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Full Length Research Paper

Antibiotic activity of two *Anabaena* species against four fish pathogenic *Aeromonas* species

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Three organic extracts (chloroform, ethyl acetate and n-butanol) of ten cyanobacterial species (*Anabaena solitaria, Anabaena variabilis, Anabaena cylindrical, Anabaena spiroides, Anabaena circinalis, Oscillatoria ornate, Oscillatoria salins, Oscillatoria tenuis, Oscillatoria rubescens* and *Oscillatoria prolifica*) were investigated for their antibacterial activities against 4 fish pathogenic bacterial species belonging to genus *Aeromonas*, namely, *Aer. salmonicida, Aer. hydrophila, Aer. formicans* and *Aer. liquefaciens* using oxytetracycline as reference antibiotic. Of all cyanobacterial samples, only ethyl acetate extracts of *A. variabilis* and *A. circinalis* were proved to be the most effective against all tested *Aeromonas* species. Bioautographic investigation for 13 organic solvent systems indicate that the spot with R_f = 0.79 for *A. variabilis* and that of R_f = 0.84 for *A. circinalis* were the most effective. The median lethal doses (LD_{50}) for the purified antibacterial compounds against mice were 246.67 and 231.67 mg/kg for that extracted from *A. variabilis* and *A. circinalis,* respectively.

Key words: Cyanobacteria, *anabaena*, antibacterial activity, fish pathogenic *Aeromonas* species.

INTRODUCTION

Documentation is required to evaluate the use of bio-control system as an alternative method for inhibition the fish pathogenic bacteria in infected fish farms (Anne-Marie et al., 2003). Fishes are susceptible to a wide variety of bacterial pathogens (Schmidt et al., 2000). Many of these bacteria capable of causing disease are considered by some to be saprophytic in nature (Toranzo et al., 2005). These bacteria only become pathogens when fishes are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to proceed (Anderson, 1995).

Bacterial fish diseases caused by *Aeromonas* species or other microorganisms lead every year to considerable economic losses in aquaculture. Antibiotic treatment of bacterial diseases in fish culture has been applied for many years. The occurrence of antibiotic resistant bacteria associated with fish diseases is a worldwide problem in aquaculture, which has received considerable attention in the last years and continues to increase due to the absence of a more effective and safer use of antibiotics (McPhearson et al., 1991; Smith et al., 1994.).

New antibiotics with high activity and without side effects for human and for environment are therefore urgently needed. Some cyanobacterial species could be a prolific resource for substances with antibacterial activity. There are numerous reports concerning the inhibiting activities from Cyanobacteria against human pathogens (Kreitlow et al., 1999; Abdel-Raouf, 2004; Ibraheem and Abdel-Raouf, 2007), fungi (Moussa and Shanab, 2001; Ibraheem and Mohammed, 2002; Hassan, 2007; Sunil and Puranik, 2007), mites (Ibraheem and Abdel -Aziz, 2002; Abdel-Aziz and Abdel-Raouf, 2002), algae (Issa, 1999 and Volk and Furkert, 2006), but there is no data about effects against fish pathogenic bacteria.

Therefore, the main objective of the present study was to investigate the antibacterial activity of extracts of ten cyanobacterial species against four species belonging to genus *Aeromonas*, which are capable of causing a disease known as “Motile *Aeromonas* Septicemia” or “Bacterial Hemorrhagic Septicemia”.

MATERIALS AND METHODS

Cyanobacterial isolates

The specimens of investigated cyanobacteria used in this study were isolated from soil samples collected from desert habitats of

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Koom Osheem of El-Fayoum city. Soil samples from different places were brought to the laboratory in aseptic condition. Ten cyanobacterial isolates had been isolated by pour plate technique on Allen's agar medium. Axenic and pure colonies were conducted according to the methods described by Fogg (1942), Felfoldy and Zsuza (1959) and Belcher and Swale (1982) and the isolated colonies had been identified according to Desikachary (1959) and revived by streaking on Allen's agar medium and incubated at 30°C until used for antibacterial screening.

Preparation of cyanobacterial extracts

Three different organic solvents were used for extraction of the bioactive compounds of the cyanobacterial species, mainly: chloroform, ethyl acetate and n-butanol.

Screening of cyanobacterial extracts for antibacterial activity

The agar diffusion assay was performed according to European Pharmacopoe (1997). Oxytetracycline was used as a positive, the solvent of each extract as a negative control.

