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Isolation, Identification and Antibacterial Activity of Alkaloid Compound N-Methylcytisine from Cyanobacterium *Hapalosiphon Aureus*

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ABSTRACT

In this study, one species of algae, belongated to cyanophyta : *Hapalosiphon aureus* west and west 1897 were isolated, identified and purified. The extracts of algal isolates were testing to clarify their ability on growth inhibition on each of gram +ve and gram –ve bacteria *E.coli* & *S. aureus*, filamentous fungi *A. fumigatus,* and yeasts *C.albican*. The extracted alkaloid of species *H.aureus* showed higher inhibition activity. In our literature survey, the alkaloid compound analog N- methylcytisine was isolated for the first time from the *H.aureus*. This compound was characterized using Thin Layer Chromatography (TLC), Ultra Violet Spectrum (UV), Infrared Spectrum (IR), Proton Nuclear Magnetic Resonance Spectroscopy (^{1}HNMR), Melting Point and Solubility in organic and inorganic solvents. The biological activity of this alkaloid was determined using the Minimal Inhibitory Concentration (MIC) against six bacterial isolates. The results showed inhibition in 33.3% growth of bacteria on 150 µg/ml concentration level, while the MIC against six fungi isolates showed inhibition of growth 33.3% at 50 µg/ml compared to standard antibiotic. The median lethal dose (LD_{50}) showed no toxicity, no cytotoxicity and no effect on human red blood cells for 18 hours.

1. Introduction

Cyanobacteria has been identified as one of the most promising groups of algae from which were isolated novel, biologically active natural products (Al-Aarajy, et.al. 2012). The prokaryotic microalgae are known to produce secondary metabolites with diverse biological activities such as antibacterial, antifungal, Pesticidal (Biondi et al., 2004; De Morais et al., 2015; Walton & Berry, 2016, Fu et al., 2017), enzyme inhibition and anticancer (Chen et al., 2003).

One such compound is calothrixin A (Chen et al., 2003), Pentacyclic indol which kills cultured human cancer and malaria cells in culture (Rickards et al., 1999). These compounds have been tested for different type of bioactivity with positive effect. The cyanobacterium *H.aureus* was exposed to some heavy metal ion to showed the effect on the growth. The results showed that the effect was depended on metal type and its concentration (Al-Hejuje, 2008). Khalaf, 2020 reported that the methanol extract from *H.aureus* was explained high activity in three concentration recording 100% of parasite death at 200 µg / ml of extract report activity against the parasite after 4-5 days. Abdulredha, et al., (2021) used the cyanobactrium Hapalosiphon *sp.* for wastewater phycoremedation that alga showed the most efficient for treatment and producing biomass after 15 days.

Two cyanobacterial strains: *Thermoleptolyngbya sp.* and *Leptolyngbya sp* isolated from Al-Ahsa were identified using molecular methods (Al Naim & El Semary, 2022; Al-Mousawi, et al., 2023). This is the first case reported on the effect of lasers on enhancing the antimicrobial profile of cyanobacterial extracts against bacterial pathogens, as well as enhancing accessory pigment content. The aim of our work was to isolate and identify active alkaloid compound from cyanobacteria and test its against some bacteria and fungal species. Furthermore, some experiments were done

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for purification of active compound and elucidation of its chemical structure.

2. Material and Methods

Isolation and Culturing of Cyanobacteria

The blue-green algae species were used: Cyanophyceae: Stigonematales:

Hapalosiphon aureus, and isolated from moist soil (Abo-Al-Khasib in Basrah city) using streaking method (Hellebust, 1985) on solid Chu-10 media, and re-streaked several time to obtain unialgal cultures. Axenic culture was obtained.

Extraction and Purification of the alkaloid

The alkaloid was extracted from axenic cultures of isolated species from *Hapalosiphon* during stationary phase for 10 days by extraction with an equal volume of 10% ammonia and chloroform, and reflexed for 1.5 hour, then 2% HCl was added. The product was then filtered. After that, 10% ammonia was added to obtain pH 9. Chloroformic layer was evaporated in room temperature. Further purification was done by TLC.

Chemical analysis of purified alkaloid

A-IR-Spectroscopy Analysis

A mixture of the test material with pure dry KBr, were pressed into a small disc. The measure was carried out between 500-4000 nm (El-Sheekh et al., 2006).

B- UV-Spectroscopy Analysis

The UV-Spectra of tested material were determined using UV-Visible spectrophotometer. The wave length is ranged from 200-500 nm.

