 6a). The morphological damage in the larvae is the lethality effect of the nanoemulsions. Sarquis (Sarquis et al. 2020) observed similar changes when using a nanoemulsion with free fatty acid from *C. guianensis* and silk fibroin, which was able to cause changes in the anal papilla and in the digestive and respiratory systems of *A. aegypti* larvae. Araújo (Araújo et al. 2020) observed morphological damage in the respiratory siphon and anal papilla in *A. aegypti* larvae when using hexanic extract from the leaves of *Acmella oleracea* solubilized in silk fibroin. There were no changes in the structures of the larvae exposed to the control (Fig. 6b).

It FABE-SF nanoemulsion is based on global trends towards sustainable development stimulate the use of renewable and biodegradable raw materials, as esters of murumuru, with higher biological performance in comparison to a synthetic-based vehicle, this case DMSO, to mosquito larval control, based drug delivery system.

4. Conclusion

This is the first study to use different esters associated with silk fibroin as larvicidal agents. The esters (FAEE, FAPE and FABE) associated with the silk fibrin solution exhibited strong larvicidal activity, especially the FABE-SF nanoemulsion, which exhibited a CL_{50} value of 18.92 μg.mL^{-1}, after 48 h of larvae exposure. It displayed greater temporal stability throughout all 21 days of monitoring, with an average particle value of 217 (± 0.85) nm, zeta potential of -25.6 (± 3.24) mV and a PDI of 0.338 (± 0.01). The FABE-SF exhibit both a hydrophobic and hydrophilic character, increasing the biodistribution and bioavailability controlled of the fatty acid esters in the aqueous medium. It also observed that the nanoemulsions caused structural changes in the larvae, affecting their development and survival. This mortality rate shows that the formulations can be used as biopesticides.

Declarations

CRediT authorship contribution statement

Victor H. Marinho, Fernando B. Neves and Fábio R. Oliveira: Conceptualization, Methodology, Validation, Software, Formal analysis, Investigation, Data curation, Writing – original draft. Sergio A. Yoshioka, Ricardo M. A. Ferreira, David E. J. Quintero, Abraão V. T. L. Santos: Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. Irlon M. Ferreira, Raimundo N. P. Souto and José C. T. Carvalho: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data material

All data generated or analysed during this study are included in this published article (and its additional files).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the study reported in this paper.

Acknowledgements

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Tables

Tables 1-4 are available in the Supplementary Information.
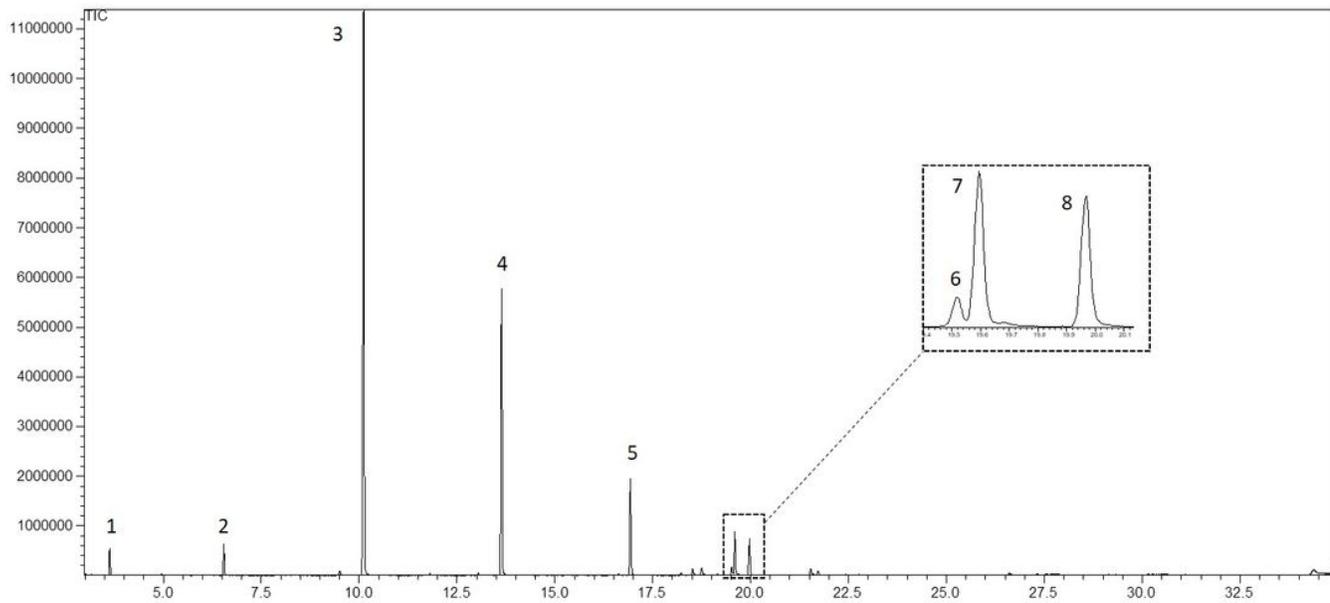
Figures

