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# Evaluation of the larvicidal and pupicidal potential on *Anopheles gambiae* sl of methanolic extracts of *Cyperus rotundus* (Cyperaceae) and *Leucas martinicensis* (Lamiaceae)

**Bouba Théophile, Saotoing Pierre and Ndjonka Dieudonné**

### Abstract

The results of biological tests carried out according to a methodology inspired by the standard protocol of the World Health Organization revealed that methanolic extracts of *Cyperus rotundus* and *Leucas martinicensis* have larvicidal and pupicidal effects. Treatment with methanolic extract of *Cyperus rotundus* induced 60% mortality in one instar larvae at 50 ppm, 100% mortality at 350 ppm in 24 hours of exposure. A similar trend was observed for all other life instar of *Anopheles gambiae* sl at different concentrations (50-450 ppm). The LC<sub>50</sub> values recorded were 61.27ppm, 70.84ppm, 85.86ppm, 104.44ppm for I to IV instars and 339.18ppm for the pupal instar, respectively. *Leucas martinicensis* induces 80% one instar mortality at 50 ppm and 100% at 250 ppm. The toxic effect of the extracts was also demonstrated with LC<sub>50</sub> values of 28.24ppm, 39.1ppm, 54.79ppm, 80.78ppm for I to IV instars and 244.3ppm for the pupal instar, respectively. The lethal hours 50 (LH<sub>50</sub>) of *Cyperus rotundus* are 10h19mn13s, 11h31mn55s, 13h31mn49s, 16h04mn13s, 36h58mn27s for first, second, third, fourth instar larvae and pupae, respectively. Those of *Leucas martinicensis* are 06h29mn37s, 08h02mn45s, 06h24mn47s, 13h29mn45s, 27h05mn50s respectively for the same larval instars.

**Keywords:** Extracts, *Cyperus rotundus*, *Leucas martinicensis*, *Anopheles gambiae* sl, Maroua, Cameroon

### 1. Introduction

In the world, the number of people affected by malaria in 2018 is estimated at 228 million compared to 219 million in 2017 with approximately 405,000 deaths, 67% of which are in children under five years of age [29]. In Cameroon, according to the Ministry of Public Health, malaria in 2018 represented 25.9% of outpatient consultations and 14.6% of deaths, including about 65% among children under five years of age [14]. Many obstacles stand in the way of the scientific community's efforts to control malaria, including the resurgence of vector resistance to synthetic insecticides, hygienic conditions in some large cities that favour human/mosquito contact and problems of access to primary health care due to the high cost of new antimalarials and insecticides [8-24]. In response to this situation, WHO is advocating for the development of new strategies to tackle malaria [28]. One method that has been shown to be effective is the reduction of vector populations through the use of vector control methods such as insecticide-treated nets and indoor residual spraying with synthetic insecticides [4]. Unfortunately, most chemicals have adverse effects on humans, other animals and the environment due to their accumulation in the natural environment [5]. In addition, these modern control methods are not available to all because of their high cost and low availability [11]. Faced with these difficulties encountered by the National Malaria Control Programme, it would also be necessary to simultaneously research and improve the use of natural insecticides which, while also active, are biologically degradable and well known by local communities [18]. Thus, many natural substances or their derivatives are commonly used as insecticides, larvicides or insect repellents by the populations of Maroua and its surroundings [20]. Previous work has shown that some plants contain in their organs (leaves, stems, roots, fruits, flowers...) substances that have acaricidal, insecticidal, bactericidal and fungicidal properties [17]. These broad-spectrum plants could be used as alternative insecticides [1]. Studies conducted by Kilani *et al.* [12]

showed that leaf extracts from *Cyperus rotundus* have larvicidal activity on *Culex quinquefasciatus*. In the work of Muhammad *et al.* [15], it is reported that adult mosquitoes are repelled by *Leucas martinicensis* leaf extract. The general objective of this work is to evaluate the larvicidal and pupicidal activity of methanolic extracts of *Cyperus rotundus* and *Leucas martinicensis* on *Anopheles gambiae* sl, in order to reduce the vector population and curb the resurgence of malaria. The specific objectives were to :

- Evaluate the mortality of the different instars of larval development according to the concentrations of *Cyperus rotundus* and *Leucas martinicensis* extracts;
- Determine the LC<sub>50</sub> and LH<sub>50</sub> of the extracts of the plants studied and to establish a correlation between these two parameters.

