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Antibacterial Effects of *Spirulina* Blue-Green Algae Aqueous and Alcoholic Extracts on *Pseudomonas aeruginosa*

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ABSTRACT

Introduction: *Pseudomonas aeruginosa*, a Gram-negative bacterium, is the cause of infections in immunocompromised individuals, resulting in various conditions, including pneumonia, and urinary tract, skin, and bloodstream infections. This pathogen produces tissue-destructing toxins, leading to significant morbidity and mortality in affected patients. Conventional antibiotics, including rifampicin and colistin, are often ineffective in treating *P. aeruginosa* infections due to the emergence of bacterial resistance within affected communities. Alternatively, algae have been explored as a promising source for controlling pathogenic bacteria. In this study, we investigated the antibacterial effects of ethanol and aqueous extracts of *Spirulina platensis* against *P. aeruginosa*. **Methods:** Ethanol and aqueous extracts of *S. platensis* were prepared at concentrations of 0.125, 0.25, 0.5, and 1 mg/mL. The antibacterial effect of *Spirulina* blue-green algae against *P. aeruginosa* was conducted using a disc diffusion test in an LB medium with 7-mm wells. We measured the inhibition zone and statistically analyzed the data by comparing the means using the Duncan multiple range test. **Results:** The ethanol extract of *S. platensis* significantly inhibited the growth of *P. aeruginosa*. Furthermore, applying the ethanol extract of *S. platensis* at a concentration of 1 mg/mL resulted in the largest inhibition zone (20 mm) compared to the control. In contrast, the *S. platensis* aqueous extract did not significantly inhibit *P. aeruginosa* growth. **Conclusions:** The ethanol extracts of *S. platensis* algae exhibited a significant antibacterial effect against *P. aeruginosa*. This alga represents a promising source of antibacterial metabolites, which could be a suitable alternative to common antibiotics. Further investigations are necessary to identify and purify the specific antibacterial substance in *S. platensis*.

INTRODUCTION

Pseudomonas aeruginosa Migula 1900 is a Gram-negative bacterium commonly in soil, water, and other moist environments [1, 2]. This opportunistic pathogen can cause infections in the blood, lungs (pneumonia), and other body areas in individuals with weakened immune systems, producing tissue-destructing toxins [3].

Algae have been considered a potential source of antimicrobial compounds in medicine for many years [4]. *P. aeruginosa* can cause various infections, including urinary tract infections, respiratory infections, skin inflammation and swelling, soft tissue infections, bacteremia (the presence of bacteria in the blood), bone and joint infections, stomach and intestinal infections, and systemic infections such as sepsis. These infections are prevalent in patients with severe burns, cancer, and AIDS, whose immune systems are suppressed [2, 5].

P. aeruginosa is resistant to common antibiotics, including rifampicin and colistin [6]. This resistance is attributed to specific genes and low membrane permeability [6]. Furthermore, various types of vaccines have been developed against this bacterium. However, these vaccines are seldom used due to their high cost [7]. Alternatively, algae have been frequently studied as a potential source for controlling pathogenic bacteria, as

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they contain metabolites with antibacterial effects [8]. Algae are commonly studied as a potential source of essential medical compounds due to their antioxidant, anticancer, and antiviral effects [8]. *Spirulina*, one of the algae with antibacterial properties, is widely studied for its potential to manage bacterial infections [9-12].

Spirulina is a type of blue-green algae from the *Cyanobacteria* phylum. *Spirulina* consists of three species: *Arthrospira maxima*, *Arthrospira platensis*, and *Arthrospira fusiformis*. This alga grows in freshwater and is taxonomically classified in the *Zygnematales* order. *Spirulina* is primarily found and cultivated in Africa, Asia, South America, and Central America. It can be a nutritional source for animals and humans [13]. *Spirulina* is primarily used as a dietary supplement in the pharmaceutical industry (in the form of tablets or powder) and in the aquaculture, aquarium, and poultry industries [14]. Dried *Spirulina* is protein-rich, containing between 51%-70% protein. This product also contains 24% carbohydrates, 8% fat and 5% water, and various vitamins and minerals [15]. *Spirulina* extracts contain two secondary metabolites responsible for their antibacterial effects: phenolic compounds, such as gallic acid, and alkaloids, such as atropine [14]. This study aimed to investigate the antibacterial effects of *Spirulina* extracts against *P. aeruginosa.*

