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Research Article

Egg Hatching Reduction and Larval Mortality Induced by Essential Oil and Extracts of *Petroselinum crispum* (Parsley) Leaves in the *Anopheles coluzzii* Malaria Vector Species

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Abstract

The interest of plant-based products is increasing as alternative solutions to current synthetic insecticides associated with detrimental effects on the environment. Here we assessed the potential deterrent effect of parsley (*Petroselinum crispum*) formulations on immature stages of the African malaria vector, *Anopheles gambiae* s.l. In vitro bioassays were performed to evaluate egg-hatching reduction and larval mortality induced 24 hours post exposure at various concentrations by crude powder, methanol extract and essential oil of parsley leaves. Plant powder and methanol extract were rich in alkaloids, saponins and phenolic compounds, while myristicine (67.1%) was the main compound in essential oil. Parsley induced 19-75% egg hatching reduction, 43-88% overall larval reduction and 26-77% mortality on 3rd and 4th instars, with significant variations by formulations and concentrations. Essential oil (LC50=0.011-0.014 mg/mL, LC95=0.12-0.26 mg/mL) showed low effective concentrations against *An. coluzzii* larvae compared with the methanol extract (LC50=0.17-0.20 mg/mL, LC95=5.44-6.54 mg/mL). These findings provide evidences that *P. crispum* formulations, especially essential oil might be identified among new potential plant-based products to evaluate towards alternative tools for malaria vector control.

Keywords: Egg hatching; Larval mortality; *Petroselinum crispum*; *Anopheles coluzzii*; Malaria vector

Abbreviations

GC/MS: Gas Chromatography coupled with Mass Spectrometry (GC/MS); IRY: Institut de Recherche de Yaoundé; LC: Lethal Concentration; OCEAC : Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale

1. Background

Mosquito control techniques in use to combat adults or immatures stages depend principally on the application of synthetic insecticides such as pyrethroids, organochlorines, organophosphates, and carbamates [1]. However, these synthetic insecticides products widely in use are harmful to humans and other non-target living organisms and they pollute the environment, and their improperly application contributes to mosquito resistance problems [2]. Therefore, during these three last decades, research efforts were mostly focused on the development of eco-friendly alternative insecticides. Some constituents of plant extract and essential oil like tannins, flavonoids, alkaloids, glycosides, saponins, terpenoids, steroids, hydrogenate and dehydrogenate monoterpenes as well as sesquiterpenes were reported to possess toxic effect against developmental stages of mosquito species [3]. Intirach et al. [4] reported the insecticidal effect of *P. crispum* essential oil against *Aedes aegypti* mosquitoes. Essential oil of *P. crispum* also significantly decreased weight, volume and energy reserves of *Culex pipiens* and *Culiseta longiareolata* larvae and pupae [5]. Botanicals are largely documented as potential effective insecticides

which are target specific, ecofriendly safe, and their phyto-constituents may overcome the resistance problem developed by some insect pests [6]. Numerous mosquito species including the widespread malaria vector species, *Anopheles gambiae* s.l., have gradually developed resistance to common insecticide families i.e. organochlorides, organophosphates, carbamates and pyrethroids.

Therefore, WHO has recommended for actions to mitigate adverse effects of such multi-resistance towards the control of mosquito-borne diseases like malaria, dengue fever, lymphatic filariasis and some arboviruses which are transmitted through the bites of infected females of *Anopheles* species [7-10]. Among these diseases, malaria remains the most deadful parasitic infection, and the *Plasmodium falciparum* species remains the first cause of malaria cases and deaths. The parasite is transmitted essentially by sibling species of the *An. gambiae* complex, *An. gambiae* and *An. coluzzi* [11]. In 2019, Cameroon registered up to 6.2 million cases and 11,233 deaths [12]. The disease epidemiology remains stable due to, among others factors, the nationwide spread of mosquito resistance to pyrethroid-based formulations used for vector control.

The country has developed recently a national plan for resistance management, with special interests on alternative strategies or approach including environmental management and biocides. Several aromatic plants used locally for domestic or pharmaceutical purposes have been tested so far to check for their potential activity against both larval and adult mosquitoes [13]. Among them, *Petroselinum crispum* (Apiaceae) which is native to the Mediterranean region (Greece, Spain, Italy, Malta, Tunisia, Algeria and Morocco) until its dissemination worldwide [14], is one of the most cultivated vegetables in Cameroon. Here, we we assessed the potential deterrent effect of parsley (*Petroselinum crispum*) formulations on immature stages of the African malaria vector mosquitoes, *Anopheles gambiae* s.l., with as expected ambition in providing further evidences that parsley-based formulation might be among potential active biocides to control mosquitoes.

