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Biocidal and Histopathological Effects of Pure *Balanites aegyptiaca* Oil on Mosquito Vectors (*Anopheles gambiae S.L*)

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Abstract | **Original Research Article**

This study aims to evaluate the effectiveness of pure *Balanites aegyptiaca* oil used on advanced stage larvae (L3 and L4) of *Anopheles gambiae s.l.* The latter remains a formidable vector of malaria in sub-Saharan Africa, particularly in Senegal. The toxicity of the oil on the larvae was tested in the laboratory according to the WHO experimental protocol, and the doses were 400, 800, 1200, 1600, and 2000 ppm, respectively. The results of our work showed that pure *Balanites aegyptiaca* oil had a lethal effect on the larvae. The lethal doses 50 and 90 were determined using the Muler Tinter formula (LD50 = 0.06618% and LD90 = 0.191%). Histological studies showed that *Balanites aegyptiaca* oil acts on different parts of the larva (head, thorax, and abdomen) but particularly on the digestive system through ingestion. Our results show that pure *Balanites aegyptiaca* oil could be recommended for the development of natural biocides against malaria-carrying mosquitoes.

Keywords: *Balanites aegyptiaca*, Histopathology, *Anopheles gambiae s.l.*

INTRODUCTION

Vector-borne diseases are caused by parasites, viruses, and bacteria that are transmitted by various vectors, including mosquitoes, sand flies, triatomine bugs, black flies, ticks, tsetse flies, mites, and lice. These diseases account for nearly 17% of the global burden of infectious diseases. Among all vector-borne diseases, malaria causes the highest global burden of disease, with approximately 405,000 deaths in 2018, most of them in children under 5 years of age (Ethics and Vector-Borne Diseases WHO Guidance, 2021). The WHO African Region continues to bear a significant and disproportionate share of the global malaria burden. In 2022, 94% of malaria cases (233 million) and 95% of deaths from the disease (580,000) were recorded in this region (WHO, 2023). In Senegal, malaria is still the major endemic disease and the leading cause of morbidity and mortality among children under five and pregnant women. In 2017, the malaria mortality rate among children under five was around 33.45%, while for pregnant women, the malaria positivity rate was 19.5% (Sadio *et al*., 2022).

The advent of insecticides raised hopes of eradicating malaria in regions where the disease was endemic. On the initiative of the WHO, a vast malaria eradication program was launched in the 1950s. The active ingredients in the insecticides used in mosquito control campaigns have led to cases of resistance. However, these preparations have proven to be very effective against Culicidae mosquitoes. In addition, the active substances in the products used have a broad spectrum of action and do not spare non-target organisms, namely humans and animals. The problem of resistance, by far the most significant, requires the use of new natural molecules with proven efficacy (Nkouandou *et al*., 2020). The complex and variable mixtures of bioactive compounds with different modes of action provided by plants offer great potential for reducing resistance in mosquitoes (Mdoe *et al*., 2014). Several studies have been conducted to evaluate the insecticidal effects of plants on mosquitoes, particularly *Anopheles gambiae* (Seye *et al*., 2006; Seye *et al*., 2012; Ndione, 2015; Badiane, 2019; Nkouandou *et al*., 2020). Studies have shown that *Balanites aegyptiaca* has a biocidal effect on insects, particularly mosquito vectors (Mano & Nana, 2022; Ali, 2023).

The objective of this study is to demonstrate the toxicity of pure Balanites aegyptiaca oil and to identify histological damage to *Anopheles gambiae s.l.* larvae.

I- MATERIALS AND METHODS

I-1 Study area

The study was conducted in the Saint Louis region (Senegal). The municipality of Saint Louis is located between 16° 1′ 52″ north latitude and 16° 28′ 52″ west longitude. The rainy season, which generally occurs between May and October in Senegal, remains the ideal period for the proliferation of malaria-carrying mosquitoes and therefore the risk of transmission. Moreover, malaria transmission follows, among other factors, the seasonal and spatial nature of rainfall (PNLP, 2021). The topography of the city of Saint Louis is very flat and at a very low altitude above mean sea level, which exposes it to coastal erosion and flooding (Ndao, 2012). The physical characteristics of the site (disjointed territory, low relief, shallow water table, soil structure with a high clay content and hydromorphic features, etc.) have led to disjointed urbanization with a series of neighborhood extensions in the agglomeration (Sall and Coly, 2017).