## 2. Materials and Methods

### 2.1. Description of the Study Area

The study was conducted in Maga (Figure 1), a locality located in the Mayo Danai Department, Far North Region of Cameroon. Maga is a town of about 170,200 inhabitants [3]. Its surface area is 2000 km<sup>2</sup> and lies between 10°32'57" and 11°58'00" North latitude and between 14°34' and 15°10'13" East longitude [13]. The Sudano-Sahelian climate is characterized by a very short rainy season that alternates with a relatively long dry season depending on the year [23]. Rainfall varies between 530 and 630 mm/year with peaks in August (250 mm) and September, the average temperature is 28°C; the hottest months are March, April and May with a maximum value of 32°C. Annual evapotranspiration is 1800 mm [10].

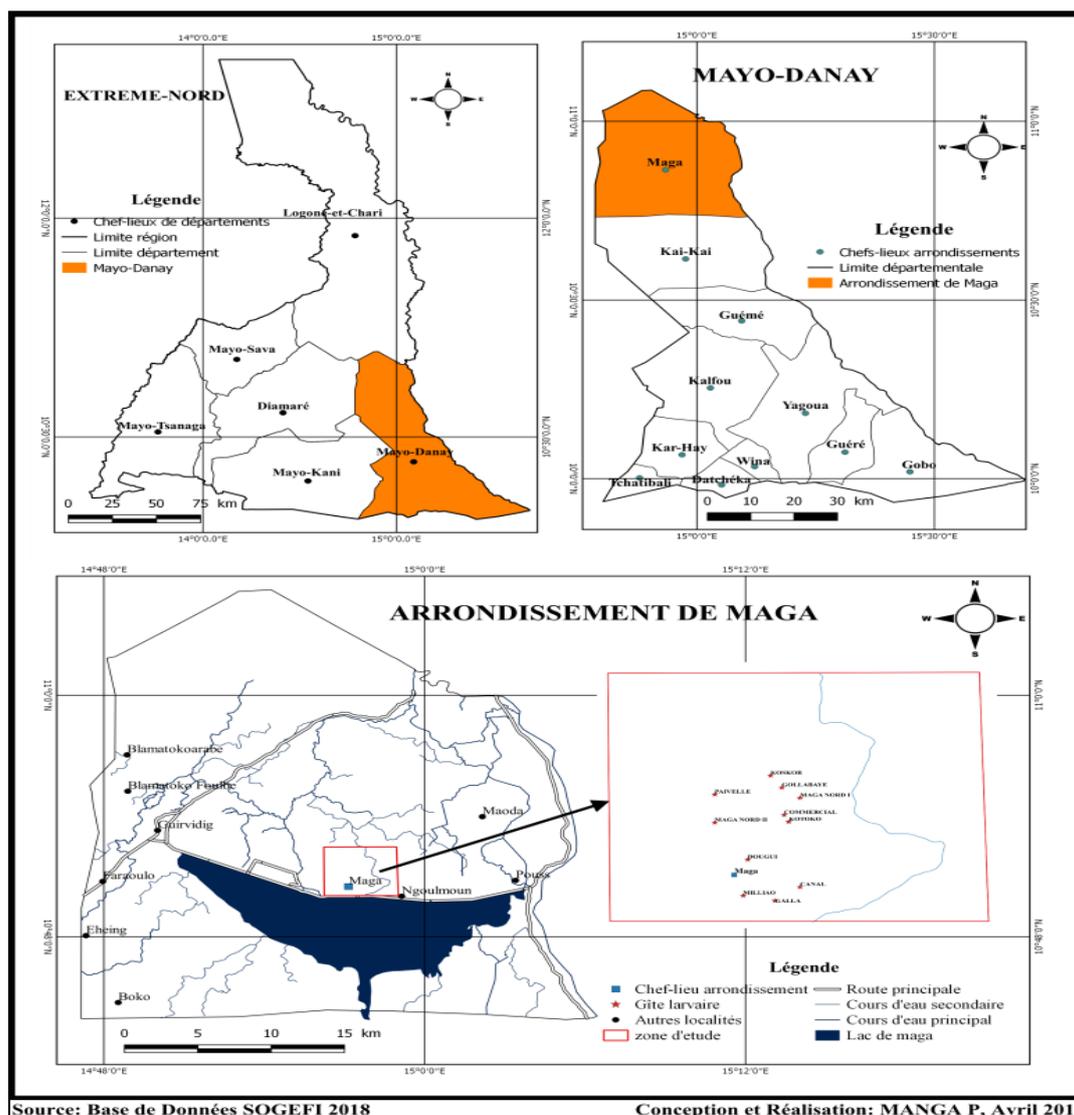


Fig 1: Location of the Study Area

### 2.2. Plant harvesting and extraction

The plant material consists of the leaves of *Cyperus rotundus* and *Leucas martinicensis*. Fractions of 250 g of powder from each plant were weighed using an electronic balance GM-300P and macerated in 1L of methanol for 24 hours. The mixture was then filtered using Whatman N°1 paper. The resulting filtrates were concentrated under a rotary evaporator set at a temperature of 65°C. The extracts thus obtained were

