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Histopathology of *Culex pipiens* (Linée, 1753) (Diptera, Culicidae) larvae exposed to the aqueous extract of *Eucalyptus globulus* l'Hér, 1789 (Myrtaceae)

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Abstract
Eucalyptus globulus was tested for its larvicidal activity against the mosquito larvae *Culex pipiens.* The application of the aqueous extract at (0.5 g / l, 2 g / l, 5 g / l and 8 g / l) concentrations caused the remarkable mortality of 83% for the larvae that were treated at 8 g / l dosage and observed after 72 hours of contact. After calculating the LD_{50} (27.6 g / l after 24hours, 13.45 g / l after 48 hours and 3.57 g / l after 72 hours of exposure).
Larvae of *Culex pipiens* treated with the aqueous extract of *Eucalyptus globulus* suffer important damages of midgut that occur over time.
After 24 hours, the cells burst and the cytoplasmic contents move to the intestinal lumen, leading to death.

Keywords: *Culex pipiens*, *Eucalyptus globulus*, LD_{50}, Larvicidal effects, histology, digestive tract

1. Introduction
The Culicidae, commonly known as mosquitoes, have more than 3200 species today as well as forty genera spread out almost everywhere in the world [1]. Mosquitoes of the genus *Culex* are the most common, with 800 species. They develop in every continent except Antarctica[2] The Culicidae insects are probably the best known and most feared because of the parasites they can inoculate during their blood meal, as well as the inconvenience and nuisance caused by their presence. [3]
In Constantine, northeast Algeria, the species *Culex pipiens* is considered the main source of nuisance in urban areas [4, 5]
Culex pipiens has the ability to adapt to all habitats and biotopes [6, 7] which gives it the role of a vector for several pathogens that cause life-threatening infectious diseases [8, 9, 10]
This species is one of the main vectors of encephalitis in St. Louis; it was also considered the main vector of the West Nile virus in Romania [11]. In Algeria, the West Nile virus has caused a major epidemic in the Timimoune region in 1994, isolated cases of encephalitis in humans with fatal cases were reported by [12].
For several years, the control methods used sporadically in Algeria consist of spraying chemicals [5]. However, the widespread use of these products was soon to know several difficulties such as the resistance phenomena, the ecosystems imbalance, the lack of specificity and the persistence effect for non-biodegradable insecticides, which are the most frequent.
To avoid these problems, research is oriented towards the discovery of new components [13]. The aqueous extracts, the powders and the essential oils of plants contain molecules that have insecticidal properties. According to [14], the insecticide that was known for centuries is pyrethrum, a powder obtained from *Chrysanthemum roseum* and *Chrysanthemum cinerariae-folium*.
Many studies have been carried out based on plant extracts. That is the case particularly for [15], who demonstrated the larvicidal activity of the aqueous extract of *Persea americana* 's leaves on *Anopheles gambiae's* larvae. On the other hand, a study was conducted by [16] and his colleagues, who used the essential oil of the Laurel leaves *Laurus nobilis* against female adults of *Culex pipiens*, which showed excellent results. In 2009, [17] tested aqueous extracts of several medicinal plants, sage (*Salvia officinalis*), marjoram (*Origanum majorana*) and rosemary (*Rosmarinus officinalis*) on *Culex pipiens*, the mortality rate was very interesting.

Furthermore, studies by [18] have shown that *Thymus vulgaris* also has an insecticidal activity against *Anopheles* and *Aedes* due to the terpenes molecules.

Other plants are promising larvicides, in the fight against mosquitoes, such as Cypress (*Tetraclinis articulata)* Common Ricin (*Ricinus communis*), Oleander (*Nerium oleander*) and fenugreek (*Trigonella foenum*) which have been tested on *Culex pipiens* larvae by [19]. The results found by [20] confirm the larvicidal activity of the essential oils of *Citrus aurantium*, *Citrus sinensis* and *Pistacia lentiscus* which have been tested on *Culex pipiens* larvae. These results were confirmed in the same year in Morocco by [21], which found that *Citrus aurantium* has an interesting larvicidal activity against *Culex pipiens* compared to the essential oil of *Citrus sinensis*.

