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3 **Efficacy and selectivity of *Sextonia rubra* wood extracts and formulation in the**
4 **control of *Aedes aegypti* strains**

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23 **Abstract:**

24 *Sextonia rubra* is an Amazonian tree known for the larvicide activity of its wood extracts
25 against *Aedes aegypti*. However, the lipophilic character of this extract presents an
26 obstacle to the development of vector management products. The purpose of this study
27 was to assess the feasibility, efficacy, and selectivity of a water-soluble formulation
28 based on a *S. rubra* wood extract characterized by its larvicidal compounds rubrenolide
29 and rubrymolide. Using reference and wild strains, larval mortality, oviposition behavior,
30 hatching response, and fitness of mosquitoes exposed to the extract were evaluated.
31 Laboratory eco- and cytotoxicity bioassays were performed concurrently. The extract
32 showed larvicidal efficacy and no repulsive effect on oviposition, thus supporting its use
33 in the treatment of breeding sites. The formulation exhibited strong acute toxicity, leading
34 to 100% larval mortality in 2-4 days in either the dry or rainy season at a test dose of 102
35 µg/ml under semi-operational conditions, with residual activity maintained during the 5
36 weeks of testing. The formulation allowed for a noticeable gain in selectivity compared
37 with the extract, with lower immobilization rates for *Daphnia magna* and *Chironomus*
38 *riparius*, and no significant toxicity towards human cell lines ($IC_{50} > 100$ µg/ml). These
39 results highlight the potential of the *S. rubra*-based formulation for the management of
40 *Ae. aegypti* larvae, including strains showing resistance to conventional insecticides.

41

42 **Keywords:** vector control, botanical insecticide, semi-field trials, non-target effects,
43 oviposition

44

45 **Key Message**

- 46 • *Sextonia rubra* wood extract formulation is easily usable to treat mosquito
47 breeding sites
- 48 • Semi-field assays highlighted acute toxicity and residual activity of the formulation
49 over 5 weeks
- 50 • The formulation is more selective than the extract in eco- and cytotoxicity assays

- 51 • *Sextonia rubra* crude extract has no effect on *Aedes aegypti* oviposition
52 behaviour
- 53 • *Sextonia rubra* sawmill waste can be valorized as an insecticide formulation
54

55 **Introduction**

56 Globalization and extensive urbanization have promoted the worldwide expansion of the
57 anthropophilic invasive mosquito *Aedes aegypti* L. (Diptera, Culicidae), notably because
58 of the proliferation of breeding sites that can retain stagnant water (Carvalho and Moreira
59 2017). This species causes numerous human casualties and is the main vector of
60 several arboviruses (Gloria-Soria et al. 2016; Jones et al. 2021). In the absence of
61 clinically approved vaccines or antiviral therapies, except for yellow fever virus, vector
62 control strategies based on the use of insecticides are of crucial importance. Dengue
63 fever, and Zika or chikungunya viruses are particularly prevalent in French Guiana,
64 where vector control strategies have followed one another (Epelboin et al. 2018; Bailly
65 et al. 2021; de Thoisy et al. 2021). Currently, this French overseas territory faces a strong
66 resistance of *Ae. aegypti* populations towards pyrethroids as adult vector-control
67 products (Dusfour et al. 2015; Faucon et al. 2017; Guidez et al. 2020; Senthil-Nathan
68 2020; Epelboin et al. 2021). *Bacillus thuringiensis* var. *israelensis* (Bti) is currently being
69 used to reduce the density of *Ae. aegypti* larvae, without the apparition of resistance
70 phenomena (Darriet et al. 2010; Kumar et al. 2012). However, the persistence of the
71 effects of this non-ecotoxic natural product in breeding sites should be explored
72 (Epelboin et al. 2018). Therefore, in an epidemiological and legislative context that is
73 particularly unsteady, it is essential to develop safe, innovative, and environmentally
74 friendly methods for vector control (George et al. 2014; Gerwick and Sparks 2014; Benelli
75 2015; Benelli and Mehlhorn 2016).

76 Due to their constant interaction with herbivores, plants synthesize a large variety of
77 molecules as part of their defense system (Agrawal and Weber 2015; Richards et al.

78 2015; Salazar et al. 2018). Thus, plants are a valuable source of novel insecticide
79 compounds (Miresmailli and Isman 2014; Pavela et al. 2019; Benelli 2020; Silvério et al.
80 2020). However, even if numerous promising results have been obtained, only few of
81 these findings have made it to commercial applications as new botanical insecticides
82 (Isman and Grieneisen 2014; Isman 2020). One of the major technological challenges
83 for the development of larvicide botanical products is to allow their homogeneous
84 dispersion in water, the natural mosquito-breeding medium, and the increase in field
85 performance through better persistence of action (Isman 2020). To overcome the
86 limitations of applicability, notably linked to the apolar nature of many natural substances
87 or extracts, and without using organic solvents exhibiting toxic profiles, micro- and
88 nanoemulsions stand in good place (de Oliveira et al. 2014; Peng et al. 2019). These
89 techniques, which lead an efficient aqueous suspension or dilution of low-water-soluble
90 substances, allow the production of usable bioactive natural products against mosquito
91 larvae (Kanis et al. 2012; Oliveira et al. 2016; Pavela 2016; Pavela et al. 2021). The
92 lower effects on non-target organisms compared to conventional products also stand
93 among the potential advantages of natural products-based micro- and nanoemulsions
94 as larvicide products, and low toxicity to humans is an important parameter to consider,
95 due to the use of such products in the household area (Oliveira et al. 2016; Pavela et al.
96 2019; Turchen et al. 2020; Faustino et al. 2020; Rodrigues et al. 2021; Machado et al.
97 2023).

98 *Sextonia rubra* (Mez.) Van der Werff is a common Lauraceae tree in the Guianas,
99 Venezuela and in Amazonian Brazil. This species of high economic importance is used
100 in the wood industry in French Guiana, where it is known as “grignon franc” (Soulères
101 2021). However, this industry produces sizeable quantities of waste, including sawdust,
102 during logs transformation. Moreover, previous studies performed by our group
103 demonstrated that the crude wood extract and the purified compounds rubrenolide and
104 rubrynlolide displayed strong activity towards *Ae. aegypti* larvae, and that commercial
105 sawmill residues of *S. rubra* can be considered as a valuable resource for the large-scale

106 production of these larvicidal extracts (Falkowski et al. 2016, 2020; Clervil et al. 2023).
107 Therefore, we characterized the larvicidal activity of the *S. rubra* wood extract on *Ae.*
108 *aegypti* iso-female strains and their parent wild strain collected in French Guiana. We
109 also evaluated the fitness of mosquitoes exposed to the extract, while concurrently
110 assessing an aqueous-based formulation containing *S. rubra* wood extract under semi-
111 field conditions. Eco- and cytotoxicity were investigated, in the objective of ascertaining
112 the selectivity of the products towards aquatic species and human cells, and display a
113 better picture of the potential of *S. rubra* material to develop novel botanical products
114 displaying larvicidal activity against insecticide-resistant *Ae. aegypti* mosquitoes.

115

116 **Materials & methods**

117 *Mosquito strains and rearing*

118 Five *Ae. aegypti* strains were used. The laboratory New Orleans (NO) strain is a
119 reference strain susceptible to all insecticides and has been maintained for decades in
120 an insectary at the Institut Pasteur de la Guyane, Cayenne, French Guiana. The
121 Cayenne (CAY) strain was collected in the eponym city, and larvae of the F1 progeny
122 were used for all experiments conducted in the laboratory and for the semi-field assays.
123 This population is resistant to both pyrethroid and organophosphate insecticides. A
124 parent population was obtained from wild-caught larvae from Ile Royale (IR), one of the
125 coastal islands of French Guiana (5.287° N; 52.590° W), in 2015, which led to the field
126 strain IR0115. Low insecticide pressure characterizes this collection area. Two isofemale
127 strains were obtained from this parent population, as previously described, after the
128 isolation of one mated female (Epelboin et al. 2021). Information on the five strains used
129 is summarized in Table 1. Rearing was performed as described previously (Touré et al.
130 2017).

