Reports of Biochemistry & Molecular Biology
Vol.13, No.1, Apr 2024

Original article

www.RBMB.net

Investigate Freshwater Algae Extract's Efficacy in Treating Diabetes Ulcers and Its Anti-Staphylococcal Properties

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Abstract

Background: Infection of diabetic foot ulcer is very common and leads in 20% of cases to amputation. Antibiotic-resistant *Staphylococcus aureus* is the main cause of severe infection. Antibiotic resistance is a major challenge to the global health system. This work aimed to investigate the antibacterial efficacy of some algae extracts against *Staphylococcus aureus* isolated from diabetic foot ulcers.

Methods: freshwater river samples were collected to isolate the algae, and PCR was used for identification. The ethanol, water, and ethyl acetate extract of these algae were prepared and analyzed using high-performance liquid chromatography-mass spectrometry to determine the key components that have antibacterial properties. The antibacterial activity of these extracts against *S. aureus* was determined by broth dilution and well diffusion methods.

Results: *Chlorella vulgaris* and *Anabaena flos*-aquae were isolated from freshwater river and identified by PCR. *Anabaena flos*-aquae has a greater antibacterial efficacy against *Staphylococcus aureus* in comparison to *Chlorella vulgaris,* and the ethanolic extract demonstrated superior outcomes compared to the aqueous and ethyl acetate extracts. The MS spectrum of both algae had a very similar pattern, but the frequency of detected peaks was different

Conclusion: Ethanolic extract of *A. flos-aquae* and *Chlorella vulgaris* can be suggested to treat and control diabetic foot ulcer infection caused by *S. aureus*. Further studies are required to explore the full potential of these algae safely and extensively.

Keywords: Algae, Bacterial Inhibition, Diabetes, Foot ulcer.

Introduction

Diabetes mellitus (DM) is a prevalent hormonal illness characterized by high blood glucose levels due to a deficiency in insulin production or effectiveness (1,2). The prevalence of diabetes mellitus has quadrupled from 1980 to 2014, and projections indicate a 51% increase by 2045 (3). Diabetes mellitus can lead to several complications, including nephropathy, neuropathy, retinopathy, atherosclerosis, and foot ulcers. These complications are responsible for more than 50% of the direct expenditures associated with diabetes. Diabetic foot ulcers (DFUs), which often occur as a result of infected wounds, can lead to the amputation of the lower leg and increased death rates. Research suggests that around 50-70% of amputations in the lower limbs are a result of DFUs. Globally, a limb is surgically removed every 30 seconds due to the presence of DFUs (4).

Chronic infections of DFUs are caused by intricate communities of several

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Received: 21 Apr, 2024; Accepted: 25 Aug, 2024

[DOI: 10.61186/rbmb.13.1.114]

microorganisms, referred to as polymicrobial biofilms, in which pathogenic bacteria thrive despite the immune system's efforts (5). Immune failure reduces the effectiveness of cellular defense systems, neuropathy affects the ability to feel pain and be aware of wounds, and angiopathy reduces and minimizes the amount of oxygen in tissues and increases the severity of infections. These attributes emphasize the complexity and severity of DFUs in individuals with diabetes (6).

The extensive use of antibiotics has led to the creation of bacteria that are resistant to antibiotics. These bacteria increase rapidly due to their fast growth and capacity to transfer genetic material (7). Individuals with diabetes who have ulcers are susceptible to bacterial infections caused by pathogens such as *Staphylococcus aureus (S. aureus)* and *Pseudomonas aeruginosa*. These infections can impede the process of wound healing (8). *Staphylococcus aureus* is a prominent pathogen known for its ability to form resilient biofilms, making treating diseases more challenging. *Staphylococcus aureus* isolated from DFUs exhibited remarkable antibiotic resistance, with only levofloxacin and ceftaroline showing efficacy (9).

Ongoing research is focused on discovering novel antibacterial compounds that have reduced side effects and enhanced efficacy. Historically, herbal remedies have been used to treat infectious diseases, specifically focusing on investigating the antibacterial properties of plant extracts and essential oils (10, 11).