I-2 Larvae collection

The larvae were collected from a natural Anopheles breeding site in Diougop (a village located near the hamlet of Sanar Peul and the S.A.E.D. neighborhood), which is located between 16° 04′ 83″ north latitude and 16° 42′ 00″ west longitude. This is a permanent natural breeding site, similar to a pond, with a surface area of more than 10 m². Vegetation is present with a vegetation cover density of 25-49. The temperature was 37.5°C, the pH was 8.63, the conductivity was 5754 S.m-1, the salinity was 2.9 ppm, and the larval density was 188.15. The latter was chosen because of its high larval density. At this site, the larvae are identified by their horizontal position in relation to the water. They are scooped up with a ladle, filtered through a net, and placed in containers filled with water.

I-3 Pure *Balanites aegyptiaca* oil

Balanites aegyptiaca oil was obtained commercially in Kébémer (Louga region). It is extracted using a traditional technique. The seeds are collected in the fields, then shelled to obtain the kernels, which are crushed. The powder obtained was steamed before the pure oil was extracted by mechanical pressing.

I-4 Toxicity tests

These tests consist of assessing the mortality of advanced stage larvae (L3 and L4) of *Anopheles gambiae* by applying doses of diluted solutions of pure *Balanites aegyptiaca* oils using a methodology inspired by the World Health Organization (WHO, 1985) protocol. To do this, we used six jars, each containing 100 mL of water and a larval population of 20 individuals. Of these six jars, one was considered a positive control (containing Tween 80) and the other five were treated. The initial preliminary tests enabled us to select a range of concentrations for the actual tests. To do this, a stock solution of pure *Balanites aegyptiaca* oil was prepared using a surfactant (Tween 80) to enable the oil to mix with water.

- For the *Balanites aegyptiaca* solution: we added 0.2% Tween 80 to the oil, i.e., 0.1 ml (100 µL) of Tween 80 to 50 ml of Balanites oil
- The Tween 80 content was chosen after preliminary tests to maximize miscibility
- The doses used for treatment were 400, 800, 1200, 1600, and 2000 ppm, respectively

The oil was collected using a micropipette. A thermohygrometer was used to measure the temperature and humidity for the treatment series. The jars containing the treated larvae were placed in WHO cylinders. Mortality observations were made every 24 hours after each treatment. These serial tests were repeated four times to ensure the reliability of the results. We also carried out a larval treatment with the LD_{90} of the oil, which we recorded 6, 12, and 24 hours after exposure in order to visualize the histological damage over time.

I-5 Calculation of corrected mortality percentages and LD_{50} and LD_{90} values (Yolidje *et al.*, 2024)

Corrected mortality percentages calculated using Abbott's formula:

%m = [(NLM - NLMT) / (NLT - NLMT)] × 100

%m = mortality percentage
NLM = number of dead larvae
NLMT = number of dead larvae in the control
NTL = total number of larvae

The lethal doses 50 and 90 are calculated using Miller and Tinter's formula:

LD_{50} = (50(X2-X1) + (X1Y2-X2Y1)) / (Y2-Y1) ; same for **LD_{90}**.

X1 = lower concentration surrounding the LD_{50}
X2 = upper concentration surrounding the LD_{50}
Y1 = percentage of mortality corresponding to X1
Y2 = percentage of mortality corresponding to X2

I-6 Statistical analyses

Excel 2016 was used to process the data obtained after collection and generate the diagrams. Mortality data were processed using R software (version 4.4.3), relying on the "tibble" package for data structuring and basic functions for statistical testing. A comparative statistical analysis of the different doses (Fisher's test) was performed. To control for the risk of type I error associated with multiple testing, a Bonferroni correction was applied to all p-values obtained. The significance threshold was set at $\alpha = 0.05$ for all analyses.