C- NMR-Analysis

The pure alkaloid was dissolved in dimethyl sulfoxide (DMSO). The different function groups could be identified using NMR (200MHZ).

D- Sodium fusion test of alkaloid isolated

This method was made according to Fieser and Williamson, 1983.

Bioassay Test of Alkaloid Isolate

Three types of bioassay methods were made to investigating the biological characters of alkaloid isolate. The first was by determination of minimum inhibition concentration (MIC) against the Gram-negative bacteria, *Escherichia coli*, *Klebsiella* the Gram-positive bacteria, *Staphylococcus aureus, Pseudomonas* sp. (Wayne, 2006), the yeast *Candida albicans; C. tropicalis,* the fungi *Aspergillus flavus,* and *A. fumigatus*.

The second method was by red blood cell for determined the cytotoxicity of the alkaloid isolate (Nair et al., 1989).

The third method was by determining of medium lethal dose concentration (LD_{50}) of alkaloid isolate on mice type albino.

Statistical Analysis

All statistical analysis were performed to detect significant differences at ($P<0.05$) using the program SPSS version II.

3. Result and Discussion

Description of Algal Isolate

The alga *Hapalosiphon* is true-branching filamentous algae in one side and the branch with a long hair (Figure 1). The importance of using bacteria-free unialgal cultures in such investigation is well known to the author. The filamentous cyanobacteria *H. aureus* was well known as a wide distribution in the world.

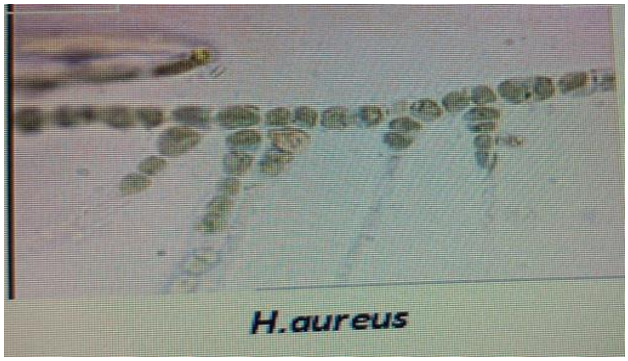

Fig. 1. Morphological characters of cyanophyta *H.aureus* isolated

Testing of Alkaloid Purity by TLC

Figure 2 shows TLC test of alkaloid. One yellow pigment with R_f =0.44 colored by the Dragendorff, and the fraction gave appositive Dragendorff; Wagner and Mayer reaction (Table 1). The results of TLC technique for purified alkaloid isolated from cyanobacterial were revealed that alkaloid compound in algal extracted as shown in Table 1. The result indicate that the alkaloid compound was differing and depends on both algal species efficiency and extraction activity. (Becher et al., 2005).

Fig. 2. TLC Test of purity alkaloid isolated from *H. aureus* in NH_4OH: Me OH(3:200) by dragendorff

Table 1
Qualitative test on alkaloid extract from *H. aurcus*

Test	Result
dragendorff	Ve+
Wagner	Ve+
Mayer	Ve+
Marqus	Ve-
Molish	Ve-
Amide	Ve+

Bioassay

The antimicrobial activity was evaluated as the diameter of inhibition zones formed as a result of disc assay and hallow well technique method in case of bacteria and fungi, respectively. Table 2 shows that the alkaloid isolate from *H. aureus* was antagonistic to all organisms examined. The highest activity against *C. albicans* (Figure 3). According to the results of primary screening of algal extract, *H. aureus* was used for alkaloid extract. These results indicate that the antimicrobial activity was strongest against Gram-positive bacteria than the Gram-negetive one. This is in agreement with Ghasemi et al., 2003; El-Sheekh et al., 2006) who found high antibacterial and antifungal activities produced from cynobacteria and chloriphyta.

Table 2
Inhibition zone of purity alkaloid extract from cyanobacteria against test pathogens

Algal isolate / Microorganisms	Inhibition zone (mm) *H.aureus*
E.col (ATCC 25922)	6
S. aureus (ATCC 25923)	8
A.fumigatus	2.Colony
C.albicans	30

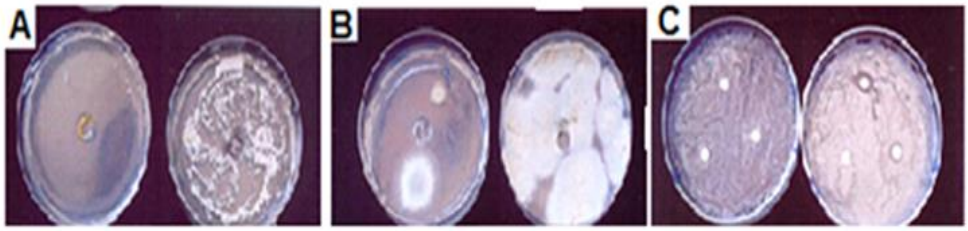

Fig. 3. The inhibition reactive of purity alkaloid extracted from *H. aureus against. A: C. albicans, B: A. fumigatus* and *C: bacteria*

