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Bioproduction, Antimicrobial and Antioxidant Activities of Compounds from *Chlorella vulgaris*

Danielli M.M. Dantas[1], Romero M.P.B. Costa[1,2], Maria G. Carneiro-da-Cunha[1,2], A. O. Galvez[3], A. R. Drummond[4], and Ranilson S. Bezerra[1]*

[1]Departamento de Bioquimica, Centro de Ciencias Biologicas, Universidade Federal de Pernambuco (UFPE), PE, Brazil.

[2]Laboratorio de Imunopatologia Keizo Asami–LIKA/UFPE, CEP 50.670-901 Recife, PE, Brazil.

[3]Departamento de Aquicultura e Pesca, Universidade Federal Rural de Pernambuco (UFRPE), PE, Brazil.

[4]Laboratorio de Fluidos, ITEP, PE, Brazil.

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***For Correspondence**

Ranilson S. Bezerra, Departamento de Bioquimica, Centro de Ciencias Biologicas, Universidade Federal de Pernambuco (UFPE), PE, Brazil, Tel: +55.81.2126.8540

E-mail: ransoube@uol.com.br

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ABSTRACT

Various crude extract preparations (ethanol, methanol, buthanol, acetone, DMSO and water) from the green alga *Chlorella vulgaris* were examined for Antioxidant activity, Phytochemical screening and Antimicrobial properties. In vitro free radical quenching and total antioxidant activity of extracts were investigated with 1, 1-diphenyl-2-picryl hydrazyl (DPPH), and compared with cathequin and Gallic acid as positive controls. In most cases, results showed a significant association between the antioxidant potency and the total phenolics content. The aqueous extract showed both the highest antioxidant activity for inhibition scavenging (68.5%) and highest phenolic content (3.45 mg/mL). Antimicrobial activities were carried out using disc diffusion assays and the broth dilution method against Gram-positive and Gram-negative bacteria. Results demonstrated activity between the aqueous extract and most specimens (*Proteus mirabilis, Klebsiella pneumoniae, Salmonella enteretidis, Bacillus subtilis and Escherichia coli*). These results suggest that the aqueous crude extract of *C. vulgaris* could be considered as a biological antioxidant and antimicrobial agent, and a valuable tool for the biotechnology field.

INTRODUCTION

Microalgae are a source of natural products and have been recently studied for biotechnological applications. The diversity of microalgae makes a potentially rich source for various chemical products with applications in nutritional, cosmetic, pharmaceutical, and medicinal industries [1]. Extracts from marine microalgae are a rich source of proteins, vitamins, and minerals. *Chlorella*, a unicellular green algae, contains various valuable proteins (40~60%) and has been widely used in aquaculture, food and biotechnology industries. The extract from Chlorella contains various biologically active compounds including growth factors, anti-inflammatory and wound healing substances, antioxidants, and emollient compounds [2].

The production of free radicals in organisms is regulated by different antioxidants molecules which may be endogenous, such as superoxide dismutase, or may come from the diet, such as ascorbic acid, α-tocopherol, carotenoids and polyphenols. When there is a limitation in the availability of antioxidants, there may be oxidative damage to the cumulative nature. Among the various classes of naturally occurring antioxidants, phenolic compounds such as simple phenols, phenolic acids (derivatives of benzoic acid and cinnamic acid), coumarins, flavonoids and others, have received much attention. According to Wang et al., the isolated indigenous *C. vulgaris* strain extract obtained from Supercritical carbon dioxide extraction exhibits significant antioxidant activities and presents dual inhibitions to lung cancer cell growth and migration ability, which is the index of cancer metastasis [1].

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Therefore, microalgae species *C. vulgaris* could have the potential for the development of antioxidant and anticancer products. A revision involving research for innovative functional food ingredients from microalgae showed the particular species of microalgae, the activity of the compounds obtained, and the type of extraction mechanisms used, showing that the unicellular algae *Chlorella vulgaris* contains many bioactive substances with medical properties. Experimental studies carried out under *Chlorella* have demonstrated its antitumor effect, cancer chemoprevention properties, anti-inflammatory activity, antioxidant activity, and antimicrobial activity [1,3-5].

