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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 formulation**  
5 **for the management of insecticide resistant *Aedes aegypti* in French Guiana**

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24 **Abstract:**

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

43

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

46

47 **Key Message**

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

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

55

## 56 **Introduction**

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

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

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

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

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

117

## 118 **Materials & method**

### 119 *Mosquito strains and rearing*

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

133

134 **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

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

136

137 *Sextonia rubra* wood extract and purified compounds

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

157 CA, USA) equipped with a 5 mm inverse probe (Auto X PGF  $^1\text{H}/^{15}\text{N}-^{13}\text{C}$ ). Samples were  
158 dissolved in deuterated chloroform ( $\text{CDCl}_3$ , Carlo Erba) in 5 mm tubes for analysis. SrM  
159 extract was characterized with  $8.4 \pm 0.2\%$  Re and  $2.0 \pm 0.0\%$  Ry (w/w), and SrK extract  
160 with  $10.5 \pm 0.1\%$  Re and  $8.9 \pm 0.2\%$  Ry (w/w) (Clervil et al. 2023).

161

#### 162 *Sextonia rubra* wood extract formulation

163 A *S. rubra*-based concentrate was firstly obtained by adding progressively a commercial  
164 rapeseed oil methyl ester (Radia<sup>TM</sup> 7961, Oleon, Ertvelde, Belgium) to *S. rubra* SrK wood  
165 extract, containing  $10.5 \pm 0.1\%$  and  $8.9 \pm 0.2\%$  (w/w) of the larvicidal compounds  
166 rubrenolide and rubrymolide, respectively, and which appears as a brownish glutinous  
167 molasses. The mixture was left in contact (3 hours) at room temperature ( $23 \pm 2^\circ\text{C}$ ) with  
168 regular manual stirring, and then heated (30 minutes,  $60^\circ\text{C}$ ) in a double-walled water  
169 bath while stirring manually until the extract is fully solubilized. This process is repeated  
170 to blend the concentrate with the emulsifying ingredients, chosen for their specific HLB  
171 value and bio-based origin, and selected as an alkylpolyglycoside (APG, Simulsol<sup>TM</sup> SL  
172 7 G, Seppic, Paris, France) and an ethoxylated rapeseed oil methyl ester (Sepigreen<sup>TM</sup>  
173 C 300, Seppic, Paris, France). An antifoaming agent was also added (acetylenic diol in  
174 ethylene glycol, Surfynol<sup>TM</sup> 104E, Evonik Industries AG, Essen, Germany). The finalized  
175 formulation was titrated in 10% of *S. rubra* extract. Eventually, 560 g of this formulation  
176 were prepared for the various assays. The stability of the final product, which is an  
177 emulsifiable concentrate (EC) aiming at obtaining spontaneous oil-in-water micro-  
178 emulsion (EW) when added to distilled water, was evaluated by visual observation at  
179 room temperature for validation before use. Composition of the matrix (defined as all  
180 formulation components without the extract) and formulation are reported in Table 2.

181



182 **Table 2** Composition (w/w) of formulation matrix and *S. rubra* wood extract emulsifiable  
 183 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>S. rubra</i> wood extract	Active substance	-	10,0
Water		10,0	-

184

185

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

187 *S. rubra* extract (SrM) was investigated using the WHO cup assay for testing mosquito  
 188 larvicides (World Health Organisation 2005). Late third or early fourth-instar larvae were  
 189 used in the test. For each bioassay, 25 larvae of each strain were transferred to cups  
 190 containing distilled water (99 ml) and the tested product (1 ml) diluted in ethanol (100%,  
 191 Sigma-Aldrich, Saint-Louis, MO, USA), at a suitable concentration. Eight cups per  
 192 concentration (8 x 25 larvae) were used, and the concentrations adapted to obtain from  
 193 0% to 100% mortality, thus allowing for LD<sub>50</sub> and LD<sub>90</sub> calculation (from 0.001 to 8 µg/ml  
 194 for NO strain and from 3 to 120 or 160 µg/ml for all other strains). Control treatments  
 195 were performed for each test with ethanol only (1 ml in 99 ml of distilled water), and larval  
 196 mortality was recorded at 24 and 48 h after exposure. Test was invalidated if mortality in  
 197 control was >20%. Lethal doses were obtained by a log-Probit regression under a  
 198 general linearized model using the MASS package in R software version 3.2.0  
 199 (<https://www.r-project.org/>) as previously described (Falkowski et al. 2016).

200

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

203 Assessment of the influence of *S. rubra* wood extract on the oviposition behavior of the  
204 mosquito was performed as described in the literature, with some modifications (El  
205 Maghrbi and Hosni 2014; Seenivasagan et al. 2019). Holding cages (30 x 30 x 30 cm)  
206 were each filled with 40 adult females and 40 adult males (6 days old) of *Ae. aegypti*  
207 New Orleans strain (3 replicates) and Cayenne strain (3 replicates). Adult mosquitoes  
208 were continuously fed on cotton soaked in 10% sugar until one day before the blood  
209 meal. Two days after the female mosquito have been artificially blood fed (beef blood),  
210 two breeding sites consisting in laying cups were placed in each cage: a control site (100  
211 ml untreated distilled water), and a site with water (100 ml) treated with SrM extract.  
212 Doses for the *S. rubra* treatment were adapted for each population and centered around  
213 the two respective 24h-DL<sub>50</sub> values (2 µg/ml of SrM *S. rubra* wood extract for New  
214 Orleans strain; 70 µg/ml of SrM *S. rubra* wood extract for Cayenne strain). A paper wick  
215 was placed in each breeding site on day 3 after the blood meal, and replaced each day  
216 during 5 days by a new one, dried, and the number of eggs laid on each paper was  
217 counted. The total number of eggs was used as a measure of oviposition response. To  
218 obtain a new generation of mosquitoes, each paper wick was put in clear water after 5  
219 days drying, and the rearing performed as previously described (Clervil et al. 2023). The  
220 numbers of hatched eggs were counted and 50 larvae coming from hatched eggs of  
221 each strain and each condition were placed into 5 different trays as five replicates. The  
222 daily survival and stage development follow-up (average number of individuals having  
223 developed to pupal and adult stages from 50 larvae according the strain and treatment)  
224 lasted until the complete emergence of all individuals. Student t-test was applied to  
225 measure the influence of the treated water on the oviposition behavior and on  
226 mosquitoes' fitness (hatching rate and mosquito development). In all analyses, a *p*-value  
227 < 0.05 was taken to indicate statistical significance.