131

132 **Table 1** Characteristics of the different *Aedes aegypti* mosquito strains used in the study

Strain	Strain type	Resistance level	Resistance mechanism ^a
New Orleans (NO)	Reference	None	None
Cayenne (CAY)	Wild	Very high	Metabolic & kdr
IR0115	Wild	Low	Metabolic
IR03F26	Isofemale	Middle high	Metabolic
IR05F26	Isofemale	High	Metabolic & kdr

133 ^a kdr: knock-down resistance

134

135 *Sextonia rubra wood extract and purified compounds*

136 *Sextonia rubra* freshly obtained commercial sawmills wastes were collected in French
137 Guiana. Sawdust was obtained from Montsinéry (4°58'34" N 52°26'45" W; September
138 2017) and Kourou sawmills (5°08'54" N 52°41'20" W; March 2020). The mass distribution
139 by particle size was measured as follows: 60% > 500 µm, 33% between 500 and 200
140 µm, and 6% < 200 µm for Montsinéry sawdust, and 74% > 500 µm, 21% between 500
141 and 200 µm, and 4% < 200 µm for Kourou sawdust. All samples were dried at room
142 temperature (21°C, 65% relative humidity) until equilibrium humidity (H<13%) was
143 reached. Any visible bark material was manually removed from crude wood powders,
144 which were then extracted using ethyl acetate (EtOAc, RPE grade, Carlo Erba Reagenti
145 SpA, Rodano, Italy) at a 1/20 ratio (w/v) at room temperature by maceration during 24h
146 under stirring. After extraction, the solution was filtered and the solvent was removed by
147 evaporation under reduced pressure using a SpeedVac™ concentrator (Savant
148 SPD121P, Thermo Scientific). The resulting crude extracts (SrM from Montsinéry
149 sawdust and SrK from Kourou sawdust) were stored in a freezer at -18°C until assayed.
150 Isolation of rubrenolide (Re) and rubrynolide (Ry) was performed using HPLC semi-
151 preparative chromatography (Waters, Milford, MA, USA), according to previously
152 described procedures (Fu et al. 2019). Their identification was confirmed and their
153 contents were evaluated using NMR. To do so, ¹H NMR spectra were recorded at 400
154 MHz and ¹³C NMR spectra at 100.6 MHz on a Varian 400 MR spectrometer (Varian, Palo

155 Alto, CA, USA) equipped with a 5 mm inverse probe (Auto X PGF $^1\text{H}/^{15}\text{N}-^{13}\text{C}$). Samples
156 were dissolved in deuterated chloroform (CDCl_3 , Carlo Erba) in 5 mm tubes for analysis.
157 SrM extract was characterized with $8.4 \pm 0.2\%$ Re and $2.0 \pm 0.0\%$ Ry (w/w), and SrK
158 extract with $10.5 \pm 0.1\%$ Re and $8.9 \pm 0.2\%$ Ry (w/w) (Clervil et al. 2023).

159

160 *Sextonia rubra* wood extract formulation

161 A *S. rubra*-based concentrate was first obtained by progressively adding a commercial
162 rapeseed oil methyl ester (RadiaTM 7961, Oleon, Ertvelde, Belgium) to *S. rubra* SrK wood
163 extract, containing $10.5 \pm 0.1\%$ and $8.9 \pm 0.2\%$ (w/w) of the larvicidal compounds
164 rubrenolide and rubrymolide, respectively, which appears as a brownish glutinous
165 molasses. The mixture was left in contact (three hours) at room temperature ($23 \pm 2^\circ\text{C}$)
166 with regular manual stirring, and then heated (30 minutes at 60°C) in a double-walled
167 water bath while stirring manually until the extract was fully solubilized. This process was
168 repeated to blend the concentrate with emulsifying ingredients, chosen for their specific
169 HLB value and bio-based origin, and selected as an alkylpolyglycoside (APG, SimulsolTM
170 SL 7 G, Seppic, Paris, France) and an ethoxylated rapeseed oil methyl ester
171 (SepigreenTM C 300, Seppic, Paris, France). An antifoaming agent (acetylenic diol in
172 ethylene glycol, SurfynolTM 104E, Evonik Industries AG, Essen, Germany) was also
173 added. The final formulation was titrated with 10% *S. rubra* extract. Finally, 560 g of this
174 formulation were prepared for various assays. The stability of the final product, which is
175 an emulsifiable concentrate (EC) aiming at obtaining spontaneous oil-in-water micro-
176 emulsion (EW) when added to distilled water, was evaluated by visual observation at
177 room temperature for validation before use. The composition of the matrix (defined as all
178 formulation components without the extract) and formulation are reported in Table 2.

179

180 **Table 2** Composition (w/w) of formulation matrix and *Sextonia rubra* wood extract
 181 emulsifiable concentrate (EC)

Ingredient	Function	Matrix	Emulsifiable concentrate
Rapeseed oil methyl ester	Non-VOC solvent	25,0	25,0
Ethoxylated rapeseed oil methyl ester	Emulsifier	34,0	34,0
Alkyl polyglycoside	Emulsifier	29,0	29,0
Acetylenic diol	Anti-foaming agent	2,0	2,0
<i>Sextonia rubra</i> wood extract	Active substance	-	10,0
Water		10,0	-

182

183

184 *Laboratory evaluation of the larvicidal activity of S. rubra wood extract and formulation*

185 The SrM *S. rubra* extract was investigated using the WHO cup assay for testing mosquito
 186 larvicides (World Health Organisation 2005). Late third- or early fourth-instar larvae were
 187 used in the test. For each bioassay, 25 larvae of each strain were transferred to cups
 188 containing distilled water (99 ml) and the tested product (1 ml) diluted in ethanol (100%,
 189 Sigma-Aldrich, Saint-Louis, MO, USA) at a suitable concentration. Eight cups per
 190 concentration (8 x 25 larvae) were used, and the concentrations were adapted to obtain
 191 from 0% to 100% mortality, thus allowing for LD₅₀ and LD₉₀ calculations (from 0.001 to 8
 192 µg/ml for the NO strain and from 3 to 120 or 160 µg/ml for all other strains). Control
 193 treatments were performed for each test with ethanol only (1 ml in 99 ml of distilled
 194 water), and larval mortality was recorded 24 and 48 h after exposure. The test was
 195 invalidated if the mortality in control group was >20%.

196

197 *Influence of S. rubra wood extract on the oviposition behavior of Ae. aegypti, hatching*
198 *response, and assessment of mosquito fitness*

199 The assessment of the influence of *S. rubra* wood extract on the oviposition behavior of
200 the mosquito was performed as described in the literature, with some modifications (El
201 Maghrbi and Hosni 2014; Seenivasagan et al. 2019). Holding cages (30 x 30 x 30 cm)
202 were filled with 40 adult females and 40 adult males (six days old) of *Ae. aegypti* New
203 Orleans strain (three replicates) and Cayenne strain (three replicates). Adult mosquitoes
204 were continuously fed on cotton soaked in 10% sugar for one day before the blood meal.
205 Two days after the female mosquito had been artificially blood-fed (beef blood), two
206 breeding sites consisting of laying cups were placed in each cage: a control site (100 ml
207 untreated distilled water) and a site with water (100 ml) treated with SrM extract. Doses
208 for the *S. rubra* treatment were adapted for each population and centered around the
209 two respective 24h-DL₅₀ values (2 µg/ml of SrM *S. rubra* wood extract for the New
210 Orleans strain; 70 µg/ml of SrM *S. rubra* wood extract for the Cayenne strain). A paper
211 wick was placed in each breeding site on day 3 after the blood meal and replaced each
212 day for 5 days with a new one, dried, and the number of eggs laid on each paper was
213 counted. The hatching rate was used as a measure of the oviposition response. To
214 obtain a new generation of mosquitoes, each paper wick was placed in clear water after
215 five days of drying, and rearing was performed as previously described (Clervil et al.
216 2023). The number of hatched eggs was counted, and 50 larvae from hatched eggs of
217 each strain and each condition were placed in five different trays in five replicates. The
218 daily survival and stage development follow-up (average number of individuals which
219 developed to the pupal and adult stages from 50 larvae according to the strain and
220 treatment) lasted until the complete emergence of all individuals.

221

222 *Simulated field trial*

223 Semi-field trials were conducted at the grounds of the Institut Pasteur de la Guyane
224 (French Guiana Pasteur Institute) in Cayenne. For the bioassays, only *Ae. aegypti*

225 natural population collected in the town of Cayenne (CAY) was used to test the
226 formulated products. The CAY strain was selected to provide a stringent context for an
227 assay that was as close to the reality of the field as possible. Experiments were carried
228 out in white plastic containers (70 l, Icko Apiculture, Bollène, France) filled with water (50
229 l) and placed under cover, out of direct sunlight and rainfall, and covered with mosquito
230 nets to prevent oviposition by wild mosquitoes or other insects in the area as well as
231 falling debris during the experiment. Four test containers were used concurrently for each
232 trial session along with four negative and four positive control containers. For the test
233 barrels, the *S. rubra* formulation (50 g), titrated at 10 % of wood extract, was added to
234 tap water (50 l) to obtain a final concentration of the active product in the barrel
235 corresponding to twice the extract LD₉₀ against *Ae. aegypti* CAY larvae after 48h of
236 exposure in the laboratory (i.e., a trial dose of 102 µg/ml). The negative control
237 containers were filled with tap water only (50 l), and the positive control containers were
238 filled with the formulation matrix (40 g) dissolved in water. During the experiment, tap
239 water was regularly added to the containers to compensate for evaporation and maintain
240 a constant volume of 50 l. In each case, tap water was given 24h hours before use for
241 dechlorination and conditioning. Late third- or early fourth-instar larvae from a first
242 generation (F1) reared from wild-caught larvae (F0) were used in the tests. A pool of 100
243 larvae was initially added to each container at the beginning of the trial, and a new pool
244 of 100 live larvae was released into each container after the total emergence of the adults
245 in the control containers (approximately every week). Larval food (yeast powder, 845
246 mg) was given to the larvae during the test when the water volume was adjusted (i.e.,
247 approximately every 3 days). Larval, and adult survival were recorded daily until the total
248 adult emergence of the controls. This experimentation was realized during both the dry
249 season (August-September 2020; average maximum temperature 33.8°C; average
250 temperature 27.8°C, average sunlight 239.5 hours; average rainfall 166.5 mm) and the
251 rainy season (January-February 2021; average maximum temperature 30.8°C; average

252 temperature 26.5°C, average sunlight 86.6 hours; average rainfall 507 mm) (infoclimat.fr,
253 2021, Cayenne).

