ALLELOPATHIC ACTIVITY OF SOME LOCAL CYANOBACTERIAL EXTRA-METABOLITES AGAINST SOME PATHOGENIC BACTERIA.

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Abstract

Ten cyanobacterial species (*Nostoc calcicola* , *N. commune, N. entophytum, N. minutum, N. palndosum, N. passerianum, N. punctiforme, Anabaena ambigua, A. amomala,* and *A. doliolum*) were isolated from the mangrove region of Ras Mohammed (Sinai, Egypt) and have been tested for their allelopathic activity that of inhibitory and / or promoting effects against two Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). Data suggested two types of allelopathic effects: one type which always appeared in cyanobacterial medium as in the case with *Nostoc minutum* (medium that inhibits the growth of all tested bacterial species). The other type is induced only when Cyanobacteria are in contact with bacteria; this is the case when the growth of both *Bacillus subtilis* and *Staphylococcus aureus* were inhibited in co-culture with *Nostoc commune.* On the other hand, promotion effects of bacterial growth were observed when grown in cyanobacterial metabolites in most of studied cyanobacterial species. The biological assays for aqueous and methanolic extracts of the two *Nostoc* species revealed that both extracts for each species were not toxic at concentrations of 0.52 and 0.59 g L^{-1} water extract for *Nostoc commune* and *N. minutum*, respectively and 0.31 and 0.425 g L^{-1} for methanolic extract for *Nostoc commune* and *N. minutum*, respectively. No mortality was observed in tested mice within 72 hours

Key words: Allelopathic activity, Cyanobacteria, Pathogenic Bacteria.

Introduction

Research activities concerning the investigation of products of metabolism of plants and other groups of organisms were undertaken not only for a better understanding of nature but also to discover metabolites of possible use for humans in different fields of interest.

Cyanobacteria are very old group of prokaryotic organisms that produce a variety of secondary metabolites of allelopathic activity (Mundt *et al.*, 2001). The screening of extracts or isolated compounds from different natural sources is a common way to discover biological active metabolites. In such research activities, Cyanobacteria were found to be a rich source for various products of commercial and pharmaceutical interest as primary metabolites such as carbohydrates, proteins, fatty acids, vitamins or pigments (Borowitzka, 1988 a, b and 1995) and/or various secondary metabolites such as phenolic compounds (De Cano *et al*.,1990; Pedersen and Da Silva, 1973 and Volk, 2005), peptides, alkaloids or

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terpenoids and Ne-glycosides (Ramamurthy, 1970 and Bonjouklian *et al*., 1991), which showed some different bioactivities as antifungal, antiviral and antibacterial (Abdel-Raouf, 2004; Volk and Furkert, 2006 and Hassan, 2007)

Most of the cyanobacterial metabolites are accumulated in the cyanobacterial biomass. Moreover, Cyanobacteria excrete various organic compounds into their environment. So, some biologically active compounds were identified among these exo-metabolites e.g. some antibacterial di-terpenoids in *Nostoc commune* (Jaki *et al*., 1999 and 2000) or antifungal peptides in *Tolypotrix byssoidea* (Jaki *et al*., 2001) and algicidal phenolic compounds (Volk, 2005). The aim of the present investigation was to quantify the allelopathic potential of the extrametabolites and/or the culture of selected ten local cyanobacterial species against two Gram positive and tow Gram negative species.

Materials and Methods

Collection of cyanobacterial isolates.

Ten cyanobacterial isolates were isolated from the mangrove regions at Ras Mohammed, Sinai, Egypt and re-cultured on artificial medium in laboratory. In this respect, Z-medium described by (Staub, 1961) and Allen's free nitrogen medium described by Hughes et al.,(1958) modified by Allen (1968), using the techniques described by Esmarsch, (1914) and EL-Ayoty and Ayyad, (1972) were used for isolation and culturing of the cyanobacterial isolates.

