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Effects of cashew nut shell liquid (CNSL) component upon *Aedes aegypti* Lin. (Diptera: Culicidae) larvae's midgut

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The cashew nut shell liquid (CNSL) has been associated with a number of biological activities. The aim of this study was to evaluate the insecticidal potential caused by CNSL from *Anacardium occidentalle* L. (Anacardiaceae) upon *Aedes aegypti* and verify histomorphological alterations in the larval midgut. The experiments were carried out using third instar *A. aegypti* larvae, exposed to CNSL at different concentrations. After 24 h, the larvae were treated and stained with hematoxylin-eosin (HE). Morphometric analyzes were performed on the *A. aegypti* larvae midgut and registered by photomicroscopy. Anacardic acid was identified in CNSL by high performance liquid chromatography (HPLC) and showed 69% purity. The minimum concentration of CNSL that promoted mortality of *A. aegypti* larvae (LC_{10}) was 0.01 mg mL^{-1}; the LC_{90} was 0.139 mg mL^{-1} and the LC_{50} was 0.07 mg mL^{-1}. Changes in the midgut were severe in larvae treated with CNSL, especially at concentrations of 1.0 to 0.01 mg mL^{-1}; degeneration of the lining, hypersecretion of epithelial cells, increased vacuoles, and separation of the epithelial cells from the basal membrane, and disintegration of the brush border and damage of the peritrophic membrane occurred. CNSL caused damage to the midgut of 3rd instars of *A. aegypti* by irreversibly disrupting their complete larval development.

Key words: Dengue fever, bioinsecticids from plants, morfology of midgut.

INTRODUCTION

Aedes aegypti (Lin, 1762) is the vector mosquito that has has the highest current dispersion in urban areas over the world and the medical importance of its vectorial capacity is to disseminate four serotypes of dengue virus as well as yellow fever. The insect's diurnal haematophagic activity, the synanthropic behavior and

anthropophilic habits associated with the physical complexity of urban centers have hampered its combat (Bessera et al., 2009). Each year infections with dengue virus are estimated to be responsible for more than 100 million of classic cases and more than 500 thousand cases of dengue hemorrhagic fever worldwide (Halstead et al., 2007). In Brazil Dengue fever has reached about 3 million cases since 1986.

The states with the highest incidence were Acre (3619.5 cases per 100 000 inhabitants), Mato Grosso do Sul (2,521.1 cases per 100 000 inhabitants), Goiás (1353.1 cases per 100 000 inhabitants), Rondônia (1256.4 cases per 100 000 inhabitants), Roraima (1146.9 cases per 100 000 inhabitants) and Mato Grosso (1,095.5 cases per 100 000 inhabitants). These six states account for 75% of cases in Brazil (Oliveira et al., 2011). Synthetic insecticides, such as pyrethroids and organophosphates, are commonly used to control mosquitoes, but this application exposes operators and environments. It also leads to the development of mosquito resistance to the compounds (Rodriguez et al., 2007). Chemical control with organophosphates was shown to be inefficient in fighting the mosquito, and among the organophosphates, temephos was reported as Aedes-resistant by Melo-Santos et al. (2010). The search for new substances such as those extracted from plants has received special attention for natural molecules. Complex extracts certainly reduces the risk of resistance and the environmental toxicity (Wandscheer et al., 2004). According to Phukerd and Soonwera (2013), the use of plants and active molecules like proteins or secondary metabolites could act against insect defense mechanisms, causing them harm by inhibiting the digestive process and/or altering their metabolisms.

Species from the botanical family Anacardiaceae, in special genus *Anacardium,* are currently being investigated for this purpose. Furthermore, *Anacardium occidentale* L. (cashew tree) stood out among the 11 species under investigation in recent years, certainly due to its high composition of phenolic lipids with antioxidant, antigenotoxic, cytostatic (Stasiuk and Kozubel, 2010) and insecticide activities (Asogwa et al., 2007), among others. These compounds, namely anacardic acids, constitute about 90% of the cashew nut shell liquid (CNSL) and is the main industrial and manufactured byproduct of this resinous plant (Mazzetto et al., 2009). A significant number of biological activities is credited to this acid, among them is the insecticidal effect on *A. aegypti* (Laurens et al., 1997; Lomonaco et al., 2009; Mukhopadhyay et al., 2010; Oliveira et al., 2011).

