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Persistence and Comparative Pesticidal Potentials of Some Constituents of *Lippia adoensis* (Hochst. ex Walp.) (Lamiales: Verbenaceae) Essential Oil against Three Life Stages of *Callosobruchus maculatus* (Fab.) (Coleoptera: Bruchidae)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MA and ENN consensually designed the project, wrote the protocol, wrote the first draft of the manuscript and performed all experiments. Author CN contributed to the reviewing and editing of the work. Authors ZC and TV performed the final experimental phase and statistical analysis. Author HC performed the literature review and plant samples collection. All authors read and approved the final manuscript.

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ABSTRACT

Over the past decades, the development of an alternative and eco-friendly pest control strategies has become a public concern for the sake of mankind. Plant essential oils are complex mixtures of volatile organic compounds, which play indispensable roles in the environment, for the plant itself, as well as for humans.
Aims: The objectives of this study were (i) to identify and report the volatile organic compounds of

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Lippia adoensis (Hochst. Ex Walp.) (Lamiales: verbenaceae) essential oil (EO, herein after) and to compare the bioactivity of its four major compounds with the crude EO at relatively low dosages on some fitness parameters of *Callosobruchus maculatus* (F.) (Coleptera: Bruchidae), (ii) to test the persistency of each compounds over time, and (iii) to evaluate the type of interaction of the major constituents of the EO (whether antagonistic, synergistic or additive) in order to project the biological interaction of the whole compounds.

Study Design: Young leaves of *L. adoensis* were randomly collected in Mbe locality (Ngaoundere-Cameroon) for EO extraction which was then analysed by the means of Gas Chromatography (GC-FID) coupled with Mass Spectrometry (GC-MS). Bioassays consisted of three shelves treatments for adult mortality, eggs laying, larval development and progeny production. A complete randomized design (CRD) with five replications was set for each treatment.

Places and Duration of Study: Laboratory of Biology of the Faculty of Science, University of Ngaoundere (Cameroon) and Pest Control laboratory of Professor Niu Changying HZAU, Wuhan (China) from February 2014 to January 2016.

Methodology: Essential oil was extracted with a Clevencher-type apparatus, hydrodistilled and air-dried. Gas Chromatography-Flame Ionization Detector (GC-FID) and Gas Chromatography coupled with Mass Spectrometry (GC-MS) were carried out to analyze the constituents. The toxicity of crude EO and its four major constituents was evaluated at 0.5, 5, 10, 20 and 40 µL/g. Ten glass jars (volume 800mL) containing 50 g of cowpea seeds were prepared. After treatment, ten couples of *C. maculatus* aged 1-7days were randomly selected and separately introduced in each glass jar and kept at 22.72±1.06℃, 83.73±1.28% RH. Control ja rs were treated only with pure acetone. Each treatment was replicated five times. The exposure lasted for six days post treatment. Data on adult's mortality, eggs laying, larvae and progeny production were assessed and monitored.

Results: The GC–MS analysis allowed the identification of 43 volatile components representing 93.54% of total oil. The major components were Thymol (22.01%), Thymol-acetate (15.21%), para-cymene (13.85%) and Triacetin (9.131%).

The crude EO suppressed adults, completely inhibited eggs laying and adult emergence at 5 and 10 μL/g after 24 h, respectively. Complete adults suppression was observed with thymol and the mixture at 20 μL/g. Para-cymene and Triacetin caused complete adult mortality at 40μL/g but did not inhibited eggs laying and progeny production. EO and the mixture have been the most potent and persistent with a higher persistency throughout the experimentation. The synergism ratios (SR) were all higher than 1, thereby suggesting a significant (P = .05) synergistic interaction of major constituents although lower than that of the crude EO. Data on behaviours at death suggests that this EO may be neurotoxic probably through the blocking of the cholinergic receptors through reversible inhibition of acetylcholinesterase.

Conclusion: The results obtained from this study revealed a significant (P = .05) insecticidal bioefficacy of EO extracted from young leaves of *L. adoensis* from Cameroon. This insecticidal properties are due to its richness in chemical constituents (43 in total) that sustained its persistency during the treatment. *L. adoensis* could therefore be a suitable topical agent to control *C. maculatus* infestations and could be of value for commercial formulations.

Keywords: Essential oil; phenolic compounds; Thymol; synergism; Callosobruchus maculates.

1. INTRODUCTION

The cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae), is a major constraint to cowpea production and storage in countries where the modern storage facilities is not well developed such as tropical and subtropical areas including Cameroon [1]. During larval stages, it causes substantial quantitative and qualitative losses (50-90%) manifested by seed perforation and reductions in weight, market value and germination ability of seeds [2,3]. The adult itself does not cause any damage to grains but lays eggs on the seeds, which then hatch and produce larvae that bore into the seed cotyledons on which they feed to occasionate the above mentioned level of damage [4-7]. Face to these constraints, farmers have been relying on the use of synthetic chemical insecticides that have greatly played a beneficial role in preserving stored products from insect infestations among which the cowpea weevil, *C. maculatus* [8], but its repeated use has posed several problems to environment and human health as the result of their persistent residues and negative effects on non-target organisms.

Some organophosphorus and organochorites insecticides are even pointed out to be carcinogenic, mutagenic and neurotoxic agents to human beings [9-11]. Also, there has been a rising number of insects developping resistance against synthetic chemicals [12,13]. However, according to Asgar Ebadollahi [14], the ideal insecticide should control target pests, be rapidly degradable and less toxic to humans and other mammals.

