ecologies

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Ecotoxicological Evaluation of Products Obtained from Technical Cashew Nutshell Liquid (tCNSL) Proposed as Larvicide to Control *Aedes aegypti* (Diptera: Culicidae)

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Abstract: The development of new insecticides for vector control that are toxicologically safe and eco-friendly (such as those obtained from industrial by-products) is an important public health concern. Previous research has shown that the obtained tCNSL (technical cashew nutshell liquid) + NatCNSLS (sodium tCNSL sulfonate mixture) emulsion displayed both surfactant properties and larvicidal activity (LC50-24 h 110.6 mg/L). Thus, the emulsion is considered a promising alternative product for the control of *Aedes aegypti*. The goal of this study was an ecotoxicological evaluation of the tCNSL + NatCNSLS mixture emulsion and its components. In addition, we compared the toxicity of the tCNSL + NatCNSLS mixture emulsion with toxicity data from larvicide currently recommended by the World Health Organization (WHO). Ecotoxicological tests were performed to assess acute toxicity, phytotoxicity, cytotoxicity, genotoxicity, and mutagenicity using *Daphnia similis*, *Pseudokirchneriella subcapitata*, *Oreochromis niloticus*, *Allium cepa*, and *Salmonella enterica* serovar Typhimurium. Regarding acute toxicity, *D. similis* was the most sensitive test organism for the three evaluated products, followed by *P. subcapitata* and *O. niloticus*. The highest acute toxicity product was tCNSL. The tCNSL + NatCNSLS mixture emulsion did not show cytotoxic, genotoxic, or mutagenic effects, and showed low acute toxicity to *D. similis*. In addition, the tCNSL + NatCNSLS mixture emulsion presented a lower or similar toxicological classification to the larvicides recommended by the WHO. Therefore, ecotoxicological tests suggest that the tCNSL + NatCNSLS mixture emulsion can be considered a larvicide environmentally safe way to control *Ae. aegypti*.

Keywords: mosquito-borne diseases; vector control; industrial waste; ecotoxicity; genotoxicity

Citation: Jorge, M.R.; Merey, F.M.; Crispim, B.d.A.; Kummrow, F.; Barufatti, A.; Silva Dantas, F.G.d.; Oliveira, K.M.P.d.; Arruda, E.J.d. Ecotoxicological Evaluation of Products Obtained from Technical Cashew Nutshell Liquid (tCNSL) Proposed as Larvicide to Control *Aedes aegypti* (Diptera: Culicidae). *Ecologies* **2022**, *3*, 161–174. https://doi.org/10.3390/ecologies3020013

Academic Editor: Valery E. Forbes

Received: 27 January 2022
Accepted: 24 May 2022
Published: 31 May 2022

1. Introduction

Aedes aegypti (Diptera: Culicidae) (Linnaeus 1762) is the main vector responsible for the propagation of diseases such as Zika, Dengue, Chikungunya, and Yellow Fever [1,2], especially in tropical and subtropical countries such as Brazil [3,4]. To restrain the spread of these mosquito-borne diseases, strategies that focus on controlling mosquito vectors (adults and larvae) are essential tools and involve the use of synthetic insecticides [4–6].

However, the use of synthetic insecticides in vector control programs has presented problems, including induced resistance in different vector species, environmental pollution, and harmful effects on non-target species (including humans). These unwanted consequences have motivated the search for new toxicologically safe and eco-friendly insecticides, such as those obtained from plant-derived industrial by-products. By-products that carry high amounts of plant bioactive compounds are cheap and are often more effective at lower concentrations than currently used synthetic insecticides [1,5,7,8]. Ideally, new products should target insects at the larval stages in the breeding sites and stimulate public use to aid domestic vector control [9,10].

In this context, the technical cashew nutshell liquid (tCNSL), mostly composed of cardanol (60% to 75%), is a by-product obtained during the industrial processing (toasting at 180 °C to 190 °C) of cashew nuts (*Anacardium Occidentale* L.). The global production of cashew nutshells is estimated at ~3 million T/year and is concentrated in tropical countries in South America, Africa, and Asia [11,12]. Approximately 30% to 35% of this amount is cashew nutshell liquid (CNSL) [11]. In Brazil, approximately 1 million T/year of the tCNSL is generated [13] at a relatively low cost of 300 USD/T [14]. As cashew nut shells are obtained as a by-product from cashew nut processing factories, they do not compete with food production [11]. The wide applicability and economic feasibility of the tCNSL in several sectors suggests that their reuse has the potential to reduce environmental impacts [11,15]. These applications include their transformation for industrial purposes and technological applications, including the production of insecticides, resins, and plastics [16–19]. This industrial by-product has proven larvicidal and insecticidal activities against *Ae. aegypti* [9,17,19]. Unfortunately, the hydrophobicity of the tCNSL limits its use in aquatic environments, which are the breeding and development sites of *Ae. aegypti*. To use the tCNSL as a larvicide in aquatic environments, Jorge et al. [9] performed a tCNSL sulfonation reaction and obtained sodium tCNSL sulfonate (NatCNSLS mixture) with surfactant properties. However, the NatCNSLS mixture lost the larvicidal activity of the precursor (tCNSL). To recover the larvicidal activity and obtain a multifunctional product (such as household cleaning products), the authors chose to produce an oil-in-water emulsion composed of tCNSL and NatCNSLS mixture (1:6 *w*/*w*). The obtained tCNSL + NatCNSLS mixture emulsion showed both surfactant properties and larvicidal activity (LC50-24 h 110.6 mg/L). This is considered a promising alternative for the development of a new commercial product for *Ae. aegypti* control. Therefore, further ecotoxicological assays are recommended to assess the environmental safety of the tCNSL + NatCNSLS mixture [9].