MATERIALS AND METHODS

Preparation of ethanolic and aqueous extracts of *Spirulina* algae. Initially, 50 g of dry *S. platensis* powder was transferred to a flask. Next, 500 mL of ethanol (Merck, Germany) and 500 mL of sterile distilled water were added separately to prepare the ethanolic and aqueous extracts. The flasks were then shaken in a dark room for 24 h. In this case, the flasks were covered with Parafilm to prevent evaporation. The flasks were placed on a stir plate and stirred for 24 h at room temperature. The upper liquid phase in the flasks was transferred to a Falcon tube, and the lower liquid phase was discarded. After centrifugation at 4000 rpm for 10 min, the upper liquid phase was filtered through Whatman filter paper No. 1 and placed in a rotary evaporator. The pellet was then transferred to a plate and incubated at 35 °C for 48 h [16]. Aqueous and alcoholic extracts of *S. platensis* were prepared at concentrations of 0.125, 0.25, 0.5, and 1 mg/mL (Figure 1) [12].

Fig. 1. Preparation of aqueous (right) and alcoholic (left) extracts from *S. platensis* algae

In vitro studies

Agar well diffusion method to determine the antibacterial effects of the extracts. Wells with a diameter of 7 mm were created on Luria Broth (LB) agar medium (Sigma-Aldrich, USA) using a Pasteur pipette. A 0.5 McFarland standard was prepared by adding 0.05 mL of 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \bullet_2 H_2O$) to 9.95 mL of 1% (vol/vol) sulfuric acid (H_2SO_4). The bacterial stock was inoculated into an LB medium and incubated at 37 °C for 24 h. The optical density of the bacterial suspension was adjusted with the 0.5 McFarland standard. Each well was filled with 100 µl of the different concentrations of extracts. The plates were then incubated at 37 °C for 24 h. The sensitivity or resistance of the bacteria to the extracts was determined by measuring the diameter (mm) of the inhibition zone (halo) around each well with a ruler. Kanamycin was utilized as the positive control, while water was the negative control in the antibiotics experiment. The experiment was triplicate, and the mean halo diameter was reported in millimeters (mm) [17].

Determination of the minimum inhibitory and lethal concentrations of the extracts. The broth microdilution method was employed for this purpose. Aqueous and alcoholic extracts of *S. platensis* were prepared and added to separate wells of a 100 µl microplate at concentrations of 0.125, 0.25, 0.5, and 1 mg/mL (four wells per concentration). All four wells were filled with 100 µl of LB medium. Half McFarland concentration of bacteria was prepared, and 50 µl of the bacterial suspension was

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added to each well. The microplate was then incubated at 37 °C for 24 h. The minimum inhibitory concentration was subsequently determined [17].

Statistical analysis. The data from the inhibition zone test were transferred to Excel software, and the mean value was subsequently calculated. The mean values were compared using the Duncan multiple range test to detect significant differences among treatments ($P < 0.05$). The statistical analysis was performed using SPSS software, ver. 22 (IBM, USA).

RESULTS

Diffusion disk assay. Exposure to *S. platensis* extract in an LB agar medium resulted in growth inhibition of *P. aeruginosa* (Figs 2 and 3). Three repetitions of the phenotypic diffusion test for aqueous and ethanol extracts demonstrated that the aqueous extract did not affect *P. aeruginosa*. Conversely, the ethanol extract exhibited growth inhibition of *P. aeruginosa*.

