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Isolation and Characterization of Antimicrobial Active Compounds from the Cyanobacterium *Nostoc commune* Vauch

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In this investigation, antimicrobial activity of *Nostoc commune* Vauch (isolated from agricultural wastewater canal, Beni Suef Governorate, Egypt) organic extracts were examined against nine selected microbial isolates. Four of them were Gram positive bacterial isolates (*Bacillus subtilis, Mycobacterium phlei, Sarcina maxima* and *Staphylococcus aureus*), four Gram negative bacteria (*Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Salmonella arizonae*) and one unicellular fungus (*Candida albicans*) were evaluated for their resistance against these extracts. Methanol was the best organic solvent for extraction of active material, rather than the other organic solvents. This material was produced, maximally, after 10 days of incubation in aerated shaken culture at 30ºC and pH 8.0 when *N. commune* was grown in Medium 18 growth medium. The antagonistic material was purified using thin layer chromatography then identified using chromatographic and spectroscopic techniques including UV, FT-IR, mass spectrophotometer and proton-NMR. Four unknown compounds were extracted that had long chain alcohol, sterol, long chain fatty acid and triterpen. These compounds were tested for their antimicrobial activity against *B. subtilis*. Only the long chain fatty acid (compound C) had an inhibitory effect on the growth of *B. subtilis* while the other compounds were not active.

Key words: Cyanobacteria, *Nostoc commune*, Antimicrobial activity, Bioactive products.

Cyanobacteria (blue-green algae) are a group of extraordinary diverse Gram-negative prokaryotes that originated 3.5 billion years ago (Kaushik *et al*., 2008). The medicinal and nutrient qualities of cyanobacteria were first appreciated as early as 1500 BC, when *Nostoc* species were used to treat gout, fistula and several forms of cancer (Liu and Chen, 2003). Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial (Falch *et al*., 1995; Mundt *et al*., 2003; Kaushik and Chauhan, 2008) and antifungal (MacMillan and Tadeusz, 2002). Some of these metabolites have potential or development of new pharmaceutical compounds. Mundt *et al*. (2003) proved that the cyanobacterium *Oscillatoria redekei* produce fatty acids which show antibacterial activity. The methanol extracts from *Chlorococcum* strain HS-101 and *Dunaliella primolecta* strongly inhibited the growth of a strain of methicillin resistant *Staphylococcus aureus*, which cause serious problems in Japanese hospitals (Ohta *et al*., 1995). Ostensvik *et al*. (1998) examined five strains of cyanobacteria for antibacterial activity and found that the methanol extracts from *Tychonema bourrellyi*, *Aphanizomenon flos-aquae* and *Cylindrospermopsis raciborskii* showed the most

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pronounced inhibitory effects against the *Bacillus cereus* and *B. subtilis*. The aim of this investigation was to isolate *N. commune* and test its antimicrobial activity against some bacterial and fungal species. Furthermore, some experiments were done for purification of the active components and elucidation of their chemical structure.

MATERIALS AND METHODS

Organism and growth conditions

N. commune Vauch was isolated from agricultural wastewater canal, Beni Suef Governorate, Egypt. The dilution culture technique adopted by Venkataraman (1969) was used for isolation and purification. The isolated species was identified according to El-Nayal (1935) and Prescott (1951 and 1969). The isolated organisms were grown autotrophically in Medium18 medium (Inthorn *et al.,* 1996) at 28±2°C and continuous light intensity of 2600 LUX for 20 days. The culture was grown in triplicate axenically by using the method recommended by Bolch and Blackburn (1996).

Effect of static and aeration growth condition on antimicrobial production by *Nostoc commune*

About 5 ml of the preculture *N. commune* was transferred into Erlenmayer flasks (100 ml) containing 50 ml of the M18 medium. The flasks were incubated under static conditions (without shaking and aeration) and aeration conditions (Nostoc culture was agitated continuously to prevent the settling of the cells at the bottom of the flasks, and bubbled with dry sterile air), for different periods (2 to 20 days). After that the antimicrobial activity was evaluated on *Bacillus subtillis* every two days of Nostoc incubation period.

Effect of different culture media on antimicrobial production by *Nostoc commune*

Four different growth media types (M18, Z, Spirulina and Allen media) were examined for the antimicrobial production from *Nostoc commune*.

