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RESEARCH ARTICLE

LYOPHILISATE OF PHYTOPLANKTON SCENEDESMUS QUADRICAUDA FOR MALARIA VECTORS ANOPHELES GAMBIAE LARVAE CONTROL IN BENIN: PRELIMINARY STUDY UNDER LABORATORY

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Abstract

The phytoplankton Scenedesmus quadricauda could be an alternative in the fight against malaria, as it has a deleterious effect on the larvae of the vectors of this disease. This phytoplankton species can be used in several forms (live or formulated). The aim of this study was to assess the effect of Scenedesmus quadricauda lyophilisate on Anopheles gambiae mosquito larvae in the laboratory. Large-scale culture trials of phytoplankton of Scenedesmus quadricauda was carried out in a semi-controlled environment. The phytoplankton cells obtained were harvested by centrifugation at 1,500 rpm for 15 min. The biomass obtained was lyophilised and its deleterious effect on stage 3 larvae of Anopheles gambiae was evaluated. For this assessment, 25 larvae were exposed to different concentrations 10 mg/l, 20 mg/l, 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l, 250 mg/l and 300 mg/l) of lyophilisate. The results showed that after two months of culture, the number of Scenedesmus quadricauda cells increased from 5,000 cells/ml to 14250000 cells/ml. The mortality rate of the larvae 10 days after exposure to the lyophilisate was 100% with concentrations 20 mg/l, 50 mg/l and 100 mg/l. With concentrations 10 mg/l, 150 mg/l, 200 mg/l, 250 mg/l and 300 mg/l, mortality rates were 88%, 98.4%, 86.4%, 84% and 67.2% respectively 11 days after exposure. The present study showed that Scenedesmus quadricauda lyophilisate could be integrated in control vector tool.

Introduction:-

Malaria remains a real public health problem, who threat more than five hundred million on people worldwide, and more specifically in Sub-Saharan African countries (OMS-Afrique, 2023; WHO, 2022). In Benin, malaria represent 44.9% of the reasons for health consultation (OMS-Bénin, 2021). Between 2015 and 2021, statistics for Benin showed that the number of deaths caused by malaria more than doubled. This number increases from 1,416 to 2,956,

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and the trend is similar for the number of malaria cases reported, which were 1,721,626 in 2015 and 2021 (OMS-Bénin, 2021; WHO, 2022).

The tools most commonly used to control this disease are Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS) (WHO 2021). These tools, based on the use of chemical products, are over time confronted with a selection of resistance within vectors populations (Chukwuekezie et al., 2020). Residues of these chemicals also affect non-target populations such as aquatic and terrestrial species, as well as a source of soil and water pollution (Chevrier, 2016; Dejoux, 1998; Everts and Koeman, 2020). To face with these problems of resistance and pollution caused by chemical control, people implicated in malaria vector control need to think about new appropriate control strategies. On the one hand, these strategies would not cause another form of resistance within vector populations, and also damage to non-target populations (WHO, 2015).

In such context, Houessou et al. (2022a) (Houessou et al., 2022a) evaluated the effect of Scenedesmus quadricauda phytoplankton on Anopheles gambiae larvae with a view to its use for biological control of malaria vectors. These authors showed that in An. gambiae breeding sites, there is a negative association between some phytoplankton species and An. gambiae larvae. The presence of these species results in the absence of mosquito larvae in the breeding sites. The species found enemies of mosquito larvae are: Chlorella vulgaris, Coelastrum reticulatum, Ankystrodesmus convolutus, Selenastrum gracile, Kirchneriella sp. and Scenedesmus quadricauda (Houessou et al., 2022a). These authors evaluated the lethal effect of alive suspension of S. quadricauda on An. gambiae larvae, and concluded that this phytoplankton species has a deleterious effect on An. gambiae larvae (Houessou et al.,2022a). Several studies showed that the cause of larval death is due to the fact that they larvae have difficulty to digest this phytoplankton species (Marten 1986; Ahmad et al. 2004; Houessou et al., 2022a, 2022b).