The detrimental effects of synthetic chemicals has therefore prompted studies in most stricken countries to develop and promote the use of plant based insecticides to control pests of agricultural importance. These botanicals can interact with most of the abundant cholinergic receptors in the insects brain and drastically reduce the probability to develop resistance [15], regardless the mode of application, be it contact (penetration through the insect cuticle), fumigant (through respiratory system) or ingestion effect (through digestive tract) [16]. It should be noted that EOs are secondary metabolites, which were first considered as phyto-metabolic waste products, but it soon became apparent that they are involved in preventing excessive evaporation, donors of hydrogen in oxidation-reduction reactions, and in the defense of plant against herbivorous insects attack. Moreover, they may attract specific insects for pollination as well as specialist-phytophagous insects [17]. Studies on the EOs are thefeore very important for initiating the implementation of an effective pest control program. Recent studies showed that they may act as fumigants, contact insecticides, repellents, deterrents and antifeedants [18-23]. In Cameroon, Ngamo et al. [24] and Nukenine *et al.* [25] have also reported the beneficial use of aromatic plants in pest management. *L. adoensis*, Hochst. ex wasp.(Lamiales: Verbenaceae) is a pantropical species widely spread in Africa (Senegal, Ethiopia, Nigeria, Cameroon etc) and central America [26] that comprises more than 200 species of herbs, shrubs, and small trees. It is an aromatic, fragant and grassy annual plant which grows in all regions of Cameroon where the annual rainfall is about 1100-1500 mm like in Ngaoundere. Its leaves and oil are extensively used as pharnaceuticals and in flavouring by the local population (Akami M. and Nukenine E-N, personal investigation). Many studies have demonstrated the contact toxicity of EOs and their constituents against numbers of stored product insects at different life stages. Seyed M-H and Seyed A-S [27] reported the toxicity of essential oil of *Platycladus orientalis (L.) Franco*(Cupressaceae) against *Sitophilus oryzea* (L.) (Coleoptera: Curculionidae), *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae) and *C. maculatus* (F.)(Coleoptera: Bruchidae). Various essential oils have also been tested against *C. maculatus* (F.) (Coleoptera: Bruchidae) [28-30]. For example, *Hura crepitans* (L.) seed oil was reported as toxic to adult and immature stages of *C. maculatus* (F.) (Coleoptera: Bruchidae) [28]. Similarly, oil of *Ziziphora clinopodioides* (Boiss), *Foeniculum vulgare*, *Teucrium polium* and *Satureja hortensis* caused significant contact toxicity against adult and eggs [29,31,32]. Olufunmilayo et al. [33] reported the fumigant toxicity of eight monoterpenoids against *C. maculatus* (F.) (Coleoptera: Bruchidae). Recent studies from our laboratory have also demonstrated high adult mortality, larvidal and ovicidal effects of *Lippia adoensis* Hochst. Ex Wasp. (Lamiales: Verbenaceae) EO against *C. maculatus* (F.) (Coleoptera: Bruchidae) [7]. The majority if not all the above studies focussed on either the contact or fumigant toxicity of crude EOs and its constituents. To the best of our knowledge, there is no previous reports on the study of the mixture of active compounds of the Cameroonian *L. adoensis* Hochst. Ex Wasp. (Lamiales: Verbenaceae) EO and the attribution of toxicity or oviposition deterrence to particular compounds through topical application (contact toxicity) against *C. maculatus* (F.) (Coleoptera: Bruchidae).

The objectives of this study were (i) to identify the compounds of *L. adoensis EO* and to compare the bioactivity of the mixture of four major compounds with the crude EO at relatively low concentrations against *C. maculatus*, (ii) to test the persistency of each compounds over time, and (iii) to evaluate the type of interaction of the major constituents of the EO (antagonism, synergism or additive). The choice of the EO constituents to be used in this experiment was based on their amount in the crude EO and their pesticidal properties against stored product pests previously reported [7,34-37]. All the possible contact effects on eggs, larvae and adults of the cowpea weevil *C. maculatus* (F.) (Coleoptera: Bruchidae) was recorded. A high-resolution monochrome CDD camera was used to record the behaviour of insects at death.

2. MATERIALS AND METHODS

2.1 Insect Rearing

Cowpea seeds were prepared as described by Akami et al. [7] with the following modification**:** the seeds assigned for the bioassays were kept in polyethylene bags in a freezer at -20℃ for three days and the seeds that were used for insect rearing were kept under the laboratory conditions for acclimatization [25]. *C. maculatus* specimens were acquired from infested white cowpea variety, *Mozongo* purchased from farmers in Lara (Far-North region, Cameroon) and reared at room temperature for three months (June, July and August 2014) under fluctuation laboratory conditions (T≈22.72±1.06℃, RH≈83.73±1.28%). 10 glass jars of 800ml in which we introduced 100 g each were set for the rearing. Twenty couples of *C. maculatus* adults were introduced in each jar, then covered with fine mesh cloth fastened with rubber bands to prevent the contamination and escape of insects. Seven days were allowed for mating and oviposition. The parent stocks were sieved out and the cowpea seeds containing eggs were left undisturbed until the new adults emerged. Only the subsequent F1 progenies of the bruchids, which emerged from the cultures and aged 1-3 days, were used for the experiment [7]. All our experiments were carried out under the same environmental conditions.

2.2 Plant Material

The plant material evaluated for insecticidal activity against *C. maculatus* were young leaves of *L. adoensis* which we collected in July 2013 from Mbe locality (601 masl, latitude 7°82N, longitude13°58E recorded with a GPS Garmin Geko 301), Adamawa region, Cameroon. The identification of the plant was confirmed at the national herbarium of Yaounde (Cameroon), where voucher specimens are deposited. The fresh leaves were rinsed in clean water to remove sand and other impurities, and then shade-dried to avoid direct exposure to sunlight and possible denaturation of the bioactive volatile compounds.