Marine habitats include algae and cyanobacteria that generate many physiologically active compounds, including several with potent antibacterial properties. Algae possess many antioxidants, pigments, fatty acids, and proteins. Cyanobacteria, including *Dolichospermum affine*, produce compounds that reduce cholesterol levels and have antibacterial properties through secondary metabolites (12). *Chlorella vulgaris* and *Dunaliella salina* are renowned microalgae species recognized for their abundant carotenoids with potent antioxidant qualities, rendering them highly sought-after in sectors such as cosmetics and nutrition (13). Microalgae, including several species of Chlorella, possess antioxidants, phenolic compounds, and flavonoids that can neutralize free radicals and combat illnesses resulting from oxidative stress (14). The health benefits offered by these natural antioxidants, particularly those derived from microalgae, underscore their potential as suitable alternatives to synthetic counterparts (15).

This research has focused on isolating and identifying two species of freshwater algae and creating extracts from them, examining the impact of the prepared extracts against *S. aureus* obtained from diabetic ulcers, and analyzing the extracts using High-performance liquid chromatography-mass spectrometry (HPLC-MS) to discover bioactive compounds.

Materials and Methods

Algae Separation and Identification

Algal sample were collected from some springs and streams water resources at Shawre valley within Ranya district Sulaymaniyah governorate, Iraq. The algal species are identified and diagnosed according to (16). Streaking technique plate method used for the purification and isolation algal sample. For the isolation of microalgal cultures 10 mL of the collected water sample were transferred to 200 mL of the sterile Bold’s basal medium (BBM) in a 500 mL conical flask. The flask was then incubated on a rotary shaker and subjected to continuous illumination with white fluorescent light at a temperature of 27 ℃ and a speed of 150 rpm for three weeks (17).

At a frequency of every other day, the flasks were inspected for the presence of algae using an optical microscope, and diluted samples were made in BBM from flasks that exhibited growth. The creation of

[DOI: 10.61186/rbmb.13.1.114]

subcultures involved the introduction of 50 mL onto petri plates with BBM that was solidified with 1.5% (w/v) of bacteriological agar. In addition, 50 mL portions of the identical dilution were added to each wells of a 96-well microtiter plate that already contained 200 mL of BBM. The Petri and microtiter plates were placed in a controlled environment at 27 ℃and exposed to constant white fluorescent light for two weeks. The integrity of the culture was maintained by iterative plating and consistent microscopic examination. Microscopic identification was conducted and subsequently validated using genetic markers and was confirmed using molecular markers.

DNA extraction and PCR amplification

A portion of cultivated cells (1 mL) was collected during the mid-to-late exponential growth phase (10-14 days) by spinning them down (13,000× g for 3 min at 4 ℃) in a sterile microcentrifuge tube. The genomic DNA was isolated using a Plant Genomic DNA extraction kit (SolGent, Daejeon, S. Korea). The DNA concentration of the extracted DNA was quantified at a wavelength of 260 nm using a spectrophotometer (HACH, DR/4000v, USA). The rDNA's D1-D2 (LSU) coding region was amplified by conducting amplification reactions on a T-Gradient thermocycler (Biometra GmbH, Gottingen, Germany). The amplification was carried out using the universal eukaryotic primers 5'-AGCGGAGGAAAAGAAACTA-'3 as the forward primer and 5'-TACTAGAAGGTTCGATTAGTC-'3 as the reverse primer. The PCR protocol described by Sonnenberg et al. was followed for the amplification process (18). Ten µL samples of the reaction mixtures were examined using 1% horizontal agarose gel electrophoresis (Sigme-Aldrich Co., Germany) to verify the product's existence. The PCR products were purified using the Gel PCR Clean-Up System (Applied Biosystems, Foster, CA). Sequencing reactions were performed using a Dye Deoxy Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and sequencing fragments were analyzed on an ABI Prism 377 DNA Sequencer.

The ribosomal RNA gene sequences from the isolates were compared to the GenBank database using the BLAST algorithm. The aligned sequences underwent human inspection and were mod ified using Genedoc (19). A phylogenetic tree was created using the neighbor-joining (NJ) approach, employing Kimura's two-parameter model of sequence evolution, as implemented in the MEGA4 program package (20).