In order to introduce the species S. quadricauda into An. gambiae natural breeding sites this study entitled "Lyophilisate of phytoplankton Scenedesmus quadricauda for malaria vectors Anopheles gambiae larvae control in Benin: preliminary study under laboratory" was initiated for assess the effect offormulation of S. quadricauda on An. gambiae larvae with a view to its use in the biological control of malaria vectors.

Methods:-

Culture, harvesting and lyophilisation of Scenedesmus quadricauda cells

Scenedesmus quadricauda cells used in this study come from a large-scale culture trial of the phytoplankton S. quadricauda carried out under semi-natural conditions at the Laboratoire de Recherches sur les Zone Humides (LRZH) of Université d'Abomey-Calavi (UAC). The cultures were carried out in the medium culture of Nichol's Bolds Basal Medium (NBBM) (Nichols and Bold 1965; Houessou et al., 2022a). To do it, a small quantity (0.5 litter) of culture realized in laboratory with a concentration of 5,000 cells/ml was transferred into a transparent white barrel with a capacity of 220 liters. Every 72 hours, the S. quadricauda cells were fed with 1.5 liter of NBBM culture. During the culture, physico-chemical parameters (temperature, transparency, salinity, conductivity, dissolved oxygen, TDS) were measured in situ periodicaly and a small quantity of the culture was sampled for microscopic observations. These observations consisted of checking the state of the culture (healthy or contaminated) and determining the phytoplankton density in the barrel using a Neubauer chamber. To harvest S. quadricauda cells, the culture was collected in 3 liter plastic food storage and the S. quadricauda cells were obtained by centrifugation at 1,500 rpm/15 min. The biomass obtained after harvesting was lyophilised. The S. quadricauda cells were dry and the large particles (lyophilisate) were ground using a porcelain mortar.

Determination of concentration of Scenedesmus quadricauda lyophilisate

The density of S. quadricauda phytoplankton at the time when all larvae had died during the bioassays was 57,000 cells/ml (Houessou et al., 2022a). On the basis of this concentration, the mass of algae in 100 ml of suspension at 57,000 cells/ml was determinate and corresponded to 6.33 mg. Algae lyophilisate concentrations for bioassays were determinate on the basis of this value. Lyophilisate concentrations used for bioassays were 1, 2, 5, 10, 15, 20, 25 and 30 mg in 100 ml of water, corresponding to 10, 20, 50, 100, 150, 200, 250 and 300 mg/l.

Bioassays

The effect of S. quadricauda was evaluated on An. gambiae larvae in the laboratory. The bioassays were carried out in order to evaluate the effect of the lyophilisate on the An. gambiae larvae survival. Overall 9 treatments were and presented as tested the follow:

- ✓ T1: 100ml of water + 44 mg/l of cat food;

- ✓ T2: 100ml of water + 44 mg/l of cat food + 10 mg/l of lyophilisate;
- ✓ T3: 100ml of water + 44 mg/l of cat food + 20 mg/l of lyophilisate;
- ✓ T4: 100ml of water + 44 mg/l of cat food + 50 mg/l of lyophilisate;
- ✓ T5: 100ml of water + 44 mg/l of cat food + 100 mg/l of lyophilisate;
- ✓ T6: 100ml of water + 44 mg/l of cat food + 150 mg/l of lyophilisate;
- ✓ T7: 100ml of water + 44 mg/l of cat food + 200 mg/l of lyophilisate;
- ✓ T8: 100ml of water + 44 mg/l of cat food + 250 mg/l of lyophilisate;
- ✓ T9: 100ml of water + 44 mg/l of cat food + 300 mg/l of lyophilisate.

The experiment was carried out in 5 replicates. Then, the experimental dispositif consisted of 45 disposables cups with a capacity of 200 ml (figure 1).