The aim of this study was to evaluate the insecticidal potential of anacardic acid, a CNSL component from *A. occidentalle* L. (Anacardiaceae), upon *A. aegypti* and verify the histomorphological alterations in the larval midgut.

MATERIALS AND METHODS

Source of plant material

The CNSL was provided by Kardol Chemical Industry, Campo Grande, Mato Grosso do Sul - Brazil, from the endocarp of the A. *occidentale*. The extraction of LCC is occurred with company standard and patented methodology.

High performance liquid chromatography (HPLC)

The CNSL was further submitted to HPLC (Varian 210, Diode Arrangement Detector) and software Star WS (workstation) was used. The columns used were reverse phase C-18 (Phenomenex) and the pre-column C-18. The elution process was carried out with a graded program of solvent A: acetonitrile/water/acetic acid; and B: 100% of tetrahydrofurane. The pump flow rate was 1 mL min^{-1} and 20 µL were injected (22°C).

Identification of the compound with the aid of DAD detector scanning in the spectral range of 200 to 800 nm did not reveal interferences in retention time of the sample in HPLC by the developed elution method. Standard was easily identified and quantified based on their absorption spectra in the UV region and in retention time. Standard found in extracts were unambiguously identified by performing co-injection experiments in which aliquots of the extract and standard were mixed and diluted to a known volume, and analyzed through HPLC. The content estimation of the anacardic acid (≥85%, Sigma Aldrich) in the CNSL was performed by external calibration.

Bioassays

The experiments were performed in the Laboratories of Entomology of Dom Bosco Catholic University (UCDB), Campo Grande, MS. Larvae of late 3^{rd} and early 4^{th} instar of *A. aegypti* were used in the tests, from mass rearing of eggs aged from two to three months after oviposition, obtained from successive generations *in vitro* maintained with blood meal for one hour and half every two alternate days in the presence of partial light and temperature between 25 and 30°C according to a validated method of Porto et al. (2013) for conditions established by the use of biological oxygen demand (BOD).

In order to confirm, susceptibility tests were performed with concurrent positive control of Rotenone concentrations ranging from 0.005 to 0.020 mg mL^{-1}. The larvicidal biotest was performed in quadruplicates using four different concentrations of CNSL: 1.0, 0.1, 0.01 and 0.001 mg mL^{-1}, in batteries of tests at different times, because the product is only 69% of anacardic acid and a mixture of

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Abbreviations: CNSL, Cashew nut shell liquid; **HE,** hematoxylin-eosin; **HPLC,** High performance liquid chromatography; **BOD,** biological oxygen demand; **LD,** lethal doses.