228

229 *Simulated field trial*

230 Semi-field trials were conducted on the grounds of the Institut Pasteur de la Guyane  
231 (French Guiana Pasteur Institute) in Cayenne. For the bioassays, only the *Ae. aegypti*  
232 natural population collected in Cayenne town (CAY) was used for testing the formulated  
233 products. The CAY strain was selected to provide a stringent context for an assay as  
234 close to the reality of the field as possible. Experiments were carried out in white plastic  
235 containers (70 l, Icko Apiculture, Bollène, France) filled with water (50 l) and placed  
236 undercover, out of direct sunlight and rainfall, and covered with mosquito nets to prevent  
237 oviposition by wild mosquitoes or other insects of the area, as well as falling debris during  
238 the experiment. Four test containers were used concurrently for each trial session,  
239 alongside with four negatives and four positive controls containers. For the test barrels,  
240 *S. rubra* formulation (50 g), titrated at 10 % of wood extract, were added to tap water (50  
241 l), to obtain a final concentration of the active product in the barrel corresponding to twice  
242 the extract LD<sub>90</sub> against *Ae. aegypti* CAY larvae after 48h of exposure in the laboratory,  
243 i.e., a trial dose of 102 µg/ml. Negative control containers were filled with tap water only  
244 (50 l), and positive control containers were filled with the formulation matrix (40 g)  
245 dissolved in the water. During the experiment, tap water was regularly added in the  
246 containers to compensate evaporation and maintain a constant 50 l-volume. In each  
247 case, tap water was given 24h hours before use for dechlorinating and conditioning. Late  
248 third or early fourth-instar larvae from a first generation (F1) reared from wild-caught  
249 larvae (F0) were used in the tests. A pool of 100 larvae was initially added to each  
250 container at the beginning of the trial, and a new pool of 100 live larvae was released in  
251 each container after total emergence of the adults in the control containers  
252 (approximately every week). Larval food (yeast powder, 845 mg) was given to the larvae  
253 during the test. The larval, and adult survival were recorded every day until the total adult  
254 emergence of the controls. This experimentation was realized during both the dry season  
255 (August-September 2020; average maximum temperature 33.8°C; average temperature  
256 27.8°C, average sunlight 239.5 hours; average rainfall 166.5 mm) and the rainy season

257 (January-February 2021; average maximum temperature 30.8°C; average temperature  
258 26.5°C, average sunlight 86.6 hours; average rainfall 507 mm) (infoclimat.fr, 2021,  
259 Cayenne). For data analysis, the time (in weeks) is recorded for the inhibition of adult  
260 emergence (%IE) to pass below the threshold value of 80%. For each treatment, %IE  
261 was estimated according to control containers by the following formula (World Health  
262 Organisation 2005; Darriet et al. 2010):

$$263 \quad \%IE = \frac{C - T}{C} \times 100$$

264 Where C represents the average percentage emergence in the untreated (control)  
265 containers at a particular time and T represents the average percentage emergence in  
266 the treated containers in the same period.

267

268 *Ecotoxicological bioassays on non-target species: Daphnia magna and Chironomus*  
269 *riparius*

270 Ecotoxicity assays were adapted from the guidelines of the “Immediate Immobilization  
271 Test” (OECD No. 202) for *Daphnia magna* (Straus, 1820) and the “Immediate  
272 Immobilization Test” (OECD No. 235) for *Chironomus riparius* (Meigen, 1804). Each  
273 product (crude extract, purified compounds rubrenolide and rubrynlolide, formulation, and  
274 formulation matrix) was tested at doses based on the beforehand established LD<sub>90</sub> (51.2  
275 µg/ml) and LD<sub>50</sub> (18.6 µg/ml) of SrK extract used to obtain the formulation, and measured  
276 on the CAY wild strain after 48h of exposure. Formulation and formulation matrix effect  
277 on non-target species were compared at the semi-field trial dose of twice the SrK crude  
278 extract LD<sub>90</sub> (102 µg/ml). The impact of formulation, formulation matrix and the crude  
279 extract were concurrently assessed at the reference LD<sub>50</sub> of the crude extract.  
280 Eventually, the purified compounds rubrenolide and rubrynlolide were tested at 2 µg/mL,  
281 representing around 10% of the extract LD<sub>50</sub> value, as each compound represented in  
282 average 10% of the extract (Clervil et al. 2023). Three conditions were tested: water  
283 control, water and solvent control, and the dose of analysis, with five replicates per

284 condition. The physicochemical measurements (pH, dissolved oxygen, conductivity)  
285 were performed with measuring devices (sensors). The remaining measures (chlorine,  
286 nitrites, nitrates, phosphates) were performed in the laboratory using colorimetric  
287 detection (spectrophotometer). Photoperiod and temperature were recorded using a  
288 “templight” recorder throughout the test period, from clutch incubation until the end of the  
289 exposure.

290

### 291 *Cytotoxicity assays*

#### 292 *Cell culture*

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

#### 301 *Cytotoxic assays*

302 Chemicals were solubilized in DMSO (Sigma, D4540) at a concentration of 20 mM or  
303 100 mg/ml (stock solution) and diluted in culture medium to the desired final  
304 concentrations. The doses effect cytotoxic assays (IC<sub>50</sub> determination) were performed  
305 by increasing concentration of each chemical (final well concentrations: 0.3 μM – 0.9 μM  
306 – 3 μM – 9 μM – 25 μM – 50 μM or 1 μg/ml – 3 μg/ml – 9 μg/ml – 28 μg/ml – 83 μg/ml –  
307 250 μg/ml). Cells were plated in 96 wells (4000 cells/well). Twenty-four hours after  
308 seeding, cells were exposed to chemicals. After 48h of treatment, cells were washed in  
309 PBS (Sigma D8537) and fixed in cooled 90% ethanol (Sigma 32221) / 5% acetic acid  
310 (Sigma A6283) for 20 minutes. Then, the nuclei were stained with Hoechst 33342 (B2261  
311 Sigma). Image acquisition and analysis were performed using a Cellomics ArrayScan

312 VTI/HCS Reader (ThermoScientific, Waltham, MA, USA). The survival percentages were  
313 calculated as the percentage of cell number after compound treatment over cell number  
314 after DMSO treatment. To evaluate the selectivity of the formulation, the selectivity index  
315 was used to highlight the results more clearly, as  $IC_{50}$  were available in this case for the  
316 non-target cells. This value can be defined as the ratio of the toxic concentration of a  
317 product (considering in our case the 48h-  $IC_{50}$  against human cell lines) against its  
318 effective bioactive (in our case, the larvicidal activity defined by the products 48h- $LD_{50}$ )  
319 concentration (Indrayanto et al. 2021).

