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Journal
African Entomology, 29(2)

ISSN
0013-8789

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Publication Date
2021

DOI
10.4001/003.029.0507

Biocontrol of the brown cocoa mirids using neem oil and an ethanolic extract from neem under laboratory conditions

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The African mirid bug (*Sahlbergella singularis*) is the most economically important insect pest in cocoa farms. Pesticide management, although controversial due to the adverse effects of these substances on the environment and on human health, remains the main option used for controlling this pest. In the recent decades, the development of alternative approaches to synthetic pesticides is a requirement. Therefore, we used neem oil (NO) and ethanolic extracts (EE) from leaves at different concentrations to evaluate, *in vitro*, their insecticidal potentials against mirids. Mirid mortality increased significantly with increase in concentrations, values ranged from 32.5 to 92.5 % for EE and 52.5 to 97.5 % for NO. Apart from negative controls, Tween 80 and distilled water, that showed significant low mortality rates, both extracts revealed effectiveness comparable to the reference insecticide used in controlling mirids, except for EE by ingestion. Mirids treated by contact showed significantly high mortality rates (72.5 to 97.5 %) compared to those treated by ingestion (32.5 to 70.0 %). The greatest biological effectiveness values were obtained at a concentration of 8 % by contact exposure: 0.88 ml/ml (NO) and 0.73 g/ml (EE) for LC50 and ≈1 day to both extracts for LT_{50}. Given effectiveness comparable to that of the insecticide, both tested extracts should be considered as effective biopesticides for IPM against mirids, especially *S. singularis*.

Key words: *Sahlbergella singularis*, *Azadirachta indica*, biopesticide, integrated pest management (IPM), cocoa agroforestry systems.

INTRODUCTION

In West and Central Africa, both mirid bugs, *Sahlbergella singularis* Hagl. and *Distantiella theobrama (*Dist.) (Heteroptera: Miridae) are the major insect pests of cocoa agroforestry systems, causing up to 40 % losses in cocoa yields (Adu-Acheampong *et al*. 2014; Awudzi *et al*. 2016; Babin 2018). Due to its omnipresence, abundance and damage in plantations, *S. singularis* is the most economically important insect pest in Cameroon (Yede *et al*. 2012; Yede 2016; Mboussi *et al*. 2018; Mahot *et al*. 2019; Mahob *et al*. 2020; Mahot 2020). This species feeds on pods, stems and soft branches of the host plants (Anikwe *et al*. 2009; Mahob *et al*. 2020). During the feeding activity, known as feeding punctures or lesions, the insects inoculate phytotoxic saliva into feeding sites which can lead to several damages such as cells/organ destruction, appearance of black spots in the affected organs or dry leaves, and annual production losses (Anikwe *et al*. 2009; N'Guessan *et al*. 2008, 2010; Yede *et al*. 2012; Mahob *et al*. 2015, 2019, 2020). Moreover, the feeding sites are reported as entry points for opportunistic fungi such as *Lasiodiplodia* spp., *Albonectria* spp. and *Fusarium* spp.; the synergistic action of both biological groups (mirids and fungi) generally results in the dieback of infected cacao trees (Adu-Acheampong *et al*. 2012; Anikwe & Otuonye 2015; Voula *et al*. 2018).

The most efficient strategies to reduce damage caused by mirids on the cacao trees under economic threshold relies essentially on the use of synthetic insecticides of the neonicotinoid family, such as lambda-cyhalothrin and imidacloprid

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Received 18 December 2020. Accepted 23 August 2021

ISSN 1021-3589 [Print]; 2224-8854 [Online]
DOI: https://doi.org/10.4001/003.029.0507

African Entomology **29**(2): 507–521 (2021)

(Ayenor *et al*. 2007; Mahob 2013; Mahob *et al*. 2014). Although effective, chemical control has led to insecticide resistance of targeted and/or non-targeted pests; it also has negative effects on the environment, human health and other living organisms such as beneficial arthropods, especially cocoa flower pollinators (Sarmah *et al*. 2004; Geiger *et al*. 2010; Manfo Tsagué *et al*. 2010; Fosu-Mensah *et al*. 2016; Kibria 2016; Lehmann *et al*. 2017; Humann-Guilleminot *et al*. 2019). Risks due to the use of synthetic pesticides, coupled with the stringent legislation of cocoa-importing countries (*e.g*. members of the European Union) and the recent high demand for food safety and quality (Cilas *et al*. 2018; Damalas & Koutroubas 2018), requires more efforts to develop alternative phytosanitary control measures against insect pests. These methods include varietal resistance/tolerance (Sounigo *et al*. 2003; Anikwe *et al*. 2009; N'Guessan *et al*. 2008, 2010), cultural control through shade management or pruning and/or plant diversity (Babin *et al*. 2010; Ratnadass *et al*. 2012; Mahob *et al*. 2020), biological control using living organisms or their derived products (Padi 2000; Bagny Beilhe *et al*. 2018; Mahot *et al*. 2019), and semiochemicals and biopesticides *via* the use of sex pheromone traps and entomopathogenic fungi (Ayenor *et al*. 2007; Posada *et al*. 2010; Mahob *et al*. 2011; Anikwe & Makanjuola 2013; Sarfo *et al*. 2018a, b; Mahot *et al*. 2020).

