Revista Brasileira de Farmacognosia 28 (2018) 618–625

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Nanosuspension of quercetin: preparation, characterization and effects against *Aedes aegypti* larvae

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ARTICLE INFO

Article history:
Received 5 February 2018
Accepted 9 July 2018
Available online 22 August 2018

Keywords:
Culicidae
Ecotoxicity
Larvicide
Nanocarrier
Flavonoid

ABSTRACT

Aedes aegypti (Diptera: Culicidae) is the main vector of some neglected diseases, including dengue. It is very important to develop formulations that increase effectiveness of vector control with low toxicity. Quercetin is a plant-derived flavonoid that modulates the development of some insects. The low water solubility of quercetin impairs the development of water-dispersible commercial products. To circumvent this problem, the preparation of nanoformulations is considered promising. Thus, this study aimed to evaluate the effect of bulk and quercetin nanosuspension against *A. aegypti* larvae and also to investigate their ecotoxicity. Quercetin nanosuspension was produced by a solvent displacement method followed by solvent evaporation and was maintained in two different temperatures (4 and 25 °C). Its size distribution and zeta potential were monitored along 30 days. The influence of quercetin nanosuspension and bulk-quercetin was investigated at various concentrations against *A. aegypti* and the green algae *Chlorella vulgaris*. The quercetin nanosuspension presented higher stability at 4 °C and negative zeta potential values. Quercetin nanosuspension and bulk-quercetin adversely affected the larvae development, especially at the highest concentrations. Larvae mortality was between 44% and 100% (48 h) for quercetin nanosuspension at 100 and 500 ppm, respectively. The bulk-quercetin induced around 50% mortality regardless the concentration used at this same time-period. Absence of emerging mosquitoes from water was observed on the survival larvae of all the treated groups. Quercetin nanosuspension was less toxic than bulk-quercetin against *C. vulgaris*, especially at higher concentrations. These data indicate that quercetin nanosuspension may represent a potential larvicide for *A. aegypti* control, once they induced larvae death and inhibited the survival ones to emerge from water. In addition, it did not demonstrated ecotoxicity against a non-target organism, highlighting its better properties, when compared to the bulk-quercetin.

Introduction

The *Aedes aegypti* (Linnaeus, 1762) mosquito is responsible for the transmission of some arboviruses neglected diseases, including dengue, chikungunya and Zika. These diseases are common in tropical and subtropical countries. The mosquito is adapted to the urban environment and it preferably uses containers with clean water for the development of its larval phase (Lindsay et al., 2017). In the last decades, the incidence of this disease has increased, reaching new countries and are now under the concern of the Center of Diseases Control and Prevention (CDC) of USA, and by Brazilian Government (Patterson et al., 2016; Petersen et al., 2016).

Dengue is endemic in more than one hundred countries and the most seriously affected regions are the Western Pacific,

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https://doi.org/10.1016/j.bjp.2018.07.003

South-Eastern Asia and the Americas. The sum of notifications in these three regions was higher than three million cases at least twice in the last five years. The increase in global cases may be associated to increasing efforts to record dengue cases, especially from countries associated to the aforementioned regions. However, the high degree of expected unreported cases should be mentioned. On this context, even some hundred million cases may occur every year (WHO, 2017). The Americas had around 1.2 million cases in 2014, while Brazil alone exhibited around 600,000 cases in this year. In the year 2016, the total dengue cases increased approximately two-fold on the Americas and three-fold in Brazil, when compared to 2014. Despite some tendency for decrease in the regional and local registered cases was observed recently, it must be highlighted that a five-fold increase over the past decade occurred (PAHO, 2018).

More recently, the less studied chikungunya and Zika infections have gained attention due to an increase in their incidence and the association of Zika infection with cases of microcephaly from 2014 to 2015 in northeast Brazil (1761 cases until December 2015) (Carneiro and Travassos, 2016). These three infections have similar symptoms that complicate the diagnosis, including fever, headache, nausea and joint pain. In some cases, dengue infection develops into a dengue hemorrhagic fever. The last is characterized by bleeding and a reduction in platelet number in the blood that can lead to death (Patterson et al., 2016).

The expansion of these diseases is related to the increase in the incidence of their vectors and the unavailability of the vaccines. The treatment is preventive and the control is focused especially in reducing the population density of the vector with synthetic insecticides (e.g. pyrethroids) in water, where the larvae develops, or spraying in the air, for mosquito control (Guzman et al., 2010; Smith et al., 2016; Ministério da Saúde, 2014). The insecticides that act on larvae stage have received attention since they prevent the emergence of the adult form, limiting them to their breeding sites (Farnesi and Valle, 2013). However, the mosquito and larvae resistance to pyrethroids (Smith et al., 2016) and environment toxicity (Bradberry et al., 2005; Antwi and Reddy, 2015) of the synthetic chemicals are limiting their use. To circumvent this fact, in the last years the researchers have focused on natural products as alternative insecticides (biopesticides), since they are biodegradable and potentially present very low or absence of toxic effects on non-target organism, when compared to the synthetic insecticides (Oliveira et al., 2017). The biopesticides can lead to death due to their ability to act by different mechanism, for example by acting on GABA or mitochondrial systems, or inhibiting acetylcholinesterase (Rattan, 2010). Moreover, they may act through interference on the growth and reproduction of the pest (Copping and Menn, 2000).