320

## 321 **Results**

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

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

331

332 **Table 3** Lethal doses ( $\mu\text{g/ml}$ ) for 50% and 90% ( $LD_{50}$  and  $LD_{90}$  respectively) of the  
333 population of *Aedes aegypti* strains, obtained with SrM extract, with associated  
334 confidence interval (CI), after 24 and 48h of exposure

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

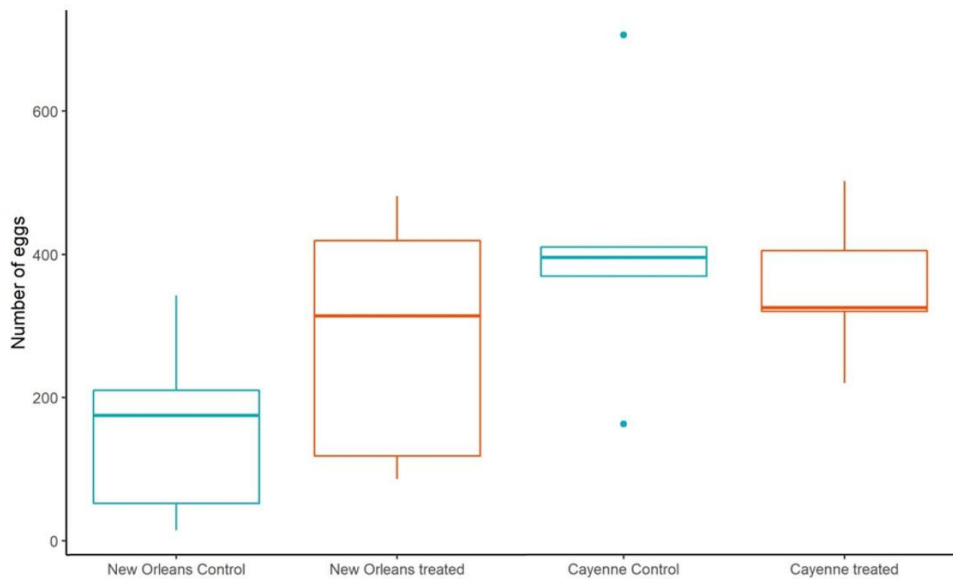
335

336

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

339 Parameters related to the mosquito fitness and life-history were assayed on respective  
 340 DL<sub>50</sub> doses of SrM extract using the reference NO strain and the wild-type CAY strain.

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



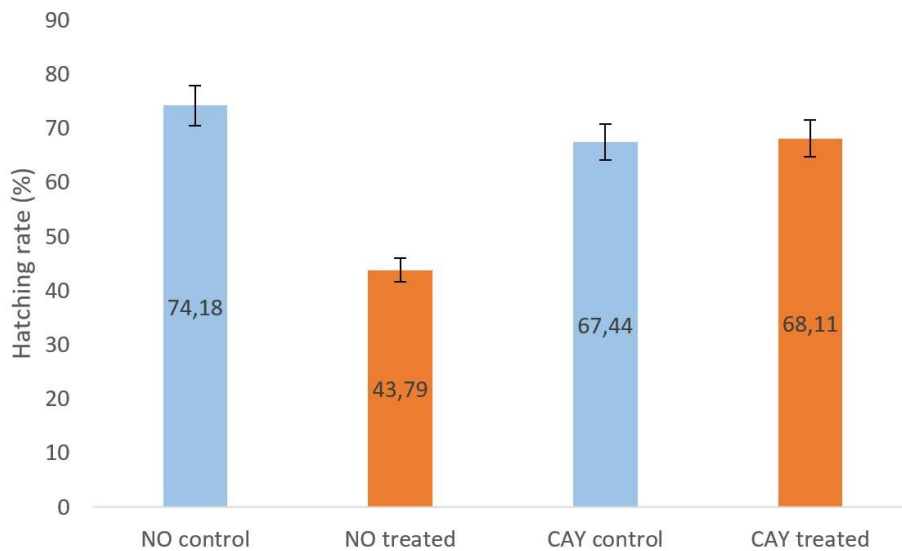
348

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

355

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





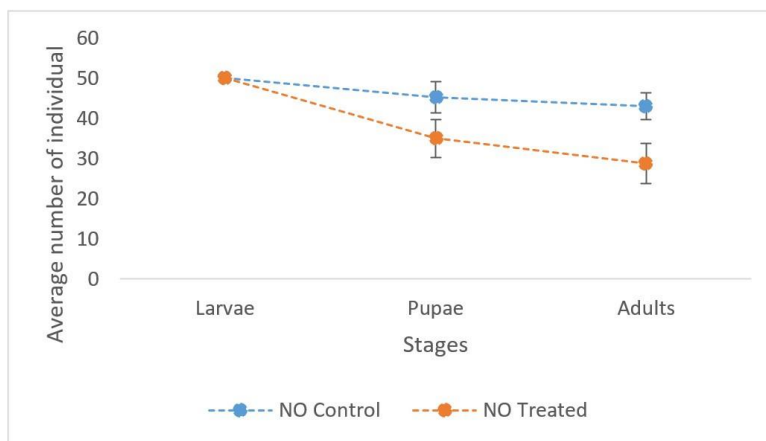
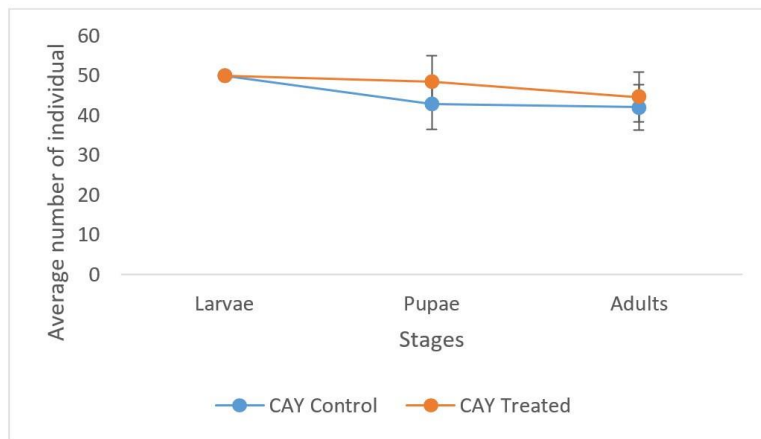
364