The myrtle family has 100 genera with about 3,000 species [22]. Eucalyptus is one of the most cultivated genera in the world; the species *Eucalyptus globulus* was introduced successfully in Algeria [23]. This tree is known for its anesthetic, antiseptic, inhaling fumigation, insects repellent, deworming and other therapeutic effects. Biological properties have already been assigned to the genus Eucalyptus, including larvicide and repellent activity against Culicidae mosquitoes, Insecticide effect on ground beetles and repellent action against *Phlebotomus papatasi* [22].

In the present work, we studied the larvicide power *of Eucalyptus globulus.* Therefore, after completing the bio tests to evaluate the larvicidal effect of this plant's aqueous extract on *Culex pipiens*' larvae (L_3 and L_4), that were reared in the Biosystematics and Arthropods ecology laboratory, we performed a histopathologic study on the midgut in order to specify the stages of intoxication in the larvae's mesenteric cells induced by this extract.

2. Materials and methods

2.1 Study period and area

This study was conducted in the summer season, from May 2015 to July 2015. It was carried between the laboratory of Biosystematics and Ecology of Arthropods, and the CHU Constantine hospital located in Constantine in the north east of Algeria (36°20'16.20"N; 6°37'33.32"E) at the altitude of 571 m.

2.2 Mosquitoes Breeding

The *Culex pipiens* Larvae used for breeding were collected in untreated sites located in the Constantine 1 University Campus. The collected larvae were transported to the laboratory for sorting according to the stages of development; their food is composed of a mixture of biscuits and yeast (75% and 25%). After transformation into pupae, they are placed in wooden cages covered in tulle where a blood meal is provided for the females. Nest boxes are introduced inside the cages for egg incubation, taking care to change the water every three days.

2.3 Preparation of the aqueous extract

Eucalyptus globulus is a species that belongs to the Myrtaceae family. This tree originated in Australia and is characterized by its blue-gray leaves in the juvenile stage, which join at the base forming a circular disc around the branch. The mature tree leaves are alternate, narrow, scythe-shaped and a shiny dark green. They grow on cylindrical rods and are 15 to 35 cm long. The buds are top-shaped, ribbed and covered with a flattened operculum bearing a central button. The cream colored flowers are solitary, in the axils of the leaves, and produce abundant nectar that bees transform into a deep flavored honey. The ligneous fruits are 1.5 to 2.5 cm in diameter and have a very hard capsule. Many small seeds escape through valves that open up on top of the fruit.

The Eucalyptus leaves are harvested, washed, dried in the open air, placed in an oven for 3 days and then ground.

An amount of 100 g of the obtained powder is extracted using a Soxhlet extractor with methanol as the extraction solution. The resulting extraction solution goes through the Rotavapor to obtain the dry active material, from which 4 concentrations are prepared (0.5g / l, 2 g / l, 5 g / l, 8g / l).

2.4 Realization of the biological tests

The methodology and formulas used are inspired from standardized methods by the World Health Organization (WHO). The tests were done on the3rd and 4th stages larvae, and for this, they were separated beforehand from the others, in a tank containing distilled water.

For each concentration, we used 4 cups (250ml), containing 99ml of spring water and 1ml of the prepared concentrations, in which 25 larvae were introduced. For each concentration, a series of 4 control cups was prepared. The mortality rate of *Culex pipiens* larvae in the cups was determined after 24, 48, and 72 hours.

2.5 Histological study

The methods followed in the histological study were inspired by those of [15]. The histological study was conducted on 4th stage *Culex pipiens* larvae, brought into contact with the aqueous extract of *Eucalyptus globulus* at a 27g / l concentration (LD_{50}). They were then systematically sampled after the given time intervals: 2, 4, 6, 12 and 24 hours, to be immediately fixed in alcoholic Bouin solution. Control larvae, placed in distilled water, were sampled at the same time intervals and were also fixed.