I-7 Histological study

After treatment, the dead larvae are fixed in Carnoy II for 72 hours before undergoing a series of steps: dehydration, clearing, and impregnation using an automated dehydration machine. Embedding using an

embedding station to block the cassettes. Cutting of the blocks obtained using a semi-automatic microtome with a thickness of 3µm. Hematoxylin-eosin staining using an automatic staining machine. The prepared slides are observed under an optical microscope. Histological photos are taken at different magnifications (X40, X100, and X400) using computer software (Euromex).

III- RESULTS AND DISCUSSION

III-1 Results

III-1-1-1 Biocidal effects of pure *Balanites aegyptiaca* oil on stage 3 and 4 larvae of *Anopheles gambiae s.l.*

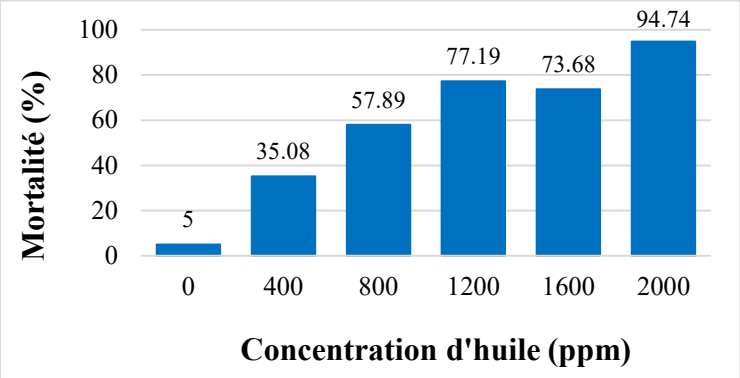

Figure 1: Variation in the cumulative mortality rate of advanced-stage larvae as a function of the concentration of pure *Balanites aegyptiaca oil* after 24 hours of exposure

The results revealed the toxicity of pure *Balanites aegyptiaca* oil on Anopheles larvae within 24 hours (Figure 1). Mortality was low (5%) in the control group, while it reached 94.74% at the maximum dose (2000 ppm). The increase in mortality was particularly marked between 800 ppm and 1200 ppm, where it rose from 57.89% to 77.19%. This suggests a dose-dependent effect. Comparative statistical analysis of mortality rates in pairs between the different treatment groups revealed several trends. Highly significant differences were detected between the control (0 ppm) and doses ≥ 0.08% ($p = 0.001$), confirming the increasing lethal effect of pure *Balanites aegyptiaca* oil. However, Fisher's test shows that there is no significant difference between the high doses (1200 ppm vs. 1600 ppm; 1200 ppm vs. 2000 ppm; 1600 ppm vs. 2000 ppm; $p = 1$). Similarly, the comparison between the 0 ppm control and 400 ppm did not reveal any significant difference ($p = 0.29$). The lethal doses LD_{50} (661.8 ppm) and LD_{90} (1910 ppm) were determined using the Muller and Tinter formula.

III-1-1-2 Histological sections of *Anopheles gambiae s.l.* larvae treated with pure *Balanites aegyptiaca* oil

The histology of the control larva shows a normal appearance of the three regions (head, thorax, and abdomen) with a well-defined cuticular layer and intact organs (Figures 2A, 3A, and 4A). However, 24 hours after treatment, the larvae show damage to the three regions mentioned above.

- Head: complete disorganization of the organs was observed, with destruction of the pharyngeal duct and part of the esophagus (Figure 2B).
- Thorax: destruction of the integument, muscle tissue, and mesenteron epithelium was observed (Figure 3B).
- Abdomen: destruction of the integument, muscle tissue, adipose tissue, and epithelium of the midgut with lysis of epithelial cells is also observed (Figure 4B).