365 **Fig. 2** Hatching rate for the New Orleans strain (NO) and the Cayenne strain (CAY)  
 366 according to the treatment applied in the breeding site (Control or treated). Control:  
 367 untreated tap water, Treated: 2 µg/ml of *S. rubra* wood extract (SrM) for NO strain and  
 368 70 µg/ml of *S. rubra* wood extract (SrM) for CAY strain

369

370 To determine whether the maturation of *Ae. aegypti* eggs on a *S. rubra* extract-  
 371 impregnated substrate impact the fitness of the mosquitoes at larval, pupal and adult  
 372 stage, the average number of individuals having developed at each stage from 50 larvae  
 373 was counted (Fig. 3). For the NO strain, there is a significant decrease between the  
 374 number of adults obtained from eggs laid in treated breeding sites (Student t-test:  $t =$   
 375  $5.2774$ ,  $df = 6.9332$ ,  $p\text{-value} = 0.0005935$ ). For the CAY strain, the decrease of the  
 376 number of adults obtained from eggs laid in treated breeding sites is not significant  
 377 (Student t-test:  $t = -0.68374$ ,  $df = 7.8959$ ,  $p\text{-value} = 0.7431$ ).

378



379

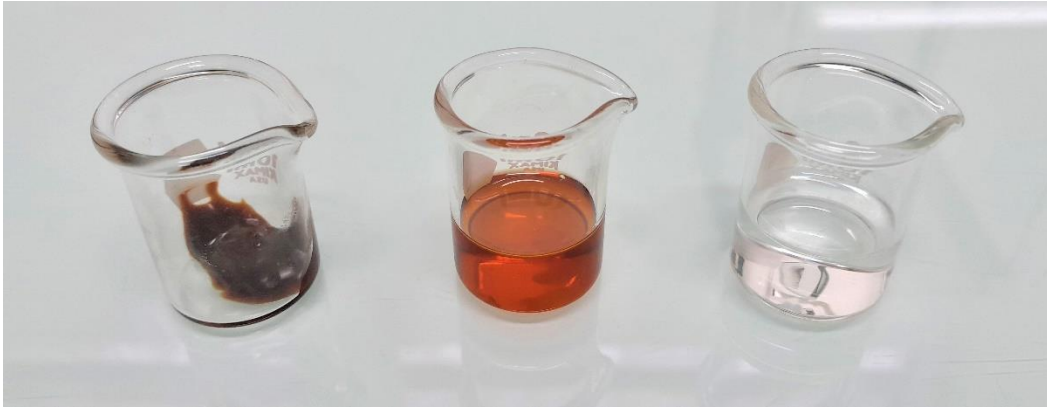
380 **Fig. 3** Average number of individuals having developed to pupal and adult stages from  
 381 50 larvae (x five replicates) according the lineage and treatment. CAY: Cayenne wild  
 382 strain (upper graph), NO: New Orleans strain (lower graph), Control: individuals from  
 383 eggs laid in control breeding site, Treated: individuals from eggs laid in treated breeding  
 384 site)

385

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

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

392 of observation, without the apparition of a cloudy louche or insoluble. This concentrate  
393 was also easily dispersed in distilled water in 0.10% solution to reach a concentration of  
394 100 µg/ml of *S. rubra* extract, leading to a homogeneous clear mixture even after 10  
395 days. The visual aspect of the extract, emulsifiable concentrate (EC) and emulsion in  
396 distilled water (EW) are represented in Figure 4.



397

398 **Fig. 4** *S. rubra*-derived larvicidal products: A) wood ethyl acetate crude extract; B)  
399 emulsifiable concentrate (EC); C) micro-emulsion in distilled water (EW), 0.1% solution  
400

401 The formulation was developed using the SrK extract (sawdust collected in Kourou  
402 sawmill), which was known to exhibit LD<sub>50</sub> values of 39.6 and 18.6 µg/mL at 24h and 48h,  
403 respectively, and LD<sub>90</sub> values of 118.2 µg/ml and 51.2 µg/ml at 24h and 48h, respectively,  
404 against the CAY strain, and to contain 10.5% and 8.9% (w/w) of the bioactive compounds  
405 rubrenolide and rubrynlolide respectively (Clervil et al. 2023). When tested in the  
406 laboratory against the CAY strain, the formulation led to LD<sub>50</sub> values of 48.0 ± 0.01 and  
407 9.3 ± 0.05 µg/mL at 24h and 48h, respectively, and LD<sub>90</sub> values of 75.1 ± 0.02 and 39.6  
408 ± 0.04 µg/ml at 24h and 48h, respectively. To ensure that this effect was not due to a  
409 toxic activity of the formulation matrix against *Ae. aegypti* larvae, we assessed the matrix  
410 and the formulation at 102 µg/ml prior to their evaluation in semi-field conditions. The  
411 results obtained for the matrix showed a mortality rate of 9.5 ± 4.8% after 24h of

412 exposure, and  $14 \pm 2.1\%$  after 48h, whereas the formulation led to 100% of larval  
413 mortality in each case.