Before initiating the histological technique steps, the heads and respiratory siphons were cut with a scalpel blade. The parts were then placed in little boxes which were then passed successively through seven ethanol baths of 70° to 100°, in order to dehydrate them, or in other words remove the intra- and extracellular water. They were then immersed in three xylene containers in order to remove traces of ethanol and lighten the parts. Once dehydrated, the parts were inclosed in paraffin. And that is to achieve permeation into the studied tissue, as complete as possible, of the paraffin that is considered a homogeneous substance, solidifying and chemically neutral. The impregnation of paraffin takes place in an oven at a temperature of 58° to 60°C.

The blocks were then prepared and then cut using a microtome in 5µ thickness. The obtained sections were stained, fixed between slide and cover slip and observed under an optical microscope to determine the various anomalies that may have appeared in the mesentery of the processed *Culex pipiens* larvae.

3. Results

3.1 Study of the toxicity of the *Eucalyptus globulus* aqueous extracts on the *Culex pipiens* larvae

The mortality of *Culex pipiens* larvae exposed to different doses of *Eucalyptus globulus,* varied with the exposure time (Table 1). After 24 hours of contact with the extract, 16% of larval mortalities were obtained for the highest concentration (8g / l) compared to 1% dead larvae for 0.5g / l. After 48 hours of exposure, the concentration 8g / l gave 32% of larval mortalities. The highest mortalities rates (83%) were observed after 72 hours of exposure at a dose of 8 g / l.

Table 1: Toxic effect *of Eucalyptus globulus* on *Culex pipiens*

Exposure time	Concentrations			
	0.5g / l	2 g / l	5 g / l	8g / l
	Mortality rate (%)			
24h	1%	2%	6%	16%
48h	5%	19%	23%	32%
72h	28%	46%	51%	83%

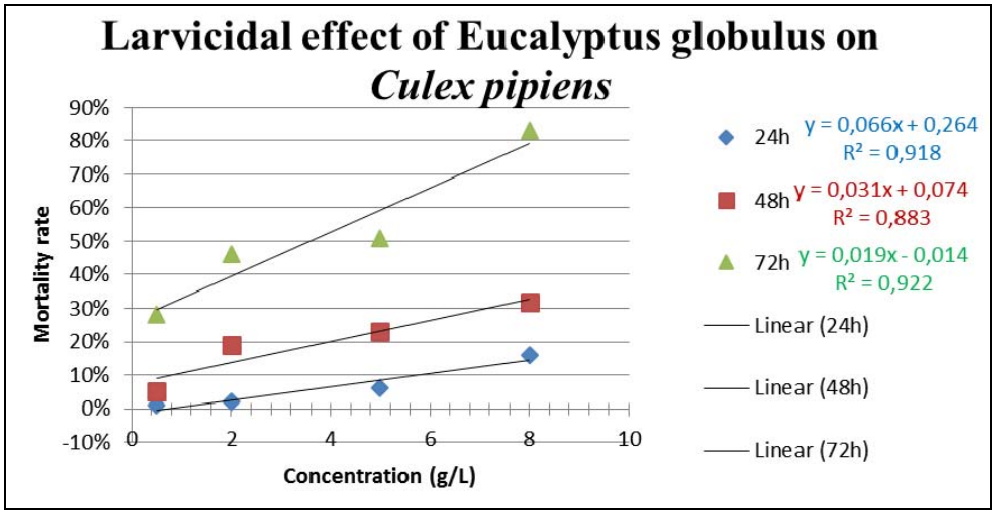

Fig 1: Laboratory evaluation of the larvicidal activity of *Eucalyptus globulus* aqueous extract on *Culex pipiens* larvae.

The mortality rate of *Culex pipiens* larvae treated with *Eucalyptus globulus* extract is shown in Table 2 with the lethal doses LD_{50} and LD_{95}, which were 27.6g / l and 52.1g / l respectively after 24 hours of exposure. After 48 hours, the lethal dose LD_{50} is 13.45g / l and the LD_{95} equals 27.69g / l; after 72 hours the lethal doses LD_{50} and LD_{95} are lower with respectively 3.57g / l and 10.39g / l.

