STUDIES ON SOME BLUE-GREEN ALGAL EXTRACTS AS ANTIMICROBIAL AGENTS OF *OREOCHROMIS NILOTICUS*

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Abstract

Oscillatoria curviceps and *Anabaena wisconsinense* (blue-green algae - Cyanobacteria) were isolated from Abbassa fish ponds. The growth of *O. curviceps* showed an exponential increase through the first 14 days, and *.A. wisconsinense* increased exponentially through the first 16 days. *O. curviceps* and *A. wisconsinense* were cultured, harvested, dried and extracted by methanol and ethanol using Soxhlet extractor. The biological activity of both algae showed that, methanolic *O. curviceps* extract had an inhibitory effect with *Lactobacillus* sp., *Aspergillus niger* and *Pseudomonas anguilliseptica*. Ethanolic *O. curviceps* extract had an inhibitory effect with *Lactobacillus sp., Aeromonas hydrophilia, Ps. anguilliseptica, Bacillus firmus, Ps. fluorecence* and *Aspergillus niger* compared with control groups. Methanolic *A. wisconsinense* extract inhibited *Ps. anguilliseptica, A. hydrophila, B. firmus, Ps. fluoresence* and *Aspergillus niger*. Ethanolic *A. wisconsinense* extract had an inhibitory effect on *A. hydrophilia, Aspergillus niger, B. firmus* and *Ps. fluoresence* compared with control groups.Methanolic *A. wisconsinense* extract was highly efficient against *Ps. anguilliseptica* where the inhibition zone 50 mm in diameter. Methanolic *A. wisconsinense* extract decreased the mortality of *O. niloticus* from 50 to 15% at 72 hours post methanolic *A. wisconsinense* extract injection to *Ps. anguilliseptica* infected *O. niloticus.*

INTRODUCTION

Algae are important components of aquatic ecosystems and are used as primary source for fish feeding. The blue-green algae have protein, vitamin B12, vitamin E, carbohydrates as rhamnose, fractose, ribose, mannose and some minerals like copper, magnesium, zinc, potassium and iron, and γ-linolenic acid. Also, it contains phycomycin, polysaccharides and toxins which have biological activity (Ray *et al.*, 2007). Marine and/or freshwater algae are one of the richest sources of bioactive compounds. These compounds may be used as antimicrobial agents. These compounds are simply referring to all types of natural, semi synthetic or synthetic substances which are capable of killing or inhibiting the growth of microorganisms. These antimicrobial agents include antibiotics, antiviral, antifungal, probiotics and feed preservatives (Katircioglu, *et al.,* 2006).

Fish diseases are major problems for the fish farming industry. Bacterial infections are considered the major causes of mortality in fish hatcheries (Grisez and Ollevier,1995). The use of antibiotics in aquaculture may cause potential hazard to public health and environment by the emergence of drug-resistant microorganisms and antibiotic residues. Furthermore, the normal microbial flora in digestive tract, which is beneficial to fish, may also kill or inhibited by oral chemotherapy (Sugita *et al.*, 1991).

Cyanobacteria have proved to produce antifungal and antibacterial substances. Bioactive substances from Cyanobacteria can be extracted from the biomass using organic solvents. The antimicrobial effects are visualized in bioassay using selected microorganisms (Frakmölle *et al.*, 1992).

The aim of present work is concerning with the antibacterial and antifungal effect from the isolated algae in Abbassa Fish Farm. The extracts from such algae were used as natural source as antibiotic against the pathogenic bacteria in cultured fish.

MATERIALS AND METHODS

Isolation and identification of Cyanobacteria

Six samples of ponds water were collected randomly from Abbassa Fish Farm for isolation and purification of Cyanobacteria. The medium used for algal isolation was BG11 medium with Trace metal solution (Rippka *et al.*, 1979). The identification of algae was undertaken microscopically for shape and their structure according to Komarek and Fott (1983).

Dry weight determination of Cyanobacteria

A definite volume of algal suspension (20 ml) was centrifuged at 3000 rpm for 10 minutes. The cells after being precipitated were washed twice with distilled water. Biomass was transferred to a pre-weighed dry filter paper then placed in an oven at 60 ^{o}C overnight to reach a fixed weight using the method of Leganes *et al.* (1987).

