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

Emmanuelle Clervil, Jean-Bernard Duchemin, Nadine Amusant, Edmond Wozniak, Didier Azam, Maïra Coke, Dominique Huteau, Rémy Le Guével, Hélène Solhi, Isabelle Dusfour, et al.

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4 **Efficacy and selectivity of *Sextonia rubra* wood extracts and bio-based**  
5 **formulation for the management of insecticide resistant *Aedes aegypti* in French**  
6 **Guiana**

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25 **Abstract:**

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

44

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

47

48 **Key Message**

- 49 • *S. rubra* wood extract formulation is easily usable in aqueous media, hence to  
50 treat breeding sites
- 51 • Semi-field assays highlighted acute toxicity and residual activity of the  
52 formulation over 5 weeks

- 53 • The formulation is more selective than the extract in eco- and cytotoxicity
- 54 assays
- 55 • *S. rubra* crude extract has no effect on *Ae. aegypti* oviposition behaviour
- 56 • *S. rubra* sawmill waste can be valorized as an insecticide formulation

57

## 58 **Introduction**

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

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

101 *S. rubra* (Mez.) Van der Werff is a common Lauraceae tree in the Guianas, Venezuela  
102 and in Amazonian Brazil. This species of high economic importance is used in the  
103 wood industry in French Guiana, where it is known as “grignon franc” (Soulères 2021).  
104 This industry however produces sizeable quantities of wastes, including sawdust during  
105 logs transformation. Moreover, previous studies performed by our group demonstrated  
106 that the crude wood extract and the purified compounds rubrenolide and rubrynlolide

107 displayed strong activity towards *Ae. aegypti* larvae, and that commercial sawmill  
108 residues of *S. rubra* can be considered as a valuable resource for the large-scale  
109 production of these larvicidal extracts (Falkowski et al. 2016, 2020; Clervil et al. 2023).  
110 We therefore embarked upon characterizing the larvicidal activity of the *S. rubra* wood  
111 extract on *Ae. aegypti* iso-female strains and their parent wild strain collected in French  
112 Guiana. We also evaluated the fitness of mosquitoes exposed to the extract, while  
113 concurrently assessing an aqueous-based formulation containing *S. rubra* wood extract  
114 in semi-field conditions. Eco- and cytotoxicity were investigated, in the objective of  
115 ascertaining the selectivity of the products towards aquatic species and human cells,  
116 and display a better picture of the potential of *S. rubra* material to develop novel  
117 botanical products displaying larvicidal activity against insecticide-resistant *Ae. aegypti*  
118 mosquitoes.

119

## 120 **Materials & method**

### 121 *Mosquito strains and rearing*

122 Five *Ae. aegypti* (Diptera: Culicidae) strains were used. The laboratory New Orleans  
123 (NO) strain is a reference strain susceptible to all insecticides and maintained for  
124 decades in an insectary at the Institut Pasteur de la Guyane, Cayenne, French Guiana.  
125 The Cayenne (CAY) strain was collected in the eponym city, and larvae of the F1  
126 progeny were used for all of the experimentations made in the laboratory and for the  
127 semi-field experimentations. This population is resistant to both pyrethroid and  
128 organophosphate insecticides. A parent population was obtained from wild-caught  
129 larvae from Ile Royale (IR), one of the coastal islands of French Guiana (5.287° N;  
130 52.590° W), in 2015, and led to the field strain IR0115. A low insecticide pressure  
131 characterizes this area of collection. Two isofemale strains were obtained from this  
132 parent population, as previously described after isolation of one mated female  
133 (Epelboin et al. 2021). The information concerning the five used strains are

134 summarized in Table 1. Rearing was performed as previously described (Touré et al.  
135 2017).

136

137 **Table 1** Characteristics of the different *Ae. aegypti* mosquito strains used in the study

Strain	Strain type	Resistance level	Resistance mechanism <sup>a</sup>
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

138 <sup>a</sup> kdr: knock-down resistance

139

140 *Sextonia rubra* wood extract and purified compounds

141 *S. rubra* (Mez.) Van der Werff (Lauraceae) freshly obtained commercial sawmills  
142 wastes were collected in French Guiana. Sawdusts were obtained from Montsinéry  
143 (4°58'34" N 52°26'45" W; September 2017) and Kourou sawmills (5°08'54" N 52°41'20"  
144 W; March 2020) respectively. Mass distribution by particle size was measured as  
145 follows: 60% > 500 µm, 33% between 500 and 200 µm, and 6% < 200 µm for  
146 Montsinéry sawdust, and 74% > 500 µm, 21% between 500 and 200 µm, and 4% <  
147 200 µm for Kourou sawdust. All samples were dried at room temperature (21°C, 65%  
148 relative humidity) until equilibrium humidity (H<13%). Any visible bark material was  
149 manually removed from crude wood powders, which were then extracted using ethyl  
150 acetate (EtOAc, RPE grade, Carlo Erba Reagenti SpA, Rodano, Italy) at a 1/20 ratio  
151 (w/v) at room temperature by maceration during 24h under stirring. After extraction, the  
152 solution was filtered and the solvent removed by evaporation under reduced pressure  
153 with a SpeedVac™ concentrator (Savant SPD121P, Thermo Scientific). The resulting  
154 crude extracts (SrM, from Montsinéry sawdust, and SrK from Kourou sawdust) were  
155 stored in a freezer at -18°C until assayed. Isolation of rubrenolide (Re) and rubrynlolide

156 (Ry) was performed using HPLC semi-preparative chromatography (Waters, Milford,  
157 MA, USA) according to previously described procedures (Fu et al. 2019). Their  
158 identification was confirmed and their content evaluated by NMR. To do so, <sup>1</sup>H NMR  
159 spectra were recorded at 400 MHz and <sup>13</sup>C NMR spectra at 100.6 MHz on a Varian  
160 400 MR spectrometer (Varian, Palo Alto, CA, USA) equipped with a 5 mm inverse  
161 probe (Auto X PGF <sup>1</sup>H/<sup>15</sup>N-<sup>13</sup>C). Samples were dissolved in deuterated chloroform  
162 (CDCl<sub>3</sub>, Carlo Erba) in 5 mm tubes for analysis. SrM extract was characterized with 8.4  
163 ± 0.2% Re and 2.0 ± 0.0% Ry (w/w), and SrK extract with 10.5 ± 0.1% Re and 8.9 ±  
164 0.2% Ry (w/w) (Clervil et al. 2023).

165

#### 166 *Sextonia rubra* wood extract formulation

167 A *S. rubra*-based concentrate was firstly obtained by adding progressively a  
168 commercial rapeseed oil methyl ester (Radia™ 7961, Oleon, Ertvelde, Belgium) to *S.*  
169 *rubra* SrK wood extract, containing 10.5 ± 0.1% and 8.9 ± 0.2% (w/w) of the larvicidal  
170 compounds rubrenolide and rubrynolide, respectively, and which appears as a  
171 brownish glutinous molasses. The mixture was left in contact (3 hours) at room  
172 temperature (23 ± 2°C) with regular manual stirring, and then heated (30 minutes,  
173 60°C) in a double-walled water bath while stirring manually until the extract is fully  
174 solubilized. This process is repeated to blend the concentrate with the emulsifying  
175 ingredients, chosen for their specific HLB value and bio-based origin, and selected as  
176 an alkylpolyglycoside (APG, Simulsol™ SL 7 G, Seppic, Paris, France) and an  
177 ethoxylated rapeseed oil methyl ester (Sepigreen™ C 300, Seppic, Paris, France). An  
178 antifoaming agent was also added (acetylenic diol in ethylene glycol, Surfynol™ 104E,  
179 Evonik Industries AG, Essen, Germany). The finalized formulation was titrated in 10%  
180 of *S. rubra* extract. Eventually, 560 g of this formulation were prepared for the various  
181 assays. The stability of the final product, which is an emulsifiable concentrate (EC)  
182 aiming at obtaining spontaneous oil-in-water micro-emulsion (EW) when added to



183 distilled water, was evaluated by visual observation at room temperature for validation  
 184 before use. Composition of the matrix (defined as all formulation components without  
 185 the extract) and formulation are reported in Table 2.