Preparation of the Algal Extracts

The extraction process with solvent consisting of ethanol, water, and ethyl acetate was carried out at a room temperature of 25 °C for 30 minutes, utilizing an ultrasonic wave with a power output of 900 W. The specimens, one g for each sample in 10 ml of solvent were immersed in an ultrasonic bath (Takta Co., Iran) for 30 minutes to facilitate extraction. The extracts underwent filtration using a 0.22 µm filter (Millipore Co, MA, USA) and were then dried using a freeze-dryer (Pishtaz Co., Iran).

Sampling and Bacterial Strains Identification

Pus samples were collected from patients with DFU and referred to the microbiology lab using transport media for analysis. Each sample was inoculated on blood agar and MacConkey agar at 37^0 for forty-eight hours, *S. aureus* isolates were identified by biochemical tests and were confirmed using API staph tests (bioMérieux, Marcy-l'Étoile, France). The age of the patients ranged from 25 to 70, and 68% were females.

There were no exclusion criteria for patients, any patient with DFU with purulent discharge was eligible to

[DOI: 10.61186/rbmb.13.1.114]

participate in this study. The study received ethics approval from the Shahid Beheshti Medical University Health District Research and Ethics Committee (IR.SBMU.RETECH.REC.1402.652 and IR.IAU.ARDABIL.REC.1402.123), and all subjects gave informed written permission.

Antibacterial activity of selected algae

Micro broth dilution methods evaluated the algae anti-staphylococcus according to standard protocols recommended by the Clinical and Laboratory Standards (21) to determine each algae's minimum inhibition concentration (MIC). Fresh cultured *S. aureus* was adjusted to 0.5 McFarland standard turbidity using normal saline and then further diluted (1:100) with sterile Mueller-Hinton broth (Sigma-Aldrich Co., Germany) before adding to the trays. Algal extract serial dilutions were made in 1024 mg/ml concentrations in sterile 96-well plastic microdilution trays containing Mueller-Hinton broth. MICs were recorded after incubation for 24 h at 37 °C.

The antibacterial activity of the algal extract was assessed using the well diffusion technique. 0.5 McFarland S. aureus suspension was spread with swabs onto Mueller–Hinton agar (MHA) cultures (Sigma-Aldrich Co., Germany). Afterward, 100 µl of the algae extract was applied onto a 6 mm measuring well in the plate. In the next step, the inoculated Petri dishes were incubated for 24 h at 37 °C, and the resulting inhibition zones (IZ) were measured. All tests were performed in triplicate. Two antibiotic swere u sed aspositive control which arelevofloxacin .and ceftazidime the water was used asanegative control

HPLC-MS analysis of Algae extract

HPLC-MS was performed to study the algae extract composition. For this, the HPLC (Knauer Co., Germany) equipped binary pump and C18 column (100 Å × 3 µm × 200 cm × 1.5 mm) for 10 µm injection were used for chromatographic analysis. The mobile phase included a mixture of water with 2% acetonitrile and 0.1% trifluoroacetic acid (Phase A) and acetonitrile with 20% water and 0.08% trifluoroacetic acid (Phase B) (Merck Co., MA, USA). Column elutes using phase A and within 5 min up to 75%. In the next 5 min, the percentage of solvent A reached 0%, and washing continued for 10 min. After that, the phase A% was increased to 100% in 5 min and kept for 10 min. The flow rate was 200 nl/min, and the total separation time was 45 min using a PDA-UV detection. The Mass spectrometry apparatus model Finigan LTQFT Ultra (Thermo Fisher Scientific., Germany) is equipped with (an NESI) ion generator, Nano Electro Spray Ionization, Ion Trap (IT) mass detector with ability separation of FWHM 100 Da and accuracy higher than 0.3 ppm were connected to HPLC and used to identify metabolites. The Xcalibur software conducted instrument control, data acquisition, and processing. Typical negative ESI-MS conditions were a capillary voltage of 3.0 kV and a skimmer cone voltage of 30 V.

Results

Algea identification and extraction

In the present study, two microalgal isolated based on their morphologies (namely, their cell shape and size) and their ability to be cultured successfully in pure form under our test conditions. Figure 1 displays light microscopic pictures of the identified species that were isolated throughout this investigation.