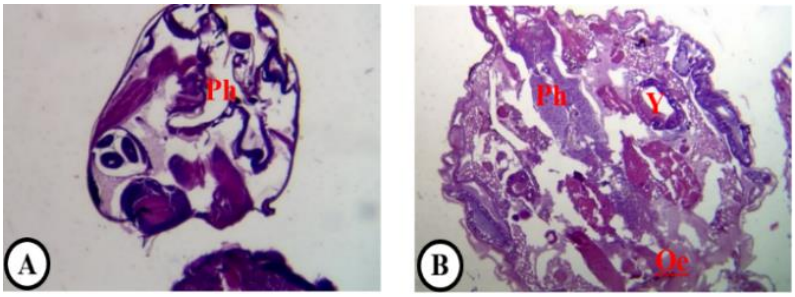

Figure 2: Longitudinal section of the head of an advanced stage larva of *Anopheles gambiae s.l.*: A) Control; B) Treated with *Balanites aegyptiaca* oil. Ph: Pharynx; Y: Eyes; Oe: Esophagus. X100

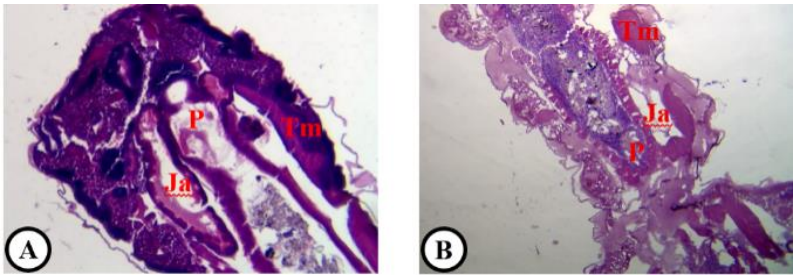

Figure 3: Longitudinal section of the thorax of an advanced stage larva of *Anopheles gambiae s.l.* : A) Control; B) Treated with Balanites aegyptiaca oil. P: Proventriculus; Ja: Crop; Tm: Muscle tissue. X100

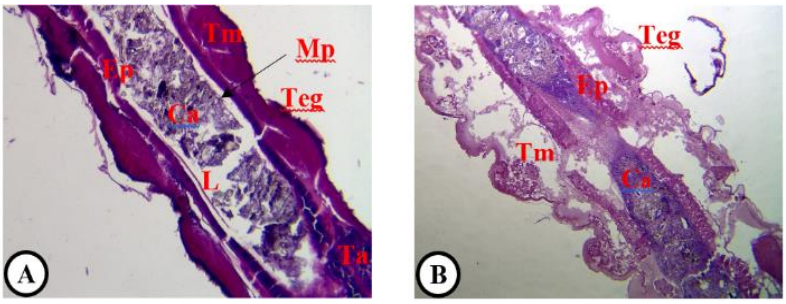

Figure 4: Longitudinal section of the abdomen of an advanced stage larva of *Anopheles gambiae s.l.*: A) Control ; B) Treated with *Balanites aegyptiaca* oil. Teg: Integument; Tm: Muscle tissue; Ta: Adipose tissue; Ep: Epithelium; L: Lumen; Ca: Alimentary column; Mp: Peritrophic membrane. X100

Histological results from larvae treated with the LD_{90} of pure *Balanites aegyptiaca* oil show that, at the level of the midgut wall, 6 hours after treatment, the integument and muscle tissues are degraded by the oil. After 12 hours, the epithelium and the peritrophic membrane surrounding the alimentary column are damaged. Twenty-four hours after exposure, in addition to the destruction of muscle tissue and adipose tissue, lysis of the epithelial cells of the mesenteron is observed (Figure 5).

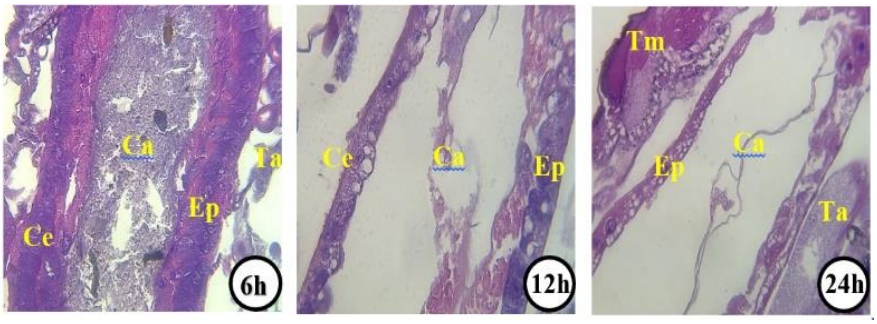

Figure 5: Longitudinal section of the mesenteron wall of late-stage *Anopheles gambiae s.l.* larvae treated with the LD_{90} of pure *Balanites aegyptiaca* oil 6h, 12h, and 24h after exposure. X400. Hematoxylin-Eosin (HE) staining.