Elucidation of the chemical structure of the purity alkaloid

The suggested structure has been confirmed by the following:

IR-Spectra

In the IR spectrum (Table 1), absorption at the 2916 and 2954 cm^{-1} represent the CH_3 and CH_2 stretching, respectively. The peaks at the 1735 cm^{-1} referred to the C=O group. The C=C and C-N stretching band located at1430 and 1025-1150 cm^1, respectively.

Table 3
Absorption and chemical groups in infrared of alkaloid isolated from *H. aureus*

Absorption (cm^{-1})	Chemical groups
2916	CH_3 St.
2954	CH_2 St.
1735	C=O St.
1650	C = O St. Conjugated
1400-1430	C = C St.
1025-1150	C-N St.
600-980	C-H b.

St = Stretching ، b = bending

UV-Spectra

The UV-spectra of the purified alkaloid isolated from *H. aureus*. This spectrum shows highest absorption at 251 and 385 nm may be assigned to types of π→π* and n→π* transitions, respectively.

^{1}HNMR Spectra

The ^{1}HNMR Spectra of alkaloid extracted from *H. aureus* using DMSO as a solvent were summarized in Table 4.

Table 4
Chemical groups in 1HNMR of alkaloid extracted from *H. aureus*

ppm	Chemical groups
0.8 – 1.2	CH_3
3.44 – 3.5	CH_2
4.3 – 4.4	CH_2 – N
6.8 – 7.0	H in aromatic ring
7.6 – 7.8	=CH
1.9 – 2.5	DMSO
3.2 – 3.4	Protons of water in DMSO

Sodium fusion test

Table 5 shows the qualitative test on alkaloid. The ability of soluble clearly indicated that the alkaloid isolated was moderate polarity and the positive amide test recorded reveals appearance of amide group in its structure.

Table 5
Qualitative test on alkaloid

Test	Result
Nitrogen test	+ve
Halogen test	-ve
Melting point	155°C
pH	6.8
Solubility	Soluble in acetone, methanol, ethanol, chloroform, ether and water

Chemical structure of *H. aureus* is $C_{12}H_{16}N_2O$ which similar with N- Methylcytisine (Figure 4) (Ma et al. 2002). Isolation of this compound from root of *Sophora flavescens* were tested for antiviral activity. In addition, the cyanophyta seem to be a source of potential new active substance that could use in pharmacological properties.

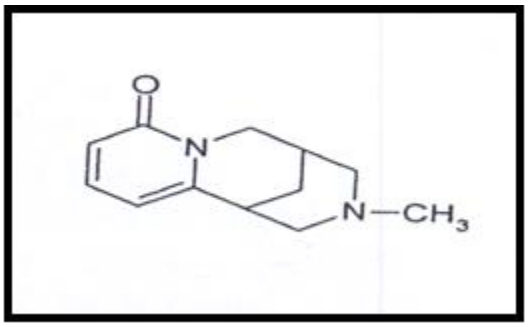

Fig. 4. Chemical structure of alkaloid N-methylcytisine

Antibacterial activity test of purity alkaloid isolated from *H. aureus*

Determination of MIC

Tables (6 and 7) summarizes the MIC of the purity alkaloid isolated from *H. aureus* against various microorganisms that were determined and compared with those of standard antibiotic Gentamycin and Tetracycline. The alkaloid was shown to have strong activity toward Gram-positive bacteria, such as *S. aureus* at 150 µg/ml concentration of alkaloid. However, the action was weak toward Gram-negative bacteria, especially when compared with Gentamycin (Table 6). The MIC is defined as the lowest concentration of antibiotic at which there is no visible growth with Ignore hazy growth and one or two colonies on the "spot" with the agar methods. The results show a strong activity toward Gram-positive bacteria (*S. aureus*) however, the action was weak toward Gram-negative bacteria (*Klebsiella* sp.). While the action of standard antibiotic was stronger than alkaloid isolated against Gram-positive and Gram-negative bacteria (Chetsumon et al., 1998).

