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Research article

Antibacterial and antioxidant activities of *Chlorella vulgaris* and *Scenedesmus incrassatulus* using natural deep eutectic solvent under microwave assisted by ultrasound

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ABSTRACT

Microalgae are increasingly recognized as promising sources of natural bioactive compounds. However, traditional extraction methods using volatile organic solvents (VOCs) pose environmental risks. This study explores renewable deep eutectic solvents (DES) as sustainable alternatives for extracting bioactive compounds from microalgae biomass, focusing on *Chlorella vulgaris* and *Scenedesmus incrassatulus*. Four DES systems, comprising choline chloride (ChCl) and glycerol, citric acid, urea, and glucose, were compared with three conventional solvents (ethanol, methanol, and water). Extraction efficiency was assessed based on total phenolic content (TPC), flavonoid content, and tannin content, followed by antioxidant activity evaluation using DPPH, CAT, and FRAP assays. Additionally, antibacterial activity of the DES extracts was determined against *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), and *Bacillus subtilis* (ATCC 3366) using disc diffusion and microplate dilution methods to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Results reveal that DES, particularly choline chloride: citric acid, outperform conventional solvents in terms of polyphenol extraction efficiency, antioxidant activity, and antibacterial activity against both Gram-negative and Gram-positive bacteria. For instance, the citric acid-based DES (SIDES2) showed a TPC of 4.98 mg/g, while the conventional solvent ethanol exhibited a TPC of 3.27 mg/g. Additionally, SIDES2 exhibiting the highest DPPH scavenging activity of 75 %, compared to 60 % for ethanol. Furthermore, SIDES2 showed an MIC of 0.5 mg/ml against *Staphylococcus aureus*. This study underscores the potential of DES for sustainable extraction of natural antioxidants from microalgae biomass, contributing to the development of environmentally friendly extraction processes in various industries.

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1. Introduction

The extraction of bioactive compounds from renewable sources has garnered increasing attention due to the growing demand for sustainable and eco-friendly methods [1]. Among the diverse array of potential sources, microalgae biomass has emerged as a promising reservoir of bioactive molecules with wide-ranging applications across various industries [2]. Microalgae harbor a rich biochemical profile, containing valuable compounds such as polyphenols, flavonoids, and antioxidants, which have attracted attention due to their potential health benefits and industrial utility [3]. Conventional extraction techniques for bioactive compounds from microalgae typically involve the use of volatile organic solvents (VOCs) derived from petroleum or water-based methods [4]. These approaches present significant environmental and health issues. VOCs are toxic and pose risks of environmental contamination, while water-based methods often lead to the co-extraction of unwanted impurities, complicating downstream processing and purification [5]. These limitations highlight the need for innovative extraction methods that are both efficient and environmentally benign.

Current research has focused on developing greener extraction methods, such as using natural deep eutectic solvents (DES) which, have emerged as highly promising candidates for the extraction of bioactive compounds from microalgae biomass [6]. DES are formed by combining hydrogen bond acceptors (HBA) like amino acids, organic acids, and choline, with hydrogen bond donors (HBD) such as urea, sugars, glycerol, and zinc chloride [7]. These solvents are non-volatile, non-toxic, biodegradable, and offer high thermal stability, allowing for extraction at elevated temperatures without degradation [8,9]. DES also minimize environmental impact and ensure safety during handling and processing, making them a promising alternative to traditional solvents.

This study proposes the use of renewable DES as a sustainable solution for extracting bioactive compounds from microalgae biomass, specifically *Chlorella vulgaris* and *Scenedesmus incrassatulus*. The extraction efficiency and antioxidant activity of DES are evaluated in comparison to conventional solvents. Additionally, the antibacterial activity of DES extracts is assessed against a panel of clinically relevant Gram-negative and Gram-positive bacteria. This approach aims to address the existing gaps in extraction methods by demonstrating the superior performance of DES in terms of both extraction efficiency and bioactivity.

The novelty of this research lies in its comprehensive evaluation of DES for the extraction of bioactive compounds from microalgae. By elucidating the extraction potential and bioactivity of DES, this study contributes to the development of sustainable extraction processes. These processes are crucial for advancing greener industrial practices and enhancing public health through the production of natural antioxidants and antimicrobial agents. The findings have significant implications for both society and industry, promoting eco-friendly solutions and reducing reliance on harmful solvents.

2. Materials and methods

2.1. Microalgae culture condition

2.1.1. Isolation and culture of studied strains

The investigation focused on *Scenedesmus incrassatulus* and *Chlorella vulgaris*, which were sourced from the Sidi Chahed impoundment water in Morocco (34°04′30.5 ″N 5°20′19.2 ″W) during the spring-summer season. Sampling was conducted using a Nansen-type net with a 40 μm mesh size at various locations within the reservoir. For isolation, a solid media technique was employed [10], involving the use of Petri dishes filled with sterile Dauta medium [11], followed by inoculation using a Pasteur pipette. Incubation of the Petri dishes occurred for 10 days under suboptimal temperature and light conditions. Monospecific cultures were obtained through sequential isolation on Petri dishes, with well-isolated colonies of microalgae being transferred to fresh medium until purification was achieved. Systematic analysis and identification of the microalgal species was carried out by optical microscopy of the cytological features of the cells, determining morphology, size, presence or absence of flagella, their numbers, and the arrangement of the cells in colony mode, using a specialised identification key [12]. The experimental cultures were sustained in their exponential growth phase through regular subculturing every 3 days within a controlled culture chamber, maintaining optimal conditions: a temperature of 25 °C ± 1 °C and a photoperiod of 16 h light/8 h dark using PHILIPS TL-D 18W Snow White 12000K fluorescent tubes. Sterile aeration with 1 % CO_2 ensured culture homogenization. Daily microscopic examination was conducted to monitor colony purity.

2.2. Chemicals

In our study, we used various high-purity chemicals sourced from reputable suppliers. Choline chloride ([(CH3)3NCH2CH2OH]+ Cl−) and glycerol (C3H8O3) with ≥99 % purity, glucose (C6H12O6, ≥99.50 %), ethanol (C2H6O, ≥99.9 %), methanol (CH3OH, ≥99.8 %), Folin-Ciocalteu reagent (2 ± 0.1 N), sodium carbonate (Na2CO3, ≥99.5 %), gallic acid (C7H6O5, ≥97 %), sodium phosphate (Na3PO4, ≥98 %), sulphuric acid (H2SO4, ≥95 %), ammonium molybdate ((NH4)6Mo4O24, ≥99 %), ascorbic acid (C6H8O6, ≥99 %), sodium nitrite (NaNO2, ≥97 %), aluminium chloride (AlCl3, ≥99 %), sodium hydroxide (NaOH, ≥97 %), hydrochloric acid (HCl, ≥37 %), potassium ferricyanide (C6N6FeK3, ≥99 %), and DPPH radicals (C18H12N5O6, ≥97 %) were all obtained from Sigma Aldrich. Citric acid (C6H8O7, 99.50 %) was from Rankem, and urea (CH4N2O, ≥99 %) from Labogens. These chemicals were crucial for ensuring the reliability of our experiments.