Before introducing the larvae, the cups and their contents were left during 2 hours in order to acclimate them to the room temperature. Into each of these forty-five (45) cups were introduced batches of 25 third instar (L3) larvae of An. gambiae (Kisumu strain). These larvae were previously left to starve for 12 hours. The larvae were monitored every day until they emerged. The number of dead larvae per 24 hours was determinate by counting and mortality rate were calculated. The dead larvae were dissected and the contents of their gut were observed under a microscope at 100X or 400X magnification.

Figure 1:- Experimental set-up for larval tests.

Data analysis

The mortality rate of An. gambiae larvae exposed to S. quadricauda lyophilisate was calculated with 95% confidence intervals every day until all mosquitoes emergence. Analysis of variance (ANOVA) was used to comprare mortality rates during the bioassays.

Results:-

Scenedesmus quadricauda density in semi-naturel conditions

The suspension of S. quadricauda seeded was 0.5 ml with a density of 5,000 cells/ml. After one week of culture, the density was 35,000 cells/ml. It was 120,000 cells/ml one month after seeding before reaching a density of 13,125,000 cells/ml after two months. Phytoplankton density became almost constant after three months of culture and was 14,250,000 cells/ml after 13 weeks of culture (Figure 2). The average temperature of the culture environment was 31.61°C and the pH was 10.46. The conductivity measured was 1,033 µS/cm and the salinity 0.50‰. With regard to Total Dissolved Solute (TDS) and Transparency, they were 513 ppm and 18cm respectively.

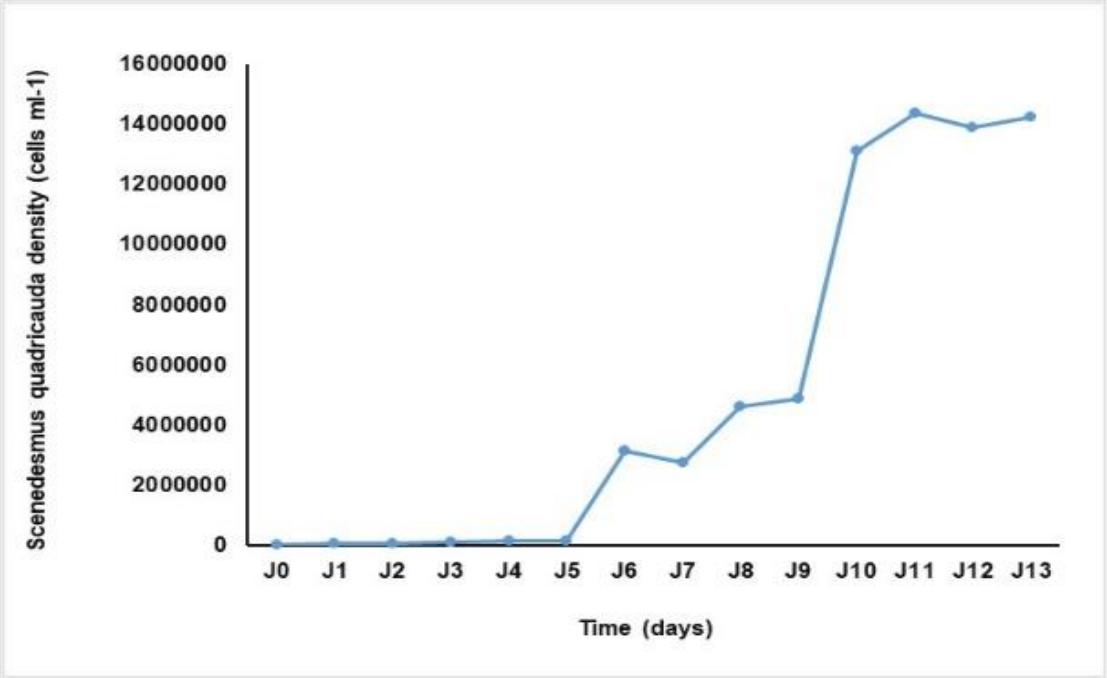

Figure 2:- Variation of S. quadricauda over time.

Lyophilisate of Scenedesmus quadricauda

From 20 litres of algal suspension, 128g of fresh S. quadricauda were collected. After lyophilisation, the dry matter obtained was 14.83g, composite of large particles, who were reduced to a powder for bioassays (Figure 3).