The co-evolution between plants and their natural enemies has produced a great variety of herbal defensive secondary metabolites. In fact, plant extracts, essential oils and isolated compounds, such terpenoids (Botas et al., 2017), alkaloids (Kim and Ahn, 2017) and flavonoids (Perumalsamy et al., 2015) have been described as larvicides and mosquito repellents. Flavonoids are polyphenolic compounds which are related to the plant defense against UV radiation, fungi, bacteria and insects (Treutter, 2005).

Quercetin (QUE) is a naturally occurring flavonol that is abundantly present in food and medicinal plants (Petersen et al., 2016; Garzón et al., 2017). Several studies pointed out the beneficial biological activities of quercetin, which includes antioxidant, anti-inflammatory and antitumor properties (Naderi et al., 2003; Mamani-Matsuda et al., 2006). It also presented larvicidal properties against bollworm (*Helicoverpa armigera*) and melon fruit fly (*Bactrocera cucurbitae*) (Li et al., 2016; Sharma and Sohal, 2013). However, several natural compounds, including non-glycosylated flavonoids such quercetin, present low solubility in water and therefore the development of water-soluble products is a critical point.

A strategy to allow the dispersion of this product in water is the preparation of nanotechnology-based formulations, such as nanosuspensions (NS). They are defined as active solid substances nanometrically dispersed in aqueous media using surfactants as stabilizers (Lefevre et al., 2016) and can be produced by different methods. In the bottom up technology, the compound is dissolved using an organic solvent and it is subsequently precipitated through the addition of an anti-solvent in the presence of a stabilizer. This method is similar to the emulsification method and a further step for removal of the organic solvent is need. The top down technology involves the disintegration of the bulk material into nanostructures and a high-energy input (e.g. high-pressure homogenization) (Patel and Agrawal, 2011). Recent studies in the literature shows the high insecticidal efficacy of aqueous nanodispersions containing herbal bioactive compounds against mosquito larvae produced by a bottom-up method (Botas et al., 2017).

Therefore, the preparation of a quercetin nanosuspension (NS-QUE) constitutes an alternative to the synthetic insecticides that are used in the pest control. However, to the best of our knowledge, there is no data concerning the effects of NS-QUE on *A. aegypti*, despite the insecticidal activity of this compound against other pest insects. Thus, it is worth mentioning the relevance of investigating the effects of bulk-QUE (named as "free-quercetin", which can also be defined as its non-nanostructured form and being a typical and common flavonol aglycon with no further modification) and NS-QUE on *A. aegypti* and evaluating their environmental toxicity in a non-target organism (green algae *Chlorella vulgaris*), which were the aims of the present study.

Materials and methods

Chemicals

Quercetin was obtained from Henrifarma (SP, Brazil). The non-ionic surfactant polyethylene glycol 400 monooleate (PM 400) was obtained from Praid (SP, Brazil). The hydrophile-lipophile balance value of this surfactant is 11. Ethanol was purchased from Vetec (RJ, Brazil) and dimethyl sulfoxide (DMSO) was purchased from Labsynth (SP, Brazil).

Preparation of NS-QUE

The NS-QUE was prepared as described elsewhere (Lefevre et al., 2016) with some modifications. The organic to aqueous phase ratio was 1:10. Briefly, the organic phase was constituted by an ethanolic solution of quercetin. The organic phase was added at a constant flow rate with a syringe into the pre-heated (55 °C) aqueous phase containing 3% (w/v) of PM 400 under magnetic stirring for 10 min. Then, the organic solvent was evaporated under reduced pressure and the final volume was adjusted with water to a final quercetin content of 1 mg/ml.

Physico-chemical characterization

Quasi-elastic light scattering (Zetasizer 3000 HS, Malvern Instruments, UK) analysis of NS-QUE was performed at 90° in order to determine the mean hydrodynamic diameter and the polydispersity index of the particle population in three different NS-QUE batches. The zeta potential (ζ) analysis was also performed, using a Laser Doppler Anemometry (LDA) (Zetasizer 3000 HS, Malvern Instruments, UK). NS-QUE was diluted with deionized water (200-fold) prior to the measurements and the effect of storage temperature on NS-QUE particle size distribution and ζ was monitored at 4 and 25 °C for 30 days.