186

187 **Table 2** Composition (w/w) of formulation matrix and *S. rubra* wood extract emulsifiable

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>S. rubra</i> wood extract	Active substance	-	10,0
Water		10,0	-

188 concentrate (EC)

189

190

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

192 *S. rubra* extract (SrM) was investigated using the WHO cup assay for testing mosquito  
 193 larvicides (World Health Organisation 2005). Late third or early fourth-instar larvae  
 194 were used in the test. For each bioassay, 25 larvae of each strain were transferred to  
 195 cups containing distilled water (99 ml) and the tested product (1 ml) diluted in ethanol  
 196 (100%, Sigma-Aldrich, Saint-Louis, MO, USA), at a suitable concentration. Eight cups  
 197 per concentration (8 x 25 larvae) were used, and the concentrations adapted to obtain  
 198 from 0% to 100% mortality, thus allowing for LD<sub>50</sub> and LD<sub>90</sub> calculation (from 0.001 to 8  
 199 µg/ml for NO strain and from 3 to 120 or 160 µg/ml for all other strains). Control  
 200 treatments were performed for each test with ethanol only (1 ml in 99 ml of distilled

201 water), and larval mortality was recorded at 24 and 48 h after exposure. Test was  
202 invalidated if mortality in control was >20%. Lethal doses were obtained by a log-Probit  
203 regression under a general linearized model using the MASS package in R software  
204 version 3.2.0 (<https://www.r-project.org/>) as previously described (Falkowski et al.  
205 2016).

206

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

209 Assessment of the influence of *S. rubra* wood extract on the oviposition behavior of the  
210 mosquito was performed as described in the literature, with some modifications (El  
211 Maghrbi and Hosni 2014; Seenivasagan et al. 2019). Holding cages (30 x 30 x 30 cm)  
212 were each filled with 40 adult females and 40 adult males (6 days old) of *Ae. aegypti*  
213 New Orleans strain (3 replicates) and Cayenne strain (3 replicates). Adult mosquitoes  
214 were continuously fed on cotton soaked in 10% sugar until one day before the blood  
215 meal. Two days after the female mosquito have been artificially blood fed (beef blood),  
216 two breeding sites consisting in laying cups were placed in each cage: a control site  
217 (100 ml untreated distilled water), and a site with water (100 ml) treated with SrM  
218 extract. Doses for the *S. rubra* treatment were adapted for each population and  
219 centered around the two respective 24h-DL<sub>50</sub> values (2 µg/ml of SrM *S. rubra* wood  
220 extract for New Orleans strain; 70 µg/ml of SrM *S. rubra* wood extract for Cayenne  
221 strain). A paper wick was placed in each breeding site on day 3 after the blood meal,  
222 and replaced each day during 5 days by a new one, dried, and the number of eggs laid  
223 on each paper was counted. The total number of eggs was used as a measure of  
224 oviposition response. To obtain a new generation of mosquitoes, each paper wick was  
225 put in clear water after 5 days drying, and the rearing performed as previously  
226 described (Clervil et al. 2023). The numbers of hatched eggs were counted and 50  
227 larvae coming from hatched eggs of each strain and each condition were placed into 5  
228 different trays as five replicates. The daily survival and stage development follow-up

229 (average number of individuals having developed to pupal and adult stages from 50  
230 larvae according the strain and treatment) lasted until the complete emergence of all  
231 individuals. Student t-test was applied to measure the influence of the treated water on  
232 the oviposition behavior and on mosquitoes' fitness (hatching rate and mosquito  
233 development). In all analyses, a  $p$ -value < 0.05 was taken to indicate statistical  
234 significance.

235

#### 236 *Simulated field trial*

237 Semi-field trials were conducted on the grounds of the Institut Pasteur de la Guyane  
238 (French Guiana Pasteur Institute) in Cayenne. For the bioassays, only the *Ae. aegypti*  
239 natural population collected in Cayenne town (CAY) was used for testing the  
240 formulated products. The CAY strain was selected to provide a stringent context for an  
241 assay as close to the reality of the field as possible. Experiments were carried out in  
242 white plastic containers (70 l, Icko Apiculture, Bollène, France) filled with water (50 l)  
243 and placed undercover, out of direct sunlight and rainfall, and covered with mosquito  
244 nets to prevent oviposition by wild mosquitoes or other insects of the area, as well as  
245 falling debris during the experiment. Four test containers were used concurrently for  
246 each trial session, alongside with four negatives and four positive controls containers.  
247 For the test barrels, *S. rubra* formulation (50 g), titrated at 10 % of wood extract, were  
248 added to tap water (50 l), to obtain a final concentration of the active product in the  
249 barrel corresponding to twice the extract LD<sub>90</sub> against *Ae. aegypti* CAY larvae after 48h  
250 of exposure in the laboratory, i.e., a trial dose of 102 µg/ml. Negative control containers  
251 were filled with tap water only (50 l), and positive control containers were filled with the  
252 formulation matrix (40 g) dissolved in the water. During the experiment, tap water was  
253 regularly added in the containers to compensate evaporation and maintain a constant  
254 50 l-volume. In each case, tap water was given 24h hours before use for dechlorinating  
255 and conditioning. Late third or early fourth-instar larvae from a first generation (F1)  
256 reared from wild-caught larvae (F0) were used in the tests. A pool of 100 larvae was

257 initially added to each container at the beginning of the trial, and a new pool of 100 live  
258 larvae was released in each container after total emergence of the adults in the control  
259 containers (approximately every week). Larval food (yeast powder, 845 mg) was given  
260 to the larvae during the test. The larval, and adult survival were recorded every day  
261 until the total adult emergence of the controls. This experimentation was realized  
262 during both the dry season (August-September 2020; average maximum temperature  
263 33.8°C; average temperature 27.8°C, average sunlight 239.5 hours; average rainfall  
264 166.5 mm) and the rainy season (January-February 2021; average maximum  
265 temperature 30.8°C; average temperature 26.5°C, average sunlight 86.6 hours;  
266 average rainfall 507 mm) (infoclimat.fr, 2021, Cayenne). For data analysis, the time (in  
267 weeks) is recorded for the inhibition of adult emergence (%IE) to pass below the  
268 threshold value of 80%. For each treatment, %IE was estimated according to control  
269 containers by the following formula (World Health Organisation 2005; Darriet et al.  
270 2010):

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

271 Where C represents the average percentage emergence in the untreated (control)  
272 containers at a particular time and T represents the average percentage emergence in  
273 the treated containers in the same period.

274

275 *Ecotoxicological bioassays on non-target species: Daphnia magna and Chironomus*  
276 *riparius*

277 Ecotoxicity assays were adapted from the guidelines of the “Immediate Immobilization  
278 Test” (OECD No. 202) for *Daphnia magna* (Straus, 1820) and the “Immediate  
279 Immobilization Test” (OECD No. 235) for *Chironomus riparius* (Meigen, 1804). Each  
280 product (crude extract, purified compounds rubrenolide and rubrynlide, formulation,  
281 and formulation matrix) was tested at doses based on the beforehand established LD<sub>90</sub>  
282 (51.2 µg/ml) and LD<sub>50</sub> (18.6 µg/ml) of SrK extract used to obtain the formulation, and

283 measured on the CAY wild strain after 48h of exposure. Formulation and formulation  
284 matrix effect on non-target species were compared at the semi-field trial dose of twice  
285 the SrK crude extract LD<sub>90</sub> (102 µg/ml). The impact of formulation, formulation matrix  
286 and the crude extract were concurrently assessed at the reference LD<sub>50</sub> of the crude  
287 extract. Eventually, the purified compounds rubrenolide and rubrynolide were tested at  
288 2 µg/mL, representing around 10% of the extract LD<sub>50</sub> value, as each compound  
289 represented in average 10% of the extract (Clervil et al. 2023). Three conditions were  
290 tested: water control, water and solvent control, and the dose of analysis, with five  
291 replicates per condition. The physicochemical measurements (pH, dissolved oxygen,  
292 conductivity) were performed with measuring devices (sensors). The remaining  
293 measures (chlorine, nitrites, nitrates, phosphates) were performed in the laboratory  
294 using colorimetric detection (spectrophotometer). Photoperiod and temperature were  
295 recorded using a “templight” recorder throughout the test period, from clutch incubation  
296 until the end of the exposure.

