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The bactericidal effect of green macroalgae *Cladophora* sp. from freshwater toward Gram-negative and Gram-positive bacteria

Amel Saoudi[1], Mabrouka Bouacha[2]*, Sandra Amri[3], Houneida Benbouzid[4], Khedidja Boufligha[1], Saber Belhaoues[1], Karima Boutarfa[1], Meriem Abdelkrim[1], Iness Abdouni[1] and Mourad Bensouilah[1]

[1]Ecobiology Laboratory for Marine Environments and Coastal Areas, Faculty of Sciences, Badji Mokhtar University, BP 12 Sidi Amar, 23000 Annaba, Algeria.
[2]Laboratory of Biochemistry and Environmental Toxicology, Faculty of Sciences, Badji Mokhtar University, Annaba, Algeria.
[3]Department of Biology, Faculty of Natural Sciences and Life and Earth Sciences and the Universe, University 08 Mai 1945 Guelma, Algeria.
[4]Laboratory of Cell Toxicology, General Direction of Scientific Research and Technological Development, Badji Mokhtar University, Annaba, Algeria.
Email: bouacha.mabrouka@yahoo.fr, mabrouka.bouacha@univ-annaba.dz

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ABSTRACT

Aims: The development of new antimicrobial agents towards multidrug-resistant bacteria is one of the most significant challenges facing the healthcare system today. The continuous increase of antimicrobial resistance rates worldwide is a significant threat to public health. Therefore, this study aimed to investigate the antibacterial effect of filamentous macroalgae *Cladophora* sp. The sample was collected from an Algerian fountainhead of fresh water. A crude hydromethanolic extract (methanol-water) was tested against two standard Gram-negative bacteria: *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853, and two standard Gram-positive bacteria: *Staphylococcus aureus* ATCC25923 and *Enterococcus faecalis* ATCC 2921.
Methodology and results: The antibacterial effect of the hydromethanolic extract of *Cladophora* sp. was investigated using the well diffusion method to determine the inhibitory diameters and the dilution methods to determine the minimum inhibitory concentration (MIC) and the bactericidal inhibitory concentration (MBC). The results indicated that the hydromethanolic extract of *Cladophora* sp. is more effective towards Gram-positive bacteria, with a significant effect on *S. aureus* ATCC 25923, where an inhibitory diameter of 35 mm was recorded. For the Gram-negative, *E. coli* ATCC25922 was more susceptible with inhibitory diameters of 46 mm, followed by *P. aeruginosa* ATCC 27853 with 28 mm. The MIC value of hydromethanolic extract of *Cladophora* sp. was 50 µg/mL for Gram-positive bacteria (*S. aureus* ATCC25923 and *E. faecalis* ATCC29212). However, it was 100 µg/mL for Gram-negative bacteria (*E. coli* ATCC25922 and *P. aeruginosa* ATCC 27853). The best bactericidal effect was observed with Gram-positive with an MBC of 100 µg/mL. The MBC for Gram-negative bacteria was 150 µg/mL.
Conclusion, significance and impact of study: The *Cladophora* sp. macroalgae represent a potential source of bioactive compounds, which could be used in the management and treatment of various microbial infections.

Keywords: Antibacterial activity, *Cladophora* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*

INTRODUCTION

Antimicrobial resistance is one of this century's most serious global public health threats (Prestinaci *et al.*, 2015). Many bacteria developed resistance to most of the conventional antibiotics available on the market (Bouacha *et al.*, 2015; 2018). The emergence of bacterial multi-resistance has become a worldwide public health problem. Bacterial resistance procreates a supplement expense, hospitalizations stay and the number of deceased (Prestinaci *et al.*, 2015). To overcome the problem of multidrug resistance, scientists need to find new molecules of natural origin whose development cost is lower than that of conventional antibiotics without adverse effects on health. The bacteria concerned by this multidrug resistance are mainly those involved in

***Corresponding author**

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nosocomial infections (Amri *et al.*, 2022; Bouacha *et al.*, 2022; Salehi, 2022), including *E. faecalis*, *E. coli*, *P. aeruginosa* and *S. aureus* (Bouacha *et al.*, 2018).