Aedes aegypti *larvae bioassay*

The tests were performed on the Laboratory of Arthropoda (Amapá Federal University, Brazil) in a room with standardized climatic parameters, kept at a 25 ± 2 °C temperature, relative humidity of 75 ± 5% and a 12-h photoperiod. The experimental evaluation was performed according to the World Health Organization protocol (WHO, 2005) with some modifications. NS-QUE or bulk-QUE was serially diluted with distilled water to final concentrations of 100, 175, 250, 375 and 500 ppm. Once bulk-QUE is not soluble in water, it was dissolved in DMSO prior to the assays. Each experiment (treated groups and control group) was conducted with (alongside) five replicates using ten early fourth-instar *A. aegypti* larvae in each container. The control group consisted of distilled water and larvae. Observations were made daily and the dead larvae were counted and removed after 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h of exposure.

Morphological assay

After the incubation with NS-QUE or bulk-QUE, died *A. aegypti* larvae were fixed on ethanol 70% and dried in the air. The dried larvae were observed using a low vacuum Tabletop Microscope TM3030Plus (Hitachi, Japan). The morphology of the *A. aegypti* larvae was also acquired by an optical microscope (Mod. BX41, Olympus) and photographed with a MDCE 5C camera.

Environment toxicology assay

The green algae *C. vulgaris* was isolated from water samples obtained at Lagoa dos Índios, situated on the municipality of Macapá (latitude 0.031368 and longitude 51.102559) and preserved in the LACAL (Unifap, Amapá, Brazil). Serial dilutions were carried out in order to isolate the colony and the cells were inoculated into nitrogen/phosphorus/potassium (NPK) media. The algae was incubated under cool white fluorescent light (12 h light:12 h dark) at a temperature of 20 °C and therefore the cells were counted using a Neubauer chamber. This organism was used as a non-target model for the environmental toxicology assay. The *C. vulgaris* inoculum was cultivated in NPK aqueous solution with initial cell density of 1×10^5 cell/ml for all tested groups. *C. vulgaris* was treated with different concentrations (100, 250, 375 and 500 ppm) of bulk-QUE or NS-QUE. The control group was constituted by NPK aqueous solution and the cell count was performed after 24, 48, 72, 96, 216, 240 h of incubation. The percentage of viable cells (%VC) was calculated as follows: $\%VC = (D/D_0) \times 100$, where: D is cell density before formulation addition, D_0 is cell density after at each specific day (Oliveira et al., 2017).

Statistics

Statistical analysis was carried out using the GraphPad Prism 6.0 Software (GraphPad, San Diego, CA, USA). The quantitative data were expressed as mean ± standard deviation. The means were compared using analysis of variance (ANOVA), followed by Tukey multiple-comparisons. Difference was considered significant when $p \leq 0.05$.

Results and discussion

NS-QUE physico-chemical characterization

Several plant-derived substances present low solubility in water, including quercetin, impairing their use in aqueous media. To circumvent this problem, nanosuspensions have been developed, due to the fact that reducing particle size is a promising way to improve dispersability of poorly soluble substances (Gao et al., 2011; Sun et al., 2010). NS-QUE was produced here by solvent displacement followed by solvent evaporation, a simple, rapid and low energy method of obtaining nanodispersions (Lefevre et al., 2016). The particle size distribution and zeta potential of NS-QUE were monitored at 25 and 4 °C just afterwards preparation (day 0) and after 1, 7, 14, 21 and 30 days of storage. The results are presented in Table 1. The particle size of the NS-QUE increased significantly after all time-points at 25 °C, ranging from 124.0 ± 1.1 nm just afterwards preparation to 347.2 ± 5.8 nm after 30 days of storage. These results are in accordance with the literature data for other quercetin nanosuspensions (Anarjan and Tan, 2013; Lefevre et al., 2016). However, when NS-QUE was kept at 4 °C the sizes remained around 123 nm for most time-points. The above results indicate better storage stability of NS-QUE at 4 °C than 25 °C. The increased stability at 4 °C storage condition may be due to the increased viscosity of the surfactant at this room temperature, therefore reducing the mobility the dispersed phase. Moreover, the fact that this surfactant can act as a plasticizer contributes to the lower aggregation along the time. Compounds with higher water insolubility would be more stable toward the Ostwald ripening phenomenon. Thus, considering that at a lower temperature the solubility will not be optimal on the external phase, a higher stability would occur. This interpretation is in accordance with our results. It can be also seen in the literature that the use of other methods, stabilizers and quercetin concentrations can alter the physico-chemical characteristics of the NS-QUE. Gao et al. (2011) obtained different nanosuspensions of QUE using more complex methods. The evaporation/precipitation into aqueous solution (EPAS) and the high pressure homogenization (HPH) process revealed sizes greater than 200 nm at the moment of production. Also, Sun et al. (2010) employed the tandem of nanoprecipitation (NP) and HPH method and obtained nanosuspensions of quercetin with sizes around 390 nm, indicating that the results found in this work are in accordance with the literature. A main advantage of the method used in the present study is related to the absence of high energy method and utilization of a green-solvent, instead of more toxic solvents, which were the main strategies on the aforementioned literature. Thus, it is in accordance with ecofriendly needs for vector control.