Minimum inhibitory concentration. At concentrations of 0.125, 0.25, 0.5, and 1 mg/mL, the diameter of the inhibition zone for the ethanol extract was 7.33, 11.23, 15.50, and 20 mm, respectively (Figs. 2-4). The results indicate that the ethanol extract exhibited growth inhibition at a minimum concentration of 0.5 mg/mL as determined by the minimum inhibitory concentration assay. The inhibition zone mean values in response to *S. platensis* ethanol extracts were statistically analyzed using the Duncan multiple range test, and the results are presented in Figure 4. The maximum inhibition was observed with 1 mg/mL of *S. platensis* ethanol extract. Conversely, the inhibition zone diameter was minimal, with a 0.125 mg/mL concentration of *S. platensis* ethanol extract. The 0.5 and 0.25 mg/mL concentrations produced inhibition zones with 15.5 and 11.23 mm diameters, respectively (Fig. 4).

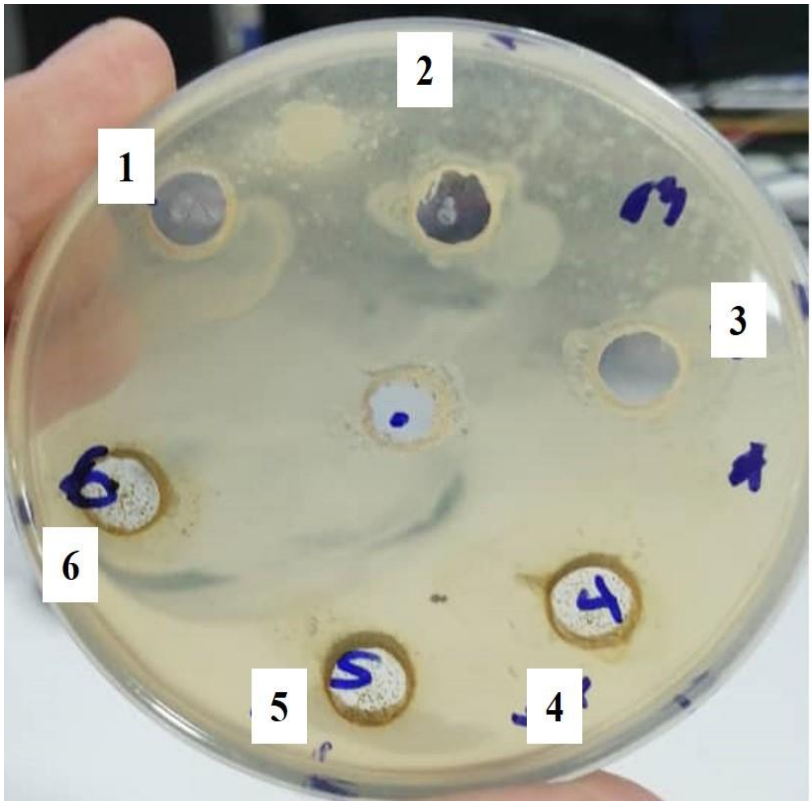

Fig. 2. Antibacterial effects of aqueous and alcoholic extracts of *S. platensis* against *P. aeruginosa* were assessed using the well diffusion method (wells 1-3: aqueous extract at 1 mg/mL, wells 4-6: alcoholic extract at 1 mg/mL). Kanamycin was used as a positive control (middle)

DISCUSSION

Algae have historically been considered photosynthetic organisms and utilized in traditional medicine for potential health benefits. Researchers and the general public have been interested in the effect of plants on infectious agents since ancient times [18]. Experiments have confirmed the impact of many plants. Herbal compounds, which contain naturally active biological components and chemical compounds, have been used to treat both infectious and non-infectious diseases [19].