placed in transparent plastic bottles of 100ml capacity. The extraction yield was calculated in relation to the weight of the dried plant material before extraction according to the following formula:

$$\text{Yield} = \frac{\text{Mass of the extract obtained}}{\text{Mass of leaf powder}} \times 100$$

### 2.3 Collection of larvae of *Anopheles gambiae* sl

Harvesting of the larvae took place during the months of April and May 2019 between 9:00 and 10:00 am on sunny days. Standing water was systematically surveyed at 12 different sites (Figure 1). The larvae of *Anopheles gambiae* sl were identified by their horizontal position on the water surface. During each sampling, rapid blows with a (plastic) ladle were made into the water of the lodges and the harvested larvae were introduced into 1.5-litre bottles, then transported to the Life and Earth Sciences laboratory at IRAD, where they were identified, separated according to size and fed with a nutritive powder composed of cat food (Brand: Frisky) and shrimps (*Penaeus monodon*) in equal quantities (2 g of powder per 500 ml can containing spring water) for 24 hours before the experiment.

## 2.4. Bioassays

Preliminary experiments have allowed the selection of a range of concentrations for the actual tests. To do this, extract stock solutions of each sample were prepared in methanol, from which dilutions were made in well water to obtain final experimental concentrations of : 50ppm, 150ppm, 250ppm, 350ppm and 450ppm. Three replicates were performed for each dilution. The tests consisted in evaluating the mortality of larvae and pupae of *Anopheles gambiae* sl in the presence of diluted solutions of the extracts following a methodology inspired by the WHO [26] protocol. Indeed, 25 larvae of different instars were taken with a pasteur pipette and put in small transparent plastic boxes of dimension: 10 x 6 x 3.6 cm, each containing a volume of the mother solution (diluted extract solution) supplemented with a volume of spring water up to 100mL, total volume.

Two control boxes were also used under the same conditions as the test boxes. The negative control contained only bed water, which is the natural habitat for the aquatic instars of mosquito larvae. The positive control contained spinosad, a biolarvicide (chemical family of naturalites) composed of a mixture of two metabolites (spinosynes A and D) synthesized

by the bacterium *Saccharopolyspora spinosa*, from the group of actinomycetes, marketed as EC (Emulsifiable Concentrate), at the WHO [27] recommended dose of 0.5mg/L. Counting of dead larvae was carried out after 24 hours of exposure to the different concentrations of extracts. A larva is considered dead when it remains immobilized at the bottom of the can and does not react to the touch of a needle. Observations were made in hourly intervals for 12 hours and the device was maintained until 24 hours, at which time mortality was assessed.

## 2.5. Analysis of the data

Data analysis was done using:

- Descriptive statistics to calculate averages and percentages;
- Z-tests to compare the observed averages to a theoretical value;
- Chi-square tests ( $\chi^2$ ) to compare observed proportions to a theoretical distribution [21];
- Excel software for plotting curves, histograms and regression lines;
- Pearson's r-correlation test to evaluate the degree of relationship between two parameters;
- Finney's [9] formulas for calculating LC<sub>50</sub> and LH<sub>50</sub>.

## 3. Results and Discussion

### 3.1 Extract yield

From 250g of powder of *Cyperus rotundus* and *Leucas martinicensis*, mass of 3.42g and 8.10g respectively were obtained, giving a yield of 1.36% and 3.24% (Table 1). According to Azevodo *et al.* [2], the ecology of a plant has a quantitative influence on the synthesis of its secondary metabolites and the yield of a plant extract depends on several factors including the nature of the soil, the climate, the harvesting period and the condition of the individual during harvest.

