The Larvicidal effect of four fungal aflatoxins against fourth instar larvae of the mosquito *Culex quinquefasciatus* Say (Culicidae: Diptera)

التأثير السمي لأربع سموم فطرية ضد يرقات العمر الرابع لبعوض *Culex quinquefasciatus* Say (Culicidae: Diptera)

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Abstract.

The aflatoxins of four different soil fungi Aspergillus terreus, A. niger, A. oryazae, and Penicillium chrysogenum were tested for the larvicidal activity against fourth instar larvae of mosquito vector Culex quinquefasciatus. The concentrations of fungal culture filtrates used in the study ranged from 0.25 to 1 mg/ml. The results showed that the larval mortality percent gradually increased with the exposure periods 24, 48 and 72 hours. Among the four fungal species tested aflatoxin extracted from A. terreus was highly toxic to larvae of Cx. quinquefasciatus, at concentration 1mg/ml the larval mortality percent was (90%) when compared to other three species of fungal culture filtrates. A. niger, P. chrysogenum and A. oryazae were (70.56, 25.56, and 0 %) after 48h respectively. The Electronic Microscopic tests of death larvae indicated that disorganized, shortened and damaged surface cuticle appeared after 48 h in fourth instar larvae of mosquito Cx. quinquefasciatus when treated with A. terreus aflatoxin compared to control treatments.

الملخص:-

تم اختبار اربع سموم فطرية مستخلصة من اربع انواع مختلفة من فطريات التربة Aspergillus terreus , A. niger A. oryazae, و Penicillium chrysogenum لأختبار الفعالية السمية ضد يرقات العمر الرابع للنوع Culex quinquefasciatus اذ تراوحت تراكيز المبيد الفطري المستخدمة في هذه الدراسة من 0.25 mg/ml الى 1 mg/ ml. بينت النتائج ان النسبة المئوية للهلاك اليرقي تزداد تدريجيا مع ازدياد فترات التعرض ولوحظ بعد 24,48 ,72 ساعة. ومن بين الانواع الاربعة التي تم اختبارها وجد ان السم المستخلص من النوع A. terreus هو الأكثر سميا ليرقات الطور الرابع للنوع Cx. quinquefasciatus اذ ففي التركيز 1mg/ml كانت نسبة الهلاك اليرقي % 90 مقارنة بالأنواع الاخرى تحت الدراسة حيث كانت نسبة الهلاك اليرقي %(70.56، 25.56 و 0)عند المعاملة للأنواع A. niger, P. chrysogenum, A. oryazae على التوالي بعد 48 ساعة. دلت نتائج الفحص المايكروسكوبي بالمجهر الالكتروني الماسح لليرقات الميتة الى ظهور خلل وانكماش و تلف الكيوتكل السطحي ليرقات الطور الرابع عند المعاملة بالمبيد الفطري للنوع A. terreus مقارنة بمعاملات السيطرة.

Introduction:-

Mosquitoes which are responsible for the transmission of more diseases than any other groups of arthropods play an important role as vectors of malaria, filariasis, dengue, yellow fever, Japanese encephalitis and other viral diseases (1). The management of larvae through the use of larvicides is an ideal method for controlling mosquitoes by reducing mosquito breeding (2). Since “adulticides” may only reduce the adult population temporarily, most mosquito control programmes target the larval stage in their breeding sites with larvicides (3, 4). *Culex quinquefasciatus* transmits filariasis and is predominantly found in the tropics and the warm temperate regions. The indiscriminate uses of chemical insecticides have adverse effects based on interference measures for the control of mosquito vectors. This has received wide public fear because of several problems like insecticide resistance, resurgence of pest species, environmental pollution, toxic hazards to humans and non-

target organisms. These problems have necessitated the need to explore and develop alternative strategies using ecofriendly, bio-degradable bio-products which are less toxic to non-target organisms too. Fungi and fungus-derived products are highly toxic to mosquitoes, yet have low toxicity to non-target organisms. Accordingly the use of entomophagous fungi and their derived products may be a promising approach for biological control of mosquitoes (5). Extracellular secondary metabolites from many fungi have been screened for larvicidal activity against mosquitoes (6). The present study has been conducted to determine the toxicity of extracellular secondary metabolites or aflatoxins of four fungal culture filtrates against *Cx. quinquefasciatus* mosquito larvae under laboratory conditions.