In the recent past decades, botanically derived products, *i.e*. secondary natural substances extracted from plants, also called biopesticides (Regnault-Roger & Philogène 2008; Sithisut *et al*. 2011) have attracted a lot of interest from researchers worldwide and have been used as alternatives to synthetic and/or chemical pesticides to control pests (Talukder *et al*. 2004; Talukder 2006; Ayenor *et al*. 2007; Regnault-Roger & Philogene 2008; Jaastad *et al*. 2009; Asogwa *et al*. 2010; Sithisut *et al*. 2011; Adesina 2014; Isman 2006, 2015; Hikal *et al*. 2017; Damalas & Koutroubas 2018; Mboussi *et al*. 2018). Thus, numerous studies used aqueous extracts from seeds and stem bark of *Azadiractha indica* A. Juss. (Sapindales: Meliaceae) against targeted mirids and revealed that these substances are effective to control this insect pest (Adu-acheampong 1997; Padi *et al*. 2003; N'Guessan & Kouassi 2006; Asogwa *et al*. 2010; Mbousi*et al*. 2018). Nonetheless, none of the previous investigations has determined the effects of other derived products from neem on cocoa mirids. The large-scale research of the insecticidal potential of diverse neem organs could ultimately provide strong solutions to the development of biopesticides market against hemipteran insects such as mirids. Neem seeds possess several mixtures of seven tetranortriterpenoid isomers which contain more than a dozen insecticide analogues including azadirachtin the most active ingredient (Schmutterer 1990; Isman 2006). *Azadiractha indica* and/or its derived products produce varied effects on insect pests such as toxicity, antifeedant, repellent, disruption of morphogenesis, development, natural mating behaviour and reproductive fitness (Schmutterer 1990; Copping 2004; Sithisut *et al*. 2011; Isman 2006, 2015; Ahmad *et al*. 2015; Hikal *et al*. 2017; Damalas & Koutroubas 2018). With regard to the insecticidal potential of neem as a promising alternative substance against insect pests (Schmutterer & Singh 2002; Isman 2006; Ayenor *et al*. 2007; Roy *et al*. 2010; Zanuncio *et al*. 2016), data on the insecticidal properties of other derived products from this plant such as neem oil (NO) and/or neem leaves ethanolic extracts (EE) need to be elucidated. Therefore, optimisation of the use of neem extracts to control mirid populations could decrease the use of synthetic insecticides and substantially contribute to the promotion of organic cocoa, the preservation of biodiversity/environment, predominantly the cocoa flower insect pollinators that are critical to improve cacao tree productivity. Accordingly, the main goal of this study was to evaluate the insecticidal effects of EE and NO on *S. singularis* populations.

MATERIAL AND METHODS

Study sites

This study was carried out in the Institute of Agricultural Research for Development (IRAD) and in the International Institute of Tropical Agriculture (IITA) from June 2019 to January 2020 for bioassays and mirids rearing, respectively; this period includes the pullulating time of mirids in plantations (Mahob *et al*. 2015, 2020). These institutes are located in the same area at Nkolbisson (3°51′N 11°30′E, 760 m a.s.l.), a suburb of Yaounde, the political city of Cameroun (Fig. 1). The pedoclimatic characteristics of Yaounde have been widely documented (Molua 2006; Kemka *et al*. 2009; Djoufack 2011; Nola *et al*. 2011; Ajeagah *et al*. 2014; Wirmvem *et al*. 2016; Monamele *et al*. 2017).

Source of mirids

Mirids were captured monthly (≈20 insects) early in the morning (07:00 and 09:30) from the cocoa plantations of Nkolbisson and surroundings, because of their photophobia and intense activity at this time of the day (Bisseleua *et al*. 2011; Mahob *et al*. 2015, 2020). After each capture, and during their transport to the laboratory, specimens were preserved in aerated rectangular Perspex boxes (9 × 7 × 1.5 cm) containing fresh segments of cocoa tree branches (5–6 cm each). Fourth and fifth instar nymphs used for bioassays were reared following the methodology proposed by Babin *et al*. (2008).

Source of neem extracts

Fresh neem leaves were manually collected from trees in August 2019, at Mvan district (3°48′37.4″N 11°30′40.6″E, altitude: 700 m a.s.l.), located in the Yaounde 4 municipality. They were preserved in a plastic bag (60 × 40 cm) and transported to the Cameroon National Herbarium (HNC) for the taxonomic confirmation of the plant identification as *Azadirachta indica* A. Juss registered under number 4447/SRFK (YA). After identification, the neem leaves were transported to the Regional Laboratory for Biological Control and Applied Microbiology of IRAD-Yaounde-Nkolbisson. Once in the laboratory, leaf samples were washed thoroughly, spread out on a wooden plate, and dried protected from sunlight at room temperature for about 20 days to avoid photo-reactions that could destroy the physicochemical properties of secondary metabolites (Oomah *et al*. 2010). Then, they were ground using an electric grinder (GEEPAS®GBS 5080, India) to obtain powder used for the ethanolic extract preparation.

One litre of neem oil extracted from the seeds (cold extraction) without addition of solvents or pesticides (Zanuncio *et al*. 2016) was gracefully given to us by the IITA Biological Control Laboratory against Pests and Diseases.