Cyanobacteria mass culture

The culture volume ranged from 2 liter flasks, to 20 liter carboys. The temperature of the algae room was 25 ± 2 $^{\circ}$C and illumination was constant, provided by florescent tubes (3000 lux). The BG11 medium was used for all stages of cultures for the organisms that were used in extraction according to Rippka *et al.* (1979).

Algal culture was obtained by cultivation of Cyanobacteria in 250 ml Erlenmeyer flasks, where each flask contained 150 ml of medium. The culture flasks were stoppered with cotton plugs and sterilized in autoclave at 121.5 $^{\circ}$C for 20 minutes. After cooling the Erlenmeyer flasks they were inoculated with 30 ml of the pre-culture of blue green-algae. The culture flasks were aerated by air pumps and incubated at 25 ± 2 $^{\circ}$C under continuous illumination provided by white fluorescent lambs (3000 lux). Other one liter flasks were prepared containing 500 ml. of the BG11 medium, the culture flasks were stoppered and sterilized. After

cooling, inoculated with 100 ml of pre-culture and this process were repeated till it reached 1500 ml from pre-culture enough to inoculate the carboy.

Carboy culture

These cultures were originally housed in glass carboys which hold 20-liter. The carboys were filled with 19 liters of tap water. The water and carboy were disinfected with commercials Clorox (5 ml) and neutralized with sodium thiosulfate (0.2 g) after 4 hours.

Processing the algal biomass, Harvesting and extraction

In stationary phase, the algal culture reached maximum growth. Before this stage the circulation provided by the pumping system was stopped and the algal cells were harvested by filtrating the carboy using a cheese cloth of cotton or nylon. The algal cells were dried in oven at 60°C and ground in an electric coffee mill. Resulting powder was submitted to lipid-soluble extraction with ethanol and methanol 1:15 (algal powder: solvent volume) using a Soxhlet Extractor at 55-60°C. All samples were refluxed until saturation (24 hours) and the respective extracts were dried in an oven at 50°C (José Vitor *et al.,* 2002).

The antimicrobial activity of extractions (In vitro)

The algal extractions were examined for its antimicrobial activity against fungi (*Saprolegnia parasitica* and *Aspergillus niger*) and pathogenic bacteria (*Lactobacillus* species, *Bacillus firmus*, *Pseudomonas anguilliseptica*, *Ps. fluorecence* and *Aeromonas hydrophila*). The pathogenic bacteria were isolated from diseased *Oreochromis niloticus* and will identify according to Austin and Austin (1993). *Aspergillus niger* was isolated from musculature of apparently healthy *Cypriuus carpio*. The pathogenic bacteria were examined for its pathogencity. *Saprolegnia parasitica* was kindly obtained from Fish Diseases Department, Central Lab for Aquaculture Research in Abbassa, Agriculture Research Center, Egypt. It was isolated previously from diseased *O. niloticus*.

1-Paper disk assay

Under aseptic conditions, the Petri dishes of tryptic soy agar were used for testing the algal extracts activity against bacteria. The plates were inoculated with 0.1ml of fresh bacterial suspension (24 hrs), immediately sterilized paper discs impregnated with 30 ml of each algal extract and air dried. The paper discs were placed over the agar surface. The plates incubated at 28°C for 24 hrs. and examined for inhibition zones. Also, sterilized paper discs were impregnated in the solvents and used as a control according to Bauer *et al.* (1966)

2-Hollow well technique (agar-well)

This method was used for fungi. In sterilized Petri dish, about three spherical parts of sterilized glass beads, were placed and the medium of Tetracycline glucose of fungi was added around the beads. After solidification, the glass beads were removed leaving a hole. In each hole, about 250 µl from the algal extract was poured. Then the plates were incubated at 28°C for 72 hrs. and examined for inhibition zones. Also, holes containing only the solvent were used as a control according to Amer (2002).

Efficiency of algal extract in vivo

Algal extract was chosen according to the result of in-vitro sensitivity test. Eighty *O. niloticus* were allotted into 4 equal groups. The first group was injected intraperitoneally **(**I/P**)** with 0.2 ml of saline suspension containing10^7/ml *Ps. anguilliseptica* and kept for 18 hr. prior to *Anabaena wisconsinense* methanolic extract once I/P injection at a dose of 10 mg / Kg body weight. (Abd El-Rhaman and El-Ashram, 2005). The second group was kept as infected by *Ps. anguilliseptica* and non-treated by the *A. wisconsinense* methanolic extract. The third group was left as non-infected and injected I/P by 10 mg / Kg body weight of such extract. The fourth group was maintained as non-infected with *Ps. anguilliseptica* and non-treated by *A. wisconsinense* methanolic extract. All groups were observed for 21 days post-injection.

