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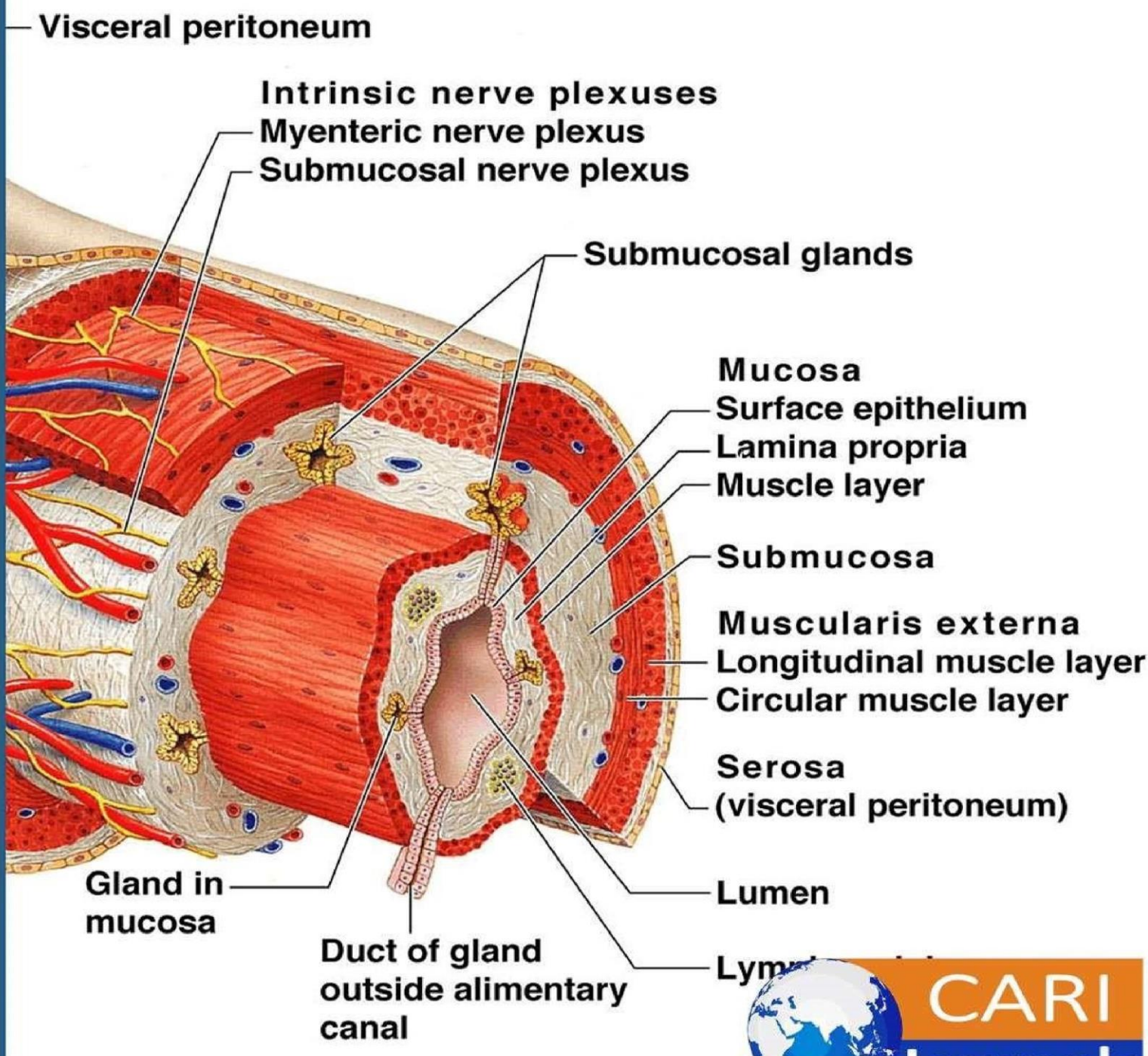

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Physical Properties of *Bacillus* Toxins and Their Larvicidal Activity Against *Anopheles Arabiensis* Mosquito Larvae.

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Abstract

Purpose: Biological control has been strongly encouraged by using of entomopathoginic bacteria *Bacillus thuringiensis* and *Bacillus sphaericus*. The present study monitored the larvicidal effect of *Bacillus thuringiesis* and *Bacillus sphaericus* against Anopheles mosquito larvae and the effect of physical factors on the larvicidal potency on mosquito larvae.