Isolation of antibacterial compounds

According to Moncheva et al. (2002), the antibacterial compound was recovered from the filtrate by solvent extraction method. Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 h for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 80-90°C and the residue obtained was weighed. Thus obtained compound was used to determine the minimum inhibitory concentration and to perform bioautography.

Determination of minimum inhibition concentration

It has been determined by standard serial broth microdilution assay (European Pharmacopoe, 1997).

Thin layer chromatography and bioautography

As described by Moncheva et al. (2002), further purification was tried by thin layer chromatography (procoated TLC plates, silica gel 60 F 254). Ten microliters of the ethyl acetate fractions were applied on the plates and the chromatogram was developed using chloroform: methanol (4:1) as solvent system. The active bands (R_f) were detected and visualized under UV light.

Bioautography of the purified antibacterial compounds

Migration of the purified antibacterial agents on paper chromatographic strips when different solvent systems (13 solvent) were used and bioautographed according to the method described by Weinstein and Wagman (1978) using *Bacillus subtilis* as standard test organism. These solvents were (A) petrolium ether, (B) benzene saturated with water, (C) chloroform saturated with water, (D) methanol, (E) n-butanol saturated with water, (F) acetone, (G) diethyl ether, (H) ethyl acetate, (I) amyl acetate, (J) n-butanol : pyridine : water (1:0.6:1), (K) n-butanol : acetic acid : water (2:1:1), (L) distilled water and (M) methylene chloride : water (9:1).

The developing chromatographic strips were air-dried and carefully placed on the surface of the seeded agar layer. The glass plates were then left in a refrigerator for about 4 h in order to permit the diffusion of the active compound. Then, the plates were incubated at 37°C for 24 h. The developing clear zones of inhibition which observed on the glasses were indicated the positions of the active compounds on strips. R_f values of these active compounds were determined.

Determination of the medium lethal dose (LD_{50})

The LD_{50} of the antibacterial compounds under investigation were calculated according to the method described by Kerber (1941) and the calculation of LD_{50} was determined according to the following equation (cited in Hozzein, 2000):

$$LD_{50} = T - \sum (Z \times d) / n$$

Where T = the toxic dose, Z = the average of mortality between each two successive groups, d = the interval between each two doses, and n = the total number of used mice.

Statistical analysis

The data were statistically analysed by applying one-way ANOVA.

RESULTS AND DISCUSSION

Table 1 summarizes all cyanobacterial isolates that showed activity against at least one of the test organisms except that of *Anabaena cylindrica* and *Oscillatoria rubescens* which have no activity against the tested fish pathogenic bacterial species. Among the n-butanol extracts, only that from the *Oscillatoria prolifica* was effective against *Aeromonas salmonicida* and *Aeromonas hydrophila* with week activities (2.3 and 5.7 mm, respectively).

It can be seen that ethyl acetate extracts from both *Anabaena variabilis* and *Anabaena circinalis* gave the widest spectrum activities that inhibited the growth of all studied pathogenic bacterial fishes with the widest inhibition zone; 23 mm for *A. variabilis* against *Aer. salmonicida* and 19 mm for *A. circinalis* against *Aer. liquefaciens*, respectively. Only two bacterial species, *Aer. formicans* and *Aer. Hydrophila*, were inhibited by ethyl acetate extracts of *A. solitaria* (moderate activities) with inhibition zones 10.9 and 12.1 mm, respectively. On the other hand no inhibitory effects appeared with the other ethyl acetate extracts of the other cyanobacterial species.

Considering the chloroform extract, only three bacterial species (*Aer. liquefaciens, Aer. salmonicida and Aer. hydrophila*) were inhibited by chloroform extracts of *A. circinalis* (moderate activities) with inhibition zones 9, 10.5 and 12.2 mm, respectively. On the other hand the chloroform extracts of *A. solitaria, Anabaena spiroides*, *Oscillatoria ornate*, *Oscillatoria salins* and *Oscillatoria tenuis* inhibited notably the growth of at least two of the tested fish pathogenic bacteria but with week activities (3 - 7 mm) as measured in our screening system. However, the chloroform extracts of both *A. variabilis* and *O. prolifica* have no activities against the target pathogenic bacterial fish.