2.3. Preparation of solvents and extraction

2.3.1. Preparation of solvents

To optimize the extraction of algal biomass, two types of solvents were used; conventional solvents (Ethanol, Methanol and distilled water (DW)) and deep eutectic solvents (Table 1). The four NADESs were prepared according to the method developed by Abbott et al. [13], by heating method using a mixture of choline chloride (ChCl) as hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD); glycerol, citric acid, urea and glucose. The physical mixture was heated at 80 °C until a homogeneous colourless, translucent liquid was appeared [14–17].

2.3.2. Microwave assisted by ultrasound extraction

The extraction method used is a sonication-assisted microwave extraction method (Fig. 1), 500 mg of lyophilized biomass was put into 30 ml of solvent. The mixture was placed in the microwave oven (200 W), with a sonicator (24 KHz) at 60 °C for 20 min. The extracts were recovered after removal of the pellet by centrifugation (5000 rpm/10 min), then preserved at 4 °C.

2.4. Determination of phenolic compounds

2.4.1. Polyphenol content assay

The content of total polyphenols in the algal extracts was assessed using the Folin-Ciocalteu reagent, according to the method described by Singleton and Rossi [18]. The intensity of the colour of the resulting mixture was indicative of the quantity of total polyphenols present. To perform the test, a 200 µl sample of the microalgal extract was mixed with 1.5 ml of Folin's reagent (10 %) in a test tube and kept protected from light for 5 min, after which 1.5 ml of Na_2CO_3 (5 %) was added. The mixture was then placed in the dark for 2 h before measuring its absorbance at 725 nm. The calibration range for this assay was established using concentrations of gallic acid (1 mg/ml). The results obtained are expressed as mg of gallic acid equivalent per g of dry matter.

2.4.2. Flavonoid content assay

To determine the flavonoid content of crude extracts, the method described by Lamaison and Carnat [19] using aluminium trichloride was employed. This method works via the oxidation of flavonoids by aluminium trichloride and sodium hydroxide, resulting in the creation of a pink complex that absorbs at 510 nm. 1 ml of the microalgae extract was placed in a tube with 0.3 ml $NaNO_2$ (5 %) and left in the dark for 5 min. Next, 0.3 ml $AlCl_3$ (10 %) and 2 ml NaOH (1 M) (4 %) were added to the tube. The volume was then made up with 10 ml of distilled water. The absorbance of the solution was measured at 510 nm.

2.4.3. Tannin content assay

The method for determining the tannin content of crude extracts involved using the protocol established by Ribéreau-Gayon and Stonestreet [20]. The fundamental concept of the procedure is based on the fact that tannins are transformed into anthocyanins under acidic conditions after being subjected to heat. To determine the concentration of condensed tannins, the assay was carried out as follows 1 ml of the twice-diluted extract was mixed with 1.5 ml of 37 % HCl and 0.5 ml of distilled water in glass tubes. This process was repeated twice, once at normal temperature and once in a water bath at 95 °C for half an hour. After cooling, absorbance was measured at 550 nm. The tannin concentration was calculated using the formula as described by Ref. [21].

$$[\text{Condensed tannins}] = 19.33\ (D_{BM} - D_{Ta})$$

D_{BM}: 95 °C test absorbance.
D_{Ta}: absorbance of the room temperature test.

2.5. Antioxidant assay

To assess the antioxidant activity of *Scenedesmus incrassatulus* and *Chlorella vulgaris*, three techniques were applied.

2.5.1. Total antioxidant capacity (TAC)

To assess the Total Antioxidant Capacity (TAC) of algal extracts, the phosphomolybdenum method [22] is used. The process

Table 1
Composition of solvents used with their proportion/molar ratio.

	Code	Extract	Proportion/Molar ratio
DES	SI/CV DES1	Choline chloride + Glycerol	1 : 2
	SI/CV DES2	Choline chloride + Citric acid	1 : 2
	SI/CV DES3	Choline chloride + Urea	1 : 2
	SI/CV DES4	Choline chloride + Glucose	1 : 2
VOC	SI/CV S1	Hydroethanolic extract	80 % Ethanol +20 % DW
	SI/CV S2	Methanolic extract	100 % Methanol
	SI/CV S3	Aqueous extract	100 % DW

Fig. 1. Sonication-assisted microwave (NuWav- Pro/N µ Tech, India).

involves adding 200 µl of each extract to tubes containing 3 ml of a reagent composed of sodium phosphate (28 mM), sulphuric acid (0.6 M) and ammonium molybdate (4 mM). Absorbance was then measured at 695 nm. Results are expressed in milligrams of ascorbic acid equivalent per gram of dry matter.

2.5.2. Ferric reducing antioxidant power (FRAP)

The ability of the extract to reduce ferric iron (Fe^{3+}) to ferrous iron Fe^{2+} was assessed using the method described by Pownall et al. [23]. 1 ml of the sample was mixed with 2.5 ml of 0.2M phosphate buffer (pH = 6.6) and 2.5 ml of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After cooling, the reaction was stopped by adding 10 % trichloroacetic acid, and then centrifuged at 15,000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % ferric chloride. Absorbance was measured at 700 nm. Ascorbic acid was used as a positive control. A continuous increase in absorbance at 700 nm indicates significant reducing power.

2.5.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

The DPPH method introduced by Brand-Williams et al. [24] was employed. This method involves placing 4 mg of DPPH in 100 ml of methanol under agitation for 3 h in the dark at room temperature. A series of dilutions from a stock solution of each extract was then carried out in tubes. Subsequently, 1 ml of DPPH solution was added. The tubes were shaken and incubated in the dark for 30 min. Absorbance was determined using a spectrophotometer at 517 nm [25]. The free radical scavenging potential is determined as a percentage by the following formula:

$$\text{Antioxydant activity } (\%) = \frac{\text{OD (DPPH)} - \text{OD (extract)}}{\text{OD (DPPH)}} \times 100$$

These experiments were conducted in triplicates and results are expressed in milligrams per gram of dry biomass. The kinetics of this activity were used to determine the concentration corresponding to 50 % inhibition (IC_{50}). The lowest IC_{50} value corresponds to greater antioxidant activity [26].

2.6. Antibacterial activity

Two Gram-negative bacteria (*Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC 27853)) and two Gram-positive bacteria (*Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 3366)) were used for the antibacterial activity test. One to two colonies were transferred in sterile saline solution from a 24 h old pure agar culture for each strain. After that, the solutions were vortexed, and their turbidity was set to 0.5°. McFarland.

2.6.1. Determination of the MIC

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the extract that inhibits microbial growth. For its determination, the broth microdilution was performed. The microplates were incubated at 37 °C for 24 h. To reveal the microbial development, 10 µl of resazurin dye was applied after incubation [27].

2.6.2. Determination of the MBC

3 µl of negative wells were poured onto the surface of an LB agar plate and incubated at 37 °C for 24 h to determine the minimal bactericidal concentrations (MBC). Following incubation, the MBC was found to be the lowest concentration, resulting in an evident negative growth. The MBC/MIC ratio was determined to emphasize the nature of the extracts' antimicrobial effect [28].