Methodology: Mid-guts of Anopheles mosquito larvae were inoculated on both Blood Agar and MacConkey Agar culture media. Depending on Gram stain to determine gram positive and gram-negative bacteria, and to identify the bacterial cells` morphology. A 1000µl of Bacillus thuringiensis. Sample was heated at 80°C for 12 min and diluted from 10 -1 to l0-8 with sterile normal saline. Bacillus strains were inoculated into Müller Hinton broth media and incubated over-night at 37°C. This time was quite enough to form the spores and crystals. The supernatant fluid was discarded and the cell suspensions were washed with sterile distilled water the process was repeated up to 3 times until spores and crystals were free from debris by using phase contrast microscopy. Collected spores and crystals were kept in sterile containers in fridge until used.

Results: Fifty-three mid-gut contents of *Anopheles arabiensis* larvae were identified morphologically and biochemically. *Bacillus* species were the most prevalent bacteria. The highest larval mortality rate was recorded by 5ml of Bt1 and Bt2 after 24 hrs. of treatment and by 150µl of *Bacillus* species spore.100% mortality was recorded by the *Bacillus* spore-crystal mixture. A mortality of 60% was recorded at pH 8.0, by 40^0 C the mortality rate was 20%at 24 hrs. After 12 hours, the exposure to UV light, larvicidal activity reduced to 40 %.

Unique Contribution to Theory Policy and Practices: These findings revealed that both *B.thuringiensis* and *Bacillus sphaericus* are a good control agent for Anopheles mosquitoes. More deep investigations are needed to verify the effect of the entomopathogens, which has biological and economic importance in mosquito control. In addition, Intensive studies must be needed to validate reliability.

Keywords: *Bacillus thuringiensis, Bacillus sphaericus, toxins, Physical properties, larvicidal activity, mosquito larvae.*

BACKGROUND

According to the 2019 World Malaria Report, malaria is a major public health concern. There were 228 million cases and 405,000 deaths occurring worldwide in 2018 **[36]**. In the same year, sub-Saharan Africa accounted for 93% of global malaria incidence, South-East Asia for 3.4% and the Eastern Mediterranean region for 2.1% **[36].**

Controlling mosquito can be realized by numerous ways: chemical, physical and biological methods **[19]** Using of predators like fish that feed on larvae, parasites and/or pathogens is type of biological control. While, some predators can also feed on useful organisms. **[22].** In recent years vector resistance results where there are chemicals used for mosquito control **[22].**

It has previously been observed that two bacteria, named as entomopathogenic bacteria: *Bacillus thuringiensis* subsp. *israelensis* de Barjac and *Bacillus sphaericus* Neide are used to control mosquitoes is a promising environmentally friendly alternative to chemical insecticides **[30].**

In the past decade, the genus *Bacillus* is classified as a gram-positive variable bacillus, which may be facultative anaerobic or aerobic**,** capable of endospore formation.

They are rod-shaped, endospore-forming bacterium. They form a cylindrical, or ellipsoidal, or oval or round endospore they located as terminal, sub-terminal, central, para-central or lateral position within the sporangium, **[31, 32]**. During sporulation, a major characteristic of *B. thuningiensis* is its ability to produce crystalline inclusions of insecticidal proteins

(8-endotoxins, Cry proteins, or ICPs) Its larvicidal activity resides in four major (of 27, 72, 128 and 134 kDa) and two minor (of 78 and 29 kDa) these polypeptides encoded respectively by cyt1Aa, cry11Aa, cry4Ba, cry4Aa , cry10Aa and cyt2Ba.

In addition, *Bacillus sphaericus* is a mesophilic, aerobic gram-positive bacterium that occurs worldwide in aquatic habitats and soil. A characteristic of spherical spore, which located at one end of the swollen sporangium. Crystal toxins and Mtx toxins are two types of *Bacillus sphaericus* proteins**, [6, 17, 30].**

In general, larvicidal activity of *B. sphaericus* is due to the binary toxin (Bin). It's composed of two proteins of molecular masses 42 kDa (BinA) and 51 kDa (BinB), that co-crystallize during late stage of bacterial growth**,** to form a single parasporal inclusion that is encapsulated with the spore [**9,17,30]**. Primarily the toxicity is due to a binary toxin (Bin) that binds to a specific receptor on the midgut microvilli of susceptible mosquitoes **[15, 9, 13].**

Surveys such as that conducted by **[7]** have been shown that**,** *B. thuringiensis* present many advantages such as safety for non-target organisms, derived from high specificity and low environmental pollution. Therefore, the most widely microbial pesticides used in the world are derived *B. thuringiensis* and *B.sphaericus*.