Table 1. Antibacterial activity of the investigated chloroform, ethyl acetate and n-butanol extracts of ten cyanobacterial species against four *Aeromonas* species using the agar plate by diffusion assay method.

Antibiotic			Inhibition zones (mm)			
			Aeromonas formicans	***Aeromonas liquefaciens***	***Aeromonas salmonicida***	***Aeromonas hydrophila***
Oxytetracycline 20 µg/disc			15.6 ± 1.7	-	17.2 ± 1.0	15.7 ± .5
Cyanobacterial extracts	*Anabaena solitaria*	Chloroform	4.1 ± 0.3	7 ± 0.8	-	-
		Ethyl acetate	10.9 ± 1.6	-	-	12.1 ± 3.1
		n-butanol	-	-	-	-
	Anabaena variabilis	Chloroform	-	-	-	-
		Ethyl acetate	13 .2 ± 2.8	11.4 ± 0.8	23 ± 3.7	15 ± 3.0
		n-butanol	-	-	-	-
	Anabaena cylindrica	Chloroform	-	-	-	-
		Ethyl acetate	-	-	-	-
		n-butanol	-	-	-	-
	Anabaena spiroides	Chloroform	-	-	3.5 ± 0.7	5 ± 0.6
		Ethyl acetate	-	-	-	-
		n-butanol	-	-	-	-
	Anabaena circinalis	Chloroform	-	9 ± 1.1	10.5 ± 1.5	12.2 ± 0.9
		Ethyl acetate	8.4 ± 2.0	19 ± 3.0	10.6 ± 1.1	11 ± 0.6
		n-butanol	-	-	-	-
	Oscillatoria ornata	Chloroform	6 ± 1.4	4.5 ± 1.7	4 ± 0.4	-
		Ethyl acetate	-	-	-	-
		n-butanol	-	-	-	-
	Oscillatoria salins	Chloroform	-	-	3.5 ± 0.3	5 ± 0.5
		Ethyl acetate	-	-	-	-
		n-butanol	-	-	-	-
	Oscillatoria tenuis	Chloroform	-	-	5.5 ± 1.3	3 ± 1.6
		Ethyl acetate	-	-	-	-
		n-butanol	-	-	-	-
	Oscillatoria rubescens	Chloroform	-	-	-	-
		Ethyl acetate	-	-	-	-
		n-butanol	-	-	-	-
	Oscillatoria prolifica	Chloroform	-	-	-	-
		Ethyl acetate	-	-	-	-
		n-butanol	-	-	2.3 ± 0.8	5.7 ± 0.9

- = No inhibitory effect; width 1 to 8 mm = week activity; width 8 to15 mm = moderate activities; width > 15 mm = strong activity.

Our data revealed that the strong inhibition zones were only observed for ethyl acetate extracts. From the above mention results, it could be concluded that ethyl acetate proved to be the most suitable organic solvent for the extraction of the investigated antibacterial compounds in both *Anabaena* species. In agreement with our results, Ostensvik et al. (1988) has found that antibacterial substances showed different solubility depending on the extract solvent used. Similarly, Filch et al. (1955) used successively extractions with solvents of increased polarity. These extracts showed different antibacterial effects in bioautographic assay.

The MIC values are presented in Table 2. The antibacterial compound extracted from *A. variabilis* can be

Table 2. Minimal inhibition concentration (MICs) (µg ml^{-1}) of ethyl acetate extracts for *Anabaena variabilis* and *Anabaena circinalis* against 4 tested *Aeromonas* species.

Pathogenic bacteria	MIC (µg ml^{-1})	
	A. variabilis	***A. circinalis***
Aer. formicans	55.30	>100
Aer. liquefaciens	>100	40
Aer. salmonicida	85	>100
Aer. hydrophila	>100	70