297

#### 298 *Cytotoxicity assays*

##### 299 *Cell culture*

300 Skin normal fibroblastic cells and HUVEC-Umbil Vein (umbilical cord cells) were  
301 purchased from Lonza (Basel, Switzerland), Caco-2 (colon), SHY5Y (human bone  
302 marrow neuroblasts), NCI-H295R (adrenal gland), HaCaT (skin keratinocytes) and  
303 HuH7D12 (liver) cancer cells lines were obtained from the ECACC collection (Porton,  
304 OK, USA). Cells were grown according to ECACC recommendations at 37°C and 5%  
305 CO<sub>2</sub>. All cultures were added with 10 % FBS (HyClone FetalClone III; Cytiva,  
306 H30109.03), penicillin-1% streptomycin (Sigma, P4333) and 2mM glutamine (Sigma,  
307 G7513).

##### 308 *Cytotoxic assays*

309 Chemicals were solubilized in DMSO (Sigma, D4540) at a concentration of 20 mM or  
310 100 mg/ml (stock solution) and diluted in culture medium to the desired final

311 concentrations. The doses effect cytotoxic assays ( $IC_{50}$  determination) were performed  
312 by increasing concentration of each chemical (final well concentrations: 0.3  $\mu$ M – 0.9  
313  $\mu$ M – 3  $\mu$ M – 9  $\mu$ M – 25  $\mu$ M – 50  $\mu$ M or 1  $\mu$ g/ml – 3  $\mu$ g/ml – 9  $\mu$ g/ml – 28  $\mu$ g/ml – 83  
314  $\mu$ g/ml – 250  $\mu$ g/ml). Cells were plated in 96 wells (4000 cells/well). Twenty-four hours  
315 after seeding, cells were exposed to chemicals. After 48h of treatment, cells were  
316 washed in PBS (Sigma D8537) and fixed in cooled 90% ethanol (Sigma 32221) / 5%  
317 acetic acid (Sigma A6283) for 20 minutes. Then, the nuclei were stained with Hoechst  
318 33342 (B2261 Sigma). Image acquisition and analysis were performed using a  
319 Cellomics ArrayScan VTI/HCS Reader (ThermoScientific, Waltham, MA, USA). The  
320 survival percentages were calculated as the percentage of cell number after compound  
321 treatment over cell number after DMSO treatment. To evaluate the selectivity of the  
322 formulation, the selectivity index was used to highlight the results more clearly, as  $IC_{50}$   
323 were available in this case for the non-target cells. This value can be defined as the  
324 ratio of the toxic concentration of a product (considering in our case the 48h-  $IC_{50}$   
325 against human cell lines) against its effective bioactive (in our case, the larvicidal  
326 activity defined by the products 48h- $LD_{50}$ ) concentration (Indrayanto et al. 2021).

327

## 328 **Results**

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

331 The investigation of the possibility of cross-resistance of *S. rubra* extract with  
332 pyrethroids was performed using the SrM extract (*S. rubra* sawdust collected in  
333 Montsinéry sawmill). Susceptibility tests on *Ae. aegypti* larvae indicated the lethal  
334 concentrations presented in Table 3. A strong larvicidal activity of *S. rubra* wood  
335 extracts against the susceptible *Ae. aegypti* strain NO was observed. Characterized  
336 resistant strains showed higher LD values than the susceptible strain. The insecticide-  
337 selected isofemale strains showed, as CAY wild strain, the highest LD values.

338

339 **Table 3** Lethal doses ( $\mu\text{g/ml}$ ) for 50% and 90% ( $\text{LD}_{50}$  and  $\text{LD}_{90}$  respectively) of the  
340 population of *Aedes aegypti* strains, obtained with SrM extract, with associated

Strain	Lethal doses ( $\mu\text{g/ml}$ ) at 24 h		Lethal doses ( $\mu\text{g/ml}$ ) at 48 h	
	$\text{LD}_{50}$ (CI)	$\text{LD}_{90}$ (CI)	$\text{LD}_{50}$ (CI)	$\text{LD}_{90}$ (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)

341 confidence interval (CI), after 24 and 48h of exposure

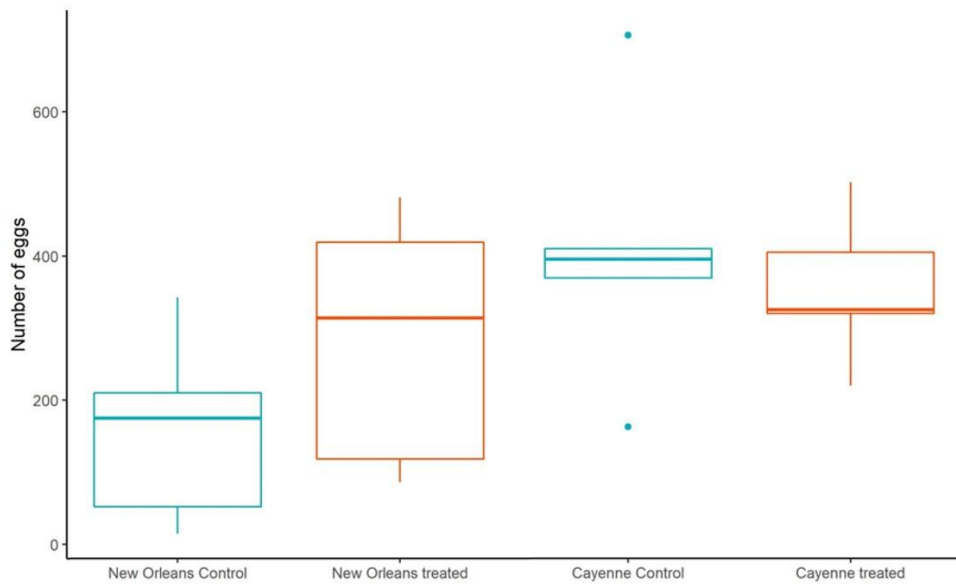
342

343

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

346 Parameters related to the mosquito fitness and life-history were assayed on respective  
347  $\text{DL}_{50}$  doses of SrM extract using the reference NO strain and the wild-type CAY strain.

348 Figure 1 represents oviposition preferences of *Ae. aegypti* females in the presence of  
349 two eggs deposition sites (control, and treated with a sub-lethal dose ( $\text{DL}_{50}$ ) of *S. rubra*  
350 wood extract). The obtained results show that no statistically significant difference is  
351 observed for the oviposition behavior of *Ae. aegypti* within each strain, and notably the  
352 wild CAY strain, between the control breeding site and the treated breeding site (NO  
353 strain Student t-test:  $t = -1.2697$ ,  $df = 7.3768$ ,  $p\text{-value} = 0.2428$ ; CAY strain Student t-  
354 test:  $t = 0.54894$ ,  $df = 6.1733$ ,  $p\text{-value} = 0.6023$ ).