The polydispersity index (PI) reflects the homogeneity of particle size. It can be seen in Table 1 that significant effect on PI occurred along most time-points for the QUE-NS kept at 25 °C. This difference was not observed for most time-points at 4 °C. PI varied significantly from 0.328 ± 0.03 just afterwards preparation to 0.738 ± 0.02 after 30 days of storage at 25 °C. However, no major changes were observed in this parameter when NS-QUE was maintained at 4 °C, with PI around 0.35. These data indicate that the lower temperature is the best analyzed condition for NS-QUE stability. Despite the absence of PI data of NS-QUE stored at 4 °C, our results (PI < 0.500) are in accordance with Lefevre et al. (2016), who obtained PI values of 0.173 ± 0.013 after 7 days of storage of NS-QUE that were produced by emulsification/evaporation technique with ethyl acetate as organic phase and quercetin content ten-fold lower that those used in the present study.

In addition, the potential zeta was also monitored during storage time, presenting negative values. It is in accordance with the literature for others nanosuspensions of quercetin using different stabilizers and preparation methodology (Gao et al., 2011). The zeta potential of the NS-QUE increased significantly (regarding the absolute values) after all time-points at 25 °C, from −26.36 ± 0.61 just afterwards preparation to −31.26 ± 0.15 mV after 30 days of storage. However, it could not be seen significantly changes for its values at most time-points, when stored at 4 °C. The negative zeta potential may be due to the chemical nature of the particle/water interface composed of QUE and PM 400, once they possesses hydroxyl groups that can be deprotonated (Weiss-Angeli

Table 1
Physicochemical characterization of NS-QUE during storage at different temperatures.

Day	Size[a] ± SD(nm)25 °C	Size[a] ± SD(nm)4 °C	PI ± SD 25 °C	PI ± SD 4 °C	Zeta potential[b] ± SD(mV)25 °C	Zeta potential[b] ± SD(mV)4 °C
0	124.0 ± 1.1	123.8 ± 3.8	0.328 ± 0.03	0.332 ± 0.05	−26.36 ± 0.61	−25.83 ± 0.45
1	95.98 ± 0.2[c]	120.0 ± 1.1[c]	0.644 ± 0.00[c]	0.274 ± 0.00	−27.06 ± 0.41[c]	−28.9 ± 0.46[c]
7	136.7 ± 1.8[c]	126.5 ± 5.5	0.477 ± 0.04[c]	0.421 ± 0.01[c]	−28.3 ± 0.87[c]	−28.16 ± 4.63
14	168.0 ± 1.6[c]	133.2 ± 9.3	0.478 ± 0.05[c]	0.433 ± 0.03	−35.2 ± 1.60[c]	−28.66 ± 7.57
21	229.1 ± 9.2[c]	128.3 ± 8.8	0.367 ± 0.08	0.427 ± 0.00	−36.4 ± 1.46[c]	−25.96 ± 1.25
30	347.2 ± 5.8[c]	122.1 ± 6.8	0.738 ± 0.02[c]	0.401 ± 0.03	−31.26 ± 0.15[c]	−23.6 ± 3.34

SD, standard deviation.
[a] Standard deviation ($n = 3$) of the population that was reported by the instrument.
[b] Measurement after 1:200 dilution in water.
[c] Statistically significant compared to day 0 in the same group.

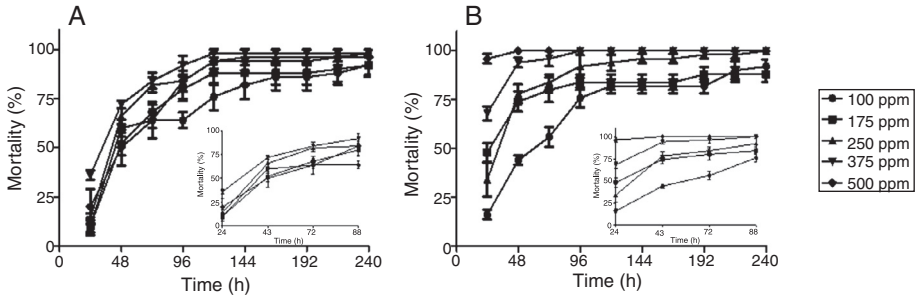

Fig. 1. Effect of bulk-QUE (A) and NS-QUE (B) on *Aedes aegypti* larvae after exposure to different concentrations (100, 175, 250, 375 and 500 ppm). Data are presented as mean ± SEM from three independent experiments.

et al., 2012). ZP value measured in this study was between −25 and −35 mV, being higher than 25 mV and therefore being in accordance with satisfactorily stability of colloidal systems (Patel and Agrawal, 2011).