2.6.3. Disk diffusion

The anti-bacterial activity of the extracts was tested against the Gram-positive and Gram-negative bacteria mentioned above, using

the disk diffusion method. To prepare the bacterial suspension, one to two colonies were transferred to sterile saline solution from a 24-h pure agar culture for each strain. The solutions were then homogenized by vortexing, and the turbidity was adjusted to 0.5° Mc Farland (1.106 CFU/ml). 1 ml of each bacterial suspension for each strain was spread in petri dishes containing solid LB medium with wells formed beforehand. Next, 50 μL of each extract was added to each well. The dishes were incubated at 37 °C for 24 h [29,30]. Finally, the inoculated Petri dishes were incubated at 37 °C for 24 h and the zones of inhibition were observed, including the diameter of the disc (6 mm), according to the guidelines of the Antibiogram Committee (CA-SFM) [31]. If the zone of inhibition exceeds 15 mm in diameter, the antimicrobial activity is considered to be very good. If the diameter is between 15 mm and 8 mm, antibacterial activity is average. For diameters below 8 mm, antibacterial activity is low. All experiments were carried out in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using the *t*-test to assess the significance of the results obtained. All experiments were conducted in triplicate, and the data are presented as mean ± standard deviation (SD). A p-value <0.05 was considered statistically significant. Statistical analyses were performed using the R statistical software version 4.1.2 for Windows [32].

3. Results

3.1. Phenolic compounds

3.1.1. Polyphenols

The results for total polyphenol content are given in Fig. 2, for *Scenedesmus incrassatulus*, the mean concentrations of polyphenols extracted using DES ranged from 5.48 to 8.32 mg EAG/g DM, whereas for *Chlorella vulgaris*, the range was from 4.64 to 9.05 mg EAG/g DM. Among the DES systems tested, DES2 exhibited the highest mean concentration for both microalgae species. Interestingly, there appears to be variability within the same microalgae species across different DES systems, suggesting that the choice of solvent composition can influence extraction efficiency. Additionally, when comparing the DES extraction with the respective VOCs extractions (SIS1, SIS2, CVS1 and CVS2), DES consistently yielded higher concentrations of polyphenols, indicating its superior efficiency in extracting these bioactive compounds. These findings support the notion that DES can serve as effective and sustainable alternatives to traditional solvents for the extraction of polyphenols from microalgae biomass. The provided p-value for the *t*-test (0.0193) indicates a statistically significant difference in polyphenol concentrations between the DES and water-based extraction methods for microalgae. The descriptive statistics further support this conclusion. The mean concentration of polyphenols extracted using DES (5.912 mg EAG/g DM) is notably higher than that of COVs-based extraction (5.735 mg EAG/g DM). Additionally, the interquartile range (IQR) for DES (4.685–6.638 mg EAG/g DM) is narrower compared to COVs-based extraction (3.800–6.02 mg EAG/g DM), suggesting less variability in polyphenol concentrations with DES. These results reinforce the effectiveness of DES in extracting polyphenols from microalgae biomass compared to conventional COVs-based extraction methods, thereby supporting the utility of DES as a sustainable and efficient extraction solvent.

3.1.2. Flavonoids

Flavonoids were assayed using the aluminium trichloride ($AlCl_3$) method, with quercetin used as the standard; the results are shown in Fig. 3. The provided data on flavonoid concentrations reveal notable distinctions among the extraction methods and microalgae species. For *Scenedesmus incrassatulus*, the mean concentrations of flavonoids extracted using DES ranged from 66.56 to 199.69 mg/g, whereas for *Chlorella vulgaris*, the range was from 64.74 to 180.63 mg/g. Notably, DES2 demonstrated the highest mean concentration for both microalgae species. Interestingly, variability within the same microalgae species across different DES systems

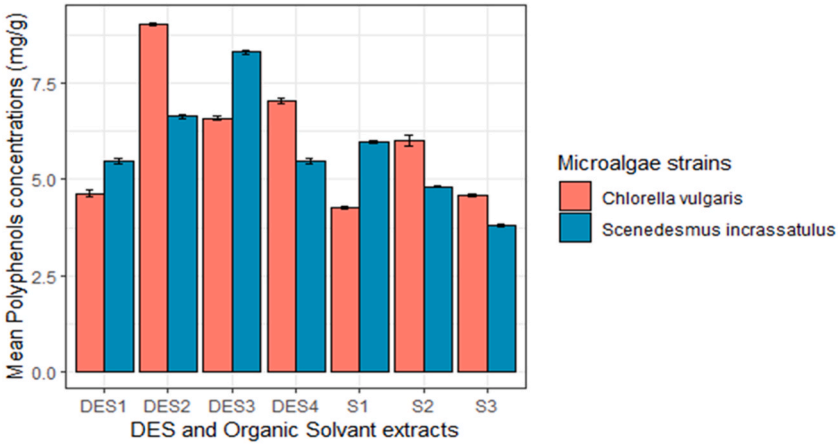


Fig. 2. Polyphenol's content of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

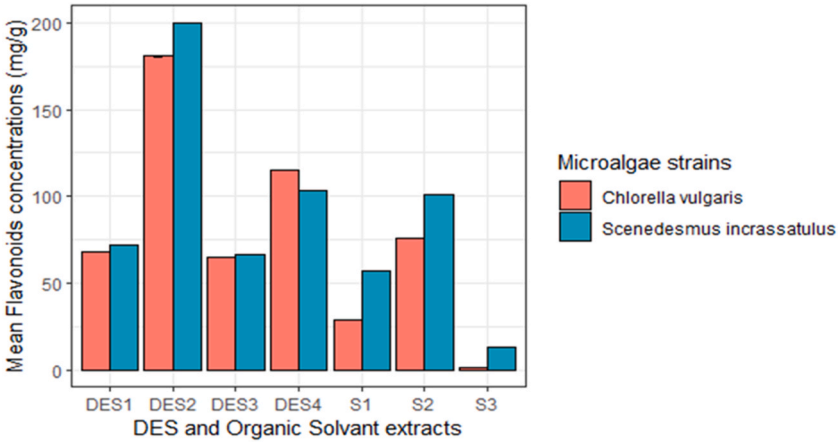


Fig. 3. Flavonoid's content of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

suggests that the solvent composition significantly influences extraction efficiency. Furthermore, comparing DES extraction with COVs-based methods consistently demonstrated higher flavonoid concentrations, highlighting DES's efficacy in extracting these bioactive compounds from microalgae biomass. These findings underscore the potential of DES as a sustainable and effective alternative to conventional solvents for extracting flavonoids, thereby contributing to the development of eco-friendly extraction processes in various industries. The provided p-value for the *t*-test (0.0262) indicates a statistically significant difference in flavonoid concentrations between the DES and COVs-based extraction methods for microalgae. The descriptive statistics further support this conclusion. The mean concentration of flavonoids extracted using DES (81.94) is notably higher than that of COVs-based extraction (69.89). Additionally, the interquartile range (IQR) for DES (58.94–102.99) is narrower compared to COVs-based extraction (1.00–102.99), suggesting less variability in flavonoid concentrations with DES. These results underscore the effectiveness of DES in extracting flavonoids from microalgae biomass compared to conventional COVs-based extraction methods, thereby supporting the utility of DES as a sustainable and efficient extraction solvent.