Table 1 : Extract Yield

Plant Species	Mass of leaf powder (g)	Mass of the extract obtained (g)	Yield (%)±ET*
<i>Cyperus rotundus</i>	250	3,42	1,36± 0,03
<i>Leucas martinicensis</i>	250	8,10	3,24± 0,05

\*standard deviation calculated over three replicates for *Cyperus rotundus* and *Leucas martinicensis*

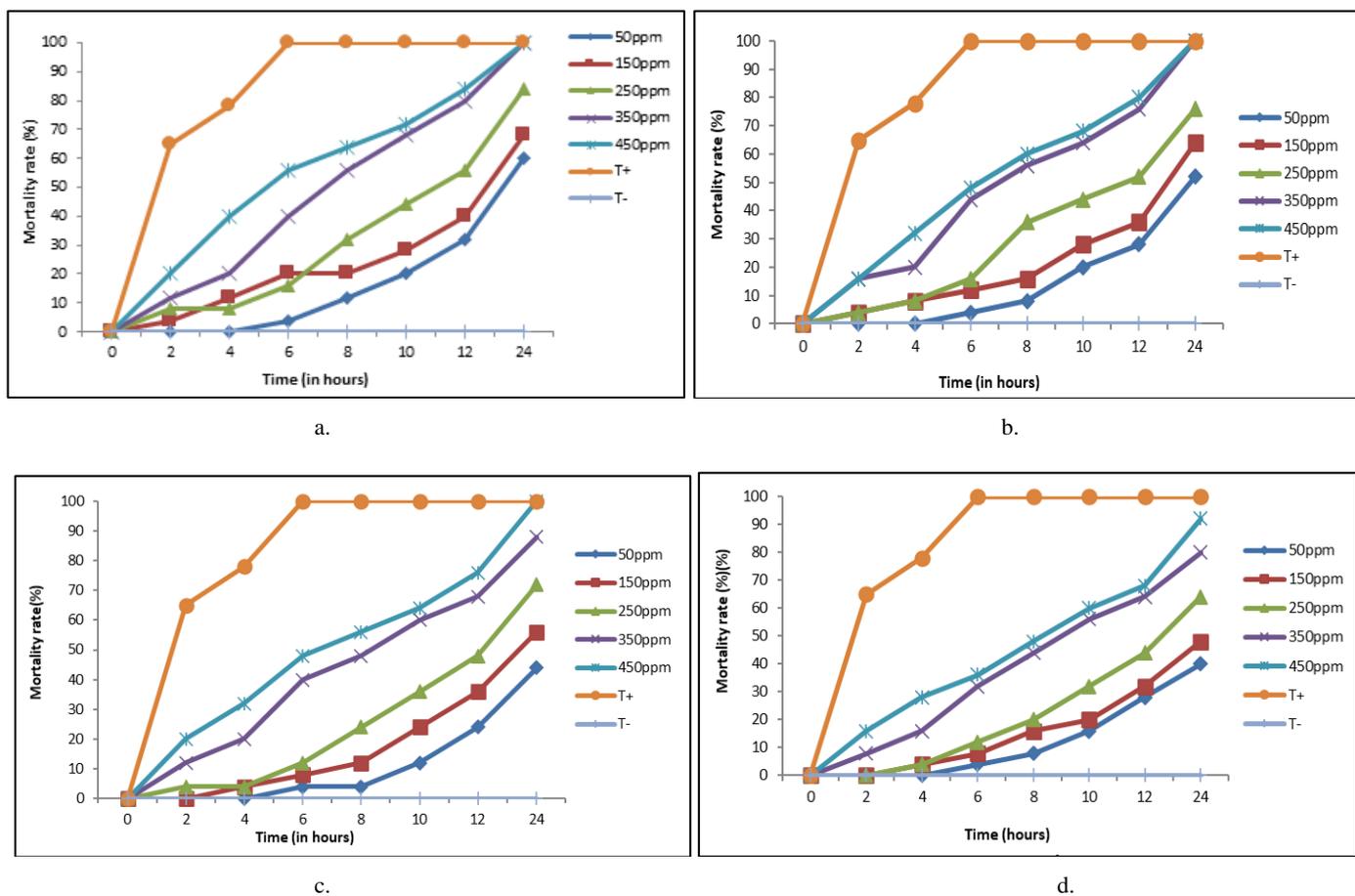
## 3.2. Mortality of the different instars of larval development

Experiments conducted revealed that the negative control (bed water) showed 0% mortality after 24 hours of exposure in both extracts at all instars of larval development. This allows us to say that the deposit water used had no influence on larval mortality. The positive control (spinosad) has a very high mortality rate reaching 100% mortality at the 6th hour of exposure. This confirms the powerful larvicidal effect of spinosad on all larvae of *Anopheles gambiae* sl. The low mortality rate observed in the pupae at different concentrations can be explained by the nature of their membrane which limits contact with the external environment on the one hand and by the fact that this instar does not feed on the other hand [25].