The legal requirements for the registration of pesticides, which include eco/toxicological methodologies considered internationally valid and a minimum dataset necessary to carry out the environmental risk assessment (ERA) process, differ among countries [20]. However, one of the main shortcomings of the ecotoxicological dataset obtained using only internationally validated testing protocols (e.g., OECD Guidelines) is the assumption that few surrogate species are sensitive enough to protect other species in an environment [21]. Thus, ecotoxicological studies published in international peer-reviewed literature can be an important source of data for ERA that exploit novel test designs and report mechanistic information, as well as individual-, population-, and community-level responses in a range of different test organisms and environments [22].

Considering that tCNSL + NatCNSLS mixture presented larvicidal activities and surfactant proprieties, the next step to develop a new commercial product to control *Ae. aegypti* was to evaluate its ecotoxicological safety. Our main objective was to conduct an ecotoxicological assessment of the tCNSL + NatCNSLS mixture and its constituents separately. We performed acute toxicity, phytotoxicity, cytotoxicity, and genotoxicity tests using different test organisms. In addition, we compared the toxicity of the tCNSL + NatCNSLS mixture with toxicity data from the larvicides recommended by the World Health Organization (WHO).

2. Materials and Methods

2.1. Source of Tested Products

In this work, we evaluated the tCNSL + NatCNSLS mixture emulsion (1:6 parts active ingredient to the surfactant *w*/*w*), which were obtained as previously by Jorge et al. [9]. We also evaluated the individual components tCNSL and NatCNSLS mixture using the same ecotoxicological tests. The tCNSL was provided by Resibrás Cashol (Fortaleza, CE, Brazil). tCNSL is an industrial by-product, and the exact quantitative composition may vary, but its main constituents are cardanol (60% to 75%), cardol (15% to 20%), and methylcardol and polymeric materials (5% to 10%) [19,23,24].

2.2. Ecotoxicological Tests

2.2.1. *Pseudokirchneriella subcapitata* Growth Inhibition Test

The maintenance of cultures and toxicity tests with *P. subcapitata* were performed according to ABNT NBR 12.648 [25] and OECD 201 [26] guidelines. Algae cultures were maintained in an LC Oligo medium. Glass flasks containing 45 mL of culture media and algae inoculums (1×10^5 cells/mL) were exposed to five test concentrations of tCNSL (ranging from 0.01 to 10 mg/L), of a NatCNSLS mixture (ranging from 0.1 to 100 mg/L), and of a tCNSL + NatCNSLS mixture (ranging from 0.70 to 70 mg/L). Three replicates were obtained for each concentration. Negative (LC Oligo medium + deionized water) and solvent (LC Oligo medium + dimethyl sulfoxide-DMSO 1%) controls were included in each test. The experiments were performed in triplicate, under continuous agitation (160 rpm) and with continuous fluorescent light (4500 lux) at 25 ± 2 °C for 72 h. The endpoint measured was growth inhibition used to calculate the 50% inhibition concentrations (IC50-72 h).

2.2.2. Daphnia similis Acute Toxicity Test

Cultures were maintained, and acute toxicity tests with *D. similis* were performed following the ABNT NBR 12.713 [27] and OECD 202 [28] guidelines. The microcrustacean culture was maintained in an MS medium in climatic chambers at 20 ± 2 °C and 16 h light:8 h dark photoperiod, with a light intensity of 700 lux, and fed with *P. subcapitata* three times per week. The culture parameters were maintained as follows: dissolved oxygen, 7 ± 1 mg/L; hardness, 44 ± 4 mg/L $CaCO_3$; conductivity, 200 ± 50 µS cm; pH 7.0–7.6. Neonates aged <24 h from the same culture were selected as test organisms for the analysis. The neonates were isolated from the *Daphnia* culture and five organisms were placed in individual screw cap tubes filled with 10 mL of solution, and then incubated at 20 ± 2 °C for 16 h light:8 h dark photoperiods. The neonates were exposed to five test concentrations of tCNSL ranging from 0.0003 to 0.3 mg/L, of NatCNSLS mixture ranging from 0.1 to 15 mg/L and of tCNSL + NatCNSLS mixture emulsion ranging from 0.0021 to 2.1 mg/L. Negative (MS medium) and solvent (MS medium + DMSO 0.5%) controls were included in each test. All tests were performed with 20 neonates randomly distributed across four replicates (n = 5). All tests were performed with four replicates. Three independent tests were conducted and validated after 48 h of exposure. The number of immobile organisms in each test concentration was counted and used to calculate the effective concentration, 50% (EC50-48 h).