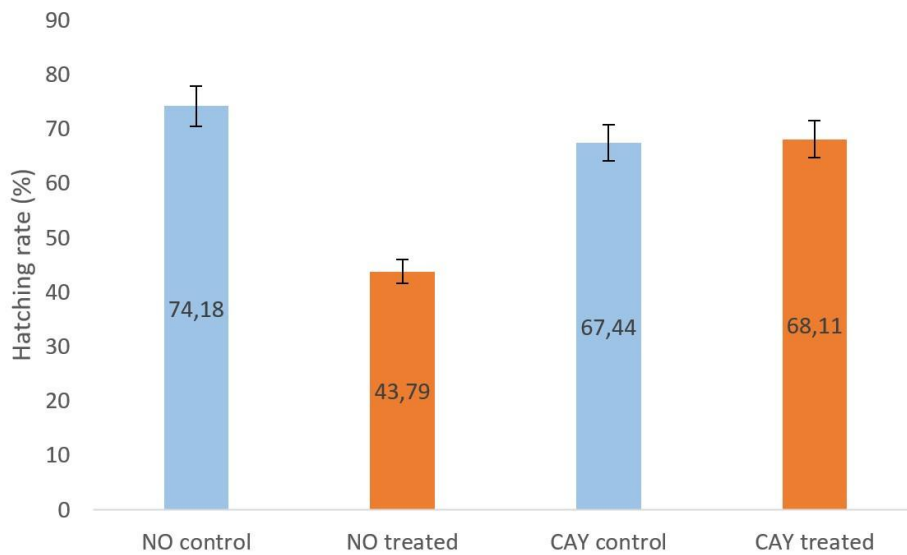
355

356 **Fig. 1** Oviposition responses of gravid females of *Ae. aegypti* to *Sextonia rubra* wood  
 357 extract. Values correspond to the daily total number of eggs laid in breeding sites for  
 358 each mosquito strain (NO and CAY) and in relation to treatment, with three replicates.  
 359 Control: untreated tap water, treated: 2 µg/ml of *S. rubra* wood extract (SrM) for NO  
 360 strain and 70 µg/ml of *S. rubra* wood extract (SrM) for CAY strain, corresponding to the  
 361 respective DL<sub>50</sub> values for each strain

362

363 In a second step, the effects of sub-lethal doses of *S. rubra* extract were assessed on  
 364 the fitness of *Ae. aegypti* eggs laid on treated sites from oviposition tests, and on the  
 365 mosquitoes originating from these eggs. The first assay aimed at measuring whether  
 366 the hatching success of *Ae. aegypti* eggs (NO and CAY strains) is affected by their  
 367 previous exposure to *S. rubra* wood extracts (Fig. 2). Statistical difference was  
 368 observed concerning the hatching success of the different strains depending on the  
 369 treatment of the breeding site (Chi<sup>2</sup>: X-squared = 4.4986, df = 1, *p*-value = 0.03392).  
 370 However, the hatching rate measured for the CAY strain eggs was not affected by the  
 371 treatment.





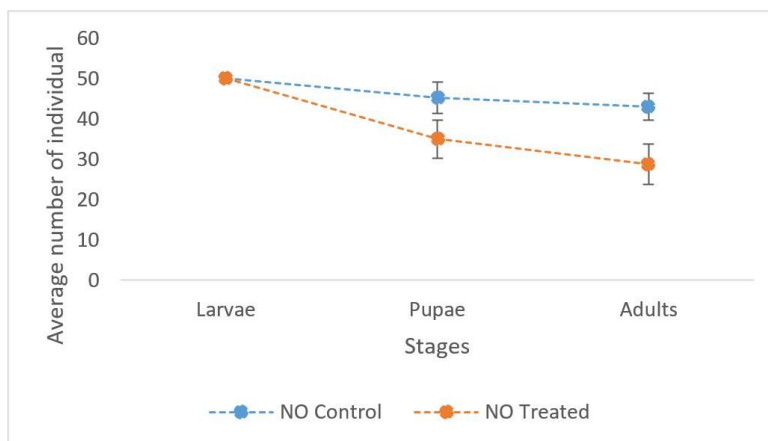
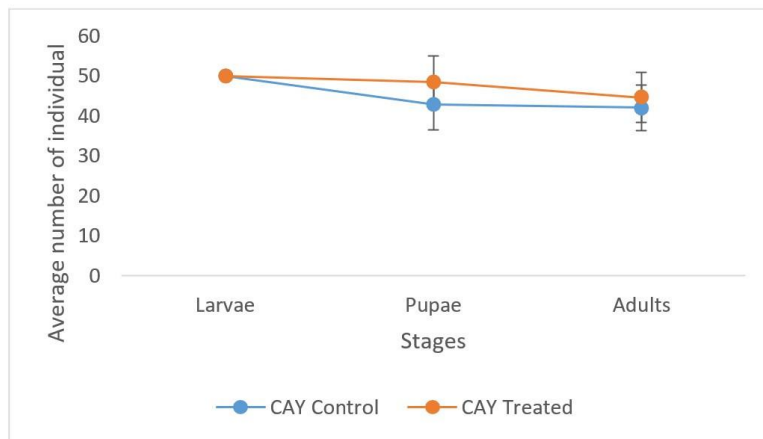
372

373 **Fig. 2** Hatching rate for the New Orleans strain (NO) and the Cayenne strain (CAY)  
 374 according to the treatment applied in the breeding site (Control or treated). Control:  
 375 untreated tap water, Treated: 2 µg/ml of *S. rubra* wood extract (SrM) for NO strain and  
 376 70 µg/ml of *S. rubra* wood extract (SrM) for CAY strain

377

378 To determine whether the maturation of *Ae. aegypti* eggs on a *S. rubra* extract-  
 379 impregnated substrate impact the fitness of the mosquitoes at larval, pupal and adult  
 380 stage, the average number of individuals having developed at each stage from 50  
 381 larvae was counted (Fig. 3). For the NO strain, there is a significant decrease between  
 382 the number of adults obtained from eggs laid in treated breeding sites (Student t-test: t  
 383 = 5.2774, df = 6.9332, *p*-value = 0.0005935). For the CAY strain, the decrease of the  
 384 number of adults obtained from eggs laid in treated breeding sites is not significant  
 385 (Student t-test: t = - 0.68374, df = 7.8959, *p*-value = 0.7431).

386



387

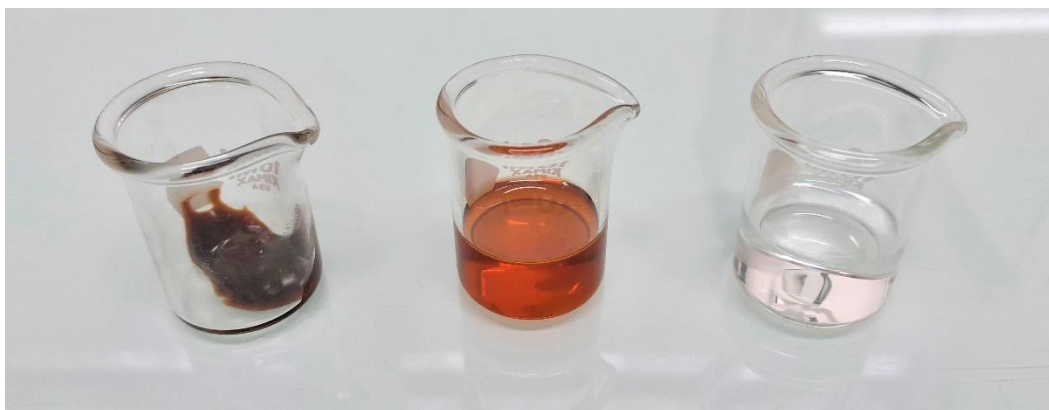
388 **Fig. 3** Average number of individuals having developed to pupal and adult stages from  
 389 50 larvae (x five replicates) according the lineage and treatment. CAY: Cayenne wild  
 390 strain (upper graph), NO: New Orleans strain (lower graph), Control: individuals from  
 391 eggs laid in control breeding site, Treated: individuals from eggs laid in treated  
 392 breeding site)

393

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

395 A prototype emulsifiable concentrate (EC) was developed to enhance the crude extract  
 396 water solubility. The EC was titrated in 10% of *S. rubra* extract alongside with  
 397 stabilizing and dispersing ingredients originating from green chemistry, as described in  
 398 Table 2. The obtained product was visually stable, the clear brown viscous aspect  
 399 obtained directly after mixing the ingredients and the extract was maintained during the

400 two weeks of observation, without the apparition of a cloudy louche or insoluble. This  
401 concentrate was also easily dispersed in distilled water in 0.10% solution to reach a  
402 concentration of 100 µg/ml of *S. rubra* extract, leading to a homogeneous clear mixture  
403 even after 10 days. The visual aspect of the extract, emulsifiable concentrate (EC) and  
404 emulsion in distilled water (EW) are represented in Figure 4.



