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The mixture of cashew nut shell liquid and castor oil results in an efficient larvicide against *Aedes aegypti* that does not alter embryo-fetal development, reproductive performance or DNA integrity

Juliana Miron Vani[1,2,3], Maria Tereza Ferreira Duenhas Monreal[2], Sarah Alves Auharek[4], Andréa Luiza Cunha-Laura[2], Eduardo José de Arruda[5], Alessandra Ramos Lima[5], Cicera Maria da Silva[5], Andréia Conceição Milan Brochado Antoniolli-Silva[1,3], Dênis Pires de Lima[2,6], Adilson Beatriz[2,6]*, Rodrigo Juliano Oliveira[1,2,3,7]*

1 Centro de Estudos em Células Tronco, Terapia Celular e Genética Toxicológica—CeTroGen, Hospital Universitário Maria Aparecida Pedrossian–HUMAP, Universidade Federal de Mato Grosso do Sul—UFMS, Campo Grande, MS, Brasil, **2** Programa de Mestrado em Farmácia, Faculdade de Ciências Farmacêuticas Alimentos e Nutrição—FACFAN, Universidade Federal de Mato Grosso do Sul–UFMS, Campo Grande, MS, Brasil, **3** Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina "Dr. Hélio Mandetta"–FAMED, Universidade Federal de Mato Grosso do Sul–UFMS, Campo Grande, MS, Brasil, **4** Faculdade de Medicina do Mucuri, Universidade Federal dos Vales do Jequitinhonha e do Mucuri–UFVJM, Teófilo Otoni, MG, Brasil, **5** Faculdade de Ciências Exatas e Tecnologia–FACET, Universidade Federal da Grande Dourados–UFGD, Dourados, MS, Brasil, **6** Instituto de Química—INQUI, Universidade Federal de Mato Grosso do Sul–UFMS, Campo Grande, MS, Brasil, **7** Programa de Pós-graduação em Genética e Biologia Molecular, Centro de Ciências Biológicas–CCB, Universidade Estadual de Londrina, Londrina, PR, Brasil

* adilson.beatriz@ufms.br (AB); rodrigo.oliveira@ufms.br (RJO)

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Abstract

Dengue fever, chikungunya fever and Zika virus are epidemics in Brazil that are transmitted by mosquitoes, such as *Aedes aegypti* or *Aedes albopictus*. The liquid from shells of cashew nuts is attractive for its important biological and therapeutic activities, which include toxicity to mosquitoes of the genus *Aedes*. The present study evaluated the effects of a mixture of surfactants from natural cashew nutshell liquid and castor oil (named TaLCC-20) on the mortality of larvae and on the reproductive performance, embryonic and fetal development and genetic stability of Swiss mice. A total of 400 *Ae. aegypti* larvae (third larval stage) were treated with TaLCC-20 concentrations of 0.05 mg/L, 0.5 mg/L, or 5 mg/L (ppm). Twenty pregnant female mice were also orally administered TaLCC-20 at doses of 5 mg/kg and 50 mg/kg body weight (b.w.), and 10 animals were given only drinking water at 0.1 mL/10 g b. w. (orally). The results of a larvicide test demonstrated that 5 mg/mL TaLCC-20 killed 100% of larvae within three hours, which is comparable to the gold standard indicated by the Ministry of Health. Overall, these results show that TaLCC-20 is an efficient larvicide that does not induce genetic damage. In addition, changes in reproductive performance and embryo-fetal development appear positive, and the formulation is cost effective. Therefore, TaLCC-20 is an important product in the exploration of natural larvicides and can assist in fighting mosquitos as vectors for dengue fever, chikungunya fever and Zika virus, which are

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emerging/re-emerging and require proper management to ensure minimal harm to the human population. Therefore, TaLCC-20 can be considered a key alternative to commercial products, which are effective yet toxigenic.

Introduction

Dengue is an epidemic in Brazil and worldwide. Coupled with chikungunya fever and Zika virus, these three diseases have caused extensive public health problems. In 2016, there were approximately 1,426,005 probable cases of dengue, including 798 severe cases and 7,105 suspected cases. In addition, dengue was responsible for 509 deaths [1].

In Brazil, 3,657 cases of chikungunya fever were diagnosed in 2014. In 2016, the number of cases increased to 216,102 autochthonous cases. Zika virus was first diagnosed in Brazil in 2015, when 1,248 cases were reported in the northeast, in addition to 739 cases of microcephaly in newborns, which characterizes the most serious form of the disease. In 2016, there were approximately 196, 976 probable cases of the disease [1–3].

Transmission of these diseases occurs through the mosquitoes *Aedes aegypti* and *Aedes albopictus* after a bite from an infected female. There is currently no effective vaccine for these diseases. Thus, the prevention of these diseases depends exclusively on the elimination of mosquito foci [4–6].

According to Machado et al. [7], the most promising results recorded to date regarding the prevention of increasing cases of dengue, chikungunya fever and Zika virus in Brazil were obtained with the use of diflubenzuron and, more recently, with pyriproxifen. Therefore, these are the compounds of choice indicated by the Brazilian Ministry of Health for use in fumigation and deposition in areas containing standing water, including water tanks used for human consumption. However, this practice is not recommended and should be discontinued, as the consumption of diflubenzuron causes genetic/genomic instability and increases predisposition to chronic diseases, such as cancer [8]. For pyriproxifen, there are no available studies on its mutagenicity and teratogenicity in the literature.

Accordingly, there is a critical need for new larvicidal compounds that do not exhibit toxicity to humans or the environment. One attractive organic raw material is cashew nut (*Anarcadium occidentale* L.) shell liquid (CNSL), which contains phenolic compounds with great biological potential, such as in treatments for asthenia, respiratory problems, genital infections, and skin diseases [9–12] and for use in larvicidal compounds [11, 13–16].

Larvicide activities against *Ae*. *aegypti* of technical CNSL, its main constituents cardanol and cardol, and their products of hydrogenation were evaluated. Structure-activity relationship studies revealed significant differences in larvicidal activity against *Ae*. *Aegypti* between technical CNSL and its main constituents. Technical CNSL presented an LC_{50} value of 51 ppm (μg/mL), whereas isolated cardol and cardanol showed LC_{50} values of 14.2 and 32.9 ppm, respectively. Therefore, we show that cardol is the constituent primarily responsible for the activity demonstrated by technical CNSL [13, 16].

Although the phenolic lipids isolated from CNSL show larvicide activity, these compounds do not dissolve in water. To solve this problem, Farias et al. [17] used sodium anacardate, isolated from natural CNSL, against *Ae*. *aegypti* larvae, obtaining an LC_{50} of 55.47 ppm. Mukhopadhyay et al. [18] tested cardanol emulsified in liquid vegetable soap against larvae of the same mosquito and calculated an LC_{50} of 12 ppm and an LC_{50} of 38 ppm for larvae of the *Anopheles subpictus*, while Raraswati et al. [19] studied the larvicide effect of an emulsion of natural CNSL with an extract of nut fruit soap (*Sapindus rarak* DC) against *Ae*. *aegypti* larvae in the 3rd instar stage and found an LC_{50} of 14.12 ppm.