### 3.2.1. Larvicidal effect of extracts of *Cyperus rotundus*

In *Cyperus rotundus*, at the lowest concentration (50ppm) all larvae become susceptible after 6 hours where it induces 4%

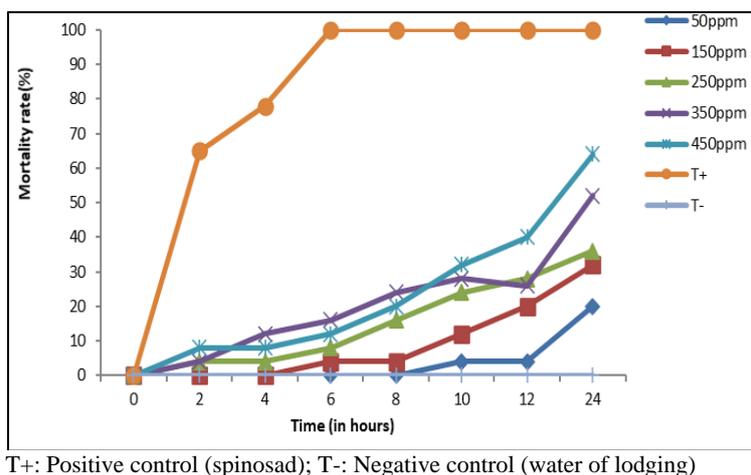
mortality (Fig. 2a, 2b, 2c and 2d). Concentrations of 150 and 250ppm killed 68 and 84% of first instar larvae within 24 hours of exposure (Figure 2a), 64 and 76% of second instar larvae (Figure 2b), 44 and 56% of third instar larvae (Figure 2c), 40 and 48% of fourth instar larvae (Figure 2d), 20 and 32% of pupae (Figure 3), respectively. Significant difference is noted between these mortality rates ( $\chi^2= 8.07$ ,  $df = 4$ ,  $\alpha = 0.05$ ). Concentrations 350 and 450ppm caused 100% mortality of 1, 2 and 3 instar larvae at the 24th hour of exposure. Our results are similar to those obtained by Shaza *et al.* [22] who demonstrated that *Cyperus rotundus* extract resulted in increasing larval mortality ranging from 20% at 50ppm to 100% at 500ppm. According to Costa [6], *Cyperus rotundus* is rich in alkaloids, anthraquinones, coumarins, steroids, triterpenes, flavonoids, saponins, tannins. These substances would be responsible for the observed larvicidal and pupicidal effect.



T+: Positive control (spinosad); T-: Negative control (water of lodging)

**Fig 2(a, b, c, d):** Mortality rates as a function of time at different concentrations of *Cyperus rotundus* in 1, 2, 3, 4 instar larvae respectively.

However, no pupal mortality was observed (Figure 3) at the same time (6 hours).