2.2.3. *Oreochromis niloticus* Acute Toxicity Test

Nile tilapia (*O. niloticus*) juveniles were acclimated for seven days in individual aquariums (10 L) with constant aeration. The parameters were maintained at a temperature of 26 ± 2 °C and a photoperiod of 12 h light: 12 h dark, and the fish were fed twice a day with granulated feed containing 28% crude protein (Laguna®, Lot 05EX180067109). Acute toxicity tests with *O. niloticus* were performed according to the ABNT NBR 15.088 [29] and OECD 203 [30] guidelines. The juvenile *O. niloticus* (average weight 1.0 ± 0.71 g) were exposed to five test concentrations ranging from 12 to 27 mg/L of tCNSL, 200 to 275 mg/L of NatCNSLS mixture, and 35 to 105 mg/L of tCNSL + NatCNSLS mixture emulsion. Negative (dechlorinated water) and solvent (dechlorinated water + DMSO 1%)

controls were included in each test. The test concentrations were randomly arranged into three replicates containing 10 fish each. The experiment was conducted in a static system without food for 96 h. After the exposure period, the number of dead organisms at each test concentration was counted and used to calculate the 50% lethal concentration (LC50-96 h).

2.3. Allium cepa Phytotoxicity, Cytotoxicity, and Genotoxicity Tests

The tests were performed according to Fiskejö [31] using seeds without pesticide treatment of *A. cepa* cv. Baia Periforme (Isla Sementes Ltd., Porto Alegre, Brazil). The seeds were exposed to five concentrations of tCNSL ranging from 27.5 to 440 mg/L, NatCNSLS mixture ranging from 165 to 2640 mg/L, and tCNSL + NatCNSLS mixture emulsion ranging from 192.5 to 3080 mg/L. For tests using the NatCNSLS mixture, dechlorinated water was used as the negative control. Tests with the tCNSL and tCNSL + NatCNSLS mixture emulsion included a negative control of dechlorinated water and 1% DMSO. For each concentration, 30 seeds of *A. cepa* were exposed and placed to germinate in a temperature of 23 ± 2 °C with a photoperiod of 12 h light:12 h dark for 96 h. All tests were performed in triplicate, totaling 90 seeds per concentration. Phytotoxicity was identified using mean root growth (MRG), measured with a Digimess® digital caliper after 96 h of germination. To determine the mitotic index (MI), chromosomal alteration index (CAI) (observed alterations: multipolar anaphases, chromosome breaks, metaphase C, adhesions, chromosome losses, and bridges), and mutagenicity index (MTI), the roots were collected and fixed in a Carnoy Solution 3:1 (alcohol/acetic acid) for 8 h, then transferred to a new Carnoy fixative, and stored at 4 °C. The fixed roots were washed with water and hydrolyzed in 1N HCl at 60 °C for 10 min. Afterwards, the cells were washed again and stained with Schiff's reagent for 2 h. After staining, the root meristems of *A. cepa* were placed on a slide and a drop of 45% acetic carmine was added, then the roots were covered with a coverslip and lightly smashed. For each concentration, five slides were prepared and photographed using a Nikon optical microscope at 400 x magnification and 1000 cells per slide were analyzed (n = 5000 per concentration). To calculate all indices, equations were used according to Francisco et al. [32].

2.4. O. niloticus Genotoxicity Test

For the genotoxicity evaluation with *O. niloticus*, eight juveniles (average weight 17 ± 10 g) were exposed for 72 h to test the tCNSL (6.36, 12.73, and 19.09 mg/L), NatCNSLS mixture (62.57, 125.15 and 187. 72 mg/L) and tCNSL + NatCNSLS mixture emulsion (10.36, 20.72 and to 31.08 mg/L). These doses corresponded to 25, 50, and 75% of the obtained LC50-96 h for each of the tested products. For the NatCNSLS mixture, the negative control used was dechlorinated water, and for the tCNSL and the tCNSL + NatCNSLS mixture emulsion, the negative control used was dechlorinated water + DMSO 1%. The positive control was cyclophosphamide (40 mg/kg) administered parenterally. Blood samples were collected in the caudal region, and blood smear slides were each prepared in triplicate. The slides were air dried for 15 min, fixed in absolute alcohol for 10 min, stained with Schiff's reagent and left overnight, and then counter stained with fast green for 10 s. For each site sample, 24,000 cells were analyzed. For the genotoxicity index (GI), we observed alterations, such as nuclear invagination, nuclear sprouting, pyknosis, binucleated cells, and lobulated nuclei. The frequency of micronuclei was counted and determined to obtain the mutagenicity index (MTI) [33]. Blood smear slides were analyzed using an optical light microscope (Nikon, at 400 × magnification. The comet assay was performed according to the methodology described by Singht et al. [34] with modifications. A 3 µL blood sample was collected from each fish (n = 8) at each concentration and diluted in 100 µL of PBS. We added 40 µL of this mixture to 240 µL low-melting agarose. After homogenization, two slides covered with 1.5% agarose were prepared for each sample. The slides were then placed in a lysis solution. Slides were denatured in an electrophoresis vat. Electrophoresis was performed at 37 V and 300 mA for 25 min. The slides were removed from the vat and immersed in a neutralization buffer. The slides were fixed in ethanol and in the sequence;

they were stained with ethidium bromide (2 mg/L). Five hundred nucleoids from each tested concentration were analyzed. The software Lucia Comet Assay v. 7.02 was used to evaluate the comets using the following parameters: percentage of DNA in the tail (CP) and size of the tail (TC).