Figure 1

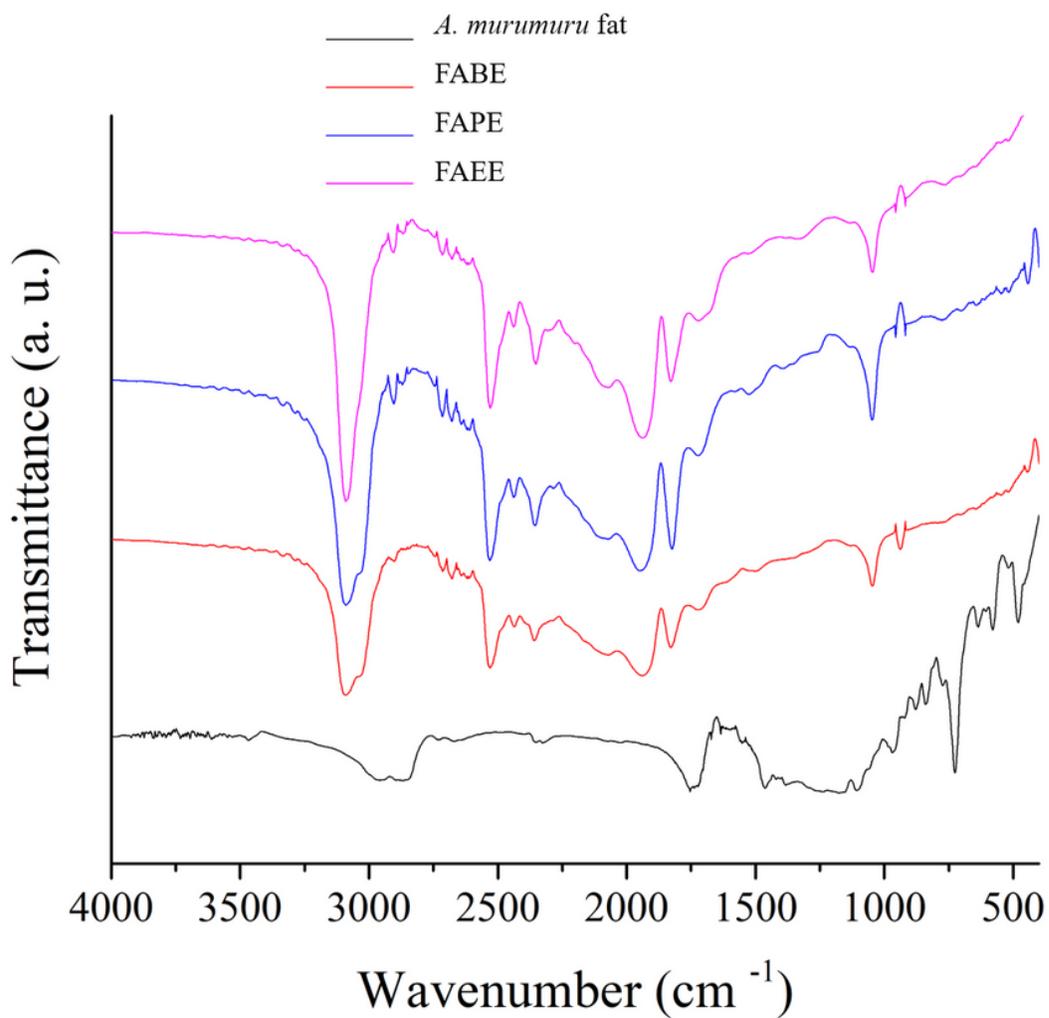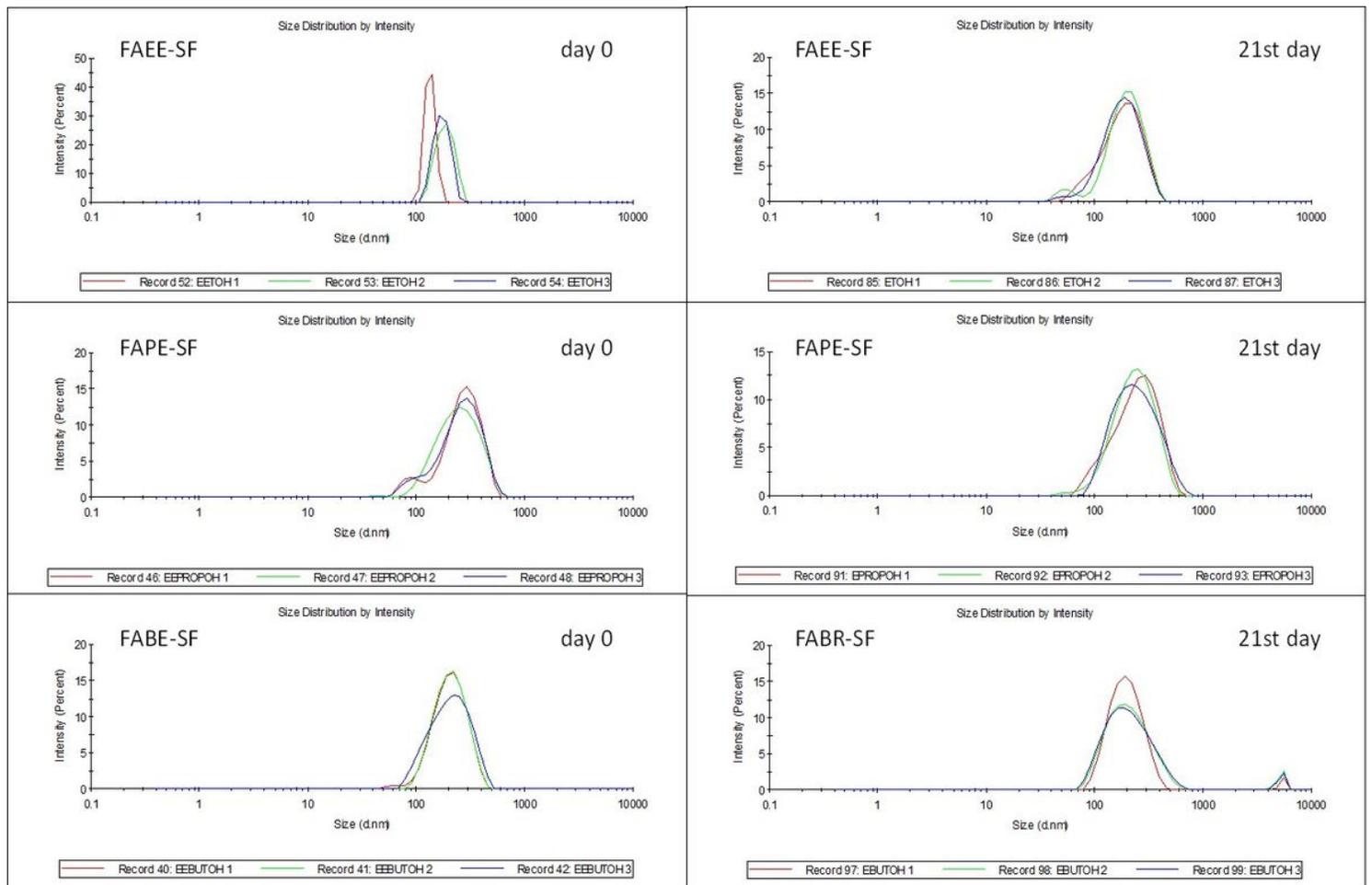Note: a figure caption was not provided in this version.