254

255 *Ecotoxicological bioassays on non-target species: Daphnia magna and Chironomus*
256 *riparius*

257 Ecotoxicity assays were adapted from the guidelines of the “Immediate Immobilization
258 Test” (OECD No. 202) for *Daphnia magna* (Straus, 1820) and the “Immediate
259 Immobilization Test” (OECD No. 235) for *Chironomus riparius* (Meigen, 1804). The
260 tested animals were at the neonate’s stage as recommended by OECD protocols. Each
261 product (crude extract, purified compounds rubrenolide and rubrynlolide, formulation, and
262 formulation matrix) was tested at doses based on the previously established LD₉₀ (51.2
263 µg/ml) and LD₅₀ (18.6 µg/ml) of the SrK extract used to obtain the formulation, and
264 measured in the CAY wild strain after 48h of exposure. The formulation and formulation
265 matrix effects on non-target species were compared at a semi-field trial dose of twice the
266 SrK crude extract LD₉₀ (102 µg/ml). The impacts of the formulation, formulation matrix
267 and crude extract were concurrently assessed at the reference LD₅₀ of the crude extract.
268 Finally, the purified compounds rubrenolide and rubrynlolide were tested at 2 µg/mL,
269 representing approximately 10% of the extract LD₅₀ value, as each compound
270 represented, on average, 10% of the extract (Clervil et al. 2023). Three conditions were
271 tested: water control, water and solvent control, and the analysis dose, with five
272 replicates per condition. Physicochemical measurements (pH, dissolved oxygen, and
273 conductivity) were performed using measurement devices (sensors). The remaining
274 measurements (chlorine, nitrites, nitrates, and phosphates) were performed in the
275 laboratory using colorimetric detection (spectrophotometer). The photoperiod and
276 temperature were recorded using a “templight” recorder throughout the test period, from
277 clutch incubation until the end of the exposure.

278

279 *Cytotoxicity assays*

280 *Cell culture*

281 Skin normal fibroblastic cells and HUVEC-Umbil Vein (umbilical cord cells) were
282 purchased from Lonza (Basel, Switzerland), Caco-2 (colon), SHY5Y (human bone
283 marrow neuroblasts), NCI-H295R (adrenal gland), HaCaT (skin keratinocytes) and
284 HuH7D12 (liver) cancer cells lines were obtained from the ECACC collection (Porton,
285 OK, USA). Cells were grown according to ECACC recommendations at 37°C and 5%
286 CO₂. All cultures were supplemented with 10 % FBS (HyClone FetalClone III; Cytiva,
287 H30109.03), penicillin-1% streptomycin (Sigma, P4333) and 2mM glutamine (Sigma,
288 G7513).

289 *Cytotoxic assays*

290 Chemicals were solubilized in DMSO (Sigma, D4540) at a concentration of 20 mM or
291 100 mg/ml (stock solution) and diluted in the culture medium to the desired final
292 concentrations. The doses effect cytotoxic assays (IC₅₀ determination) were performed
293 by increasing concentration of each chemical (final well concentrations: 0.3 µM – 0.9 µM
294 – 3 µM – 9 µM – 25 µM – 50 µM or 1 µg/ml – 3 µg/ml – 9 µg/ml – 28 µg/ml – 83 µg/ml –
295 250 µg/ml). Cells were plated in 96 wells (4000 cells/well). Twenty-four hours after
296 seeding, the cells were exposed to chemicals. After 48h of treatment, cells were washed
297 in PBS (Sigma D8537) and fixed in cooled 90% ethanol (Sigma 32221) / 5% acetic acid
298 (Sigma A6283) for 20 minutes. Nuclei were stained with Hoechst 33342 (B2261 Sigma).
299 Image acquisition and analysis were performed using a Cellomics ArrayScan VTI/HCS
300 Reader (ThermoScientific, Waltham, MA, USA). The survival percentage was calculated
301 as the percentage of cell number after compound treatment over the cell number after
302 DMSO treatment. To evaluate the selectivity of the formulation, the selectivity index was
303 used to highlight the results more clearly, as IC₅₀ values were available in this case for
304 non-target cells. This value can be defined as the ratio of the toxic concentration of a
305 product (in our case the 48h- IC₅₀ against human cell lines) against its effective bioactive

306 (in our case, the larvicidal activity defined by the products 48h-LD₅₀) concentration
307 (Indrayanto et al. 2021).

308

309 *Statistical analysis*

310 Lethal doses for evaluation of the larvicidal activity of *S. rubra* wood extract and
311 formulation were obtained by a log-probit regression under a general linearized model
312 using the MASS package in R software version 3.2.0 (<https://www.r-project.org/>) as
313 previously described (Falkowski et al. 2016).

314 Student's t-test (MASS package in R software version 4.1.3; <https://www.r-project.org/>)
315 was used to measure the influence of the treated water on oviposition behavior and
316 mosquito fitness (hatching rate and mosquito development). In all analyses, a *p*-value <
317 0.05 was taken to indicate statistically significant.

318 For data analysis of simulated field trials, the time (in weeks) taken for the inhibition of
319 adult emergence (%IE) to pass below the threshold value of 80% was recorded. For
320 each treatment, %IE was estimated according to the control containers using the
321 following formula (World Health Organization 2005; Darriet et al. 2010):

$$322 \quad \%IE = \frac{C - T}{C} \times 100$$

323 Where C represents the average percentage emergence in the untreated (control)
324 containers at a particular time and T represents the average percentage emergence in
325 the treated containers in the same period.

326 For the bioassays on *D. magna* and *C. riparius*, Kruskal-Wallis tests were performed
327 using the rstatix package in R software version 4.1.3 (<https://www.r-project.org/>).

328

329 **Results**

330 *Activity of S. rubra wood extract on Ae. aegypti strains displaying various degrees and*
 331 *mechanisms of resistance to deltamethrin*

332 The possibility of cross-resistance of *S. rubra* extract with pyrethroids was investigated
 333 using SrM extract (*S. rubra* sawdust collected in Montsinéry sawmill). Susceptibility tests
 334 for *Ae. aegypti* larvae indicated the lethal concentrations, as presented in Table 3. A
 335 strong larvicidal activity of *S. rubra* wood extracts against susceptible *Ae. aegypti* strain
 336 NO was observed (LD₅₀ and LD₉₀ values of 0.7 (-3.8-5.1) µg/ml and 1.2 (-7.8-10.3) µg/ml,
 337 respectively). Characterized resistant strains showed higher LD values than the
 338 susceptible strain. The insecticide-selected isofemale strains showed the highest LD
 339 values such as for the CAY wild strain.

340

341 **Table 3** Lethal doses (µg/ml) for 50% and 90% (LD₅₀ and LD₉₀ respectively) of the
 342 population of *Aedes aegypti* strains, obtained with *Sextonia rubra* SrM extract, with
 343 associated confidence interval (CI), after 24 and 48h of exposure

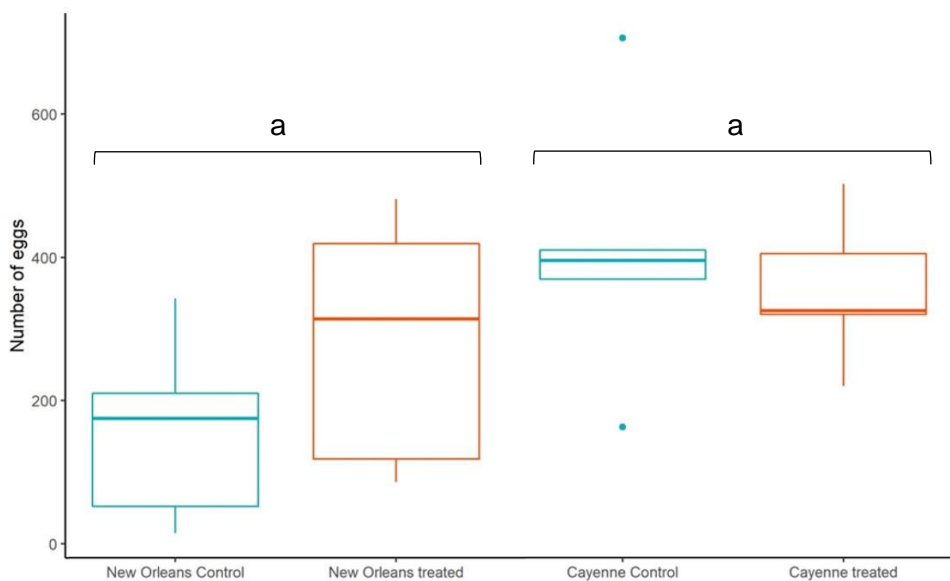
Strain	Lethal doses (µg/ml) at 24 h		Lethal doses (µg/ml) at 48 h	
	LD ₅₀ (CI)	LD ₉₀ (CI)	LD ₅₀ (CI)	LD ₉₀ (CI)
NO	2.2 (1.7-2.7)	5.3 (4.2-6.5)	0.7 (-3.8-5.1)	1.2 (-7.8-10.3)
CAY	62.9 (59.9-68.9)	96.3 (91.8-100.8)	40.1 (36.9-43.3)	62.0 (56.6-67.4)
IR0115	38.8 (33.1-44.5)	72.7 (62.4-83.1)	26.3 (16.3-36.1)	46.7 (28.4-64.9)
IR03F26	58.7 (54.8-62.5)	93.4 (87.3-99.4)	52.1 (48.0-56.1)	83.8 (77.4-90.3)
IR05F26	48.0 (41.7-54.2)	85.6 (74.9-96.2)	34.2 (29.2-39.3)	63.0 (53.6-72.3)