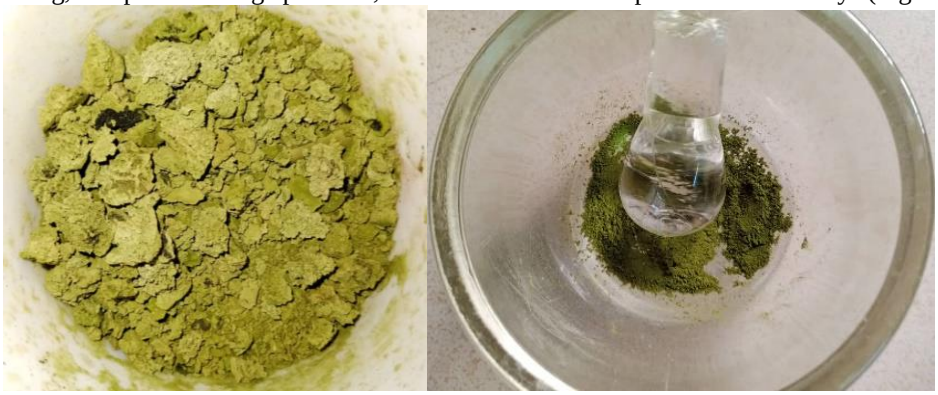

Figure 3:- Lyophilisate of S. quadricauda.

Effect of Scenedesmus quadricauda lyophilisate on Anopheles gambiae larvae survival

During the bioassay the mortality rate of An. gambiae larvae fed with cat food only (T1) was 4% five days after exposure. Used of S. quadricauda lyophilisate caused a 88% of mortality rate when larvae where exposure to 10 mg/l concentration of lyophilisate (T2). All larvae died within seven days with the 50 mg/l of lyophilisate concentration and nine days with the 20 mg/l, and 100 mg/l respectively when larvae were exposure to lyophilisate (T3, T4 and T5). Larval mortality decreased significantly when larvae were exposed to lyophilisate at concentrations above 100 mg/l. The larval mortality rates recorded with lyophilisate concentrations of 150 mg/l, 200 mg/l and 250 mg/l were 98.4%, 86.4 and 84% respectively over a period of twelve days after exposure of the larvae (T6, T7, T8). The lowest mortality rate was recorded with the 300 mg/l concentration. This rate was 67.2% (T9) twelve days after exposure of the larvae (Figure 4). A significant difference was observed between the different mortality rates (Anova, $P < 0.01$).

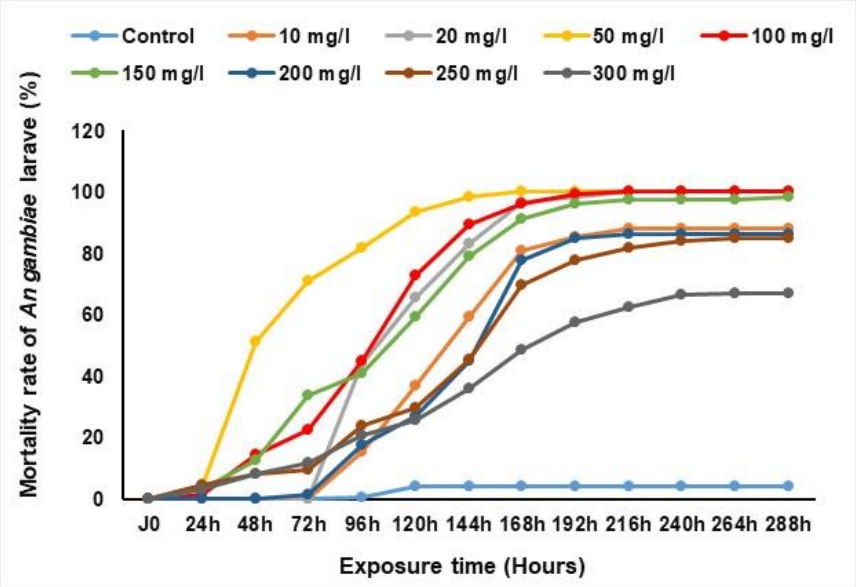

Figure 4:- Mortality of An. gambiae exposures to concentrations S. quadricauda lyophilisate.