405

406 **Fig. 4** *S. rubra*-derived larvicidal products: A) wood ethyl acetate crude extract; B)  
407 emulsifiable concentrate (EC); C) micro-emulsion in distilled water (EW), 0.1% solution  
408

409 The formulation was developed using the SrK extract (sawdust collected in Kourou  
410 sawmill), which was known to exhibit LD<sub>50</sub> values of 39.6 and 18.6 µg/mL at 24h and  
411 48h, respectively, and LD<sub>90</sub> values of 118.2 µg/ml and 51.2 µg/ml at 24h and 48h,  
412 respectively, against the CAY strain, and to contain 10.5% and 8.9% (w/w) of the  
413 bioactive compounds rubrenolide and rubrynlide respectively (Clervil et al. 2023).  
414 When tested in the laboratory against the CAY strain, the formulation led to LD<sub>50</sub> values  
415 of 48.0 ± 0.01 and 9.3 ± 0.05 µg/mL at 24h and 48h, respectively, and LD<sub>90</sub> values of  
416 75.1 ± 0.02 and 39.6 ± 0.04 µg/ml at 24h and 48h, respectively. To ensure that this  
417 effect was not due to a toxic activity of the formulation matrix against *Ae. aegypti*  
418 larvae, we assessed the matrix and the formulation at 102 µg/ml prior to their  
419 evaluation in semi-field conditions. The results obtained for the matrix showed a

420 mortality rate of  $9.5 \pm 4.8\%$  after 24h of exposure, and  $14 \pm 2.1\%$  after 48h, whereas  
421 the formulation led to 100% of larval mortality in each case.

422

#### 423 *Semi-field bioassays*

424 The formulation was tested at the trial dose of  $102 \mu\text{g/ml}$  for the final product,  
425 corresponding to  $2x\text{DL}_{90}$  of the crude extract. The obtained dispersion was  
426 homogeneous, without the formation of any oily layer under the water surface, residue,  
427 or formation of distinct phases during the whole duration of the assay, despite a certain  
428 milky appearance (Fig. 5).

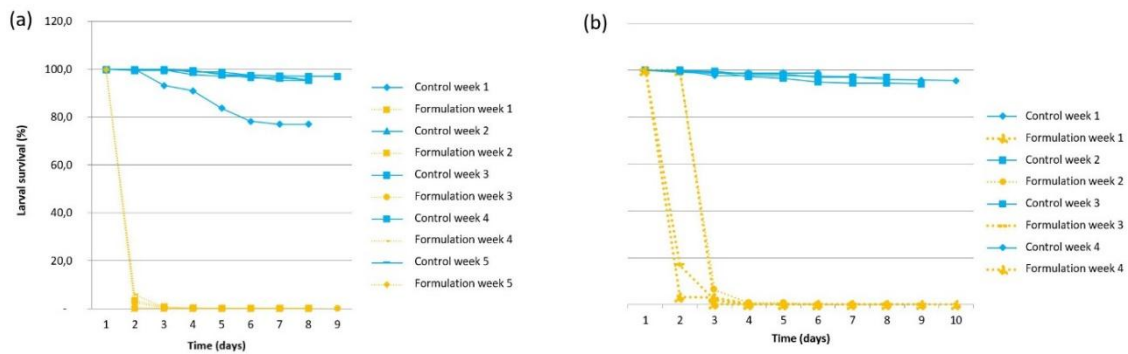


429

430 **Fig. 5** Comparison of the visual aspects of control (left) and treated (right) container  
431 when testing a *S. rubra* wood extract-based formulation against *Ae. aegypti* larvae in  
432 semi-field conditions

433

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

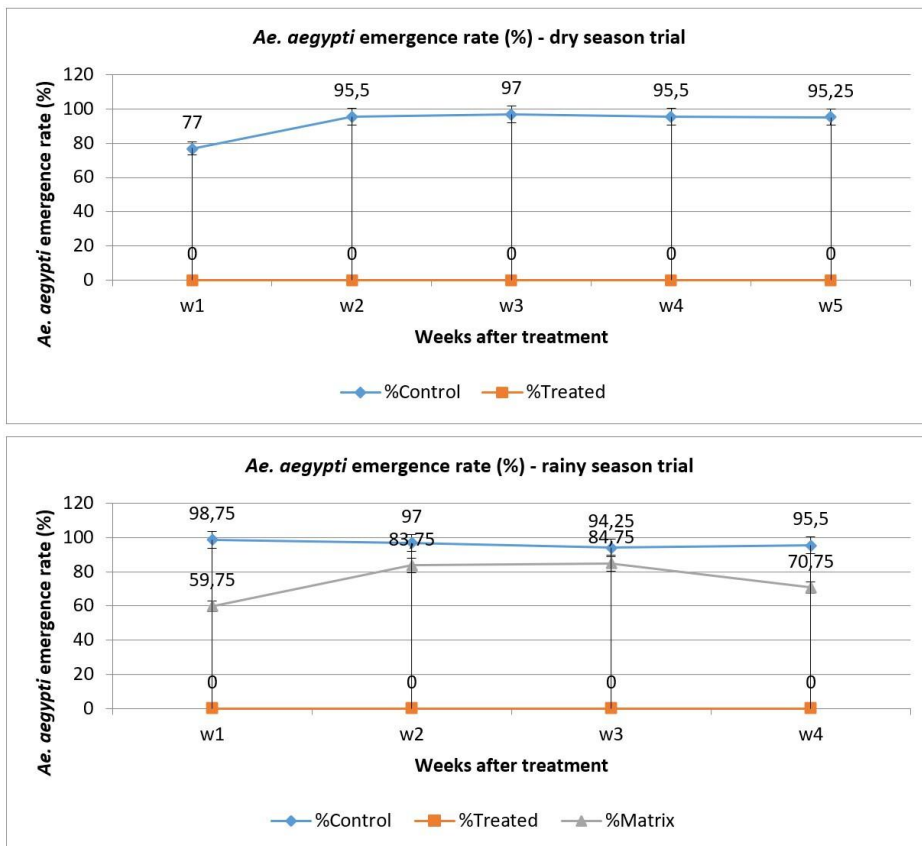


439

440 **Fig. 6** Survival of *Ae. aegypti* mosquito larvae exposed or not to a formulation based on  
 441 *S. rubra* wood extract under semi-operational test conditions, during (a) the dry season  
 442 (August-September 2020) and (b) the rainy season (January-February 2021). Each  
 443 curve corresponds to one pool of 100 larvae (average value of the four replicates).  
 444 Control = untreated water, Formulation = EC at a trial dose of 102 µg/ml

445

446 Figure 7 shows that the emergence rate of adult *Ae. aegypti* stayed above 80% during  
 447 all the experimentation (5 weeks i.e., 35 days) for control containers, with a mean  
 448 emergence rate of  $92 \pm 8\%$  in the dry season and  $96 \pm 2\%$  in the rainy season. On the  
 449 contrary, no emergence was observed for the treated containers over all the 5 weeks  
 450 duration of the experiment. The matrix was tested only once, in the rainy season,  
 451 exhibiting a slight inhibition of the emergence rate of approximately 15-40%, depending  
 452 on the time of measurement (Fig. 7).



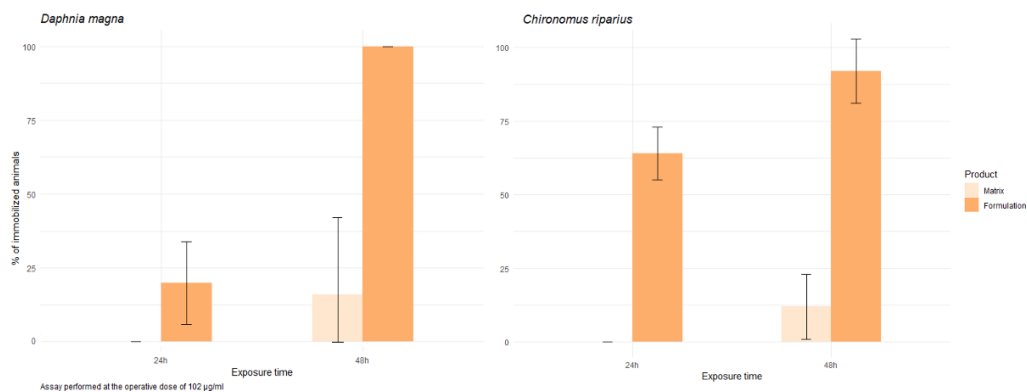
453

454 **Fig. 7** Simulated field trial performed during dry (upper graph) and rainy (lower graph)  
 455 seasons respectively: residual activity of *S. rubra* wood extract formulation on the  
 456 emergence of *Ae. aegypti* ( $\pm$  s.e.)