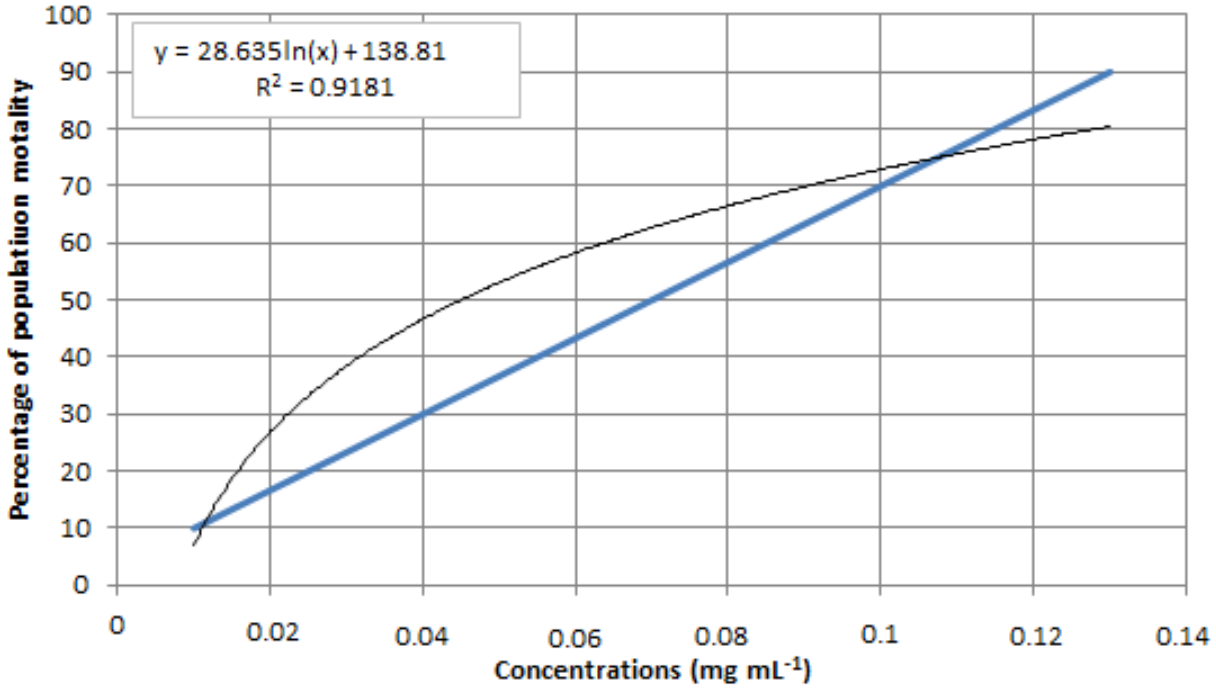

Figure 1. Lethal concentrations (mg mL^{-1}) of different concentration of CNSL.

several minor components. Twenty-five larvae were exposed to each specimen in the test solutions (25 mL) for 24 h, this is an acute test; after this period, the number of dead insects and survivors through stimulation with Pasteur pipette was observed. Individuals that did not move after stimulation were considered dead. Concentrations with the highest mortality were submitted to calculate the concentrations of larvicide Lethal Doses (LD).

Statistical analyses

The results obtained were statistically analyzed by the Probit method (Mclaughlin et al., 1991), using software Leora® (Polo 9735947870655352). The following standard doses prior dilution 1:10 considering the highest and the lowest toxic dose and four doses between 0 and 100% effect were used. Then, parametric analysis was performed by the probit method, using a random variable associated with a cumulative probability of response between binary zero (0) and one (1). Once the calculation is set at X2 or chi-square where defines the occurrence of two different variables, and calculating to compare proportions by dispersing 90, 95 and 99% confidence.

Histology

The larvae were left standing in their respective concentrations for 24 h before further analysis was done at the darkened cephalic capsule at room temperature in formaldehyde (4%) for a period of 2 h. These samples were processed in increasing concentrations of ethanol and embedded in paraffin, sectioned at 5 µm and stained with hematoxylin-eosin (HE). The morphometric analyzes were performed in the midgut of the *A. aegypti* larval digestive system, and captured in photomicroscope by Samsung® video camera, coupled to microscope Bioval L2000C, using software IMAGELAB version 2.4.

RESULTS AND DISCUSSION

Composition of the CNSL and calculation of lethal concentrations (LC)

The product was provided by Kardol, in which HPLC analysis showed the presence of 69% of anacardic acid and the remainder as a pool of substances according to benchmark analysis. From the larval mortality curve ($Y=28.635\ln(x) + 138.8$, $R^2 = 0.9181$) in relation to increasing concentrations of CNSL (Figure 1), the minimum concentration capable of producing mortality (LD_{10}) was 0.01 mg mL^{-1} and the maximum concentration of toxicity (LD_{90}) was 0.13 mg mL^{-1}. The LC_{50} was calculated in the dilution range of about 0.07 mg mL^{-1} (Figure 2). Tests carried out with diluted CNSL caused mortality of dilution from 0.01 to 0.1 mg mL^{-1}, and the confidence interval for mortality is a significance level of $P < 0.05$. Anacardic acids and derivatives have been reported in a number of works as having insecticidal action, particularly for *A. aegypti* (Lomonaco et al., 2009). The results of this study demonstrate that *A. aegypti* larvae and pupae are highly susceptible to 0.012 mg mL^{-1} CNSL. Larvae and pupae of *Anopheles subpictus*, however, are susceptible to 0.038 mg mL^{-1} Cardanol/CNSL solution (Mukhopadhyay et al., 2010). The cardol (LC_{50} = 14.20 ± 0.62 mg mL^{-1}) and cardanol (LC_{50} = 32.90 ± 0.2 mg mL^{-1}) obtained from CNLS showed activity for *A. aegypti* (Lomonaco et al., 2009). According to Oliveira et al. (2011), the three components of CNSL demonstrated good larvicidal activity against A.