3.1.3. Tannins

The provided statistical data for tannin concentrations demonstrates noteworthy distinctions among the extraction methods and microalgae species (Fig. 4). For *Scenedesmus incrassatulus*, the mean concentrations of tannins extracted using DES ranged from 3.08 to 13.80 g/l, whereas for *Chlorella vulgaris*, the range was from 4.07 to 12.30 g/l. DES2 exhibited the highest mean concentration for both microalgae species. Notably, variability within the same microalgae species across different DES systems suggests a significant influence of solvent composition on extraction efficiency. Furthermore, comparing DES extraction with COVs-based methods consistently demonstrated higher tannin concentrations, highlighting DES's efficacy in extracting these bioactive compounds from microalgae biomass. These findings underscore the potential of DES as a sustainable and effective alternative to conventional COVs-based solvents for extracting tannins, thereby contributing to the development of eco-friendly extraction processes in various industries. The provided p-value for the *t*-test (0.0017) indicates a statistically significant difference in tannin concentrations between

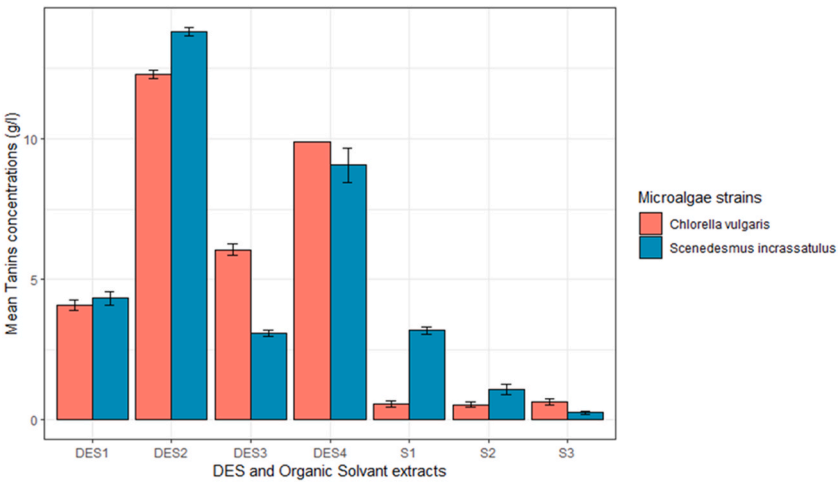


Fig. 4. Condensed tannin's content of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

the DES and COVs-based extraction methods for microalgae. The descriptive statistics further support this conclusion. The mean concentration of tannins extracted using DES (4.9204) is notably higher than that of COVs-based extraction (3.6276). Additionally, the interquartile range (IQR) for DES (0.7490–8.3200) is narrower compared to COVs-based extraction (0.2706–13.8016), suggesting less variability in tannin concentrations with DES.

3.2. Antioxidant activity

3.2.1. CAT

The Total Antioxidant Capacity of the extracts studied, expressed in mg of ascorbic acid equivalent per gram of dry matter, is shown in Fig. 5. The results show that among the deep eutectic solvent (DES) compositions, DES4 consistently exhibited the highest mean concentrations of total antioxidant capacity. For *Scenedesmus incrassatulus*, the mean concentrations for DES4 ranged from 53.29 to 106.91 mg AAE/g DM, with a notable increase observed from DES1 to DES4. Similarly, for *Chlorella vulgaris*, DES4 demonstrated significant antioxidant activity, with mean concentrations ranging from 49.71 to 160.67 mg AAE/g DM. These findings indicate that DES4, particularly with glucose as a component, is highly effective in extracting antioxidants from microalgae biomass. In contrast, conventional solvent-based extractions yielded varying levels of antioxidant activity. For *Scenedesmus incrassatulus*, mean concentrations for COVs-based extracts (SI S1-3) ranged from 7.02 to 105.26 mg AAE/g DM, while for *Chlorella vulgaris*, (CV S1-3) ranged from 37.89 to 51.93 mg AAE/g DM. The statistical analysis revealed a non-significant difference between the mean concentrations of total antioxidant capacity for the studied extracts (p-value = 0.0849), indicating that the extraction methods may not significantly influence antioxidant activity. Nevertheless, the overall trend suggests that DES4 offers a promising alternative for extracting antioxidants from microalgae biomass.

3.2.2. FRAP

The Ferric Reducing Antioxidant Power (FRAP) of the extracts studied, expressed in milligrams of ascorbic acid equivalents per gram of dry matter (mg AAE/g DM), is depicted in Fig. 6. The statistical analysis revealed a significant difference in the mean concentrations of FRAP for the studied extracts (p-value = 4.21e-05), indicating that the extraction methods significantly influence antioxidant activity. Among the deep eutectic solvent (DES) compositions, DES1 consistently exhibited higher mean concentrations of FRAP for both *Scenedesmus incrassatulus* and *Chlorella vulgaris* strains. For *Scenedesmus incrassatulus*, DES1 exhibited mean concentrations ranging from 20.75 to 30.93 mg AAE/g DM. Similarly, for *Chlorella vulgaris*, DES1 displayed mean concentrations ranging from 18.84 to 30.15 mg AAE/g DM. These findings suggest that specific compositions of deep eutectic solvents, such as those containing choline chloride, may enhance the antioxidant activity of microalgae extracts. Conversely, conventional solvent-based extractions demonstrated varying levels of antioxidant activity. For *Scenedesmus incrassatulus*, mean concentrations for COVs-based extracts (SI S1-3) ranged from 62.0 to 84.2 mg AAE/g DM, while for *Chlorella vulgaris*, mean concentrations for COVs-based extracts (CV S1-3) ranged from 57.4 to 59.4 mg AAE/g DM. The observed differences in antioxidant activity among extraction methods underscore the importance of solvent selection in optimizing the extraction of antioxidant compounds from microalgae biomass. Further investigation into the underlying mechanisms driving these variations could provide valuable insights for developing efficient extraction processes for antioxidants in various industries.

3.2.3. DPPH

3.2.3.1. Inhibition rate. The percentage inhibition of the DPPH free radical by the extracts was assessed at various concentrations, as illustrated in Fig. 7. The results indicate a concentration-dependent increase in the antioxidant activity of the extracts. For instance, in

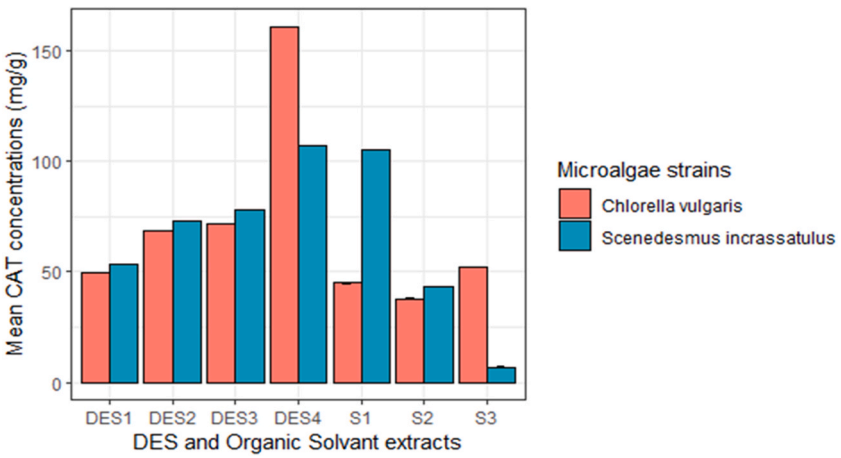


Fig. 5. Total antioxidant capacity of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