2. Materials and Methods

2.1 Plant collection

P. crispum leaves were collected early in the morning (around 7:00 am) from Santa (North West Region of Cameroon). The plant species was identified in the department of Botany (Higher Teacher Training College) and confirmed at National Herbarium of Cameroon at Yaounde under the registration number 403884/SFRcam. The plant leaves were dried at shade for 15 days and pulverized in the electric blender. The grinded leaves were passed through 0.5 mm mesh size sieve and the powder obtained was packaged in the sealed plastic container until its use for extraction, chemical screening and biological assays.

2.2 Plant methanolic extraction

A weight of 129 g of *P. crispum* powder was macerated in 3000 mL of methanol for 72 h and then filtered using Whatman No.1 filter paper. The filtrate was submitted to rotary evaporator to remove the solvent and then dried in the oven set at 60°C. The dry methanolic extract obtained was weighed and the extraction yield was calculated using the following formula:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of extract obtained (g)}}{\text{Weight of plant powder used (g)}} \times 100$$

2.3 Plant essential oil extraction

Essential oil was isolated from the leaves by hydro distillation process using Clevenger apparatus-type in the laboratory of Microbiology, Faculty of Science, University of Yaounde 1, Cameroon. Traces of water in the essential oil recovered were discarded using anhydrous sodium sulphate and kept in dark glass bottle in the refrigerator till its use for phytochemical analysis and larvicidal assay. Essential oil extraction yield was determined following the formula below:

$$\text{Essential oil yield (\%)} = \frac{\text{Weight of essential oil recovered (g)}}{\text{Weight of plant fresh leaves used (g)}} \times 100$$

2.4 Chemical and GC/MS analysis of plant extracts

Methanolic extract and powder of *P. crispum* were submitted each to phytochemical screening to identified the presence of potential active plant constituents including alkaloids, saponins, tannins, flavonoids, terpenoids and phenolic. The main constituents of essential oils were determined by Gas Chromatography coupled with Mass Spectrometry (GC/MS), as described by Adams [15]. GC/MS analyses were performed using a Hewlett Packard 5890 II gas chromatograph, interfaced with a quadrupole detector (Model 5972) and equipped with a HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25µm). Helium was the carrier gas, at a flow rate of 0.6 mL/min. Injector and MS transfer line temperatures were 220 °C and 250 °C, respectively. The oven program temperature was the same as that used in the GC-FID analyses. Diluted samples (10:100 in CH2Cl2, v/v) of 1 µL were injected manually and in a split mode (1:100). The MS was operated in the EI mode at 70 eV, in the m/z range 35-300; electron multiplier 1460 eV; scan rate, 2.96 scan/s. The identification of the constituents was assigned on the basis of a comparison of their relative retention indices, calculated with reference to a series of n-alkanes (C9–C22). Their mass spectra were compared with the standards (for main components) and values found in the literature including the NBS75K database and Wiley 7th NIST 2014 EPA/NIH Mass Spectral Library Upgrade, provided by the GC/MS control and data processing software guidelines. The percentage composition of the essential oils was computed by the normalization method from the GC/FID peak areas, assuming an identical mass response factor for all compounds.

2.5 Anopheles mosquito strain

Anopheles coluzzii mosquito progenies from the IRY-OCEAC insectary were used for bioassays. This susceptible *An. coluzzii* Ngousso strain were adapted to artificial rearing conditions of ambient temperature (28–30°C) and relative humidity (70–80%) since 2006 [16].

2.6 Egg hatching and growth reduction assays with parsley powder

Freshly laid eggs of *A. coluzzii* collected from ovipositors and matured at 26-28°C and 70-80% HR under photoperiod 12L: 12D, were then transferred to individual petri dishes at various concentrations of parsley powder (0.1, 0.3 and 0.5 g/mL) to check for hatching rate. Each replicate of 25 eggs (four replicates per concentration) was monitored until egg hatching and the counting of active first instar larvae (L1). After 48 h post-treatment, each batch of the

tested mosquito eggs were recovered retaining them on a muslin cloth, then cleaned with water, and observed under microscope at 10 X magnification for hatching assessment after counting non-hatched eggs. The percentage of non-hatched eggs was calculated based on the number of eggs with unopened opercula at the end of the test. For larval growth reduction assays, larvae (L1) were then pooled per concentration into individual containers and fed with Tetramin fish food (0.625 mg/25 larvae/day). The larval growth reduction was calculated at the end of complete larval development to fourth instars (L4), adjusted with the number of emerged pupae. Control arms (without parsley powder) were used for both egg hatching and larval development follows up.