Non-monotonic dose-response effect of lyophilisate

The effect of lyophilisate on An. gambiae mosquito larvae decreased considerably as the applied masses became higher. The results were explained by the curve of variation in mortality as a function of concentration (figure 5). This curve reflects a Monotonic Dose-Response effect when the larvae were exposed to the lyophilisate at concentrations of 10 mg/l and 20 mg/l. From 20 mg/l to 300 mg/l, the effect induced by the lyophilisate is characteristic of a non-monotonic dose-response effect (figure 5).

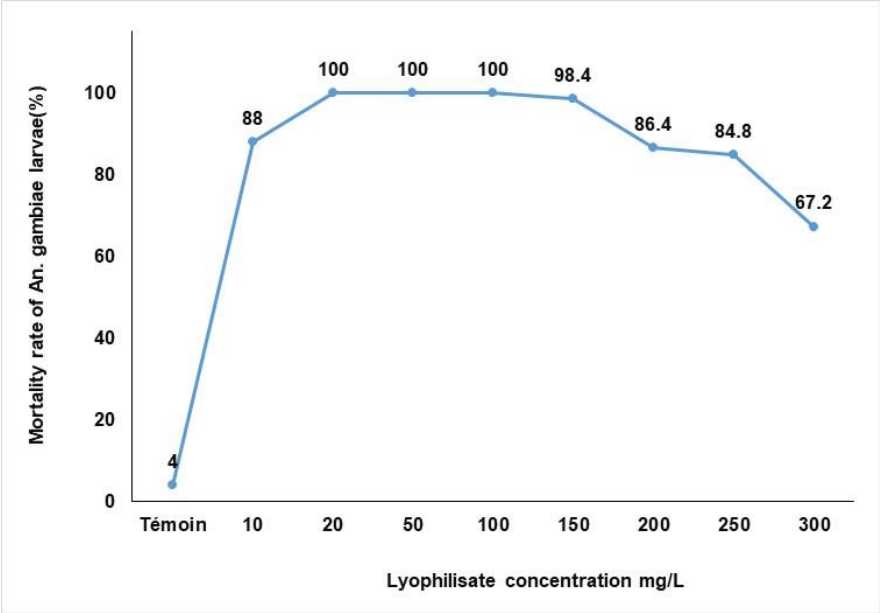

Figure 5:- Mortality of An. gambiae larvae as a function of different concentrations of S. quadricauda lyophilisate.

Effect of Scenedesmus quadricauda lyophilisate on the growth and development of Anopheles gambiae larvae

Larvae dead in treatments containing phytoplankton lyophilisate showed a shrunken body (Figure 6A) compared to control larvae (Figure 6B). A morphological malformation characterised by an increase in head-thorax area (elephantoids) was observed in pupae obtained from larvae exposed to the lyophilisate (figure 6D) compared to control larvae, i.e. those fed cat food only (figure 6C). Larval growth was delayed after exposure. The appearance of adults emergence was observed 4 days after exposure of the An. gambiae larvae to cat food (control). In the

disposables cups contains S. quadricauda lyophilisate, adult's emergence were observed around 6 days after exposure.

Figure 6:-Morpholgic malformation of dead An. gambiae larvae and pupae after exposure to S. quadricauda lyophilisate.

A: Larva exposed to cat food; B: Body shrinkage observed in larvae exposed to S. quadricauda lyophilisate; C: Control pupal; D: pupal with malformations and dead from elephantoïds.
Microscopic observation (100X).

Digestibility of Scenedesmus quadricauda lyophilisate

Observation of the guts of dead larvae after dissection reveals the presence of a green mass in the larval digestive tract. This mass represents an accumulation of undigested phytoplankton (Figure 8).