Examination of algal isolates under a microscope showed that they existed in colonies and were free from impurities. The diverse morphology of algae poses challenges for microscopic identification. Hence, both the whole DNA and the PCR-amplified rRNA (LSU) were extracted to validate the species identifications based on morphology.

[DOI: 10.61186/rbmb.13.1.114]

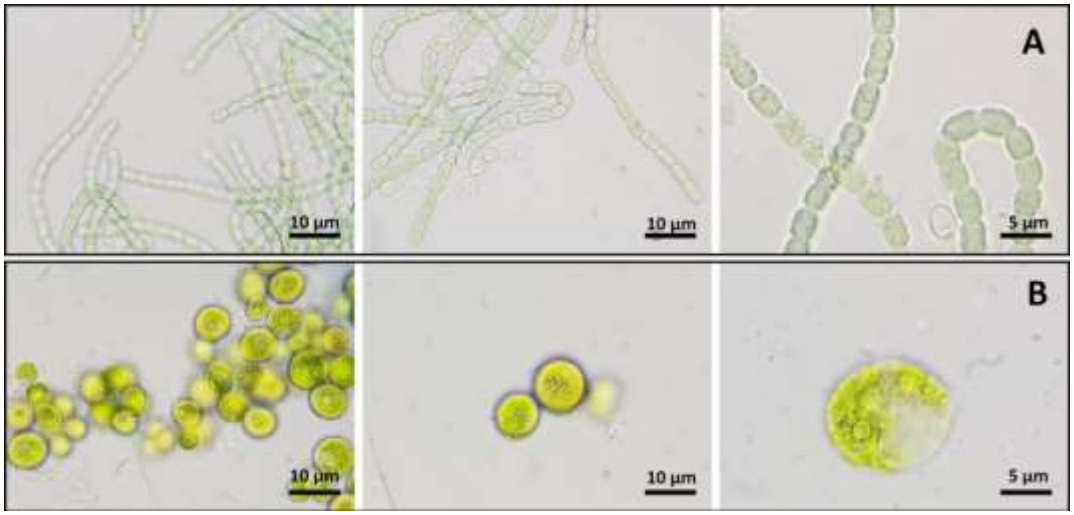

Fig. 1. Light microscope images with 40 and 100 magnifications related to algae *Chlorella vulgaris* (A) and *Anabaena flos*-aquae (B).

All isolates showed a single amplified LSU rDNA product with a size of around 850 bp. The LSU rRNA gene has a higher evolutionary rate than the SSU rRNA gene and should, therefore, allow for better discrimination between closely related species using short diagnostic sequences. Based on the LSU rDNA sequences, we concluded that microalgal isolates were closely related to *Chlorella vulgaris (C. vulgaris)* and *Anabaena flos*-aquae (*A. flos*-aquae). The results from the phylogenetic analysis of the LSU rDNA sequence in the phylogram (Fig. 2) further confirmed the identification of the microalgal strains.