Extract preparation

Eight days old *N. commune* culture was centrifuged and the pellets were dried in hot air oven (60°C) till constant weight and used for extraction of antimicrobial agents. Twenty gram of *Nostoc* dry weight was extracted separately by chloroform, acetone, ethanol or methanol using Soxhlet extractor. After 6 h of extraction the solvents were evaporated from crude extract by rotary evaporator and collected in pre-weighed test tubes and then preserved at 4°C till use (Solomon *et al*. 2005).

Antimicrobial activity test

The test organisms used for antimicrobial evaluation were nine, four of them were Gram positive bacterial isolates (*Bacillus subtilis, Mycobacterium phlei, Sarcina maxima* and *Staphylococcus aureus*), four Gram negative bacteria (*Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Salmonella arizonae*) and one unicellular fungus (*Candida albicans*). These test organisms were deposited as culture collection at Botany and Microbiology Dept., Faculty of Science, Al-Azhar University, Cairo, Egypt. Screening for antibiotic activity of the tested cyanobacterial extracts was carried out by the agar diffusion assay according to European Pharmacopoeia (1997). One loop full of each test organism was suspended in 3 ml 0.85% sterile NaCl solution, separately. Nutrient agar (Difeco, UK) was inoculated with this suspension of the respective organism and poured into a sterile Petri dish. According to preliminary test for the most effective dose, 10 µl of dimethyl sulfo-oxide (DMSO) contained 2 mg of each extract was placed on sterilized paper disc (6 mm diameter). The loaded discs were placed apart from each other on the inoculated agar plate aseptically. Sterilized discs that loaded with DMSO only served as negative control and antibiotic discs (Ampicillin 10 µg, Ciprocin, 30 µg and Cephalothin, 30 µg) served as positive control. A pre-diffusion for 3h was carried out at 10°C (Bansemir *et al*., 2006). Inhibition zones were measured after 24h incubation period at 37°C for bacteria and at 30°C after 48h for the fungus species. The inhibition zones were measured in mm, triplicates were maintained and the mean values were obtainable.

Column chromatography

The dried methanol extract of *N. commune* (one gm) from 20 gm dry weight was dissolved in a small amount of methanol with 5 g silica gel then dried under vacuum and inserted on the top of silica gel column (150 g, 120x5 cm) packed by wet method with n- hexane. The column was eluted initially with n-hexane followed by gradient

elution from 100 % n- hexane to 100 % methanol. The effluent was collected in fractions (50 ml each). Each fraction was concentrated under reduced pressure and then subjected to TLC examination. The spots were located by means of para anisaldehyde acid followed by heating at 110 ºC for 5 minutes. The purified fraction was lyophilized and subjected to the following analyses in order to recognize its structure as much as possible.

1. Shimadzu- 265 spectrophotometer for determination of ultraviolet absorption spectra.
2. Shimadzu-IR-435 infrared spectrophotometer.
3. Joel NMR spectrometer, 500 MHZ.
4. Joel mass spectrometer, 70 eV.

Statistical analysis

The obtained results were analyzed for statistical significant between control and treated groups by using one-way analysis of variance (ANOVA, SPSS 16.0.Ink program). Values were expressed as mean (±) standard deviation and values of $P \leq 0.05$ were statistically significant.

RESULTS

Antimicrobial activities of *Nostoc commune*

The antimicrobial activity was evaluated as the diameters of the inhibition zones formed as a result of disc assay method in case of bacteria and fungi. Table 1 showed that the methanol extract of *N. commune* had more activity for most of test organisms than ethanol extract that recorded 13 mm of inhibition zone in case of *Bacillus subtilis*. On the other hand, this extract didn't show any activity against *Salmonella arizonae* and *Proteus mirabilis*. Meanwhile, chloroform and acetone extracts were not active against the all investigated microbes (data not shown).

Influence of different culture conditions on the production of the antimicrobial material

Effect of aeration

Figure 1 illustrated the effect of methanol extract of *N. commune* when it was grown at aeration growth condition on *B. subtilis* to produce an inhibition zone of 12 mm after 8 days of incubation period. While in static condition (without air bubbling) the inhibition zone reached its maximum (10 mm) after 10 days of *Nostoc* incubation period (Fig. 1)

Effect of different culture media

N. commune exhibited its antimicrobial activity against *B. subtilis* when incubated in various growth media but in different pattern ranged between 6 to 13 mm inhibition zones (Fig. 2). The highest zone diameter was obtained in M18 medium.