The knowledge of the chemical composition of the different microalgae species is mandatory as a first step (considering a screening methodology) since it will help to target the valuable compounds, antioxidants, suphated polysaccharides, PUFAs, etc., in the studied microalgae. As a second step, the growing conditions (salinity, luminosity and nutrient availability) could be optimized to maximize the production of the compound of interest. The next step, once the biomass in the target compound (or compounds) is enriched, is to optimize the conditions to extract the valuable components with high yield and activities. Therefore, it is necessary to know not only the selectivity of the process but also the impact of such processes in the global definition of a sustainable process; aspects related to the extract such as yield, quality, and bioactivity should be considered but also other factors such as sustainability, environmental pollution, residues, cost effectiveness, etc. and should also contribute to the final selection of the most appropriate extraction process (sub- and supercritical fluids like CO2, ethanol, water, and combinations). The development of such processes is a bet that is becoming everyday more and more urgent in our society [6]. Hence, in the present study we focused on different crude extracts from *C. vulgaris* for its antioxidant and antimicrobial properties.

MATERIAL AND METHODS

Microalgae Production

The microalgal species, Chlorella vulgaris, was obtained from culture Collections of the Laboratory of the Production of live food of the Federal Rural of Pernambuco University. The microalgae was cultivated in a semi continuous culture system, both indoors and outdoors. The indoor cultures of microalgae were grown with autoclaved freshwater with Provasoli medium in 2 L bottles bubbled with air, and incubated in a temperature controlled room (24˚C). The bottles were irradiated with daylight fluorescent tubes (light intensity, 4000Lux) for 72 hours. For outdoor cultivation, freshwater was used with the fertilizer NPK (20:10:20), constant aeration, and a natural photoperiod (12:12) in 10 L, 100 L, and 500 L containers of fiberglass under a light intensity of approximately 100.000 Lux. Algal biomass was estimated with use of a Neubauer chamber. The separation of microalgae biomass was obtained by flocculation with NaOH (1M), dried, and sent to the Biotechnology Laboratory of the Federal University of Pernambuco for processing.

Preparation of the Extracts

The extracts were obtained from different solvents: ethanol, methanol, buthanol, acetone, DMSO and water. Samples of dried microalgae biomass (1g) were suspended in 10 mL for each solvent. The samples were extracted under 30 minutes of sonication (40 kHz) in an ultrasonic batch (model Ultra Cleaner 1400, Ultrasonic Unique, Brazil) followed by camera shake for 2 hours and centrifugation (4000rpm) for 10 minutes to obtain the supernatant liquid **(Figure 1)**. These extracts were analyzed for activity antioxidant, antibacterial, antifungal and phytochemical compounds.

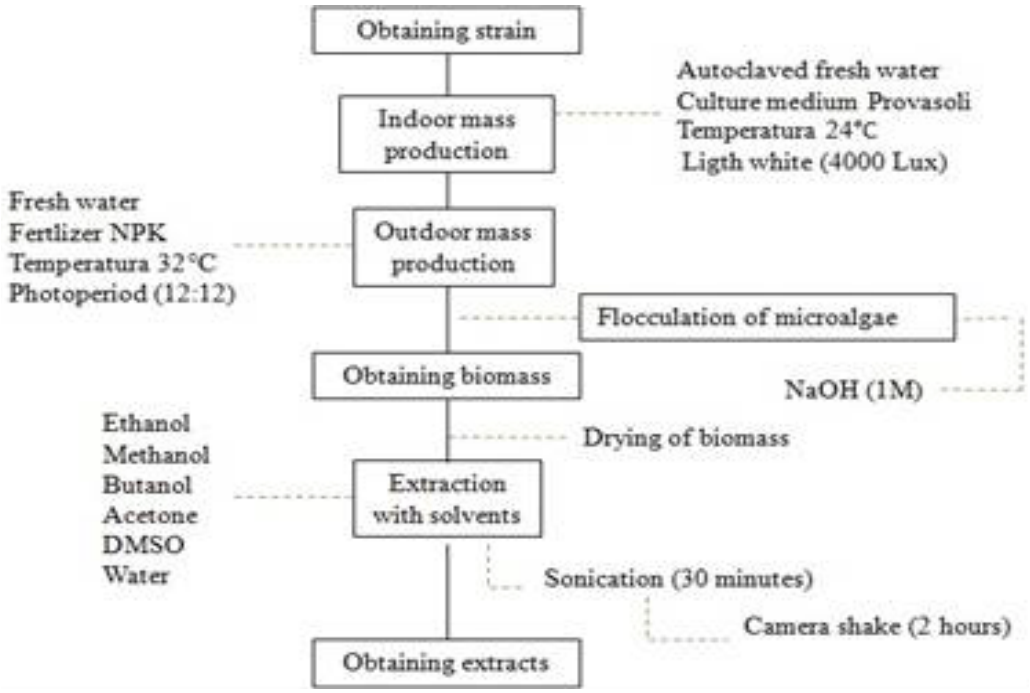

Figure 1. Full scheme of bio production and preparation of extracts from the microalgae *C.vulgaris.*