RESULTS AND DISCUSSION

Algae are important components of aquatic ecosystems and are used as primary source for feeding fish. Cyanobacteria which isolated from Abbassa Fish ponds were identified as *Oscillatoria curviceps* and *Anabaena wisconsinense.*

O. curviceps is an unbranched filament composed of disk-shaped cells arranged in a single series (photo, 1). *Oscillatoria* often grows in mats of intermoven filaments.

A. wisconsinense is a typical heterocystous Cyanobacterium, filaments in bundles surrounded by a mucilaginous sheath, tapering filaments with heterocyst at their base, *Anabaena* is unbranched filament sometimes give the appearance of a string of beads (photo, 2) as described by Komarek and Fott (1983**).** While Abd El-Tawwab (1994) isolated and purified two kinds of Cyanobacteria, *Anabeana variabilis* and *Oscillatoria* sp. from water of fish ponds of (CLAR) at Abbassa, Sharkia.

The growth curve of *Oscillatoria curviceps* and *Anabaena wisconsinense*

It is evident from Fig. (1) that, the growth of *O. curviceps* showed an exponential increase through the first 14 days, thereafter, decline phase began and continued till the 16th day. On the other hand, *A. wisconsinense* increased exponentially through the first 16 days. After that the growth phase declined and continued till the 18^{th} day.

Microalgae are rich in many specific and attractive compounds, some of which are very interesting as nutritional supplements, such as long-chain polyunsaturated fatty acids. Other nutriceuticals derived from algae are vitamins and antioxidants, such as β-carotene and astaxanthin. Microalgae are also used in the pharmaceutical market as they contain sterols, which can be used as building blocks for pharmaceuticals (hormones). Furthermore, Cyanobacteria are a potential source of compounds with biomedical applications, such as antimicrobial, antiviral and anticancer compounds (AlgaeLink, 2007).

Antimicrobial activities of *O. curviceps* extracts

Methanolic extract of *O. curviceps* caused an inhibition zones 34, 0.0, 4.0, 16, 0.0, 20, and 0.0 mm in diameter with *Lactobacillus* sp., *Pseudomonas fluorescence, Aeromonas hydrophila, Pseudomonas anguilliseptica, Bacillus firmus, Aspergillus niger* and *Saprolegnia parasitica* respectively. While, the inhibition zone with methanol alone as control were 4.0, 0.0, 6.0, 10, 8.0, 0.0 and 0.0 mm in diameter in the same manner respectively as shown in (Table, 1).

Ethanolic extract of *O. curviceps* caused inhibition zones 30, 6, 30, 20, 14, 28 and 0.0 mm diameter with *Lactobacillus* sp., *Ps. fluorescence, A. hydrophilic, Ps. anguilliseptica, B. firmus, Aspergillus niger* and *S. parasitica* respectively. The inhibition zone with Ethanol alone as control were 6.0, 6.0, 6.0, 6.0, 8.0, 0.0 and 0.0 mm in diameter respectively as shown in (Table,1).

Antimicrobial activities of *A. wisconsinense* extracts

The diameter of inhibition zones due to methanolic *A. wisconsinense* extract were 0.0, 30, 32, 50, 32, 20 and 0.0 mm with *Lactobacillus* sp., *Ps. fluorescence, A. hydrophila, Ps. anguilliseptica,* (photo, 3), *B. firmus, Aspergillus niger* and *S. parasitica* respectively. While, the inhibition zone with methanol alone as control were 4.0, 0.0, 6.0, 10, 8.0, 0.0 and 0.0 mm in diameters respectively as shown in (Table, 2).

Ethanolic extract of *A. wisconsinense* gave inhibition zones 0.0, 12 , 34, 0.0, 24, 26 and 0.0 mm diameter with *Lactobacillus* sp., *Ps. fluorescence, A. hydrophila, Ps. anguilliseptica, B. firmus Aspergillus niger,* and *S parasitica* respectively. The control groups of ethanol gave inhibition zones 6.0, 6.0, 6.0, 6.0, 8.0, 0.0 and 0.0 mm in diameter respectively as shown in (Table, 2).