Effect of the pure extracted compounds from *N. commune* against *B. subtilis*

Four pure fractions were extracted from the crude methanol extract of *N. commune*. These fractions were labeled A, B, C and D. Fractions A, B and D didn't have any inhibition effect on *B. subtilis*. Whereas fraction C recorded 16 mm inhibition zone at concentration of 1 mg loaded disc. The reference antibiotic Ciprocin (30 µg) showed more activity against *B. subtilis* and gave

Table 1. The antimicrobial activities of *Nostoc commune* extracts

Test microbes	Organic extracts		Reference antibiotics		
	Ethanol	Methanol	Ampicillin 10µg	Ciprocin 30µg	Cephalothin 30µg
Bacillus subtilis	10	13	-	20	-
Mycobacterium phlei	11	12	-	20	-
Sarcina maxima	7	-	-	20	-
Staphylococcus aureus	10	12	-	24	-
Escherichia coli	9	12	-	20	-
Proteus mirabilis	-	-	-	20	-
Pseudomonas aeruginosa	9	11	-	23	-
Salmonella arizonae	-	-	-	16	-
Candida albicans	7	12	-	18	-

(-) not detected inhibition zone

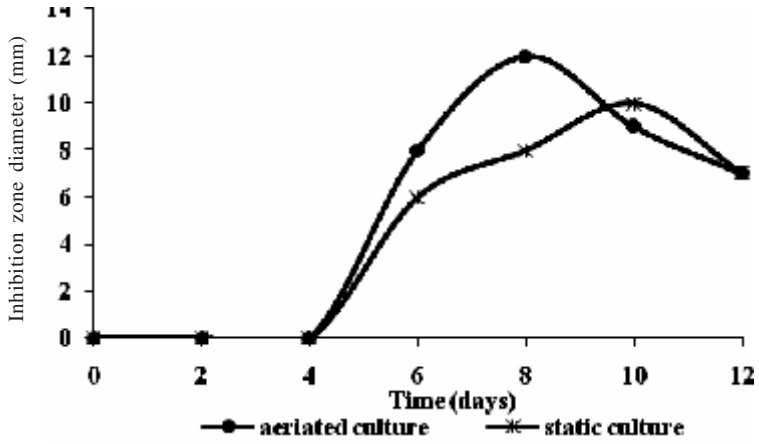

Fig. 1. Effect of static and aerated condition on the antimicrobial activity production of *Nostoc commune*

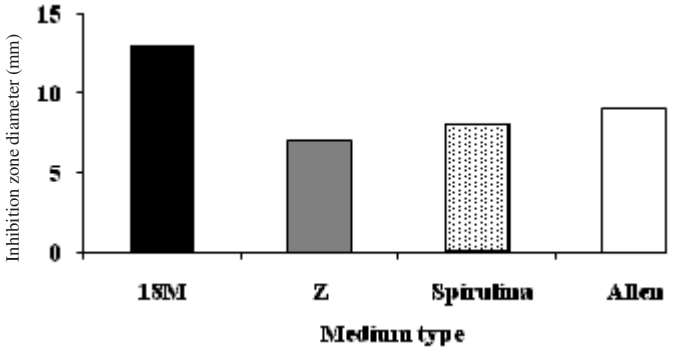

Fig. 2. Antimicrobial activities by *Nostoc commune* grown in different growth media

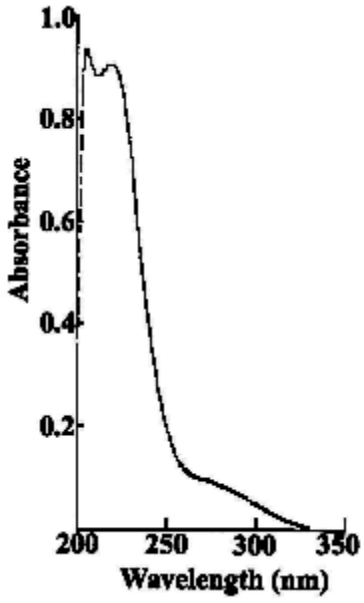

Fig. 3. UV spectrum of the bioactive compound (C) of *Nostoc commune*

20 mm inhibition zone, on the other hand Cephalothin (30 µg) and Ampicillin (10 µg) didn't show any activity.

Elucidation of the chemical structure of the isolated pure compound (C) from *N. commune*

The suggested structures of the purified extracted compound have been confirmed by Physical, chromatographic and spectroscopic studies (UV, FT-IR, proton magnetic resonance and mass spectroscopy).