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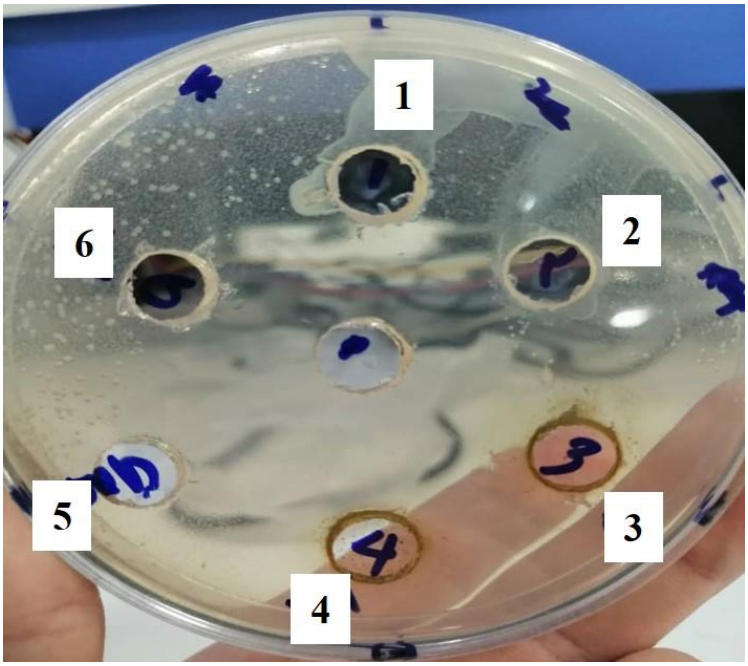

Fig. 3. The antibacterial effects of aqueous and alcoholic extracts of *S. platensis* against *P. aeruginosa* were evaluated using the well diffusion method (wells 1-2: aqueous extract at 1 mg/mL, wells 3-4: alcoholic extract at 1 mg/mL, wells 5-6: absolute ethanol). Kanamycin was used as a positive control (middle).

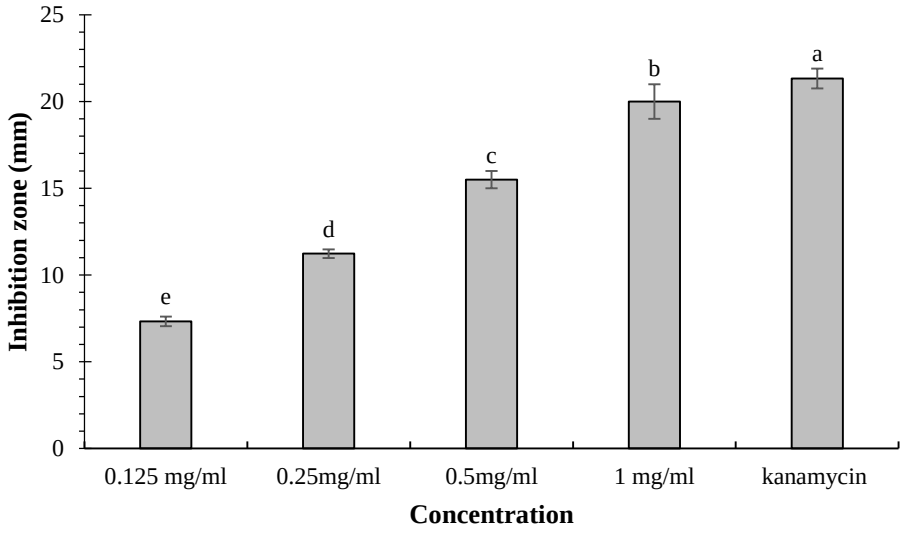

Fig. 4. The graph displays the growth inhibition diameter of *P. aeruginosa* exposed to varying concentrations of *S. platensis* ethanol extracts. Significant differences between the treatments were determined using Duncan's multiple range test, and the letters on the bars represent these differences.