Different pH values (6, 7 and 8), different light / dark periods (12/12, 16/8 and 24/0.0) and different light intensities (2000, 3000 and 4000 Lux) were used to optimize the growth conditions for all tested Cyanobacteria for more productivity.

Unialgal and axenic cultures were obtained as described by Pringsheim (1949). These cultures were subjected to different trials to employ bacteria free cultures according to Felfoldy and Zsuzsa (1959) and Hoshaw & Rosewski, (1973).

These purified cyanobacterial species were identified according to Smith, (1950) and Desikachary (1959).

Logarithmic growth phase of each cyanobacterial species was detected to obtain healthy growth mass and vigorous extracellular products to be used in the allelopathic activity.

Bacterial test organisms.

1. **Gram positive***: Bacillus subtilis, NCTC 1040* and *Staphylococcus aureus, NCTC 7447*
2. **Gram negative**: *Escherichia coli*, NCTC 10416 and *Pseudomonas aeruginosa*, ATCC 10145.

All test organisms were kindly supplied by Biotechnological Research Center, Al-Azhar University (For Boys), Cairo, Egypt. These organisms were sub-cultured on specific culture media until used in the experiments.

Determination of antagonistic activity.

1. Detection of cyanobacterial supernatants. According to the method described by Safonova and Reisser (2005), Cyanobacteria of exponentially growing cultures were separated from their culture medium by centrifugation (14000 r.p.m. for 10 min). The supernatant was checked microscopically for remaining cells and to 1000 µL of the cell free supernatant 10 µL of bacterial suspension were added (OD_{670} = 0.3-0.4). An OD_{670} of 0.3 corresponded to 0.4 x 10^6 c.f.u. ml^{-1} for *Escherichia coli,* 0.15 x 10^5 c.f.u. ml^{-1} for *Pseudomonas aeruginosa* , 0.6 x 10^6 c.f.u. ml^{-1} for *Bacillus subtilis* and 1.5 x 10^6 c.f.u. ml^{-1} for *Staphylococcus aureus* (c.f.u. = colony forming units).

After incubation at pH 7 for 48 hours in the dark at 30^o C, the number of viable bacteria was determined by a plate test by counting colony forming units per milliliter. Controls were done with bacteria in plain cyanobacterial media. Screening tests were run two times. Data for c.f.u. are obtained from three parallel platings.

2. Detection of mixed cultures. Suspension of the four tested bacteria (10 µL**,** OD_{670} = 0.3 - 0.4) were added separately into the two *Nostoc* species cultures (50 ml, exponential phase of growth). An aliquot of the mixture was taken for determination of c.f.u. at the start of the experiment and every 48 hours for 16 days. The pH was routinely checked.

Toxicity test. Mouse bioassay for toxicity test of both aqueous and methanolic extracts of the dry matter of the two *Nostoc* species to mice was evaluated by injecting mice with different concentrations for these extracts. It was conducted in Animal Laboratory, Faculty of Science, Beni-Suef University.

Statistical analysis. Analysis of variance (one-way ANOVA) was employed to determine if treatments were significantly different from each other (Zar, 1984). Results were seemed significantly different at the levels of 5 and 1 %. All data were of three replicates.

Results

The most common filamentous Cyanobacteria inhabiting the mangrove samples have been detected either by direct observation or by the culture method. The greatest abundance percentage was recorded by two genera *Nostoc* and *Anabaena*. From which we isolated seven species belonging to *Nostoc* sp. (*Nostoc calcicola*, *N. commune, N. entophytum, N. minutum, N. palndosum, N. passerianum and N. punctiforme*) and three species belonging to *Anabaena sp.* (*Anabaena ambigua*, *A. amomala* and *A. doliolum*).

When the supernatant of the ten cyanobacterial cultures were screened for allelopathic activity against bacteria, in most assays growth promoting effects were observed. At maximum, this led to a 20.7-fold increase of c.f.u. of *Pseudomonas aeruginosa* with supernatant of *Nostoc palndosum* compared with control.