Preparation of the ethanolic extract

The EE of *A. indica* was obtained following the modified method of Oomah *et al*. (2010). A quantity of 100 g of neem leaves powder was added to 750 ml of 80 % ethanol contained in an Erlenmeyer

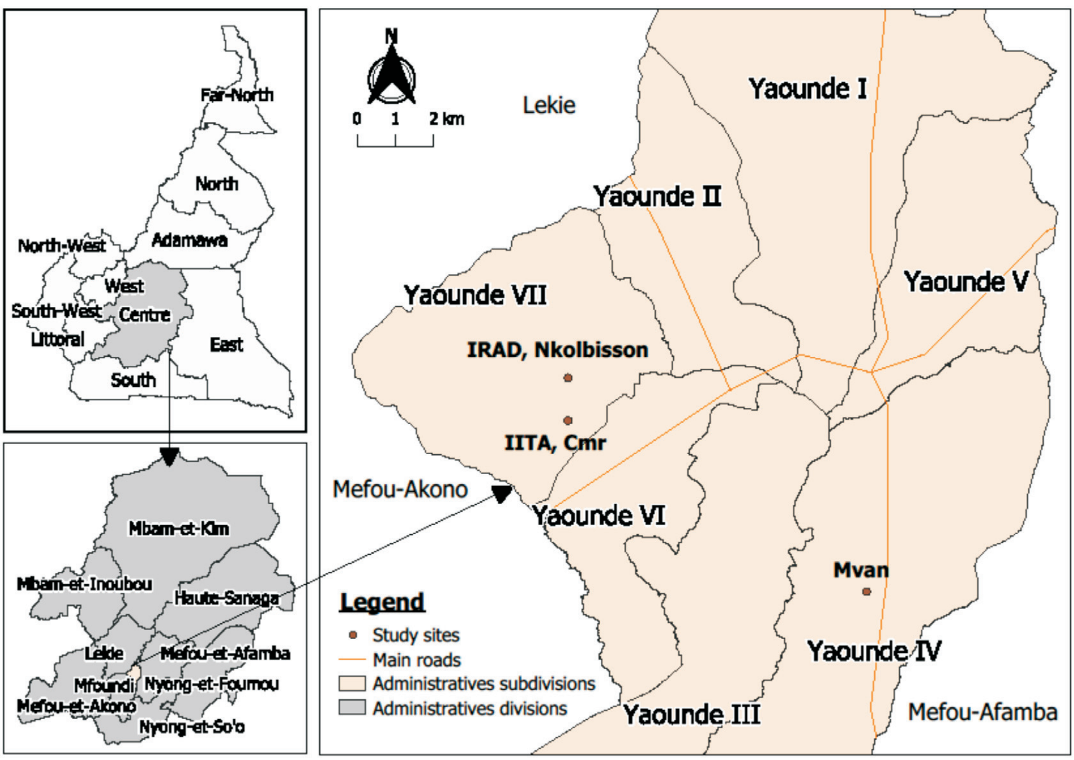

Fig. 1. Geographical localisation of the sampling locality and study sites.

flask. The mixture was homogenised for 2 h using a magnetic stirrer (Pyro-Multi-Magnestir, England) at room temperature; it was then centrifuged at 300 rpm for 30 min using a centrifuge (Rotofix 32 A, Japan). The supernatant was collected in an Erlenmeyer flask, while the pellet was taken up in 250 ml of 80 % ethanol, then stirred and centrifuged again. The second supernatant was concentrated by evaporation using a rotary evaporator, to free it from ethanol; the subsequent product was frozen at –80 °C for 24 h and freeze-dried at –200 °C during 72 h. The final product was a dry residue whose quantity was expressed in grams. The latter was stored in the refrigerator at 4 °C until further use. The calculation of the extraction yield was carried out according to the following equation:

Yield (%) = [mass of powder obtained (g) / mass of initial powder (g)] × 100 (Basdevant *et al*. 2007).

Preparation of extract concentrations

Ethanolic extracts

Three concentrations were prepared for the treatments. Indeed, 2 g, 5 g and 8 g of the test substance (EE) were taken with a spatula and weighed, using an electronic balance with nearest milligram precision (Kern PLB-20002, Japan). Each collected quantity was introduced into an Erlenmeyer flask containing 100 ml of distilled water (DW) to obtain respective concentrations of 2 % (1/50 % w/v), 5 % (1/20 % w/v) and 8 % (1/12.5 % w/v) (Zanuncio *et al*. 2016; Mboussi *et al*. 2018).

Neem oil

Three concentrations, 2 % (1/50% v/v), 5 % (1/20 % v/v) and 8 % (1/12.5 % v/v), of NO were also prepared for the treatments, according to the modified methods of Adu-Acheampong (1997); Anikwe (2013); Zanuncio *et al*. (2016). For this purpose, respective volumes of 2 ml, 5 ml and 8 ml of neem oil were taken using a micropipette, and introduced into Erlenmeyer flasks containing, respectively, 98 ml, 95 ml and 92 ml of Tween 80 solution, obtained by mixing 0.4 μl of Tween 80 with 99.6 μl of DW.

Laboratory bioassays

Laboratory bioassay tests were carried out at the Regional Laboratory for Biological Control and Applied Microbiology of IRAD-Nkolbisson according to Adu-Acheampong (1997); Zanuncio *et al*. (2016); Mboussi *et al*. (2018); Mahot *et al*. (2019). Except mirids, the experiments included two main treatments: one positive control based on the use of a common commercial insecticide, imidacloprid (20 g/l) + lambda-cyalothrin (20 g/l), which is a benchmark insecticide in decreasing mirid populations in the field (Mahob *et al*. 2014); two negative controls (Tween 80 solution and DW), EE and NO at the different concentrations listed above, which were completely randomised in design for each treatment and conducted under laboratory conditions at 25 °C. Each treatment involved 80 fourth and fifth instar nymphs of *S. singularis*, due to the ease with which they can be manipulated compared to other development stages (N'Guessan *et al*. 2010; Mahob *et al*. 2019; Mahot *et al*. 2019, 2020). They were subdivided into four replicates. The insecticidal effects of the tested products on the mirids was assessed directly (contact approach), by spraying specimens with the tested products, and indirectly (or ingestion approach) by immersing in advance in the test products, cocoa pods used to feed mirids (Mahot *et al*. 2019, 2020).