The present results showed that the alcoholic extract especially methanolic extract had antimicrobial activity against Gram-negative and Gram-positive bacteria (*Ps. anguilliseptica, Ps. fluorecence,. A. hydrophila, B. firmus* and *Lactobacillus sp.*) and also against *Aspergillus niger.* These results are in agreement with the results found by Katircioglu, *et al.* (2006) who recorded that the alcoholic algal extracts of

Anabaena sp. and *Oscllatoria* sp. which isolated from freshwater in Turkey had antimicrobial activity against Gram-negative and Gram-positive bacteria. The antimicrobial activity of microalgae could be explained by the presence of cyclic peptides, alkaloids and lipopolysaccharides. Also, Zienb *et al.* (2004) and Prashantkumar *et al.* (2006) recorded that the methanolic and ethanolic extracts had inhibitory effect against Gram-negative and Gram-postive bacteria and fungi from marine algae. Sastry and Rao (1994) found that the Choloroformic algal extract had greatest antibacterial activity which disagreed with our results.

Carmichael *et al.* (1990) and Oufdou *et al.,* (2001) recorded that natural toxins from Cyanobacteria *(Lyngbya, Oscillatoria* and *Anabaena)* have antifungal, antibacterial, antiprotozoan, and antineoplastic activity. The antimicrobial activity to methanolic algal extract attributed to their phenolic compounds (Decano *et al.*, 1990).

Efficiency of *A. wisconsinense* methanolic extract against the pathogenic *Ps. anguilliseptica* in *O. niloticus*

According to the invitro activity of the results of algal extracts, we chose methanolic *A. wisconsinense* extract in the treatment of *O. niloticus* infected by *Ps. anguilliseptica*. From table (3) methanolic *A. wisconsinense* extract decreased the mortalities of *O. niloticus* from 50% during infection to 15% at 72 hours post treatment. No bacteria were isolated from any fish that survived to the end of the trial. On the contrary, *Ps. anguilliseptica* was isolated from dead fish. Neither mortalities nor *Ps. anguilliseptica* were detected in the non-infected and treated group. While the mortality rate of non-treated and infected group was 80% throughout the experiment. This means that the algal extracts were active invitro and invivo.

Successful control of bacterial diseases of cultured fish is a major management and preventing economic problems for aquaculture industry (Plumb, 1999). Recently, probiotic and prebiotic are receiving attention as a candidate to improve the fish health and the prevention of bacterial diseases for environment

friendly aquaculture. Austin *et al.* (1992) indicated that extracts derived from *Tetraselmis suecica* were observed to inhibit Gram-negative bacteria invitro, and when used as a food supplement, the algal cells inhibited laboratory –induced infections in Atlantic salmon.

So, the biological control is essential for success of aquaculture operations, while the inadequate management is the principle factor in triggering disease outbreaks (Plumb 1999).

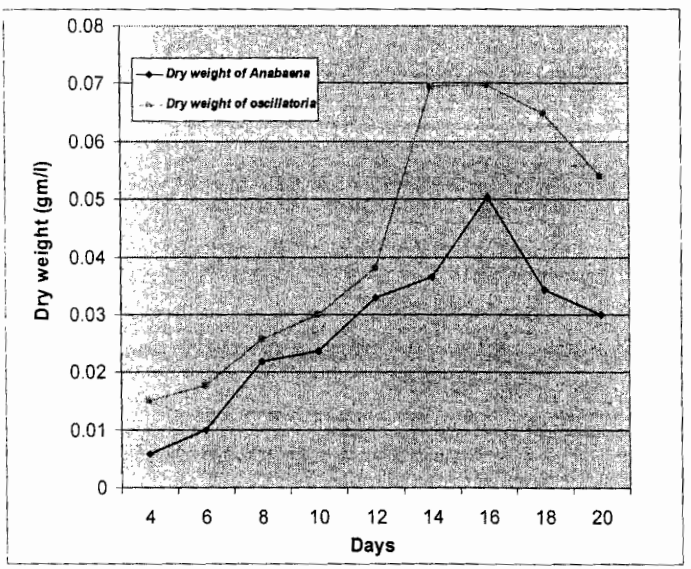


Figure 1. Growth curve of *Anabaena wisconsinense* and *Oscillatoria curviceps*

Table 1. The antimicrobial activities of *Oscillatoria curviceps* extract against different pathogen microbs.

