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Effect of aqueous extract of lemon (*Citrus limon)* on *Anopheles gambiae sensu lato* (Diptera: Culicidae) larvae tolerance in malaria vector control in Dogbo district in south-western Republic of Benin, West Africa

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Abstract

The use of chemical insecticides causes important damages to environment and human health and there is a need to search for alternative solutions. This study aims to investigate on the effect of aqueous extract of lemon in *Anopheles gambiae s.l.* larvae tolerance in couffo department in south-western Republic of Benin, West Africa. Larvae of *Anopheles gambiae s.l* mosquitoes were collected from breeding sites using the dipping method in July 2020 during the rainy season in Dogbo district. A batch of 15 larvae of fourth instar were exposed to a mixture of aqueous extract of lemon with distilled water saturated with oxygen containing in each of five glass jars or test cups of same dimensions contained each 48 ml distilled water saturated with oxygen plus 2 ml of aqueous extract of lemon (*Citrus limon)* and one control jar containing no trace of aqueous extract of lemon. Larval mortality was recorded after 24hours, 48hours and 72hours exposure. The results show that the use of aqueous extract of lemon causes full-grown Anopheles larvae to die by suffocation. After the application of this mixture, the larvae of four instars cannot breathe. The use of aqueous extract of lemon is effective method for disturbing the siphonal respiration of mosquito larvae. Aqueous extract of lemon is effective method for mosquito larvae control.

Keywords: *Citrus limon* (L) *Osbeck*; Siphonal respiration; Larvae of *Anopheles gambiae s.l.*; Malaria control; Republic of Benin

1. Introduction

The increase in the use of World Health Organization approved vector control methods between 2000 and 2015 contributed to a large decline in the number of malaria cases globally. But progress against malaria has stalled. There was no significant reduction in global malaria between 2015 and 2017. An estimated 219 million malaria cases were reported in 2017 (versus 213 million in 2015). There were more than 435 000 deaths in 2017 [1].

Today, there is renewed interest in Larval Source Management (LSM) [2-11]. and its practical application in Africa as a complementary intervention to Long-Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS), especially

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where outdoor biting by malaria vectors is problematic or where there is resistance to the insecticides used for LLINs or IRS [5]. Field trials in different eco-epidemiological settings in Africa and Asia (where larval habitats were few, fixed and findable) have shown that larviciding can reduce the density of adult vectors and consequently malaria transmission and morbidity [4-5]. However, in other field trials it has been shown that LSM does not work in every ecosystem, for instance it performed poorly in areas with extensive flooding, where larvicides were applied by ground teams [6].

The control measures for mosquitoes involve chemical control [12-14], biological control, environmental management, genetic control, and physical control [15]. Among the control measures, several methods have been controversial because of ecosystem disturbance and the tolerance development of mosquitoes against the given control methods [15-17]. However, mosquitoes have not acquired tolerance against physical control methods [15]. Oil, surface film, and polystyrene beads have been introduced to disturb the respiration of mosquito larvae and pupae submerged in water [15-16, 18-21].

Very few researches were published on the use of essential oils in *Anopheles gambiae s.l.* larvae tolerance in Benin. Therefore, there is a need to carry out new researches for this purpose.

The goal of this study was to measure the effect of aqueous extract of lemon on *Anopheles gambiae s.l.* larvae tolerance in Couffo department in south-western Benin.