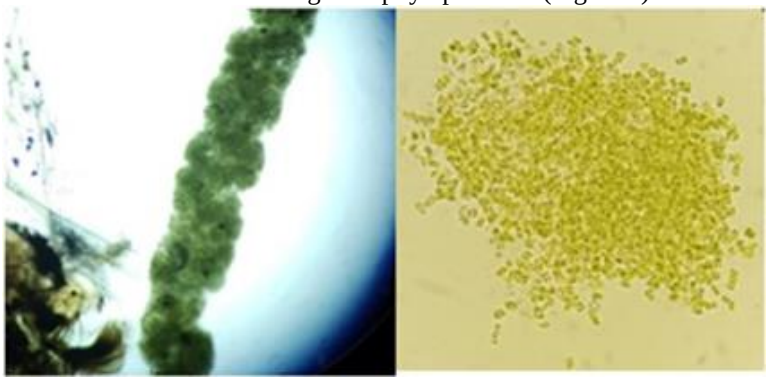

Figure 8:- State of the gut of dead larvae after ingestion of S. quadricauda lyophilisate, (observation with a miscroscope at 400X).

Discussion:-

The aim of this study was to evaluate the effect of lyophilisate of phytoplankton Scenedesmus quadricauda on Anopheles gambiae larvae. The study showed that S. quadricauda lyophilisate have a deleterious effect on An. gambiae larvae. The larval mortality rates who were induced were high when larvae were exposed to low concentrations of the S. quadricauda lyophilisate and low when lyophilisate concentrations were higher. A delay of larval growth was observed in larvae exposed to the lyophilisate, and dead larvae and pupae exhibited a shrunken body and morphological malformation of the body, respectively.

Assessing the effect of S. quadricauda lyophilisate on larvae required large-scale culture. Microalgae, specifically species of the genus Scenedesmus, were cultivated around the 1980 years and used in food, livestock and agropisciculture (Borowitzka, 2013 ; Borowitzka, 1995, 2018; Liady et al., 2020) because of their richness in protein, amino acids, fatty acids and vitamins (Becker 1994). Various large-scale cultivation systems for the genus Scenedesmus have been developed, varying from one species to another (Becker and Venkataraman 1984; Gross et al. 1986). The cultivation of S. quadricauda requires the influence of physicochemical parameters to be taken into account. According to Tahiri et al. (Tahiri et al., 2000a, 2000b), the most important factor is temperature. For these authors, temperature has an effect on the biochemical composition of the species. The average temperature of the culture medium in this study was 31.6°C, which proves that the phytoplankton cells were in contact with the sun's

rays. Sunlight is an important factor in the growth of microalgae (Kaufman et al., 2006). Here, the phytoplankton S. quadricauda was applied in the biological control of mosquito larvae. Several studies used phytoplankton to control mosquito larvae of the genera of Culex, Aedes and Anopheles (Dhillon et al., 1982; Marten 1986, 1987; Ahmad et al., 2001, 2004; Ahmad et al., 2018; Marten and Reid 2007; Houessou et al., 2022a, 2022b; Marten et al., 2022; Marten et al., 1989). The difference with previous studies reside in the way the phytoplankton species were used.

The deleterious effect of S. quadricauda phytoplankton on mosquito larvae has been the subject of several studies (Ahmad et al., 2001, 2004; Houessou et al., 2022a). In these studies, the mortality rate of larvae increased significantly with increasing phytoplankton density. This is generally observed in toxicology or pharmacology studies. The higher the concentration applied, the greater the effect induced (monotonic effect). The opposite phenomenon was observed with S. quadricauda lyophilisate, where high concentrations induced low mortality. According to the literature, there are substances for which the toxic responses are greater at low concentrations, known as non-monotonic dose-response effects (Lanphear et al., 2005; Wallet, 2012).

The results obtained in this study were consistent with this phenomenon. Concentrations of 50 mg/l and 100 mg/l induced a higher mortality than concentrations of 150 mg/l, 200 mg/l, 250 mg/l and 300 mg/l respectively. The lowest mortality (67.2%) was obtained with the 300 mg/l concentration.