344

345

346 *Influence of S. rubra wood extract on the oviposition behavior of Ae. aegypti, hatching*
347 *response, and assessment of mosquito fitness*

348 Parameters related to mosquito fitness and life history were assayed on respective DL₅₀
349 doses of SrM extract using the reference NO strain and the wild-type CAY strain. Figure
350 1 shows the oviposition preferences of *Ae. aegypti* females in the presence of two egg
351 deposition sites (control and treated with a sublethal dose (DL₅₀) of *S. rubra* wood
352 extract). The obtained results show that no statistically significant difference is observed
353 for the oviposition behavior of *Ae. aegypti* within each strain, and notably the wild CAY
354 strain, between the control breeding site and the treated breeding site (NO strain
355 Student's t-test: $t = -1.2697$, $df = 7.3768$, $p\text{-value} = 0.2428$; CAY strain Student's t-test: t
356 $= 0.54894$, $df = 6.1733$, $p\text{-value} = 0.6023$).

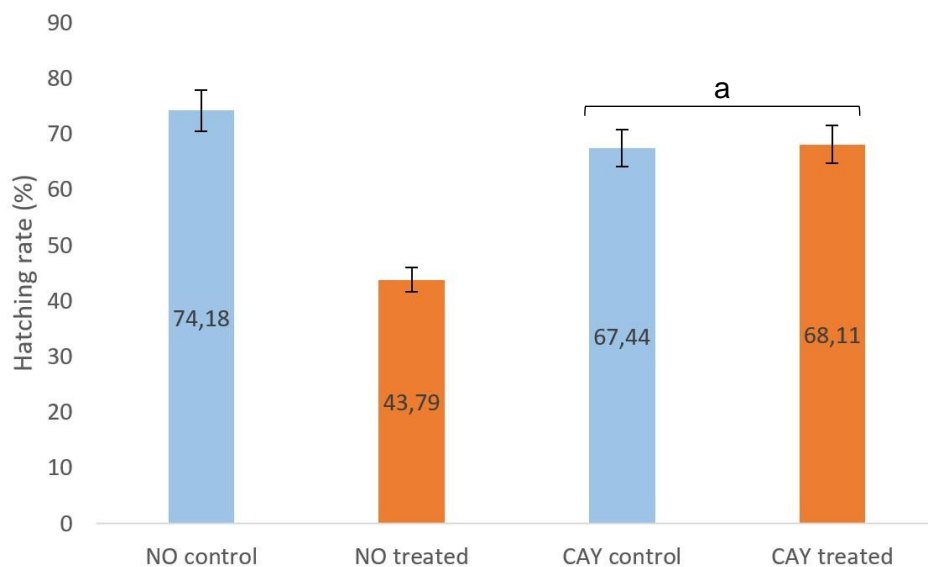


357

358 **Fig. 1** Oviposition responses of gravid females of *Aedes aegypti* to *Sextonia rubra* wood
359 extract. Values correspond to the daily total number of eggs laid in breeding sites for
360 each mosquito strain (NO and CAY) and in relation to treatment, with three replicates.
361 Control: untreated tap water, treated: 2 $\mu\text{g/ml}$ of *S. rubra* wood extract (SrM) for NO strain
362 and 70 $\mu\text{g/ml}$ of *S. rubra* wood extract (SrM) for CAY strain, corresponding to the
363 respective DL₅₀ values for each strain. **a** indicates no statistical difference.

364

365 In the second step, the effects of sublethal doses of *S. rubra* extract were assessed on
366 the fitness of *Ae. aegypti* eggs laid on treated sites from oviposition tests and on
367 mosquitoes originating from these eggs. The first assay aimed to measure whether the
368 hatching success of *Ae. aegypti* eggs (NO and CAY strains) was affected by previous
369 exposure to *S. rubra* wood extracts (Fig. 2). Statistical differences were observed in the
370 hatching success of the different strains depending on the treatment at the breeding site
371 (Chi²: X-squared = 4.4986, df = 1, *p*-value = 0.03392). However, the hatching rate of the
372 CAY strain eggs was not affected by the treatment.



373

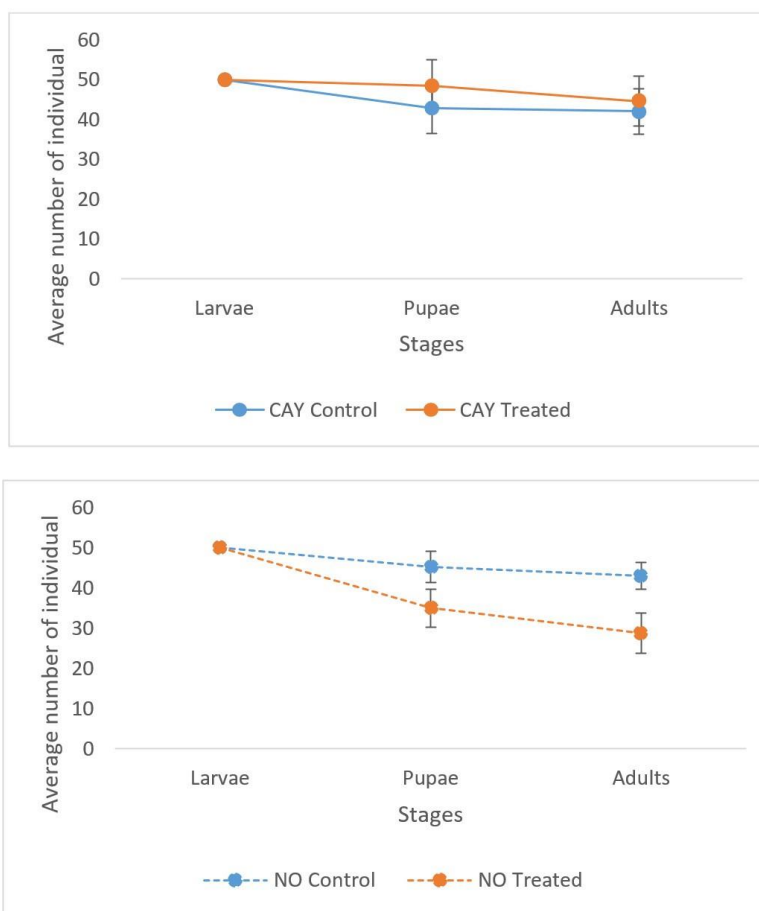
374 **Fig. 2** Hatching rate of the *Aedes aegypti* New Orleans (NO) and Cayenne (CAY) strains
375 according to the treatment applied at the breeding site (control or treated). Control:
376 untreated tap water, Treated: 2 µg/ml of *Sextonia rubra rubra* wood extract (SrM) for NO
377 strain and 70 µg/ml of *Sextonia rubra* wood extract (SrM) for CAY strain. **a** indicates no
378 statistical difference.

379

380 To determine whether the maturation of *Ae. aegypti* eggs on a *S. rubra* extract-
381 impregnated substrate affected the fitness of the mosquitoes at the larval, pupal, and

382 adult stages, the average number of individuals having developed at each stage from 50
383 larvae was counted (Fig. 3). For the NO strain, there was a significant decrease in the
384 number of adults obtained from eggs laid in treated breeding sites (Student's t-test: $t =$
385 5.2774 , $df = 6.9332$, $p\text{-value} = 0.0005935$). For the CAY strain, the decrease in the
386 number of adults obtained from eggs laid in the treated breeding sites is not significant
387 (Student's t-test: $t = -0.68374$, $df = 7.8959$, $p\text{-value} = 0.7431$).

388



389

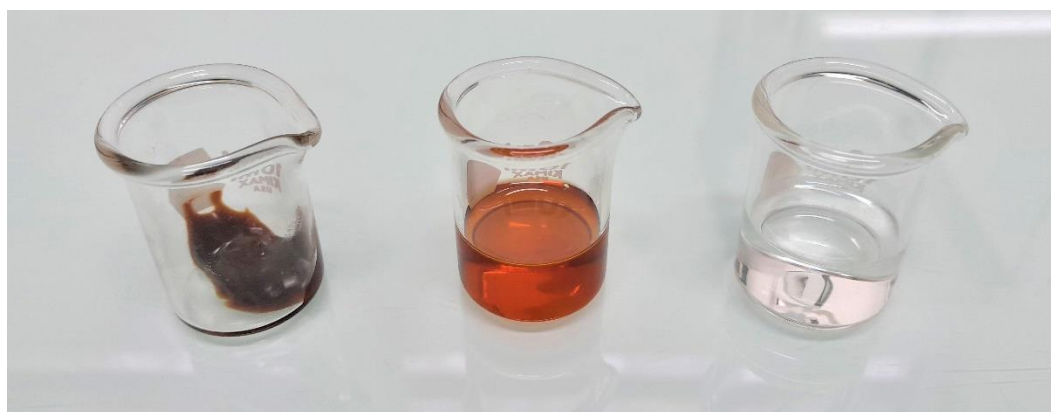
390 **Fig. 3** Average number of *Aedes aegypti* individuals having developed to the pupal and
391 adult stages from 50 larvae (five replicates) according to lineage and treatment. CAY:
392 Cayenne wild strain (upper graph), NO: New Orleans strain (lower graph), Control:
393 individuals from eggs laid in control breeding site, Treated: individuals from eggs laid in
394 treated breeding site). The number of adults obtained from eggs laid in treated breeding

395 sites is significant for the NO strain only (Student's t-test: $t = 5.2774$, $df = 6.9332$, p -value
396 = 0.0005935).