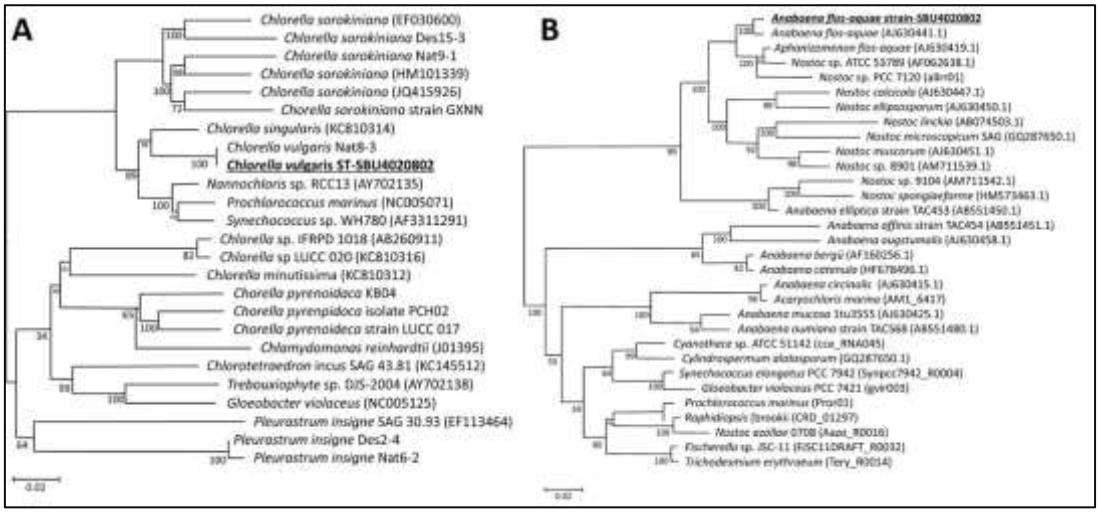

Fig. 2. Phylogenetic tree generated by the neighbor-joining method based on partial 16S rRNA sequences showing the phylogenetic relationships between SBU isolates and closely related species of the genus Bootstrap values (expressed as percentages of 1000 replications) are shown at significant branching points.

Antibacterial effect of Algae extracts

The ethanolic extract demonstrated superior outcomes for *S. aureus* compared to the aqueous and Ethyl acetate extracts (Table 1). The aqueous extract of both types of algae had a negligible impact. Compared with positive control, we found the ethanolic extract of *Anabaena flos-aquae* had an efficacy less than levofloxacin but close to the efficacy of ceftazidime. However, the antibacterial activity of *Anabaena flos-aquae* was higher than *Chlorella vulgaris.* (Fig. 3.)

[DOI: 10.61186/rbmb.13.1.114]

Table 1. MICs and IZ of algae extracts, positive control (levofloxacin and ceftazidime), and negative control (water) against *S. aureus.*

Extract & control conditions		*S. aureus*			
		MIC[1] (µg/ml)	SD[2]	IZ[3] (cm)	SD[2]
Chlorella vulgaris	**Ethanol EX.**	64	0.0206	1.14	0.0058
	Water EX.	512	0.0116	ND[4]	0.0194
	Ethyl acetate EX.	128	0.0189	1.08	0.0221
Anabaena flos-aquae	**Ethanol EX.**	16	0.0057	1.52	0.0655
	Water EX.	256	0.0208	ND[4]	0.0178
	Ethyl acetate EX.	128	0.0202	1.03	0.0189
Positive control	**Levofloxacin**	8	0.0189	2.28	0.0177
	Ceftazidime	16	0.0179	2.08	0.0137
Negative control	**Water**	ND[4]	ND[4]	ND[4]	ND[4]

[1] Minimum Inhibitory Concentration (MIC), [2] Standard deviations (±), [3] Inhibition zone diameter (IZ), [4] ND: Not detected.

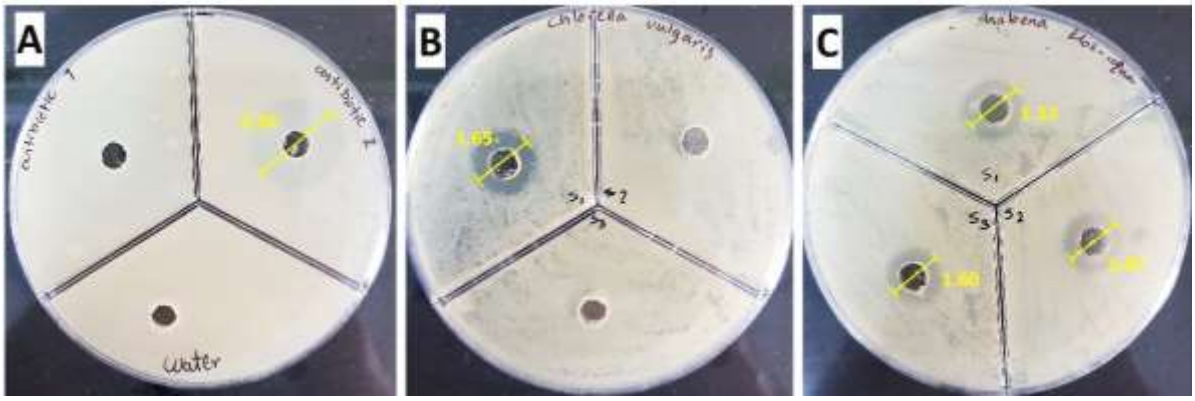

Fig. 3. The inhibition zone results of ethanolic extracts of selected *Chlorella vulgaris* (B) and *Anabaena flos*-aquae (C) against diabetic foot ulcer-isolated *S. aureus* compared to the antibiotics (A) Ceftazidime (No. 1) and Levofloxacin (No. 2) as a positive control and water as a negative control.