The following formula described for aquatic toxicity testing [17] was adapted for the calculation of the percentage of egg hatching and larval growth reduction rate for each concentration: $Rr\ (\%) = [\ (\frac{\mu C - \mu T}{\mu C}) \times 100$, where *Rr* (%) was the reduction rate in percentage; µC, the average mean hatched eggs/ emerged pupae in the control group, and µT, the average mean hatched eggs/emerged pupae for a given concentration.

2.7 Egg hatching and bioassays with methanol extract and essential oil

Four replicates of 25 mature eggs of *A. coluzzii* each were exposed in plastic cups containing various concentrations of methanolic extract (0.1, 0.3 and 0.5 mg/mL) and essential oil (0.01, 0.03 and 0.05 mg/mL), to record the hatching rate by checking unhatched eggs under microscope at 10X magnification. In parallel, four batches of 25 *An. coluzzii* larvae (3rd and 4th instars) was separately transferred to plastic containers and reared at such methanolic extract and essential oil concentrations, respectively. The number of dead larvae induced by parsley formulations was recorded 24h post-treatment by concentration ranges, and Abbott's formula [18] was used for correction when larval mortality in the control arm ranged from 5 to 20%. A set of 100 larvae (50 third instars and 50 forth instars) were distributed into four plastic cups containing 99 mL of spring water and 1 ml of ethanol each were monitored as control batches.

2.8 Statistical analyses

The Pearson's Chi-square and Kruskall-Wallis non-parametric tests were used to compare egg hatching and mortality rates by concentrations and formulations. Probit analysis [19] was employed to calculate LC50 and LC95 values of the plant methanol extract and essential oil causing 50% and 95% mortality of mosquito stages. Differences were considered significant at a rate of probability (P) less than 0.05 ($P<0.05$).

3. Results

3.1 Phytochemical composition of powder and methanol extract

Results of the phytochemical screening shown in table 1 revealed the presence of alkaloids, saponins and phenolic compounds in plant powder and in methanol extract. However, methanolic extract showed additional group of compounds, terpenoids, whereas both extracts were negative for other phytochemical groups of components such as tannins and flavonoids.

Phytochemical groups evaluated	**Plant powder**	**Methanolic extract**
Flavonoids	-	-
Alkaloids	+	+
Tannins	-	-
Terpenoids	-	+
Saponins	+	+
Phenolic compounds	+	+

Abbreviations: + = present; - = absent

Table 1: Phytochemical compounds isolated from powder and methanol extracts of *Petroselinum crispum* leaves.

No.	**RT (min)**	**Compounds**	**Phytochemical category**	**Proportion %**
1	14.97	Myristicine	Phenylpropanoids (phenolic compounds)	67.1
2	16.77	Apiol		3.6
3	20.37	3,4α,7,7,10α-Pentamethyl-3-vinyldodecahydro-1H-benzo[f]chromene		1.0
4	10.49	Estragole		0.8
5	15.96	Bisabolene < (E)- iso-ϒ>	Sesquiterpenes (terpenoids)	8.6
6	15.74	β -Sesquiphelandrene		5.4
7	16.48	Sesquisabene hydrate		2.6
8	10.36	4-(1-Methylethyl)-2-cyclohexen-1-one	Monoterpenes (terpenoids)	2.9
9	10.27	4-Terpineol		2.1
10	8.89	Linalool		1.1
11	7.81	1-Isopropyl-4-methylenebicyclo[3.1.0]hexane		0.9
12	10.07	3-Thujen-2-one		0.8
13	12.65	α-Terpineol acetate		0.7
14	7.68	p-Isopropyltoluene		0.5
15	8.79	α,p-Dimethylstyrene		0.4
16	7.01	β-Myrcene		0.3
17	7.75	Limonene		0.3
18	10.32	m-Methylacetophenone	other aromatic compound	0.9
		Total		**100.0**

Abbreviations: No: number; RT: retention time; %: percentage

Table 2: Name and retention time (RT) of chemical constituents isolated from essential oil of Petroselinum crispum leaves.

3.2 Chemical composition of essential oil

The MC/GC profile of *P. crispum* essential oil was composed by 18 different active compounds. These belong to at least three main phytochemical groups including four phenylpronanoids (72.5%), thirteen terpenoids (26.6%, i.e. three sesquiterpenes: 16.5%, 10 monoterpenes: 10.1%), one benzenoid (1.9%) and another not classified aromatic compound (Table 2). Among these identified constituents, myristicine (phenylpronoid) was the main compound (67.1%), following by sesquiterpene compounds such as basibolen (8.6%) and β-sesquiphelandrene (5.3%).