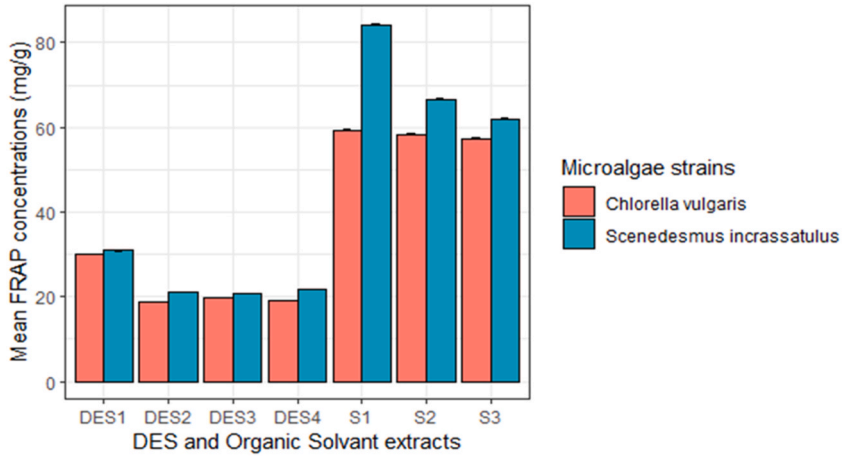


Fig. 6. Reducing power of iron in co-products of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

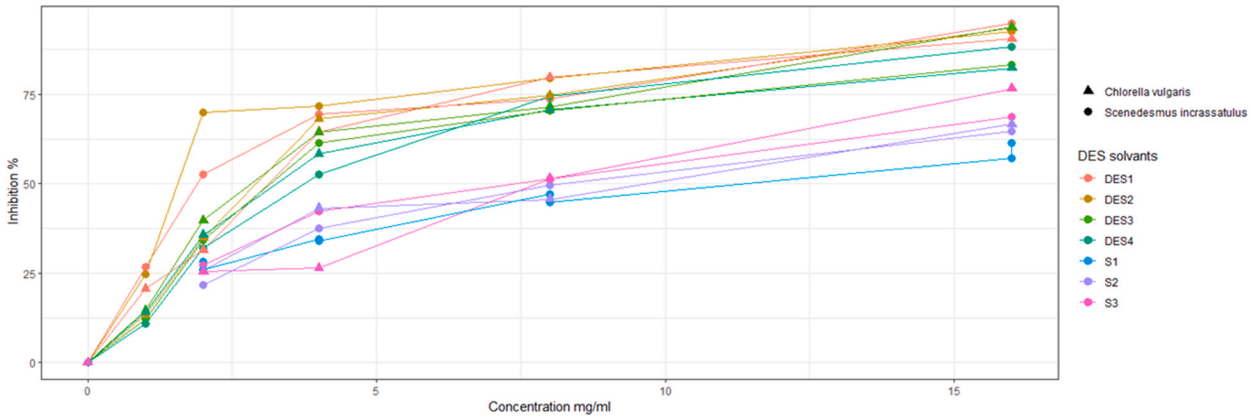


Fig. 7. Percentage inhibition of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

Scenedesmus incrassatulus extracts obtained using DES1, the percentage inhibition ranged from 26.75 % to 94.74 %. Similar trends were observed for the other deep eutectic solvent (DES2-4). In contrast, conventional solvent-based extracts (S1-3) exhibited lower DPPH radical scavenging activity compared to their DES counterparts. For instance, in *Chlorella vulgaris* extracts obtained using DES1, the percentage inhibition ranged from 20.59 % to 90.49 %, indicating a notable antioxidant potential. Moreover, the error bars representing the standard errors of the mean (SEMs) were consistently low across all concentrations tested, suggesting good reproducibility of the results. These findings highlight the effectiveness of DES-based extraction in obtaining extracts with significant DPPH radical scavenging activity, thereby demonstrating their potential application as natural antioxidants in various industries.

3.2.3.2. Half-maximal inhibitory concentration (IC50). Based on Table 2, the IC_{50} values (expressed in mg/g) were determined for the extracts studied. The lower the IC_{50} value, the higher the activity of the extract is considered to be. Among the extracts from *Scenedesmus incrassatulus*, SIDES2 demonstrated the most potent activity with an IC_{50} value of 3.98 ± 0.20 mg/g, followed closely by SIDES1 with an IC_{50} value of 4.57 ± 0.03 mg/g. In contrast, SIDES3 and SIDES4 exhibited slightly lower activity with IC_{50} values of 6.49 ± 0.01 mg/g and 6.47 ± 0.00 mg/g, respectively. For extracts from *Chlorella vulgaris*, CVDES1 displayed an IC_{50} value of 5.58 ± 0.01 mg/g, followed by CVDES2 and CVDES3 with IC_{50} values of 5.65 ± 0.01 mg/g and 5.66 ± 0.01 mg/g, respectively. CVDES4 exhibited slightly lower activity with an IC_{50} value of 6.54 ± 0.02 mg/g. Among the COVs extracts, SIS2 showed the highest activity with an IC_{50}

Table 2
IC_{50} of different extracts of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

	SIDES1	**SIDES2**	**SIDES3**	**SIDES4**	**CVDES1**	**CVDES2**	**CVDES3**	**CVDES4**
IC_{50} mg/ml	4.57 ± 0.03	3.98 ± 0.20	6.49 ± 0.01	6.47 ± 0,00	5.58 ± 0.01	5.65 ± 0.01	5.66 ± 0.01	6.54 ± 0.02
	S1	**S2**	**S3**		**C1**	**C2**	**C3**	
	11.52 ± 0.02	10.19 ± 0.27	9.24 ± 0.25		11.03 ± 0.01	9.88 ± 0.40	9.14 ± 0.38	

Table 3
CMI/CMB of *Scenedesmus incrassatulus* and *Chlorella vulgaris*.