On the other hand, supernatant of *Nostoc minutum* did not show comparable growth promoting effects, but there were significant inhibitory effects on all studied bacterial species.

Table (1): Effect of cyanobacterial supernatants on the growth of *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus.* Data for c.f.u. are obtained from three parallel platings after 48 hours of incubation in dark at 30°C.

Species	*Escherichia coli* 10^6 c.f.u. ml^{-1}	*Pseudomonas aeruginosa* 10^5 c.f.u. ml^{-1}	*Bacillus subtilis* 10^6 c.f.u.ml^{-1}	*Staphylococcus aureus* 10^6 c.f.u. ml^{-1}
Control	**0.5 ± 0.02**	**0.3 ± 0.004**	**1 ± 0.02**	**2.0 ± 0 .55**
Nostoc calcicola	**1.0 ± 0.30**	**1.2 ± 0.075**	**1.9 ± 0.07**	**3.1 ± 0. 37**
Nostoc commune	**0.4 ± 0.005**	**0.3 ± 0.07**	**1.2 ± 0.08**	**2.06 ± 0.09**
Nostoc entophytum	**1.2 ± 0.17**	**2.0 ± 0.37**	**3.0 ± 0.31**	**2.4 ± 0.14**
Nostoc minutum	**0.02 ± 0.005**	**0.1 ± 0.05**	**0.2 ± 0.095**	**0.5 ± 0.008**
Nostoc palndosum	**2.7 ± 0.11**	**6.2 ± 1.51.**	**4.0 ± 0.38**	**3.5 ± 0. 2**
Nostoc passerianum	**1.4 ± 0.11**	**4.0 ± 0.96**	**2.4 ± 0.22**	**3.7 ± 0.50**
Nostoc punctiforme	**5.0 ± 0.24**	**2.0 ± 0.24**	**3.4 ± 0.74**	**2.3 ± 0.35**
Anabaena ambigua	**2.3 ± 0.32**	**1.0 ± 0.074**	**1.5 ± 0.06**	**3.2 ± 0.19**
Anabaena amomala	**4.2 ± 0.81**	**5 .0± 0.51**	**2.0 ± 0.3**	**6.1 ± 1. 09**
Anabaena doliolum	**3.0 ± 0.41**	**2.1 ± 0.09**	**1.36 ± 0.07**	**2.7 ± 0.04**

Concerning the effect of *Nostoc commune* culture on the tested bacteria, there was slightly inhibitory effect on *E. coli* and slightly promoting effect on *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*.

By a second series of experiments we tested whether bacteria might induce the formation of bioactive compounds in *Nostoc minutum* and *N. commune*. Thus, we co-cultured the two cyanobacterial species with the four bacteria and monitored the amount of viable bacteria by the c.f.u. assay.

In co-culture, *Nostoc minutum* inhibited the growth of all tested bacterial species (Fig. 1). This is in agreement to the effect of the cyanobacterial supernatant alone (Table 1). A most interesting resistance effect was observed with *E. coli* after an initial decrease in count, it have been increased again and reached the original level (Fig. $1A_1$).

Nostoc commune inhibited the growth of both *E. coli* (Fig. $2B_1$) and *Pseudomonas aeruginosa* (Fig. $2B_2$). This is in contrast to the effect of cyanobacterial supernatant alone which did not show any effect on growth of *Pseudomonas aeruginosa* after 48 hours incubation period (Table 1).