Table 2: Toxic effect *of Eucalyptus globulus* on *Culex pipiens*

Exposure time	Regression line	LD_{50}	LD_{95}	Slope	R^2	F(3,12)	P
24h	Y = 0.485x-0.382	27.6g / l	52.1g / l	0.485	0.882	16.71	0.00013
48h	Y= 0.793X+1.861	13.45g / l	27.69g / l	0.793	0.883	45.91	7.5^{E-07}
72h	Y=4.25X+2.375	3.57g / l	10.39g / l	4.25	0.918	149.9	8.9^{E-10}

3.2 Histological examination of a control larva (untreated)

The histology of a control larvae's gastric caeca showed the gastric caeca's cells which have variable shapes and a brush border (Fig. 2). The midgut of untreated 4th stage larvae consists of epithelial tissues with a nucleus in the central position (Fig. 3).

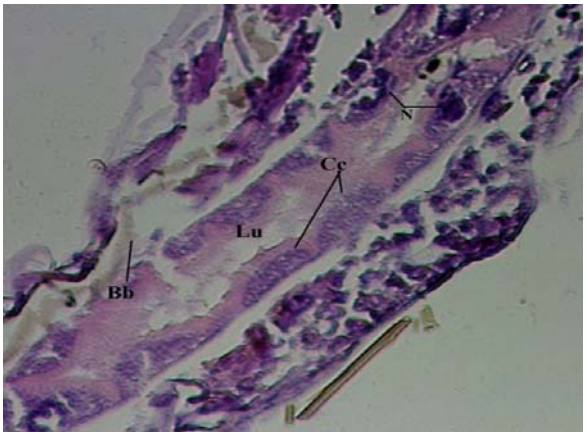

Fig 2: Longitudinal section of the gastric caeca cells of a control *Culex pipiens* larva. Abbreviations: Bb: Brush borders. Lu: Gastric caeca lumen. Cc: Gastric caeca cells. N: Nucleus.

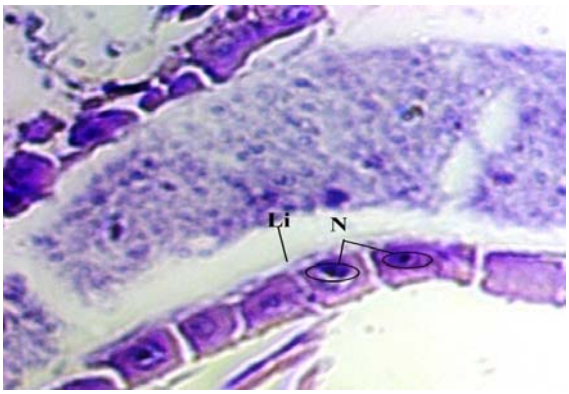

Fig 3: Longitudinal section of control *Culex pipiens* larvae midgut cells. Abbreviations: Li: Intestinal Lumen. N: Nucleus

3.3 Histological examination of the larvae treated with *Eucalyptus globulus* aqueous extract

3.3.1 Gastric caeca

After 2 h exposure of the *Culex pipiens* larvae to the LD_{50} dose of *Eucalyptus globulus* aqueous extract, the cells of the gastric caeca suffered a slight cell hypertrophy (Fig. 4). The cells of this area were morphologically altered and completely disorganized after 4 hours of contact (Fig. 5). After 6 hours of treatment, gastric caeca cells burst, and discharges of cytoplasmic debris in the gastric caeca lumen were observed

(Fig. 6). Cells started to degenerate after 12h of exposure to the aqueous extract (Fig. 7) until their almost complete destruction after 24 h (Fig. 8).

3.3.2 The larval midgut

The first changes were observed after 4 hours of exposure to the Eucalyptus aqueous extract. There was a hypertrophy of intestinal cells which began to pull away from each other (Fig.9). This is explained by the fact that the intercellular connections 'junctions' were broken.