Nugud [26] in his first trials of controlling mosquito larvae by an insect bacterial pathogen. He has used a commercial preparation of *B.thuringiensis* Berliner (H-14), with a significant mortality of larvae. Moreover, in Sudan, [33] did an effective control of Anopheles and Culex larvae, which had resulted in a small-scale field application of B.alvei and B.thuringiensis**.**

Different physical factors like ultraviolet radiation, rain, pH and temperature are known to influence efficacy of Bt formulations.

The objective of this research was to evaluate the toxicity of *Bacillus thuringiensis* and *Bacillus sphaericus* against *Anopheles arabiensis* mosquito larvae and to determine whether the physical factors, such as pH concentration, Ultra Violet light and temperature, effect on the toxicity of *Bacillus* suspensions against *anopheles* mosquito larvae in Sudan.

MATERIALS AND METHODS

Collection of samples:

Alive and dead larvae were collected from the insectary and were removed from the water and washed several times with tap water followed by sterile distilled water then sterile normal saline. Finally, they were dipped in 70% alcohol for a few seconds, then washed, and transferred to a sterile slide, for dissection.

Cultivation of samples:

Mid-guts of Anopheles mosquito larvae were inoculated on both Blood Agar and MacConkey Agar culture media. Culture were performed under aseptic condition then plates were incubated at 37^0 C under aerobic condition for 24-48 hrs. After that, colonies with significant growth were selected for purification and then kept in slants and saved in the refrigerator for identification tests.

Isolation of spore-forming bacteria:

Isolation was conducted by comparing morphology of the grown bacterial colonies.

Morphologically different colonies were considered as different colonies. Depending **on Gram stain** to determine gram positive and gram-negative bacteria, and to identify the bacterial cells` morphology. Isolated bacteria were identified according to [3]**.**

Gram staining:

Gram's stain was done whereby the method described by [12]. Bacteria violet colored were labeled as Gram- positive organisms and red colored were labeled as Gram- negative organisms, **Spore stain:**

The smear was then examined microscopically to show the shape of the cell, the presence of the spores, their position and the dimensions of the cell. Schaeffer-Fulton method [25]**,** was used for

endospore staining, where endospores appear as blue green structures while vegetative cells are brownish red.

Spore counts:

A 1000µl of *Bacillus thuringiensis.* Sample was heated at 80°C for 12 min and diluted from 10^{-1} to $l0^{-8}$ with sterile normal saline. Three plates were cultured for each dilution (100µl) at 30°C for 48hrs. Spores were counted by the spreading technique. *Bacillus thuringiensis* ***l00*** µl of sample was suspended in 900µl of sterile Normal saline and vortexed. Then, the other steps of the procedure were performed in a similar manner.

Production of spore-crystal mixture suspension:

Bacillus strains were inoculated into Müller Hinton broth media and incubated over-night at 37°C.Then the suspensions were inoculated on Nutrient agar and incubated at 37°C for over-night. Then 0.05 ml of the inoculum was transferred to 10ml of T3 broth to simulate the sporulation. The flasks were shaken from 2-10 days with shaking at 200rpm.this time was quite enough to form the spores and crystals.

Then 10ml of broth suspension was centrifuged at 3000rpm/15mins. The supernatant fluid was discarded and the cell suspensions were washed with sterile distilled water the process was repeated up to 3 times until spores and crystals were free from debris by using phase contrast microscopy.

Collected spores and crystals were kept in sterile containers in fridge until used. [8,34]**.**

Larvicidal Activity:

Bioassays were performed to observe the larvicidal activity of *Bacillus thuringiensis* spore. 2^{nd} and 3^{rd} instars larvae (20) were placed in a flask containing 20ml of tap water. A few finely ground fish food granules have been added to feed the fry.