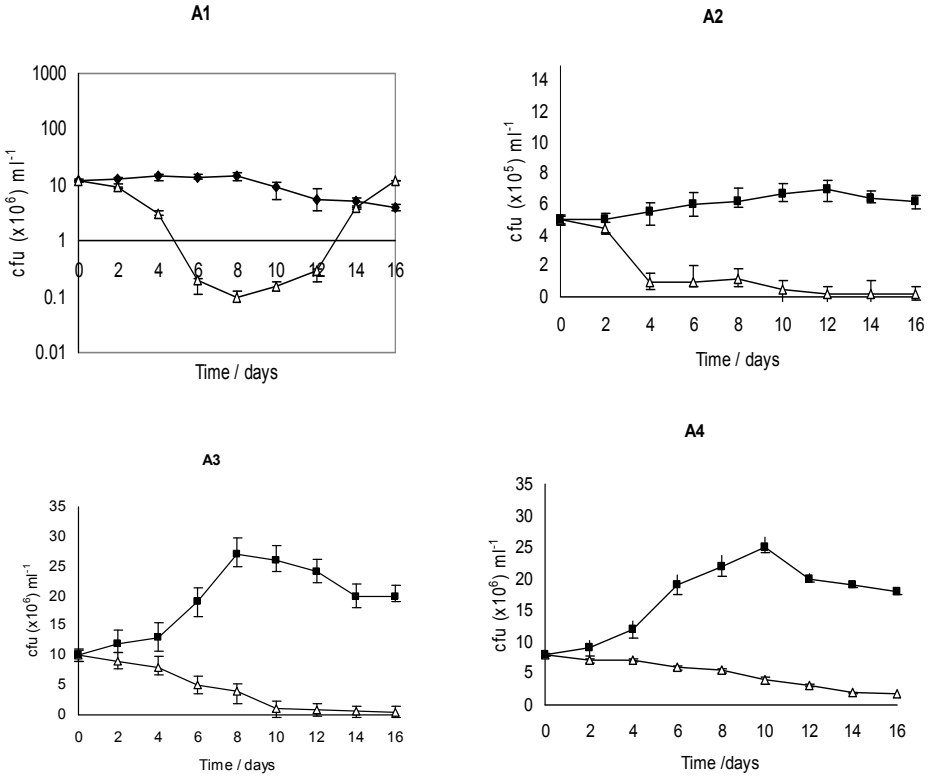

Figure (1): Continuous growth phase of *Escherichia coli* (A1), *Pseudomonas aeruginosa* (A2), *Bacillus subtilis* (A3) and *Staphylococcus aureus* (A4) in co-culture with *Nostoc minutum* (Δ) and without Cyanobacteria (■). Data are the means of triplicate tests ± SD

In the presence of *Nostoc commune* the growth of *Bacillus subtilis* was enhanced (Fig. $2B_3$). This is in agreement with the effect of *Nostoc commune* supernatant (Table 1). In a co-culture of *Nostoc commune* and *Staphylococcus aureus*, however, bacterial growth was inhibited (Fig. $2B_4$). This is in striking contrast to the slightly promoting effect of the cyanobacterial growth as shown in Table 1.

The biological assays for aqueous and methanolic extracts of the two *Nostoc* species revealed that both extracts for each species were not toxic at concentrations of 0.52 and 0.59 g L^{-1} water extract for *Nostoc commune* and *N. minutum*, respectively and 0.31 and 0.425 g L^{-1} for methanolic extract for *Nostoc commune* and *N. minutum*, respectively. No mortality was observed in tested mice within 72 hours.