HPLC-MS of Extracts

The MS spectrum of both algae has a very similar pattern, but the frequency of detected peaks is different (Fig. 4). The most important identified adduct ion peaks are $[M+Na]^{+\bullet}$, $[M+H]^{+\bullet}$, $[M+H-H_2O]^{+\bullet}$, $[M+H-2H_2O]^{+\bullet}$. Vulgaxanthin I containing the ions of $[M+Na]^{+\bullet}$: 363.22 m/z, $[M+H]^{+\bullet}$: 341.19 m/z, $[M+H-H_2O]^{+\bullet}$: 322.24 m/z and $[M+H-2H_2O]^{+\bullet}$: 305.21 was identified as a separated peak in 32.03 min in *C. vulgaris* ethanolic extract. In the case of *A. flos-aquae* the most important identified adduct ion peaks for Anatoxin-a are $[M+Na]^{+\bullet}$: 188.11 m/z, $[M+H]^{+\bullet}$: 166.11 m/z, $[M+H-H_2O]^{+\bullet}$: 148.19 m/z and $[M+H-3H_2O]^{+\bullet}$: 130.11 as a separated peak in 29.00 min

[DOI: 10.61186/rbmb.13.1.114]

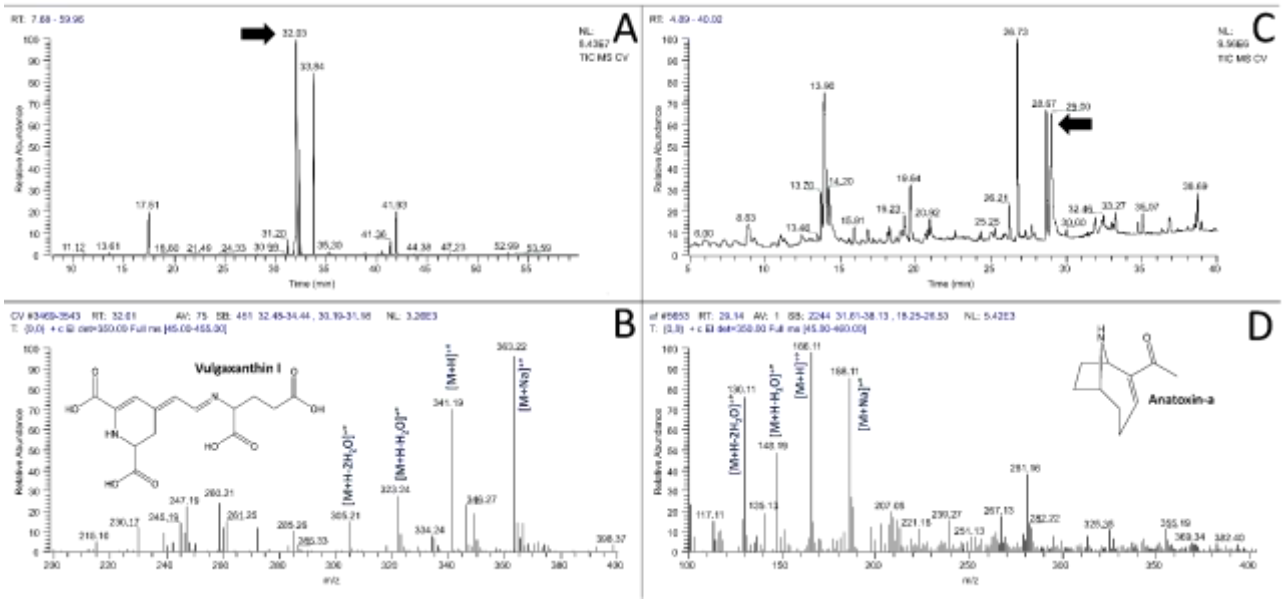

Fig. 4. HPLC-MS analysis of ethanolic extract and the Identified compound mass spectrum of *Chlorella vulgaris* (A and B) and *Anabaena flos-aquae* (C and D).