Physical and chromatographic studies

The active extracted compound (C) characterized by white powder, was soluble in hexane and chloroform and was sparingly soluble in methanol. The compound appeared as a single spot on the TLC plate and when sprayed by para anisaldehyde sulphuric acid reagent gave gray color with R_f =0.1. This spot was scratched and dissolved in solvent system of n-hexan: chloroform (9:1, v/v). This extract was centrifuged to remove

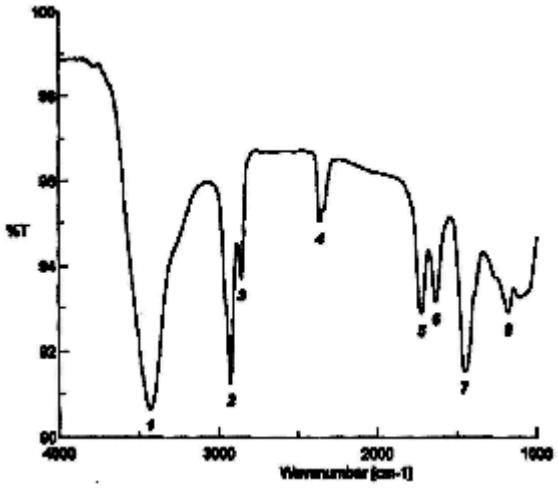

Fig. 4. FT-IR spectrum of the bioactive compound (C) of *Nostoc commune*

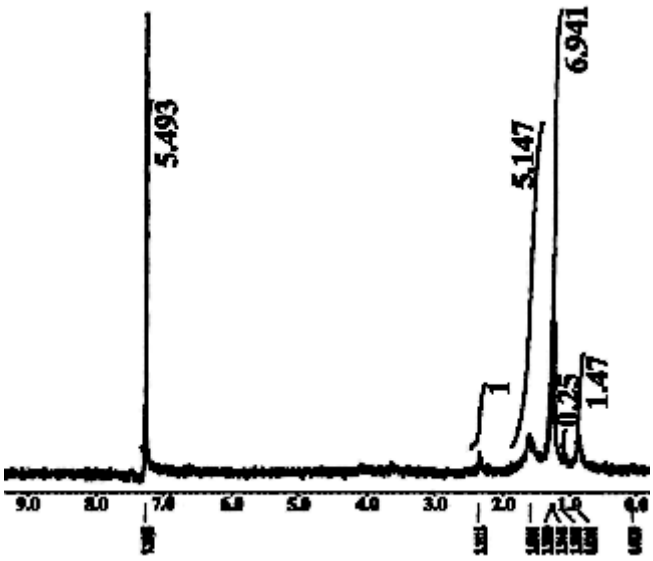

Fig. 5. [1]HNMR spectrum of the bioactive compound (C) of *Nostoc commune*

Fig. 6. The suggested chemical structure of the purified compound (C) isolated from *Nostoc commune*

any unwanted material from the TLC silica gel. The supernatant was dried and stored at 4 ºC till use.

Spectroscopic studies

UV spectral analysis

It showed UV absorption with » max below 230 nm (H" 209 nm) (Fig. 3)

FT-IR spectral analysis

The IR (cm^{-1}) spectrum of compound (C) revealed presence of the following absorption bands at ½ max (cm^{-1}): 3430, 2924, 1630 and 1453, 1178 that were characteristic of COOH stretching, CH stretching, C=O and CH bending, respectively (Fig. 4).

^{1}H NMR studies ($CDCL_3$, 500 MHz)

^{1}HNMR showed several peaks in the aliphatic region [1- 2ppm] and one peak for CH proton at 2.35 for CH group adjacent to COOH group (Fig. 5). So compound (C) was suggested to be a long chain of a fatty acid.

Mass spectroscopic analysis

The mass spectrum of compound (C) showed a molecular ion peak at 636 m/z (30%), base peak at 621 m/z, in addition to several peaks at 565, 551, 423 and 382 m/z. So we could suggest a molecular formula of the extracted compound (C) was $C_{43}H_{86}O_2$ and its suggested chemical structure was illustrated by Fig. 6.