397

398 *Preparation and preliminary evaluation of a S. rubra-based formulation*

399 A prototype emulsifiable concentrate (EC) was developed to enhance the water solubility
400 of the crude extract. Emulsifiable concentrate was titrated in 10% *S. rubra* extract along
401 with stabilizing and dispersing ingredients originating from green chemistry, as described
402 in Table 2. The obtained product was visually stable, and the clear brown viscous aspect
403 obtained directly after mixing the ingredients and the extract was maintained during the
404 two weeks of observation, without the appearance of a cloudy louche or insoluble. This
405 concentrate was also easily dispersed in distilled water in a 0.10% solution to reach a
406 concentration of 100 $\mu\text{g/ml}$ of the *S. rubra* extract, leading to a homogeneous clear
407 mixture even after 10 days. Visual aspect of the extract, emulsifiable concentrate (EC)
408 and emulsion in distilled water (EW) are shown in Figure 4.



409

410 **Fig. 4** *Sextonia rubra*-derived larvicidal products: A) wood ethyl acetate crude extract; B)
411 emulsifiable concentrate (EC); C) micro-emulsion in distilled water (EW), 0.1% solution

412

413 The formulation was developed using the SrK extract (sawdust collected in Kourou
414 sawmill), which was known to exhibit LD_{50} values of 39.6 and 18.6 $\mu\text{g/mL}$ at 24h and 48h,

415 respectively, and LD₉₀ values of 118.2 µg/ml and 51.2 µg/ml at 24h and 48h, respectively,
416 against the CAY strain, and to contain 10.5% and 8.9% (w/w) of the bioactive compounds
417 rubrenolide and rubrynolide respectively (Clervil et al. 2023). When tested in the
418 laboratory against the CAY strain, the formulation led to LD₅₀ values of 48.0 ± 0.01 and
419 9.3 ± 0.05 µg/mL at 24h and 48h, respectively, and LD₉₀ values of 75.1 ± 0.02 and 39.6
420 ± 0.04 µg/ml at 24h and 48h, respectively. To ensure that this effect was not due to the
421 toxicity of the formulation matrix against *Ae. aegypti* larvae, we assessed the matrix and
422 the formulation at 102 µg/ml prior to their evaluation in semi-field conditions. The results
423 obtained for the matrix showed a mortality rate of 9.5 ± 4.8% after 24h of exposure and
424 14 ± 2.1% after 48h, whereas the formulation led to 100% larval mortality in each case.

425

426 *Semi-field bioassays*

427 The formulation was tested at a trial dose of 102 µg/ml for the final product,
428 corresponding to 2xDL₉₀ of the crude extract. The obtained dispersion was
429 homogeneous, without the formation of any oily layer under the water surface, residue,
430 or distinct phases during the entire duration of the assay, despite a certain milky
431 appearance (Fig. 5).

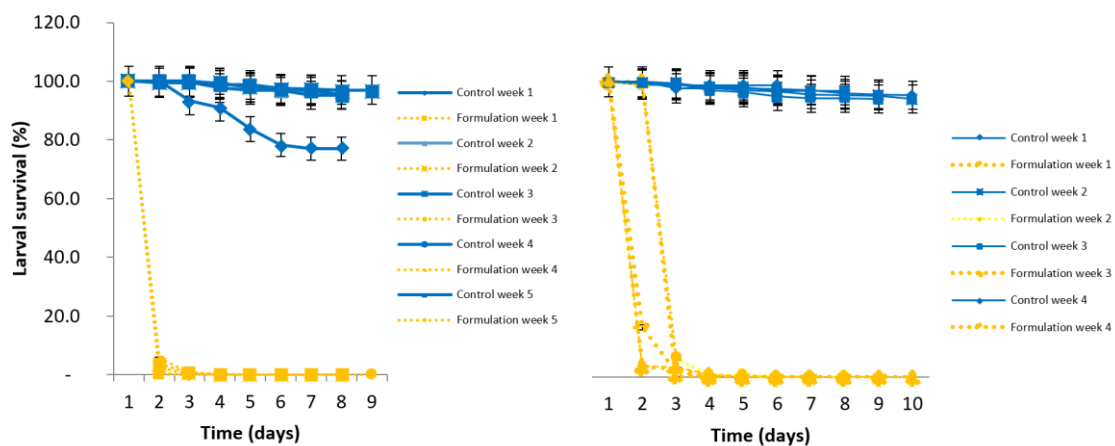


432

433 **Fig. 5** Comparison of the visual aspects of control (left) and treated (right) container when
434 testing a *Sextonia rubra* wood extract-based formulation against *Aedes aegypti* larvae in
435 semi-field conditions

436

437 The results of the semi-field bioassays are presented in Figures 6 and 7. Figure 6 shows
438 the survival of the 3rd-instar larvae of the CAY strain from *Ae. aegypti* mosquitoes. The
439 formulation killed larvae mosquito in less than three days during the dry season, and
440 in less than four days during the rainy season, without any loss of activity during the nine-
441 ten days of observation.



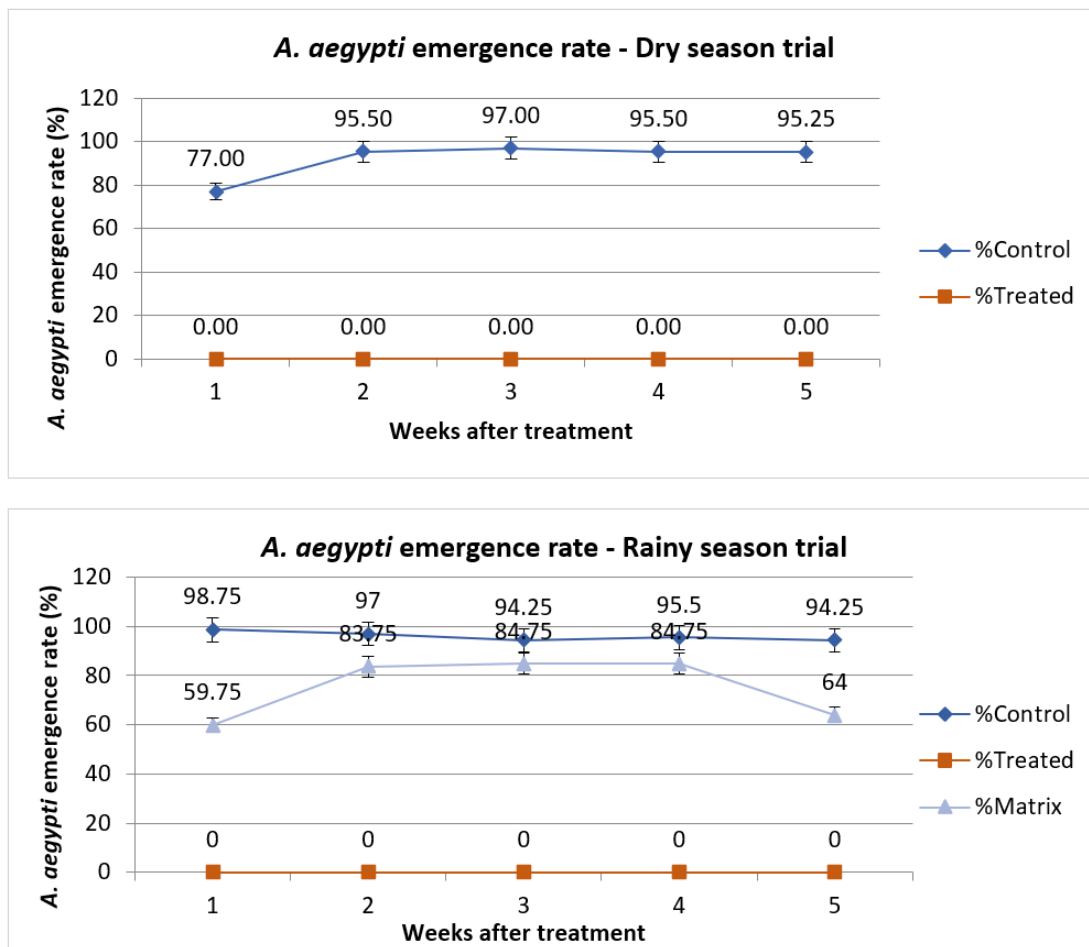
442

443 **Fig. 6** Survival of *Aedes aegypti* mosquito larvae exposed or not to a formulation based
444 on *Sextonia rubra* wood extract under semi-operational test conditions during (a) the dry
445 season (August-September 2020) and (b) the rainy season (January-February 2021).
446 Each curve corresponds to one pool of 100 larvae (average value of four replicates).
447 Control = untreated water, Formulation = EC at a trial dose of 102 $\mu\text{g/ml}$

448

449 Figure 7 shows the emergence rate of adult *Ae. aegypti* remained above 80% during all
450 experiment (5 weeks, i.e., 35 days) for control containers, with a mean emergence rate
451 of $92 \pm 8\%$ in the dry season and $96 \pm 2\%$ in the rainy season. On the contrary, no
452 emergence was observed for the treated containers over all the 5 weeks duration of the
453 experiment. The matrix was tested only once, in the rainy season, exhibiting a slight

454 inhibition of the emergence rate of approximately 15-40%, depending on the time of
455 measurement (Fig. 7).