In preliminary studies, Galdino; Beatriz [14] and Beatriz et al. [15] devised saponification reactions with these natural products to obtain water-soluble salts, aiming to develop a mixture of surfactants from CNSL and castor oil. The castor oil was employed together with CNSL as a vehicle to boost the surfactant effect of the mixture. Subsequently, the larvicide tests against *Ae. aegypti* showed that all compounds were active. The product termed Tensoativo do Líquido da Casca da Castanha do Caju (TaLCC-20) (CNSL:Castor oil 20:80 w/w) exhibited more effective activity at a concentration of 0.2 ppm, killing 97% of larvae within the first 24 hours, while the surfactant from only castor oil showed the lowest activity. That mixture presented efficient larvicide action and resulted in the registration of a patent [15]. Considering these results, the present study evaluated the effects of TaLCC-20 on the mortality of larvae of *Ae. aegypti*, Rockefeller lineage (*Ae*-Rockefeller), and on the reproductive performance, embryo development and genetic stability of Swiss mice.

Materials and methods

Extraction of cashew nut shell liquid

Cashew nut shell (CNS) was donated by Kardol Indústria Química in 2014. The plant material was verified by Msc. Juliana Miron Vani, and a voucher specimen was deposited (No. 51838) in the herbarium of the Federal University of Mato Grosso do Sul (UFMS). The extraction of natural CNSL from CNS was performed using the procedures of Gandhi et al. [20]. First, 250 mL of 95% ethanol was added to the round bottom flask of a Soxhlet apparatus. Then, 30 g crushed CNS was Soxhlet extracted for 6 h, after which the solvent in the thimble of the Soxhlet apparatus was colorless. Finally, the solvent was recovered from a simple distillation method. The natural CNSL was obtained as reddish brown phenolic oil (40% yields). The HPLC analysis was performed using a Shimadzu LC-6AD apparatus with a Diode Array Detector (SPD-M10Avp, Shimadzu). The analytical column was a Phenomenex Luna C18 column (4.6 × 250 mm, 5 μm). The mobile phase was methanol/water (95:5) at a flow rate of 1.5 mL/min. ^{1}H-NMR spectra were recorded in $CDCl_3$ (Tedia Brazil, Brazil) solution on a Bruker DPX300 spectrometer (300 MHz), and spectra was referenced to TMS using residual solvent signals as secondary standards. The ethanol employed for extraction of CNSL was AR grade (Vetec, Brazil), and the methanol used in HPLC was HPLC grade (Mallinckrodt, USA). HPLC-grade water (18 mW) was prepared using a Milli-Q system (Millipore). Solvents were filtered with spare membrane filters (0.2 μm, sterile).

Preparation of the sodium surfactant from a mixture of cashew nutshell liquid and castor bean oil (TaLCC-20)

For these procedures, the following reagents were used: absolute ethanol VTEC, p.a., ACS reagent, 99% purity; castor bean oil EXP type 01, batch M-05-04 Celtic; sodium hydroxide Dinâmica, p.a., ACS reagent, 97% purity; distilled water; and natural CNSL obtained via Soxhlet extraction. The common procedures for producing soaps from vegetable oils were used [15]. Approximately 33.0 mL of an aqueous solution of sodium hydroxide (12.6 g) was slowly added to an ethanolic solution of 20 g natural CNSL and 80 g castor bean oil (66.00 mL ethanol) while stirring. The resulting solution was stirred for 15 minutes. After this period, the reaction medium was protected from light until complete solidification.

Establishment and maintenance of *Ae. aegypti* Rockefeller colony

The prime matrices of *Ae. aegypti* eggs (Rockefeller line) were supplied by the Animal Biology Department, UNICAMP, Campinas-SP and Control of Endemia Superintendency (SUCEN),

Marilia-SP. The establishment and maintenance of the *Ae. aegypti* colony and the production of eggs were realized in a creation room of the LIVe laboratory (LIVe, Laboratory of Vector Insects–Biological Science University–FCBA, Federal University of Grande Dourados) at a controlled temperature of ± 28˚C, relative humidity of ± 60% and a programed photoperiod of 10 hours of dark and 14 hours of light.

For biological assay execution, *Ae. aegypti* eggs were supplied by LIVe.

Larvicide assay

The larvicide bioassay was carried out with 3rd-stage *Ae. aegypti* larvae of the Rockefeller lineage (*Ae*-Rockefeller). First, eggs (on filter paper) were placed in plastic trays containing a volume of distilled water greater than 1 mL *per* larva and macerated fish feed for larval hatching (Alcon Basic® Lot 162). After five days, the larvae reached the 3rd developmental stage. With a polyethylene Pasteur pipette, 20 *Ae*-Rockefeller larvae were transferred to a 50-mL beaker containing 20 mL of solution and 20 larvae. Four-hundred larvae divided into four replicates were subjected to each treatment: a negative control containing only distilled water, a positive control containing temephos at a concentration of 0.012 mg/L, and three concentrations of TaLCC-20 (0.05 mg/L, 0.5 mg/L, and 5 mg/L).

Residual effect test

The residual effect of TaLCC was assessed for the larvicide concentration (5 ppm—5 mg/L) and for a 10× higher dose (50 ppm—50 mg/kg). Drinking water was used for the control group, which was the TaLCC dilution vehicle.

The tests were performed in triplicate. Beakers were used with 30 mL of each solution containing 20 *Ae. aegypti* larvae of the Rockefeller line (Ae-Rockefeller) in the 3rd stage.

Larval mortality was assessed every 24 hours of exposure. Daily larval counting and replenishment continued until complete loss of the larvicide effect in the solution.

Preclinical trial

Experimental animals. Swiss mice (*Mus musculus*) (30 females and 15 males) of reproductive age (8–10 weeks) with an average weight of 30 g, obtained from the State Bureau of Animal and Plant Health Protection (Agência Estadual de Defesa Sanitária Animal e Vegetal—IAGRO), were used. This study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Mato Grosso do Sul (No. 401/2012).

The animals were maintained in propylene boxes; males were housed in insolation, and females were housed in pairs. The mice were allowed an adaptation period of seven days. The housing environment was an ALESCO® ventilated cabinet that was light and temperature controlled, with a photoperiod of 12 hours (12 hours of light: 12 hours of dark) and a temperature of 22 ± 2˚C. The mice were provided with commercial feed (Nuvital ®) and filtered water.

Overnight mating was performed at a ratio of 1 male: 2 females, and detection of pregnancy was based on vaginal plug formation (considered day zero of gestation) [21–25].

After vaginal plug formation, health and well-being of the animals were evaluated daily. The following clinical signs of toxicity were observed: mucosal dryness, walking alterations (locomotor hypo-activity and hyperactivity), behavioral changes, diarrhea, decreased food and water intake, eye opacity, hair bristling, tremors, and morbidity [26–28].

Experimental design

The pregnant females were divided into three groups (n = 10): control group animals received drinking water at 0.1 mL/10 g body weight (b.w.) orally (gavage) on each day of pregnancy (1st

to 18th); gestational group animals received TaLCC-20 at doses of 5 mg/kg (Gest. D1) and 50 mg/kg (Gest. D2) b.w. orally on all days of gestation (Fig 1).

The dose of 5 mg/kg (p.c., v.o.) was based on the larvicide dose, and the security dose was defined as 10× greater than the indicated for guidelines, i.e., 50 mg/kg (p.c., v.o.) [29,30].