Materials and Methods:-

1. Sample collection:-

Four fungal species namely Aspergillus terreus, Aspergillus niger, Aspergillus oryazae, and Penicillium chrysogenum isolated from the various soil samples collected from Al- kufa, Al-Manathera, Al-Abbassai and Al-Quzwini were evaluated.

2. Cultivation of fungi:-

Potato dextrose broth (200 g sucrose, 20 g/L dextrose, 15 g agarose, 40 mg/L Chloromphenchol, , 1000 ml distilled water) was used to grow the fungi(7). A loopful of fungal growth from an agar slant was transferred into 100 ml of growth medium in a 250 ml conical flask and incubated for 7 days at 25 C° (8). The culture was filtered through Whatman No-1 filter paper, the mycelial mass was discarded and the culture filtrate was used as a test material for larvicidal activity. The filterate was stored under refrigerator conditions. All four fungi were identified depending on (9, 10, and 8).

3. Tests of the ability of fungi to produce of Aflatoxins:-

Four fungal species were tested by cultivated and isolated on nutmeg media (100 g nutmeg, 300 ml distilled water, 1.5% agar and 40 mg/L Chloramphenicol) and incubated for 7 days (11).After incubation all plates were inverted with 0.1 ml ammonium solution 10% and closed then incubated for 4 days at 25 C°. Fungal colonies were observed with pale yellow or red indicated as aflatoxins productivity (12).Isolates of fungi were cultured in 100 ml PDA broth flasks and incubated in 25 $^{\circ}C$ for two weeks (13). The procedure of extraction was carried out depending on (14) and modified by (15).

4. Detection of aflatoxins in TLC plates:-

The TLC plates were used (provided from Silica Gel 60) for detect aflatoxins on fungal cultures with standard aflatoxins B1, B2 at concentration 5 ppm (provided from Fluka AG, Fabrik CII .9470 Buchs-switzerland company) used with chloroform/methanol 3/97. The standards and the samples were spotted at 1.0 cm from the bottom of the silica gel plates, and spaced 1.5 cm between each one. The aflatoxins detected by the presence of fluorescent rays and with compatible RF, colour and intensity of standard aflatoxins B1, B2 (16).

5. Chemical analysis:-

The chemical analysis techniques were performed according to those described by (17) and (16) for certified from the presence of aflatoxin B1 and B2 and used mixed of sulfuric acid 20% and ethanol alcohol for color test.

6. Collection and rearing of Mosquitoes:-

Culex quinquefasciatus larvae were collected from stagnant surface water of pools by a standard mosquito larval dipper with extendable handle and reared in plastic cups with water from the public water supply, under laboratory conditions of (27 ± 2) C°, (70 ± 5%) RH and 12 photoperiod. The larvae were reared in dechlorinated water and fed daily with finely grinded bread and yeast extract at a ratio of 3:2.Adults were maintained on a 10% sugar solution and females were also fed on pigeon (18). The identification of *Cx. quinquefasciatus* was carried out by PCR

technique and also certified by Natural History Museum / Baghdad University. The fourth instar larvae were used in bioassay tests.

7. Bioassays and larval mortality:-

In four replicates, ten larvae were pipetted into each 20 ml volume and observed for 24, 48 and 72 h when mortality was recorded. Different concentrations 1, 0.75, 0.5 and 0.25 mg/ml of test sample were used. Larvae maintained in distilled water served as a control. Statistical analysis of the data was carried out according to the method of Factorial Experiments within Completely Randomized Design (C.R.D) and means were compared by using Least Significant Difference Test (L.S.D) at (P= 0.05 %) level for showing the significant of results (19).When necessary, percentage mortality in the treatments was corrected for mortality in the controls using Abbott method (20).

8. Electron Microscopic test: -

The treated and untreated newly fourth larvae of *Cx. quinquefasciatus* were isolated from the standard laboratory colonies which were incorporated with 1mg/ml concentration of fungal aflatoxins after 24 h after exposure. The larvae were scanned using Scanning electron microscope (Inspect S50 / FEI Company / Netherlands) and photographed with a photomicroscope.

Results and discussion:-

1. Toxicity of fungal aflatoxins:-

The results of the larval susceptibility of *Cx quinquefasciatus* using four aflatoxins are presented in and Table (1) .The results of the present study revealed that that among four fungal aflatoxins tested, the aflatoxins of *A. terreus* were found to be more effective than *A. niger*, the percentage of mortality was reached (69.2%) compared with A. *niger,* was (57.4 %) whereas *P. chrysogenum* and *A. oryazae* were 25.5%, 0 % respectively.