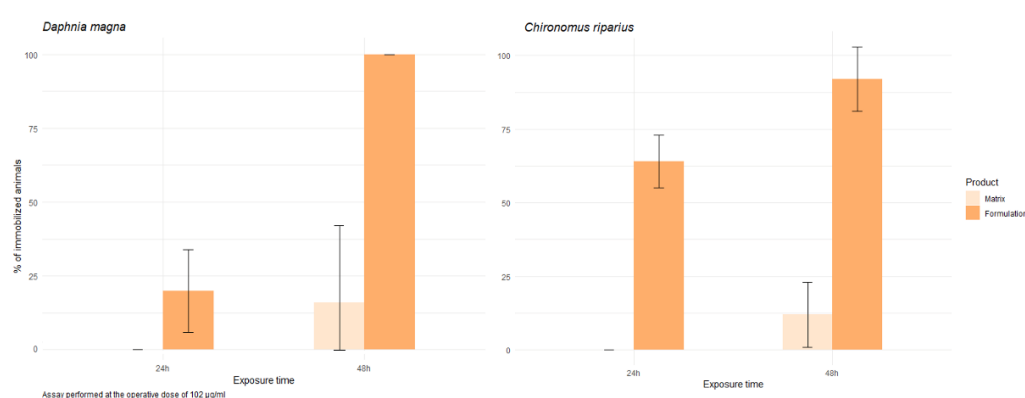
456
457 **Fig. 7** Simulated field trial performed during dry (upper graph) and rainy (lower graph)
458 seasons respectively: residual activity of *Sextonia rubra* wood extract formulation on the
459 emergence of *Aedes aegypti* (\pm s.e.)

460

461 *Impact on aquatic non-target organisms and human cell lines*

462 The matrix and formulation were tested at the dose used in the semi-field trial
463 (102 μ g/ml). At 24h and 48h, the matrix displayed in our test conditions no (at 24h) to
464 low toxicity towards both organisms (16 \pm 26% immobilization for *D. magna*, and 12 \pm
465 11% immobilization for *C. riparius* at 48h) (Figure 8). For the formulation, significant
466 immobilization rates were observed after both 24h and 48h of exposure in the case of

467 *C. riparius*, with $64 \pm 2\%$ and $92 \pm 2\%$ immobilization respectively. The toxic effect
468 appeared more slowly towards *D. magna*, with only $20 \pm 3\%$ immobilization after 24h of
469 exposure, but 100% of immobilized animals after 48h. Despite high standard deviations,
470 Kruskal-Wallis tests showed significant differences between matrix and formulation for
471 both exposure times for *D. magna* ($\text{Chi}^2 = 6$, $\text{df} = 2$, $p\text{-value} = 0.04979$ at 24h, and Chi^2
472 $= 9$, $\text{df} = 3$, $p\text{-value} = 0.02929$ at 48h) and for *C. riparius* ($\text{Chi}^2 = 9$, $\text{df} = 2$, $p\text{-value} =$
473 0.01111 and $\text{Chi}^2 = 9$, $\text{df} = 3$, $p\text{-value} = 0.02929$ at 24h and 48h, respectively).



474

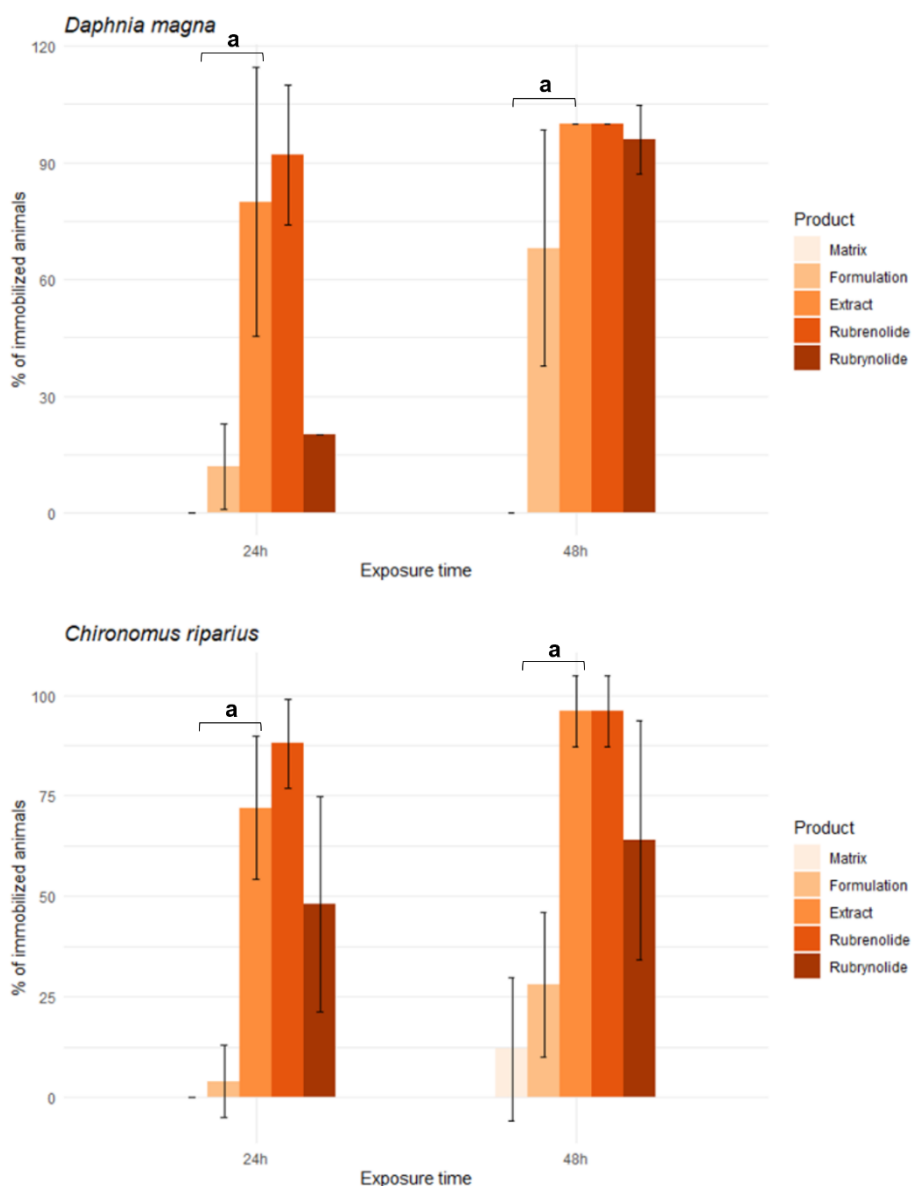
475 **Fig. 8** Comparative assessment of the immobilization percentage (and standard error)
476 of non-target aquatic organisms *Daphnia magna* (left) and *Chironomus riparius* (right)
477 after 24h and 48h of exposure to the formulation matrix (light orange) and full formulation
478 (*Sextonia rubra* extract emulsifiable concentrate; dark orange) at an operative dose of
479 $102 \mu\text{g/ml}$. Ecotoxicity assays were performed using 5 animals per condition according
480 to OECD guidelines, hence the high standard deviations observed

481

482 The effect of extract, matrix and formulation was then assessed at a lower dose, chosen
483 as the 48h-LD₅₀ ($18.6 \mu\text{g/ml}$) of the SrK extract used to prepare the emulsifiable
484 concentrate. The compounds rubrenolide and rubrymolide were tested at a concentration
485 of $2 \mu\text{g/ml}$, which corresponds to approximately 10% of the 48h-LD₅₀, consistent with the
486 extract composition, which contained 10.5% rubrenolide and 8.9% rubrymolide (Clervil et

487 al. 2023). The results are shown in Fig. 9. No to very low effects were observed for the
488 matrix towards both non-target organisms. The environmental effect of the formulation
489 could be considered low in the case of *C. riparius*, with a maximum immobilization rate
490 of only $28 \pm 18\%$ after 48h of exposure at a dose corresponding to the 48-h LD_{50} of the
491 extract. The effect of EC on *D. magna* was more important, with $68 \pm 30\%$ immobilized
492 animal after 48h. The results obtained for the pure compounds rubrenolide and
493 rubrynlolide indicated strong toxicity against non-target organisms at a dose as low as 2
494 $\mu\text{g/ml}$. Rubrenolide was the most toxic compound, with $92 \pm 18\%$ immobilized *D. magna*
495 and $88 \pm 11\%$ immobilized *C. riparius* as soon as 24h after exposure. At 48 h, rubrenolide
496 caused almost complete mortality of the two non-target organisms (100% for *D. magna*
497 and $96 \pm 9\%$ for *C. riparius*). Rubrynlolide acted more slowly, with immobilization values
498 of $20 \pm 0\%$ and $48 \pm 27\%$ at 24h, and $96 \pm 9\%$ and $64 \pm 30\%$ at 48h for *D. magna* and
499 *C. riparius*, respectively. However, when comparing the effects of EC and crude extract
500 on non-target organisms for statistical significance, no difference was found between the
501 two products at 24 h and 48 h on *D. magna* (Kruskal-Wallis test, $\text{Chi}^2 = 6.3$, $\text{df} = 3$, p -
502 value = 0.09789 and $\text{Chi}^2 = 3.8571$, $\text{df} = 2$, p -value = 0.1454) and *C. riparius* (Kruskal-
503 Wallis test, $\text{Chi}^2 = 9$, $\text{df} = 4$, p -value = 0.0611 in both cases).