Then 100µl about 10^7,10^6 spore of sample were added to the flasks. (Final concentration: about 10^6,$10^5$and 10^4 spore/ml respectively) Mortality rate was scored at 24 and 48 hrs after treatment.

Larvicidal activity of *Bacillus* metabolites on mosquito larvae:

Mosquito larval toxicity of *Bacillus* metabolites was determined by adding a known amount of *Bacillus* metabolites to 25ml of tap water and then 2^{nd} and 3^{rd} instars of larvae of *An.arabiensis* (20) were placed to each flask.

A few grains of finely ground fish food pellets were mixed with feed the larvae. Observations were made at 90min, 180min, and 360min.

Assays were performed in triplicate, and mortality was scored after 24 and 48 hrs. in incubation at 28°C.Control larvae incubated in the absence of *Bacillus* metabolites and had no mortality in this period.

Larvicidal activity of *Bacillus* spore-crystal mixture on mosquito larvae:

Purified spore-crystal mixture of two *Bacillus thuringienisis* strain and *Bacillus sphaericus* were examined for quantitative larvicidal activity against mosquito larvae. Bioassay against 2nd and 3rd instars *An.arabiensis* larvae was applied to four different concentrations with three replicates per concentrations.

Ten 2nd and 3rd instars *An.arabiensis* larvae placed in 5ml of tap water were added to each well of 20 well tissue culture plates.

A few grains of finely ground fish food pellets were mixed with feed the larvae. Observations were made at 90min, 180min, and 360min.

Assays were performed in triplicate, and mortality was scored after 24, and 48 hrs. at an incubation of 28°C. Control larvae were incubated in the absence of *Bacillus* spore-crystal mixture and had no mortality in this period.

PHYSICAL PROPERTIES:

Effect of UV irradiation:

In this study, A 12W germicidal lamp (General Electric, 257nm) was used. The solution of *Bacillus thuringiensis* was exposed to UV light directly for duration of 1, 6 and 12 hrs. after UV exposure, samples were taken and a spore count was determined. 2nd and 3rd instars larvae (20) were placed in a flask containing 25ml of tap water. A few grains of finely ground fish food pellets were added to feed the larvae. Bacillus metabolites were added, and two flasks were left untreated as control. Changes were record daily up to 48 hours. Bioassays were replicated three times for each concentration.

Effect of pH:

In this study the pH concentration were used 4.0, 6.0 and 8.0. The solution containing *Basillus thuringiensis* metabolites were adjusted by using an electric pH meter following standardization with appropriate buffers (0.1 HCL and 0.1M NaOH) to decrease or increase pH respectively, to obtain values of pH 4.0, 6.0 and 8.0. 2nd and 3rd instars larvae (20) were placed in a flask containing 25ml of tap water. A few grains of finely ground fish food pellets were added to feed the larvae. *Bacillus thuringiensis* metabolites with different values were added, and two flasks were left untreated as control. Changes were recorded daily up to 48 hours. Bioassays were replicated three times for each concentration.

Effect of temperature:

Bacillus thuringiensis metabolites were heated through water-bath at different temperature 40°C, 60°C and 80°C. 2nd and 3rd instars larvae (20) were placed in a flask containing 25ml of tap water. A few grains of finely ground fish food pellets were mixed with feed the larvae. *Bacillus*

metabolites with different values were added, and the control temperature (28±2°C) Changes were recorded daily up to 48hours. Bioassays were replicated three times for each concentration.

RESULTS

Fifty-three bacteria were isolated, these isolates were characterized, morphologically and biochemically. Table (1) shows that all *Bacillus* species. The percentage of mortality of mosquito larvae on treating with different concentration of Bacillus spores at 24 and 48 hrs. Table (2) revealed that the 85 and 100% mortality rates were recorded using 10^7 and 10^8 concentrations of *Bacillus* spore at both 24 and 48 hrs.

Larvicidal activity of *Bacillus* metabolites on mosquito larvae:

Three strains of *Bacillus* species were chosen to study the larvicidal activity against the mosquito larvae. They were *Bacillus thuringiensis* (1), *Bacillus thuringiensis* (2) and *Bacillus sphaericus*. Table 3 shows the toxicity of *Bacillus* metabolites when added to different doses to the of Anopheles mosquito larvae.

The potency of the bacterial metabolites was measured in terms of mortality percentage of the mosquito larvae after 24 and 48 hrs.