Biological assays

Reproductive performance and embryonic and fetal development (teratogenicity). On the 18th day of gestation, the animals were euthanized, followed by laparotomy, hysterectomy and oophorectomy. Analgesia was not used to euthanize mice, as analgesia could affect the frequency of damaged cells in the micronucleus assay according to studies of Hoerauf et al. [31], Heine et al. [32], Kotani et al. [33] and Souza [34]. Thus, the pregnant female mice were euthanized by cervical dislocation.

The fetuses were euthanized using isoflurane, as only the tissues and bones needed to be preserved for subsequent analysis. The micronucleus assay was not performed for the fetuses.

The spleen, heart, liver, lungs and kidneys were collected and weighed, and the fetuses and placentas were also weighed. An external systematic analysis of the fetuses was performed to detect possible external malformations, and the sex of the fetuses was determined. The number of implantations, resorptions, and live and dead fetuses was recorded. Based on these data, fetal viability (number of live fetuses/number of implantations x 100), the post-implantation loss rate (number of implantations–number of live fetuses x 100/number of implantations), the resorption rate (number of resorptions x 100/number of implantations), the placental index (placental weight/fetal weight) and the sex ratio (number of male fetuses/number of female fetuses) were obtained [21, 23–25]. Then, the suitability of the observed fetal weight for the gestational age was determined according to Oliveira et al. [21], and the fetuses were

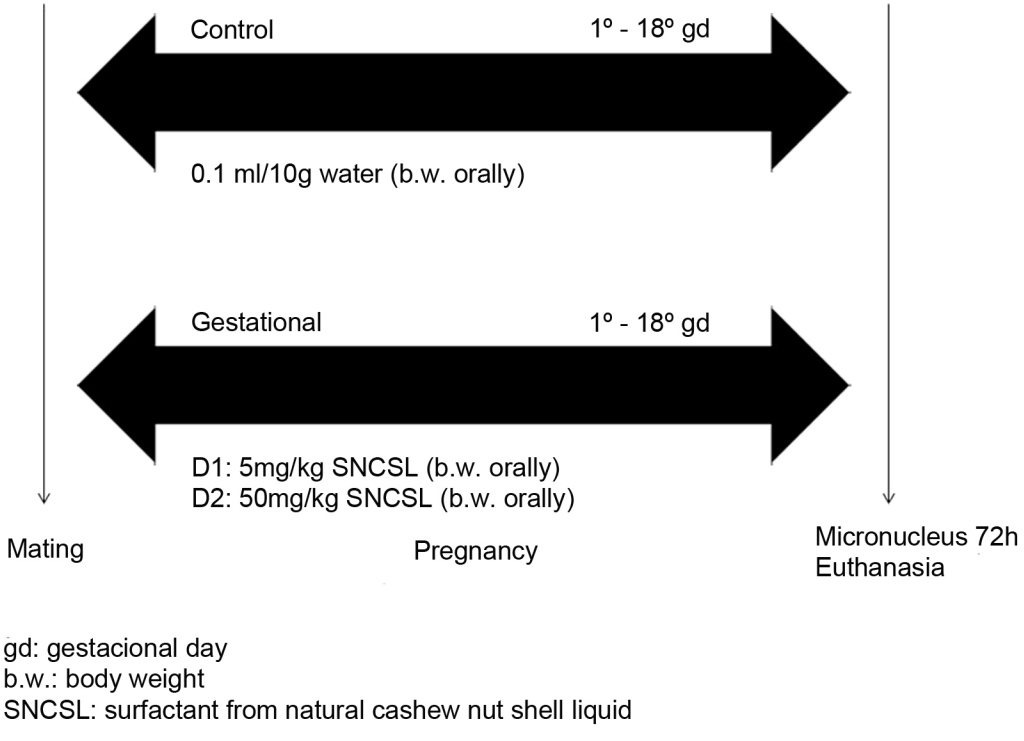

Fig 1. Treatment period and experimental design.

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classified as follows: fetuses with an appropriate weight for their gestational age (AWGA), with a body weight within the mean weight of the control group of fetuses plus or minus the standard deviation; fetuses with a low weight for their gestational age (LWGA), with a body weight lower than the mean weight of the control group fetuses minus the standard deviation of the same group; or fetuses overweight for their gestational age (OVGA), with a body weight higher than the mean weight of control group fetuses plus the standard deviation of the same group.

Subsequently, the fetuses were randomly divided into two subgroups. The first subgroup underwent visceral analysis, for which the fetuses were fixed in Bodian's solution (distilled water (142 mL), acetic acid (50 mL), formaldehyde (50 mL) and 95% ethanol (758 mL)) for at least seven days. Visceral analysis was performed via microdissection with strategic cuts to examine the chest and abdomen, according to Barrow and Taylor [35], and to examine the head, according to Wilson [36], as modified by Oliveira et al. [21]. Visceral changes were described based on the studies by Taylor [37], Manson and Kang [38], Damasceno et al. [26] and Oliveira et al. [21]. The second subgroup of fetuses was intended for skeletal analysis using the alizarin red technique proposed by Staples and Schnell [39], as modified by Oliveira et al. [21]. The fetuses were fixed in acetone for at least seven days. For the diaphonization process, the fetuses were eviscerated and placed in a solution of KOH (0.8%). Then, four drops of alizarin were added. This solution was replaced every 24 hours over four days. After this period, the KOH solution was discarded, and the fetuses were placed in a bleaching solution (1 L glycerin:1 L of ethyl alcohol: 0.5 L of benzyl alcohol), which was replaced every 24 hours for seven days. Skeletal changes were classified according to Taylor [37], Manson and Kang [38], Damasceno et al. [26] and Oliveira et al. [21].

All analyses were performed under a stereomicroscope (NIKON SMZ745T).

Micronucleus assay

The technique used for the micronucleus assay was based on Hayashi et al. [40], as modified by Oliveira et al. [21]. A total of 20 μL peripheral blood was collected via tail vein puncture, deposited on a slide that was previously stained with acridine orange (1 mg/mL) and then covered with a coverslip. Samples were collected on the 18th gestational day (i.e., at the end of the experiment) to assess whether TaLCC-20 had the ability to cause cumulative damage. The slides were stored in a freezer at -20˚C for at least 15 days. A total of 2,000 cells/animal were analyzed under an epifluorescence microscope (Motic®; Model BA 410) at a magnification of 400×).

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) and were evaluated according to the nature of their distribution (parametric: ANOVA/Tukey test; nonparametric: Kruskal-Wallis/Dunn test). The chi-square test was used to compare frequencies (percents) between the control and experimental groups. The level of significance was set at $p<0.05$.