Negative development effects of NS-QUE on Aedes aegypti *larvae*

The toxic effects of NS-QUE were compared with the bulk-QUE at various concentrations (100, 175, 250, 375 and 500 ppm) against the *A. aegypti* larvae and monitored for 240 h period. Our findings did not demonstrate a statistical correlation between the concentration of bulk-QUE and the time necessary to achieve the maximum effect (Fig. 1A). In addition, only in few cases a significant difference was detected between the activities of the different concentrations of bulk-QUE for each time. On the other hand, the required time to achieve the maximum larvicidal activity for NS-QUE was reduced as function of increased concentrations (Fig. 1B). In fact, while NS-QUE required 72 h to achieve maximum activity at 100 ppm, it induced this level of mortality in the first 24 h at 500 ppm. There was no statistical difference of the larvae mortality achieved between NS-QUE and the bulk-QUE on the concentrations lower than 375 ppm. However, NS-QUE at 375 and 500 ppm induced mortality values significantly higher than bulk-QUE. The significance of this difference was reduced with increasing exposure time until 120 h, being no observed difference after this period. NS-QUE showed a larvicidal action significantly higher than bulk-QUE in a time-dependent manner. The results indicate that the main differences between NS-QUE and bulk-QUE are not based on their maximum activities, but on the ability of NS-QUE to induce mortality faster than the bulk-QUE. In the case of the control group (water), no mortality was observed within 24 h and the development of larvae into pupae, and then adults, were considered normal (data not shown). *A. aegypti* larvae mortalities after 24 h of exposure to bulk-QUE at concentrations below 375 ppm were lower than 20%. However, at 500 ppm, the mortalities after 24 h and 48 h were 36.0 ± 5.5% and 72.0 ± 4.4%, respectively. For NS-QUE, these values were around 96% and 100% after the same time-points. Regarding 250 ppm, the values were 66.0 ± 8.9% and 78 ± 10% for bulk-QUE and NS-QUE, respectively. However no statistical significance was observed.

Satisfactory results for natural products-based larvicides in the literature are associated to samples that induce mortality levels higher than 75% at 250 ppm (Pimenta et al., 2006; Botas et al., 2017), as it can be seen for NS-QUE. The efficacy of products derived from plants against mosquito larvae can vary significantly depending on vegetal species, if the substances are isolated or not, the concentration of the active compound in the extract or oil and also the tested mosquito species. Sumroiphon et al. (2006) tested the *Citrus reticulate*, Rutaceae, ethanolic extract against *A. aegypti* and observed 75% of mortality after 24 h of exposure to a concentration around 3000 ppm. On the other way, Chaithong et al. (2006) found 76% of mortality of *A. aegypti* after 24 h of exposure to the ethanolic extract of *Piper longum* at 3 ppm.

Despite the fact that there is no data about the effect of nanocarriers prepared with QUE against *A. aegypti*, the higher mortality observed, when compared to bulk-QUE, is in accordance with Sugumar et al. (2014) and Anjali et al. (2010). They also found higher mortality levels for nanoemulsions, another type of nanostructure, against *Culex quinquefasciatus* mosquito larvae, when compared to their bulk formulations. This can be explained by the enhanced contact provided by the higher surface area of the nanostructured systems. The highest mortality was observed at the highest concentrations (375 and 500 ppm). For all surviving larvae that were incubated with bulk-QUE or NS-QUE, it was found that the samples adversely affected their development, avoiding the pupation and mosquito emergence. This fact is in accordance with literature data that revealed a development delay on larvae from *B. cucurbitae* after treatment with QUE (Sharma and Sohal, 2013).

The growth inhibitory effects on larvae incubated with QUE may be associated to problems on food consumption (Sharma and Sohal, 2013; Li et al., 2016). It was suggested that QUE oxidation by larvae generates reactive oxygen species that can degrade the nutritional quality of food present in gut lumen of the insects. It also affects transhydrogenase activity, impacting negatively their growth and