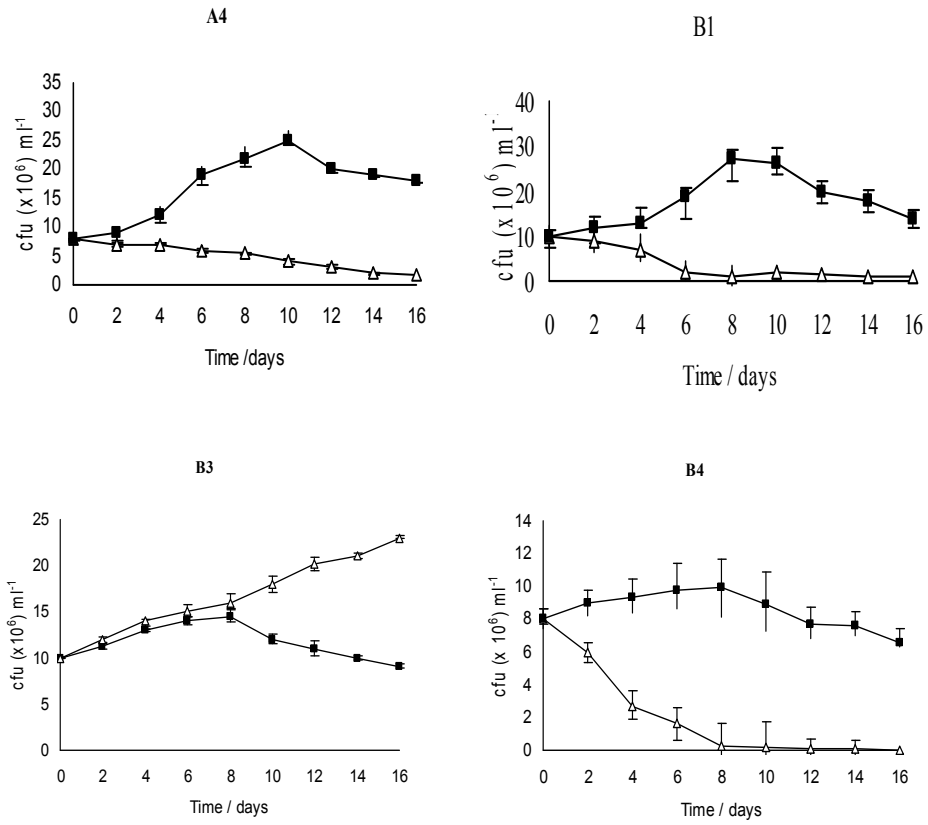

Figure (2): Continuous growth phase of *Escherichia coli* (A1), *Pseudomonas aeruginosa* (A2), *Bacillus subtilis* (A3) and *Staphylococcus aureus* (A4) in co-culture with *Nostoc commune* (Δ) and without Cyanobacteria (■). Data are the means of triplicate tests ± SD

Discussion

Interactions between microalgae and bacteria in aqueous or co-cultures have been studied repeatedly. In most cases, growth of microalgae is enhanced in the presence of bacteria (de Bashan *et al*., 2002), although the mechanism remain to be clarified. Mouget *et al*., (1995) suggested that growth promoting effect of bacteria as a result of oxygen reduction of the surface of microalgae. Only few reports dealt with an impairment of microalgal growth by bacteria (Gozales-Bashan *et al*., 2000) or with growth promotion of bacteria by microalgal substances as shown by the present data of this investigation.

Gäumann and Jaag (1950) reported on the production of vitamin A by various microalgae, which therefore might support the growth of microorganisms. The release of organic compounds is a well established feature of different kinds of microalgae and has been studied repeatedly (Hellebust, 1974).

The promoting effects of supernatants of cyanobacterial cultures on the growth of heterotrophic bacteria, as shown in Table 1, were most probably a result of a general accumulation of organic material that is derived from sporulation processes, dying cells, cell wall remnants and diffusing slime materials. However, it is interesting that in some cases promoting effects can act selectively on Gram negative or Gram positive bacteria or are even lacking (Safonova and Reisser, 2005). Therefore, in our experiments, the release of substances that specifically promote the growth of bacteria cannot be ruled out.

Comparably few studies deal with antibacterial compounds that are released by living microalgae (Jones 1988). For Cyanobacteria the release of antibacterial compounds was shown for *Scytonema* sp, *Nostoc muscorum* and *Chroococcus turgidus* (Chetsumon *et al*., 1993). In the present study, we observed a slightly inhibiting effect of *Nostoc commune* on *E. coli*, but no inhibitory effects were detected against the other tested bacteria. On the other hand *Nostoc minutum* has shown significant inhibiting effects against all tested bacteria.