Phytochemical Screening

The total phenolic content in the extracts was determined according to the method described by Julkunen-Titto with some modifications [7]. An aliquot (50 µL) of each extract or standard solution was mixed with 1 mL of H2O and 500 µL of Folin–Ciocalteu's phenol reagent. Afterwards 2.5 mL of 20% Na2CO3 solution were added to the mixture, followed by incubating at ambient temperature in the dark for 45 min. The absorbance against a blank was measured at 735 nm (Spectro UV–vis auto spectrophotometer, Labomed Inc., Culver City, CA). Gallic acid was used to prepare a standard curve (0.025–0.6 mg/mL). The results were expressed as mg gallic acid equivalents (GAE)/g extract (dw). The total of flavonoid content was determined according to the method of Zhishen et al [8]. An aliquot (250 µL) of each extract or standard solution was mixed with 1.25 mL of H2O and 75 µL of 5% NaNO2 solution. After 6 min, 150 µL of 10% AlCl3 H2O solution were added. After 5 min, 0.5 mL of 1 M NaOH solution was added and then the total volume was made up to 2.5 mL with H2O. Following the thorough mixing of the solution, the absorbance against a blank was determined at 510 nm. (+)-Catechin was utilized for constructing the standard curve (0.05–0.5 mg/mL). The results were expressed as mg catechin equivalents (CE)/g extracted (dw). The condensed tannins were determined according to the method of Julkunen-Titto [7]. An aliquot (50 µL) of each extract or standard solution was mixed with 1.5 mL of 4% vanillin (prepared with MeOH) and then 750 µL of HCl were added. The well-mixed solution was incubated at ambient temperature in the dark for 20 min. The absorbance against blank was read at 500 nm. (+) - Catechin was used to make the standard curve (0 – 1 mg/mL). The results were expressed as mg catechin equivalents (CE)/g extracted (dw).

Antioxidant Assay: DPPH (1,1-diphenil-2-picrylhydazyl) free-radical Scavenging Activity

The antioxidant activity of the extracts from *P. pyrifolia* leaves was evaluated by DPPH free-radical scavenging activity according to the method of Soler-Rivas et al and Moure et al [9,10]. The experiments were performed using the SmartSpec 3000 spectrophotometer (Bio-Rad). Briefly, an aliquot (20 µL) of buthanol, ether, ethyl acetate and aqueous extracts were mixed separately with 90 µM methanolic DPPH radical solution to a final volume of 1 mL. Analytical grade methanol was used as negative control. (+)-catechin, ascorbate and pirocatechin in methanol were used as a positive control. The DPPH radical concentration in the reaction mixture was calculated by the calibration curve according to the following nonlinear regression equation (R= 0.9983): A515 nm = 0.0362 [DPPH] – 0.055, where [DPPH] is expressed in mg mL^{-1}. The percentage of remaining DPPH (%DPPHREM) was calculated according to Brand-Williams et al. (1995), as follows:

%DPPHREM = [DPPH]T /[DPPH]To x 100

Where T is the time when absorbance was determined (1–30 min) and T0 is the time zero. For determination of IP, an aliquot (50 µL) of each extract was added to 2 mL of 90 µM methanolic solution of the DPPH radical and the absorbance was determined at 515 nm at the steady state (20 min).

Reducing Power

Reducing power was determined according to the method of Costa et al. [11]. Extracts prepared in different solvents (10% w/v) were centrifuged 10,000xg, 20ºC, for 15 min, and the supernatant dried, after 5mg (dried weight) were suspended with 1mL MeOH. An aliquot of sample (250 µL) was mixed with 250 µL of sodium phosphate buffer (0.2 mol/L, pH 6.6) and 250 µL of K3Fe(CN)6: H2O (1:99, w/v) incubated at 50°C for 20 min. After adding 250 µL of Trichloroacetic acid: H2O (10:90, w/v), the mixture was centrifuged at 3750g for 10 min. The supernatant (100 µL) was then collected and immediately mixed with MeOH (100 µL) and 25 µL of Ferric chloride: H2O (0.1:99.9, w/v). After incubation for 10 min, the absorbance was determined at 700 nm. The IC_{50} value is the concentration at which the absorbance is 0.5. Ascorbic acid was utilized as standard.