2.3 Essential Oil Extraction

The fresh leaves earlier prepared were used for the extraction of the essential oil by water distillation for 4 h using a Clevenger-type apparatus exactly as described by Akami et al. [7]. Each sample consisted of 50 g of shade-dried leaves. The extracted oil was dehydrated with anhydrous sodium sulfate (10 min) and immediately stored in airtight transparent glass bottle in refrigerator at 4℃ until used for analysis and bioassays.

2.4 Analysis of the *Lippia adoensis* Hochst. Ex Wasp. (Lamiales: Verbenaceae) Essential Oil

2.4.1 Gas Chromatography (GC)

The Gas Chromatography (GC) analysis was carried out using a Perkin-Elmer Sigma-115 gas chromatograph equipped with a data handling system and a flame ionization detector (FID) as described by Laura De Martino et al. [38] with the following modifications. Separation was achieved by a fused-silica capillary column HP 5MS, 30m length, 0.25 mm internal diameter, 0.25 µm film thickness at a constant helium (99.99%) flow rate of 1.2 mL/min as carrier gas. The operating conditions were as follows: injector and detector temperatures, 250℃ and 280℃, respectively; oven temperature programme: 5 min isothermal at 40℃, subsequently at 2℃/min up to 250℃ and finally raised to 270℃ at 10℃/min. Diluted samples (1/100 v/v, in n-pentane) of 1 µL were manually injected at 250℃, and in the splitless mode. Analysis was also made by using a fused silica HP Innowax polyethylenglycol capillary column (50 m x 0.20 mm i.d.; 0.20 µm film thickness). The quantification of each constituent was computed by the normalization method from the GC peak areas and they were arranged in order of GC elution.

2.4.2 Gas chromatography - mass spectrometry

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed using an Agilent 6850 Ser. A apparatus, equipped with a fused silica HP-5 capillary column (30 m x 0.25 mm i.d.; film thickness 0.33 µm), linked on line with an Agilent MS D5973 Mass Selective Detector; ionization energy 70eV, multiplier voltage 2000 V. Mass spectrawere scanned in the range 35-450 amu, scan time 5 scans/s. Gas chromatographic conditions were as given above, transfer line was kept at 295℃.

The oil components were identified from their GC retention indices, with either those of the literature [39-40] or with those of authentic compounds available in our laboratories. The identity of the components was assigned by

comparing their linear retention indices, relative to C_9H_{20}–$C_{19}H_{40}$ n-alkanes, under the same operating conditions. Further identification was made by comparison of their MS spectra on both columns, with either stored in NIST 02 or Wiley 275 libraries or with mass spectra from the literature [39-41]. Retention Indices (RI) were obtained with equation proposed by Vandendool and Kratz [42].

2.5 Essential Chemicals

Thymol was obtained from Sigma-Aldrich (Milwaukee, WI, USA), Thymol-acetate from Tokyo Kasei (Tokyo, Japan), para-cymene from Fluka (Buchs, Switzerland) and Triacetin from Sigma-Aldrich (St. Louis, MO, Italy). The purity of the oil components ranged between 95 and 99%. These major compounds and crude EO were dissolved in acetone for topical application.

2.6 Bioassay Tests

2.6.1 Toxicity

The toxicity of crude EO and its four major constituents was evaluated at 0.5, 5, 10, 20 and 40 µL/g. Ten glass jars (volume 800mL) containing 50g of cowpea seeds were prepared. After treatment with differents products (Triacetin, Para-cymene, Thymol-acetate, Thymol, crude EO and Mixture) at different concentrations (0.5, 5, 10, 20 and 40 µL/g), ten couples of *C. maculatus* aged 1-7days were randomly selected and separately introduced in each glass jar and kept at 22.72±1.06℃, 83.73±1.28% RH. Control jars were treated only with pure acetone. Each treatment was replicated five times. The exposure days lasted for six days post treatment. Mortality of control was evaluated by using Abbott's formula [43]. Insects were considered dead when no signs of leg or antennal movement were observed after being touched with a camel hair brush [7].

To determine the bioefficacy on eggs and larvae, we counted and recorded the number of eggs laid on cowpea seeds of each replicate at 1, 2, 4 and 6 day exposure. Adult emergence was monitored daily for two weeks starting from the 32nd day post treatment. The emerged adults were removed daily for 14 days after the first emergence was observed [44]. The percentage of adult emergence (progeny development) was calculated using the formula:

$$PF1 = \left[\frac{Nem.}{Neg.}\right] x\ 100$$

Where ***Nem*** and ***Neg***. were the number of adults that emerged and the number of eggs laid, respectively.

2.6.2 Evaluation of the persistency of pesticidal actions

Persistence of EO and major compounds was evaluated through their individual biopotency that can be defined as their capacity to cause mortality and/or to inhibit eggs laying and progeny production over time. It is a time-depending measurable parameter regardless the life stage of the insect; different from the potency which is simply the apparent and predictable capacity to produce effects. We used the method described by Heydarzade and Moravvej [31] with modifications to evaluate the persistency of different products according to rising concentrations.

The biopotency of each product based on their LC_{50} values was evaluated by diluting each LC_{50} obtained in 1 mL acetone and pipetted onto filter paper discs (Whatman No.1) (9 cm diameter) in Petri dishes. Twenty minutes later after complete evaporation of the solvent, twenty adults of mix sex aged one-day old were introduced separately into each Petri dish and then numbers of dead insects were recorded over time at 24 h intervals till the end of the experiment. For each interval, separate series of Petri dishes were set up with five replications. The dead insects over time were recorded and removed until no mortality was observed.