2.5. Salmonella/Microsome Assay

Salmonella/microsome assay was performed using the microsuspension protocol [35]. Strains of *Salmonella enterica* serovar Typhimurium TA97a, TA98, TA100, TA1535, and TA102 were used. The samples were tested in the absence and presence of a metabolic activation system (S9 mix; Moltox Molecular Toxicology Inc., Boone, NC, USA). The tCNSL was dissolved in ultrapure water and 5% DMSO. The NatCNSLS mixture and the tCNSL + NatCNSLS mixture emulsion were both dissolved in ultrapure water and 1% DMSO. The tested concentrations of the three products ranged from 2 to 2000 mg/plate. For products demonstrating cytotoxicity, concentrations of 0.5 and 1 mg/plate were also tested. The negative controls used were ultrapure water + 5% DMSO (tCNSL) and ultrapure water + 1% DMSO (NatCNSLS mixture and tCNSL + NatCNSLS mixture). 2-aminoanthracene (2.5 µg/plate) was used as a positive control for all the strains in the tests performed with the S9 mix. In the tests performed in the absence of S9 mix, the positive controls used were 4-nitro-o-phenylenediamine (10 µg/plate) (TA97a and TA98), sodium azide (2.5 µg/plate) (TA100 and TA1535), and mitomycin C (0.5 µg/plate) (TA102).

2.6. Ecotoxicological Evaluation and Statistical Analysis

The results of the *P. subcapitata* (IC50-72 h) toxicity tests were analyzed using ICp software (version 2.0) [36]. The results of the *D. similis* and *O. niloticus* (EC50-48 h and LC50-96 h) toxicity tests were analyzed using the Trimmed Spearman-Karber statistical method [37] using the JSPEAR software. The I/E/LC50 values obtained were converted to toxicological units (TU) (TU = 100/I/E/LC50) [38]. The toxicological classification of the tested products was performed using the OECD protocol (2002) [39]. This methodology relies on the values of IC50, EC50, and LC50 for each test-organism and classifies the product under one of the three categories from the most to less toxic: Acute I ($\leq$1.0 mg/L); Acute II (>1.0–10.0 mg/L); and Acute III (>10.0–100.0 mg/L). To evaluate the cytotoxic and genotoxic effects obtained in the tests with *A. cepa* and *O. niloticus*, the normality of data was tested using the Shapiro–Wilk test. The Kruskal–Wallis non-parametric test with Dunn's posteriori was used to analyze the data. Differences between treatments were considered significant at $p < 0.05$. Statistical analyses were performed using the R platform (R-Development Core Team, 2021) [40]. The results of the *Salmonella*/microsome assay were analyzed using Salanal software (Integrated Laboratory Systems, Research Triangle Park, NC, USA). The mutagenicity ratio (MR) was calculated from the number of colonies induced by the sample and the number of colonies in the negative control. The samples were considered mutagenic when they had an MR $\geq$ 2 for at least one of the tested concentrations. Samples with MR < 2 at all tested concentrations were considered non-mutagenic, and the tested concentrations (MR < 0.7) were considered cytotoxic.

2.7. Classification of Acute Toxicity of the Larvicides Recommended by WHO and the tCNSL + NatCNSLS Mixture Emulsion

The search for scientific studies containing ecotoxicological data on larvicides recommended by the WHO was performed with the aid of the databases SciELO (Scientific Electronic Library Online), Scopus, ScienceDirect, and Google scholar. Only studies that presented results referring to acute toxicity (I/E/LC50) in different test organisms (Tables S1–S5) were included for comparison. The toxicity data retrieved from the literature were converted to TU. Toxicity classification was performed following the OECD protocol (2002) [39].

3. Results and Discussion

3.1. Acute Toxicity Evaluation with P. subcapitata, D. similis, and O. niloticus

The tCNSL showed the highest toxicity in all three test organisms. The NatCNSLS mixture was the least toxic product to *P. subcapitata* and *O. niloticus*. The tCNSL + NatCNSLS mixture emulsion was the least toxic product for *D. similis* and presented an intermediate toxicity for the other two test organisms (Table 1).

Table 1. The tCNSL, NatCNSLS mixture and tCNSL + NatCNSLS mixture Inhibitory (IC), effective (EC) and lethal (LC) concentrations 50% obtained with the three test-organisms used. In brackets the 95% confidence intervals.