Results

The natural CNSL used in this analysis is predominately composed of anacardic acid, as verified in the ^{1}H NMR spectrum (Fig 2). The spectrum shows signals corresponding to aromatic, olefinic, methylenic, and methyl hydrogens, in agreement with reports in the literature [41]. Signals corresponding to methylcardol are not shown in the spectrum. Aromatic hydrogens are observed from 6.10–7.35 ppm. The signals between 4.95–5.87 ppm are attributed to olefinic hydrogens. The signals between 1.15–2.99 ppm are assigned to methylene hydrogens. The three aromatic hydrogens of anacardic acid are a doublet of doublets at 7.33 ppm (J = 9 and 6 Hz), integrated for 1H, and, two doublets at 6.85 ppm (J = 9 Hz) and 6.74 (J = 6 Hz),

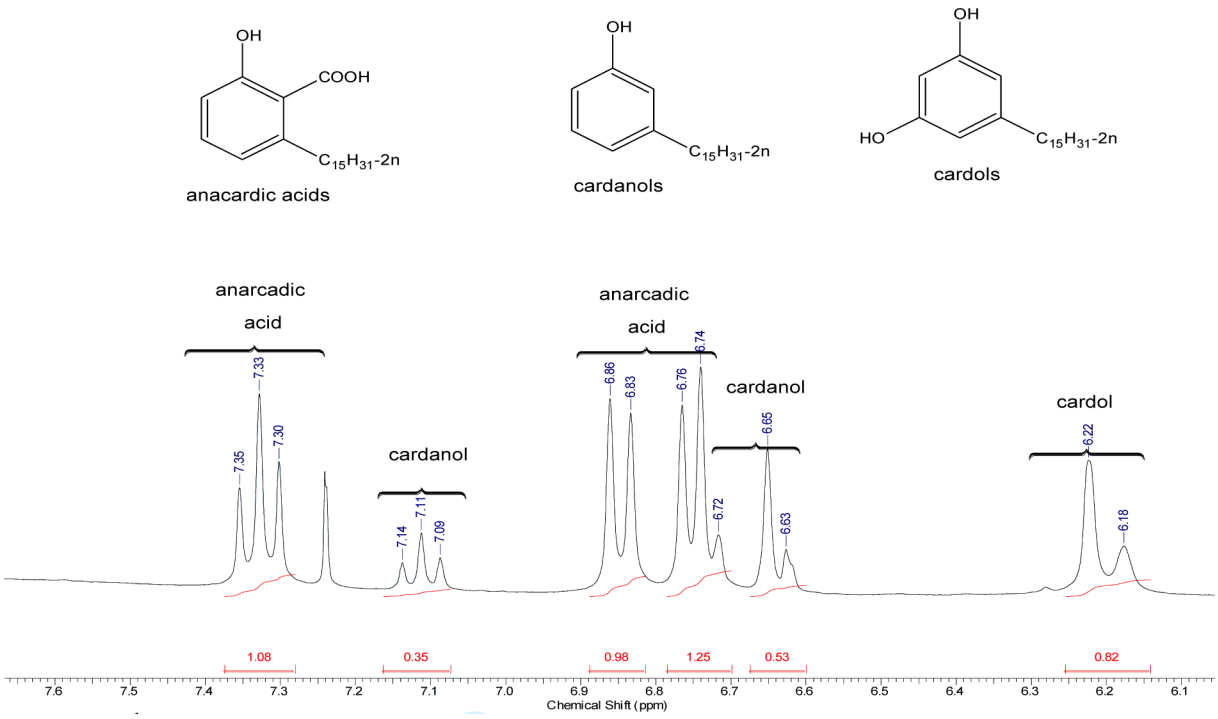

Fig 2. ^{1}H-NMR spectra of natural CNSL (expanded region for aromatic protons).

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integrated for 1H each. Aromatic hydrogens of cardol are observed at 6.15–6.25 ppm, and those for cardanol are observed at 6.60–6.75 ppm together with a doublet of doublets at 7.11 ppm. In accordance with the integration of the signals for aromatic hydrogens, our findings suggest that the ratio of anacardic acid/cardanol/cardol is 4:1.36:1, i.e., the approximate composition of natural CNSL is 62.3% anacardic acid, 21.4% cardanol and 15.7% cardol, shown in the expanded view of the aromatic region in Fig 2.

Shows the HPLC chromatogram obtained for natural CNSL. Peaks 1, 2 and 3 correspond to monoene-, diene- and triene-cardol, peaks 4, 6 and 8 correspond to monoene-, diene- and triene-cardanol, and peaks 5, 7 and 9 correspond to monoene-, diene- and triene anacardic acid (Fig 3) [42,43].

CNSL evaluation

In this study, we developed a mixture of surfactants with natural CNSL and sodium ricinoleate. Common procedures for producing surfactants from vegetable oils were used [15]. The substrates were reacted with sodium hydroxide using pure castor bean oil in the presence of the newly extracted CNSL, resulting in a mixture of anionic surfactants containing sodium ricinoleate (largely originating from the triglyceride of ricinoleic acid) and sodium anacardate and phenolates (originating from CNSL).

This surfactant was prepared via a saponification reaction with NaOH solidified after 10 days at room temperature protected from light.

Evaluation of larvicide potential

Temephos causes 100% death of larvae within three hours ($p < 0.05$). This result is considered the gold standard and was therefore used as a positive control (Table 1). TaLCC-20 exhibited

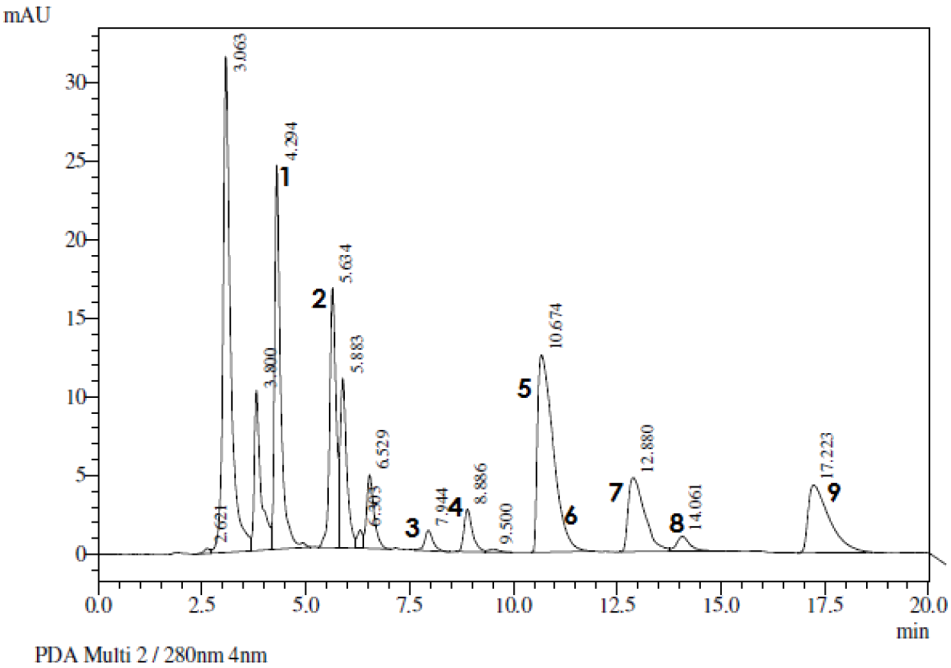

Fig 3. HPLC chromatogram obtained from natural CNLS, cardol (1, 2 and 3), cardanol (4, 6 and 8), and anacardic acid (6, 7 and 9).

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larvicidal potential compared with the negative control, as there was a decrease ($p<0.05$) in larval viability observed at all the tested concentrations. Compared with the positive control, the two lowest concentrations exhibited lower larvicidal activity ($p<0.05$), while the highest concentration presented the same effectiveness as temephos (i.e., causing death of the larvae at the same frequency over the same time interval) (Fig 4). These results demonstrate that temephos and TaLCC-20, administered at a dose of 5 mg/L, result in the same gold-standard response.