After 6 hours of treatment; the cells were separated, and took an elongated shape. We also observed that they were detached from their basal lamina (Fig. 10). Figure 11 shows the state of the larval intestinal cells after 12 hours of exposure. Cell lysis was observed with a cytoplasmic debris discharge in the intestinal lumen. After 24 hours all the cells of the area were destroyed (Fig. 12).

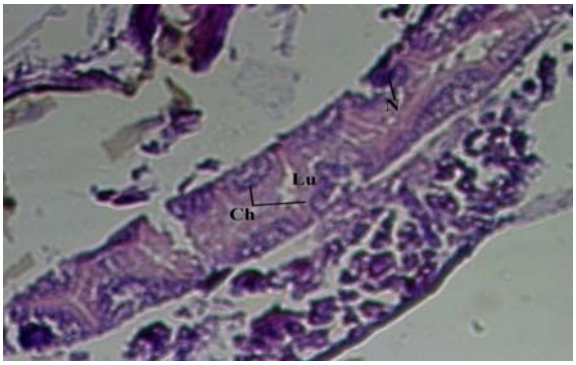

Fig 4: Longitudinal section of the gastric caeca cells after 2h of exposure Abbreviations: Ch: Hypertrophied Cells; Lu: Gastric caeca lumen. N: Nucleus.

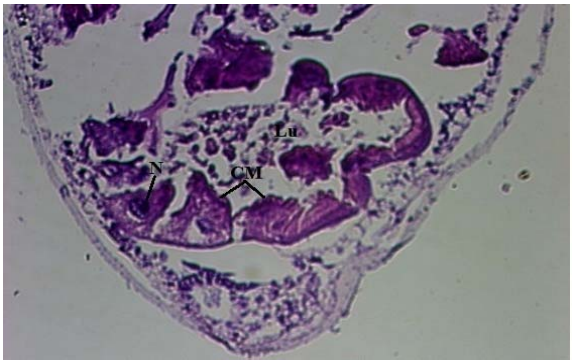

Fig 5: Longitudinal section of the gastric caeca cells after 4 h of exposure. Abbreviations: Cm: malformed cells; Lu: Gastric caeca lumen; N: Nucleus.

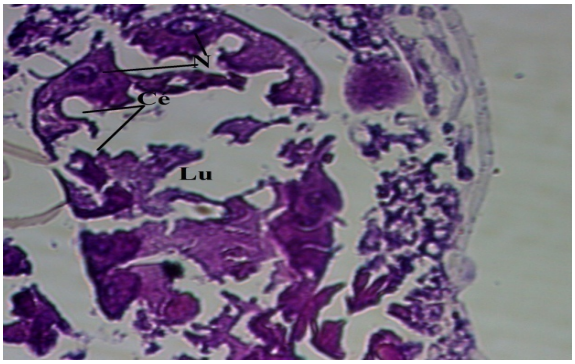

Fig 6: Longitudinal section of the gastric caeca cells after 6h of exposure. Abbreviations: Lu: Gastric caeca lumen; Ce: Broken cells; N: Nucleus.

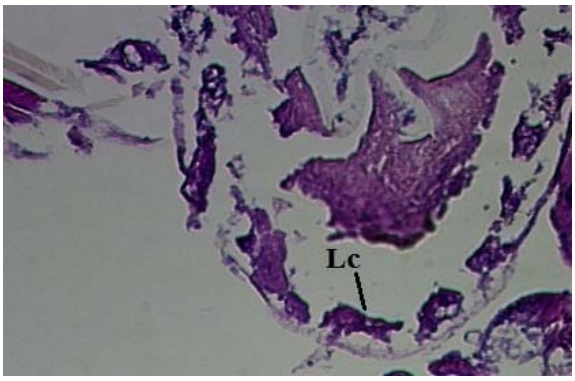

Fig 7: Longitudinal section of the gastric caeca cells after 12h of exposure. Abbreviations: Lc: cell lysis.