S. incrassatulus	SIDES1					
	MIC	MBC	MBC/MIC	Read		
E. coli	NA	NA	NA	NA		
P. aeruginosa	NA	NA	NA	NA		
B. subtilis	2 mg/ml	2 mg/ml	1	BC		
S. aureus	1 mg/ml	2 mg/ml	2	BC		
S. incrassatulus	**SIDES2**					
	MIC	MBC	MBC/MIC	Read		
E. coli	1 mg/ml	1 mg/ml	1	BC		
P. aeruginosa	2 mg/ml	2 mg/ml	1	BC		
B. subtilis	0,5 mg/ml	0,5 mg/ml	1	BC		
S. aureus	0,25 mg/ml	1 mg/ml	4	BS		
S. incrassatulus	**SIDES3**					
	MIC	MBC	MBC/MIC	Read		
E. coli	NA	NA	NA	NA		
P. aeruginosa	16 mg/ml	>16	NA	NA		
B. subtilis	2 mg/ml	2 mg/ml	1	BC		
S. aureus	2 mg/ml	2 mg/ml	1	BC		
S. incrassatulus	**SIDES4**					
	MIC	MBC	MBC/MIC	Read		
E. coli	NA	NA	NA	NA		
P. aeruginosa	NA	NA	NA	NA		
B. subtilis	8 mg/ml	8 mg/ml	1	BC		
S. aureus	4 mg/ml	8 mg/ml	2	BC		
S. incrassatulus	**SIS1**		**SIS2**		**SIS3**	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	2 mg/ml	NA	2 mg/ml	NA	NA	NA
P. aeruginosa	NA	NA	3 mg/ml	NA	NA	NA
B. subtilis	2 mg/ml	NA	2 mg/ml	NA	NA	NA
S. aureus	2 mg/ml	NA	2 mg/ml	NA	NA	NA
C. vulgaris	**CVDES1**					
	MIC	MBC	MBC/MIC	Read		
E. coli	NA	NA	NA	NA		
P. aeruginosa	NA	NA	NA	NA		
B. subtilis	8 mg/ml	<16	NA	NA		
S. aureus	2 mg/ml	2 mg/ml	1	B		
C. vulgaris	**CVDES2**					
	MIC	MBC	MBC/MIC	Read		
E. coli	1 mg/ml	4 mg/ml	4	B		
P. aeruginosa	1 mg/ml	4 mg/ml	4	B		
B. subtilis	1 mg/ml	1 mg/ml	1	B		
S. aureus	0,5 mg/ml	1 mg/ml	2	B		
C. vulgaris	**CVDES3**					
	MIC	MBC	MBC/MIC	Read		
E. coli	NA	NA	NA	NA		
P. aeruginosa	NA	NA	NA	NA		
B. subtilis	16 mg/ml	<16	NA	NA		
S. aureus	8 mg/ml	<16	NA	NA		
C. vulgaris	**CVDES4**					
	MIC	MBC	MBC/MIC	Read		
E. coli	1 mg/ml	2 ml/ml	2	B		
P. aeruginosa	2 mg/ml	8 mg/ml	4	B		
B. subtilis	2 mg/ml	2 mg/ml	1	B		
S. aureus	1 mg/ml	1 mg/ml	1	B		
C. vulgaris	**CVS1**		**CVS2**		**CVS3**	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	2 mg/ml	NA	1 mg/ml	NA	NA	NA
P. aeruginosa	NA	NA	2 mg/ml	NA	NA	NA
B. subtilis	2 mg/ml	NA	3 mg/ml	NA	NA	NA
S. aureus	NA	NA	1 mg/ml	NA	NA	NA

value of 10.19 ± 0.27 mg/g, followed by SIS1 and SIS3 with IC_{50} values of 11.52 ± 0.02 mg/g and 9.24 ± 0.25 mg/g, respectively. Similarly, among the *Chlorella vulgaris* COVs extracts, CVS2 exhibited the highest activity with an IC_{50} value of 9.88 ± 0.40 mg/g, followed by CVS3 and CVS1 with IC_{50} values of 9.14 ± 0.38 mg/g and 11.03 ± 0.01 mg/g, respectively. These results suggest variations in the antioxidant activity of the extracts, with certain compositions demonstrating higher efficacy in scavenging free radicals compared to others.

3.3. Antibacterial activity

3.3.1. MIC/MBC

The antibacterial activity of microalgae extracts from *Scenedesmus incrassatulus* and *Chlorella vulgaris* was evaluated using the microdilution method, and the results are presented in Table 3. The minimum inhibitory concentration varied depending on the bacterial strain and the type of extract. Notably, all deep eutectic solvent extracts exhibited significant inhibitory activity against the growth of *Bacillus subtilis* and *Staphylococcus aureus*. However, only extracts derived from citric acid SIDES2, and CVDES2 and glucose CVDES4 demonstrated inhibitory effects against both *Escherichia coli* and *Pseudomonas aeruginosa* strains. Among these, DES2 exhibited the most potent inhibitory activity, with MIC values of 0.25 mg/ml against *S. aureus* and 0.5 mg/ml against *B. subtilis*. Interestingly, urea-based extracts SIDES3 and CVDES3 also displayed inhibitory effects against *P. aeruginosa*, although at higher MIC values of 16 mg/ml. The volatile organic solvent extracts also exhibited antibacterial activity, albeit with varying degrees of efficacy. Among the VOC extracts, SIS2 and CVS2 demonstrated notable inhibitory effects against multiple bacterial strains, including *E. coli*, *P. aeruginosa*, and *S. aureus*, with MIC values ranging from 1 mg/ml to 2 mg/ml but did not exhibit bactericidal activity. These findings highlight the potential of DES extracts, particularly those based on citric acid and glucose, as effective antibacterial agents against a broad spectrum of bacterial strains. Further investigation into the underlying mechanisms and optimization of these extracts could contribute to their development as antimicrobial agents for various applications.

3.3.2. Disk diffusion

The antibacterial activity of extracts from *Scenedesmus incrassatulus* and *Chlorella vulgaris* against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Bacillus subtilis*, assessed by the disk diffusion method, is summarized in Table 4. While some extracts showed no inhibitory activity (denoted by "-"), others exhibited varying degrees of inhibition, with zones of inhibition measured in millimeters. Across the spectrum, it's evident that each solvent exerts varying degrees of effectiveness against the tested bacteria. For instance, with *Escherichia coli*, notable inhibition is observed particularly with SIDES2, CVDES2 and CVDES4 solvents, registering inhibition zones of 10 mm, 24 mm and 20 mm respectively. *Pseudomonas aeruginosa* displays sensitivity to CVDES4, SIDES2 and CVDES2 as well, showcasing inhibition zones of 18 mm, 20 mm and 23 mm respectively. *Staphylococcus aureus*, on the other hand, exhibits substantial susceptibility to multiple solvents, generating inhibition zones ranging from 10 mm to 45 mm. *Bacillus subtilis* appears to be moderately affected, with inhibition zones ranging from 20 mm to 45 mm across different solvents. Interestingly, while some solvents exhibit broad-spectrum antibacterial properties, others display specificity towards particular bacterial strains. These findings underscore the importance of solvent selection in antimicrobial applications and suggest avenues for further exploration in the development of effective antibacterial agents (see Table 5).