414

#### 415 *Semi-field bioassays*

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

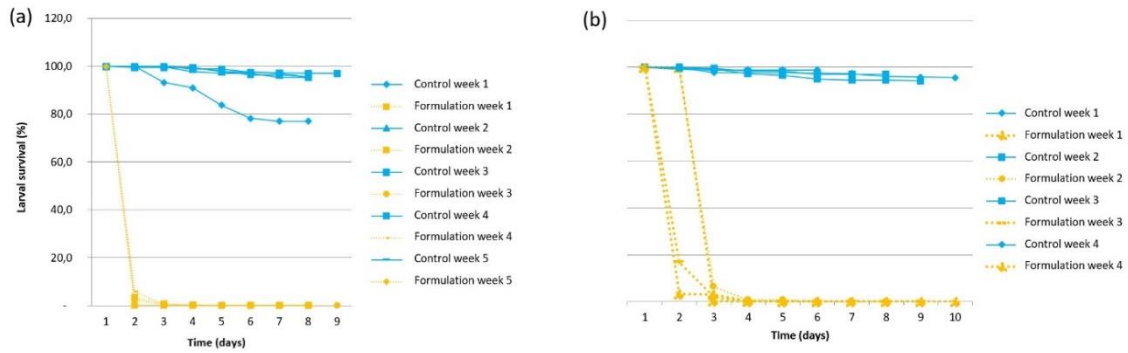


421

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

425

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

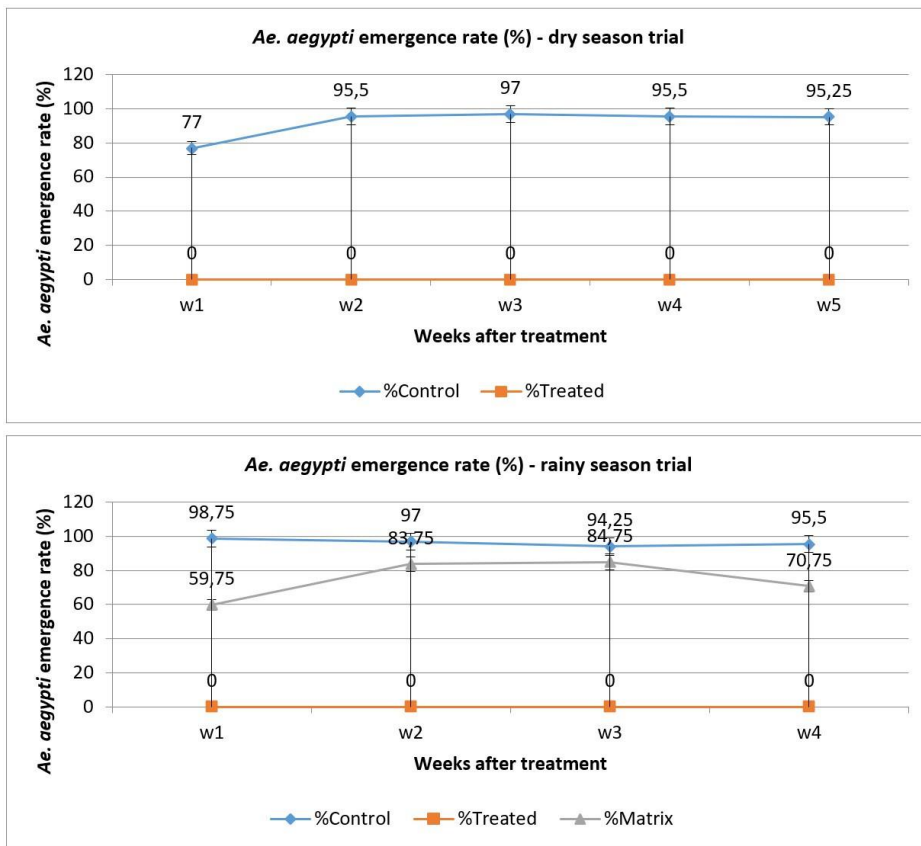


431

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

437

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



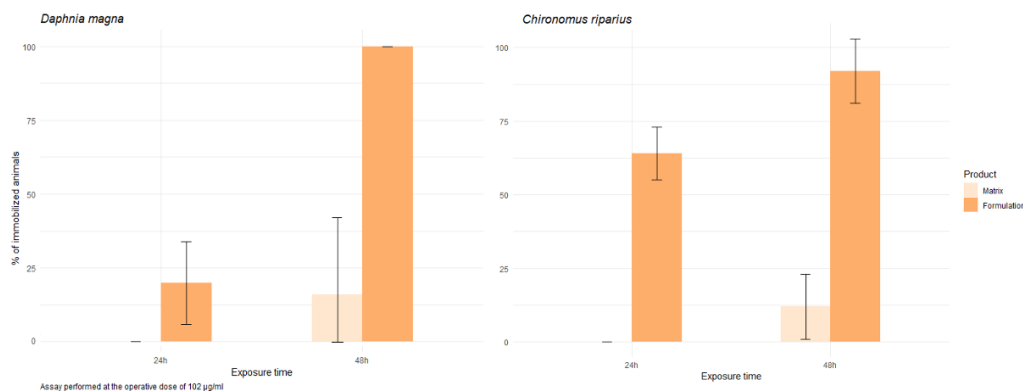
445

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

449

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

451 The matrix and the formulation were tested at the dose used for the semi-field trial  
 452 (102  $\mu$ g/ml). At 24h and 48h, the matrix displayed in our test conditions no (at 24h) to  
 453 low toxicity towards both organisms (16  $\pm$  26% of immobilization for *D. magna*, and 12  $\pm$   
 454 11% immobilization for *C. riparius* at 48h) (Figure 8). For the formulation, significant  
 455 immobilization rates were observed after both 24h and 48h of exposure in the case of  
 456 *C. riparius*, with 64  $\pm$  2% and 92  $\pm$  2% of immobilization respectively. The toxic effect  
 457 appeared more slowly towards *D. magna*, with only 20  $\pm$  3% of immobilization after 24h  
 458 of exposure, but 100% of immobilized animals after 48h.