Direct and indirect toxicity trials

The toxicity effects of the different prepared solutions of test products were evaluated on mirid populations for both approaches by using a 1000 ml mini-sprayer and Petri dishes (≈9 cm φ-LC × 1 cm). Each Petri dish was lined with sterile absorbent paper to avoid *ex situ* contamination and covered with a ventilated cloth sleeve (Mahob *et al*. 2019; Mahot *et al*. 2019). Twenty insects were exposed to the test products for each treatment. During the contact approach, specimens received five manual spraying pulses of different concentrations of the neem extracts (NO and EE) and target insecticide (imidacloprid + lambda-cyhalothrin) used following the manufacturer's recommendations, *i.e*. insecticide was diluted in 5 l of water to produce a stock solution by adjusting 12.5 ml of insecticide to obtain the required concentrations. At ≈10 sec after spraying products, the treated insects were transferred into the experimental arena, described above, using a fine brush. During the indirect approach or ingestion, the insect samples were exposed to cocoa pods (two per replication) previously immersed, for 5 min, in the different prepared concentrations of test substances; these feeding cocoa pods were maintained throughout the experimental arena of bioassays according to the modified protocols of Mboussi *et al*. (2018) and Mahot *et al*. (2019). Then, for each replication, the number of dead insects was recorded daily and removed from the experimental arena for a duration of 7 days (Mboussi *et al*. 2018; Mahot *et al*. 2019). For each treatment, distilled water was used as a negative control for EE *versus* Tween 80 solution for NO.

Assessment of the insecticidal effects of the extracts

The insecticidal effects of the testing extracts on mirids were estimated based on the lethal concentration 50 (LC_{50}) and lethal time 50 (LT_{50}) which were determined using the probit analysis program, based on the logistic regression *via* times and concentrations/treatments probit-mortality (Finney 1971). Probit-mortality data were obtained after corrected mortality *CM* (%) following Abbott's (1925) equation.

$$CM = \frac{Mce - Mt}{100 - Mt} \times 100$$

where *Mce* is the mortality obtained during the trial and *Mt* the mortality registered in the negative controls.

Data analysis

Cumulative numbers of dead mirids during treatments were log-transformed before each analysis to correct unequal variances inherent in count data. The analysis of variance (ANOVA) was performed for multiple comparisons of means between the different treatments. When the ANOVA revealed statistical differences, Tukey's HSD was used for *post hoc* comparison of means. The Student's *t*-test was also used to compare mean numbers of dead mirids between both tested methods (direct and indirect toxicity) for each treatment. The effects of the two main factors (test method and treatment) were tested using an ANOVA with two factors which verified whether they interact or not on the mirids' mortality under our experimental conditions. All calculations were performed with SPSS version 21.0 for probit analysis and R version 3.5.1 software for ANOVA, at the 5 % confidence level.

RESULTS

Assessment of mirid mortality

Direct toxicity

From the first day of exposure, the mortality rate of mirids due to the insecticide was 100 %. The toxicity of the different extracts on these insects was concentration-dependent; it increased as a function of the tested concentrations. At the concentrations of 2 %, 5% and 8 %, the mortality rate raised from 17.5 to 75.0 %, 20.0 to 87.5 % and 60.0 to 97.5 % for NO (Fig. 2A) and 5.0 to 72.5 %, 15.0 to 85.0 % and 60.0 to 92.5 % for EE (Fig. 2B). Both negative controls (Tween 80 solution and DW) recorded first mirids mortalities 4 days post-treatment (PT); values ranged from 2.5 to 15.0 % for Tween 80 solution and 5.0 to 12.5 % for DW. In addition, values obtained with the negative controls were significantly low ($F_{4,395} = 54.94$, $P < 0.001$ for NO and $F_{4,395} = 55.34$, $P < 0.001$ for EE) compared to others, but no statistical difference of mirid mortality rates was observed between both substances tested and the insecticide (Table 1).

Indirect toxicity

The insecticidal effect of imidacloprid + lambda-cyhalothrin gradually increased from 45 % on the first day of exposure to 100 % on the third day PT. However, the biocidal activity after ingestion of NO and EE by mirids was also concentration-dependent; with the increasing concentrations of 2 %, 5 % and 8 %, mirid mortality rates ranged from 0 to 52.5 %, 0 to 62.5 % and 7.5 to 70.0 % for NO (Fig. 3A) against 0 to 32.5 %, 7.5 to 42.5 % and 12.5 to 65 % for EE (Fig. 3B), and from 0 to 12.5 % and 0 to 15.0 % for the Tween 80 solution and DW, respectively. The comparison of the average mirid mortalities between the test substances revealed that, apart from controls (Tween 80 and DW) which caused very low mortalities, values recorded did not differ significantly between the tested extracts and insecticide, except for EE by ingestion (Table 2).