Residual effects

The control group did not exhibit larvae lethality throughout the experiment. At the 5 ppm (5 mg/kg) concentration, 100% of larval mortality was observed through the 3rd day of exposure. After the 4th day, a decreased larvicidal effect was observed, which was completely lost by the 13th day. The concentration 10× higher than the larvicide dose (50 ppm-50 mg/kg) caused

Table 1. Percentage of dead larvae at different exposure times to treatment.

	Treatments				
Exposure Time	**Control**	**Temephos**	**TaLCC 0.05 mg/L**	**TaLCC 0.5 mg/L**	**TaLCC 5 mg/L**
	Mortality (%)				
3 hours	1.25	100	0	1.25	100
6 hours	0	0	0	7.5	0
9 hours	0	0	0	10	0
12 hours	0	0	0	18.75	0
24 hours	0	0	0	16.25	0
48 hours	0	0	2.5	18.75	0
72 hours	0	0	0	12.5	0

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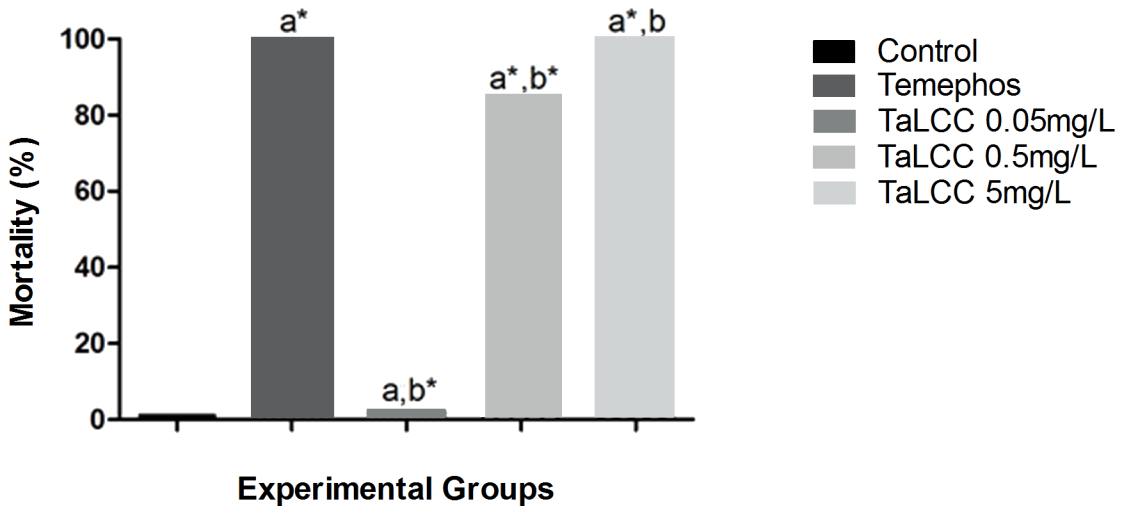

Fig 4. Mortality of larvae at the end of treatment exposure. Treatment exposures were statistically compared to the negative control (a) and the positive control (b). * Significant difference (chi-square test, $p < 0.05$).

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100% mortality of the larvae through the 22nd day of exposure. After this time, larval mortality decreased until no morality was noted on the 36th day (Fig 5).

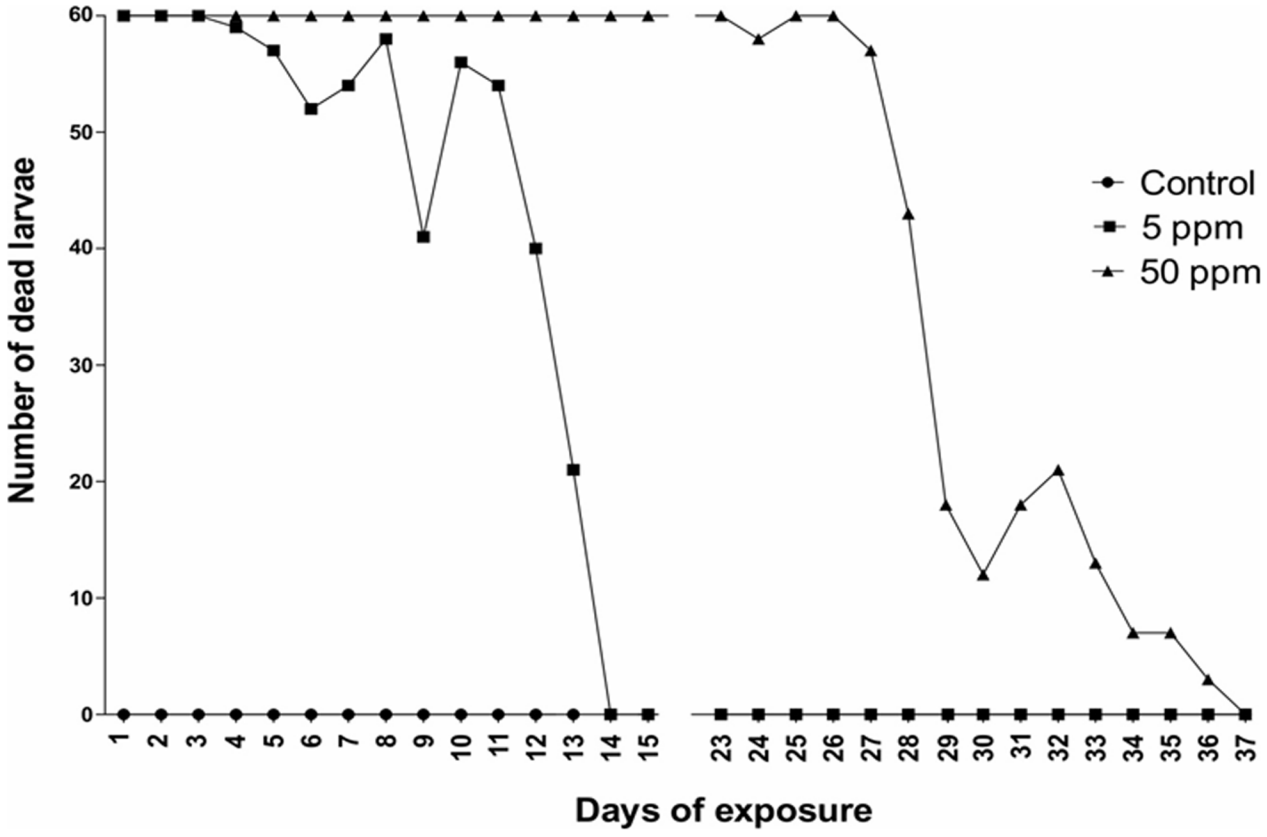

Fig 5. Residual effect of the mixture of cashew nut shell liquid and castor oil on 3rd instar larvae of *Aedes aegypti*.

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Table 2. Parameters related to growth development and organ weight of the females treated with TaLCC.