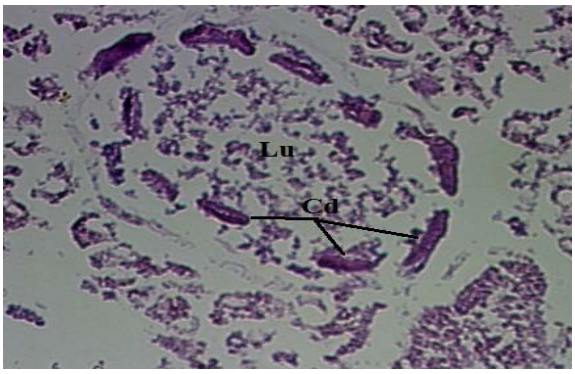

Fig 8: Longitudinal section of the gastric caeca cells after 24h of exposure. Abbreviations: Lu: Gastric caeca lumen; Cd: destroyed cells.

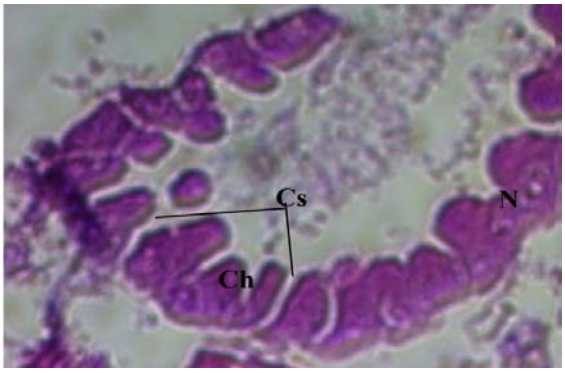

Fig 9: Longitudinal section of *Culex pipiens'* larvae midgut cells after 4h of exposure. Abbreviations: Ch: Hypertrophied Cells; Cs: separated cells; N: Nucleus.

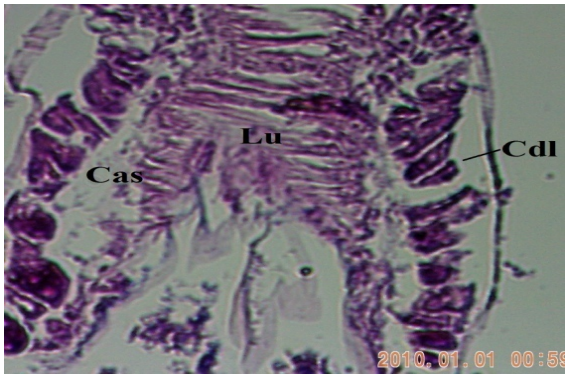

Fig 10: Longitudinal section of *Culex pipiens'* larvae midgut cells after 6h of exposure. Abbreviations: Cas: elongated separated cells; Lu: Intestinal Lumen; Cdl: Detached cells from the basal lamina.

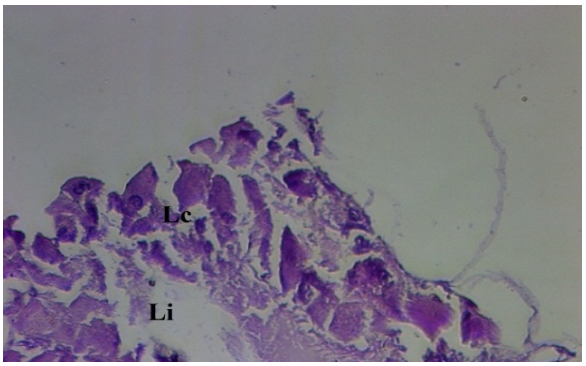

Fig 11: Longitudinal section of *Culex pipiens'* larvae midgut cells after 12h of exposure. Abbreviations: Lc: cell lysis. Li: Intestinal lumen.