2. Material and methods

2.1. Study area

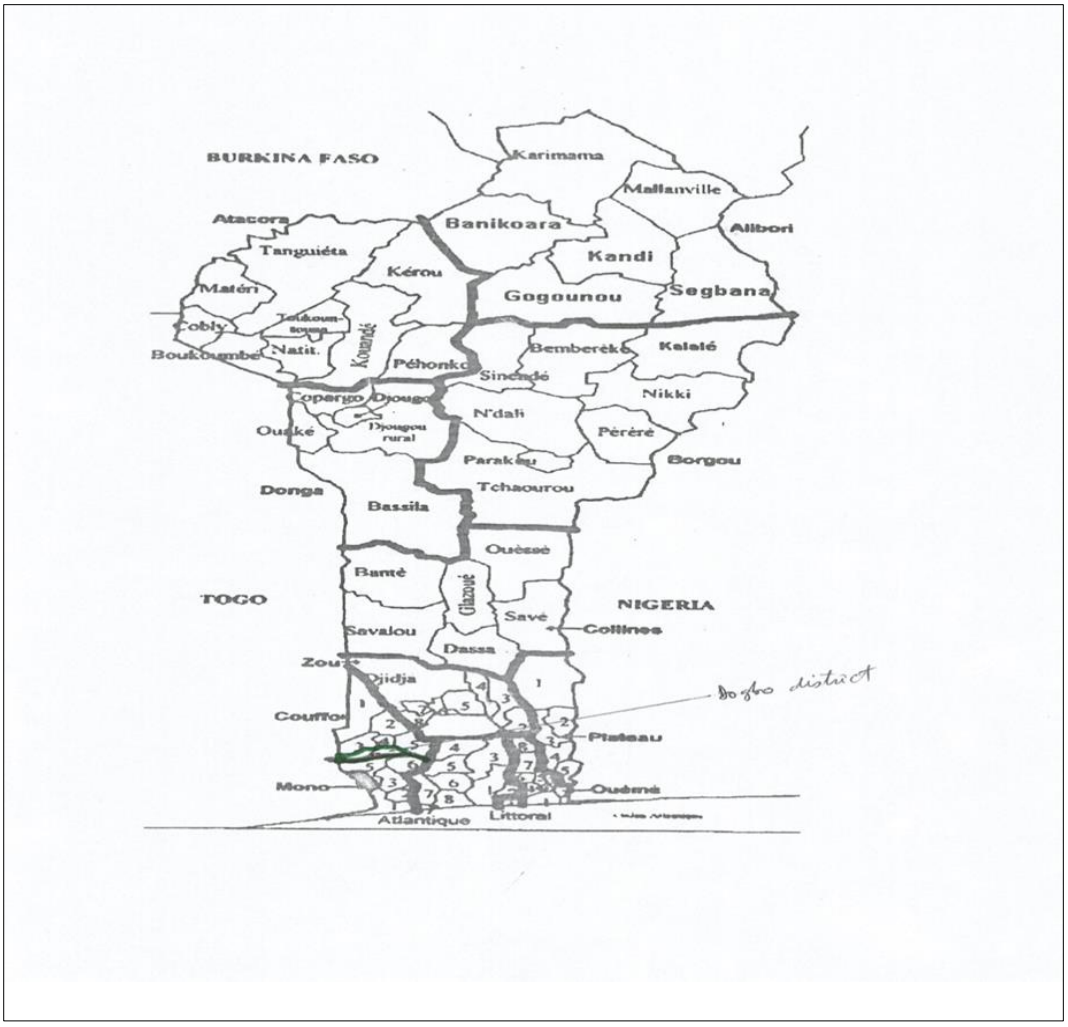

Figure 1 Map of Republic of Benin showing Dogbo District

The study area is located in Republic of Benin (West Africa) and includes the department of Couffo. Couffo department is located in the south-western Benin and the study was carried out more precisely in Dogbo district (Fig.1). The southern borders of this district are Lokossa and Bopa districts. The northern border is Djakotomey district. The eastern border is Lalo district and the western border of Dogbo district is Togo republic. Dogbo district covered 475 km^2 and belongs to geographic region of ADJA.The choice of the study site took into account the economic activities of populations, their usual protection practices against mosquito bites and peasant practices to control farming pests. We took these factors into account to study the effect of aqueous extract of lemon (*Citrus limon*) on *Anopheles gambiae s.l.* larvae tolerance in Dogbo district in Couffo department. Couffo has a climate with four seasons, two rainy seasons (March to July and August to November) and two dry seasons (November to March and July to August). The temperature ranges from 25 to 30°C with the annual mean rainfall between 900 and 1100 mm.