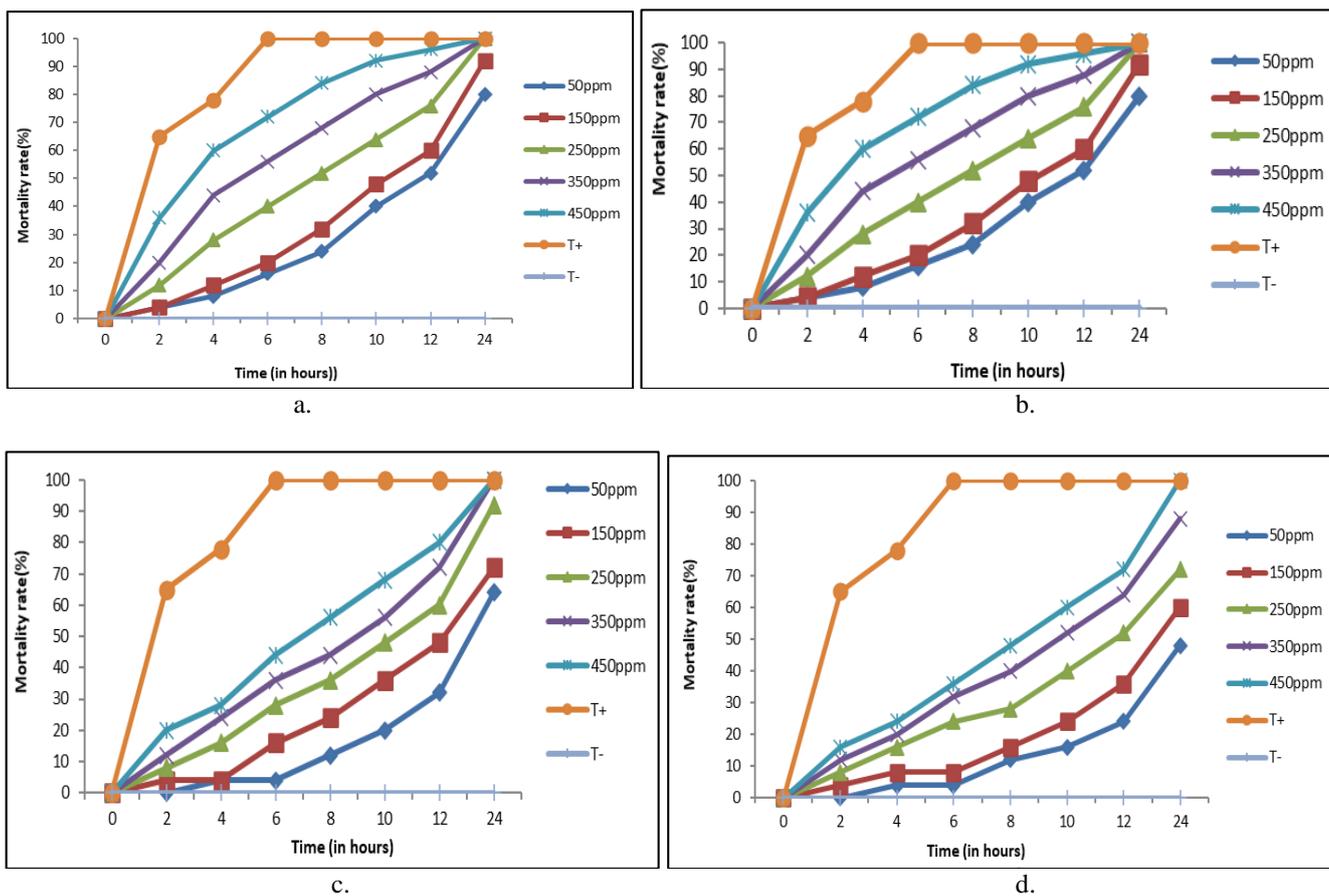
T+: Positive control (spinosad); T-: Negative control (water of lodging)

**Fig 3:** Mortality rate as a function of time at different concentrations of *Cyperus rotundus* in pupae.

**3.2.2. Larvicidal effect of extracts of *Leucas martinicensis***

The methanolic extract of *Leucas martinicensis* resulted in mortality of 16%, 12%, 4% and 4% respectively in 1, 2, 3 and 4 instars after 6 hours of exposure to 50ppm (Figure 4a, 4b, 4c and 4d). Concentrations of 150ppm and 250ppm induced 92 and 100% mortality of first instar larvae within 24 hours of exposure (Figure 4a), 84 and 100% mortality of second instar larvae (Figure 4b), 72 and 92% mortality of third instar larvae (Figure 4c), 60 and 72% mortality of fourth instar larvae (Figure 4d), 36 and 48% mortality of pupae (Figure 5),

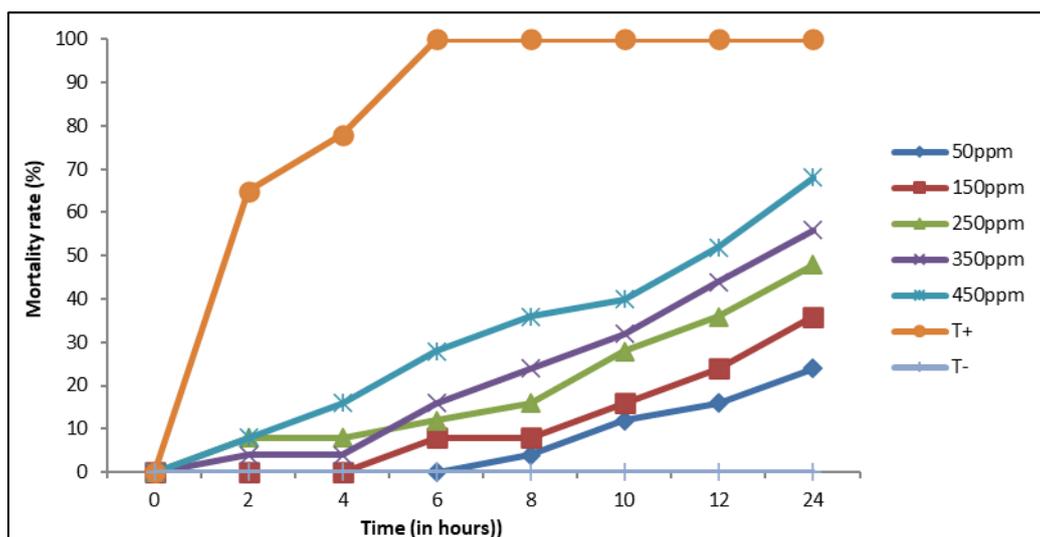
respectively. A significant difference is noted between these mortality rates ( $\chi^2 = 7.41$   $df = 4$ ,  $\alpha = 0.05$ ). Our results are consistent with those obtained by D. Elumalai *et al.* [7] who showed that *Leucas martinicensis* extracts caused 35% mortality at a minimum concentration of 50 ppm when exposed for 24 hours. A phytochemical study conducted on *Leucas martinicensis* by Muhammad *et al.* [15] revealed that this plant contains saponins, alkaloids, flavonoids, tannins, resins, steroids and triterpenes that would be responsible for the observed larvicidal effect.