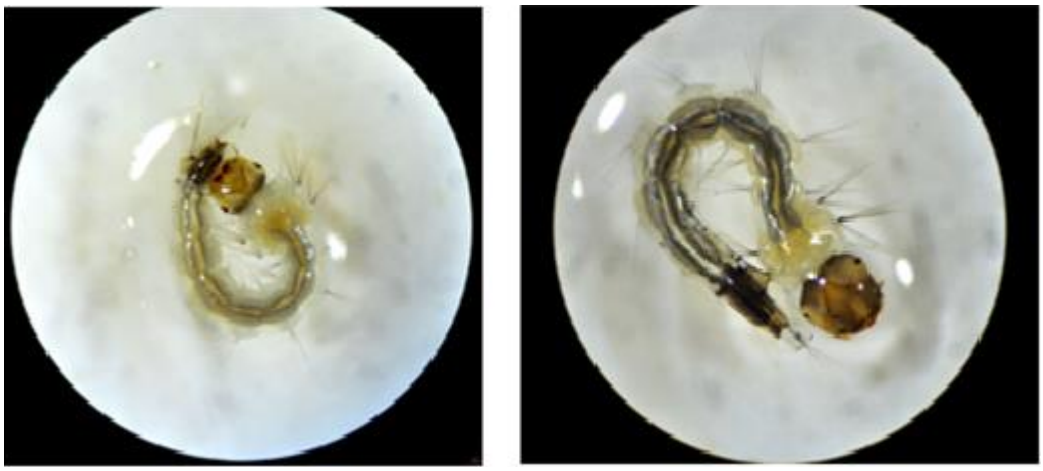

Figure 2. Larvae without treatment (A) and subjected to exposure of larvae active CNSL (B), 40x magnification.

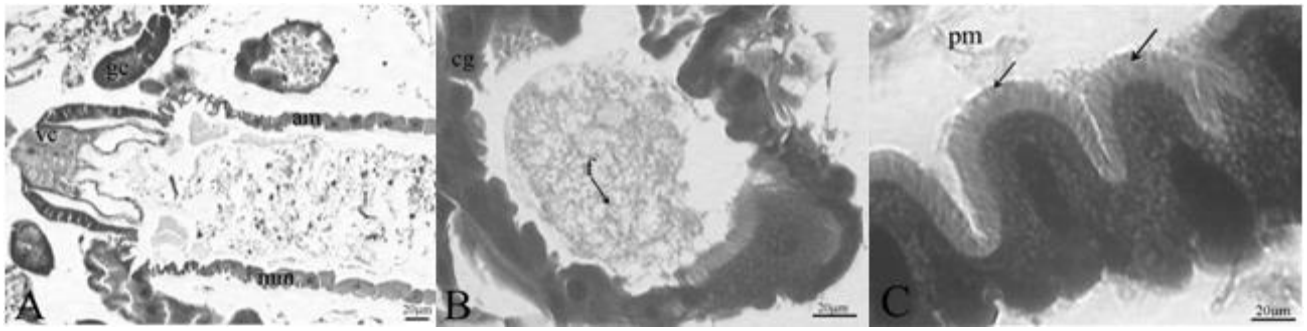

Figure 3. Photomicrography of 3rd instar larvae *Aedes aegypti* digestive system tissue. Control group. **A)** longitudinal section of gastric cecum, cardia valve (cv), anterior midgut (am), middle midgut (mm), lumen (L). HE. 100X. **B)** Transversal section of gastric cecum, ingesta (i), brush border (arrows), HE. 400X. **C)** Longitudinal section of posterior midgut (pm) and its brush borders (arrows), HE. 400X.

aegypti (LC_{50} = 12.40 mg L^{-1} for anacardic acid; 10.22 mg L^{-1} for cardol; and 14.45 mg L^{-1} for cardanol).