504



505

506 **Fig. 9** Immobilization percentage of non-target aquatic organisms *Daphnia magna*
 507 (upper graph) and *Chironomus riparius* (lower graph) after 24h and 48h of exposure.
 508 Formulation matrix, full formulation (*Sextonia rubra* extract emulsifiable concentrate) and
 509 *Sextonia rubra* crude extract were tested at 18.6 µg/ml (*Sextonia rubra* SrK extract 48-h
 510 LD₅₀ against *Aedes aegypti*), and the pure compounds rubrenolide and rubrynlolide at 2
 511 µg/ml. **a** indicates no statistical difference when comparing the formulation and the
 512 extract (Kruskal-Wallis test, *p*-value > 0.05)

513

514 The cytotoxicity of all the products was concurrently assessed in a panel of human cell
 515 lines (Table 4).

516

517 **Table 4** Cytotoxic effects on human cell lines after 48h of exposure to the formulation
 518 matrix, full formulation (*Sextonia rubra* emulsifiable concentrate), *Sextonia rubra* crude
 519 extract, and the pure compounds rubrenolide and rubrymolide. IC₅₀ are expressed in
 520 µg/ml for mixtures, and in µM for pure compounds. Standard errors (SE) are indicated

IC ₅₀ ± SE (µg/ml ^a or µM ^b)	Liver (HuH7)	Kidney (NCI- H295R)	Colon (Caco2)	Skin fibrobl asts	Skin keratinocytes (HaCaT)	Bone marrow neuroblasts (SHY5YSY)	Umbilical cord (HUVEC)
Matrix	143 ± 16	87 ± 7	120 ± 25	> 250	184 ± 24	> 250	214 ± 11
Formulation (EC) ^c	102 ± 32	94 ± 13	172 ± 74	> 250	187 ± 21	156 ± 20	170 ± 14
<i>Sextonia rubra</i> extract	32 ± 8	93 ± 23	91 ± 16	> 250	135 ± 6	> 250	70 ± 5
Rubrenolide	35 ± 4	31 ± 9	> 50	> 50	> 50	> 50	34 ± 3
Rubrymolide	12 ± 4	> 50	> 50	> 50	> 50	> 50	> 50

^a For mixtures

^b For pure compounds

^c EC: emulsifiable concentrate

521

522 According to the standards proposed in the literature, the matrix, formulation, and crude
 523 extracts could be considered nontoxic when exhibiting IC₅₀ values > 100 µg/ml; this
 524 endpoint was set at 25 µM in the case of pure compounds (Cos et al. 2006). From this
 525 perspective, both the matrix and the formulation displayed only slight cytotoxic effects
 526 against kidney NCI-H295R cells (human carcinoma), with IC₅₀ values of 87 and 94 µg/ml,
 527 respectively. The extract, however, could be considered cytotoxic against liver HuH7
 528 cells (human hepatocellular carcinoma, IC₅₀ value of 32 µg/ml), and also led to a
 529 moderate effect against human umbilical vein endothelial cells (HUVEC), kidney, and
 530 colon Caco2 (human colorectal adenocarcinoma) cells, with IC₅₀ values in the 70-93

531 µg/ml range. Among the pure compounds, rubrynolide did not display any cytotoxicity
 532 except against liver cells (IC₅₀ of 12 µg/ml). This was the only case in which this
 533 compound was more toxic than rubrenolide, which exhibited a measurable effect on liver,
 534 kidney, and umbilical cord cells, even if above the 25 µM criterion (IC₅₀ in the 31-35 µM)
 535 range. Notably, no toxicity was observed against any of the products in the skin
 536 fibroblasts.

537 The selectivity index was calculated (Table 5), except for human skin fibroblasts and
 538 human bone marrow neuroblasts in the case of the extract, as the obtained IC₅₀ were
 539 > 250 µg/ml. Overall, EC exhibited higher selectivity indices than the crude extract for
 540 all tested cell lines, with values ranging from 10 (kidney human carcinoma NCI-H295R
 541 cells) to 20 (skin human immortalized keratinocytes HaCat).

542

543 **Table 5** Selectivity indices calculated as SI = IC₅₀ (nontarget cells) / LD₅₀ (*Aedes aegypti*
 544 Cayenne strain larvae) after 48 h of exposure, for the *Sextonia rubra* extract and the
 545 formulation. n.a.: not applicable

SI	Liver (HuH7)	Kidney (NCI- H295R)	Colon (Caco2)	Skin fibroblasts	Skin keratinocytes (HaCaT)	Bone marrow neuroblasts (SHY5YSY)	Umbilical cord (HUVEC)
<i>Sextonia rubra</i> extract	2	5	5	n.a.	7	n.a.	4
Formulation (EC) ^a	11	10	19	n.a.	20	17	18

^a EC: emulsifiable concentrate

546

547 Discussion

548 In the first step, the activity of the *S. rubra* wood extract was tested against *Ae. aegypti*
 549 strains with different levels and mechanisms of resistance to deltamethrin. These new
 550 results corroborated the previous efficacy measured against the susceptible Paea strain

551 and the preliminary data obtained for the wild strain Cayenne (Falkowski et al. 2016,
552 2020). The overall results were in accordance with the pattern of resistance to pyrethroids
553 described in French Guiana (Dusfour et al. 2015; Faucon et al. 2017; Guidez et al. 2020;
554 Senthil-Nathan 2020; Epelboin et al. 2021). In addition to lethality, the effects of active
555 products on oviposition deterrence and repellency, as well as on the development,
556 longevity, and vitality of the target insect, are also of great importance in the field of vector
557 control (Borges et al. 2019; Turchen et al. 2020; Pavela et al. 2021). Plants and plant-
558 derived substances are often known to potentially interfere with oviposition, resulting in
559 either repellent or attractant effects, especially at concentrations greater above 50 µg/ml
560 (Kanis et al. 2013; El Maghrbi and Hosni 2014; Satho et al. 2015; Seenivasagan et al.
561 2019). In our case, the absence of repulsive effects on the oviposition behavior of *Ae.*
562 *aegypti* is important when evaluating the potential of *S. rubra* extract as a larvicide used
563 in stagnant water. Indeed, a repellent effect regarding oviposition would induce
564 mosquitoes to lay eggs in other, untreated sites, thus rendering such treatment irrelevant
565 and favouring the spread of the vector (Kanis et al. 2013). At the same time, we observed
566 that the larval hatching response measured for the CAY strain eggs did not vary
567 significantly depending on the medium type of the breeding site. Such a result had
568 previously been observed for *Culex molestus* eggs collected in standing water pools in
569 Australia, and placed in breeding sites with different treatments (methoprene and QN50,
570 a sesquiterpene alcohol derived from Australian sandalwood *Santalum spicatum* A. DC.)
571 (Spafford et al. 2007). Furthermore, the probability of obtaining pupae and adult *Ae.*
572 *aegypti* mosquitoes is lowered when eggs are laid in treated sites is reduced only in the
573 case of the NO strain, as no statistical difference was obtained in the case of the
574 Cayenne wild strain. It would therefore be interesting to evaluate such an effect on the
575 formulation and on other wild-type strains collected in French Guiana, in order to assess
576 the existence of a potential secondary beneficial effect of *S. rubra* extract and formulation
577 in cases of sublethal exposure of the larvae (Silva et al. 2019).

578 The ingredients of the formulation were chosen to comply with EU Regulation 528/2012
579 and Directive 98/8/EC (Biocidal Products directive) on the placing on the market and use
580 of biocidal products, or Regulation (EC) 1107/2009 on the placing on the market of plant
581 protection products. Regulatory approvals represent an important barrier to the industrial
582 implementation of botanical insecticides, and early consideration of them is therefore
583 crucial (Villaverde et al. 2014; Isman 2020). In particular, natural-oil-based esters,
584 including methyl esters, have been documented and patented for their remarkable
585 solvency properties when used to solubilize different insecticides and obtain oil-in-water
586 (EW) emulsions (Taranta et al. 2002; Salimon et al. 2012; Tan et al. 2013). Surfactants
587 were selected among products derived from renewable raw materials, with non-ecotoxic
588 properties under environmental conditions as well as high biodegradability properties
589 (Rybinski and Hill 1998; Johansson and Svensson 2001; Renkin et al. 2005; Zhang et
590 al. 2011; Geetha and Tyagi 2012; Peng et al. 2019).