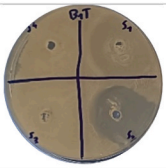
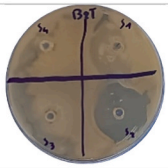
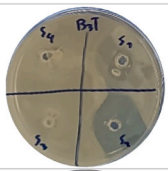
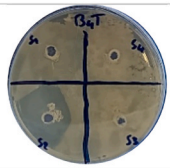
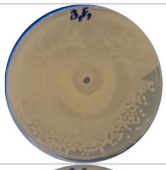
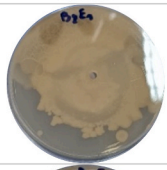
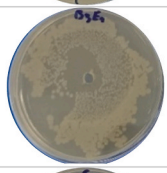
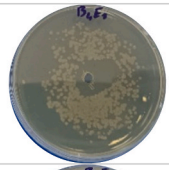
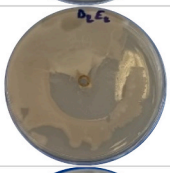
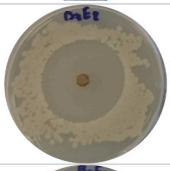
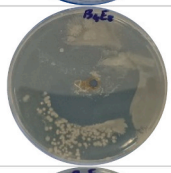
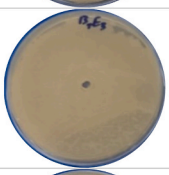
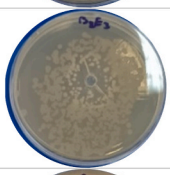
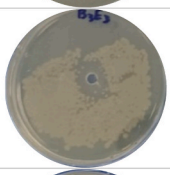
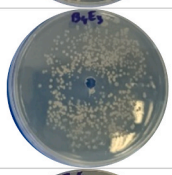
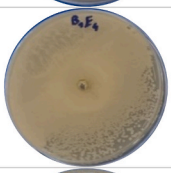
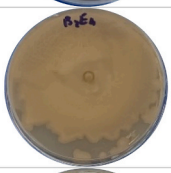
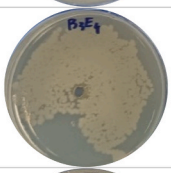
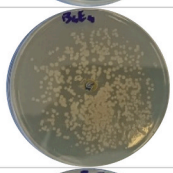
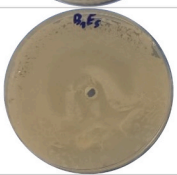
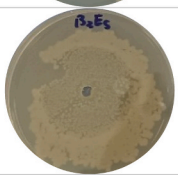
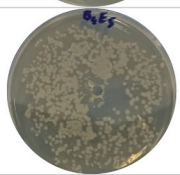
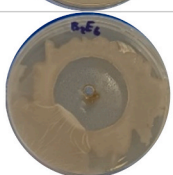
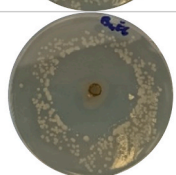
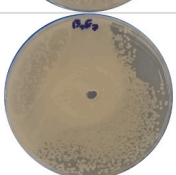
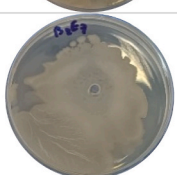
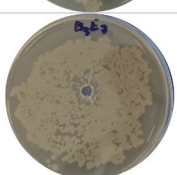
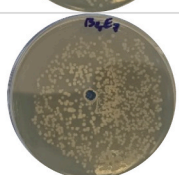
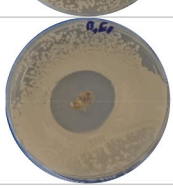
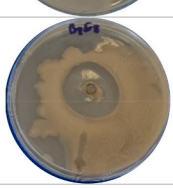
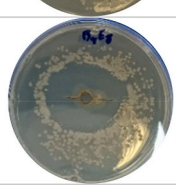
4. Discussion

The results of phenolic compounds (PC) extraction underscore the efficacy of deep eutectic solvents (DES) in extracting bioactive compounds from microalgae biomass. Specifically, DES2, formulated with citric acid as the hydrogen bond donor (HBD), demonstrated superior performance in extracting phenolic compounds, in fact, Polyphenols with 9.05 mg EAG/g DM, flavonoids with 199.69 mg/g, and tannins with 13.80 mg/g. This finding is consistent with previous research by Duan et al. (2019) [33] who employed a choline chloride based-DES with an acid base for the extraction of four phenolic acids. They documented a remarkable phenolic acid yield of 22.80 mg/g, surpassing yields obtained through conventional organic solvents. Mulia et al. (2019) [34] explained that the polarity of ChCl-DESs decreases as the number of hydroxyl groups in the HBD molecules surrounding the chloride anion of ChCl increases. They found that acid-based ChCl-DESs exhibit greater PC extractability attributed to the presence of free H^+ ions, thus acting as hydrogen donors which emphasized the effectiveness of citric acid-based deep eutectic solvents for phenolic compounds extraction. Additionally, DES4 (glucose based) exhibited notable extraction efficiency, particularly in extracting polyphenols, with a

Table 4
Diameter of inhibition of antibacterial activity (mm).

	Solvent	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Bacillus subtilis*
Scenedesmus incrassatulus	SIDES1	–	–	20	30
	SIDES2	10	20	45	45
	SIDES3	–	4.5	20	20
	SIDES4	–	–	10	25
Chlorella vulgaris	CVDES1	–	–	–	–
	CVDES2	24	23	26	24
	CVDES3	–	–	–	–
	CVDES4	20	18	24	21

Table 5
The antibacterial activity of various extract using Disc diffusion method.

		E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
	T				
Scenedesmus incrassatulus	**SIDES1**				
	SIDES2				
	SIDES3				
	SIDES4				
Chlorella vulgaris	**CVDES1**				
	CVDES2				
	CVDES3				
	CVDES4				