Given the emerging resistance of pathogenic bacteria to antibiotics [6], algae appear to be a promising alternative for controlling bacterial infections. Several studies have investigated the antibacterial effects of algae, including *Cystoseira mediterranea*, *Enteromorpha linza*, *Ulva rigida*, *Ulva lactuca*, *Gracilaria gracilis*, *Pyropia yezoensis*, and *Ectocarpus siliculosus*, against *P. aeruginosa* [20-22]. The ethanol extract of *S. platensis* caused significant growth inhibition in *P. aeruginosa* in the present study. Rao *et al*. (2020) demonstrated that the methanol extract of S. platensis exhibits antibacterial activity against *Klebsiella pneumoniae*, a bacterium associated with pneumonia. The algal extract at a 5 mg/mL concentration produced an inhibition zone of 12.65 mm. The variation in inhibition zone size may be attributed to differences in the bacterial species (*K. pneumoniae*) and the type of alcohol (methanol) employed for extraction. In addition to the methanol extract, the authors evaluated other algal extracts, such as hexane, chloroform, ethyl acetate, and acetone. They

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found that the methanol extract produced the largest inhibition zone [12]. Our study yielded similar results, as only the methanol extract of *S. platensis* was found to inhibit the growth of P. aeruginosa significantly. Furthermore, our study employed a lower concentration (1 mg/mL) of the algal extract compared to the concentration used by Rao *et al.* (2020), yet still demonstrated a relatively high antibacterial effect against *P. aeruginosa*. Yousefzadi *et al*. (2011) conducted a disk diffusion test and determined the minimum inhibitory concentration for the methanol and chloroform extracts of *Laurencia snyderiae* seaweed and brown algae *Sargassum angastophyllium*. The results showed that these extracts possess antibacterial activity against several Gram-positive bacteria, including *Streptococcus mutans*, *Streptococcus salivaris*, and *Streptococcus sanguis*, as well as four Gram-negative bacteria, namely *Salmonella typhi*, *Proteus vulgaris*, *Shigella flexniu*, and *Micrococcus luteus*. The methanol and chloroform extracts exhibited the most potent antibacterial activity against the Gram-negative bacterium *S. typhi*, with growth inhibition zone diameters of 18 mm and 20 mm, respectively. In our study, the ethanol extract of *S. platensis* blue-green algae (1 mg/mL) produced the largest growth inhibition zone diameter against P. aeruginosa, consistent with the findings of a previous study [23]. A separate study indicated that the ethyl acetate extract of red algae (*Gelidiella acerosa*) displays antibacterial activity against certain Gram-positive and Gram-negative bacteria, such as *K. pneumoniae*, *Escherichia coli*, *Photobacterium damselae*, and *Lactococcus garvieae* [24]. Variations in the methods of plant extract preparation and application and the use of diverse antibacterial assays can lead to differences in the observed antibacterial activity of natural products. The sensitivity of microorganisms to antimicrobial substances varies depending on their cell wall composition and structure. The presence of lipopolysaccharides in the cell wall of Gram-negative bacteria can hinder the penetration of active compounds to the cytoplasmic membrane, resulting in resistance to antimicrobial substances [25]. Previous studies have suggested that the antibacterial activity of *S. platensis* is closely linked to the presence and quantity of phenolic compounds [12]. In addition to phenolic compounds, fatty acids, hydroxyl unsaturated fatty acids, and glycolipids have also been identified as contributing to the antimicrobial activity of microalgae [14]. Additional biochemical analyses are necessary to identify the specific compounds responsible for the antibacterial activity exhibited by the algal extract. Our findings demonstrate the antibacterial effect of the ethanol extract in this study. It is promising that extracts from *S. platensis* blue-green algae could serve as a potential substitute or supplement for antibiotics.

Overall, algae are a valuable source of bioactive secondary metabolites that could be utilized in developing antimicrobial agents. The current study evaluated the antibacterial activity of aqueous and ethanolic extracts of *S. platensis* algae at various concentrations and subsequently determined the minimum lethal concentration. The data obtained indicated that the ethanolic extract of *S. platensis* exhibited a significant antibacterial effect against *P. aeruginosa*. The findings of this study provide a basis for further research and in-depth investigations aimed at identifying and purifying the active antibacterial substances present in *S. platensis*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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