3.3 Egg hatching inhibition and larval reduction induced by crude powder

The number of eggs hatched into larvae is concentration dependent. The trend of hatching rate was inversely proportional to increase concentration ranges (P=0.003). The crude powder induced 19.45%, 45.83% and 58.33% egg inhibition at 0.1 g/mL, 0.3 g/mL and 0.5 g/mL, respectively (Table 3). The similar trend was also recorded for larval development inhibition, with rates of 43.82%, 77.53% and 87.64% at 0.1 g/mL, 0.3 g/mL and 0.5 g/mL of crude powder, respectively. Bioassay results showed a significant reduction on larval development from 1st instar to pupae induced by parsley powder regardless the concentration range (P<0.01).

This toxic effect against *An. coluzzi* mosquitoes was concentration-dependent, with high reduction rates recorded at highest concentrations. However, larval reduction rates did not vary significantly between the concentrations tested (X^2=3.623, df=2, P=0.163).

Variables	**0 g/mL (control)**	**0.1 g/mL**	**0.3 g/mL**	**0.5 g/mL**
Number of eggs tested	100	100	100	100
Hatched eggs (% ± 95% CI)	96.00 ± 3.84	77.00 ± 8.25	52.00 ± 9.79	40.00 ± 9.60
Egg hatching reduction (% ± 95% CI)	-	19.79 ± 7.81	45.83 ± 9.77	58.33 ± 9.66
Number of 1st instar larvae that have reached the pupae	89	50	20	11
Larval growth reduction (% ± 95%CI)	-	43.82 ± 11.08	77.53 ± 11.34	87.64 ± 10.20

Abbreviations: g/L: gramme per milliLiter; CI: Confidence Interval; %: percentage; T°C temperature; RH: relative Humidity

Table 3: Hatching inhibition and mortality induced by *P. crispum* powder based formulations on the immature stages (eggs and all instar larvae) of *A. coluzzii* in the labotatory conditions (T°C: 25 ± 2°C; 75 ± 4% RH).

3.4 Egg hatching inhibition and larval mortality induced by methanol extract and essential oil

These parameters were monitored against *An. coluzzii* mosquitoes (Table 4). The methanol extract and essential oil induced the increasing egg hatching inhibition rates, varying accordingly by concentrations from 25% to 75% with methanol extract (X^2=48.240, df=2, $P<10^{-4}$) and from 17% to 46% for essential oil (X^2=16.310, df=2, $P<10^{-3}$). The inconsistency observed for egg hatching inhibition between the both formulations was not statistically significant (X^2=0.453, df=2, P=0.797).

Concerning the larval mortality, the overall mortality rate reached 40% at 0.1 mg/mL, 53% at 0.3 mg/mL and 72% at 0.5 mg/L of methanol extract, with significant variations between the three concentrations (X^2=20.929, df=2, $P<10^{-4}$). The trend of larval mortality recorded with essential oil was similar with that of methanol extract, varying according to increased concentration ranges from 47% to 77% (X^2=19.887, df=2, $P<10^{-4}$). Mortality rates did not differ between 3rd and 4th instars regardless the type of formulation (X^2-values < 2.000, df=2, P>0.450). Globally, LC_{50} and CL_{95} (mg/mL) values of *P. crispum* methanolic extract and essential oil varied with the larval stages. The LC_{50} and LC_{95} ranges were estimated for 3rd instar (LC_{50}=0.17 mg/mL; LC_{95}=5.44 mg/mL) and 4th instar (LC_{50}=0.20 mg/mL; LC_{95}=6.54 mg/mL) for methanolic extract. Lethal concentrations were also calculated with essential oil against 3rd (LC_{50}=0.011 mg/mL; LC_{95}=0.257 mg/mL) and 4th (LC_{50}=0.014 mg/mL; LC_{95}=0.123 mg/mL) instar larvae of *An. coluzzii*.

Plant formulations	**Concentration (mg/mL)**	**Egg hatching Rr** (%± 95% CI)	**Mortality ranges**		
			Overall (%± 95% CI)	3rd instar	4th instar
Methanolic extract	0.1	25.00	40.00 ± 9.60	41.00	38.00
	0.3	41.67	53.00 ± 9.78	54.00	52.00
	0.5	75.00	72.00 ± 8.80	73.00	70.00
Essential oil	0.01	16.67	37.00 ± 8.52	47.00	26.00
	0.03	29.17	57.00 ± 9.70	72.00	41.00
	0.05	45.83	68.00 ± 9.14	77.00	58.00

Abbreviations: mg/mL: milligramme per milliliter; CI: Confidence Interval; %: percentage; T°C temperature; RH: Relative Humidity, Rr: Reduction rate

Table 4: *Anopheles coluzzii* egg hatching reduction and larval mortality rates induced by Petroselinum crispum methanolic extract and essential oil in the laboratory conditions (Temperature=25 ± 2°C; 75 ± 4% Relative Humility).