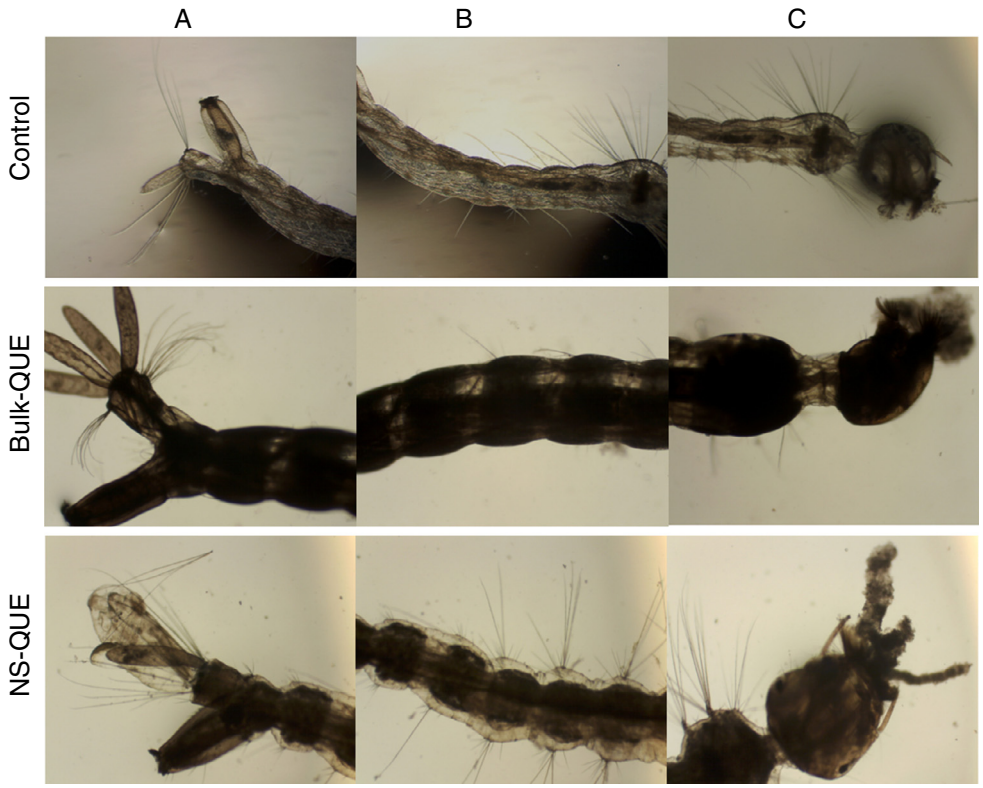

Fig. 2. Light micrograph of *Aedes aegypti* larvae. Control showing no alteration. Larvae treated with bulk-QUE or NS-QUE at 500 ppm showing alterations on cuticle of abdomen and anal papillae.

therefore leading them to death (Barbehenn et al., 2005; Vandock et al., 2012).

There is a lack of studies in the literature regarding comparison of the larvicidal activity of isolated substances and their nanostructure-based systems. To our knowledge, there is no data about herbal-derived larvicidal nanosuspensions containing only active compound/surfactant until now. However, other studies aimed to generate larvicidal herbal nanodispersions, including nanoemulsions, through the use of plant oils against *A. aegypti* (Rodrigues et al., 2014; Duarte et al., 2015; Oliveira et al., 2017; Botas et al., 2017). These studies demonstrated mortality levels higher than 80% after 48 h of incubation with concentrations ranging from 200 to 500 ppm. Thus, our results are in accordance with mortality levels of larvicidal herbal nanostructures.

Morphological evaluation of the larvae treated with 500 ppm of NS-QUE or bulk QUE revealed different degrees of toxicity, as can be seen in Figs. 2 and 3. *A. aegypti* larvae incubated with NS-QUE and bulk QUE exhibited morphological alterations, such as damaged anal papillae, distorted body, shrunken and darken body (Fig. 2). However, it can be seen that larvae incubated with NS-QUE was more intensely damaged than that incubated with bulk-QUE, while the larvae of the control group showed elongated and vermiform appearance with the well-defined body. *A. aegypti* darken body was also observed by Oliveira et al. (2013) when they were incubated with the essential oil of *Piper aduncum*, being this observation attributed to overlap of cuticle segments. Another study with pepper plants on *A. aegypti* also demonstrated extensive damage and shrunken cuticle of the anal papillae, which were associated to the larvae death (Chaithong et al., 2006). It is demonstrated in the literature that *C. quinquefasciatus* and *A. aegypti* larvae incubated with larvicidal nanoemulsions have been reported to have shrunken cuticle after the treatment (Botas et al., 2017; Oliveira et al., 2017). The observed alterations here may affect larvae development, contributing to the observed mortality and morphological changes in the larvae subjected to treatments. However, other factors can contribute for the larvicidal activity on larvae, such the degradation of food by reactive oxygen species in gut lumen induced by QUE (Li et al., 2016; Sharma and Sohal, 2013; Alves et al., 2010).