The metabolites of the three *Bacillus* strains produced 75-100% mortality rate to mosquito larvae after 24hrs. In *Bacillus thuringiensis* (1), 75% mortality rate was produced by 3ml, 90% by 4ml, and 100% by 5ml of the *Bacillus* metabolites. Moreover, in *Bacillus thuringiensis* (2), 100% mortality rate was produced by 3ml of the *Bacillus* metabolites. In *Bacillus sphaericus* 80% mortality rate was produced by 3ml, 85%by 4ml and 100%by 5ml of the *Bacillus* metabolites.

Table 1 Prevalence Rate of Bacillus species among An. arabeinsis Mosquito Larvae:

Bacillus species	**Total No. of larvae**	**No. of Isolates**	**Percentage**
B. thuringiensis	30	10	33%
B. cereus	30	7	23%
B. sphaericus	30	7	23%
B. lentus	30	5	16%
B. brevis	30	4	13%
B. popilliae	30	3	10%
B. mycoides	30	3	10%
B. laterosporus	30	2	6.7%
B. pantithenticus	30	2	6.7%
B. alvei	30	2	6.7%

B. larvae	30	1	3.3%
B. polymyxa	30	1	3.3%
B. firmus	30	1	3.3%
B. badius	30	1	3.3%
B. megaterium	30	1	3.3%
B. circulans	30	1	3.3%
B. coagulans	30	1	3.3%
B. lentimorbus	30	1	3.3%

Table 2 Bioassay of Larvicidal Activity of Bacillus spores at 24, 48 hrs.

Spore loading	After 24 hrs.	% mortality	After 48 hrs.	% mortality
106	5 die 15 alive	25%	14 die 6 alive	70%
107	13 die 7 alive	65%	7 die	100%
108	17 die 3 alive	85%	3 die	100%

Larvicidal activity of *Bacillus* spore-crystal mixture on mosquito larvae:

Tables 3 and 4 show the effects of the *Bacillus* spore-crystal mixture when added in different doses to the mosquito larvae.

The spore-crystal mixture of *Bacillus* species produced 80-90 and 100% mortality rates to *An.arabiensis* mosquito larvae. After 12 hrs. the spore-mixture of *Bacillus* species produced 100% mortality rate to mosquito larvae by 100,150 and 200µ.l. Moreover, in *Bacillus thuringiensis* (1) 80% was produced by 50µl, 90% was produced by 100µl and 100% was produced by 150µl. However, in *Bacillus thuringiensis* (2), 90% mortality rate was produced by 50 µl,100% was produced by 100 µl. In contrast, in *Bacillus sphaericus* 80% mortality rate was produced by 50 µl, 100% was produced by 100 µl.

Table 3 Toxicity of the Three Bacillus Metabolites on An.arabiensis Mosquito Larvae According to Time.

Isolate	Doses Ml	Results 24hrs	%	Results 48hrs	%	Results 72hr	%
B.thuringiensis(1)	1	No death	0%	3 died	15%	7 died	35%
	2	9 died, 11 alive	45%	14died, 6 alive	70%	20died	100%
	3	15 died, 5alive	75%	20died	100%		
	4	18 died, 2 alive	90%	20died	100%		
	5	20died	100%				
B.thuringiensis(2)	1	No death	0%	6 died, 14alive	30%	8 died	40%
	2	15died, 5alive	75%	4died, 1alive	95%	20died	100%
	3	20died	100%				
	4	20died	100%				
	5	20died	100%				
B. sphaecius	1	No death	0%	2 died, 18alive	10%	5 died	25%
	2	10 died, 10alive	50%	15died, 5alive	75%	20died	100%
	3	16 died, 4alive	80%	20died	100%		
	4	17died, 3alive	85%	20died	100%		
	5	20died	100%				
Control	-	No death	0%	No death	0%	Pupae	0%

Table 4 Toxicity of the Bacillus Spores of Three Bacillus Strains on An.arabiensis Mosquito Larvae After 12 and 24hrs.