2.6.3 Determination of synergistic ratio (SR) of the mixture

In order to determine the potential synergism among the four chosen major constituents from the essential oil, a unique quaternary mixture of triacetin (Hexane and octane derivatives), Para-cymene (hydrocarbon monoterpenes), thymol-acetate and thymol both oxygenated monoterpenes and phenolic compounds prepared in the proportion in quaternary mixture (1:1:1:1). Acetone was used as solvent for solution preparation.

To determine the effect of different mixture among thymol, thymyl acetate, para-cymene and triacetin, Metcalf model [45] with modification was used to estimate the synergistic ratio (SR), as follows:

$$\mathrm{SR} = \frac{\mathrm{LC}_{50}\mathrm{A}}{\mathrm{LC}_{50}\mathrm{M}}$$

Where LC_{50} A is the average of LC_{50} of each compound alone and LC_{50} M is LC_{50} of the mixture.

According to this model when the SR value is 1, the toxicity of a mixture and their compounds isolated was equal (additive effect); for values exceeding 1, synergism between compounds occurs; and for lower values than 1, the mixture of compounds showed an antagonism.

2.7 Data Analysis

Data were analyzed at three levels for the toxicity test (mortality, eggs laying, oviposition deterrence and adult emergence).

For the adult mortality, data were arcsine transformed and analyzed using one-way analysis of variance (ANOVA). Tukey's HSD test (P = .05) was used for mean separations. Mortality of control was evaluated by using Abbott's formula [43]. The LC_{50} was calculated by probit analysis [46]. Multiple comparisons were done on least square means. Data on adult emergence were monitored to determine the future of the eggs laid during bioassay. Logarithmic transformation was performed on oviposition deterrence and adult emergence. ANOVA and Tukey's HSD test were used to compare the number of eggs laid and number of adults that emerged. Chemical compounds of the EO were identified using the NIST Mass Spectral Database and by comparison to pure samples. All the comparisons were considered significant when p≤.05.

3. RESULTS

3.1 Chemical Composition of Essential oil of *L. adoensis* Hochst. Ex Wasp. (Lamiales: Verbenaceae)

The chemical constituents of *L. adoensis* Hochst, ex Wasp. (Lamiales: Verbenaceae) EO from Cameroon are summarized in Table 1. The GC–MS analysis allowed the identification of 43 volatile components representing 93.54% of total oil. The major components were Thymol (22.01%), Thymol-acetate (15.21%), para-cymene (13.85%) and Triacetin (9.131%).

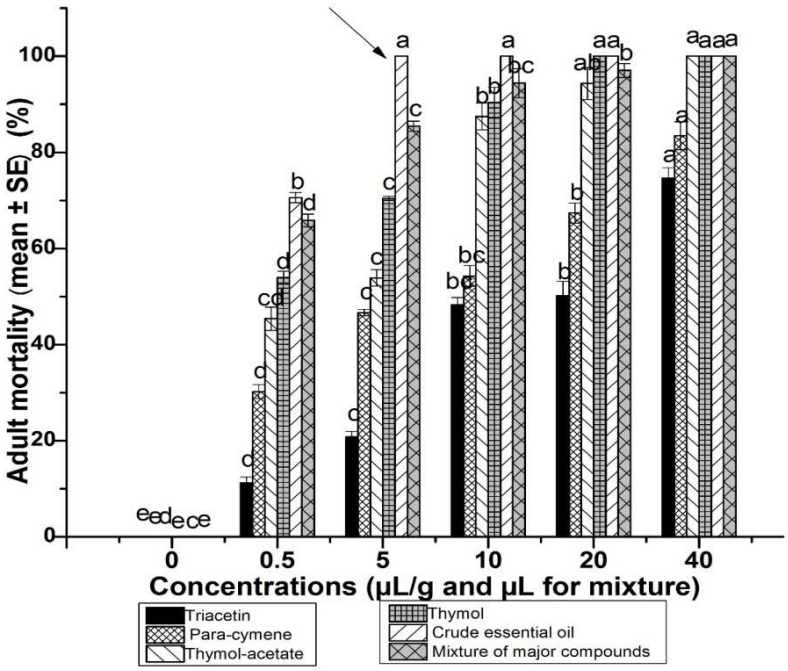

Fig. 1. Adult mortality of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) exposed to five dosages of crude and constituents of *Lippia adoensis* Hochst, ex Wasp. (Verbenaceae) essential oil. NB: Means with the same letter are not significantly different after comparison between concentrations with Tukey's test at (P<.05)

Table 1. Chemical constituents (%) of the essential oil from young leaves of *L. adoensis* Hochst. Ex Wasp. (Lamiales: Verbenaceae) collected from Mbe, Adamawa region of cameroon