Ecotoxicity effects of NS-QUE on Chlorella vulgaris

The environmental toxicity test was performed using the green algae *C. vulgaris* in order to determine whether NS-QUE or bulk QUE would affect a non-target organism. It develops in aquatic ecosystems, being a useful model to evaluate NS-QUE and bulk QUE. This alga is a single-cell organism of rapid multiplication, being an excellent model of eukaryotic organism for environmental toxicity studies. It has green photosynthesis pigments and it is easy to grow and well-adapted to various conditions of development in fresh water (Simmons and Wallschläger, 2011; Zhang et al., 2016). The toxic effect is determined by the inhibition of algal biomass growth. The algae were submitted to different concentrations of bulk-QUE and NS-QUE and the cells were monitored during the period of 10 days. The algae culture treated with different concentrations of bulk-QUE and NS-QUE are shown in Fig. 4.

The algae growth ranged according to the sample (NS-QUE or bulk-QUE) and also the tested concentration. Lower toxicity was observed for the NS-QUE, while highest toxicity was observed for the bulk-QUE, especially at 375 and 500 ppm. It could not be seen significant statistical difference on algae viability when they were incubated with samples at 100 and 250 ppm. In addition, when *C. vulgaris* was incubated with NS-QUE at 375 or 500 ppm, the viability did not change significantly along the time. On the other hand, when the algae were incubated with bulk-QUE at 375 or 500 ppm, the viability decreased to approximately 16% after 10 days. It can be observed a statistically significant difference ($p < 0.01$) on algae viability when they were treated at 375 and 500 ppm of bulk-QUE

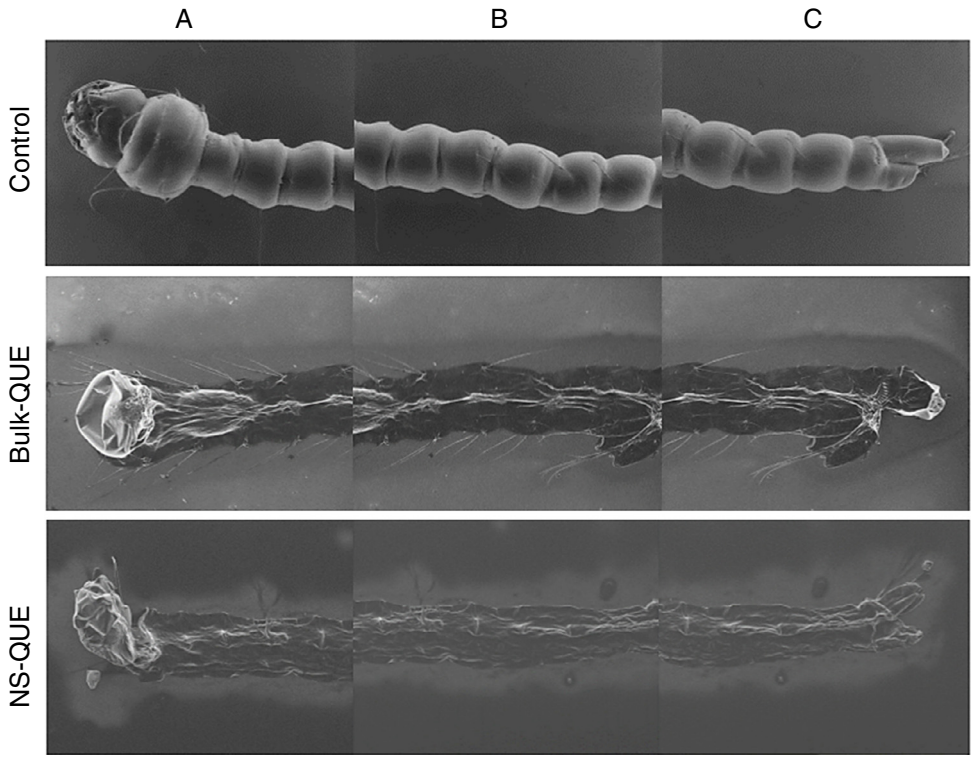

Fig. 3. Scanning electron micrograph of *Aedes aegypti* larvae. Control showing no alteration. Larvae treated with bulk-QUE or NS-QUE at 500 ppm showing alterations on cuticle of abdomen and anal papillae.