T+: Positive control (spinosad); T-: Negative control (water of lodging)

**Fig 4 (a, b, c, d and c):** Mortality rates as a function of time at different concentrations of *Leucis martinicensis* in 1, 2, 3 and 4 instar larvae respectively.

No sensitivity was observed in the pupae (Figure 5) at the same time (6 hours).



T+: Positive control (spinosad); T-: Negative control (water of lodging)

**Fig 5:** Mortality rate as a function of time at different concentrations of *Leucis martinicensis* in pupae.

### 3.3 Evaluation of LC<sub>50</sub> of extracts of *Cyperus rotundus* and *Leucis martinicensis*

To calculate lethal concentrations 50, mortality rates were transformed to probit after 24 hours of exposure as the ordinate and the decimal logarithm of the concentrations as

the x-axis. From these two parameters, the regression lines and the determination coefficients were automatically established. The lethal concentrations obtained are shown in Table 2. The table shows that *Cyperus rotundus* and *Leucis martinicensis* have LC<sub>50</sub> of 61.27ppm and 28.24ppm in first

instar, 70.84ppm and 39.1ppm in second instar, 85.86ppm and 54.79ppm in third instar, 104.44ppm and 80.78ppm in fourth instar, 339.18ppm and 244.3ppm in the pupal instar, respectively. While Spinosad has LC<sub>50</sub> of 3.98ppm, 4.27ppm, 4.13ppm, 4.38ppm and 4.3ppm in 1, 2, 3, 4 instar and pupae, respectively. At the LC<sub>50</sub> level, Spinosad (control +) is by far more effective than the two extracts. This seems normal since methanolic extracts of *Cyperus rotundus* and *Leucas martinicensis* would contain impurities that could influence

the results compared to Spinosad, which is a well-purified larvicide [18]. Table 2 indicates that LC<sub>50</sub> vary depending on the extract and the of larval development instar. Our results corroborate the observations of Aouinty *et al.* [1] who showed in their work that LC<sub>50</sub> vary according to extracts, Culicid species and larval instar. *Leucas martinicensis* had lower LC<sub>50</sub> than *Cyperus rotundus*. Their extract would contain more active molecules than that of *Cyperus rotundus*.

**Table 2:** Lethal concentrations 50 (LC<sub>50</sub>) of *Cyperus rotundus*, *Leucas martinicensis* and Spinosad (positive control).

Mosquito larval Instars and pupae	<i>Cyperus rotundus</i>			<i>Leucas martinicensis</i>			Spinosad		
	Regression equation	R	LC <sub>50</sub> (ppm)	Regression equation	R	LC <sub>50</sub> (ppm)	Regression equation	R	LC <sub>50</sub> (ppm)
L1	y= 3,15x – 0,63	0,83	61,27 <sup>a</sup>	y= 2,75x + 1,01	0,92	28,24 <sup>a</sup>	y= 5,25x + 1,85	0,92	3,98
L2	y= 3,34x – 1,18	0,82	70,84 <sup>b</sup>	y= 3,09x + 0,08	0,90	39,1 <sup>b</sup>	y= 5,34x + 1,63	0,93	4,27
L3	y= 2,72x – 0,26	0,78	85,86 <sup>c</sup>	y= 3,1x – 0,39	0,87	54,79 <sup>c</sup>	y= 5,19x + 1,80	0,92	4,13
L4	y= 1,59x + 1,79	0,88	104,44 <sup>d</sup>	y= 2,59x + 0,06	0,77	80,78 <sup>d</sup>	y= 5,42x + 1,52	0,93	4,38
N	y= 1,15x + 2,09	0,93	339,18 <sup>e</sup>	y= 1,16x + 2,23	0,96	244,3 <sup>e</sup>	y= 5x + 1,83	0,94	4,3

R: correlation coefficient; Values followed by different letters in the same column are significantly different at the 5% threshold.