Biometrics Parameters					
Experimental Groups	**Initial Weight**	**Final Weight**	**Weight Gain**	**Weight Utero**	**Liquid Weight Gain**
Control	35.8±1.45[b]	61.01±1.88[b]	25.21±1.24[a]	20.48±0.99[b]	4.73±1.12[a]
Gest. D1	27.8±1.32[a]	52.46±2.46[a, b]	24.66±3.20[a]	19.48±0.50[a, b]	5.25±2.84[a]
Gest. D2	31.4±1.15[a, b]	49.00±2.61[a]	17.60±2.70[a]	15.94±1.70[a]	1.66±1.75[a]
Absolute Weight Organs (g)					
	Heart	**Lung**	**Spleen**	**Kidney**	**Liver**
Control	0.19±0.011[a]	0.23±0.01[a]	0.17±0.02[a]	0.42±0.01[b]	2.77±0.06[b]
Gest. D1	0.17±0.011[a]	0.36±0.03[b]	0.15±0.01[a]	0.24±0.02[a]	0.60±0.29[a]
Gest. D2	0.16±0.008[a]	0.19±0.02[a]	0.16±0.02[a]	0.35±0.01[b]	2.13±0.09[b]
Relative Weight Organs (g)					
	Heart	**Lung**	**Spleen**	**Kidney**	**Liver**
Control	0.003±0.0002[a]	0.004±0.0002[a]	0.003±0.0006[a]	0.007±0.0002[b]	0.04±0.002[b]
Gest. D1	0.003±0.0002[a]	0.007±0.0006[b]	0.003±0.0006[a]	0.005±0.0004[a]	0.01±0.005[a]
Gest. D2	0.003±0.0002[a]	0.004±0.0006[a]	0.003±0.0002[a]	0.007±0.0004[b]	0.04±0.001[b]

Different letters (a and b) indicate statistically significant differences: $p<0.05$ (Test a: Analysis of Variance/Tukey; Test b: Kruskal-Wallis/Dunn).

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Evaluation of reproductive toxicology

Evaluation of biometric parameters. Despite the random distribution of animals in the experimental groups, the animals of the group control corresponded to higher initial weights than animals of the experimental groups. This weight difference was maintained until the end of pregnancy. However, the weight gain and net weight gain were similar among all groups ($p>0.05$). A reduction in uterus weight ($p<0.05$) was observed in the Gest. D2 group compared with the control group (Table 2).

Regarding the relative weights of organs, an increased weight ($p<0.05$) was observed for the lungs and a decreased weight ($p<0.05$) for the kidneys and the liver in the Gest. D1 group compared with the control group (Table 2).

Evaluation of reproductive performance

The number of implants, live and dead fetuses, mean number of fetuses, resorption rate, placental weight and sex ratio did not differ ($p>0.05$) among the experimental groups. However, there were decreases ($p<0.05$) in the fetal viability rate, post-implantation losses and fetal weight and increases ($p<0.05$) in the number of resorptions and placental index. Despite these differences, the weight of the fetuses in the experimental groups was considered suitable for the gestational age (Table 3).

Evaluation of embryonic and fetal development: external, visceral and skeletal malformations

Malformations of the external limbs and tail were observed in all experimental groups, though at a low frequency that did not significantly differ among groups (Table 4).

Visceral observed malformations included hydrocephaly and hydronephrosis. These conditions occurred at the same frequency in all experimental groups (Table 5).

Regarding the detected skeletal malformations, an absence of or reduced ossification was observed in the phalanges, metacarpals, metatarsals, sternal centers, palate, presphenoid, parietal and ribs. The occurrence of these malformations was similar in all experimental groups.

Table 3. Reproductive parameters for females treated with TaLCC.

	Experimental Groups		
Parameter	**Control**	**Gest. D1**	**Gest. D2**
Implants	14.00±0.60[a]	12.80±0.42[a]	13.70±0.67[a]
Live Fetuses	13.50±0.70[a]	12.60±0.30[a]	11.10±1.34[a]
Dead Fetuses	0.00±0.00[a]	0.10±0.10[a]	0.30±0.21[a]
Average Number Fetuses	13.4±0.72[a]	12.6±0.30[a]	11.1±1.34[a]
VF	96.46±3.20[b]	98.67±0.89[b]	79.58±7.77[a]
TPPI	82.46±3.27[a,b]	85.87±1.27[b]	65.88±7.51[a]
Reabsorption	0.70±0.40[a]	0.10±0.10[a]	2.30±0.97[b]
TR	5.21±2.93[a,b]	0.67±0.67[a]	18.36±8.10[b]
PP (g)	0.09±0.002[a]	0.09±0.002[b]	0.08±0.002[a]
IP	0.07±0.002[a]	0.07±0.001[a]	0,08±0,002[b]
PF (g)	1.21±0.01[b]	1.24±0.01[b]	1.09±0.01[a]
APIP		PAIP	PAIP
RS	0.94±0.20[a]	1.31±0.45[a]	1.01±0.16[a]

Different letters (a and b) indicate statistically significant differences: $p<0.05$ (Test a: Analysis of Variance/Tukey; Test b: Kruskal-Wallis/Dunn). Fetal viability; TPPI: rate of post-implantation losses; TR: resorption rate; PP: placental weight; PF: fetal weight; IP: placental index; APIP: adequacy of weight to age of pregnancy; PAIP: proper weight to age of pregnancy; RS: sex ratio.

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Table 4. Relationship and frequency of external malformations in the offspring of females treated with TaLCC.

	Experimental Groups		
Parameters	**Control**	**Gest.D1**	**Gest.D2**
Members			
Analyzed Fetuses	134	126	111
Normal Fetuses	125	121	109
Retr.Post.Unilateral	5	5	2
Retr.Pos.Bilateral	2	0	0
Retr.Ant.Unilateral	1	0	0
Phocomelia	1	0	0
Freq.Malf.	**9**	**5**	**2**
%M.F.	**6.2**	**3.97**	**1.80**
Tail			
Normal Fetuses	132	124	108
Rolled up tail	2	2	3
Freq.Malf.	**2**	**2**	**3**
%M.F.	**1.49**	**1.59**	**2.78**
Nose			
Normal Fetuses	134	126	110
Hematoma	0	0	1
Freq.Malf.	**0**	**0**	**1**
%M.F.	**0**	**0**	**0.91**

Freq.Malf.: frequency of malformations; %M.F.: average value percentage of malformation; Retr.: retroversion; Ant.: anterior; Post.: posterior. Statistically compared with the control (chi-square test, $p>0.05$).

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Table 5. Relationship and frequency of visceral malformations in the offspring of females treated with TaLCC.

Experimental Groups			
Parameters	**Control**	**Gest. D1**	**Gest. D2**
Cerebro-Hydrocephalus			
Analyzed Fetuses	67	63	56
Normal Fetuses	20	26	18
Hidro.Light	44	33	38
Hidro.Severe	0	4	0
Freq.Malf.	**44**	**37**	**38**
%M.F.	**65.67**	**58.73**	**67.86**
Region Urogenital—Hydronephrosis			
Normal Fetuses	64	60	52
Hidro.Light	3	3	4
Freq.Malf.	**3**	**3**	**4**
%M.F.	**4.48**	**4.76**	**7.14**

Freq.Malf.: frequency of malformations; %M.F.: average-value percentage of malformation; Hidro.: hydronephrosis; Hidro.: hydrocephalus. Statistically compared with the control (chi-square test, $p > 0.05$).

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However, there was an increase ($p<0.05$) in the degree of ossification reduction recorded in the palate and presphenoid in the TaLCC-20-treated groups compared with the control group (Table 6).