According to Viguié et al. (Viguié et al. 2012); Vandenberg (Vandenberg, 2014); Zoeller and Vandenberg (Zoeller and Vandenberg, 2015); Gillot (Gillot, 2017), this phenomenon is observed much more frequently with endocrine disruptors. The lyophilisate would therefore act as an endocrine disruptor once in contact with the larvae. Endocrine disruptors are compounds capable of interacting with the hormonal system (Lanphear et al., 2005; Wallet, 2012). An endocrine system consists of organs that secrete hormones. In insects, this includes the prothoracic gland, the fat body, etc. The normal function of this hormonal system is to release chemical mediators able of acting specifically on certain body functions: growth, metabolism, sexual development, brain development, etc. (Wallet, 2012). When they enter the body, endocrine disruptors can interact with the hormonal system via hormone synthesis, transport, mode of action or degradation.

Thus, the lyophilisate could have the same modes of action as endocrine disruptors. Once ingested by the An. gambiae larva, the lyophilisate could have a disruptive effect on the production or regulation of hormones or their receptors (Pascal, 2018). Even at low concentrations, it could have a very significant toxic effect (Mancini, 2021).At high concentrations, S. quadricauda lyophilisate inhibits the production of the juvenile hormone responsible for maintaining the larval character, resulting in the development and transformation of larvae into adults (Mulla and Su 1999). The opposite phenomenon is observed with low concentrations of lyophilisate, leading to the death of the larvae. The endocrine disruptor could alter the function (s) of the endocrine system in an organism, its offspring or (sub) populations. The lyophilisate is thought to have an impact on larvae that reach adults old. This study did not take into account the fate of the larvae once they become adults. Further experiments will be needed for this.

The shrinkage and deformations observed in the bodies of An. gambiae larvae before death could be a response to the effect of the lyophilisate on the effector organs. According to Mekhlif and Khudhair (Mekhlif and Khudhair 2016), the malformations observed in larvae and pupae exposed to lyophilisate are characteristic ofthe clinical signs of elephantoids. The presence of undigested S. quadricauda cells in the gut of dead larvae has been observed by other authors (Marten 1986; Ahmad et al., 2004; Houessou et al., 2022a, 2022b). Indigestion of the lyophilisate may also be one of the causes of larval death. Burczyk et al. (Burczyk et al., 1981) revealed that the indigestibility of S. quadricauda was due to the presence of a layer of pectin (trilaminar) in the cell wall of this species. This is sporopollenin, a carotenoid that the digestive enzymes of the larvae cannot degrade (Atkinson et al., 1972; Mahdy et al., 2016). Even if all the possible explanations for the observed phenomenon were hypotheses, this study clearly shows that low concentrations can induce higher mortality rates than those induced by high concentrations. It should be noted that the notion of low dose is not an absolute concept and depends on the substance or product studied, the target/toxic agent combination and the means of observation (Chateauraynaud et al., 2011).

Conclusion:-

This study showed that the lyophilisate induced a non-monotonic response once in contact with An. gambiae larvae. Mortality rates of 100% were obtained when stage 3 larvae of the Kisumu strain were exposed to S. quadricauda lyophilisate at low concentrations. Above 100 mg/l, mortality rates dropped considerably. Lyophilisate is thought to

act as an endocrine disruptor. These results indicate that the use of lyophilisate would be an effective way of controlling An. gambiae mosquito larvae.

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Authors' contributions
DA, LMND, BA, SE, TOC, SC, FMG, AM, FED and HYL designed and developed the study. DA, SE and LMND coordinated the study. LMND, FMG and HYL carried out the Senedesmus quadricauda phytoplankton culture. DA, BA, SC, TOC, FMG and HL participated in the lyophilisation of Senedesmus quadricauda and laboratory bioassays. DA, LMND and HL analysed and interpreted the data and prepared the draft manuscript. HL wrote the draft. All authors read and approved the final manuscript.

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