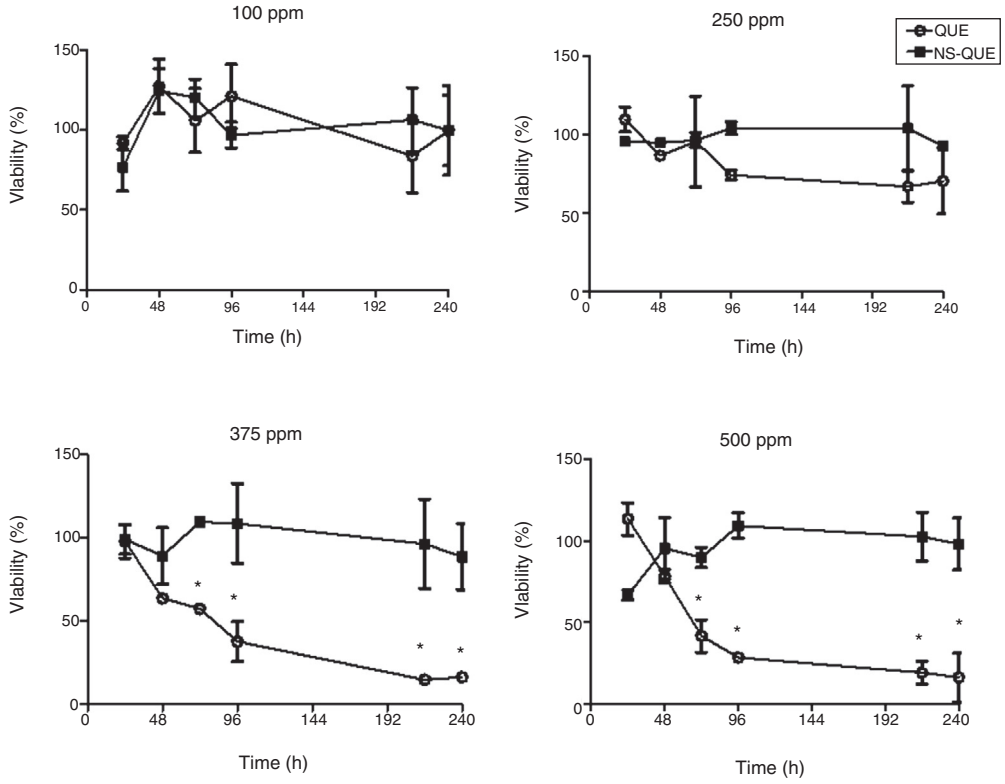

Fig. 4. Effect of NS-QUE or bulk-QUE on cell viability of *Chlorella vulgaris* algae after exposure to different concentrations (100, 250, 375 and 500 ppm). Data are presented as mean ± SEM from three independent experiments. Groups significantly different from the control group (QUE) are shown by $^{*}p < 0.05$.

(48 and 72 h), when compared to NS-QUE. These data are in accordance with Kovačević and Matulić (2013), who reported cell mortality from 10% to 46% when QUE was incubated for 48 h with other algae (green *Hydra*) at concentrations between 200 and 300 ppm. This mortality could be attributed to pro-oxidative processes and also to a decrease in the cell diameter, as well a change in chloroplast characteristics. These authors also observed that this flavonoid can stimulate or inhibit some effects on

organisms depending on its concentration (Kovačević and Matulić, 2013).

After 48 h of incubation with bulk-QUE at 500 ppm, it was observed the presence of a green precipitate. This may be an indicative of cell death, once it was observed a loss of the typical green color of algae. The group undergoing treatment with NS-QUE at the same concentrations kept the cell population viable along the time. Although most of studies focuses on the advantages of the nanostructured products, their toxicity may cause greater impacts on the ecosystem (Polonini et al., 2015). Previous studies carried out with *Pterodon emarginatus* oleoresin-based nanoemulsion with pesticide activity also demonstrated some toxic effects on *C. vulgaris* algae with concentrations above 100 ppm (Oliveira et al., 2017). Suman et al. (2015) demonstrated that zinc oxide nanoparticles induced mortality on *C. vulgaris* with concentrations ranging from 50 to 300 ppm. These toxic effects can be due the release of polar molecules or ions with biological activity on these algae. In the present study, it was verified that the use of the NS-QUE induced lower toxic effects when compared to bulk-QUE against the *C. vulgaris* species, indicating that this nanoproduct would be more advantageous.

Conclusion

In conclusion, a NS-QUE was produced using a green solvent and by a low energy method, being in accordance with sustainability concepts. The homogeneity, the adequate size and the negative zeta potential along the time indicate that this formulation may be very potential for utilization as a system for better dispersability of QUE in water. NS-QUE altered the development of *A. aegypti* at all investigated time-points, inducing them to death especially at higher concentrations. Thus, this nanocarrier can be considered a promising candidate for insects control once it is made of a natural product and well-studied substance. It may be applied without lasting environmental toxic effects on algae once this formulation demonstrated to be target specific and therefore it may be useful in the tropical vectors control.

Authors contribution

LZSP (MSc student) contributed in running the laboratory work, JLD contributed in nanosuspension characterization, RMAF contributed in the larvicidal assay, RASC and JCTC contributed in performing a critical reading of the manuscript, SMMF and AEMFMO contributed in the green algae assay design and analysis of this data, CPF contributed in analysis of the data and revision of the manuscript, RPS and RSA contributed as advisors of the MSc student, designing the study, supervising the laboratory work, analyses of data and drafted the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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