DISCUSSION

This study was done to evaluate the strength of *Nostoc commune* to generate antimicrobial active compounds against some microbes. The production of biologically active compounds by any organism culture requires studying the culture conditions that enhancing this activity. Almost all of the biologically active compounds of interest are secondary metabolites and thus are usually most abundant in stationary phase or in slow-growing cultures (Borowitzka, 1995). The present investigation indicated that, *N. commune* produced the antimicrobial material optimally at the stationary phase when it was cultured under shacked conditions and reached the maximal values after eight days of incubation. The antibiotic cyanobacterin LU1 from *N. linckia* was synthesized throughout the growth cycle (Gromov *et al*., 1991). Oufdou *et al*. (2001) found that, the extracellular and intracellular products released by the cyanobacterium *Pseudanabaena* sp. in the stationary growth phase, reduced the survival of *E. coli*, *Salmonella* sp., *S. aureus* and *C. albicans*. Our results were in agreement with Bloor and England (1991) who found that, the highest antimicrobial activity of *N. muscorum* achieved by day 14 of the cultivation.

We aimed to optimize suitable conditions to maximize the secondary metabolites production by *N. commune*. This organism was subjected to further studies to obtain the most favorable culture conditions under which it produced high values of the antagonistic material. This activity was indicated by the formation of inhibition zones against Gram-positive bacterium (*B. subtilis*).

The highest antimicrobial activity was recorded under aerated shacking condition and this finding was in agreement with Piccardi *et al*. (2000), who found that high antibacterial and antifungal activities were produced from *Nostoc* sp. when incubated in an orbital shaker flushed with a mixture of air:CO_2 (95:5, v:v). M18 medium proved to be the best for the production of antimicrobial substance and formation of inhibition zones.

Methanol was the best solvent for extraction of the active material that produced by *N. commune*. Ostensvik *et al*. (1998) have found that methanol extracts made from *Tychonema bourrellyi*, *Anabaena flos-aquae* and *Cylindospermopsis raciborskii* showed the most pronounced inhibitory effects where an aqueous extracts made from *Microcystis aeruginosa* and *T. bourrellyi* possessed evident antibacterial properties. Mynderse and Moore (1977) extracted debromoaplysiatoxin (alkalylphenol) from the cyanobacteria *Lyngbya* sp. and *N. muscorum* with a mixture of chloroform and methanol (1:2, v/v). The antimicrobial activity produced by the experimental organism *(N. commune)* was subjected to series of experiments aiming to identify its chemical composition. The antimicrobial material was purified by using preparative TLC and silica gel column chromatography. The chromatographic studies of one gram total methanol extract of *N. commune* resulted in the isolation and purification of four compounds (A, B, C and D). In the present work we used UV spectra, NMR, IR and mass spectra data to elucidate the chemical composition of the antimicrobial active compound produced by

Nostoc commune. The IR spectrum indicated the presence of OH and ester functionalities and ^{1}H-NMR spectrums indicated the presence of 13-glycosidic linkage. Gromov *et al.* (1991) showed that cyanobacterin LU-l produced by *N. linckia* had a characteristic UV spectrum with maximum absorbance at 210 nm, 265 nm and 300 nm. Juttner *et al.* (2001) elucidated the chemical structure of Nostocyclamide M, a cyanobacterial cyclic peptide with allelopathic activity from *Nostoc* 31, by chemical degradation detailed NMR and mass spectroscopic analyses. The purified extracted compounds were long chain alcohol, sterol, triterpen and long chain fatty acids. These compounds were tested for their biological activities against *B. subtilis* and showed that compound (C) produced the highest growth inhibition with *B. subtilis* while compounds A, B and D were not active. The spectroscopic studies suggested that compound (C) was a long chain of fatty acid. The saturated fatty acids caprylic, capric, lauric, myrestic and the un-saturated ones, palmitaleic, oleic, linoleic and linolenic acids separated in *Oscillatoria* extracts were demonstrated to have antimicrobial activities against Gram - ve, Gram + ve bacteria and pathogenic fungi as reported by different studies (Fei *et al*., 2002, Ghazala *et al*., 2004, Krasnoff *et al*., 2005). Sabine *et al*. (2003) founded that fatty acids inhibited the growth of *B. subtilis* SBUG 14, *Micrococcus flavus* SBUG 16, *Staphylococcus aureus* SBUG 11 and ATCC 25923, but no activity was observed against multi resistant *S. aureus* strains.

In conclusion, more chemical analyses must be carried out to elucidate the complete structure of the obtained and purified compound from our isolate as the amount obtained from these fractions in *Nostoc commune* was very little.

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