Indeed, several studies have focused on bioactive substances extracted from natural products, more particularly on medicinal green algae (Ghania *et al.*, 2019; Munir *et al.*, 2019) known for their inexhaustible potential in bioactive secondary metabolites such as chlorophyll, carotenoids, phycocyanin, phycoerythrin, phenolics and flavonoid. The bioactive substances are likely to be used in the development of new pharmaceutical agents with antibacterial properties (Munir *et al.*, 2019). In general, the number of studies reserved for marine algae was greater than for freshwater algae (Unpaprom *et al.*, 2020). However, both types of algae are known to possess a wide variety of bioactive molecules with antibacterial, antifungal and anti-inflammatory effects (Munir *et al.*, 2019). *Cladophora* sp. has a branched filamentous reticulated thallus, classified among the Chlorophyceae autotrophs and photosynthetic organisms. *Cladophora* sp. represents a cosmopolitan genus found in fresh and marine waters. Depending on the habitat and environmental conditions, it can show a significant variation in appearance and chemical composition (Piotrowicz *et al.*, 2022). It is used as food because of its protein, fibre and vitamin richness. It is also a rich source of biologically active compounds, including saturated and unsaturated fatty acids, sterols, terpenoids and phenolic compounds (Unpaprom *et al.*, 2020; Piotrowicz *et al.*, 2022).

Despite the abundance of the Algerian aquatic environment with *Cladophora* sp., however, very few studies have been done on *Cladophora* sp. and to the best of our knowledge, this is the first study in Algeria that showed the clear effect of *Cladophora* sp. on the growth and viability of bacteria. Therefore, this study aims to evaluate the effect of hydromethanolic extract of *Cladophora* sp. collected from the fountainhead of fresh water in the region of Guelma, Algeria, on the growth and viability of standard Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*S. aureus* and *E. faecalis*).

MATERIALS AND METHODS

Identification of *Cladophora* sp.

Samples were collected from the fountainhead of fresh water of Selmoun El Hachemi, Guelma, Algeria. Macroalgae samples were collected manually from the rocks. Harvested macroalgae were stored in plastic bags, transported to the laboratory and identified with the help of classical algal (Wong and Wainwright, 1993). The samples were washed with fresh water to remove epiphytes, animal castings, attached debris and shells, then rinsed with distilled water and dried under shade. The dried samples were put in a mill to obtain powder and stored at 4 °C until use. The morphology of *Cladophora* sp. was determined by direct examinations between slide and lamella under an optical microscope (Carl Zeiss model Axiostar plus) equipped with a digital uEye32 camera. The morphological observation was made at ×1000 magnification to visualize the maximum of morpho-anatomical characters.

Preparation of hydromethanolic extract of *Cladophora* sp.

A 67 g of dried macroalgal sample was extracted by maceration with 10 mL of methanol (80%) at room temperature. The mixture was filtered through a Whatman No. 1 paper. The resulting filtrate was evaporated under reduced pressure and at a temperature of 43 °C until a viscous extract was obtained. The dried extract was stored at 4 °C until use. Three concentrations were prepared in 2% dimethyl sulfoxide (40, 80 and 100 µg/mL).

Bacterial cultures

The antibacterial effect of the hydromethanolic extract of *Cladophora* sp. was assessed towards two Gram-positive bacteria: *S. aureus* ATCC 25923 and *E. faecalis* ATCC29212, and two Gram-negative bacteria: *E. coli* ATCC25922 and *P. aeruginosa* ATCC 27853. The bacterial species were obtained from the Pasteur Institute of Algeria. Each bacterial strain was incubated in nutrient broth (Bio-Rad Laboratories, Inc. France) at 37 °C for 24 h in a shaking incubator. The bacterial suspension was prepared according to the recommendations of the Clinical and Laboratory Standard Institute (Humphries *et al.*, 2021).