It is clear from table (1,2 and 3) that the exposure periods of fungal aflatoxins were significantly affect the larval mortality of fourth instar larvae of *Cx. quinquefasciatus*, the mean of highest mortality percent (85.14%) was recorded after 72h when exposured to aflatoxin of *A. terreus*, and the mean of mortality percent were (72.6,50%) observed after 24,48h respectively.

Larval mortality percent also significantly increased with treated in aflatoxins of A. *niger,* and exposure period attribute to increase of larval mortality percent from (42.7%) to (50.9 %) after 24, 48h respectively. After 72 h larval mortality percent increased to (78.75%). The lower larval mortality percent was observed when treated with *P. chrysogenum* it was (18, 25.5%) after 48,72h respectively, Furthermore no larval mortality were recorded when treated with *A. oryazae* against fourth instar larvae of *Cx. quinquefasciatus* after 24,48, and 72 h table(1,2 and 3).

From data given in table (1) indicated the larvicidal activity of aflatoxins extract from *A. terreus,* against the fourth instar larvae of *Cx quinquefasciatus* was high toxic. High larval mortality percent was (76.17, 90, and 90%) caused at the highest concentrations (1mg/ ml) after 24, 48 72h respectively. Meanwhile, the larval mortality percent decreased to 25.56, 39.23 and70.56 % at the lowest concentrations (0.25 mg/ml) after 24, 48 72h respectively compared to 0.0% for the untreated larvae or control treatments.

As shown in table (1,2 and 3), when treated with aflatoxins of A. *niger,* the highest mortality percent (56.79, 70.56 and90%) was recorded at the concentration (1mg/ml) after 24, 48 and 72h respectively and the lowest mortality percent (18.44, 25.56 and 45%) was observed at the lowest concentration (0.25mg/ml) compared to 0.0% for the untreated larvae or control treatments. *P. chrysogenum* aflatoxins were less toxic against fourth instar larvae *Cx quinquefasciatus*. The larval mortality percent were (18, 25.56%) after 24,27h respectively. Furthermore no larval mortality was recorded when treated with *A. oryazae* against fourth instar larvae of *Cx. quinquefasciatus* after 24, 48, and 72 h.

Table (1) Larvicidal activity of four fungal toxins against fourth instar larvae of Cx. quinquefasciatus after 24 h. ,

Fungal toxins	Observed mortality in percentage after 24 hrs			
	1mg/ml	0.75 mg/ml	0.5mg/ml	0.25mg/ml
A. terreus	76.17	56.79	45	25.56
A. niger	56.79	50.77	45	18.44
A. oryazae	0	0	0	0
P. chrysogenum	18.44	0	0	0
Control	0	0	0	0

L.S.D. value at 0.05 =1.7

Table (2) Larvicidal activity of four fungal toxins against fourth instar larvae of Cx. quinquefasciatus after 48 h.

Fungal toxins	Observed mortality in percentage after 48 hrs			
	1mg/ml	0.75 mg/ml	0.5mg/ml	0.25mg/ml
A. terreus	90	90	71.56	39.23
A. niger	70.56	56.79	50.77	25.56
A. oryazae	0	0	0	0
P. chrysogenum	18.44	0	0	0
Control	0	0	0	0

L.S.D. value at 0.05 =1.7

Table (3) Larvicidal activity of four fungal toxins against fourth instar larvae of Cx. quinquefasciatus after 72h.

Fungal toxins	Observed mortality in percentage after 72 hrs			
	1mg/ml	0.75 mg/ml	0.5mg/ml	0.25mg/ml
A. terreus	90	90	90	70.56
A. niger	90	90	90	45
A. oryazae	0	0	0	0
P. chrysogenum	25.56	25.56	25.56	25.56
Control	0	0	0	0

L.S.D. value at 0.05 =1.7

2. Indication of Scanning electron microscope:-

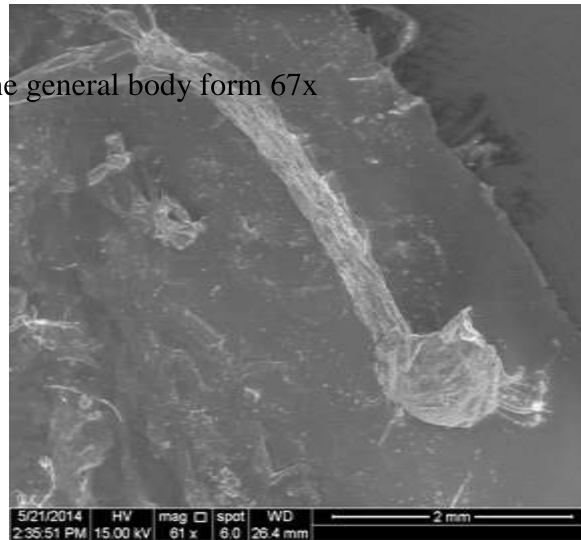
The present study examined a series of normal untreated and treated fourth instar larvae of *Cx. quinquefasciatus*. The normal general form of the head, thoracic and abdominal regions were scanned. The microscopic test was indicated that the outer membrane or cuticle and body form were normal and dilated (Photograph 1, 2).