2.2. Mosquito sampling

Anopheles gambiae s.l. mosquitoes were collected in July2020 during the rainy season in Dogbo district. Larvae were collected from breeding sites using the dipping method and kept in labeled bottles (Fig.2). The samples were then carried out to the Laboratory of Applied Entomology and Vector Control (LAEVC) of the Department of Sciences and Agricultural Techniques located in Dogbo district.

Figure 2 An *Anopheles gambiae s.l.* larvae breeding site surveyed in Dogbo district

2.3. Purchase of lemons

The lemons (*Citrus limon*) (Fig.3) used in the current study were bought in Dogbo market. This market is not far (about 500 meters) from the Laboratory of Applied Entomology and Vector Control (LAEVC) of the Department of Sciences and Agricultural Techniques of Normal High School of Technical Teaching (ENSET) of Lokossa. These limons (*Citrus limon*) are held in a bag and carried out to Laboratory.

Figure 3 Lemons (*Citrus limon*)

2.4. Bioassays

A batch of 15 larvae of fourth instar reared in the insectary of the Laboratory of Applied Entomology and Vector Control (LAEVC) was added in each of five glass jars or test cups of same dimensions contained each 48 ml distilled water saturated with oxygen plus 2 ml of aqueous extract of lemon (*Citrus limon*) and one control jar containing no trace of aqueous extract of lemon (*Citrus limon*). Otherwise, the control jar or control cup containing only 50 ml distilled water saturated with oxygen and 15 larvae of fourth instar.

Four replicates were set up and an equal number of controls were set up simultaneously with distilled water. Each test was run three times on different days. The test containers were held at 25-28°C.

Larval mortality was recorded after 24 hours, 48 hours and 72hours exposure. Moribund larvae were counted and added to dead larvae for calculating percentage mortality. Dead larvae were those that could not be induced to move when they were probed with a needle in the siphon or the cervical region. Moribund larvae were those incapable of rising to the surface or not showing the characteristic diving reaction when the water was disturbed.

2.5. Statistical analysis

Analysis using t-test was performed with 95% confidence interval in SPSS version 16.0 (SPSS Inc., Chicago, IL). The p-value acquired by t-test for all cases of this study is less than 5%. Abbott's formula was not used in this study for the correction of mortality rates in test jars because the mortality rates in all controls was always less than 5% [22].

3. Results

The recording of the number of dead larvae was done after 24hours, 48 hours and 72hours exposure. The analysis of Table 1 shows that no dead larvae was registered in control jar or control cup during the different bioassays. After 24hours exposure, there was no alive larvae in test cups, but six (6), four (04) and two (2) moribund larvae respectively were registered during the bioassay 1, 2 and 3.

Table 1 Recording the number of dead larvae after 24 hours exposure

Control				**Bioassay 1**				**Bioassay 2**				**Bioassay 3**			
Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead
15	15	0	0	15	0	6	9	15	0	4	11	15	0	2	13

In the same way, the analysis of Table 2 shows that no dead larvae was registered in control jar or control cup during the different bioassays. After 48hours exposure, there still was no alive larvae in test cups, but two (2), one (1) and Zero (0) moribund larvae respectively were registered during the bioassay 1, 2 and 3. These results show that some of moribund larvae were died after 24 hours exposure to the mixture of aqueous extract of lemon with distilled water saturated with oxygen.

Table 2 Recording the number of dead larvae after 48 hours exposure

Control				**Bioassay 1**				**Bioassay 2**				**Bioassay 3**			
Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead
15	15	0	0	15	0	2	13	15	0	1	14	15	0	0	15

The same remark, was made when we analyze the Table 3. In fact, after 72 hours exposure, there was no alive and no moribund larvae in the test cups of the different bioassays. They were all died due to the effect of the mixture of aqueous extract of lemon (*Citrus limon*) with distilled water saturated with oxygen.

The analysis of Table 4 shows that there are many advantages in the use of aqueous extract of lemon to control mosquito larvae. But, also there are very few disadvantages.