Impact of methods of exposure on mirid mortality

High mirid mortality was obtained by contact exposure to NO (86.6 % on average) than by ingestion (61.6 % on average). A significant difference ($t = 3.67$, $P < 0.01$) was noticed between both methods only at the concentration of 8 % (Fig. 4). Similarly with treatments based on all concentrations of EE, mirid mortality was also significantly ($P < 0.05$) higher by contact exposure (83.3 % on average) compared to the ingestion method (46.6 % on average) as shown in Fig. 5. In addition, our findings showed the existence of test method and treatment effects on mirid mortality (Table 3).

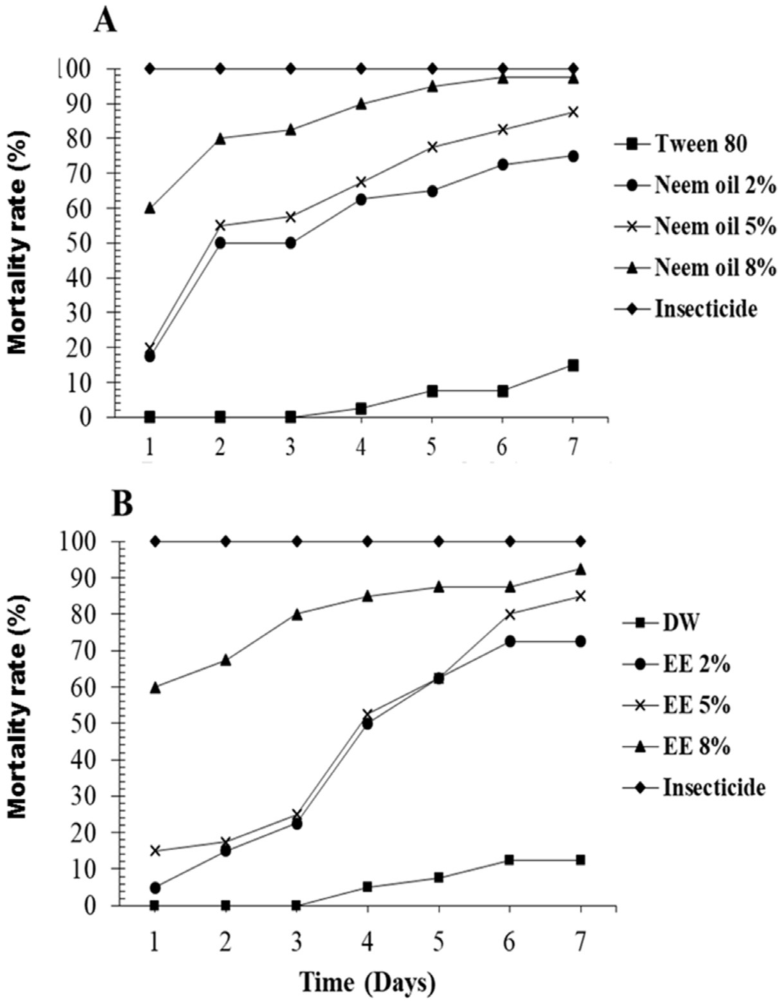

Fig. 2. Daily assessment of the mirid mortality due to the contact exposure to test substances. EE: ethanolic extract from neem leaves; DW: distilled water.

Table 1. Comparison of mirid mortalities (average cumulative rates± S.E.) by direct toxicity of the test substances.

Treatments		Mirid mortality (average cumulative rate ± S.E.)	
		Neem oil	Ethanolic extract
Insecticide (lambda-cyhalothrin + imidacloprid)		100 ± 0.0[a]	100 ± 0.0[a]
Concentrations:	8 %	97.5 ± 5.0[a]	92.5 ± 9.6[a]
	5 %	87.5 ± 18.9[a]	85.0 ± 5.7[a]
	2 %	75.0 ± 17.3[a]	72.5 ± 23.6[a]
Control	Tween 80	15.0 ± 5.7[b]	–
	DW	–	12.5 ± 5.0[b]
Statistics		$F_{4,395} = 54.94$, $P < 0.001$	$F_{4,395} = 55.34$, $P < 0.001$

Within columns, values followed by the same letter are not significantly different at the 5 % level, according to Tukey's test. S.E.: standard error; DW: distilled water.