Toxicogenic evaluation: micronucleus assay

TaLCC-20 showed no mutagenic activity (Fig 6), as there was no difference ($p<0.05$) in the frequency of micronuclei between the various experimental groups. The mean frequency of micronuclei was 5.20 ± 1.31 in the control group and 4.50 ± 0.73 and 3.89 ± 1.02 in the Gest. D1 and Gest. D2 groups, respectively, on the 18th gestational day (after treatment throughout the gestational period), demonstrating that TaLCC-20 does not cause cumulative genetic damage.

Discussion

Larvicides are generally used in fumigation and/or deposited in water tanks (regardless of whether they contain drinkable water) [44]. Thus, important concerns regarding the use of larvicides include their inhalation, accidental poisoning and contamination of drinking water consumed by the population, especially for cooking and hydration.

According to Machado et al. [7], the best results recorded to date in terms of preventing an increase in the number of dengue cases in Brazil have been obtained using diflubenzuron and, more recently, the juvenile hormone analogue pyriproxifen, which is indicated and distributed by the Brazilian Ministry of Health [45,46]. Thus, these two commercial products have largely been used to restrain the reproduction of the vector mosquitoes responsible not only for dengue but also for chikungunya fever and Zika viruses, which are re-emerging in Brazil and other parts of the world [47,48].

It is well established in the literature that the use of pesticides, insecticides, larvicides and/or growth inhibitors is directly linked to genetic/genomic instability, which can increase predisposition to cancer [8,49–55], in addition to altering reproductive performance and causing hormonal disorders and male infertility [56,57].

Table 6. Relationship and frequency of skeletal malformations in the offspring of females treated with TaLCC.

		Experimental Groups		
Parameters		**Control**	**Gest.D1**	**Gest. D2**
Members				
Analyzed Fetuses		67	63	55
Normal Fetuses		0	2	0
Phalanges.	Absente	60	52	51
	O.R.	1	0	4
Metac.Metat.	Absent	6	7	0
	O.R.	0	2	0
Freq.Malf.		**67**	**61**	**55**
%M.F.		**100**	**92.82**	**100**
Sternum				
Normal Fetuses		56	43	45
Sternal centers	Absent	3	2	3
	O.R.	8	18	7
Freq.Malf.		**11**	**20**	**10**
%M.F.		**16.41**	**31.75**	**18.18**
Head and Jaw				
Normal Fetuses		57	13	37
Pal.Sph.	Absent	0	0	0
	O.R.	10	50	17
Parietal	Absent	0	0	1
Freq.Malf.		**10**	**50**	**18**
%M.F.		**14.92**	**79.36***	**32.73***
Column				
Normal Fetuses		66	63	55
Rib Agenesis		1	0	0
Freq. Malf.		**1**	**0**	**0**
%M.F.		**1.49**	**0**	**0**

Freq.Malf.: frequency of malformations; %M.F.: average-value percentage of malformation; Metac.: metacarpus; Metat.: metatarsal;; Pal: palate Presf.: sphenoid O.R.: reduced ossification.

* Statistically significant difference compared with the control (chi-square test, $p < 0.05$)

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According to Barros et al. [57], the aforementioned effects may be caused by diflubenzuron, which has been widely used in the recent epidemics and is efficient in combating mosquitoes/vectors but toxic. No information on pyriproxifen is available in the literature regarding its mutagenic and/or teratogenic effects. In the absence of such data, pyriproxifen must be used with caution and considered potentially toxic, calling for the need of biomonitoring. Given these concerns, the development of less toxic products with available safety information regarding their use is urgently needed.

For a suitable product to be developed, the product must exhibit equal or better efficiency than commercially available products in combating larvae and/or mosquitoes/vectors in other life stages and be more selective (i.e., be able to control vector survival and reproduction without causing genetic and/or reproductive toxicity to animals or humans).

Developing such a product would solve important public health issues without causing harm to the population that could potentially require further investments to treat chronic non-degenerative diseases, such as cancer, subfertility and infertility.

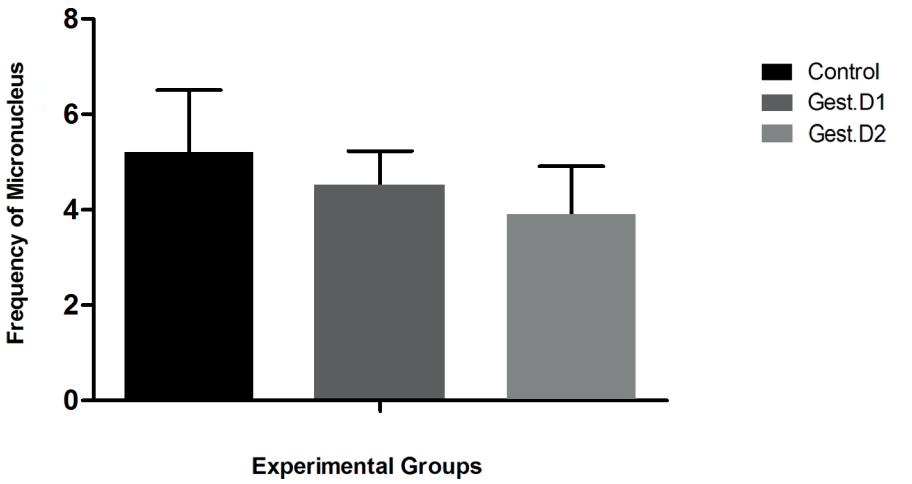

Fig 6. Micronucleus frequency after treatment. (Test: analysis of variance/Tukey, $p<0.05$).

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CNSL exhibits larvicidal activity, as previously described [11,13,16]. In addition, preliminary data from Galdino and Beatriz [14] and Beatriz et al. [15] demonstrated that mixtures of sodium surfactants from natural CNSL and castor bean oil exhibit larvicidal activity; among the various tests that were performed, the best results were observed at a ratio of 20:80. Thus, the same mass/mass relationship was chosen for this pilot study. We present a larvicide with natural peculiarities in its composition that was obtained from a combination of natural CNSL and castor bean oil and takes the form of a sodium surfactant. This formulation increases the solubility of CNSL, allowing further dispersion of the product at mosquito/vector breeding sites, offering further larvicidal potential.

New tests performed with TaLCC have demonstrated its ability to kill 100% of *Ae. aegypti* larvae within a mean time of three hours at a dose of 5 mg/L (ppm). These results are considered by the Ministry of Health to represent the gold standard for commercial compounds, such as temephos, which also induces the death of 100% of larvae within a few hours of exposure [58,59]. In addition to achieving the recommended gold standard, our results suggest that the TaLCC-20 product has no side effects in mammals, including humans. These findings confirm the better cost/benefit ratio of TaLCC-20 compared, for example, with temephos, because although the latter product shows high efficiency, it is toxic to both the environment and humans [51].