Isolate	Doses	Results 12hrs	%	Results 24hrs	%
B.thuringiensis (1)	50 µl	8 die, 2 alive	80%	2 die	100%
	100 µl	9 die, 1alive	90%	1 die	100%
	150µl	10 die	100%		
	200 µl	10 die	100%		
B.thuringiensis (2)	50 µl	9 die, 1alive	90%	1die	100%
	100 µl	10 die	100%		
	150 µl	10 die	100%		
	200 µl	10 die	100%		
B.sphaericus	50 µl	8 die, 2alive	80%	2 die	100%
	100 µl	10 die	100%		
	150 µl	10 die	100%		
	200 µl	10 die	100%		
Control	-	No death	0%	No death	0%

Effect of UV light:

The effects of the UV lights treatment of *Bacillus* metabolites on *Anopheles* larvae was explain in Figure 1.The results and rates of mortalities were taken after 24 and 48 hrs. of treatment. After 24hrs.40%, 50% and 30% were recorded. And 40% mortality rate was produced with *Bacillus* metabolites which exposed to 12hrs. of UV light.

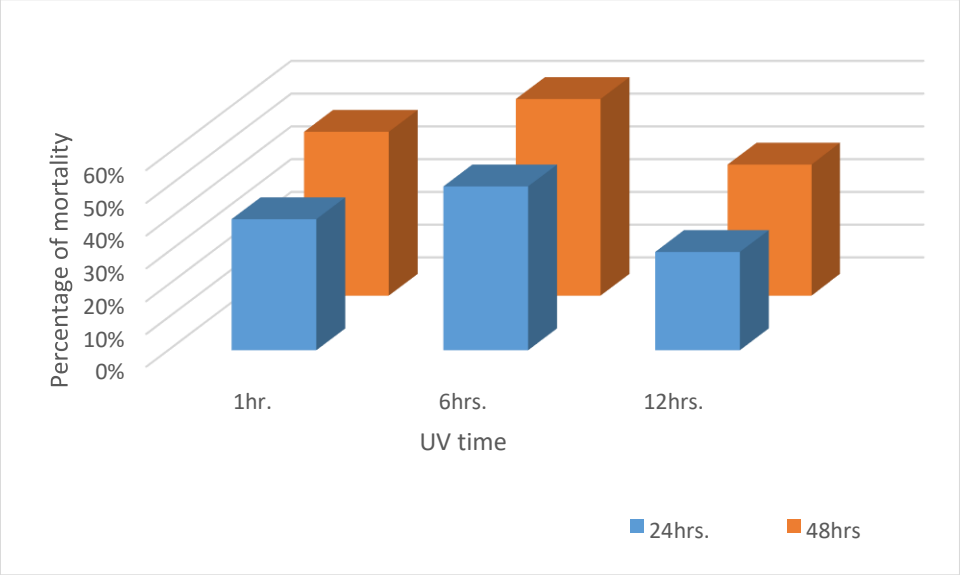

Fig 1: Effect of UV light treatment of *Bacillus* metabolites on the mortality rate of *Anopheles* mosquito larvae at various time.

Effect of pH treatment:

Figure (2) expressed the effect of pH on the larvicidal potency of the Bacillus metabolites. As observed in this experiment, the percentage mortality recorded at 24 and 48hrs of treatment. The mortality rate of mosquito larvae ranged from 20-60% in 24hrs. However, the mortality rates were very low 20-30% at various pH's (4.0, 6.0 and 8.0). After 48hrs. The mortality rate recorded 60% with pH8.0 value.

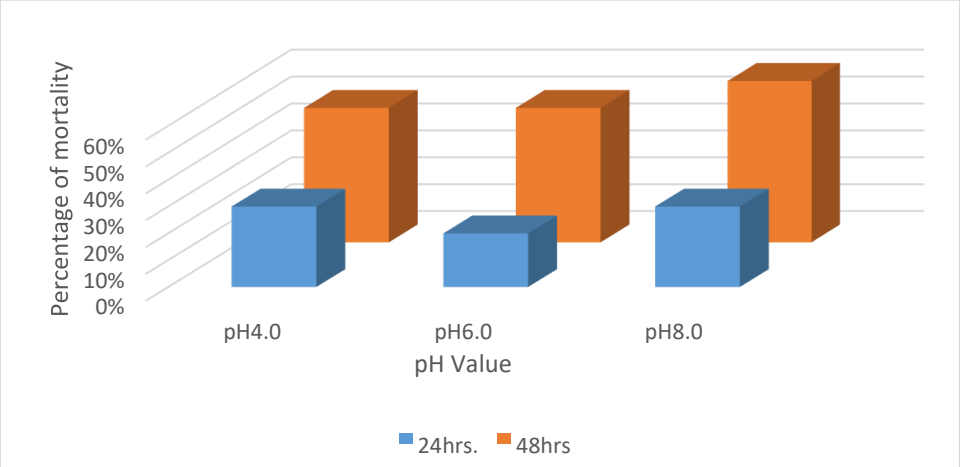

Fig 2: Mortality rate in *Anopheles* mosquito larvae by *Bacillus* metabolites at various pH and time.