Table 3 Recording the number of dead larvae after 72 hours exposure

Control				**Bioassay 1**				**Bioassay 2**				**Bioassay 3**			
Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead	Number tested	Alive	Moribund	Dead
15	15	0	0	15	0	0	15	15	0	0	15	15	0	0	15

Table 4 Advantages and disadvantages of the use of aqueous extract of lemon (*Citrus limon*)

Advantages	**Disadvantages**
Citrus limon (L.) Osbeck (Family of Rutaceae) is cultivated in many regions in Benin country	Limited effectiveness of aqueous extract of lemon (*Citrus limon*) in the presence of vegetation and floating debris (is the maindisadvantage)
Aqueous extract of lemon (*Citrus limon*) is a cheap and easy method of larval control for some breeding sites such as borrow-pits, pools and so on	
Mosquitoes may not develop resistance to aqueous extract of lemon (*Citrus limon*)	
Aqueous extract of lemon (*Citrus limon*) is not toxic to most non-target organisms including mammals and fish.	

4. Discussion

The results obtained in the current study shows that the aqueous extract of lemon (*Citrus limon*) causes full-grown Anopheles larvae to die: by suffocation, due to a mechanical barrier being formed between them and the air and also by suffocation, due to the essential oil entering their breathing siphons to an extent sufficient to physically block the passage of air. But also, by poisoning, due to the toxic properties of the volatile portions of this oil penetrating the tracheal tissues.

Our obtained results also show that mosquito larvae fail to do siphonal respiration with the application of aqueous extract of lemon (*Citrus limon*). Consequently, mosquito larvae mainly depend on the dissolved oxygen in water. Certain species of mosquito larvae breathe underwater by piercing their air tube called a siphon [21].

Given that mosquitoes in the immature stages (eggs, larvae, and pupae) are restricted to small-scale aquatic habitats, avoiding the control measures is difficult for them [16, 23-24].

The application of plant oils to water is one of the forms of larval control. Due to their relatively high cost in comparison with some other larvicides and because they have limited persistence, their use has declined in mosquito control. Monomolecular films (MMFs) were developed during the 1980s and while several isostearyl alcohol products are available, these have not been used extensively in mosquito control programmes in Africa. The application of aqueous extract of lemon (*Citrus limon*) to water containing *Anopheles gambaie s.l.* larvae has many advantages. *Citrus limon (L.) Osbeck* is cultivated in many regions in the Benin country and therefore lemons (*Citrus limon*) are available on the markets. Aqueous extract of lemon (*Citrus limon*) is miscible with water after mixture and therefore cannot soil the earth after its action or effect where it has been applied. The use of aqueous extract of lemon (*Citrus limon*) is a cheap and easy method of larval control for several breeding sites such as brick pits, pools, marshes, streams, ditches, pits dug for plastering traditional huts, puddles of water, water pockets caused by the gutters. In addition, mosquitoes may not develop resistance to aqueous extract of lemon (*Citrus limon*). It is not toxic to most non-target organisms including mammals and fish. But, the application of aqueous extract of lemon (*Citrus limon*) also presents a few disadvantages and the main is that its effectiveness is limited in the presence of vegetation and floating debris.

Some agents include petroleum distillates and monomolecular surface films (MMF) such as isostearyl alcohol made from renewable plant oils act by suffocating larvae or disrupting surface tension, inhibiting the ability of larvae to rest and breathe at the surface of the water causing them to drown and interfering with adult emergence. They are

considered effective in control of *Anopheles* larvae, but may be impacted by wind or absorbed by vegetation. These agents will affect any aquatic invertebrate requiring use of the air-water interface for breathing, resting or egg-laying. Re-treatment is needed weekly [25].

5. Conclusion

In this work, we have showed that the use of aqueous extract of lemon (*Citrus limon*) disallows mosquito larvae to acquire tolerance. It directly disturbs their siphonal respiration. The use of aqueous extract of lemon (*Citrus limon*) is effective method for mosquito larvae control. After the exposure to the mixture of aqueous extract of lemon (*Citrus limon*) with distilled water saturated with oxygen, the larvae of fourth instar cannot breathe. However, this study was conducted in laboratory conditions and there is also a need to carry it out in field conditions for better conclusions.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Statement of ethical approval

The study follows proper ethical procedures.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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