So from the obtained results we suggest that there exist two types of antibacterial effects of Cyanobacteria: the constitutive type when antibacterial substances are generally released by Cyanobacteria into their culture media, such as, for example substances released by *Nostoc minutum* and acting on all tested bacteria (Table 1) and the induced type when antibacterial substances are only formed by Cyanobacteria in the presence of bacteria, such as induced by *Nostoc commune* against *E. coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The chemical nature of those constitutive and induced substances is not clear. As has been shown here, constitutive substances might be water soluble molecules. Whether synthesis of induced substances depends on special chemical signals produced by bacteria or requires cell-to-cell contact between bacteria and Cyanobacteria as shown in the co-culture of *N. minutum* and tested bacteria (Fig. 2).

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النشاط المثبط للمنتجات الأيضية لبعض أنواع السيانوبكتيريا المحلية ضد بعض أنواع البكتيريا الممرضة

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تم في هذه الدراسة عزل وتعريف وتنقية عشرة أنواع من السيانوبكتيريا وهي نوستك كلاسيكولا، نوستك كوميون، نوستك انتوفيتم ، نوستك منيتم، نوستك بالندوسم، نوستك باسيريانم، نوستك بانكتيفورم، أنابينا امبيجوا، أنابينا اومالا، أنابينا دوليولم من المانجروف المنتشرة في منطقط رأس محمد جنوب سيناء ، مصر.
أجريت دراسة معملية لتقدير مدي كفاءة هذه الأنواع العشرة في تثبيط نمو أربعة أنواع من البكتيريا الممرضة ، اثنتان منها تنتمي إلي مجموعة البكتيريا الموجبة لصبغ جرام وهما باسيلس ستلس و ستافيلوكوكس أوريس، بينما تنتمي الأخرتان إلي مجموعة البكتيريا السالبة لصبغ جرام وهما إشرشيا كولاي ، سيدوموناس أريجينوزا.
تم وضع خطة ذات محوران لإتمام الاستفادة من هذه الدراسة وهما:

١. أختبار المفرز الناتج من أنواع السيانوبكتيريا في الوسط الغذائي بعد فصله عن خلاياه علي إحداث تثبيط نمو البكتيريا.
٢. أختبار كفاءة خلايا السانوبكتيريا ذاتها علي إحداث تثبيط نمو البكتيريا.

كما تم أجراء تجرب أختبارية لبعض مستخلصات السيانوبكتيريا ذات النشاط الضد بكتيري لقياس مدي سميتها علي فئران التجارب.
أثبتت النتائج الأحصائية في حالة التجربة الأولي أن الناتج الأيضي لجنس نوستك منيتم قد أعطي كفاءة عالية في تثبيط نمو الأنواع البكتيرية الأربعة المستخدمة. بينما أثبتت النتائج الأحصائية في حالة التجربة الثانية ان خلايا جنس نوستك كوميون أحدثت تثبيط لنمو باسيلس ستلس و ستافيلوكوكس أوريس. من ناحية اخري عملت باقي أنواع السيانوبكتيريا علي زيادة نمو هذة الأنواع البكتيرية.
كما أثبتت التجربة البيولوجية التي أُجريت لتقدير سمية بعض مستخلصات أكثر الأنواع السيانوبكتيرية نشاطاً علي فئران التجارب ، أن حقن هذه الفئران بـ ٠,٥٢ ، ٠,٥٩ جم / لتر من المستخلص المائي لكل من نوستك كوميون، نوستك منيتم وكذلك حقن الفئران بـ ٠,٣١ ، ٠,٤٢٥ جم / لتر من مستخلص الميثانول لكل من نوستك كوميون، نوستك منيتم علي التوالي غير سام لهذه الفئران ولم يعطي أي نسبة وفاة بعد مرور ٧٢ ساعة من الحقن.