Effect of temperature:

Difference in larvicidal potency of the tested microorganism at different temperature values was observed. Figure (3) show the effect of high temperature of Bacillus metabolites on mosquito larvae. After 24hrs. mortality rate was 20-30% at 40°C,60°C, 80°C. After 48hrs. mortality rate reached 60,50 and 40% with 40°C,60°C and 80°C respectively.

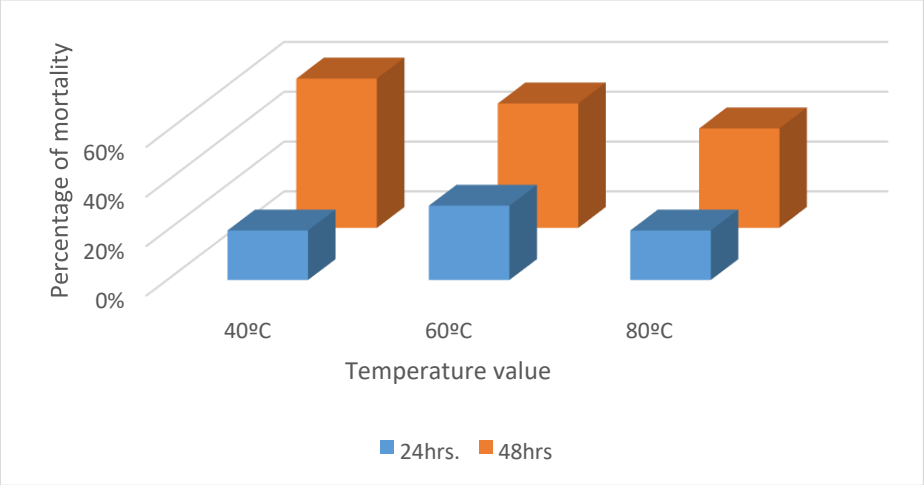

Fig 3: Effect of temperature treatment of *Bacillus* metabolites on the mortality rate of *Anopheles* mosquito larvae according to time.

DISCUSSION

Hence, increasing of the resistance of mosquitoes to chemical pesticides, an eco-friendly method has been promoted to control the mosquitoes. In the present study *Bacillus thuringiensis* and *Bacillus sphaericus* were confirmed characteristically, morphologically and biochemically according to **[32].**

The results of this study demonstrated that after 24hrs. 100% was achieved by 5ml of Bt1, Bt2 and Bs 3,4ml of Bt2. After 48hrs.100% mortality rate by 3,4 ml of both Bt1 and 2ml of Bt2. Similar results were obtained by **[1]** who reported high efficiency of *B.thuringiensis* preparation on Anopheles larvae reach 100% within 48hrs of treatment. While in a study conducted by **[18**] 95% mortality rate to Anopheles mosquito larvae was observed in less than 48hrs.Most of the previous studies carries out about potential mosquitocidal activity of entomopathogenic *B.thuringiensis* and *B.sphaericus* and their ability to control the Anopheles larvae **[2,4,14,19,23]**.

It was also observed that the increased concentrations led to increase in mortality rate of various isolates both in the 24hrs. and 48hrs. of treatment. after adding the *Bacillus* spore-crystal mixture to the *An.arabiensis* larvae, 100% mortality rate was produced by 150µl of *B.thuringiensis*(1), 100 µl of both *B.thuringiensis* (2) and *B.sphaericus*.80% mortality rate was produced with the doses 50 µl of both *B.thuringiensis* and *B.sphaericus*. This results fits well with those from **[38]** they demonstrated that, 100% mortality of the mosquito larvae was at its peak within 24h post-treatment

by endotoxins of two strain of *B.thuringiensis* 1and 2 isolates and within 48h for endotoxins of *B.sphaericus1*.