	System method column	Group	RT/ MS2 FM3.M INNOWax	Relative content (%) Y_*L. adoensis*
	Substance		**RT (min)**	
1	2-Methyl-1-butanol	Hod		x
2	α-Pinene	Mth	6.43	0.404
3	α-Thuiene	Mth	6.63	1.138
4	β-Pinene	Mth	9.72	t
5	1-Octen-3-ole	Hod		x
6	Sabinene	Mto	10.36	0.312
7	3-Carene	Mth	11.60	0.107
8	α-Phellandrene	Mth	12.32	t
9	α-Terpinene	Mth	12.98	1.327
10	Limonene	Mth	13.87	3.212
11	B-Phellandrene	Mth	14.28	0.361
12	γ-Terpinene	Mth	16.13	6.419
13	Z-Ocimene	Mth	16.50	0.082
14	**para-Cymene**	**Mth**	17.33	**13.854**
15	Terpinolene	Mth	17.84	0.079
16	Fenchone	Mto	22.91	0.099
17	1-Octen-3-ole	Hod	25.39	0.071
18	E-Limonen oxide	Mto	25.64	0.180
19	Z-β-Terpineole	Mto	25.94	0.190
20	Camphor	Mto	28.00	0.022
21	Linalool	Mto	29.47	0.377
22	α-copaene	Sth		x
23	3-Aminopyrazole	Hod	31.05	3.304
24	Caryophyllene	Sth	31.29	3.508
25	Terpinen-4-ole	Mto	31.64	0.628
26	Umbellulone	Mto	33.16	0.126
27	α-Terpineole	Mto		x
28	Myrtenale	Mto		X
29	β-Farnesene	Sth	34.07	2.640
30	α-Caryophyllene	Sth	34.11	0.076
31	m-tert-Butylphenol	Hod	35.21	1.186
32	β-Cubebene	Sth	35.64	0.112
33	Verbenone	Mto	35.95	0.722
34	Carvone	Mto	36.72	1.883
35	**Thymol acetate**	**Mto**	41.03	**15.207**
36	para-Thymol	Mto	41.80	0.955
37	Piperitone	Mto	43.40	0.077
38	Caryophyllene oxide	Sto	45.32	0.389
39	**Triacetin**	**Hod**	48.59	**9.131**
40	Eugenol	Mto	51.42	0.079
41	**Thymol**	**Mto**	51.98	**22.0147**
42	Carvacrol	Mto	52.83	3.264
43	Triacetin	Hod		x
	Total			93.54

***RT:** Retention time; **Hod**: Hexane and octane derivatives (13.69%); **Mth:** Monoterpene hydrocarbons (26.98%); **Mto:** Oxygenated monoterpenes (46.14%); Sum of monoterpenes (Mt)**:** 73.12%; **Sth**: Sesquiterpene hydrocarbons (6.34%); **Sto:** oxygenated sesquiterpenes (0.39%); Sum of sesquiterpenes (St)**:** 6.73%; t: trace; **x**: Not quantify, **x**: Not determined; **Y_L:** Young Leaves*

3.2 Bioassay Tests

3.2.1 Adults

During this study, no mortality was recorded in untreated cowpea seeds (controls). *L. adoensis* oil demonstrated a strong toxicity against *C. maculatus* adults. The mortality was dose-dependent and increased with rising concentration from 0.50 to 40 µL/g (Fig. 1). Thymol and thymol-acetate were more toxic than para-cymene and triacetin because they caused ≥80% mortality to adult weevils at the third dose 10 μL/g within 1 day but their biopotency curves dropped after 2 days (Fig. 2). Their combination had more toxic effects than their single applications (Fig. 1). However, the crude EO was the most efficient by causing complete mortality (100%) to adults as from 5μL/g within 24 hours (arrow Fig. 1).

The results of ANOVA revealed that the potency of each product on their various applications are significantly correlated to their activities over time (Fig. 2) {R=0.899; $F_{1,8}$=357.44, df=4; P<0.001 (because p=0.0004338)}. There was a significant negative and linear association between the death rate caused by each product and exposure periods. Triacetin, para-cymene, thymol-acetate and thymol had their peak of activity before the second day post exposure and then decreased completely to zero before the 4th day except that of thymol (Fig. 2). This parameter was evaluated based on the mean number of adults death recorded, the inhibition of eggs laid as well as that of progeny production (More details are found in Tables 1s and 2s of the supplementary materials). The crude EO was the most persistent in terms of toxicity sustainability.

Table 2 presents the behavior of insects at death has been recorded by eye observation and with a high-resolution monochrome CDD camera during off experimental periods after topical application. Four various types of behaviors have recurrently been observed before the insects died. Table 2 presents the results obtained as from the 1st day post exposure.

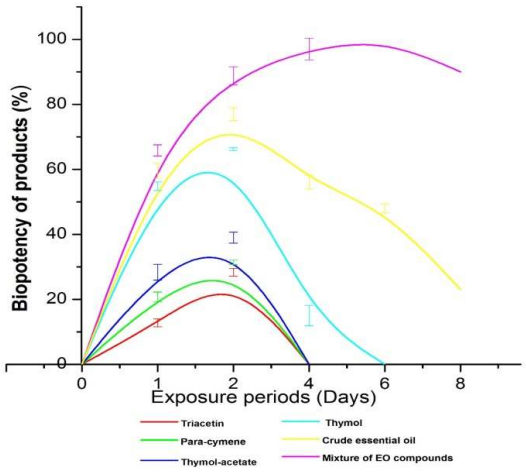

Fig. 2. Mean persistence activity of crude and constituents of *Lippia adoensis* essential oil on all fitness parameters measured. It was calculated by correlating the effects produced based on the LC_{50} of products and the corresponding death rate over time until no effect was recorded. As such, data were assessed every day for 8 days

3.2.2 Larvae and eggs

Fig. 3 presents the bioefficay of *L. adoensis* EO and its major compounds against *C. maculatus* eggs and larvae.