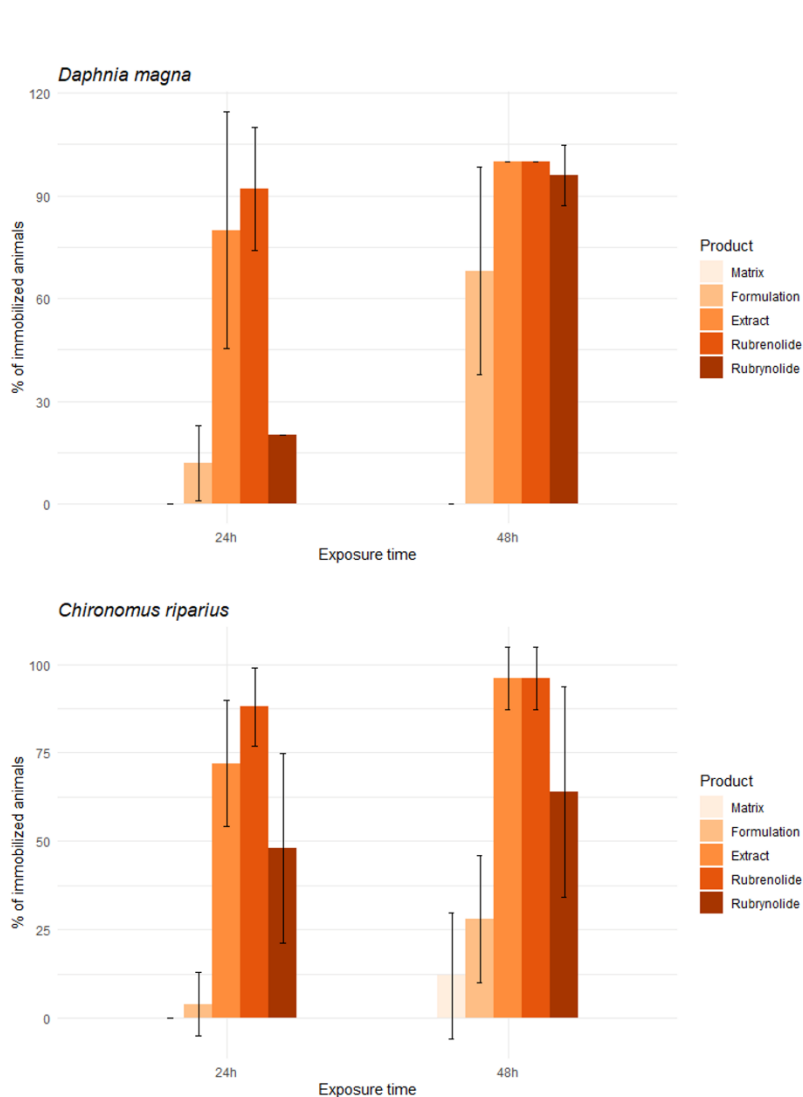
459

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

466

467 The effect of extract, matrix and formulation was then assessed at a lower dose, chosen  
 468 as the 48h-LD<sub>50</sub> (18.6  $\mu\text{g/ml}$ ) of the SrK extract used to prepare the emulsifiable  
 469 concentrate. The compounds rubrenolide and rubrynlide were tested at a concentration  
 470 of 2  $\mu\text{g/ml}$ , which corresponds to around 10% of the 48h-LD<sub>50</sub> in consistency with the  
 471 extract composition, which contains 10.5% of rubrenolide and 8.9% of rubrynlide  
 472 (Clervil et al. 2023). The overall obtained results are showed in Figure 9. No to very low  
 473 effect was observed for the matrix towards both non-target organisms. The  
 474 environmental effect of the formulation could be considered low in the case of *C. riparius*,  
 475 with a maximum immobilization rate of only  $28 \pm 18\%$  after 48h of exposure at a dose  
 476 corresponding to the 48-h LD<sub>50</sub> of the extract. The effect of the EC towards *D. magna*  
 477 was more important, with  $68 \pm 30\%$  of immobilized animal after 48h. The results obtained  
 478 for the pure compounds rubrenolide and rubrynlide indicated a strong toxicity against

479 non-target organisms, at a dose as low as 2 µg/ml. Rubrenolide was the most toxic  
480 compound, with 92 ± 18% immobilized *D. magna* and 88 ± 11% immobilized *C. riparius*  
481 as soon as 24h after exposure. At 48 hours, rubrenolide led to almost complete mortality  
482 of the two non-target organisms (100% for *D. magna* and 96 ± 9% for *C. riparius*).  
483 Rubrynlolide acted more slowly, with immobilization values of 20 ± 0% and 48 ± 27% at  
484 24h, and 96 ± 9% and 64 ± 30% at 48h for *D. magna* and *C. riparius* respectively.



485

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



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

491

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

494

495 **Table 4** Cytotoxic effect towards human cell lines after 48h of exposure to the formulation  
 496 matrix, full formulation (*S. rubra* emulsifiable concentrate), *S. rubra* crude extract, and  
 497 pure compounds rubrenolide and rubrymolide. IC<sub>50</sub> are expressed in µg/ml for mixtures,  
 498 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

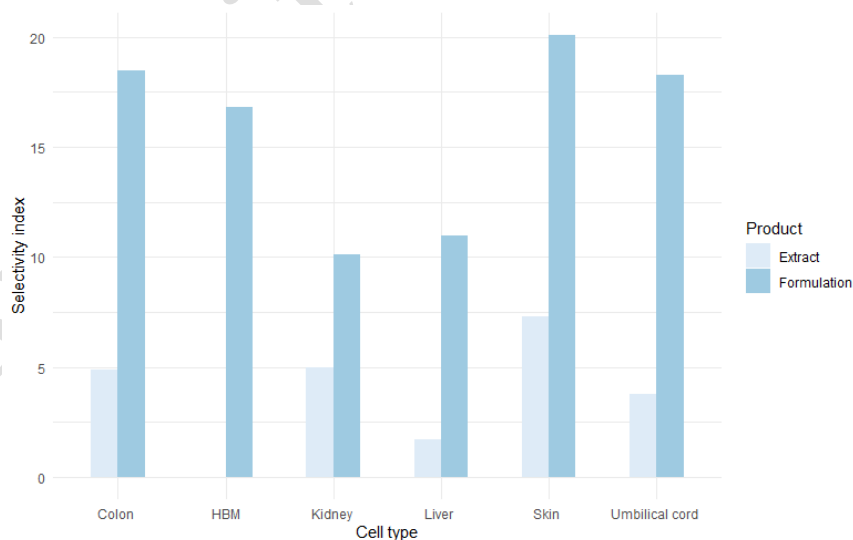
<sup>c</sup> EC: emulsifiable concentrate