Microbes	Quantity of extract (ml)	Inhibition zone due to the algal extracts (mm)			
		Methanol		Ethanol	
		A	C	A	C
Pathogenic bacteria					
Lactobacillus sp.	30	34.0	4.0	30.0	6.0
Pseudomonas fluoresence	30	0.0	0.0	6.0	6.0
Aeromonas hydrophila	30	4.0	6.0	30.0	6.0
Pseudomonas anguilliseptica	30	16.0	10.0	20.0	6.0
Bacillus firmus	30	0.0	8.0	14.0	8.0
Pathogenic Fungi					
Aspergillus niger	250	20.0	0.0	28.0	0.0
Saprolegnia parasitica	250	0.0	0.0	0.0	0.0

A= the algal extract C= the control.

Table 2. The antimicrobial activities of *Anabaena wisconsinense* extracts against different pathogenic microbes.

Microbes	Quantity of extract (ml)	Inhibition zone due to the algal extracts(mm)			
		Methanol		Ethanol	
		A	C	A	C
Pathogenic bacteria					
Lactobacillus sp.	30	0.0	4.0	0.0	6.0
Pseudomonas fluorecence	30	30.0	0.0	12.0	6.0
Aeromonas hydrophilia	30	32.0	6.0	34.0	6.0
Pseudomonas anguilliseptica	30	50.0	10.0	0.0	6.0
Bacillus firmus	30	32.0	8.0	24.0	8.0
Pathogenic Fungi					
Aspergillus niger	250	20.0	0.0	26.0	0.0
Saprolegnia parasitica	250	0.0	0.0	0.0	0.0

Table 3. Mortality rate of the experimentally *O. niloticus* treated with methanolic *A. wisconsinense* extract for controlling *Ps. anguilliseptica* infection.

parameters		Treated & infected	Non treated &infected	Treated & no infected	Non treated & non infected
No. of examined fish		20	20	20	20
Mortality rate	before treatment	50%			
	after treatment	15%	50%	0.0	0.0

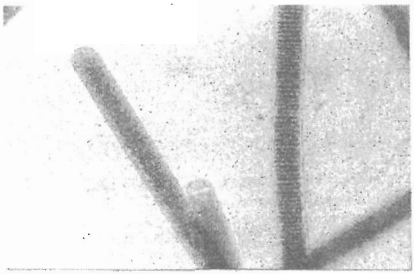


Photo 1. Showing *Oscillatoria curvicep wisconsinense* 400x

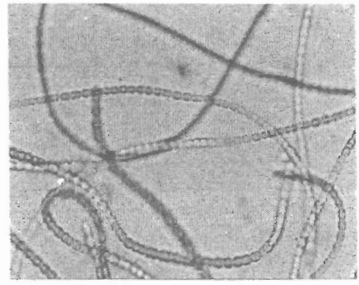
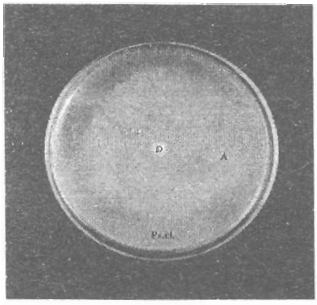


Photo 2. Showing *Anabaena* 400x



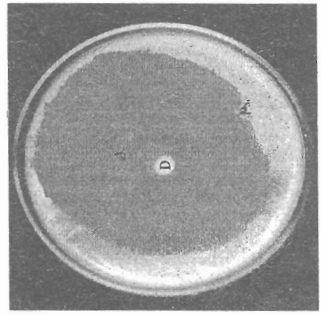


Photo 3. Methanolic *Anabaena wisconsinense* extract against a= *Pseudomonas anguilliseptica* and b= *Pseudomonas fluorecence*
D: Methanolic *Anabaena wisconsinense* extract paper disc.
A: inhibition zone measured as mm. Ps.: *Pseudomonas* species growth.