### 3.4. Evaluation of Lethal Hours 50 (LH<sub>50</sub>).

The determination of lethal hours 50 (HL<sub>50</sub>) of extracts of *Cyperus rotundus* and *Leucas martinicensis* on larvae and pupae of *Anopheles gambiae* sl was made from the regression

lines obtained by transforming the percentages of mortality into probit after 24 hours of exposure according to the decimal logarithm of the hours. The results are reported in Table 3.

**Table 3:** Lethal hours 50 (LH<sub>50</sub>) of *Cyperus rotundus*, *Leucas martinicensis* and Spinosad.

Mosquito larval Instars and pupae	<i>Cyperus rotundus</i>		<i>Leucas martinicensis</i>		Spinosad	
	Régression equation	LH <sub>50</sub>	Régression equation	LH <sub>50</sub>	Régression equation	LH <sub>50</sub>
L1	y= 2,19x + 2,78	10h19min13sec	y= 2,4x + 3,05	06h29min37sec	y= 5,25x + 1,85	3h58mn04s
L2	y= 2,1x + 2,77	11h31min55sec	y= 2,33x + 2,89	08h02min45sec	y= 5,34x + 1,63	4h16mn22s
L3	y= 1,98x + 2,76	13h31min49sec	y= 3,68x + 2,03	06h24min47sec	y= 5,19x + 1,80	4h11mn58s
L4	y= 1,99x + 2,6	16h04min13sec	y= 1,92x + 2,83	13h29min45sec	y= 5,42x + 1,52	4h22mn06s
N	y= 1,62x + 2,46	36h58min27sec	y= 1,64x + 2,56	27h05min50sec	y= 5x + 1,83	4h17mn04s

It can be seen from this table that lethal hours 50 (LH<sub>50</sub>) vary according to the larval instar. Indeed, the lethal hours 50 of the methanolic extract of *Cyperus rotundus* are 10h19mn13s, 11h31mn55s, 13h31mn49s, 16h04mn13s, 36h58mn27s respectively for 1, 2, 3, 4 instars larvae and pupae. Those of *Leucas martinicensis* are 06h29mn37s, 08h02mn45s, 06h24mn47s, 13h29mn45s, 27h05mn50s respectively for the same larval instars. *Leucas martinicensis* has a low HL<sub>50</sub> on all larval instars of *Anopheles gambiae* sl compared with *Cyperus rotundus*. In view of this observation, there would be a correlation between the efficacy of the extracts (LC<sub>50</sub>) and their lethal hours (LH<sub>50</sub>). Generally, an extract that is highly effective also has a low LH<sub>50</sub>. Our results corroborate those of Njan Nlôga *et al.* [16] who find in their work that the essential oil of *Ocimum canum* is very effective (LC<sub>50</sub>=11.95mg.m<sup>-2</sup>) and has a very low LH<sub>50</sub> (6h36mn36s) compared to other plants. A study carried out by Saotoing [19] based on acetone extracts from the leaves of *Calotropis procera* and *Boswellia dalzielii* revealed that these two plants show remarkable efficacy with an LC<sub>50</sub> of 3.03g.m<sup>-2</sup> and an LH<sub>50</sub> of 7h31mn08s for *Calotropis procera* and an LC<sub>50</sub> of 3.94g.m<sup>-2</sup> and an LH<sub>50</sub> of 9h52mn25s for *Boswellia dalzielii*.

### Conclusion

The present work has shown that our two samples have larvicidal and pupicidal properties on *Anopheles gambiae* sl. Higher concentrations induced higher mortality rates. However, the methanolic extract of *Leucas martinicensis* is

the most effective sample with LC<sub>50</sub> of 28.24ppm, 39.1ppm, 54.79ppm, 80.78ppm for I to IV instars and 244.3ppm for the pupae, respectively. The results of this research showed the existence of local means for successful larval control in the Far North Region of Cameroon. Given the availability of these extracts, their effectiveness and even their toxicity tolerance for humans and the environment, the popularization of the virtues of these plant species could be a solution in vector control programs. In the future, it will be necessary to determine the nature of the compounds responsible for this larvicidal and pupicidal activity by fractionation carried out in parallel with bioassays.

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### Conflicts of Interest

The authors confirm that the content of this article does not present any conflict of interest.

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