Discussion

Current research is dedicated to finding new antibacterial substances with less adverse effects and improved effectiveness. Historically, several alternative substances have been utilized to treat infectious illnesses, focusing on studying the antibacterial characteristics of herbal extracts and essential oils (22). This work involved purifying and identifying two algae, namely; *C. vulgaris* and *A. flos-aquae* to detect their antibacterial effect against *S. aureus*, the one responsible for most DFU infection. The algae extract affects the synthesis of enzymes such as superoxide dismutase, catalase, peroxidases, and scavengers such as vitamins B, C, D, and E, cysteine, and glutathione. Additionally, it can counteract or eliminate excited states and reactive oxygen species (23).

The present study found antibacterial activity against S. aureus for both algae, with *C. vulgaris* outperforming *A. flos-aquae* compared with some antibiotics as positive control.

The *C. vulgaris* showed the existence of active phytochemicals. The presence of phenolics, flavonoids, and tannins in the sample exhibited antioxidant properties, consistent with the results reported in the previous study (24). Due to the increasing interest in using natural-based components in cosmetic and nutraceutical formulations, there is a strong desire to explore the full potential of *C. vulgaris* safely and extensively (25). Several studies have suggested that incorporating *C. vulgaris* into one's diet as a supplement can effectively decrease inflammation and oxidative stress (26). Various human studies have demonstrated that *C. vulgaris* can reduce oxidative stress and inflammation, as well as improve lipid profiles and blood sugar levels. Nevertheless, it is crucial to acknowledge that these effects have not been uniformly seen in all studies (27). Participating in endurance exercise and using C. vulgaris resulted in a reduction in symptoms in patients with diabetes (28). Although the favorable benefits of *C. vulgaris* on hematological indicators, inflammation, oxidative stress, and body composition indicators have been shown, the precise molecular and cellular mechanisms underlying these effects remain incompletely known (29).

This work (to our knowledge) is the first study to detect the antibacterial effect of *A. flos-aquae.* The efficacy of *A. flos-aquae* was higher than *C. vulgaris* (MIC= 16 vc 64 µg/ml, IZ= 1.52 vs 1.14 cm). Anabaena is a type of filamentous cyanobacterium that is capable of taking in nitrogen and producing oxygen

[DOI: 10.61186/rbmb.13.1.114]

through photosynthesis (30). Approximately ten percent of the photosynthetic vegetative cells undergo terminal differentiation into nitrogen-fixing heterocysts at regular intervals along filaments when they are exposed to an environment that does not contain a combined nitrogen source (31). several compounds were isolated from *Cyanobacterial* the phylum of Anabaena with antibacterial impact against *S. aureus* such as Aeruginazole A (32), Ambiguine (33), and Anaephenes A, B and C inhibit (34, 35). Further investigation should be carried out to identify the active compounds of *A. flos-aquae* that hold the most promise for future research endeavors.

This investigation used ethanol and ethyl acetate as supplementary solvents, finding that ethanolic extract from algae had superior inhibitory effects on model bacteria. Alcohol is the most effective solvent for extracting phenols, with high efficiency. Methanol is unsuitable due to toxicity and solvent loss (36). Ethyl acetate is recommended as a safer alternative.

This study demonstrates that the ethanol extract from *C. vulgaris* and *A. flos-aquae* exhibits antibacterial properties against *S. aureus* isolated from DFU. Further studies are required to explore the full potential of *C. vulgaris* and *A. flos-aquae* safely and extensively

Financial support
No grant or financial support was used to conduct this research.

Conflict of interest
Authors have no conflict of interest to declare.

Acknowledgment
The authors want to acknowledge the cooperation of Shahid Beheshti University colleagues and university staff.

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[DOI: 10.61186/rbmb.13.1.114]

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[DOI: 10.61186/rbmb.13.1.114]

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[DOI: 10.61186/rbmb.13.1.114]