Test-Organism		tCNSL (mg/L)	NatCNSLS Mixture (mg/L)	tCNSL + NatCNSLS Mixture (mg/L)
P. subcapitata	IC_{50}	0.33 (0.30–0.37)	7.10 (5.60–15.40)	2.10 (1.96–2.17)
D. similis	EC_{50}	0.12 (0.09–0.15)	0.75 (0.64–0.89)	1.05 (0.91–1.26)
O. niloticus	LC_{50}	25.46 (23.63–27.42)	250.30 (242.24–258.62)	41.44 (39.06–43.89)

The higher toxicity of tCNSL in relation to the other two tested products may be due to the unsaturation in the lipid phenol nonpolar tail, allowing them to interfere with cell membrane permeation [10,41]. The toxicity reduction observed by the NatCNSLS mixture in relation to tCNSL seems to be related to an alteration/reduction of nonpolar tail unsaturation after the sulfonation reaction. In the 1:6 (*w*/*w*) tCNSL + NatCNSLS mixture emulsion, there was dilution of the active ingredient tCNSL in the surfactant NatCNSLS mixture, which explains the lower toxicity observed for non-target test organisms.

To compare the acute toxicity data from different ecotoxicological tests with different aquatic organisms, the results were converted to TU [38]. This conversion made it possible to verify that *D. similis* was the test organism most sensitive to the toxic effects of all three tested products. The organism with the intermediate sensitivity was *P. subcapitata*. *O. niloticus* was the most resistant to all tested products (Figure 1).

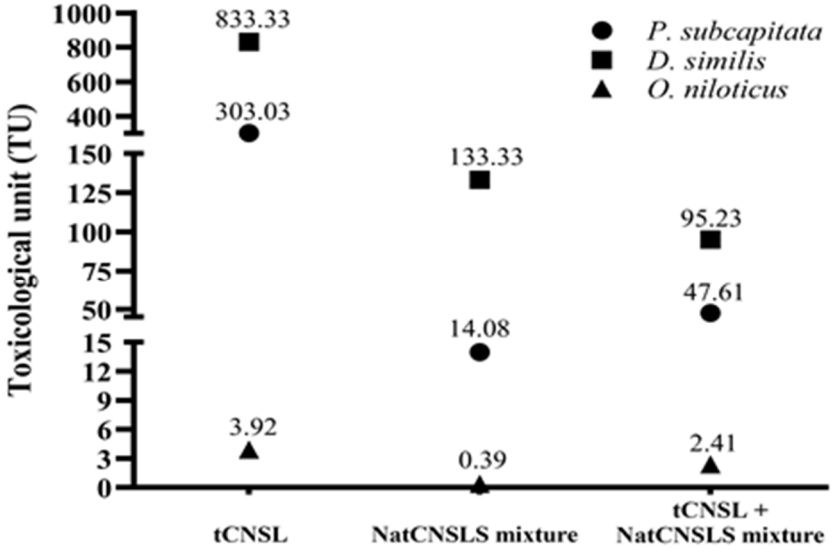

Figure 1. Comparison of the sensitivity of the test-organisms *P. subcapitata*, *D. similis* and *O. niloticus*. For this comparison, the acute toxicity data obtained for the three products tested were converted to toxicological units.

According to the classification from the OECD protocol (2002) [39], the tCNSL was classified as Acute I for *P. subcapitata* and *D. similis* and Acute III for *O. niloticus*. The NatCNSLS mixture was classified into Acute II for *P. subcapitata*, Acute I for *D. similis*, and Acute III for *O. niloticus*. The tCNSL + NatCNSLS mixture was classified as Acute II for

P. subcapitata and *D. similis* and Acute III for *O. niloticus*. Ecotoxicological evaluation of tCNSL and its products derived for aquatic organisms is scarce. However, Leite et al. [42] showed that the tCNSL is non-toxic to the marine organism *Artemia salina*. In general, the tCNSL + NatCNSLS mixture can be considered a less toxic product for nontarget aquatic organisms (Table 2).

Table 2. Acute ecotoxicological classification (OECD, 2002) comparison of the tCNSL + NatCNSLS mixture with the larvicides recommended by WHO.

Products	Acute Classification OECD (2002) in Each Biological Group		
	Algae	Crustacean	Fish
tCNSL + NatCNSLS mixture	II	II	III
Diflubenzuron	III	I	I and II
Novaluron	I and II	I	I, II and III
Pirimiphos-methyl	I	I	I and II
Pyriproxyfen	I	I	I
Temephos	-	I	I, II and III

- Data not found in the literature.

Our literature search retrieved 84 ecotoxicological datasets for algae, microcrustaceans, or fish of the larvicides recommended by the WHO [43] for use in combating *Ae. aegypti* larvae (Tables S1–S5). Of these, we refined our search to only acute toxicity data and classified them according to the OECD protocol (2002) [39]. Then, we compared the ecotoxicological classes of the tCNSL + NatCNSLS mixture emulsion obtained for algae, microcrustaceans, and fish with the ecotoxicological classes of larvicides recommended by the WHO [44] for each test organism.