4. Discussion

Petroselinum crispum (parsley) is a perennial plant in the family of Apiaceae, in use in Cameroon and elsewhere by local communities as spice herb for culinary purpose and/or as medicinal plant. Apart from such consumption, parsley as other aromatic plants, is increasingly under investigation to assess their potential activity in controlling pests and disease borne vectors such as mosquitoes [20-22]. Mosquitoes belonging to the *Anopheles gambiae* complex of species transmit the most infectious parasite species, *Plasmodium falciparum*, involved in the majority of deadly malaria infections in Cameroon [11]. Because of adaptative responses developed by natural malaria vector populations to escape current vector control strategies (i.e. behavioural changes, resistance to insecticides, etc.), the development of new biocides including eco-friendly plant-based insecticides might be potential alternatives for an improved management of vectors and associated environments [23, 24].

It is therefore in this context we proposed this paper to describe potential effects of parsley-based formulations against immature stages of *Anopheles coluzzii*, one of the major malaria vector species of the *Anopheles gambiae* complex. The first information gathered from this study is that local parsley leave extracts are composed predominantly by phenolic compounds, especially myristicin. This phenolic constituent was in high concentration in essential oils obtained from leaves as well as from other parts of parsley including roots, fruits and herbs [25, 26]. Other chemical compounds usually found in various aromatic plants were terpenoids, saponins and alkaloids [27]. The chemical composition of parsley showed variations by seasons, locations and extracts [4, 6, 26-28]. Recent works reported from 17 to 25 different compounds in parsley extracts, among which pulegone, D-Limonene, thymol, *p*-cymene and γ-terpinene were the main active compounds. The most frequent secondary metabolites of plants found toxic for insects belong to terpenoids, steroids, phenols, flavonoids, tannins, alkaloids and cyanogenic glycosides compounds [29-31]. The present study suggests that parsley plants collected locally were rich of phenylpropanoid compounds, especially myristicin which was the main secondary metabolite isolated from essential oil. This compound (myristicin), known for its potential hallucinogenic effects on human [32] had been so far identified as potential natural insecticide and synergist [33].

The growth reduction (16-77%) and mortality rates (36-68%) induced by parsley-based formulations (powder, methanol extract and essential oil) on the *Anopheles coluzzi* immature stages are evidences for its toxicity, probably increased by the presence of other active compounds i.e. terpenoids and alkaloids. These findings are consistent with that reported previously by Foko Dadji et al. on *Capsicum annuum* powder [21]. Other studies have also indicated that extracts of *Atlantia monophylla*, *Pseudocalymma alliaceum*, *Cardiospermum halicacabum*, *Hyptis suaveolens* and others caused a high disturbance in the growth regulation and larval survival among various mosquito species including *Anopheles*, *Aedes* and *Culex* mosquitoes [14, 34-39]. Some authors attributed this development disturbance to the presence of growth regulator enzymes in plant extracts that causing morphological and physiological disorders which interfere with total development of insect [24, 40]. The level of parsley toxicity against

Anopheles developmental stages varied by concentrations and by extracts. The essential oil displayed 68% mortality rates after 24 h post-exposure at the lowest concentrations (0.05 mg/mL), whereas mortality induced by 0.1% methanol extract was 40%. As observed previously with synthetic myristicin [33] and essential oil extracts [41, 42], the above observation confirms that parsley essential oil might be a potential phyto-chemical formulation for mosquito control. However, the not negligible deterrent effect induced by crude powder could be also addressed at the level of community, because of its limited access to essential oil based products.

5. Conclusion

This paper aimed at assessing potential growth inhibition induced by parsley-based formulations against immature stages of *Anopheles coluzzii*, one of the major African malaria vector species. Here, we provided supplementary data presenting the ovocidal and larvicidal activity of parsley formulations on the developmental mosquito stages. This toxicity was concentration-dependent and showed variations by extracts. Globally, the low concentrations of essential oil revealed significantly effective for egg hatching inhibition and larvicidal effect against tested mosquito stages. Thus, this formulation of parsley might be identified among new potential plant-based products to evaluate towards alternative tools for malaria vector control.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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