Antibacterial assay

The antibacterial activity of the crude was evaluated using the agar well diffusion method in Petri dishes (Perez *et al.*, 1990). Wells of 6 mm in diameter are prepared in Mueller Hinton agar plate (Bio-Rad, Inc. France). Then, the plates were inoculated with the bacterial strain. A volume of 30 µL of each concentration of the hydromethanolic extract of *Cladophora* sp. (40, 80 and 100 µg/mL) was introduced into the well. A well filled with 2% dimethyl sulfoxide served as a negative control. Plates were incubated at 37 °C for 24 h. The antibacterial activity of the hydromethanolic extract of *Cladophora* sp. was expressed as the inhibitory diameters around the wells. All assays were performed in triplicates.

Minimum inhibitory concentration (MIC) was determined by the broth dilution method according to the Clinical and Laboratory Standard Institute recommendations (CLSI, 2017). MIC was defined as the lowest concentration of the hydromethanolic extract of *Cladophora* sp., which inhibits the growth of the bacteria strains. Briefly, 2 mL of hydromethanolic extract of *Cladophora* sp. at each concentration (200, 150, 100, 80, 50, 40, 20 and 10 µg/mL) were added to 2 mL of Mueller Hinton broth and inoculated with the tested bacteria. The tubes were placed in a shaking incubator at 37 °C for 24 h. Data was recorded to compare with the negative

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control (Muller Hinton broth). The lowest concentration of the hydromethanolic extract of *Cladophora* sp. required to inhibit bacterial growth is recorded as MIC value. 10 µL of the tubes that did not show bacterial growth were inoculated on nutrient agar plates to determine the minimum bactericidal concentration (MBC). The cultures were incubated for 24 h at 37 °C. The MBC value was determined as the minimum concentration of the hydromethanolic extract of *Cladophora* sp. that did not show bacterial growth on the agar plates. All experiments were performed in triplicate.

Data analysis

The statistical analysis of the results was carried out by the GraphPad Prism software version 7.00 for Windows (GraphPad Software, La Jolla, California, USA). Data were subjected to a one-way analysis of variance for mean comparison. In all assays, the results are expressed as the mean of triplicate experiences ± standard deviations (SD). Differences at $p \leq 0.05$ were considered to be statistically significant.

RESULTS

Identification of *Cladophora* sp.

Macroalgae collected from Algerian fountainheads were filamentous in form, attached to the rock and coarse-to-touch mats. Microscopically (Figure 1), the branches are dichotomous and the talli were composed of joint cylindrical cells with an average length of 623 µm and an average width of 198 µm. Cell walls were robust, and the chloroplast was net-like (reticulate) and parietal with numerous pyrenoids.

Antibacterial activity

The results of the inhibitory diameters of the hydromethanolic extract of *Cladophora* sp. are shown in Figure 2 and Figure 3. The antibacterial activity of Gram-positive bacteria ranged between (27.66 and 35.33 mm), the highest inhibitory diameter was found in *S. aureus* at the concentration of 80 µg/mL and the lowest was in *S. aureus* at the concentration of 40 µg/mL. Concerning the Gram-negative strains, the antibacterial activity ranged between 22.66 and 46 mm, and the highest was in *E. coli* at the concentration of 100 µg/mL.

The results of the determination of the MIC, MBC and MBC/MIC ratio by the broth dilution assay are reported in Table 1. The hydromethanolic extract of *Cladophora* sp. shows that the extract was more active on Gram-positive strains (*S. aureus* and *E. faecalis*) with a MIC value of 50 µg/mL. This is significantly lower than the MIC value obtained with Gram-negative species (*E. coli* and *P. aeruginosa*) with a MIC of 100 µg/mL.

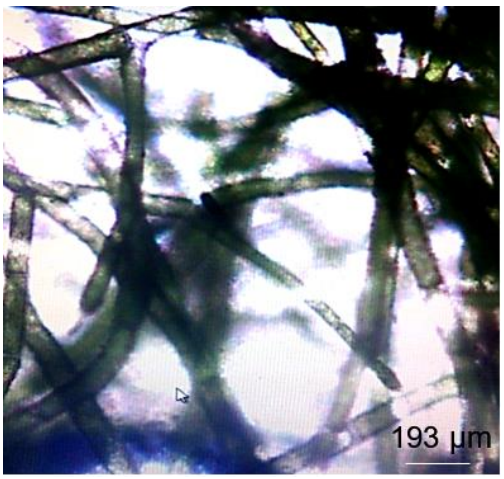

Figure 1: Filament of *Cladophora* sp. of attached thallus collected from the fountainhead of freshwater of Selmoun El Hachemi, Guelma, Algeria.