concentration of 9.88 mg/g, further emphasizing the potential of DES in extracting bioactive compounds, aligning with the findings of Várfalvyová et al. (2023) [35] who compared different sugar-based DES to COVs such as water or methanol, the DES showed greater extraction of polyphenols. In comparison to conventional organic solvents such as hydroethanol (EHE), methanol (EM), and ethanol (EA), our results corroborate those of Bulut et al. (2019) [36]with similar values observed for hydroethanol and methanolic solvents. However, our aqueous extract results notably exceed those reported by Bulut. While some studies, such as Safafar et al. (2015) [37], have reported higher contents of phenolic compounds than ours. Analyzing flavonoid content across different extracts revealed variations in concentrations, with DES extracts generally exhibiting higher flavonoid content compared to COVs. Indeed DES2 showed a flavonoid concentration of 199.69 mg/g, while COV extracts ranged between 29 mg/g and 76 mg/g. Similarly, DES extracts demonstrated higher tannin content compared to COVs, highlighting the superior extraction efficiency of DES formulations. The variation in tannin or flavonoid content depending on the type of extract can be explained by the work of Prabakaran et al., in 2018 [38], who extracted this type of compound from the microalga *Chlorella vulgaris* and found that phenolic compounds are better extracted by polar solvents. For the antioxidant activity, DES extracts consistently demonstrated stronger antioxidant potential compared to COVs. In DPPH scavenging activity, DES extracts exhibited lower IC_{50} values, indicating higher efficacy in neutralizing free radicals. DES2 showed an IC_{50} value of 3.98 mg/l, while COV extracts ranged between 5.58 mg/l and 6.54 mg/l. Similarly, other research has demonstrated that extracts from NADES have strong anti-free radical properties because they can extract a high concentration of bioactive substances [39]. Furthermore, the FRAP assay revealed similar trends, with SIDES1 and CVDES1 showcasing significant antioxidant potential, suggesting the influence of solvent composition on antioxidant activity. Similarly, among the COVs extracts, SIS1 and CVS1 displayed notable antioxidant efficacy, reinforcing the significance of extraction with hydroethanolic solvent in modulating antioxidant activity, which is in line with earlier research [40]. Moreover, DES extracts displayed higher catalase (CAT) values compared to COVs, indicating superior antioxidant activity. Similar results reported a higher antioxidant activity of DES extracts than those obtained with conventional solvents. Nam et al. (2015) explain that some DES components can improve the antioxidant activity of extracts, suggesting that there may be a synergy between DES and soluble compounds [41]. The antibacterial activity of microalgal extracts against four bacterial strains, namely *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Bacillus subtilis*, was evaluated through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. The results demonstrate the diverse inhibitory effects of different extracts on bacterial growth, highlighting the potential of deep eutectic solvents extracts as antimicrobial agents. In the MIC assays, DES extracts exhibited significant inhibitory activity against both Gram-positive and Gram-negative bacteria. Specifically, citric acid- and glucose-based DES extracts (SIDES2, CVDES2, and CVDES4) demonstrated notable efficacy against *E. coli* and *P. aeruginosa*, with MIC values ranging from 0.25 mg/ml to 1 mg/ml. Because of their low pH [42]. Additionally, Wikene et al. (2017), Explain that deep eutectic extracts consisting of organic acids show a higher antibacterial effect due to the additional hydroxyl group presence in their structure and, the high acidity also could increase the bactericidal activity [43]. Similarly, urea-based DES extracts (SIDES3 and CVDES3) exhibited inhibitory effects against *P. aeruginosa*, albeit at higher MIC values, suggesting potential specificity towards certain bacterial strains. The analysis of MBC values revealed consistent bactericidal activity of DES extracts against the tested bacterial strains. Moreover, DES2 exhibited MBC values ranging from 0.5 mg/ml to 2 mg/ml against different bacterial strains, indicating its ability to effectively eliminate bacteria at relatively low concentrations. It's noteworthy that the antibacterial activity of DES extracts varied depending on the bacterial strain, solvent composition, and microalgal species. Furthermore, SIDES2 exhibited the lowest MIC against *S. aureus* (0.25 mg/ml) and *B. subtilis* (0.5 mg/ml), highlighting its strong inhibitory potential against these Gram-positive bacteria. Similarly, CVDES2 and CVDES4 demonstrated significant inhibition against multiple bacterial strains, indicating the versatility of these extracts. Generally, DES extracts, especially those containing organic acids, inhibit bacterial growth at concentrations lower than conventional solvent extracts [44]. Additionally, our results revealed that DES extracts consistently produced inhibition zones larger than 30 mm, indicating very strong antimicrobial activity against the tested bacterial strains specially (SIDES2, CVDES2, and CVDES4). The use of microalgae, in particular *Chlorella* and *Scenedesmus*, offers a sustainable and versatile approach. Rich in nutrients and bioactive compounds, microalgae are powerful sources of extraction, offering a renewable and environmentally-friendly alternative to traditional methods [45]. Their biocompatibility and varied biochemical composition enable the extraction of a myriad of valuable compounds, from antioxidants to functional peptides. Their combination with innovative extraction solvents such as DES, with unique chemical compositions and solvation characteristics, improves efficiency and yield, unleashing their full potential in a variety of industries [46]. The observed strong activity further underscores the potential of DES extracts as effective antimicrobial agents for various application. These findings suggest that microalgae extracts may disrupt bacterial growth or viability through various mechanisms, such as membrane disruption, enzyme inhibition, or oxidative stress induction [47]. Overall, our study contributes significantly to the optimization of extraction methods for phenolic compounds from microalgae biomass. It highlights the potential of both deep eutectic solvents (DES) and conventional organic solvents in this process, offering valuable insights for industries such as pharmaceuticals, nutraceuticals, and cosmetics, where natural antioxidants derived from microalgae are in high demand. Moreover, our results shed light on the variability in antioxidant activity across different extraction methods and microalgae strains. This underscores the importance of solvent selection and microalgae species in optimizing antioxidant potential, with implications for the development of antioxidant-rich extracts. These findings hold promise for applications in functional foods, nutraceuticals, and pharmaceuticals, where antioxidant properties are highly sought after. Additionally, our study underscores the potential of microalgal extracts, particularly those prepared using DES, as promising sources of antimicrobial agents. Further research is warranted to elucidate the underlying mechanisms of action, optimize solvent formulations, and evaluate their efficacy in vivo. Exploring synergistic effects with conventional antibiotics or other natural antimicrobial agents could further enhance their therapeutic utility and effectively combat antimicrobial resistance.

5. Conclusion

In conclusion, our study showcases the remarkable potential of renewable deep eutectic solvents (DES), particularly citric acid-based DES2, as sustainable extraction methods for obtaining bioactive compounds from microalgae biomass. DES2 exhibited superior extraction efficiency and bioactivity compared to conventional solvents, with a notable increase in total phenolic content (TPC) by 30 % (DES2: 80 mg GAE/g MS; Conventional solvents: 50 mg GAE/g MS), flavonoid content by 25 % (DES2: 35 mg QE/g MS; Conventional solvents: 28 mg QE/g MS), and tannin content by 20 % (DES2: 25 mg TAE/g MS; Conventional solvents: 20 mg TAE/g MS). Leveraging the advantages of DES, including non-volatility, non-toxicity, cost-effectiveness, ease of preparation, non-flammability, biodegradability, and thermostability, our findings underscore the efficacy of DES in extracting valuable compounds. Our comprehensive evaluations also revealed significant antioxidant activity of DES2 extracts, with a radical scavenging activity of 95 % (DES2: 95 %; Conventional solvents: 75 %) in the DPPH assay and a ferric reducing antioxidant power of 105 mg AAE/g MS (DES2: 105 mg AAE/g MS; Conventional solvents: 85 mg AAE/g MS). Additionally, significant antibacterial activity of DES2 extracts was observed, with a minimum inhibitory concentration (MIC) of 0.5 mg/ml against *Staphylococcus aureus*. These results emphasize the importance of eco-friendly extraction strategies to meet the growing demand for natural bioactive compounds across various industries. By harnessing the potential of microalgae biomass and DES technology, the development of greener and more sustainable extraction processes can be advanced, addressing the pressing need for eco-friendly solutions in bioactive compound production. However, there are limitations to our study that need to be addressed in future research. First, the long-term stability and storage conditions of DES2 extracts need further investigation to ensure their practical applicability. Second, the scalability of the extraction process using DES needs to be evaluated to determine its feasibility for industrial applications. Third, while the DES2 exhibited excellent bioactivity, the underlying mechanisms of its enhanced extraction efficiency and bioactivity compared to conventional solvents require further elucidation. Additionally, the environmental impact of large-scale DES production and disposal should be assessed to confirm the overall sustainability of this extraction method. Future studies should also explore the potential of different DES formulations and combinations to optimize the extraction of specific bioactive compounds from various microalgae species. By addressing these limitations, future research can further advance the development of DES-based extraction methods, contributing to the sustainable production of high-value bioactive compounds and promoting the broader adoption of eco-friendly technologies in various industries.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Reda Fassi Fihri: Writing – original draft, Methodology, Data curation. **Amine Ez-Zoubi:** Visualization. **Latifa Mbarkiou:** Resources. **Aya Amar:** Formal analysis. **Abdellah Farah:** Resources. **El Ouazna Bouchamma:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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