591 The LD₅₀ and LD₉₀ values obtained for the formulation in the laboratory can lead to its
592 classification as a significant larvicidal agent (Machado et al. 2023). Micro- and nano-
593 encapsulation of active ingredients, especially botanical products, may lead to a slight to
594 significant increase in the lethal concentration value for the formulated products (Kanis
595 et al. 2012; Pavela et al. 2021). Such phenomena did not appear in our hands, with, on
596 the contrary, the same range of activity for both the crude extract and the emulsifiable
597 concentrate, and thus a gain in activity for the active ingredient when the EC is titrated in
598 10% *S. rubra* extract. This could be due to an increased bioavailability because of the
599 presence of tensioactive compounds in the EC, such as previously observed for
600 controlled-release systems and nano-emulsions of essential oils (Maia et al. 2019;
601 Rodrigues et al. 2021). Furthermore, the comparative mortality rates obtained for both
602 the matrix and the formulation confirmed the relative innocuousness of the matrix against
603 *Ae. aegypti*, and ensured that the semi-field bioassay would provide us with information
604 on the *S. rubra* extract in the form of an EC as the sole active product.

605 The efficacy of the tested formulation as a mosquito larvicide was demonstrated by our
606 results, particularly against the CAY *Ae. aegypti* strain, which exhibited a strong
607 resistance to conventional insecticides. The milky appearance of the aqueous media
608 treated with the emulsifiable concentrate, observed under semi-operational conditions,
609 may be due to the composition of the tap water used for the assay, although it was
610 allowed to dechlorinate for 24 h before use, or to the addition of larval food. Indeed, the
611 presence of calcium, sodium, or chloride ions can modify the spontaneous formation of
612 a micro-emulsion and lead to a basic emulsion while changing the translucent aspect for
613 a cloudy one (Egito et al. 2020). The strong and rapid acute toxicity can represent a
614 technical advantage in the field (Darriet et al. 2010). The persistent residual activity
615 suggests an effective potential of the formulation for vector population inhibition during
616 dengue transmission periods, which is a key parameter for disease control (Kanis et al.
617 2013, 2018; Maia et al. 2019). Botanical products often have to face the problem of
618 residual activity due to their high biodegradability through photodegradation, abiotic
619 oxidation, or volatilisation (Amer and Mehlhorn 2006; Isman 2020). In our case, this
620 relatively long residual activity was achieved for a simple emulsion without the use of
621 slow-release systems. This can be put into perspective with the previously demonstrated
622 stability of the crude *S. rubra* extract under ambient conditions over time (Clervil et al.
623 2023).

624 Regarding the ecotoxicity of the matrix and the formulation under our experimental
625 conditions, *D. magna* was overall more sensitive to the tested products than *C. riparius*,
626 notwithstanding the high standard deviations observed due to low number of animals
627 involved in the homologated protocol. For the matrix and the formulation at the dose
628 used in the semi-field study (102 µg/ml), our results underlined the harmlessness of the
629 green formulation base to non-targeted organisms, as observed for *Ae. aegypti* larvae.
630 These results are also consistent with supplier, regulatory, and literature data on the
631 ecotoxicological assessment of the formulation matrix components (Renkin et al. 2005;

632 Szwach and Lukosek 2017; ECHA 2022a, b). At this dose, 100% mortality of *Ae. aegypti*
633 larvae was achieved after 24 h in the laboratory evaluation of the formulation. At 102
634 µg/ml, the formulation showed a tendency towards selectivity for both non-target
635 organisms only after 24 h (five times less mortality for *C. riparius* than for *Ae. aegypti*
636 larvae at the same dose and approximately 1.5 times less mortality for *D. magna*), with
637 this advantage being lost after 48 h of exposure. However, this tendency was not
638 supported by statistical tests, and should be confirmed by complementary assays.

639 However, when testing the effect of extract, matrix, and formulation on the 48h-LD₅₀ (18.6
640 µg/ml) of the SrK extract used to prepare the emulsifiable concentrate, the formulation
641 resulted in 1.5 less toxicity to *D. magna* and 3.4 less toxicity to *C. riparius* when compared
642 to the extract. In addition to solving the technological problem of solubilising the lipophilic
643 crude extract of *S. rubra* in water, the use of the formulation also allows for lower toxicity
644 of the active ingredient at the same dose without losing larvicidal efficacy.
645 Complementary evaluations should now be carried out in collaboration with public health
646 workers to determine the useful dose of the formulation in the field and thus the precise
647 level of selectivity proposed by the *S. rubra* EC. The data obtained for rubrenolide and
648 rubrynolide are consistent with the previous evaluation of these pure compounds against
649 *Ae. aegypti* larvae, with higher toxic activity observed for rubrenolide than rubrynolide
650 against both the laboratory strain Paea and the wild strain Cayenne (Falkowski et al.
651 2016). Thus, valuing the extract and even more the formulation rather than the pure
652 compounds can add significant value when it comes to the ecotoxicological profile of the
653 larvicidal products.

654 Eventually, with the exception of the extract tested on kidney cells, the overall cytotoxic
655 effect of the tested product was moderate to insignificant in an acute toxicity assay. The
656 data obtained for the pure compounds rubrenolide and rubrynolide are consistent with a
657 previous study in which the pure compounds showed an IC₅₀ > 100 µg/ml against NIH-
658 3T3 fibroblast cells (Rodrigues et al. 2010). Although rubrenolide had been previously

659 reported to have significant toxicity (GI_{50} in the 3.3-9.9 $\mu\text{g/ml}$ range, i.e., $< 33 \mu\text{M}$) on
660 UACC-62 (human melanoma), MCF-7 (human breast cancer), HT-29 (human colorectal
661 adenocarcinoma), and PC-3 (human prostate cancer) cell lines, most of the values in our
662 assays remained outside the cytotoxic range (Tófoli et al. 2016). However,
663 complementary cytotoxic evaluation of the products in *in vivo* models would be a valuable
664 asset. The selectivity index data are of high interest, as an $SI \geq 10$ has previously been
665 reported in the literature as a valuable criterion for selective samples (Awouafack et al.
666 2013; Houël et al. 2015; Peña-Morán et al. 2016). These results therefore reinforce those
667 obtained when comparing the ecotoxicity of the crude extracts with the formulated
668 products, since in both cases (ecotoxicity and cytotoxicity), a clear advantage is obtained
669 when using the *S. rubra* EC.

670

671 **Conclusion**

672 This study aimed to evaluate *S. rubra* wood extract against *Ae. aegypti* reference,
673 laboratory, and wild strains, in parallel with the evaluation of a *S. rubra*-based formulated
674 product for application in aqueous media. We demonstrated the efficacy of the wood
675 extracts on *Ae. aegypti* larvae, particularly resistant wild strains, and highlighted the lack
676 of repellent effect of the wood extract on mosquito oviposition behavior. A simple, stable,
677 easy-to-produce, and easy-to-use formulation in the form of an emulsifiable concentrate
678 titrated with 10% *S. rubra* extract was successfully obtained. The choice of the
679 ingredients, derived from green chemistry, allowed both the use of a lipophilic plant
680 extract in water and compliance with European standards and regulations. This
681 formulation showed strong larvicidal activity (acute toxicity and residual activity) in semi-
682 field assays, along with better selectivity on non-target aquatic organisms and human
683 cell lines than the crude wood extract. Following previous promising results on the
684 larvicidal activity of *S. rubra* wood extract and the usability of sawmill residues for a large-

685 scale production of the extract, in a biorefinery perspective included in the circular
686 economy objectives, this work aims to contribute to the development of novel solutions
687 for the management of *Ae. aegypti* mosquitoes and the control of vector-borne diseases
688 such as dengue fever and Zika or chikungunya viruses, while addressing both the
689 environmental and the human health issues associated with the use of insecticide
690 products.

691

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926

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937

938 **Competing interests**

939 The authors have no relevant financial or non-financial interests to disclose.

940

941 **Author Contribution Statement**

942 EH, ID and JBD conceived and designed research. EH and NA collected the wood
943 samples and conducted the extractions and chemical analysis. EC conducted the
944 laboratory and semi-field larvicidal assays and the mosquito fitness study. EW conceived
945 the formulation. DA, MC and DH designed and conducted the ecotoxicity assays. RLG
946 and HS designed and conducted the cytotoxicity assays. EH, EC, ID and JBD analyzed
947 the data and wrote the manuscript. All authors read and approved the manuscript.

948

949 **Data availability**

950 The datasets generated during and analyzed during the current study are available from
951 the corresponding author on reasonable request.

952

953 **Ethical statements**

954 Wood material was accessed according to French regulations concerning genetic
955 resources access and benefit-sharing (French Ministry of Ecological Transition and
956 Solidarity permit n° TREL1820249A/80), and obtained the required certificate of
957 compliance delivered by the international Access and Benefit-Sharing Clearing House
958 (UID: ABSCH-IRCC-FR-246958-1).

959