Histological analyses

The third instar *A. aegypti* larvae in the control group showed elongated and wormlike external appearance. The histological sections showed a normal morphology of stomodeum stomach and intestinal cecum (Figure 3AB). In the middle region of low cylindrical cells, acidophilic cytoplasm with variable areas, central spherical nucleus and clear nucleolus were seen. The brush border is thicker and marked (previous) and the peritrophic matrix (PM) is evident (Figure 3C). Morphological analysis in the treated group based on different concentrations of CNSL showed concentration of 0.001 mg mL^{-1} in three regions, cell shedding in the midgut, food debris and fragmentation of MP. The changes were apparent in gastric cecum, such as loss of brush border, and the absence of material ingested by the larvae in the lumen (Figure 4A). In the middle midgut, lining cells were rounded, with lots of side crossings and scaling, with a decrease in brush border and profuse secretion in the lumen (Figure 4B). The epithelium hindgut cells completely lost the lateral junction of flooring and base, resulting in detachment of the cells to the lumen of the hindgut. The brush border was evidenced in these cells (Figure 4C).

The transition zone between the anterior and medial regions were formed by throttling midgut with intestinal obstruction at a concentration of 0.1 mg mL^{-1} (Figure 5A); in the posterior region, the cells had elongated brush border. The deleterious changes included total or partial destruction of the lining cells, high vacuolization of the cytoplasm and increased subperitrophic space with

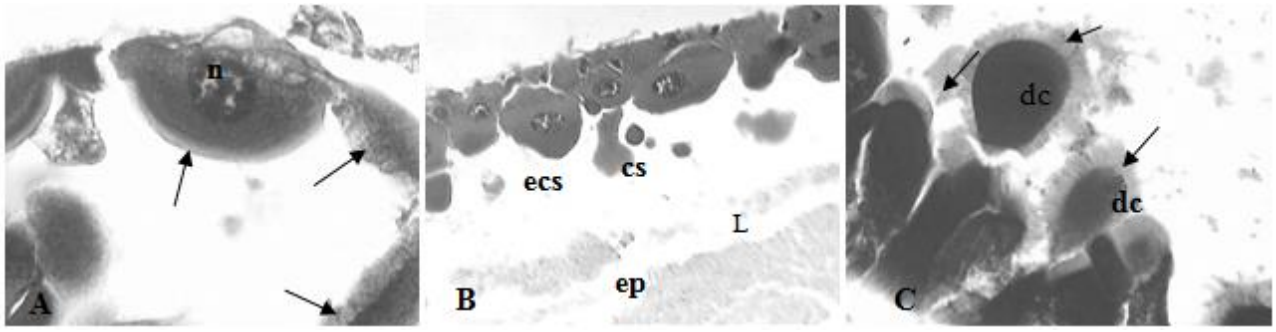

Figure 4. Photomicrography of 3rd instar *Aedes aegypti* larvae digestive system treated with *Anacardium occidentale* (Anacardic acid 0.01 mg mL^{-1}. **A)** Transversal section of gastric caecum, nucleus (n), brush border (arrows), HE. 400X. **B)** Longitudinal section of middle midgut, endoperitrophic space (ep), ectoperitrophic space (ecs), cellular secretion (cs), HE. 200x. **C)** Longitudinal section of posterior midgut, brush border (arrows), desquamation cell (dc), HE. 400x.