Photograph (1) the morphology of normal untreated Cx. quinquefasciatus fourth instar larva showing the general body form 67x.

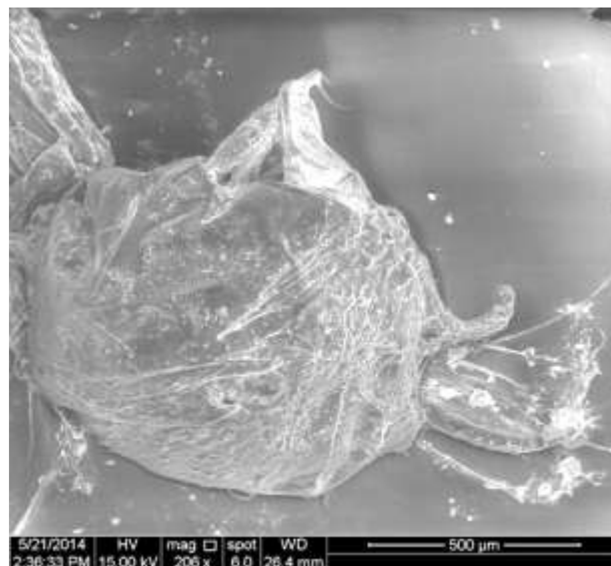
Photograph (2) the normal untreated *Cx. quinquefasciatus* fourth instar larva showing the head region 159x.

When treated with *A. terreus* aflatoxin all larvae appeared dramatic shrinkages affecting mainly the abdominal segments and head regions showing disorganized, shortened and damaged cuticle with morphology had become more destroyed after 48 h (Photograph 3, 4).

iatus fourth instar larva showing the general body form 67x



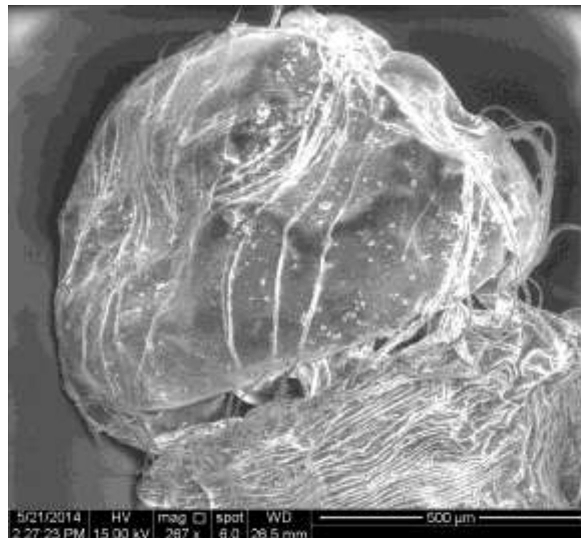
Photograph (3) Morphology of *Cx. quinquefasciatus* fourth instar larva treated with1mg/ml of *A. terreus* aflatoxin showing the effects after 48 h of exposure 61 x.



Photograph (4) destroyed head region of *Cx. quinquefasciatus* fourth instar larva treated with1mg/ml of *A. terreus* aflatoxin, showing the effects after 48 h of exposure 206 x.

When treated with *A. niger* aflatoxin all larvae appeared mild shrinkages affecting mainly the abdominal segments and head regions showing disorganized, shortened and damaged cuticle with morphology had become more destroyed after 48 h (Photograph 5, 6).

Photograph (5) Morphology of Cx. quinquefasciatus fourth instar larva treated with1mg/ml of A. niger aflatoxin showing the effects after 48 h of exposure 66 x.



Photograph (6) head region of *Cx. quinquefasciatus* fourth instar larva treated with1mg/ml of *A. niger* aflatoxin, showing the effects after 48 h of exposure 267 x.