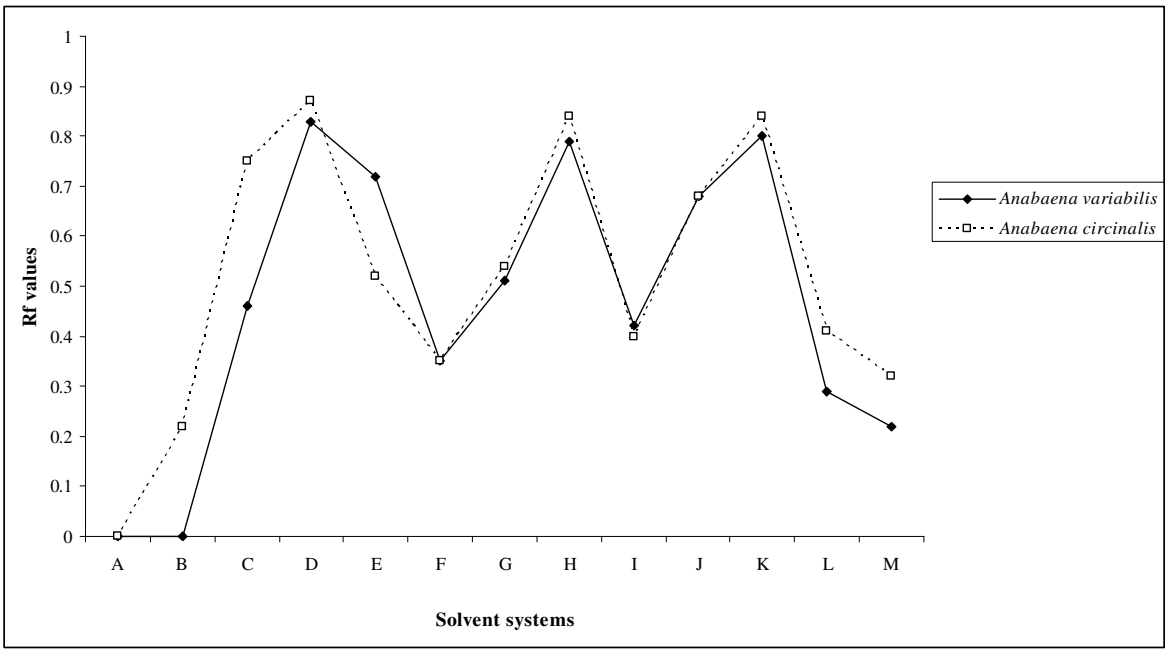

Figure 1. The migration (R_f values) of the purified active compounds isolated from *Anabaena variabilis* and *Anabaena circinalis* by ethyl acetate extract when bioautographed with various developing solvent systems.

Table 3. The median lethal dose (LD_{50}) of the purified antibacterial compound from ethyl acetate extract of *Anabaena variabilis* on mice.

Dose (mg/kg)	No. of mice/group	No. of dead mice/group	(d)	(Z)	(Z x d)
200	6	0	10	-	-
210	6	0	10	-	-
220	6	0	10	-	-
230	6	1	10	0.5	5
240	6	2	10	1.5	15
250	6	3	10	2.5	25
260	6	4	10	3.5	35

$LD_{50} = T - \sum (Z \times d) / n$
$LD_{50} = 260 - 80 / 6 = 246.67$ mg/kg.

classified as a strong antibiotic inhibitor against *Aer. formicans* and *Aer. salmonicida* and that extracted by *A. circinalis* is a strong inhibitor against *Aer. liquefaciens* and *Aer. hydrophila*.

TLC analysis of the two obtained antibacterial compounds exhibited only green spot in the case of *A. variabilis* and greenish yellow spot in the case of *A. circinalis.* Considerable bioautographyic technique was used for the detection of the R_f of the active spot (s) (Figure 1), which revealed that only one definite inhibition zone was always recorded in each paper strip chromatogram in all cases. It indicates that the obtained antibiotic is one pure compound in each case. The median lethal doses (LD_{50}) of the purified antibacterial compounds for ethyl acetate extracts of the two *Anabaena* species (*A. variabilis* and *A. circinalis*) against mice (Tables 3 and 4) were 246.67 and 231.67 mg/kg of the body weight, respectively, when the bioactive compounds were injected to the mice orally.

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Table 4. The median lethal dose (LD_{50}) of the purified antibacterial compound for ethyl acetate extract of *Anabaena circinalis* on mice.

Dose (mg/kg)	No. of mice/group	No. of dead mice/group	(d)	(Z)	(Z x d)
200	6	0	10	-	-
210	6	0	10	-	-
220	6	2	10	1.0	10
230	6	3	10	2.5	25
240	6	4	10	3.5	35
250	6	5	10	4.5	45
260	6	6	10	5.5	55

$LD_{50} = T - \sum (Z \times d) / n$
$LD_{50} = 260 - 170 / 6 = 231.67$ mg/kg.

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