In the present study, we found as the concentration of *Bacillus thuringiensis* var. *israelensis* increased the mortality rate of also Anopheles larvae increased.

Bacillus thuringiensis var. israelensis is toxic to mosquitoes and their toxicity is commonly imputed to the parasporal endotoxins, which are produced during sporulation time. The larvae to accomplish toxicity assimilate these endotoxins. *Bacillus thuringiensis* var. *israelensis* also produce different insecticidal crystal proteins (endotoxins), and their toxicity has been determined **[10, 35]**. These crystalline inclusion is solubilized in the midgut, releasing endotoxins activated by midgut protease then interact with larval apical midgut brush border membrane causing a disruption leading to insect death.[11,5].

Nevertheless, biotic and abiotic factors influence the larvicidal activity of *Bacillus* thuringiensis var. israelensis based on mosquito species, their respective feeding strategies, rate of ingestion, age and density of larvae, habitat factors (temperature, depth of water, turbidity), formulation factors (type of formulation, toxin content, and how effectively the material reaches the target), means of application and frequency of treatments**. [39]**.

The present research investigated the physical factors of *Bacillus* spore/crystal complex to pH (power of hydrogen.) change, UV (ultra violet) irradiation and temperature effect. In addition, larvicidal activity was followed against *Anopheles arabeinsis* mosquito larvae in the laboratory. In the current study, *Bacillus* metabolites were inactive after 12 hrs. of UV exposure. This observation agrees with the results of **[16]** who confirmed that the *Bacillus* formulation degraded after they were exposed to 12 hrs UV light, by decreasing in the mortality rate against mosquito larvae. [21, 27, 1] also reported similar reduction in the potency of B.thuringiensis preparation due to exposure to UV light**.** This may be attributed to damage to the DNA of spore as well as δendotoxin.

[16] have suggested that aluminum carboxymethylcellulose, carrageenan and alginate encapsulated forms of *B.sphaericus* and *B. thuringiensis* var. When *israelensis* are exposed to different high temperature, pH levels and UV radiation, they might be used more efficiently than their free forms in the control of mosquito larvae.

Moreover, this work showed effect of pH on the larvicidal activity of *Bacillus* on mosquito larvae, we found 60 % mortality rate was recorded at pH 8.0. Our results were correlated with [28] who performed maximum larvicidal efficacy of *Bacillus* species was at pH 9. 0. On the other hand, they recorded that a gradual decrease in mortality was noticed at higher pH11.0 against *Anopheles* mosquito larvae. Moreover, [26] measured the effects of pH on the crystal of *Bacillus thuringiensis*. Efficacy, by larval mortality under three pH conditions 6.1, 7.4, and 8.5. High levels of mortalities were obtained at pH7.4.

Difference in larvicidal potency of the tested *Bacillus* at different temperature values was observed, at (40^0 C) the percentage mortality recorded after 24hrs. and 48 hrs. of treatment were 20%and 60% respectively. Also [28] reported 68% mortality at 48hrs. in temperature (40^0 C) against Anopheles mosquito larvae. Besides all the above cited, we believe that three factors are contributing to the potency of *B. thuringiensis* toxins: the origin of the toxin (strain), the ability of the mid-gut juice to dissolve the crystals, and the intrinsic susceptibility of the insect to the toxin.

Therefore, an optimal relationship between the insect and the toxin is required for greater efficacy.

CONCLUSION:

In conclusion, the use of chemical insecticides to kill mosquito larvae poses harm to the environment and ecosystem at large, hence the need for an alternative, which is environmentally friendlier, cheaper and more effective. A study confirms that *B. thuringiensis* and *B. sphaericus* are highly potent against *An.arabiensis* larvae, although they have a short residual efficacy by the assessed physical parameters. Therefore, more studies should be carried out on the use of utilizing mosquitocidal bacteria as *B. thuringiensis B. sphaericus* as commercial mosquito larvicidal and biological controlling agents in the tropical countries such as Sudan where malaria is endemic to reduce the risk of malaria transmission.

Abbreviations:

Bt: *B*acillus *thuringiensis*

pH: power of hydrogen

UV: ultra violet

Cry: Crystal

ICPs: insecticidal crystal proteins

kDa: Kilo Dalton

Bin: binary

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