III-2 DISCUSSION

The use of plant extracts as insecticides is a promising alternative because plant species produce bioactive compounds that generally act synergistically. They have the advantage of being biodegradable, accessible to all, and safe for the environment (Akono *et al*., 2016). These plant extracts are very often composed of alcohols, ketones, sesquiterpenes, monoterpenes, diones, terpene aldehydes, esters, azulenes, and oxides (Angone *et al*., 2015).

The results of our study show insecticidal activity in pure *Balanites aegyptiaca* oil with a dose-response relationship. This activity increases with concentration. These results are similar to those obtained by Foko *et al.,*(2016) with essential oil from *Aframomum sulcatum* seeds on the aquatic stages of *Anopheles gambiae* Giles 1902. Similarly, Tchoumbougnang *et al.,*(2009) observed the larvicidal activity of essential oil from the leaves of four plants cultivated in Cameroon (*Cymbopogon citratus*, *Ocimum canum*, *Ocimum gratissimum*, and *Thymus vulgaris*) on stage IV larvae of *An. gambiae*.

The toxic effect of *Balanites aegyptiaca* oil is clearly demonstrated by the LD_{50} and LD_{90} values, which are 661.8 ppm and 1910 ppm, respectively.

Significant lethality of the oil was observed at concentrations of **800 ppm** and above. Furthermore, no

significant differences were observed between high doses (1200 ppm vs. 1600 ppm, 1200 ppm vs. 2000 ppm, 1600 ppm vs. 2000 ppm; p = 1). This could indicate a potentially saturated effect (no significant difference in mortality) at concentrations above **1200 ppm**.

The histological results obtained show that pure *Balanites aegyptiaca* oil acts mainly on the midgut of the larvae 24 hours after treatment. The midgut is the longest and most crucial functional component of the digestive tract, as it is responsible for digestion, nutrient absorption, and larval growth (Taha *et al.,*2010). Microscopic results show that *Balanites aegyptiaca* oil acts both by contact, degrading the cuticle, muscle, and adipose tissue, and by ingestion, damaging the peritrophic membrane surrounding the alimentary column and the epithelium while causing epithelial cell lysis. These findings are also reported by Seye *et al.,*(2006), who studied the histological effect of neem oil (*Azadirachta indica*) on *Culex quinquefasciatus* larvae, showing destroyed gastric epithelial cells invading the lumen of the cecum, a disorganized alimentary column, and absent adipose tissue compared to the control. Similarly, work carried out by Ali (2023) shows that *Balanites aegyptiaca* extract causes severe damage to the intestinal tissue, muscles, and cuticle of *Culex pipiens* larvae. Our work corroborates that of Seye *et al.,*(2021), showing that 24 hours after treatment of third-stage *Aedes aegypti* (L) larvae with *Cymbopogon citratus* (lemongrass) essential oil, the larvae's bodies, digestive tracts, and tissues are completely damaged. Our results also show that tissue deterioration occurs gradually from the outside in and is time-dependent, with the contact effect (destruction of the integument, muscle and adipose tissues) occurring first, followed by the ingestion effect, which mainly affects the digestive system (deterioration of the peritrophic membrane surrounding the bolus, of the epithelium, and lysis of epithelial cells).

CONCLUSION

The results of our study show that pure Balanites aegyptiaca oil has a lethal effect on stage III and IV larvae of *Anopheles gambiae s.l.* It acts through the intestines, causing destruction of the digestive tract, and through contact, causing deterioration of the cuticle, muscle tissue, and adipose tissue. Furthermore, this oil acts gradually over time, from the outside in. Therefore, in terms of cost and availability, we recommend the use of pure *Balanites aegyptiaca* oil as a biocide in the fight against malaria vectors.

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