499

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

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

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



520

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

524

525 **Discussion**

526 In a first step, the activity of *S. rubra* wood extract was assayed against *Ae. aegypti*  
527 strains displaying various degrees and mechanisms of resistance to deltamethrin. These  
528 new results corroborated the previous efficacy measured against the susceptible Paea  
529 strain and the preliminary data obtained about the wild strain Cayenne (Falkowski et al.  
530 2016, 2020). The overall results are in accordance to resistance pattern towards  
531 pyrethroids described in French Guiana (Dusfour et al. 2015; Faucon et al. 2017; Guidez  
532 et al. 2020; Senthil-Nathan 2020; Epelboin et al. 2021). Besides lethality, the impact of  
533 active products on oviposition deterrence and repellence, as well as development,  
534 longevity or vitality of the target insect are also of great importance in the field of vector  
535 control (Borges et al. 2019; Turchen et al. 2020; Pavela et al. 2021). Plants and plant-  
536 derived substances are often known to possibly interfere with oviposition effect, leading  
537 to either repellent or attractant effect, notably at concentrations greater than 50 µg/ml  
538 (Kanis et al. 2013; El Maghrbi and Hosni 2014; Satho et al. 2015; Seenivasagan et al.  
539 2019). In our case, the absence of repulsive effect concerning *Ae. aegypti* oviposition  
540 behavior is of importance when it comes to evaluating the potential of *S. rubra* extract as  
541 a larvicide used in stagnant water. A repulsive effect regarding oviposition would indeed  
542 lead the mosquitoes to lay eggs in other, untreated sites, and therefore at once make  
543 such treatment irrelevant and favor the dispersion of the vector (Kanis et al. 2013).  
544 Concurrently, we observed that larval hatching response measured for the CAY strain  
545 eggs did not vary significantly depending on the breeding site medium type. Such a result  
546 had previously been observed for *Culex molestus* eggs, collected in standing water pools  
547 in Australia, and placed in breeding sites with different treatments (Methoprene and  
548 QN50, a sesquiterpene alcohol derived from Australian sandalwood *Santalum spicatum*  
549 A. DC.) (Spafford et al. 2007). Moreover, the chance to obtain pupae and adult *Ae.*  
550 *aegypti* mosquitoes is lowered when eggs are laid in treated sites in the case of the NO

551 strain only, as no statistical difference was obtained in the case of the Cayenne wild  
552 strain. It would thus be interesting to assess such an effect on the formulation and on  
553 other wild-type strains collected in French Guiana, in order to evaluate the existence of  
554 a potential secondary beneficial effect of *S. rubra* extract and formulation in case of  
555 sublethal exposition of the larvae (Silva et al. 2019).

556 The ingredients of the formulation were chosen in order to fulfill the regulation (EU)  
557 528/2012 and directive 98/8/EC (Directive Biocide) concerning the making available on  
558 the market and use of biocidal products, or the regulation (EC) 1107/2009 concerning  
559 the placing of plant protection products on the market. Regulatory approvals represent  
560 an important barrier to the industrial implementation of botanical insecticides, and taking  
561 them into account in an early stage is therefore crucial (Villaverde et al. 2014; Isman  
562 2020). Notably, natural-oil based esters, including methyl esters, have been recorded  
563 and patented for their noticeable solvency properties when used to solubilize various  
564 insecticides and obtain oil-in-water (EW) emulsions (Taranta et al. 2002; Salimon et al.  
565 2012; Tan et al. 2013). Surfactants were chosen among products obtained from  
566 renewable raw material, with non-ecotoxic characters in environmental conditions as well  
567 as high biodegradability properties (Rybinski and Hill 1998; Johansson and Svensson  
568 2001; Renkin et al. 2005; Zhang et al. 2011; Geetha and Tyagi 2012; Peng et al. 2019).

569 The LD<sub>50</sub> and LD<sub>90</sub> values obtained in the laboratory for the formulation can lead to its  
570 categorization as a significant larvicidal agent (Machado et al. 2023). The micro- and  
571 nano-encapsulation of active ingredients, notably botanical products, may lead to  
572 somewhat to noticeable increase of lethal concentrations value for the formulated  
573 products (Kanis et al. 2012; Pavela et al. 2021). Such phenomena did not appear in our  
574 hands, with on the contrary the same range of activity for both the crude extract and the  
575 emulsifiable concentrate, and thus a gain of effect for the active ingredient, as the EC is  
576 titrated in 10% of *S. rubra* extract. This could be due to an increased bioavailability  
577 because of the presence of tensioactive compounds in the EC, such as previously

578 observed for controlled-release systems and nano-emulsions of essential oils (Maia et  
579 al. 2019; Rodrigues et al. 2021). Moreover, the mortality rates obtained for both the  
580 matrix and the formulation, comparatively, confirmed the relative innocuousness of the  
581 matrix against *Ae. aegypti*, and ensured that the semi-field bioassay would give us  
582 information on the *S. rubra* extract in the form of an EC as the only active product.

583 The efficiency of the tested formulation as a mosquito larvicide was demonstrated by our  
584 results, especially towards the CAY *Ae. aegypti* strain, exhibiting a strong resistance to  
585 conventional insecticides. The milky appearance of the aqueous media treated with the  
586 emulsifiable concentrate observed in semi-operational conditions can be due to the  
587 composition of the tap water used for the assay, although it was let 24h for dechlorination  
588 before use, or to the addition of larval food. Indeed, the presence calcium, sodium or  
589 chloride ions can modify the spontaneous formation of a micro-emulsion, and lead to a  
590 basic emulsion, while changing the translucent aspect for a cloudy one (Egito et al.  
591 2020). The strong and rapid acute toxicity can represent a technical advantage in the  
592 field.(Darriet et al. 2010) The lasting residual activity suggests an effective potential of  
593 the formulation for vector population inhibition during dengue transmission periods,  
594 which is a key parameter for disease control (Kanis et al. 2013, 2018; Maia et al. 2019).  
595 Botanical products often have to face the problem of residual activity, due to a high  
596 biodegradability through photodegradation, abiotic oxidation or volatilization (Amer and  
597 Mehlhorn 2006; Isman 2020). In our case, this relatively long residual activity was  
598 obtained for a simple emulsion, without using slow-release systems. This can be put in  
599 perspective with the previously demonstrated stability of the crude *S. rubra* extract in  
600 ambient conditions over time (Clervil et al. 2023).