Our comparison should be considered with caution because the effective dose as a larvicide for each product was not considered. We only considered the classification of acute toxicity for aquatic organisms, as proposed by the OECD protocol (2002) [39]. Therefore, the tCNSL + NatCNSLS mixture emulsion had a lower or similar ecotoxicological classification to the larvicides Diflubenzuron, Novaluron, Pirimiphos-methyl, Pyriproxyfen, and Temephos for algae, microcrustaceans, and fish. The only exception was algae, for which the tCNSL + NatCNSLS mixture emulsion was more toxic than Diflubenzuron (Table 2). However, ecotoxicological data on Diflubenzuron using algae are scarce, with only a single study available [43]. These results corroborate the literature that plant-derived insecticides, even those containing non-selective substances that can have a negative impact on non-target organisms, are less harmful to many non-target organisms and are more eco-friendly than synthetic insecticides [45].

3.2. Phytotoxicity, Cytotoxicity, and Genotoxicity Evaluation with A. cepa

None of the evaluated products were phytotoxic or cytotoxic to *A. cepa*. For endpoint genotoxicity, only the tCNSL + NatCNSLS mixture emulsion had a significantly lower frequency of the chromosomal alteration index (CAI) at concentrations of 192.5, 1155, and 3080 mg/L (Table 3). Despite the observed statistical difference, the tCNSL + NatCNSLS mixture emulsion is inferred not to be genotoxic because no dose–response relationship was observed and the frequency of changes in concentrations tested was lower than that in NC.

Our results corroborate the results obtained by Leite et al. [42]. Phytotoxicity, cytotoxicity, or genotoxicity was not observed in *A. cepa* bulbs exposed to tCNSL (maximum tested dose 69.5 mg/L). However, Matias et al. [46] showed that CNSL (composed of 53.2% anacardic acid) is phytotoxic to *Lactuca sativa* and *Lycopersicon esculentum* in seed germination and seedling development (root and aerial growth) and to *Senna obtusifolia* seedling development (aerial growth). These authors attributed the observed phytotoxicity to the presence of anacardic acid and cardol in the evaluated CNSL samples [46]. Thus, the absence of any phytotoxicity by the tCNSL + NatCNSLS mixture and its constituents can

be attributed, at least in part, to the composition of tCNSL (anacardic acid not detected, cardanol 51.97%, cardol 11.98%, 2-methyl cardol 2.12%) [9].

Table 3. Median and interquartile deviation from mean root length (CMR), mitotic index (IM), chromosomal alterations index (IAC) and mutagenicity index (IMT) obtained in tests with *Allium cepa* exposed to tCNSL + NatCNSLS mixture and its components (tCNSL and NatCNSLS mixture).

Products	Concentrations (mg/L)	Phytotoxicity Endpoints	Cytotoxicity Endpoints	Genotoxicity Endpoints	
		CMR (mm)	IM (%)	IAC (%)	IMT (%)
tCNSL	NC [1]	4.22 | 0.69	88.47 | 3.21	0.49 | 0.10	0.20 | 0.15
	27.5	4.42 | 0.57	95.44 | 1.64	0.10 | 0.10	0.00 | 0.05
	55	3.83 | 0.53	95.64 | 3.12	0.10 | 0.10	0.00 | 0.05
	165	3.87 | 0.96	96.79 | 0.83	0.20 | 0.14	0.30 | 0.33
	220	3.32 | 0.22	92.81 | 2.04	0.09 | 0.05	0.00 | 0.05
	440	2.52 | 0.73	95.12 | 7.72	0.00 | 0.05	0.00 | 0.05
NatCNSLS mixture	NC [2]	6.08 | 0.53	98.33 | 0.95	0.10 | 0.05	0.00 | 0.00
	165	8.13 | 1.73	93.00 | 0.98	0.20 | 0.34	0.00 | 0.39
	330	7.10 | 1.59	93.00 | 1.01	0.10 | 0.05	0.00 | 0.10
	990	5.98 | 1.24	93.00 | 0.82	0.20 | 0.19	0.20 | 0.09
	1320	7.10 | 2.24	93.72 | 1.41	0.59 | 0.15	0.30 | 0.29
	2640	6.83 | 1.20	94.26 | 1.09	0.57 | 0.24	0.10 | 0.10
tCNSL + NatCNSLS mixture	NC [1]	4.22 | 0.69	88.47 | 3.21	0.49 | 0.10	0.20 | 0.15
	192.5	5.71 | 0.75	94.08 | 2.57	0.00 | 0.05 *	0.00 | 0.05
	385	6.14 | 0.68	95.76 | 1.04	0.18 | 0.10	0.10 | 0.09
	1155	6.11 | 0.34	94.87 | 1.55	0.00 | 0.10 *	0.00 | 0.05
	1540	6.16 | 1.13	96.54 | 0.48	0.20 | 0.05	0.10 | 0.05
	3080	4.25 | 0.96	95.32 | 2.08	0.10 | 0.05 *	0.00 | 0.00

Medians followed by an asterisk (*) in the column, for each compound evaluated, represent statistical difference when compared with the negative control ($p < 0.05$). CN [1]—negative control (dechlorinated water + DMSO 1%); CN [2]—Negative control (dechlorinated water).