DISCUSSION

The emergence of antibiotic resistance represents an emergency, which is responsible for high mortality rates and may leave few effective antimicrobial options (Bouacha *et al.*, 2022). Although considerable research has been conducted previously to isolate and identify pharmaceutically important compounds from *Cladophora* sp., very few compounds are commercialized (Munir *et al.*, 2019).

Figure 2 and Table 1 show that the hydromethanolic extract of *Cladophora* sp. exhibits a good antibacterial effect on pathogenic bacteria. According to Soussy *et al.* (2000), bacterial strains with inhibition diameters greater than 17 mm are considered to be sensitive to the antibacterial agent, while bacterial strains with inhibition diameters less than 12 mm are considered to be resistant to the antibacterial agent. The antibacterial activity of the Gram-positive strains ranged between (27.66 and 35.33 mm). The highest was in *S. aureus* with a concentration of 80 µg/mL and the lowest was in *S. aureus* with a 40 µg/mL concentration. A similar observation was made in a methanol extract of green microalgae, which showed high inhibiting activity against S. *aureus* (Zbakh *et al.*, 2014). The antibacterial activity of the hydromethanolic extract of

Table 1: MIC, MBC and MBC/MIC ratio of the hydromethanolic extract of *Cladophora* sp.

Bacterial strain	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC ratio
E. coli ATCC25922	100	150	1.5
P. aeruginosa ATCC 27853	100	150	1.5
S. aureus ATCC 25923	50	100	2.0
E. faecalis ATCC 29212	50	100	2.0

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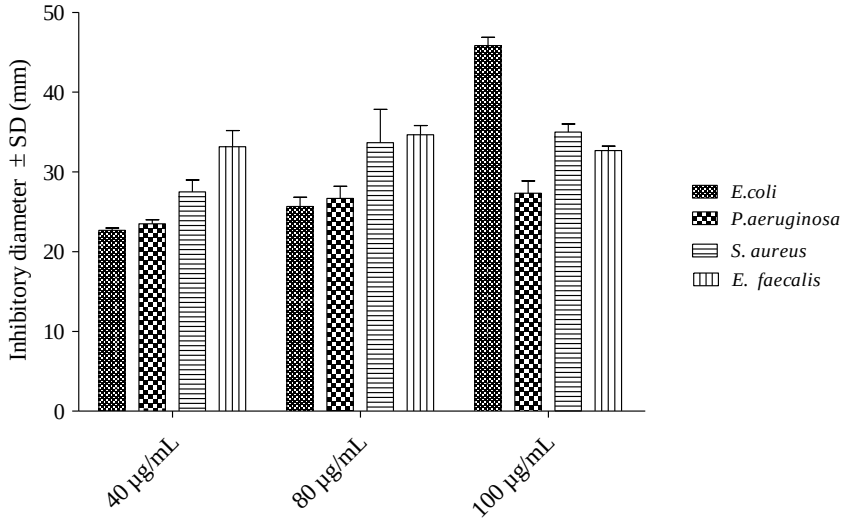

Figure 2: Antibacterial activity of the hydromethanolic extract of *Cladophora* sp. towards pathogenic bacteria (inhibitory diameter ± SD).