Antibacterial Activity

The antibacterial activity of the different extracts of *C. vulgaris* was determined according to Costa et al. [11]. Bacteria were provided by the Department of Antibiotics (DA), Universidade Federal de Pernambuco (UFPE), Brazil in DifcoTM Nutrient Agar (NA) and stored at 4˚C. Gram-positive strains were *Streptococcus faecalis and Bacillus subtilis; Gram-negative strains were Klebsiella pneumoniae, Proteus mirabilis, Salmonella enteritidis* and *Escherichia coli.* Extract preparations for antibacterial activity were investigated by the disc diffusion method. One-hundred milliliters of warm NA (43˚C) and 0.5 mL of bacteria suspensions (105–106 CFU mL^{-1}) were mixed, and 10 mL volumes were distributed in sterile Petri plates (90x15 mm) and allowed to solidify. Sterile blank paper discs (6 mm diameter) impregnated with 20 µL of sterile extracts carried out using dried algae (10%, w/v) obtained in different solvents (ethanol, methanol, buthanol, acetone, DMSO and water) were added on the center agar plates. The negative controls were discs with different solvents (20 µL). Plates were incubated at 37˚C for 24 h. A transparent ring around the paper disc revealed antibacterial activity.

Antifungal Activity

The antimicrobial activity of the different extracts of *C. vulgaris* was determined according to Costa et al. [11]. *Aspergilus niger* (URM2813), *A. flavus* (URM2814), *A. fumigatus* (URM2815), Rhizopus arrhizus (URM2816), Paecilomyces variotti (URM2818), Fusarium moniliforme (URM2463), F. lateritium (URM2665), Candida albicans (UFPE-DA1007) and *C. burnenses* (UFPEDA4674)

were obtained from the Cultures Collection —Micoteca (URM) of the Department of Mycology and from the Department of Antibiotic (DA), University Federal of Pernambuco (UFPE), Brazil.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) of four determinations. Statistical analyses were performed using a one-way analysis of variance. Differences were considered significant at P values < 0.05.

RESULTS AND DISCUSSION

Phytochemical Screening and Antioxidant Properties

Dried samples of *C. vulgaris* algae were submitted to analysis by their constituents. Throughout the phytochemical analysis of the extracts obtained from different solvents, the extraction using water as solvent showed the highest levels of total phenols (3.45 mg/mL) in higher concentrations than others, followed by the DMSO solvent (2.23 mg/mL). Buthanol and acetone extracts contained significantly less phenols and flavonoids than others. The results achieved by Wang et al., for Rhodomela confervoides algae showed that ethyl acetate solvent (minor polarity) had better extraction for TPC than others, about 74 mg/mL. According to López et al. (2011) the amount of total phenolics extracted from Stypocaulon scoparium varied from 1.23 to 3.28 mg equivalent GA/g of dry alga powder, while in our studies TPC varied from 0.110 to 3.450 mg equivalent GA/g of dry alga powder [12]. We concurrently observed that low contents of tannins were presented or undetectable in all samples. It is well known that the yield of chemical extraction depends on the type of solvents with varying polarities, pH, extraction time and temperature as well as on the chemical compositions of the sample. Earlier, solvents such as methanol, ethanol, butanol, acetone, chloroform and water have been commonly used for the extraction of phenolics from brown and red seaweeds [13,14]. Further tests were conducted for the extracts of *C. vulgaris* in methanol and water to assess the presence of coumarin, phenilpropanoglicoside compounds, terpene, alkaloids and carbohydrates. Low quantities of carbohydrates were observed in methanolic extract but not in water. The other biomolecules were undetectable. Ananthi et al., showed that in Turbinaria ornata, a major contribution of carbohydrates in quantitative estimation of compounds were present in the same extract [15] **Table 1**.

Table 1. Phytochemical screening of different extracts of *C. vulgaris.*

	*TPC (mg/mL)	*TFC (mg/mL)	*CTC (mg/mL)
Ethanol	0.380 ± 0.020	ND	ND
Methanol	0.650 ± 0.010	ND	ND
Buthanol	0.110 ± 0.010	0.06 ± 0.02	ND
Acetone	0.120 ± 0.004	0.04 ± 0.02	ND
DMSO	2.230 ± 0.200	1.12 ± 0.02	ND
Water	3.450 ± 0.260	1.48 ± 0.02	0.230 ± 0.020

* TPC: Total phenols contents; * TFC: Total flavonoids contents; * CTC: Condensed tannins contents
* ND: Not detected. Means ± standard deviation of three measurements.