The present study demonstrated larvicidal activity of fungal aflatoxins against fourth instar larvae of *Cx. quinquefasciatus*. When the mosquito larvae were treated with *A. terreus* aflatoxin a significant mortality of the larvae was noticed. However, when the concentration increases from 0.25 onwards, larval mortality was significantly increased. Among this concentration a less mortality rate was noticed in 0.25. Toxicity of the tested fungal aflatoxins against the fourth larval instar was varied according to fungal aflatoxins used and the aflatoxins concentration. The larval mortality percent increased as exposure periods increased for three fungal species *A.terreus. A. niger*, *P. chrysogenum* whereas no larval mortality were recorded when treated with *A. oryazae* against fourth instar larvae of *Cx. quinquefasciatus* after (24, 48, and 72 h). The toxicity values of

tested aflatoxins from three fungal species based on activity may be arranged in a descending order as follows: *A. terreus A. niger* and *P. chrysogenum.*

Fungi are a very complex groups with great morphological diversity and they affect or kill mosquitoes due to debilitation. Among the microbes, fungi and actinomycetes are best known for their ability to produce a great variety of secondary metabolites (21, 22). They have also proved themselves to be a source of powerful agents, which could be used in the control of pests and parasites. The biological properties of the secondary metabolites of fungi range from antibiotics to mycotoxins (21, 23). These have been reported to cause retardation of growth, low level of fecundity, loss of fertility, mortality, repellency, etc. Studied the ovicidal, larvicidal and adulticidal activities of the metabolites of fungi and actinomycetes against *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. The metabolites of 34 fungi and 3 actinomycetes, 133 fungi and 35 actinomycetes, and 17 fungi were found to kill the eggs, larvae and adults, respectively (6). The secondary metabolites of *Aspergillus*, *Penicillium*, *Fusarium*, *Paecilomyces*, *Rhizopus*, *Amanita, Syncephalastrum*, *Monilia* and *Tolypocladium* species have been reported to be toxic to mosquitoes (24, 25, and 26). (27) Reviewed the insecticidal activity of a Malaysian isolate of *A. niger*. Bioassays showed that *Ae. aegypti* larvae was the most susceptible with *An. maculatus* being the least susceptible while *Ae aegypti* and *Cx. quinquefasciatus* were susceptible to the 72 h *A. niger* supernatant culture but not to the 24 h and48 h culture and these results agree with the present results. (28) Showed that the larval mortality could be observed on 24 h and LC50 values of *A. flavus, A. parasiticus*, *P. falicum, F. vasinfectum* and *T. viride* were 38.34, 40.39, 44.97, 50.03 and 54.16 mg/L, respectively. Among the five different fungi, the culture filtrates of *A. flavus* were found to be more toxic than the other four species of fungi against *Cx. quinquefasciatus* and these findings agreement with our findings. Mycotoxins can cause toxicity to insects, including insecticidal effects and developmental delay. There is variable sensitivity of insects to aflatoxin toxicity, initially examined in the fruit fly *Drosophila melanogaster* (29) who Described toxicity of AFB1 to *Helicoverpa zea* larvae at different larval stages. For newly hatched first instar larvae, 20 ng/g AFB1 caused 50% mortality while a higher concentration, 200 ng/g AFB1, caused 100% mortality.

The principal mode of action in most cases is obstruction of air passage through the two tracheal trunks of the larvae. In mosquito larvae all air enters the insect through two spiracles at the tip of the siphon. These spiracles are surrounded by perispiracular valves regulated by muscles. *Metarrhizium* conidia adhere to these valves, germinate and penetrate through the cuticle into the hemocoel. The spiracles become obstructed in the process, and the lack of air and perhaps toxins produced by the fungus in the hemolymph, causes death of the host (22).

The results of present study revealed that *A. oryazae* aflatoxin could not be toxic against fourth instar larvae of *Cx. quinquefasciatus* and this might to the strain of *A. oryazae* did not produce aflatoxins B1, B2, G1 and G2. The non-occurrence of aflatoxins in *A. oryzae* cultures was demonstrated by other authors (30, 31, and 9). Previous work showed a number of mutations within the aflatoxin biosynthesis gene homolog cluster in *A. oryzae* relative to the *A. flavus* sequence, including deletions, frameshift mutations, and base pair substitutions, which induce inactivation at the protein level and consequently impair the aflatoxin synthesis (32, 33).

The Electronic Microscopic studies revealed the association of two fungi aflatoxins extracted from *A. terreus* and *A. niger* effect on different parts, legs, antennae and whole body surface of fourth larvae of *Cx. quinquefasciatus.*

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