Table 2. Pre-death behaviours of adult's *C. maculatus* treated with various products of *Lippia adoensis* Hochst, ex Wasp. (Verbenaceae) EO

Products	Behaviours at death			
	Turning around	Agitation	Hyperactivity	Direct knockdown
Triacetin	79.68±2.77^{c}	20.35±0.99^{b}	00^{a}	00^{a}
Para-cymene	61.36±1.98^{c}	37.03±1.30^{b}	00^{a}	00^{a}
Thymol-acetate	21.91±1.29^{b}	52.32±1.09^{c}	19.34±0.07^{b}	8.12±1.88^{a}
Thymol	5.02±0.35^{c}	88.27±0.17^{b}	92.53±2.01^{b}	27.09±1.33^{a}
Crude EO	4.27±1.01^{d}	7.03±1.2^{c}	38.92±1.03^{b}	98.17±0.37^{a}
Mixture	27.89±1.77^{c}	68.29±0.12^{b}	72.76±1.21^{b}	52.93±1.08^{a}

Means ± S.E. in the same row for the same category of insecticide, followed by the same letter are not statistically significant at P = .05 (Tukey's test). Each datum represents the mean of five replicates of 10 insects each

All the treatments reduced egg laying and inhibited adult emergence with efficiency that varied with products and concentrations. Crude EO and mixture completely deterred oviposition and suppressed adult emergence at 5 and 10 μL/g, respectively, whereas thymol and thymol-acetate performed the same efficiency at higher dosages (20 and 40 μL/g, respectively) (Figs. 3a, b, respectively). Regardless the various dosages used, no complete reduction of egg laying and adult emergence were recorded with para-cymene and triacetin (Figs. 3a, b). Numbers of eggs laid decreased with increasing concentrations in all treatments. Here again, the crude EO is still leading in terms of efficiency compared to single compounds and/or their common mixture.

Apart from triacetin and para-cymene, thymol-acetate, the mixture and crude EO deterred completely the oviposition and adult emergence at the highest dose tested (40 μL/L) (Figs. 3a, b, respectively). Adult emergence decreased with increasing dose (R=-0896). It was significantly ($F_{1,8}$=298.37, df=4; P = 0.001) higher in triacetin and para-cymene than in Thymol, and lower in EO and the mixture at 0.5–10μL/L (Figs. 3a, b, respectively). The fecundity (capacity of laying eggs) and fertility (capacity of eggs laid to produce progeny) were evaluated based on the mean number of eggs laid and adult that emerged from these eggs. The crude EO exhibited the highest inhibition rate in both parameters (Figs. 3a, b, respectively). (More details are found in Table 2s a, b, c, d, e and f of the supplementary materials).

3.3 Evaluation of LC_{50} of Treatments

It should be noted that more the LC_{50} is higher, least the product is efficient and least the LC_{50} is, most efficient is the products compared to others involved in the same treatments. On the basis of Probit analysis, the highest toxicity of Crude EO in all the treatments (eggs, larvae and adults) can therefore be justified by its lowest LC_{50} (0.06-0.40 μL/L) (Fig. 4, dash arrow), and its activity kept on increasing till the 6th day, followed by the mixture with a relatively lower LC_{50} values (0.35-0.64 μL/L). However, triacetin which appeared as the least efficient in all treatments (Fig. 4, bold arrow), despite its shortest persistency, exhibited more detrimental effect to insects than para-cymene. The steepness of slopes with the EO shows that it rapidly disrupts the physiology of insects (oviposition, larval development and pupal eclosion) compared to the other tested products. Details on this can be found in Table 3s of the supplementary materials.

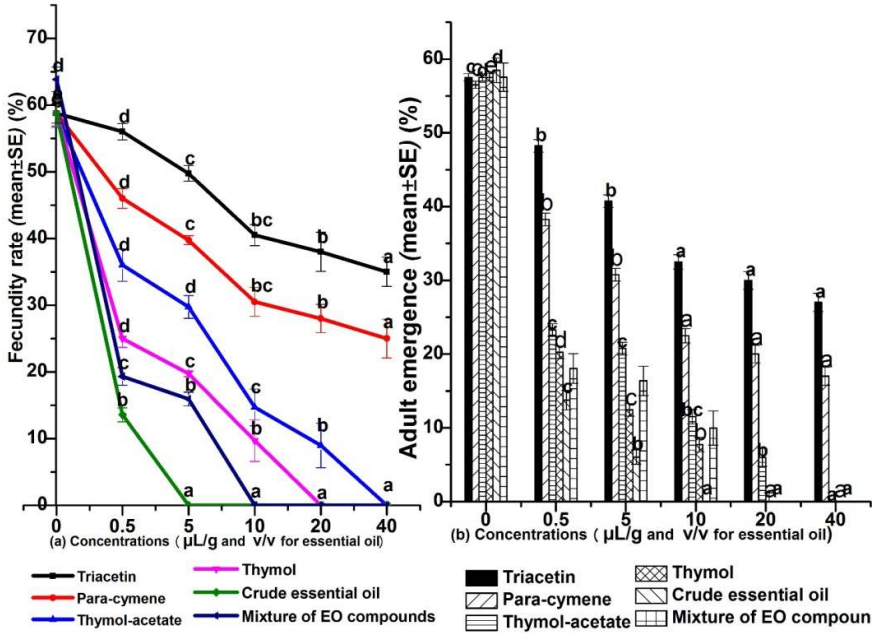

Fig. 3. Mean number of eggs laid by females exposed to five dosages of crude and constituents of *Lippia adoensis* essential oil and adults emergence. NB: Means with the same letter are not significantly different after comparison between concentrations with Tukey's test at (P<.05)