3.3. Genotoxicity Evaluation with O. niloticus

The tCNSL + NatCNSLS mixture emulsion and its constituents were not genotoxic at any of the endpoints evaluated in the *O. niloticus* tests ($p < 0.05$) (Table 4). Both micronucleus and nuclear abnormality tests are widely applied for genotoxicity assessment because of their proven suitability for different fish species.

The micronucleus test detects the aneugenic and clastogenic effects of a wide range of genotoxic chemicals. In addition, testing for nuclear abnormalities (notched, blebbed, lobbed, budding, fragmented nuclei, and binucleated cells) are considered high-quality indicators of cytotoxicity [47]. Thus, the tCNSL + NatCNSLS mixture emulsion proposed as a new larvicide is safe in comparison to the three other larvicides recommended by the WHO [44], which showed some type of genotoxic effect for different fish species. For example, Diflubenzuron (Dimilin®) that presented genotoxicity to *Prochilodus lineatus* at a concentration of 0.12 mg/L for the endpoint nuclear alterations and at a concentration of 0.25 mg/L for the endpoint micronucleus [48]. Moreover, the p-chloroaniline metabolite generated during the biotransformation of Diflubenzuron is considered potentially mutagenic and carcinogenic to humans and other animals [48]. Piriproxifen has also presented genotoxicity to *Danio rerio* embryos (96 h after fertilization) in the comet assay [49]. Temephos induced a marked increase in the frequencies of micronuclei (3-fold) and chromosomal abnormalities (10-fold in lobed nuclei) in gill erythrocytes of the fish species *Poecilia reticulata* after 168 h of exposure to a concentration of 0.02 mg/L [50].

Table 4. Median and interquartile deviation of the micronucleus index (MN), genotoxicity index (GI), DNA present in the tail (PT) and tail size (TS) data obtained in tests with *Oreochromis niloticus* exposed to tCNSL + NatCNSLS mixture and its components (tCNSL and NatCNSLS mixture).

Products	Concentrations (mg/L)	Chromosomal Alterations		DNA Damages	
		MN	GI	PT (%)	TS (μm)
tCNSL	NC [1]	0.00 | 0.00	1.79 | 1.66	38.53 | 1.91	18.37 | 0.44 *
	PC	0.03 | 0.00 *	6.86 | 0.61 *	59.95 | 4.81 *	45.22 | 1.23 *
	6.36	0.00 | 0.00	1.26 | 0.17	33.19 | 0.73	15.65 | 1.07
	12.73	0.00 | 0.00	2.89 | 0.67	33.78 | 0.17	22.15 | 1.27
	19.09	0.00 | 0.00	2.82 | 1.49	37.02 | 2.51	22.52 | 1.32
NatCNSLS mixture	NC [2]	0.00 | 0.00	1.80 | 0.37	34.45 | 1.57	10.67 | 0.52
	PC	0.03 | 0.00 *	6.86 | 0.61 *	59.95 | 4.81 *	45.22 | 1.23 *
	62.53	0.00 | 0.00	2.50 | 0.67	48.80 | 2.45	18.75 | 1.45
	125.06	0.00 | 0.00	1.97 | 0.27	49.51 | 0.66	17.69 | 0.86
	187.59	0.00 | 0.00	3.05 | 0.38	40.55 | 0.98	18.25 | 1.85
tCNSL + NatCNSLS mixture	NC [1]	0.00 | 0.00	1.79 | 1.66	38.53 | 1.91	18.37 | 0.44
	PC	0.03 | 0.00 *	6.86 | 0.61 *	59.95 | 4.81 *	45.22 | 1.23 *
	10.36	0.00 | 0.01	1.86 | 0.23	36.53 | 4.06	20.04 | 2.46
	20.72	0.00 | 0.01	2.18 | 0.03	27.23 | 0.79	19.81 | 1.02
	31.08	0.00 | 0.00	2.15 | 0.10	27.27 | 3.91	16.14 | 2.17

Medians followed by an asterisk (*) in the column, for each compound evaluated, present statistical difference when compared with the negative control ($p < 0.05$). NC [1]—negative control (DMSO 1%); NC [2]—negative control (dechlorinated water); PC-positive control (Cyclophosphamide (40 mg/kg)).

3.4. Salmonella/microsome Assay

As verified in the genotoxicity tests with *O. niloticus*, the tCNSL + NatCNSLS mixture emulsion and its constituents did not show mutagenic activity against any of the five *Salmonella* strains used (both in the absence and presence of S9 mix). For the tCNSL and NatCNSLS mixture, high toxicity was observed for TA1535, TA98, and TA102 strains. However, for the tests with the tCNSL + NatCNSLS mixture emulsion, toxicity was not observed; thus, the evaluation of the mutagenicity of the product proposed as larvicide was not impaired (Tables 5 and S5).

Table 5. Mutagenic activity of tCNSL + NatCNSLS mixture and its constituents (tCNSL and NatCNSLS mixture) expressed by the mutagenicity ratio (MR) for strains TA97a, TA98, TA100, TA102 and TA1535 in the absence (−S9) and presence (+S9) of metabolic activation.