Although the alkaloid activity against pathogenic yeast varied, depending on the strain tested, it was stronger than that of Fluconazol and Ketoconazole which inhibit the filamentous fungi at the concentration more than 100 µg/ml (Table 7). Its activity against pathogenic fungi *A. flavus ; fusarium* was also varied and stronger than that of standard antifungal. May be because of cell membrane of filamentous fungi are damage and become wrinkled (Chetsumon et al., 1998) and changing its permeability. Moreover, the activity of alkaloid isolated from *H. aureus* is due to specific carbonyl group (C=O) in its structure which interact with DNA and RNA and then reducing the growth of microorganism and killed them, (Rickards et al., 1999). On the other hand, Ghasemi et al. (2004) reported that the methanolic and hexane extracted from *Hapalosiphon* sp. had the minimum activity against the test organism.

The statistically result revealed significant differences at $P<0.01$ in the alkaloid isolate and standard antibiotic, while there were no significant differences between standard antibiotic and antifungal.

Table 6
MIC of purity alkaloid extracted from *H. aureus* against pathogen bacteria compared to standard antibiotics

Microorganism	g/mlµ		
	Tetracycline	Gentamycine	Alkaloid
E.coli (ATCC 25922)	0.5	0.25	200
E.coli	0.5	0.25	200
S.aureus (ATCC 25923)	1	0.5	150
S.aureus	1	0.5	150
Klebsiella sp.	1	1	1000
Pseudomonas sp.	1	0.25	500

Table 7
MIC of purity alkaloid extracted from *H. aureus* against pathogen fungi and compared to standard antifungal

microorganism	g/mlµ		
	Fluconazole	Ketoconazole	Alkaloid
Aspergillus flavus	100 <	100 <	50
A.fumigatus	100 <	100 <	100 <
Fusarium sp.	100 <	100 <	50
Penicillium sp.	100 <	100 <	100 <
Candida tropicales	100 <	100 <	100 <
C.albicans	100 <	100 <	100 <

Determination of (LD_{50}) for alkaloid from *H. aureus*

The mouse bioassay is widely used methods to determine the medium lethal dose (LD_{50}) the results in Table 8 referred that there is no death among the mouses used after expouring to the algal alkaloid in rang between 24-28 hours. This result isagreement with Dasgupta (2015) and Fu, et al., (2017).

Table 8
LD50 of purity alkaloid extracted from *H. aureus* against mouses

Dose g/kg	Mouse	Mortality (hour)		Death (%)
		48	24	
1	4	0	0	0
3	4	0	0	0
4	4	0	0	0
Control 0.25ml of D water	4	0	0	0

Cytotoxicity

5 revealed that the isolated alkaloid had no effect on the human blood cell at 50, 100 and 500 µg/ml concentration. The second bioassay method using human red blood cell is a suitable test to determine the cytotoxicity of cyanobacterial extract showing that the concentration from 50-500 µg/ml could not influence on RBC among the period 1-18 hours.

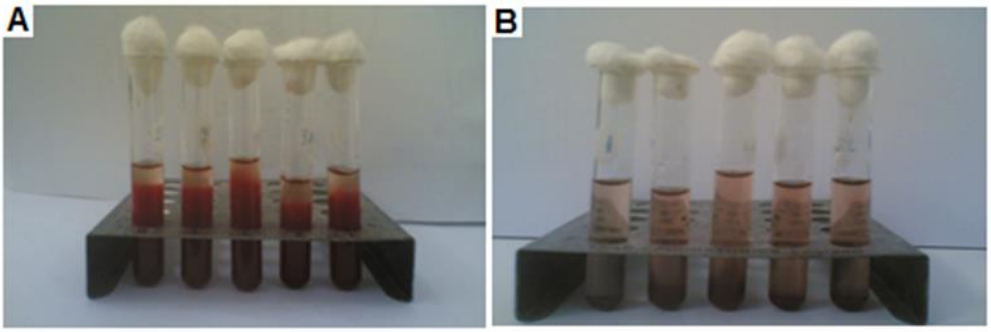

Fig. 5. Cytotoxicity of purity alkaloid extracted from *H. aureus* against human RBC A= after one hour B= after 18 hour

4. Conclusion

Alkoloid compound N-methylcytisin was isolated for the first time from cyanobacterium *H. aureus*. It showed a broad spectrum efficacy against germs and yeasts. The results of the middle lethal dose and cytotoxicity also showed no toxicity of the compound on experimental animals or against red blood cells. The concentration of the isolated alkaloid from 50-500 µg/ml couldn't influence on RBC among the period 1-18 hours.

Competing Interests

The authors have declared that no competing interests exist.

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