601 Concerning the ecotoxicity of the matrix and the formulation under our experimental  
602 conditions, *D. magna* were overall more sensitive to the tested products than *C. riparius*,  
603 with high standard deviations observed, due to the protocol involving a low number of  
604 animals. For the matrix and the formulation at the dose used for the semi-field trial

605 (102 µg/ml), our results highlighted the harmlessness of the green formulation basis  
606 against non-targeted organisms, as observed towards *Ae. aegypti* larvae. These results  
607 are also consistent with suppliers, regulatory and literature data concerning the  
608 ecotoxicological assessment of the formulation matrix components (Renkin et al. 2005;  
609 Szwach and Lukosek 2017; ECHA 2022a, b). At this dose, an *Ae. aegypti* larval mortality  
610 of 100% was obtained as soon as 24h when assessing the formulation in the laboratory.  
611 At 102 µg/ml, the formulation thus only showed selectivity towards both non-target  
612 organisms at 24h (5 times less mortality for *C. riparius* than for *Ae. aegypti* larvae at the  
613 same dose, and around 1.5 times less mortality for *D. magna*), this advantage being lost  
614 after 48h of exposure.

615 However, when testing the effect of extract, matrix and formulation at the 48h-LD<sub>50</sub> (18.6  
616 µg/ml) of the SrK extract used to prepare the emulsifiable concentrate, the formulation  
617 led to 1.5 less toxicity towards *D. magna*, and 3.4 less toxic effect towards *C. riparius*,  
618 when compared to the extract. Besides solving technological problems due to the  
619 solubilization of *S. rubra* lipophilic crude extract in water, the use of the formulation also  
620 allows to lower the toxicity of the active ingredient at the same dose, without losing  
621 larvicidal efficacy. Complementary assessments should now be performed in  
622 cooperation with public health workers to determine the useful dose of formulation in the  
623 field, and therefore identify the precise level of selectivity proposed by the *S. rubra* EC.  
624 The data obtained for rubrenolide and rubrymolide are consistent with the previous  
625 evaluation of these pure compounds against *Ae. aegypti* larvae, with higher toxic activity  
626 observed for rubrenolide than rubrymolide both on the laboratory strain Paea and the wild  
627 strain Cayenne (Falkowski et al. 2016). Thus, valuing the extract, and even more the  
628 formulation, rather than the pure compounds, can add a noticeable value when it comes  
629 to the ecotoxicological profile of the larvicidal products.

630 Eventually, except for the extract towards kidney cells, the cytotoxic effect of the tested  
631 product was overall moderate to non-significant in an acute toxicity assay. The data

632 obtained for the pure compounds rubrenolide and rubrynlolide are consistent with a  
633 previous study, in which the pure compounds exhibited  $IC_{50} > 100 \mu\text{g/ml}$  against NIH-  
634 3T3 fibroblasts cells (Rodrigues et al. 2010). Even if a noticeable toxicity had been  
635 previously reported for rubrenolide ( $GI_{50}$  in the 3.3-9.9  $\mu\text{g/ml}$  range i.e.,  $< 33 \mu\text{M}$ ) against  
636 UACC-62 (human melanoma), MCF-7 (human breast cancer), HT-29 (human colorectal  
637 adenocarcinoma) and PC-3 (human prostate cancer) cell lines, most values in our  
638 assays remained out of the cytotoxic range (Tófoli et al. 2016). However, complementary  
639 cytotoxic evaluation of the products in *in vivo* models would be a valuable asset. The  
640 selectivity index data are of high interest, as a  $SI \geq 10$  was previously cited in the literature  
641 as a valuable criterion for selective samples (Awouafack et al. 2013; Houél et al. 2015;  
642 Peña-Morán et al. 2016). These results therefore reinforce those obtained when  
643 comparatively assessing the ecotoxicity of the crude extracts towards the formulated  
644 products, as in both cases (eco- and cytotoxicity), a clear advantage is obtained when  
645 using the *S. rubra* EC.

646

## 647 **Conclusion**

648 This study aimed at the evaluation of *S. rubra* wood extract towards *Ae. aegypti*  
649 reference, laboratory and wild strains, concurrently to the assessment of a *S. rubra*-  
650 based formulated product for application in aqueous media. We demonstrated the  
651 efficiency of the wood extracts on *Ae. aegypti* larvae, notably resistant wild strains, and  
652 highlighted the absence of repulsive effect of the wood extract on the mosquitoes'  
653 oviposition behavior. A simple, stable, easy-to-produce and easy-to-use formulation, in  
654 the form of an emulsifiable concentrate titrated in 10% of *S. rubra* extract, was  
655 successfully obtained. The choice of the ingredients, derived from green chemistry,  
656 allowed both the application of a lipophilic botanical extract in water and the compliance  
657 with European standards and regulations. This formulation demonstrated strong

658 larvicidal activity (acute toxicity and residual activity) through semi-field assays alongside  
659 with better selectivity towards non-target aquatic organisms and human cell lines than  
660 the crude wood extract. Following previous promising results on the larvicidal activity of  
661 *S. rubra* wood extract and the useability of sawmill residues for a large-scale production  
662 of the extract, in a biorefinery perspective included in circular economy objectives, this  
663 work aims to contribute to the development of novel solutions for the management of *Ae.*  
664 *aegypti* mosquitoes and the control of vector-borne diseases such as dengue fever and  
665 Zika or chikungunya viruses, while taking into account environmental and human health  
666 issues associated with the use of insecticide products.

667

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902

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913

### 914 **Competing interests**

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

916

### 917 **Author Contribution Statement**

918 EH, ID and JBD conceived and designed research. EH and NA collected the wood  
919 samples and conducted the extractions and chemical analysis. EC conducted the  
920 laboratory and semi-field larvicidal assays and the mosquito fitness study. EW conceived  
921 the formulation. DA, MC and DH designed and conducted the ecotoxicity assays. RLG  
922 and HS designed and conducted the cytotoxicity assays. EH, EC, ID and JBD analyzed  
923 the data and wrote the manuscript. All authors read and approved the manuscript.

924

925 **Data availability**

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

928

929 **Ethical statements**

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

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Submitted version