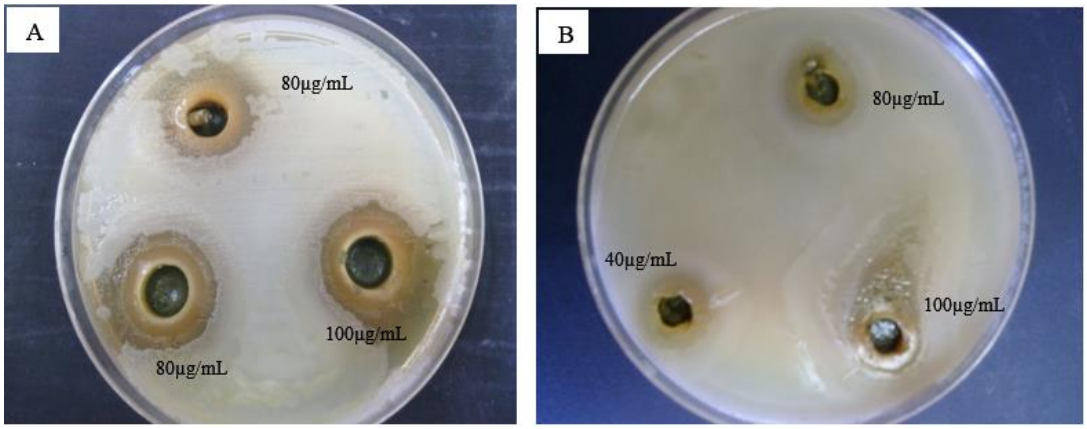

Figure 3: Growth inhibition of *S. aureus* (a) and *E. coli* (b) by the hydromethanolic extract of *Cladophora* sp.

Cladophora sp. towards Gram-negative bacteria ranged between 22.66 and 46 mm and the highest was *E. coli* with a concentration of 100 µg/mL. The inhibitory diameter of the hydromethanolic extract of *Cladophora* sp. against *P. aeruginosa* varies between 23- and 27.66-mm. Similar results were reported by Al-Saif *et al*. (2014), who obtained 23 mm of the *Cladophora* extracts prepared with ethanol and 27 mm with chloroform (Al-Saif *et al.*, 2014). However, the hydromethanolic extract of *Cladophora* sp. has no antibacterial activity against *K. pneumoniae* ATCC 700603. Regarding the bacterial species tested, it could be noticed that there was a difference in sensitivity among the bacterial strains. The Gram-positive bacteria were more sensitive than the Gram-negative. This could be related to the differences in the outer membrane. Gram-negative bacteria are surrounded by a thin peptidoglycan wall and an outer membrane containing lipopolysaccharide, which is less permeable to many antimicrobial agents (Bouacha *et al*., 2018; Sun *et al*., 2022). The negative effect of the hydroalcoholic extract of *Cladophora* sp. on the *K. pneumoniae* strain could be due to the impermeability of the capsid, which protects the bacteria from external stresses (Lin *et al.*, 2017; Priyanka *et al.*, 2020). Many authors have also reported that Gram-positive bacteria were more effectively inhibited by algal extracts than Gram-negative bacteria (Sreenivasa *et al.*, 1988; Soltani *et al.*, 2011; Soltani and Khoshrooei, 2014). Also, the MBC/MIC ratio revealed that *Cladophora* sp. hydromethanolic extract has bactericidal effects against *E. coli*, *P. aeruginosa*, *S. aureus and E. faecalis*. Indeed, O'Neill and Chopra (2004) have declared that an antimicrobial drug is regarded as bacteriostatic, which implies that it inhibits bacterial growth rather than killing the germs when the MBC/MIC ratio is higher than the number four. However, if the MBC/MIC ratio is lower or equal to four, it is qualified to be a bactericidal agent, which means that it affects the viability of the bacteria.

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The bactericidal effect of the hydromethanolic extract of *Cladophora* sp. could be related to one or more bioactive compounds, such as the subclass of simple phenols, phenolic acids and the subclass of quinones, flavonoids, flavones and flavonols. The bioactive substances may exert their effects through a variety of mechanisms, including the disruption of the plasma membrane, binding to adhesins, the formation of complexes with the wall, the inhibition of enzymes and interaction with DNA (Bhowmick *et al*., 2020).

CONCLUSION

The present investigation showed that the hydromethanolic extract of *Cladophora* sp. from the Algerian fountainhead has good bactericidal activity against non-encapsidated Gram-negative and Gram-positive bacteria. Further studies on extracting and purifying the bioactive substances responsible for the bactericidal effect from *Cladophora* sp. could improve understanding about their potential in treatmenting infectious diseases. These molecules will be used as new natural antibiotics resulting from green chemistry, which is environmentally friendly.

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