Products	Concentrations (mg/plate)	TA97a		TA98		TA100		TA102		TA1535	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
tCNSL	**0.5**										
	1										
	2										
	20										
	200										
	2000										
NatCNSLS mixture	**0.5**										
	1										
	2										
	20										
	200										
	2000										
tCNSL + NatCNSLS mixture	**1**										
	2										
	20										
	200										
	2000										

Concentrations not tested ; Cytotoxicity (MR < 0.7) ; Negative (MR < 2) .

Genotoxicity assessments are an essential component of safety assessments for all substances intended for use as pesticides. In recent years, considerable efforts have been made worldwide to optimize strategies for in vitro genotoxicity testing. Thus, the standard in vitro test includes the *Salmonella*/microsome bacterial reverse mutation assay [51]. The absence of mutagenicity in all three products evaluated using the *Salmonella*/microsome assay is especially important if we aim to implement the tCNSL + NatCNSLS mixture emulsion as a multifunctional product to control *Ae. aegypti*.

4. Conclusions

The tCNSL was the most toxic (acute toxicity tests) product for all the non-target organisms evaluated. The NatCNSLS mixture was the least toxic product to *P. subcaptata* and *O. niloticus*. The tCNSL + NatCNSLS mixture emulsion was the least toxic product for *D. similis* which was considered the most sensitive test organism and had an intermediate toxicity for *P. subcaptata* and *O. niloticus*. Although the tCNSL + NatCNSLS mixture emulsion was toxic to aquatic non-target organisms, this effect was observed at concentrations below the LC50 obtained for larvicidal effects. Toxic levels of this product are unlikely to be found in the environment because of its dispersion and dilution capabilities in aquatic environments. None of the three evaluated products showed phytotoxic, cytotoxic, genotoxic, or mutagenic effects on *A. cepa* or genotoxic effects on *O. niloticus*. Despite the cytotoxicity observed in the *Salmonella*/microsome assay, we verified that the three tested products did not show mutagenic activity.

The tCNSL + NatCNSLS mixture emulsion was an effective larvicide against *Ae. aegypti* (LC50-24 h 110.6 mg/L) and can be considered less toxic or with the same degree of toxicity when compared to the larvicides used to control vector mosquitoes, as recommended by the WHO. Our data may contribute to the registration of this new product for use in Brazil to combat *Ae. aegypti*. According to the ecotoxicological evaluation, the tCNSL + NatCNSLS mixture emulsion is an effective product for the control of larvae and is environmentally safe, providing a new strategy for the control of vectors such as *Ae. aegypti*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ecologies3020013/s1, Table S1. Ecotoxicological assessment of Diflubenzuron in aquatic organisms [43,52–61], Table S2. Ecotoxicological assessment of Novaluron in aquatic organisms [62–65], Table S3. Ecotoxicological assessment of Pirimiphos-Methyl in aquatic organisms [66–73], Table S4. Ecotoxicological assessment of Pyriproxyfen in aquatic organisms [73–80], Table S5. Ecotoxicological assessment of Temephos in aquatic organisms [55,59,61,81–88]. Table S6. Mutagenic activity expressed by the mean of reversals/plate and standard deviation for the strains TA97a, TA98, TA100, TA102 and TA1535 of *Salmonella enterica* serovar Typhimurium after treatment with the LCCt emulsion in the absence (-S9) and presence (+S9) of metabolic activation system

Author Contributions: Conceptualization, M.R.J., B.d.A.C., A.B. and E.J.d.A.; methodology, M.R.J., F.M.M., B.d.A.C., F.K., A.B., F.G.d.S.D., K.M.P.d.O. and E.J.d.A.; validation, M.R.J., F.M.M., B.d.A.C., F.K., A.B., F.G.d.S.D., K.M.P.d.O. and E.J.d.A.; formal analysis, M.R.J., F.M.M., B.d.A.C., A.B., F.G.d.S.D. and K.M.P.d.O.; investigation, M.R.J., F.M.M. and B.d.A.C.; resources, B.d.A.C., A.B. and E.J.d.A.; writing—original draft preparation, F.M.M., B.d.A.C. and F.K.; writing—review and editing, F.M.M., B.d.A.C., F.K., A.B., F.G.d.S.D., K.M.P.d.O. and E.J.d.A.; supervision, A.B. and E.J.d.A.; project administration, B.d.A.C. and A.B.; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

Funding: We thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado do Mato Grosso do Sul (FUNDECT-020/2017, SIAFEM: 027251, Processo: 59/300.058/2017) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support.

Institutional Review Board Statement: The use of *Oreochromis niloticus* for the experimental procedures in this article was developed in accordance with the Ethics Committee on the Use of Animals/Universidade Federal da Grande Dourados (CEUA/UFGD), registered under protocol number 09/2017.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank Universidade Federal da Grande Dourados (UFGD, Brazil) for logistical support. We thank the Ecotoxicology and Genotoxicity Laboratory (LAEG) and Adria Caloto de Oliveira for the realizations the acute toxicity evaluation with P. subcapitata and D. similis. We thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado do Mato Grosso do Sul (FUNDECT) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support.

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

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