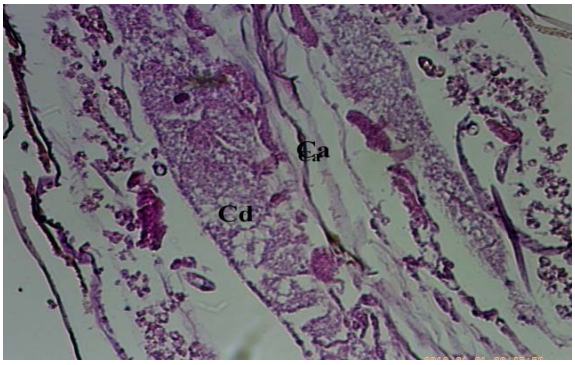

Fig 12: Longitudinal section of *Culex pipiens* larvae midgut cells after 24h of exposure. Abbreviations: Cd: Destroyed cells; Ca: Food tract.

4. Discussion

Plants (leaves, fruits, flowers, roots and bark) contain chemical complexes (active ingredients) that may have antiviral, anti-bacterial or anti-fungal properties [24]. Over 2 000 plant species have already been identified as having an insecticidal activity. A lot of work related to the use of plants as a larvicide against insects has been achieved. According to [17, 25] and [24], this larvicidal activity of aqueous extracts from plants can have a toxic effect, inhibit growth, have an effect on reproduction or even be a repellent.

Concerning the Eucalyptus leaves, which are the subject of our study, they contain a large amount of very diverse active substances such as eucalyptol, carbon hydrates, phytosterol, alkaloids, flavonoids, tannins, saponins, cardiac glycosides and terpenoids [17, 23, 26].

These results clearly illustrate The interest of *Eucalyptus globulus'* aqueous extract in larva control. This was confirmed by [24], after having conducted a study to evaluate the larvicidal activity of a Eucalyptus based aqueous extract against Aedes *aegypti* mosquito larvae. The measured lethal dose (LD_{50} and LD_{90}) of 106.21ppm and 198.76ppm show a significant toxic effect of Eucalyptus leaves on larvae of the 3rd and 4th instar.

Our results also reveal a direct relationship between the mortality rate of larvae and the dose to which they were exposed, as confirmed by [23] in his studies. The same author also showed a direct relationship between mortality and exposure time, where mortality increases from the first hour to the last. This confirms our results, where the average mortality increases from 16% after 24 hours, to 32% in the following 48 hours, to reach 83% of dead larvae after 72 hours of exposure to the aqueous extract of *Eucalyptus globulus* leaves, which is clearly shown in Figure 1.

Similarly, the results of [22] reveal a toxic effect of *Eucalyptus globulus* leaves extract against Lutzomyia *longipalpis* larvae. For his part, [27] showed a remarkable effectiveness of *Eucalyptus* against the larvae of the two fly species, *Musca domestica* and *Chrysomia megacephala*. Consequently, this plant reacts the same way against some Diptera (flies, mosquitoes and sandflies).

According to [22] the leaf extract acts by a neurotoxicity whose effect only appears after the development of the embryo's nervous system. For this, vector control is more effective against the larval stage.

Histological study on the 4th stage *Culex pipiens'* larvae treated with *Eucalyptus globulus* leaves aqueous extract shows different and progressive damage to the larvae's intestinal tissue, causing the mixing of gut cells content with hemolymph, which is responsible for larval mortality. The changes that were observed in the gastric caeca and midgut cells, are explained according to [28] by the fact that this portion of the digestive tract, which is responsible for digestion in insects, is in direct contact with the toxic elements and so causing death. The fact that this section is the major food absorption site in mosquitoes is confirmed by [29] in their study, where hungry mosquito larvae are fed with fructose and glucose; and after a few hours, massive glycogen deposits appear inside the cells of the midgut's posterior portion, indicating that it is the major absorption site.

The hypertrophied gastric caeca cells are morphologically altered and disorganized after 4 hours of contact (Fig.5).

This observation coincides with [30] who found in a study on the *Culex quiquefasciatus* larvae that were exposed to the Neem powder (*Azadirachta indica*), that his sections showed disorganization of intestinal cells. Neem was also tested on *Aedes aegypti* larvae by [31] who observed that the signs of intoxication first appear in the gastric caeca, showing severe morphological damage to the epithelial cells of this area.