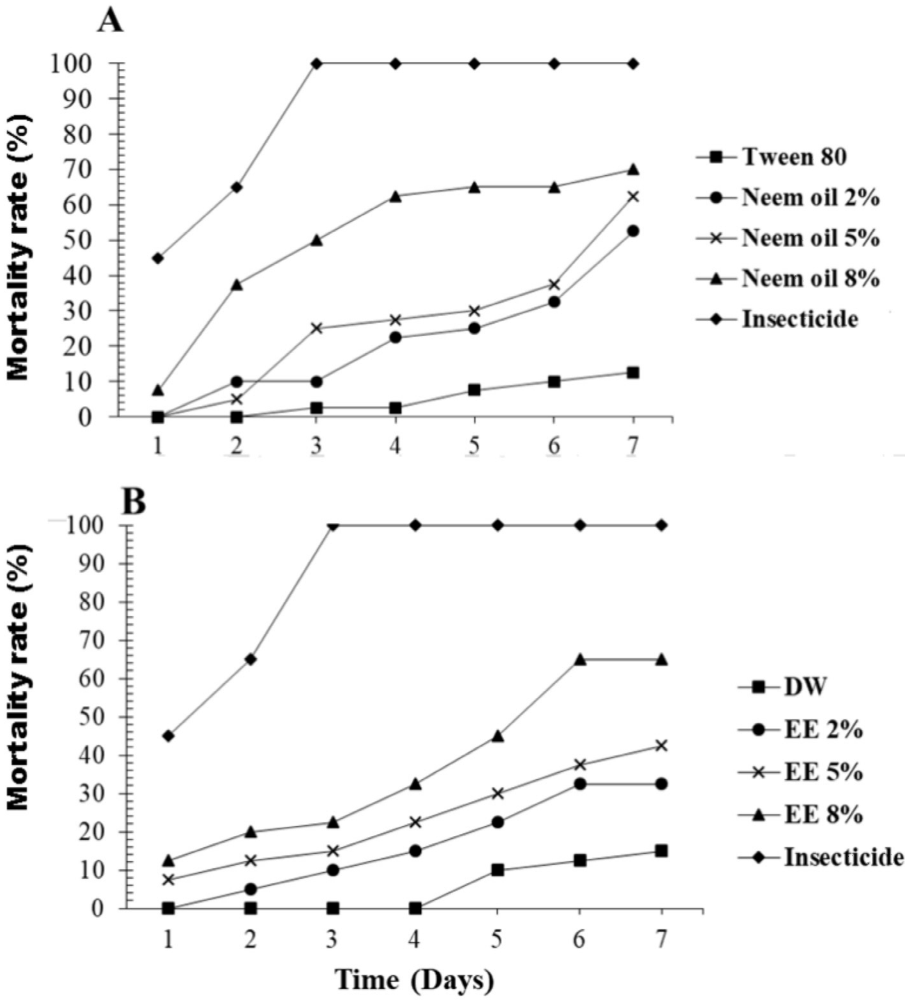

Fig. 3. Daily assessment of the mirid mortality due to the ingestion of test substances.

Table 2. Comparison of mirid mortalities (average cumulative rates± S.E.) by indirect toxicity of the test substances.

Treatments		Mirid mortality (average cumulative rate ± S.E.)	
		Neem oil	Ethanolic extract
Insecticide (lambda-cyhalothrin + imidacloprid)		100 ± 0.0^{a}	100 ± 0.0^{a}
Concentrations:	8 %	70.0 ± 14.1^{a}	65.0 ± 12.9^{ab}
	5 %	62.5 ± 17.1^{a}	42.5 ± 26.3^{abc}
	2 %	52.5 ± 18.9^{a}	32.5 ± 28.7^{bc}
Control	Tween 80	12.5 ± 9.6^{b}	–
	DW	–	12.5 ± 5.7^{c}
Statistics		$F_{4,395} = 18.07$, $P < 0.0001$	$F_{4,395} = 6.79$, $P < 0.0025$

Within columns, values followed by the same letter are not significantly different at the 5% level, according to Tukey's test.

Evaluation of LC_{50} and LT_{50}

Results indicated a significant ($P < 0.05$) linear relationship between log concentration and probit-mortality (Table 4). Between both exposure methods, the best insecticidal potential could be assigned to the contact exposure for which lowest values of LC_{50} were recorded (0.87 ml/ml for NO and 0.73 g/ml for EE) compared to the ingestion exposure. In addition, the high values of the coefficient of determination ($R^2 > 80$ %) showed a good fit of our data to a binomial regression model from the probit analysis (Table 4). Taking into account the LT_{50} of mirids, the lowest values were obtained for both treatments at the concentration of 8 % with contact exposure (0.72 day for EE and 0.78 day for NO); thus this concentration could also be considered as more efficient in controlling *S. singularis* (Table 5).

Table 3. Effect of methods of exposure and treatments on *Sahlbergella singularis* mortality.

Source of variation	d.f.	*F*	*P*-value
Method	1	33.53	<0.0001
Method × treatment	8	2.49	0.0223

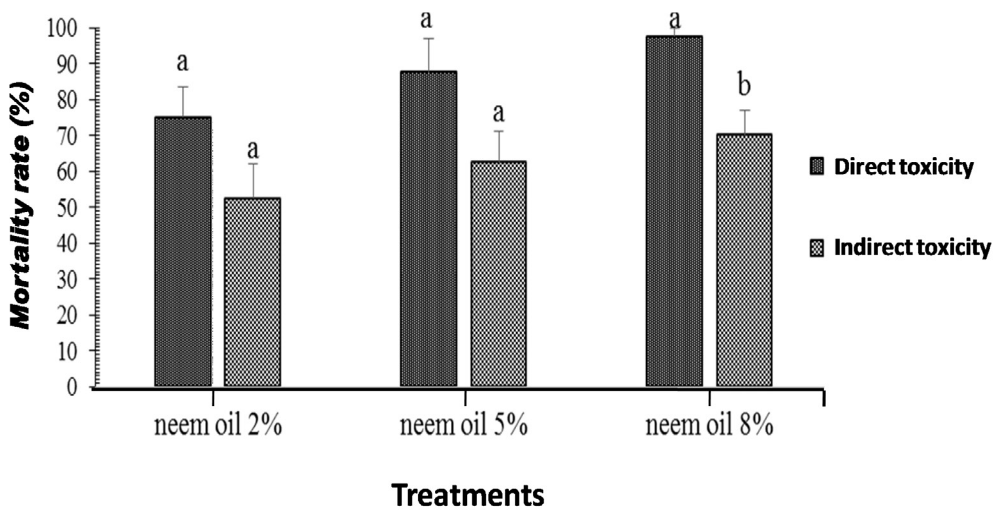

Fig. 4. Comparison of mirid mortalities (means ± S.E.) between both methods of specimen's exposure to neem oil.