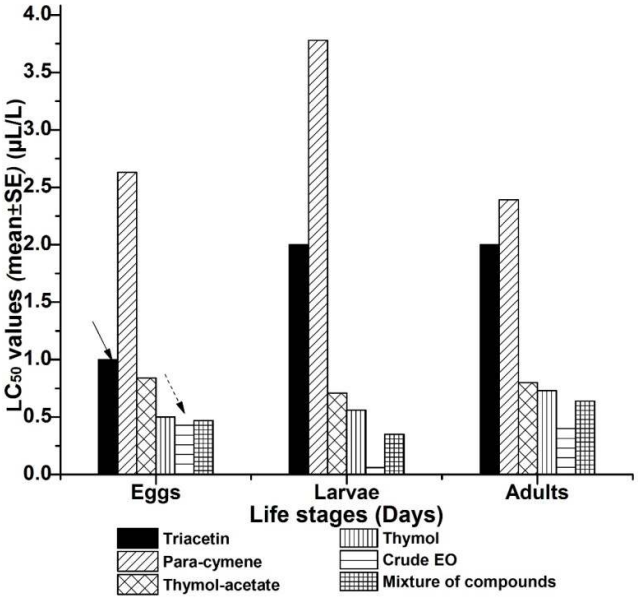

Fig. 4. Variation of mean LC_{50} according to insect life stages after probit analysis regression

Table 3. Toxicity of products on *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) life stages

Life stages	Slope values ± SE	n	R^2	LC_{50} (50% FL) (µl/l air)	SR	χ^2
Eggs	1.22±0.21	5	0.96	1.65 (1.05-36.47)	3.56	2.59^{ns}
Larvae	0.69±0.12	5	0.99	2.04 (1.04-35.70)	5.83	1.28^{ns}
Adults	1.08±0.21	5	0.99	1.76 (0.14-0.67)	2.76	2.48*

*20 individuals per replicates, 5 replications per doses, **LC** is lethal concentration (µl/L), **SR** is synergism ratio, χ^2 is chi-square values, R is linear regression and **ns** is not statiscally significant*

3.4 Evaluation of the Interactions between Combined Constituents

The Synergism Ratio (SR) values calculated and computed according to the formula defined in the methodology are all higher than 1 (Table 3), thereby suggesting a synergistic action between the constituents of EO. The LC_{50} values in this table are all means for each treatment, more detailed values are found in Table 3s of the supplementary materials.

4. DISCUSSION

Our experimental results indicate that *L. adoensis* EO and its constituents had strong toxicity against *C. maculatus* at all life stages. Previous studies have documented a lot on the toxicity of EO against a large number of noxious insects. Over 120 fragrant plants are nowadays labeled to possess insecticidal properties and used in cosmetics, medicines, flavoring food and in stored pest management [47-49]. The interest in Essential oils is rising due to their high content in secondary metabolites possessing insecticidal activities and the less stringent regulatory approval mechanisms for their exploration [50]. Within this framework, our laboratory started series of experiments toward the development of botanical insecticides through the exploration of EO vertues for the protection of pulses against the weevil *C. maculatus* attacks. Our previous work revealed a strong contact toxicity of crude *L. adoensis* EO against the same pest specie, alone and in combination with wood ash [7]. In all treatments, there was a positive relationship between EO concentration and adult mortality (Fig. 1). Our results are in accordance with Moravvej et al. [51], Nyamador et al. [52] and Heydarzade and Moravvej [31] who recorded a similar positive correlation between the death rate and concentrations on many insects with various EOs.

Out of the 43 chemical compounds isolated, 4 are major and 39 are minor (following their

amount after GC-MS test). It accounts 46.14% of oxygenated monoterpenes (mto), 26.98% of hydrocarbons monoterpenes (mth), and 13.69% of Hexanes/Octanes derivatives (Hod). *L. adoensis* accounts 73.12% of total monoterpenes, which is, to our knowledge, the first time ever discovered in a single aromatic plant such as *L. adoensis*. These phenolic compounds played important roles in the toxicity of the oil. In this experiment, when tested individually, none of the isolated major constituents had produced as higher effects as the crude EO not even their complex mixture. This highlights the useful contribution of the low amount compounds in the potentiation of the insecticidal activity of major compounds when mixed together, commonly known as synergism [24,53 and 54]. This could therefore be justified by the synergism ratio (SR) values higher than 1 in all treatments (Table 2). Thymol (22.01%) as the most abundant compound has a vital synergist role in the mixture, and the crude EO as well. The synergism among monoterpenes is found in several EO. For example, Rafaela et al. [55] got the same results when they studied the tertiary mixture (1:1:1) of monoterpenes carvacrol, 1,8-cineole and thymol isolated from *Lippia sidoides* Cham. (Lamiales: Verbenaceae) against *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae). Also, Hummelbrunner and Isman [56] found that (E)-anetol acts synergistically with thymol, citronelal and α-terpineol against the caterpillars *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae). Similarly, Fahineh et al. [57] and Chaudey [58] recorded a very low efficacy of major compounds used individually when they studied the effectiveness of the EO of *Zataria multiflora* Boiss. (Lamiales: Laminaceae) and other EOs on the cowpea weevil, *C. maculatus.* They also recorded a significant reduction in oviposition potential, an inhibition of pupation and adult emergence in larvae of *Tribolium castaneum.* It is therefore obvious that the insecticidal activity of *L. adoensis* EO is the result of interaction between all its chemical constituents, rather than the activity of single major compounds. The constituents of EOs found in low percentages in this experiment may have acted as synergists that would have increased the effectiveness of the major constituents through a variety of mechanisms [24,54 and 56].