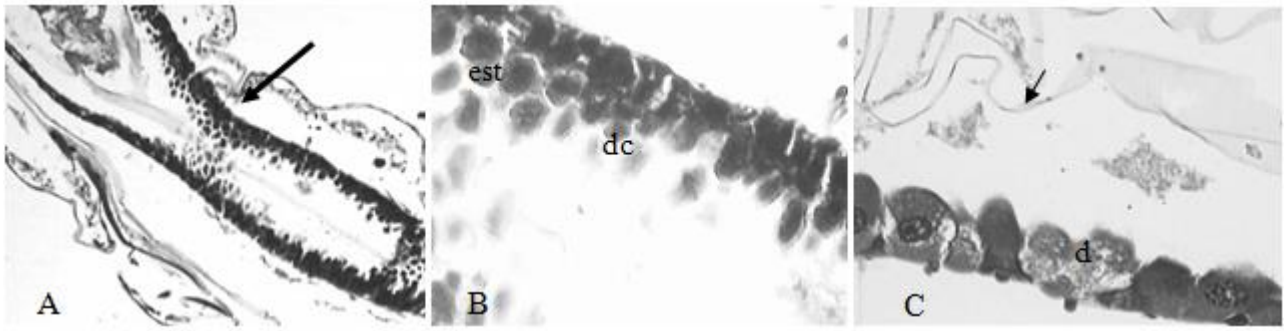

Figure 5. Photomicrography of Longitudinal sections from 3rd instar *Aedes aegypti* larvae digestive system treated with *Anacardium occidentale* - Anacardic acid 0.1 mg mL^{-1} (A, B) and 1.0 mg mL^{-1} (C). **A)** Strangulation between the anterior and middle midgut (arrow). HE. 200x. **B)** Stratification in the median midgut (est) desquamation cell (dc). HE. 400x. **C)** Degeneration of the median midgut (d), remnants of the peritrophic matrix (small arrow). 400x. HE.

accumulation of acidophilic materials (Figure 5C). The Culicidae midgut of the larvae comprises three regions (previous, middle and further) and is formed by a layer of epithelial cells lining integral supported by a cylindrical basement membrane. Like other insects, insect abdominal region, in addition to its digestive function, provides chemical defense function, mechanical protection and defense against pathogens (Roel et al., 2010). The first change caused by anacardic acid in *A. aegypti* larvae was related to movement, contrasting the control group that showed great mobility and locomotion in liquid medium. The larvae subjected to the highest concentrations of anacardic acid showed low resistance, lethargy and immobility, with an overall mortality after 24 h of exposure. By observing the tissue section, it was possible to detect the disconnection of cells lining the basement membrane and loss of intercellular junctions.

As for the mode of action of phenolic lipids against larvaer *A. aegypti*, few studies have clarified the toxicity mechanisms of these compounds in this organism. The deleterious changes caused by anacardic acid were observed in all three regions of the midgut of the larvae of *A. aegypti* and this activity may be related to the chemical structure of lipids phenolic. The aliphatic chain of anacardic acid provides a hydrophobic nature, which facilitates its permeation in the membrane of the cell wall of the larva (lipoprotein membrane), which flows through the apolar lipid bilayer of the group affecting the permeability of the cell and within groups polar (phenolic and carboxylic acid group) can act on the protein amino acid residues of the organisms disabling them, as can be observed in the changes described in Figures 3, 4 and 5, this substance affect primarily the midgut epithelium, and secondarily the gastric caeca and malpighian tubules, successfully described histopathological alterations showed that this substances affect primarily the midgut epithelium, and secondarily the gastric caeca and malpighian tubules. Successfully, described histopathological alterations after exposure of third-instar larvae of *A. aegypti* to LCC. Larval death was observed

by Valotto et al. (2010), using catechin and tannin 0.037 mg mL^{-1} of *Magonia pubescens* on larvae of *A. aegypti*. High cytoplasmic vacuolization, absence of boundaries and formation of apical vesicles were seen. The epithelial cells showed stratified, irregular nucleus and vacuoles.

Scudeler et al. (2014) define that the stratification and transformation of a simple columnar epithelium into a stratified epithelium, that is, new replacement of cell layers showed that a sophisticated defense may occur to replace the loss as a result of local lesions induced by cells acid. Costa et al. (2012), in studies on *A. aegypti* larvae exposed with methanolic extract of *Annona coriacea* (Magnoliales: Annonaceae), described serious histopathological changes, such as vacuolization of the cytoplasm, hypertrophy of epithelial cells and their nucleus, deterioration of the brush border, cell disintegration and apical vesicles formation that released their material in the intestinal lumen. In this work, we observed the same changes caused by anacardic acid, a phenolic lipid that constitutes the most of the liquid from the bark of the cashew nut. The changes described in this work are mainly caused due to a loss of control of the ionic balance and water intake that is commonly reported in treatments with oils, plant extracts and toxins (Scudeler et al., 2014). The loss of the cytoplasm due to the release of cytoplasmic protrusions may be indicative of a toxic effect of anacardic acid in the columnar cells.

Conclusion

The LCC from *A. occidentalle* caused changes in the midgut of *A. aegypti* larvae, triggering it irreversible damage. In the light of the changes caused by anacardic acid in the larvae, this product proved to be toxic and potentially able to act as a larvicide and lethal action for 90% of the population at a dose of 0.13 mg ml^{-1} after 24 h exposure *in vitro* conditions.

Conflict of interests

The authors did not declare any conflict of interest.

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