Due to the presence of different antioxidant components in the crude extracts of biological tissue samples, it is relatively difficult to measure each antioxidant component separately. Therefore, several assay methods have been developed and applied in recent years to screen and evaluate the total antioxidant activity of such samples [16]. Assays for free radical scavenging with DPPH are known by the action of antioxidants to inhibit the oxidation of products. Thus, the DPPH assay is often used as an indicator of antioxidant activity [1]. **(Figure 2)** shows the kinetic behaviour of antioxidant extracts against DPPH. According to data, DMSO and water extracts demonstrate the highest antioxidant properties. This may occur because molecules which have antioxidant properties in these extracts present a hydrophilic character. Antioxidants are able to stabilize or deactivate free radicals before they attack targets in biological cells. The radicals formed from antioxidants that are not reactive enough to propagate the chain reaction are neutralized by reactions with another radical to form stable products, or may be picked up by another antioxidant [17]. In **(figure 2)**, we observe that the extractions obtained using acetone and butanol presented the lowest antioxidant activities confirming the results reported earlier about the hydrophilic character of the compounds extracted. DPPH free radical scavenge testing system is an acknowledged mechanism by which antioxidants act to inhibit oxidation products. Hence, this DPPH assay has been widely applied as one of the indicators for antioxidant activity.

In this study the solvents with higher efficiency of extraction of compounds with antioxidant activity of the species Chlorella vulgaris are shown in **Table 2**. The solvents DMSO (dimethyl sulfoxide) and water showed the DPPH percentage of inhibition of 64.6% and 68.5%, respectively, higher than the standards used Catechin (49.6%) and Gallic acid (28.7%), showing that they are potential inhibitors of cellular oxidation by free radicals. When Wang et al. studied the same species they observed that in the DPPH assay using ultrasonic extraction with ethanol found the lowest percentage of inhibition (0.74%) than found in this study (37.2%) [1]. This fact might be related to the variation in strains of microalgae and with the appropriate constituents. The result found using aqueous extract proves satisfactory because in addition to reducing processing costs, resulting in a product without the potentially toxic residues found in other solvents.

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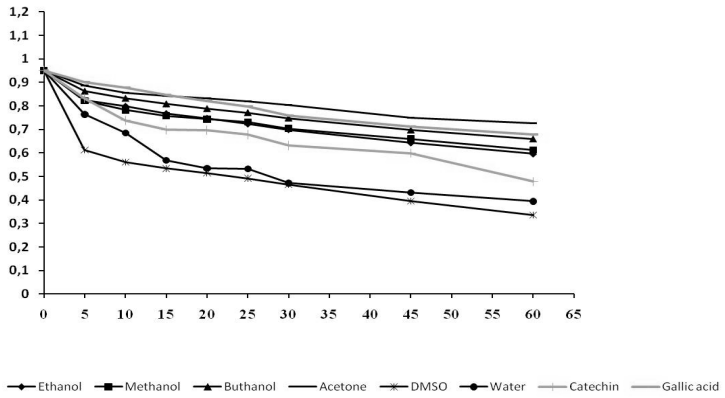

Figure 2. Kinetic behaviour of antioxidant extracts. The error in the determination of absorbance 515 nm to relative values of different doses is approximately ± 0.010, smaller than the size of the symbols. Preparations of extracts are described in the figure.

Table 2. Radical scavenging performance of extracts from *C. vulgaris.*

C. vulgaris extracts			
	DPPH scavenging		**Reducing power**
	IP (%)	**REMDPPH (%)**	**AscAE ± SD**
Ethanol extract	37.2	62.8	396 ± 1.34a
Methanol extract	35.4	64.6	384 ± 1.45a
Buthanol extract	30.6	69.4	185 ± 2.46b
Acetone extract	23.5	76.5	198 ± 3.12b
DMSO extract	64.6	35.4	595 ± 3.12c
Water extract	68.5	31.5	612 ± 2.24c
Catechin standard	49.6	50.4	412 ± 2.11a
Gallic acid standard	28.7	71.3	187 ± 3.45b

*Catechin and Gallic acid are the standards; IP: percentage inhibition; REMDPPH: percentage of remaining DPPH. IP (%) and REMDPPH were calculated at the steady state (60 min).
¨Reducing power is expressed as ascorbic acid equivalents (AscAE; mg/g seaweed dry weight of AscAE). Each value is presented as mean ± SD (n = 3). Means within each column with different letters (a–i) differ significantly ($p < 0.05$).