Figure 2

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Figure 3

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Figure 4

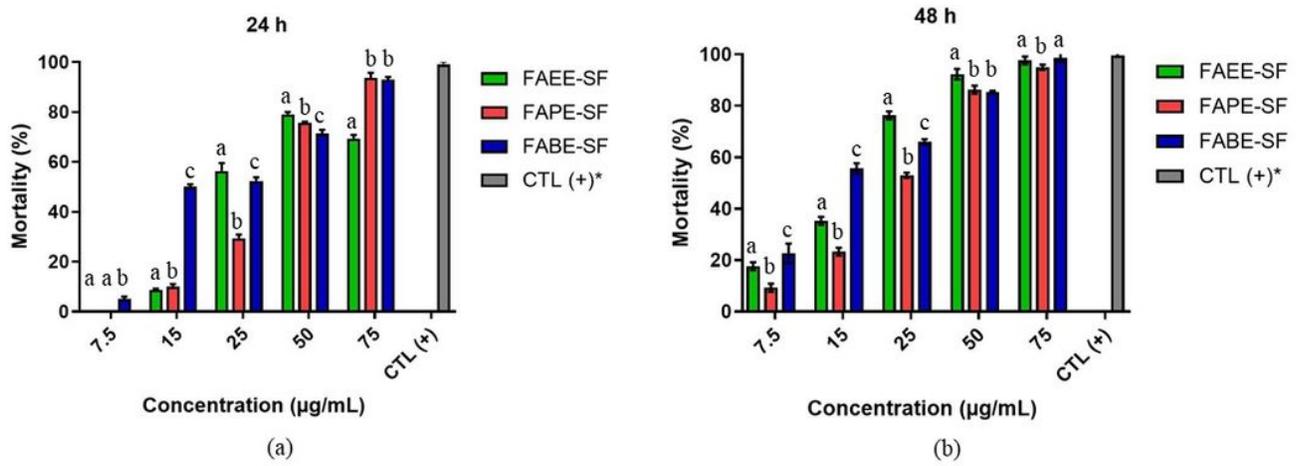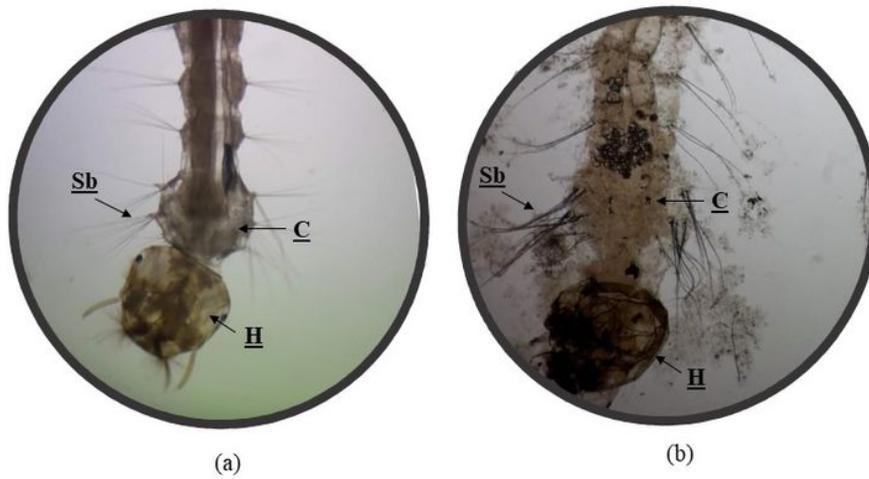Note: a figure caption was not provided in this version.

(a)

(b)

Figure 5

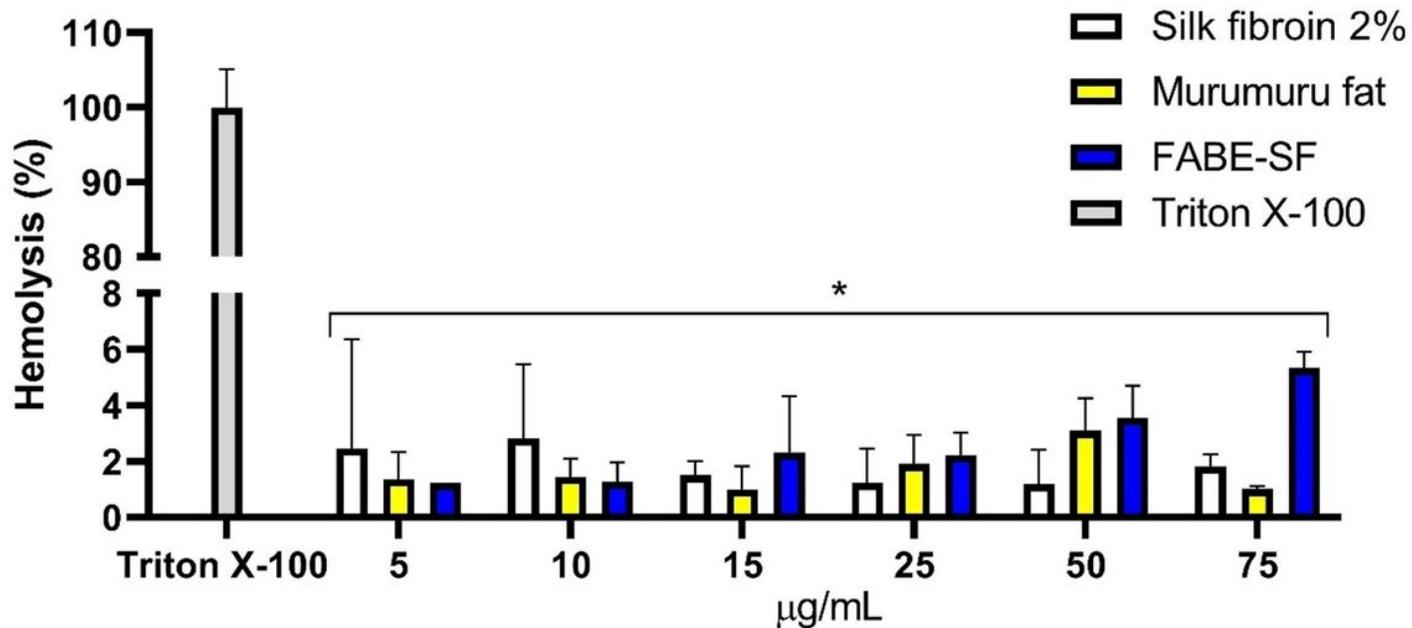Note: a figure caption was not provided in this version.

Figure 6

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