457

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

459 The matrix and the formulation were tested at the dose used for the semi-field trial  
 460 (102  $\mu$ g/ml). At 24h and 48h, the matrix displayed in our test conditions no (at 24h) to  
 461 low toxicity towards both organisms (16  $\pm$  26% of immobilization for *D. magna*, and 12  
 462  $\pm$  11% immobilization for *C. riparius* at 48h) (Figure 8). For the formulation, significant  
 463 immobilization rates were observed after both 24h and 48h of exposure in the case of  
 464 *C. riparius*, with 64  $\pm$  2% and 92  $\pm$  2% of immobilization respectively. The toxic effect  
 465 appeared more slowly towards *D. magna*, with only 20  $\pm$  3% of immobilization after 24h  
 466 of exposure, but 100% of immobilized animals after 48h.



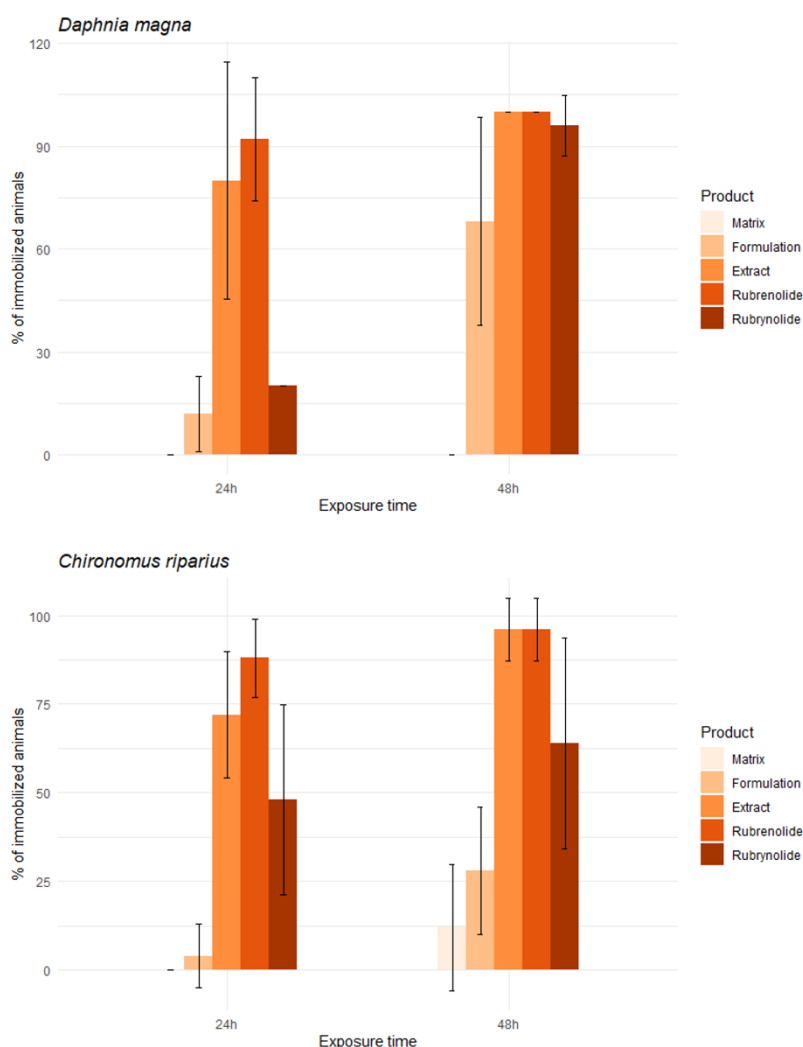
467

468 **Fig. 8** Comparative assessment of the immobilization percentage (and standard error)  
 469 of non-target aquatic organisms *Daphnia magna* (left) and *Chironomus riparius* (right)  
 470 after 24h and 48h of exposure to the formulation matrix (light orange) and full  
 471 formulation (*S. rubra* extract emulsifiable concentrate, dark orange) at the operative  
 472 dose of 102 µg/ml. Ecotoxicity assays were performed using 5 animals per condition  
 473 according to OECD guidelines, hence the high standard deviations observed

474

475 The effect of extract, matrix and formulation was then assessed at a lower dose,  
 476 chosen as the 48h-LD<sub>50</sub> (18.6 µg/ml) of the SrK extract used to prepare the emulsifiable  
 477 concentrate. The compounds rubrenolide and rubrynlolide were tested at a  
 478 concentration of 2 µg/ml, which corresponds to around 10% of the 48h-LD<sub>50</sub> in  
 479 consistency with the extract composition, which contains 10.5% of rubrenolide and  
 480 8.9% of rubrynlolide (Clervil et al. 2023). The overall obtained results are showed in  
 481 Figure 9. No to very low effect was observed for the matrix towards both non-target  
 482 organisms. The environmental effect of the formulation could be considered low in the  
 483 case of *C. riparius*, with a maximum immobilization rate of only 28 ± 18% after 48h of  
 484 exposure at a dose corresponding to the 48-h LD<sub>50</sub> of the extract. The effect of the EC  
 485 towards *D. magna* was more important, with 68 ± 30% of immobilized animal after 48h.  
 486 The results obtained for the pure compounds rubrenolide and rubrynlolide indicated a

487 strong toxicity against non-target organisms, at a dose as low as 2 µg/ml. Rubrenolide  
 488 was the most toxic compound, with  $92 \pm 18\%$  immobilized *D. magna* and  $88 \pm 11\%$   
 489 immobilized *C. riparius* as soon as 24h after exposure. At 48 hours, rubrenolide led to  
 490 almost complete mortality of the two non-target organisms (100% for *D. magna* and  $96$   
 491  $\pm 9\%$  for *C. riparius*). Rubrynlolide acted more slowly, with immobilization values of  $20 \pm$   
 492  $0\%$  and  $48 \pm 27\%$  at 24h, and  $96 \pm 9\%$  and  $64 \pm 30\%$  at 48h for *D. magna* and *C.*  
 493 *riparius* respectively.



494

495 **Fig. 9** Immobilization percentage of non-target aquatic organisms *Daphnia magna*  
 496 (upper graph) and *Chironomus riparius* (lower graph) after 24h and 48h of exposure.  
 497 Formulation matrix, full formulation (*S. rubra* extract emulsifiable concentrate) and *S.*



498 *rubra* crude extract were tested at 18.6 µg/ml (*S. rubra* SrK extract 48-h LD<sub>50</sub> against  
 499 *Ae. aegypti*), and the pure compounds rubrenolide and rubrymolide at 2 µg/ml

500

501 Eventually, the cytotoxicity of all products was concurrently assessed towards a panel  
 502 of human cell lines (Table 4).