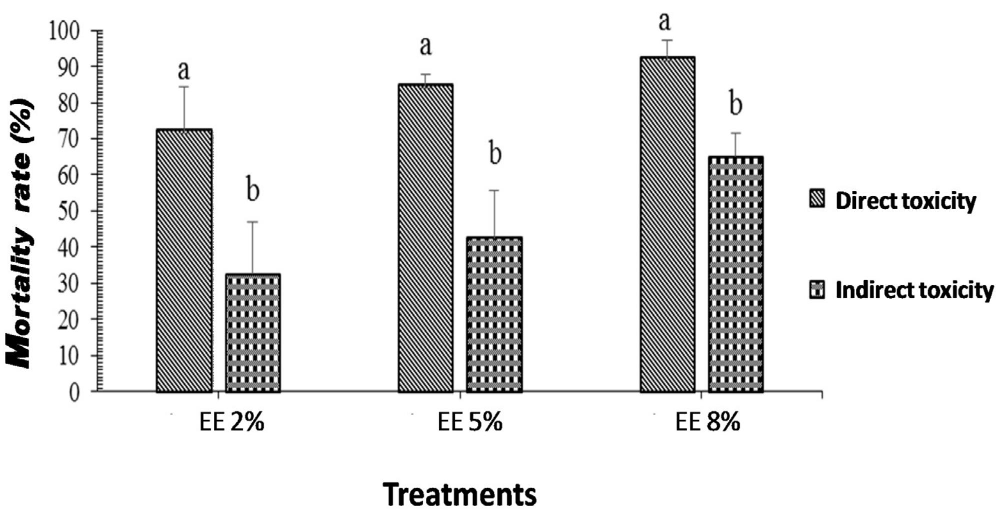

Fig. 5. Comparison of mirid mortalities (means ± S.E.) between both methods of specimen's exposure to ethanolic extract.

Table 4. Assessment of LC_{50} per test method and treatment.

Treatments	Methods	LC_{50} ml/ml	Probit parameters ± S.E.		χ^2-value	R^2
			Intercept	Slope		
Neem oil	Contact	0.87	0.10 ± 0.36	1.76 ± 0.62	0.97	0.89
	Ingestion	1.70	0.17 ± 0.31	0.75 ± 0.47	0.04	0.98
		g/ml				
Ethanolic extract	Contact	0.73	0.18 ± 0.35	1.32 ± 0.55	0.14	0.97
	Ingestion	5.05	–0.91 ± 0.32	1.29 ± 0.47	1.34	0.85

Table 5. Assessment of LT_{50} per test method and treatment.

Treatments	Concentrations	Test method	LT_{50} (days)	95 % confidence interval		χ^2-value	*P*-value Df = 3
				Lower	Upper		
Ethanolic extract	EE 2 %	Contact	4.21	3.72	4.81	3.44	0.63
		Ingestion	9.86	7.55	17.69	0.74	0.98
	EE 5 %	Contact	3.64	2.61	5.06	12.78	0.02
		Ingestion	8.37	6,60	13.30	1.38	0.93
	EE 8 %	Contact	0.72	0.20	1.20	0.94	0.97
		Ingestion	5.21	4.33	6.76	6.35	0.27
Neem oil	NO 2 %	Contact	2.75	2.13	3.37	2.29	0.81
		Ingestion	7.87	6.45	11.33	4.14	0.53
	NO 5 %	Contact	2.22	1.74	2.65	1.98	0.85
		Ingestion	6.46	5.56	8.20	6.02	0.30
	NO 8 %	Contact	0.78	0.38	1.13	1.45	0.92
		Ingestion	3.36	2.79	4.00	3.71	0.59

DISCUSSION

From this study, *Azadirachta indica* extracts (NO and EE) showed comparable biological efficiencies with imidacloprid + lambda-cyhalothrin (Parastar® 40 EC), the benchmark insecticide, in controlling mirid populations under laboratory conditions, particularly at high concentration (8 %) for the both tested methods. These findings clearly showed that the toxicity of neem extracts on mirid populations was concentration-dependent: the lethal effects of NO and EE towards *S. singularis* increased with their concentrations. This result confirms the insecticidal effects of neem extracts, especially aqueous extracts and/or neem oil, on insect pests belonging to the Hemiptera, including mirids (Adu-Acheampong 1997; N'Guessan & Kouassi 2006; Ayenor *et al*. 2007; Asogwa *et al*. 2010; Anikwe 2013; Adesina 2014; Formentini *et al*. 2016; Zanuncio *et al*. 2016; Mboussi *et al*. 2018). However, some significant divergences in mirid mortality due to ingestion of insecticide, the negative controls (Tween 80 and DW) and the ethanolic extracts were observed; they justify the fact that the susceptibility of the hemipterans such as *S. singularis* may vary with the mode of exposure and the different concentrations of neem extracts and insecticides (Schmuttetrer 1990; Ishaaya *et al*. 2007). However, additional studies in the field are needed to confirm or refute our *in vitro* results which, especially, showed that NO induced high mirid mortality compared to EE. In this work, NO was extracted from seeds and EE from leaves of *A. indica*. It is known that compared to leaves, neem seeds contain numerous active compounds, including azadirachtin which is the most important active ingredient of neem (Schmutterer 1990). Schmutterer (1990) and Mordue (Luntz) & Nisbet (2000) reported that azadirachtin has several modes of action against insect pests, amongst which is apoptosis known as the great lethal toxicity phenomenon against insects. This justifies the differences in the average mortalities of mirids observed in the present study between NO and EE treatments. Moreover, the biological effectiveness of plants and/or their derivative products on insect pests may vary considerably between plant species, even within the same species (genetic reasons), and with the time and locality of their collection as well as the organ collected; because the quantity of active compounds present in the extracts depends on several biotic and abiotic factors (Schmutterer 1990; Mordue (Luntz) & Nisbet 2000). Our results differ from those obtained by Adu-Acheampong (1997) on *Distantiella theobroma* (Hemiptera: Miridae) and both Anikwe (2013) and Mboussi *et al*. (2018) on *S. singularis* (Hemiptera: Miridae) for EE and Zanuncio *et al*. (2016) on *Podisus nigrispinus* Dallas (Heteroptera: Pentatomidae) for NO. This situation could be explained by (a) the diversity of bioactive substances contained in each extract and (b) the difference in the susceptibility of the different populations and/or insect pest species, and/or experimental conditions. It is known that the toxicity of plant extracts against these organisms vary with the type of extracts, the dose and the duration of insects' exposure to their bioactive substances (Kim *et al*. 2003; Bouchikhi *et al*. 2010; Sow & Diarra 2014; Zanuncio *et al*. 2016), and experimental conditions (Adu-Acheampong 1997; Anikwe 2013; Mboussi *et al*. 2018).