Besides, the biopotency is negatively correlated with time. Our findings corroborate that of Ngamo et al. [24], Miresmailli & Isman [59] and Heydarzade and Moravvej [31]. The biological activity of single compounds was lost within 48h except that of thymol. The crude EO is the most persistent (Fig. 2). This situation could be the result of many factors: the high volatility of the compounds which may have escaped through the perforated metal lid that covered the glass jars during the experimental period, the rapid degradation of low single compounds, and the potential oxidation of Sesquiterpene hydrocarbons (Sth). This assumption corroborates that of the abovementioned authors when they studied the persistency of crude EOs towards adult's *C. maculatus.* The high persistency of crude EO could be the result of its content in oxygenated monoterpenes (mto) (46.14%) which attribute more stability in the biological activity of EOs. Ngamo et al. [24] and Heydarzade and Moravvej [31] reported that the persistency of *Lippia. rugosa* Hochst. Ex Wasp. (Lamiales: Verbenaceae) and *Satureja hortensis* (L.) (Lamiales: Laminaceae) EOs were probably the result of its high content in oxygenated monoterpenes. An EO is therefore more unstable when it accounts more hydrogenated constituents. Therefore, the constituents of an EO are crucial in sustaining its biological activity. In this study, the crude EO was demonstrated to be the most persistent even above the experimental period (Fig. 2). The more an EO is contentful in compounds, the more it is persistent over time, and more efficiently pests will be suppressed. This assumption aligned that of Obeng-Ofori et al. [60] who suggested that the persistence of the insecticidal activity of EOs depends on its chemical composition. *L. adoensis* crude EO is by far the richest EO in terms of constituents amongst the most abundant fragrant plants that have been studied so far for their insecticidal properties and demonstrated more than 8 days of persistency, quite a good benefit to combat even resistant insects.

Table 3s of the supplementary materials showed the slope values of different toxicants. According to Robertson & Preisler [61] and Tiwari & Singh [62], a steep slope value indicates that there is a large increase in the mortality of insects with a relatively small increase in the concentration of pesticides. Based on this fact, the steepness of slope values in probit mortality regression of crude EO and mixture in all treatments justified the highest death rate recorded at 5 and 10 uL/g, respectively after a single day exposure.

The evaluation of the behaviours at death aims at giving us an idea and projection on the mode

of action of EO in topical application against insects. Four different types were recorded at death: Turning around, agitation, hyperactivity and direct knockdown (Table 2). Our findings corroborate that of Coats et al. [63] and Isman [64] who reported the same death behaviours regardless the method of administration (oral, topical, or inhalation). The EOs exhibit symptoms which are similar to toxins with a neurotoxic mode of action. Samir et al. [65] found that the toxicity of EOs from *Artemisia judaica (*L.) (Asterales: Asteraceae)*, Callistemon viminals* (Sol. Ex. Gaertn.) G. Don (Myrtales: Myrtaceae) and *Origanum vulgare* (L.)(Lamiales: Laminaceae) may be attributed to their inhibitory potency on AchE and ATPases activities. In the same light, Ryan and Byrne [66] suggested that the toxic effect of EO is due to reversible competitive inhibition of acetylcholinesterase by the occupation of the hydrophobic site of the enzyme's active site centre. In fact, Acetylcholine is thought to be the most abundant excitatory neurotransmitter in the insect's brain found in neural and non-neural tissues. Most of the current generations of insecticides are inhibitors of the enzyme acetylcholinesterase which hydrolyses acetylcholine, terminating its synaptic actions [67]. Acting through nicotinic receptors (nAchRs), it plays a functional role in the sensitivity to neonicotinoid. Lee et al. [68] revealed that the volatiles lipophylic monoterpenes can penetrate through breathing and quickly disrupts the physiology of insects. They can also act as direct neurotoxic affecting AchE activity and/or octpamine receptors [69]. The behavioral response we recorded at death support our projection together with the above cited authors that the inhibition of AChE and ATPase may be one of the main cause of massive adults mortality once *C. maculatus* get into contact with EOs at varied content and concentrations. Other evidence on the mode of action of EO in this study may be attributed to other components of cholinergic synapses which might constitute potential sites of action of insecticidally active molecules. These include the presynaptic synthesis of acetylcholine involving the enzyme choline acetyltransferase, the mechanism of transmitter release and the post-synaptically located acetylcholine receptor molecules [70].

5. CONCLUSION

The results obtained from this study revealed the significant insecticidal properties of EO extracted from young leaves of *L. adoensis* from Cameroon. The insecticidal properties are due to its richness in chemical constituents (43 in total) that sustained its persistency during the treatment. We also showed how even mix together, the major constituents, although acting synergistically, did not produce the same efficiency over all the fitness parameters of insects studied compared to the crude EO. Therefore, one can conclude by saying that the chemical compounds of *L. adoensis* EO had a synergistic interaction with the low amount compounds in the fulfillment of their biological activity. As discussed above, data on behaviours at death suggests that this EO may be neurotoxic by blocking the cholinergic receptors through reversible inhibition of acetylcholinesterase. A close system during the treatment will enhance the effectiveness of this EO by trapping all the constituents and limiting oxidative reactions. As such, this EO could be an appropriate alternative for the replacement of the synthetic insecticides currently used by farmers in stored products protection. In order to align with the policy of research for development, further studies are still needed for the manufacturing and promotion of formulations to enhance efficiency and reduce cost.

6. PERSPECTIVES

This paper article displayed partial findings of series of research undertook in our laboratory on the assessment of potential use of EO extracted from *L. adoensis* from Cameroon. Now that we got a satisfactory result concerning its bioefficacy on adults, eggs and larvae of *C. maculatus*, the third and last part of the work actually in progress aims at evaluating its physiological effects through the assessment of the responses of general esterase and Gluthation S-Transferase (GST) as well as the total contents of protein, lipid and carbohydrate of *L. adoensis* pure EO on surviving larvae.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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