The residual effect of TaLCC for maintaining lethality of 100% of larvae lasted for up to 13 days and 22 days at the used doses of 5 and 50 ppm, respectively. After these periods, larvicide action was gradually lost and ceased within 13 and 37 days for the doses of 5 and 50 ppm, respectively. These results demonstrate that a formulation of TaLCC not only improves the solubility of other products prepared based on cashew nuts but also improves the time of action. According to Guissoni et al. [11], TaLCC can kill larvae within 6 days (100% death) with a residual effect remaining for 14 days. Therefore, the TaLCC has a greater residual effect, and this period is increased by 23 days, which allows for greater action over time and is a highly desired feature of larvicides

In addition to these benefits, TaLCC-20 presents strong commercial appeal because it is easily produced, exhibits good yield and is inexpensive. Thus, TaLCC-20 may provide and inexpensive solution for a public health issue that is associated with high costs in various regions of the world. In addition, this product can prevent many deaths and improve the quality of life of millions of people who are affected by the symptoms caused by dengue,

chikungunya fever and Zika virus. These infections and their complications are also responsible for the absence of workers from their workplaces, causing major harm to the production sectors of various countries [60,61].

The toxic effects of xenobiotics on genetic material and/or reproductive health can be evaluated based on mutagenesis, reproductive performance, embryonic and fetal development and teratogenesis assays [62–64]. Products that do not induce damage according to these biological assays are favored for commercialization [62]. Thus, the present study evaluated TaLCC-20 in a preclinical model to determine whether it can be safely used and to predict the health risks of exposure to this product in mammals, including humans.

The guidelines for reproductive toxicology [30] and genetic toxicology [65,66] and the National Health Surveillance Agency [67] indicate that preclinical trials should be carried out using doses intended for use in humans (or using the doses to which humans may be exposed) and another dose 10 times higher. The lower dose can only be considered safe if the higher dose is free of adverse effects. Thus, in the present study, we evaluated a dose that has larvicidal potential (5 mg/kg) and a dose 10 times higher (50 mg/kg) to assess possible maternal effects and embryonic toxicity. For a compound to be released for use and commercialization, evidence of a lack of mutagenic and teratogenic effects and the cost/benefit ratio associated with its use must be evaluated [30, 67,68].

According to Zhang et al. [69] and Sally et al. [70], the state of pregnancy modifies an individual's metabolism, which may induce the body to become more susceptible to xenobiotic effects. Hence, also performed a mutagenesis analysis of pregnant females.

Considering the above issues, the results of the present study were promising, including 1) the high larvicidal efficiency of the product, and2) the lack of changes in embryonic and fetal development caused by the product and no detected genetic instability. Such effects can be correlated with infertility, teratogenesis/congenital malformations and cancer [8,51,53,56,57], which are important health issues that may negatively impact public health systems because, as chronic diseases, they require large investments to treat and maintain the quality of life of patients.

Regarding the mutagenic capacity of TaLCC-20, the micronucleus assay revealed no genetic damage. This result is important because some products used against mosquitoes, such as temephos and diflubenzuron, are mutagenic and/or cause changes in DNA [8,51].

The preclinical trial also indicated an absence of toxicity based on the biometric parameters that were evaluated. According to other studies, weight loss and changes in the absolute and relative weights of organs may be indicative of toxicity [71,72]. In the present study, although the animals were randomly distributed, the Gest. D1 group presented the lowest mean weight. However, the recorded weight gain and net weight gain showed no significant variation, indicating an absence of TaLCC-20 toxicity. The reduction in uterus weight recorded for the Gest. D2 group can be explained by the lower number of fetuses per litter in this group; thus, it is not indicative of toxicity. The low initial weight of the animals in the Gest. D1 group may explain the reduction in the weights of the lungs, kidneys and liver observed in this group. In general, these parameters are not considered signs of toxicity because xenobiotics causing damage to the body particularly lead to enlargement of the liver and kidneys, which are organs that are directly involved in metabolism and excretion. Furthermore, toxicity generally results in an increase in the activity of these organs, which would be consistent with their increased size.

Regarding reproductive performance and embryonic and fetal development, pregnant females may be exposed to a test compound in different stages to predict any interference with implantation (treatment from the 1st to the 4th gestational day), organogenesis (treatment from the 5th to the 15th gestational day) [23] or fetal development (treatment from the 15th to

18th gestational day) [73]. The literature also describes pregnancy treatments performed to assess whether a compound exhibits a cumulative effect or if it can affect more than one embryonic or fetal developmental stage [74,75]. In the present study, a gestational treatment protocol was used, and reductions in the fetal viability rate and fetal weight were observed. There were also increases in the number of resorptions and the placental index. Among these parameters, the reduction in fetal viability requires further attention.

The reduction in fetal weight and the increase in the placental index are not worrisome because, despite these differences, the fetuses continued to show an adequate weight for their gestational age. In general, a low weight of fetuses after birth is associated with an increased placental index, which is an adaptation of the maternal body in an attempt to increase the provision of nutrients to the fetus and, thus, allow proper development [76,77]. The increased number of resorptions is not a critical finding because the resorption rate, which represents complementary data, showed no difference among the experimental groups. Regarding embryonic and fetal development, there was no increase in the frequency of external or visceral malformations in relation to the control group.

The observed visceral malformations (hydrocephalus and hydronephrosis) may be normal variations, as they were also present in the control group [22,23,37]. Furthermore, the fetuses were collected prematurely, although the procedure was performed as indicated by the literature [22,37]. These studies also indicate that these changes may revert at the end of pregnancy or after birth [21,37], corroborating the notion that they are normal variants. In relation to skeletal malformations, the only significant changes observed in the treated groups were those related to reduced palate and presphenoid and parietal ossification. However, these fetuses were collected early, and thus, the ossification process was interrupted. This may explain the damages that were observed, while the ossification process can still be completed after birth. We suggest that this is not a factor that discourages the use of CNSL.

Thus, the cost-benefit relationship observed for issues related to reproductive performance and embryonic and fetal development is also positive and supports the use of TaLCC-20, especially because the available commercial products that are indicated for use by the government, such as temephos [51] and diflubenzuron [8], are reported to be toxic to reproductive health and are possible teratogens.

In summary, TaLCC-20 is considered an effective larvicide that does not induce genetic damage. In addition, the cost-benefit relationship associated with changes in reproductive performance and embryonic and fetal development appears to be positive. These findings indicate that it is an important product to be explored for use as a natural larvicide that can be employed against the mosquitoes/vectors responsible for dengue, chikungunya fever and Zika virus. These are emerging and/or re-emerging diseases that require proper management while minimizing harm to the population and the environment. Therefore, CNSL is considered an important alternative to commercial products that are toxigenic, although effective.

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Author Contributions

Conceptualization: Juliana Miron Vani, Andréa Luiza Cunha-Laura, Alessandra Ramos Lima, Dênis Pires de Lima.

Funding acquisition: Maria Tereza Ferreira Duenhas Monreal.

Investigation: Juliana Miron Vani, Andréia Conceição Milan Brochado Antoniolli-Silva, Dênis Pires de Lima.

Methodology: Sarah Alves Auharek, Andréa Luiza Cunha-Laura, Eduardo José de Arruda, Alessandra Ramos Lima, Cicera Maria da Silva, Andréia Conceição Milan Brochado Antoniolli-Silva, Dênis Pires de Lima, Adilson Beatriz, Rodrigo Juliano Oliveira.

Project administration: Rodrigo Juliano Oliveira.

Supervision: Adilson Beatriz, Rodrigo Juliano Oliveira.

Writing – original draft: Juliana Miron Vani.

Writing – review & editing: Sarah Alves Auharek, Eduardo José de Arruda, Adilson Beatriz, Rodrigo Juliano Oliveira.

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