Although taxonomically distant from neem (Meliaceae), toxicity/virulence of both entomopathogenic fungi, *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hypocreales) and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycota: Hypocreales), assessed *in vitro* on *S. singularis* populations by direct and/or indirect approaches (Mahot *et al*. 2019; Mahot 2020) showed similar results with those obtained in our investigations. Thus, the results of the present study coupled with those obtained by these latter authors confirm the potential of neem extracts and entomopathogenic fungi tested in the control of insect pests such as *S. singularis*. These results should be confirmed or invalidated under field conditions in order (a) to contribute in the search of a suitable Integrated Pest Management (IPM) system such as a biopesticide against *S. singularis*, and (b) even to eliminate agrochemistry in the cocoa sector by promoting organic cocoa farming, to reduce adverse effects of synthetic pesticides (insecticides) on the environment, human health, and other living organisms, particularly the pollinator insects such as those of the genera *Forcipomya* (Diptera: Ceratopogonidae) and *Drosophila* (Diptera: Drosophilidae) usually encountered in cacao trees in Cameroon (Mbondji Mbondji 2010).

In this study, the LC_{50} and LT_{50} were used to measure the biological activity of neem extracts on *S. singularis* populations. The best values of LC_{50} and LT_{50} were obtained by direct contact of insects compared to those by indirect contact or ingestion. These parameters are involved in the choice of the toxicity level of a given product, expressed as the amount of deaths that an extract or a pathogen can provoke in a batch of tested insects (Inglis *et al*. 2001; Hao & Ng 2011). The discrepancy of mirid mortality between both exposure methods of specimens can be related to the difference in the ability of these insects to metabolise active ingredients, depending on the body entrance pathways. In this respect, Gillot (2005) reported that toxic products ingested by insects first pass into the detoxification organs (middle intestine, Malpighian tubules and fatty bodies) before being distributed throughout the animal's body; in the opposite, those which are applied directly to their tegument cross the cuticle *via* the waxy canaliculi, reach the haemolymph which carries them throughout the body, particularly in the most lipophilic areas, causing a rapid lethal effect on the insects. The greater toxicicy of *A. indica* extracts (NO and aqueous extracts) at different doses applied by direct contact was also confirmed in the control of *S. singularis* (Mboussi *et al*. 2018) and the hemipteran Psyllidae *Gyropsylla spegazziniana* (Lizer & Trelles) populations (Formentini *et al*. 2016) compared to indirect toxicity. Our results depart from the observations made by Zanuncio *et al*. (2016) which obtained higher toxicity by ingestion than by contact on *P. nigrispinus* populations; it thus confirms the idea that the high mortality of insects does not depend on the exposure method of extracts (Adu-Acheampong 1997) but on their susceptibility to the different concentrations of tested substances (Bandara *et al*. 2010).

The importance of this work is based on the demonstration of the toxic effects of neem extracts (NO and EE) on mirid populations under laboratory conditions. It emerges that the toxicity of *A. indica* extracts towards mirids significantly differs as a function of the treatments and test methods. The mirid mortality rates induced by neem oil are globally higher than those obtained with EE. Whatever the type of extract and test method, the mirid mortality rates increased with the concentrations of test substances; moreover, except for the 2 % concentration of EE by ingestion, their biological effectiveness towards *S. singularis* is comparable to Parastar® 40 EC, the benchmark insecticide in controlling mirids in cocoa farms in Cameroon. The biocidal activity of our extracts on *S. singularis* populations is greater by contact than by ingestion, as clearly demonstrated by the values of LC_{50} and TL_{50}. These promising results support the use of both NO and EE as effective for IPM of the African brown cocoa mirid, *S. singularis*; but they need to be confirmed under field conditions in order to promote the cultivation of organic cocoa, and consequently the preservation of biodiversity/environment, human health and other living organisms.

ACKNOWLEDGEMENTS

This study was funded by the special research allowances from the Ministry of Higher Education and internal allowances from the University of Yaounde I. Thanks to the Institute of Agricultural Research for Development and the International Institute of Tropical Agriculture for logistic and laboratory products, and to G. Ajeagah and C. Njua for their contributions in the English proofreading.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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