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دراسات على بعض مستخلصات الطحالب الخضراء المزرقة كمضاد لميكروبات أسماك البلطي النيلي

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١- *قسم أمراض الأسماك ،المعمل المركزي لبحوث الثروة السمكية بالعباسة –مركز البحوث الزراعية*
٢- *قسم الليمنولوجي ، ،المعمل المركزي لبحوث الثروة السمكية بالعباسة –مركز البحوث الزراعية*
٣- *الطحالب- قسم النبات- كلية العلوم- جامعة بنها*
٤- *الطحالب- قسم النبات- كلية العلوم- جامعة طنطا*

تم عزل نوعين من الطحالب الخضراء المزرقة من الأحواض الترابية بالمعمل المركزي لبحوث الأسماك بالعباسة وتم تصنيفهم إلي اسيلاتوريا كرفيسبس و الانابينا وسكونينس. وكان منحنى نمو اسيلاتوريا كرفيسبس فى الفترة بين (١٤: ١٦ يوم) وبالنسبة للانابينا وسكونينس تراوحت من (١٦:١٨يوم). حيث تم تجميع الطحالب قبل هذه الفترة مباشرة وبعد التجفيف تم استخلاصها بمذيبات عضوية وهى (الإيثانول والميثانول) وذلك باستخدام جهاز السوكسليت.

وقد تم اختبار نشاط هذه المستخلصات ضد البكتيريا الممرضة للأسماك وكذلك الفطريات وكانت النتائج كما يلي:

١- مستخلص الكحول المثيلى لطحلب اسيلاتوريا كرفيسبس أدى إلي تثبيط لنمو البكتيريا (اللاكتوبسلس، السيدوموناس أنجويلى سيبتكا و الأروموناس هيدروفيلا) معطيا مناطق تثبيط قطرها ٣٤، ١٦ ،٤٠ مم على التوالي . كما أظهر نشاط ضد فطر الأسبرجلس نيجر معطيا منطقة مثبطة قطرها ٢٠مم.

٢- عند استخدام المستخلص الأيثيلى لطحلب اسيلاتوريا كرفيسبس حدث تثبيط لنمو البكتريا (اللاكتوبسلس ، الأروموناس هيدروفيلا ، السيدوموناس أنجويلى سيبتكا ، الباسلس فيرمس والسيدوموناس فلورسينس) معطيا مناطق تثبيط قطرها ٣٠ ،٣٠ ،٢٠ ،١٤ ،٦ مم على التوالي. كما أظهر نشاط ضد فطر الأسبرجلس نيجر معطيا منطقة مثبطة قطرها ٢٨مم .

٣- المستخلص المثيلى لطحلب الأنابينا وسكونينس أحدث تثبيط لنمو البكتيريا (السيدوموناس أنجويلى سيبتكا، الأروموناس هيدروفيلا ، الباسلس فيرمس والسيدوموناس فلورسينس) معطيا

مناطق تثبيط قطرها ٥٠ ،٣٠،٣٢،٣٢مم علي التوالي. كما أظهر نشاط ضد فطر الأسبرجلس نيجر معطيا منطقة مثبطة قطرها ٢٠مم.

٤- المستخلص الأيثيلى لطحلب الأنابينا وسكونينس احدث تثبيط لنمو البكتيريا (الأروموناس هيدروفيلا، الباسلس فيرمس والسيدوموناس فلورسينس) معطيا مناطق تثبيط النمو البكتيري قطرها ٣٤، ٢٤، ١٢مم على التوالي. كما أظهر نشاط ضد فطر الأسبرجلس نيجر معطيا منطقة تثبيط قطرها ٢٦مم.

مما سبق فقد تم اختيار المستخلص المثيلى لطحلب الأنابينا وسكونينس لدراسة نشاطه ضد البكتيريا بعد عمل عدوى صناعية لأسماك البلطي النيلي السليمة ظاهريا حيث تم حقنها ببكتيريا السيدوموناس أنجويلى سيبتكا في البريتون (٠,٢×١٠^{٧}خلية/مللي). وبعد ثمانية عشر ساعة تم حقن هذه الأسماك بالمستخلص المثيلى لطحلب الانابينا فأظهرت النتائج عدم ظهور أي علامات مرضية للأسماك المحقونة بالمستخلص ولكن نسبة النفوق تناقصت من ٥٠ إلى ١٥%. بينما كانت نسبة النفوق ٨٠% في أسماك البلطي النيلي المحقونة صناعيا بالبكتيريا فقط. ونوصى بمزيد الدراسة.