Antimicrobial Activity of C. vulgaris

The effects of different extracts on the antibacterial and antifungal activities were evaluated in this research. **Table 3** showed the size of zone of inhibition (mm) of *C. vulgaris* extracts against different strains. The aqueous extract showed antibacterial activity against most bacteria tested, except *S. faecalis*. The growth inhibition of *E. faecalis* was observed only when the extracts with acetone and DMSO were used, with size of zone of inhibition of 12 mm and 15 mm, respectively. The other extracts showed no inhibition against the tested bacteria. **Table 4** showed Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of different extracts from *C. vulgaris*. According to Aligiannis et al, plant materials showing MIC values up to 0.5 mg/mL are considered strong inhibitors, values of 0.6-1.5 mg/mL are moderate inhibitors, and vales above 1.6 mg/mL are weak inhibitors [18]. The results of this work showed that the MIC values of extracts of *C. vulgaris* were more satisfactory with the following solvents: water for *B. Subtilis* (0.7 mg/mL), DMSO for *S. faecalis* (0.55 mg/mL) and acetone for S. enteritidis (0.8 mg/mL). Guzman, Cat and Calejja (2001), when evaluating the anti-inflammatory activity of the methanolic extract of Chlorella stigmatophora, did not find significant results even at high concentrations of the extract, only in aqueous extracts. This result was similar to that found in the present work for antibacterial activity of *C. vulgaris* against all bacteria tested.

Table 3. Size of zone of inhibition (mm) (mean ± standard deviation) of *C. vulgaris* extracts (20 ☐L per disc) against different strains.

Extracts preparations	**S. fecalis**	**P.mirabilis**	**K.pneumoniae**	**S.enteritidis**	**B. subtilis**	**E. coli**
Ethanol	NI	NI	NI	NI	NI	NI
Methanol	NI	NI	NI	NI	NI	NI
Buthanol	NI	NI	NI	NI	NI	NI
Acetone	12 ±1	NI	NI	15 ±1	NI	NI
DMSO	15 ±1	NI	NI	NI	11 ±1	NI
Water	NI	11 ±1	11 ±1	12 ±1	15 ±1	10 ±1

NI: no inhibition; numbers represent the average diameter (in mm) ± SD of the inhibition zone (three replicates).

According to Ki-Bong et al., compounds from Odonthalia corymbifera showed potent antibacterial effect against *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Proteus vulgaris, and Salmonella typhimurium* [19]. Besides,

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the antifungal activity found to be the most active against *Candida albicans, Aspergillus fumigatus, Trichophyton rubrum and Trichophyton mentagrophytes.* Despite strong antibacterial activity, antifungal activity tests showed no effect for all extracts.

Table 4. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of different extracts from *C.*

Strains	Acetone		DMSO		Water	
	MIC	MBC	MIC	MBC	MIC	MBC
E. fecalis	1.05	>2.00	0.55	0.75	NT	NT
P.mirabilis	NT	NT	NT	NT	1.50	>2.00
K.pneumoniae	NT	NT	NT	NT	1.50	>2.00
S.enteritidis	0.80	0.95	NT	NT	1.00	1.50
B. subtilis	1.00	>2.00	1.00	>1.50	0.70	1.00
E. coli	NT	NT	NT	NT	1.00	>1.50

NT: Not Tested; Values of MIC and MBC in $mg.mL^{-1}$.

CONCLUSIONS

In conclusion, a series of extracts from *C. Vulgaris* has been prepared. The results for antioxidant activity obtained from different solvents demonstrated greater efficiency using aqueous and DMSO, and a more effective extraction of compounds with antioxidant activity than others. We conclude that the hydrophilic character of the solvent is related to these results. The antibacterial activity was also higher for the aqueous and DMSO extracts, including the acetone extract which has also demonstrated growth inhibition of some bacteria (*E. faecalis, S. enteritidis and B. subtilis*). The other extracts showed no inhibition against the tested bacteria. Microalgae did not demonstrate any inhibition of fungal growth. Our experiment demonstrates a process resulting in an efficienct isolation of an aqueous extract from microalgae using a more cost efficient protocol and avoiding the use of toxic residues found in other solvents.

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