After 6 hours, the cells burst and cytoplasmic debris are discharged into the gastric caeca lumen (Fig.6).

The observed changes, after 12 hours, are a progressive cell lysis (Fig.7), with a cytoplasmic debris discharge in the gastric caeca lumen which results in the complete destruction of the cells after 24 h (Fig.8). The aqueous extract of Eucalyptus leaves acts on *Culex pipiens* larvae in the same way as *Persea americana* on the *Anopheles gambiae* larvae, but more slowly.

According to [15], *Anopheles gambiae*'s larvae cells, treated with the extract of *Persea americana*, are destroyed after only 16h of exposure. Compared to control larvae treated with water, the midgut of the treated larvae is the most affected part.

Indeed, this portion is the first to present deep changes as a result of the larvae's intoxication with the *Eucalyptus globulus* aqueous extract. These changes begin to appear 4 hours after exposure, where intestinal cells appear hypertrophied in a remarkable way, while breaking away from one another (Fig.9). After 6 hours of treatment, the cells are well separated, and take an elongated shape. We observe also that they are detached from their basal lamina (Fig.10).

After 12h of exposure, cell lysis is observed with a discharge of cytoplasmic debris in the intestinal lumen. The cells in this area are totally destroyed after 24 hours (Fig. 12).

These findings were also made by [32] when he studied the histopathological effects of fenugreek (*Trigonella foenumgraceum*) on the *Culex quinquefasciatus* larvae. [33] reported that each region of the mesentery absorbs various

kinds of substances. For example, cells in the anterior part of the mosquitoes' stomach absorb fats, while the cells in the posterior portion predominantly absorb toxic elements, which suggest that they are the first to get damaged.

In the same context, the results reported by [34], on the effect of *Citrus limon* and *Allium sativum* oils against *Culex pipiens* larvae, reveal that there was clear damage to the epithelial cells of the midgut. Intestinal tissue, muscles and cuticle were the most severely damaged by the treatment, as well as the separation of the midgut cells from their basal membrane.

Our study is similar to that carried out by [30]. The authors evaluated the histopathological effect of the *Melia Azederach* extract against *Culex quinquefasciatus* larvae. Their results also revealed that, like the *eucalyptus* extract, *Melia azederach*acts on the *Culex* larvae by progressive destruction of mesenteric cells, causing the discharge of cytoplasmic contents into the midgut's lumen, and therefore the death of the larvae. But more slowly, because the cells of *Culex quinquefasciatus* ' larvae, treated with the extract of *Melia azederach*, degenerate after a 48 hour period.

Our results show similarities with those observed by [35] in their research on the mode of action of the *Bacillus sphaericus* bacteria against *Culex pipiens* larvae's digestive tract. The first changes are observed in the midgut. Similarly, [33] have concluded, after the histological study of *Bacillus thuringiensis* ' action on *Culex pipiens* larvae, that the first changes, in the mesentery, first concern the gastric caeca and posterior stomach.

5. Conclusion

The use of insecticide like plants extracts has been known to us for a longtime. The results presented in this work will lead us to believe that the aqueous extract of *Eucalyptus Globulus*, thanks to its larvicidal effects on *Culex pipiens*, should be considered among the natural components to be explored.

The effect on the mosquito's larvae (*Culex pipiens*) can be observed by: the destructions of the APICAL part of the stomach cells, the total destruction of stomach cells of mosquito's larvae with the rejection of some debris in the nutrition column, the relatively big distances between cells, a Hypertrophy or an intestinal cell augmentation. This would cause the death of the larvae's. This extract can be considered as a defense and control strategy against harmful insects, in fact, it is composed of many molecules that do not all apply to the larvicidal action.

A more developed study is in order to help us better understand the action's mechanism. We think that dissections and analyses of these total extracts will better explain the nature of the active molecules. This would form an interesting perspective of our research. It is also interesting to keep track of the impacts that theses aqueous plants extracts have on the intestines of larva mosquitos.

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