503

504 **Table 4** Cytotoxic effect towards human cell lines after 48h of exposure to the  
 505 formulation matrix, full formulation (*S. rubra* emulsifiable concentrate), *S. rubra* crude  
 506 extract, and pure compounds rubrenolide and rubrymolide. IC<sub>50</sub> are expressed in µg/ml  
 507 for mixtures, and in µM for pure compounds

IC <sub>50</sub> (µg/ml <sup>a</sup> or µM <sup>b</sup> )	Liver (HuH7)	Kidney (NCI- H295R)	Colon (Caco2)	Skin fibroblasts	Skin keratinocytes (HaCaT)	Bone marrow neuroblasts (SHY5YSY)	Umbilical cord (HUVEC)
Matrix	143	87	120	> 250	184	> 250	214
Formulation (EC) <sup>c</sup>	102	94	172	> 250	187	156	170
<i>S. rubra</i> extract	32	93	91	> 250	135	> 250	70
Rubrenolide	35	31	> 50	> 50	> 50	> 50	34
Rubrymolide	12	> 50	> 50	> 50	> 50	> 50	> 50

<sup>a</sup> For mixtures

<sup>b</sup> For pure compounds

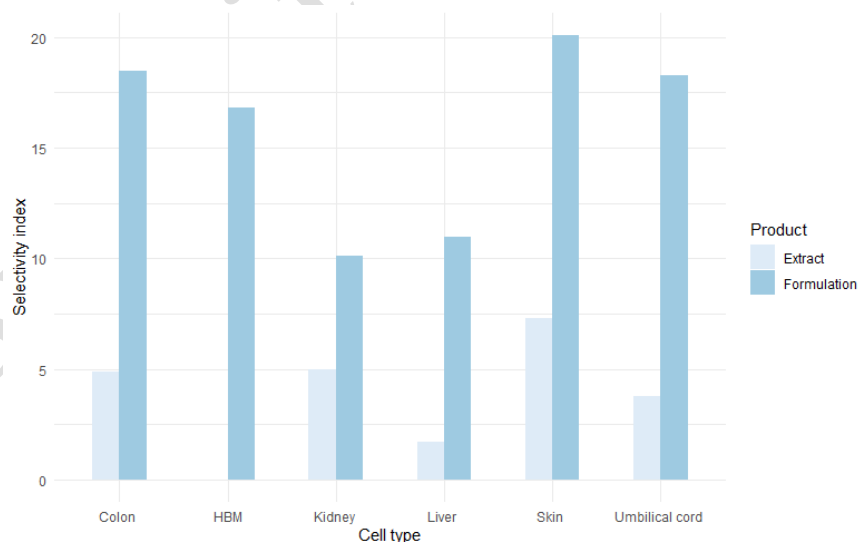
<sup>c</sup> EC: emulsifiable concentrate

508

509 According to the standards proposed in the literature, the matrix, formulation and crude  
 510 extracts could be considered non-toxic when exhibiting IC<sub>50</sub> values > 100 µg/ml, this  
 511 endpoint being set at 25 µM in the case of pure compounds (Cos et al. 2006). In this  
 512 perspective, both the matrix and the formulation only displayed slight cytotoxic effect  
 513 against kidney NCI-H295R cells (human carcinoma), with IC<sub>50</sub> values of 87 and 94

514 µg/ml respectively. The extract however could be considered cytotoxic against liver  
 515 HuH7 cells (human hepatocellular carcinoma, IC<sub>50</sub> value of 32 µg/ml), and also led to a  
 516 moderate effect against HUVEC (human umbilical vein endothelial cells), kidney and  
 517 colon Caco2 (human colorectal adenocarcinoma) cells, with IC<sub>50</sub> values in the 70-93  
 518 µg/ml range. Concerning the pure compounds, rubrynolide did not display any  
 519 cytotoxicity except against liver cells (IC<sub>50</sub> of 12 µg/ml). It was the only case when this  
 520 compound was more toxic than rubrenolide, which exhibited a measurable effect  
 521 towards liver, kidney and umbilical cord cells, even if above the 25 µM criteria (IC<sub>50</sub> in  
 522 the 31-35 µM) range. Notably, no toxicity was observed against any of the products in  
 523 the case of skin fibroblasts.

524 A selectivity index was calculated (Figure 10), except for human skin fibroblasts and  
 525 human bone marrow neuroblasts, as the obtained IC<sub>50</sub> were > 250 µg/ml. Overall, the  
 526 EC exhibited higher selectivity indices than the crude extract for all tested cell lines,  
 527 with values from 10 (kidney human carcinoma NCI-H295R cells) to 20 (skin human  
 528 immortalized keratinocytes HaCat).



529

530 **Fig. 10** Selectivity indices calculated as  $SI = IC_{50} \text{ (non-target cells)} / LD_{50} \text{ (} Ae. aegypti \text{)}$   
 531 Cayenne strain larvae) after 48 hours of exposure for *Sextonia rubra* extract (light blue)  
 532 and formulation (dark blue). HBM: human bone marrow

533

534 **Discussion**

535 In a first step, the activity of *S. rubra* wood extract was assayed against *Ae. aegypti*  
536 strains displaying various degrees and mechanisms of resistance to deltamethrin.  
537 These new results corroborated the previous efficacy measured against the susceptible  
538 Paea strain and the preliminary data obtained about the wild strain Cayenne (Falkowski  
539 et al. 2016, 2020). The overall results are in accordance to resistance pattern towards  
540 pyrethroids described in French Guiana (Dusfour et al. 2015; Faucon et al. 2017;  
541 Guidez et al. 2020; Senthil-Nathan 2020; Epelboin et al. 2021). Besides lethality, the  
542 impact of active products on oviposition deterrence and repellence, as well as  
543 development, longevity or vitality of the target insect are also of great importance in the  
544 field of vector control (Borges et al. 2019; Turchen et al. 2020; Pavela et al. 2021).  
545 Plants and plant-derived substances are often known to possibly interfere with  
546 oviposition effect, leading to either repellent or attractant effect, notably at  
547 concentrations greater than 50 µg/ml (Kanis et al. 2013; El Maghrbi and Hosni 2014;  
548 Satho et al. 2015; Seenivasagan et al. 2019). In our case, the absence of repulsive  
549 effect concerning *Ae. aegypti* oviposition behavior is of importance when it comes to  
550 evaluating the potential of *S. rubra* extract as a larvicide used in stagnant water. A  
551 repulsive effect regarding oviposition would indeed lead the mosquitoes to lay eggs in  
552 other, untreated sites, and therefore at once make such treatment irrelevant and favor  
553 the dispersion of the vector (Kanis et al. 2013). Concurrently, we observed that larval  
554 hatching response measured for the CAY strain eggs did not vary significantly  
555 depending on the breeding site medium type. Such a result had previously been  
556 observed for *Culex molestus* eggs, collected in standing water pools in Australia, and  
557 placed in breeding sites with different treatments (Methoprene and QN50, a  
558 sesquiterpene alcohol derived from Australian sandalwood *Santalum spicatum* A. DC.)  
559 (Spafford et al. 2007). Moreover, the chance to obtain pupae and adult *Ae. aegypti*

560 mosquitoes is lowered when eggs are laid in treated sites in the case of the NO strain  
561 only, as no statistical difference was obtained in the case of the Cayenne wild strain. It  
562 would thus be interesting to assess such an effect on the formulation and on other wild-  
563 type strains collected in French Guiana, in order to evaluate the existence of a potential  
564 secondary beneficial effect of *S. rubra* extract and formulation in case of sublethal  
565 exposition of the larvae (Silva et al. 2019).

566 The ingredients of the formulation were chosen in order to fulfill the regulation (EU)  
567 528/2012 and directive 98/8/EC (Directive Biocide) concerning the making available on  
568 the market and use of biocidal products, or the regulation (EC) 1107/2009 concerning  
569 the placing of plant protection products on the market. Regulatory approvals represent  
570 an important barrier to the industrial implementation of botanical insecticides, and  
571 taking them into account in an early stage is therefore crucial (Villaverde et al. 2014;  
572 Isman 2020). Notably, natural-oil based esters, including methyl esters, have been  
573 recorded and patented for their noticeable solvency properties when used to solubilize  
574 various insecticides and obtain oil-in-water (EW) emulsions (Taranta et al. 2002;  
575 Salimon et al. 2012; Tan et al. 2013). Surfactants were chosen among products  
576 obtained from renewable raw material, with non-ecotoxic characters in environmental  
577 conditions as well as high biodegradability properties (Rybinski and Hill 1998;  
578 Johansson and Svensson 2001; Renkin et al. 2005; Zhang et al. 2011; Geetha and  
579 Tyagi 2012; Peng et al. 2019).

580 The LD<sub>50</sub> and LD<sub>90</sub> values obtained in the laboratory for the formulation can lead to its  
581 categorization as a significant larvicidal agent (Machado et al. 2023). The micro- and  
582 nano-encapsulation of active ingredients, notably botanical products, may lead to  
583 somewhat to noticeable increase of lethal concentrations value for the formulated  
584 products (Kanis et al. 2012; Pavela et al. 2021). Such phenomena did not appear in our  
585 hands, with on the contrary the same range of activity for both the crude extract and  
586 the emulsifiable concentrate, and thus a gain of effect for the active ingredient, as the

587 EC is titrated in 10% of *S. rubra* extract. This could be due to an increased  
588 bioavailability because of the presence of tensioactive compounds in the EC, such as  
589 previously observed for controlled-release systems and nano-emulsions of essential  
590 oils (Maia et al. 2019; Rodrigues et al. 2021). Moreover, the mortality rates obtained for  
591 both the matrix and the formulation, comparatively, confirmed the relative  
592 innocuousness of the matrix against *Ae. aegypti*, and ensured that the semi-field  
593 bioassay would give us information on the *S. rubra* extract in the form of an EC as the  
594 only active product.

595 The efficiency of the tested formulation as a mosquito larvicide was demonstrated by  
596 our results, especially towards the CAY *Ae. aegypti* strain, exhibiting a strong  
597 resistance to conventional insecticides. The milky appearance of the aqueous media  
598 treated with the emulsifiable concentrate observed in semi-operational conditions can  
599 be due to the composition of the tap water used for the assay, although it was let 24h  
600 for dechlorination before use, or to the addition of larval food. Indeed, the presence  
601 calcium, sodium or chloride ions can modify the spontaneous formation of a micro-  
602 emulsion, and lead to a basic emulsion, while changing the translucent aspect for a  
603 cloudy one (Egito et al. 2020). The strong and rapid acute toxicity can represent a  
604 technical advantage in the field.(Darriet et al. 2010) The lasting residual activity  
605 suggests an effective potential of the formulation for vector population inhibition during  
606 dengue transmission periods, which is a key parameter for disease control (Kanis et al.  
607 2013, 2018; Maia et al. 2019). Botanical products often have to face the problem of  
608 residual activity, due to a high biodegradability through photodegradation, abiotic  
609 oxidation or volatilization (Amer and Mehlhorn 2006; Isman 2020). In our case, this  
610 relatively long residual activity was obtained for a simple emulsion, without using slow-  
611 release systems. This can be put in perspective with the previously demonstrated  
612 stability of the crude *S. rubra* extract in ambient conditions over time (Clervil et al.  
613 2023).

614 Concerning the ecotoxicity of the matrix and the formulation under our experimental  
615 conditions, *D. magna* were overall more sensitive to the tested products than *C.*  
616 *riparius*, with high standard deviations observed, due to the protocol involving a low  
617 number of animals. For the matrix and the formulation at the dose used for the semi-  
618 field trial (102 µg/ml), our results highlighted the harmlessness of the green formulation  
619 basis against non-targeted organisms, as observed towards *Ae. aegypti* larvae. These  
620 results are also consistent with suppliers, regulatory and literature data concerning the  
621 ecotoxicological assessment of the formulation matrix components (Renkin et al. 2005;  
622 Szwach and Lukosek 2017; ECHA 2022a, b). At this dose, an *Ae. aegypti* larval  
623 mortality of 100% was obtained as soon as 24h when assessing the formulation in the  
624 laboratory. At 102 µg/ml, the formulation thus only showed selectivity towards both  
625 non-target organisms at 24h (5 times less mortality for *C. riparius* than for *Ae. aegypti*  
626 larvae at the same dose, and around 1.5 times less mortality for *D. magna*), this  
627 advantage being lost after 48h of exposure.

628 However, when testing the effect of extract, matrix and formulation at the 48h-LD<sub>50</sub>  
629 (18.6 µg/ml) of the SrK extract used to prepare the emulsifiable concentrate, the  
630 formulation led to 1.5 less toxicity towards *D. magna*, and 3.4 less toxic effect towards  
631 *C. riparius*, when compared to the extract. Besides solving technological problems due  
632 to the solubilization of *S. rubra* lipophilic crude extract in water, the use of the  
633 formulation also allows to lower the toxicity of the active ingredient at the same dose,  
634 without losing larvicidal efficacy. Complementary assessments should now be  
635 performed in cooperation with public health workers to determine the useful dose of  
636 formulation in the field, and therefore identify the precise level of selectivity proposed  
637 by the *S. rubra* EC. The data obtained for rubrenolide and rubrymolide are consistent  
638 with the previous evaluation of these pure compounds against *Ae. aegypti* larvae, with  
639 higher toxic activity observed for rubrenolide than rubrymolide both on the laboratory  
640 strain Paea and the wild strain Cayenne (Falkowski et al. 2016). Thus, valuing the

641 extract, and even more the formulation, rather than the pure compounds, can add a  
642 noticeable value when it comes to the ecotoxicological profile of the larvicidal products.  
643 Eventually, except for the extract towards kidney cells, the cytotoxic effect of the tested  
644 product was overall moderate to non-significant in an acute toxicity assay. The data  
645 obtained for the pure compounds rubrenolide and rubrynlolide are consistent with a  
646 previous study, in which the pure compounds exhibited  $IC_{50} > 100 \mu\text{g/ml}$  against NIH-  
647 3T3 fibroblasts cells (Rodrigues et al. 2010). Even if a noticeable toxicity had been  
648 previously reported for rubrenolide ( $GI_{50}$  in the 3.3-9.9  $\mu\text{g/ml}$  range i.e.,  $< 33 \mu\text{M}$ )  
649 against UACC-62 (human melanoma), MCF-7 (human breast cancer), HT-29 (human  
650 colorectal adenocarcinoma) and PC-3 (human prostate cancer) cell lines, most values  
651 in our assays remained out of the cytotoxic range (Tófoli et al. 2016). However,  
652 complementary cytotoxic evaluation of the products in *in vivo* models would be a  
653 valuable asset. The selectivity index data are of high interest, as a  $SI \geq 10$  was  
654 previously cited in the literature as a valuable criterion for selective samples  
655 (Awouafack et al. 2013; Houël et al. 2015; Peña-Morán et al. 2016). These results  
656 therefore reinforce those obtained when comparatively assessing the ecotoxicity of the  
657 crude extracts towards the formulated products, as in both cases (eco- and  
658 cytotoxicity), a clear advantage is obtained when using the *S. rubra* EC.

659

## 660 **Conclusion**

661 This study aimed at the evaluation of *S. rubra* wood extract towards *Ae. aegypti*  
662 reference, laboratory and wild strains, concurrently to the assessment of a *S. rubra*-  
663 based formulated product for application in aqueous media. We demonstrated the  
664 efficiency of the wood extracts on *Ae. aegypti* larvae, notably resistant wild strains, and  
665 highlighted the absence of repulsive effect of the wood extract on the mosquitoes'  
666 oviposition behavior. A simple, stable, easy-to-produce and easy-to-use formulation, in

667 the form of an emulsifiable concentrate titrated in 10% of *S. rubra* extract, was  
668 successfully obtained. The choice of the ingredients, derived from green chemistry,  
669 allowed both the application of a lipophilic botanical extract in water and the  
670 compliance with European standards and regulations. This formulation demonstrated  
671 strong larvicidal activity (acute toxicity and residual activity) through semi-field assays  
672 alongside with better selectivity towards non-target aquatic organisms and human cell  
673 lines than the crude wood extract. Following previous promising results on the larvicidal  
674 activity of *S. rubra* wood extract and the useability of sawmill residues for a large-scale  
675 production of the extract, in a biorefinery perspective included in circular economy  
676 objectives, this work aims to contribute to the development of novel solutions for the  
677 management of *Ae. aegypti* mosquitoes and the control of vector-borne diseases such  
678 as dengue fever and Zika or chikungunya viruses, while taking into account  
679 environmental and human health issues associated with the use of insecticide  
680 products.

681

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919

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930

## 931 **Competing interests**

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

933

934 **Author Contribution Statement**

935 EH, ID and JBD conceived and designed research. EH and NA collected the wood  
936 samples and conducted the extractions and chemical analysis. EC conducted the  
937 laboratory and semi-field larvicidal assays and the mosquito fitness study. EW  
938 conceived the formulation. DA, MC and DH designed and conducted the ecotoxicity  
939 assays. RLG and HS designed and conducted the cytotoxicity assays. EH, EC, ID and  
940 JBD analyzed the data and wrote the manuscript. All authors read and approved the  
941 manuscript.

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943 **Data availability**

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

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947 **Ethical statements**

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

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