Revue Agrobiologia
www.agrobiologia.net
ISSN (Print): 2170-1652
e-ISSN (Online): 2507-7627

EVALUATION OF ULTRASOUND AND PULSED ELECTRIC FIELD ASSISTED EXTRACTIONS ON THE BIOLOGICAL ACTIVITIES OF *CHLORELLA SPP*

ATTAL Fella-Sara[1,4*], KORD Afaf [2], BERROUANE Nour El Houda [1], BENLAHRECH Samia [3], BENCHABANE Ahmed [1] and AMIALI Malek [1]

1. Laboratory of Food Technologies and Human Nutrition, Agronomic Higher National School, El- Harrach (ENSA, ES1603), Algiers, Algeria
2. National Center for Research and Development of Fisheries and Aquaculture (CNRDPA), BP 11 Bou-Ismail 42415, Tipaza, Algeria
3.University of Sciences and Technology, Houari Boumediene (USTHB), Faculty of chemistry, BP 32 El-Alia Bab-Ezzouar 16111, Algiers, Algeria
4. Institute of Applied Sciences and Techniques, Saad Dahleb University, Blida 1, Blida, Algeria

Reçu le 11/07/2022, Révisé le 23/11/2022, Accepté le 03/12/2022

Abstract

Description of the subject: Ultrasound (US) and pulsed electric field (PEF) treatments are widely used to disrupt living cells to enhance the extraction of bioactive compounds
Objective : The aim of the present work was to compare them for processing fresh water green alga *Chlorella* spp. native to Lake El-Golea in southern Algeria and grown under controlled conditions in the laboratory
Methods : Intracellular contents soluble in acetone, chloroform, ethanol, methanol, and distilled water were extracted in conjunction with US or PEF or just soaking in the solvent. The extracts were screened for phytochemical content and tested for antimicrobial and antioxidant activities.
Results : They were found to contain bioactive molecules such as tannins, carotenoids, flavonoids, alkaloids, chlorophylls, phenols, glycosides and reducing sugars. They inhibited the growth of *Listeria innocua* ,*Staphylococcus aureus,* and *E. coli* to varying degrees depending on the extraction process. *Candida albicans, Saccharomyces cerevisiae,* and *Aspergillus niger* were all sensitive to the extract obtained using chloroform.
Conclusion : Extraction with ethanol or acetone gave the best biological activity results overall. PEF/ethanol and PEF/acetone extracts had the greatest antioxidant power. Pulsed electric field was the best enhancer of extraction of the biologically active substances, followed by ultrasound.
Keywords : *Chlorella* spp; ultrasound; pulsed electric field; bioactive compounds; biological activity.

ÉVALUATION DES ACTIVITÉS BIOLOGIQUES DES EXTRAITS DE *CHLORELLA SPP* OBTENUS PAR EXTRACTION ASSISTÉE AUX ULTRASONS ET CHAMPS ÉLECTRIQUE PULSÉ

Résumé

Description du sujet : Les Ultrasons (US) et le Champ Electric Pulsé (CEP) sont des techniques très utilisées pour fragiliser les membranes des cellules vivantes pour favoriser l'extraction des composés bioactifs.
Objectifs : L'objectif de ce présent travail a été de comparer ces techniques sur une microalgue d'eau douce *Chlorella* spp isolée dans le Sud Algérien au Lac El-Goléa et cultivée sous des conditions contrôlées.
Méthodes : Le contenu intracellulaire soluble dans l'acétone, chloroforme, éthanol, méthanol et l'eau distillée, est obtenu par Ultrasons, Champ Electric Pulsé ou par une simple macération dans les solvants. Les extraits ont été analysés pour leur contenu phytochimiques, leurs activités antimicrobiennes et antioxydantes.
Résultats : Le screening phytochimique a montré la présence de molécules bioactives telles que : tannins, carotenoides, flavonoides, alkaloides, chlorophylle, phénols, glycosides and sucres réducteurs. Ces dernières ont inhibé la croissance *Listeria innocua*, Staphylococcus *aureus*, et *E. coli* à des degrés différents selon la techniques d'extraction. Aussi, *Candida albicans, Saccharomyces cerevisiae*, et *Aspergillus niger* ont été sensibles aux extraits obtenus avec le chloroforme.
Conclusion : L'Extraction utilisant le méthanol, éthanol et acétone ont donné les meilleures activités. Les combinaisons CEP/éthanol and CEP/acétone ont obtenu les activités antioxydantes les plus intéressantes. Le Champ électrique Pulsé a représenté le meilleur pré-traitement pour l'extraction des molécules bioactives, suivi par les ultrasons.
Mots clés: *Chlorella* spp; ultrasound; pulsed electric field; bioactive compounds; biological activity.

*Auteur correspondant: ATTAL Fella-Sara, E-mail: attal.fs@univ-blida.dz

INTRODUCTION

Increasing consumer demand for natural food additives and preservatives is driving the search for suitable compounds in the lithosphere (soil and plants) and in the hydrosphere, particularly in fishes and algae. Algae and microalgae found in freshwater and seawater contain numerous bioactive compounds, including proteins, enzymes, free amino acids, lipids, carbohydrates, vitamins, minerals, phenolics, flavonoids and pigments with various biological activities [1-3]. Some algae are being proposed as functional foods or nutraceutical products [4, 5]. In addition to the widely reported macronutrient value of algae [6, 7, 8], antibiotic, antifungal, antiviral, antioxidant, anti-inflammatory and antitumor activities associated with the phenolic and flavonoid portions are also being alleged [9-11].

Chlorella is a green alga consisting of spherical, non-flagellated single cells 2–8 µm in diameter [12]. It occurs as individual cells, colonies, or extended filaments in freshwater and is viewed as an excellent source of bioactive compounds [13-16]. These have been extracted using conventional methods (e.g., Soxhlet and maceration) but so far not in satisfactory quantity or quality [17]. Two alternative technologies proposed to facilitate the extraction of nutraceutical compounds from *Chlorella* are ultrasound [18-20] and pulsed electric field [21-23]. These methods work primarily by weakening the rigid cell wall. Pretreatment of microalgal biomass possessing rigid cell wall like chlorella, is a critical step for enhancing the efficiency of microalgal extraction. Majority of the microalgae components are present either in the cytoplasm or inside the organelles. Due to the rigid cell walls of the microalgae protecting the organelles, intracellular molecules are hard to be released [24]. Because of that thick and strong cell walls, which acts as a barrier for compound extraction, restricting the entry of organic solvents into the cell and limiting extraction of intracellular compounds [25, 26] extraction of the target compound(s) is greatly improved if the cells are disrupted [27, 28].

Sonication is one of the most widely employed. Ultrasound is the energy generated by sound waves at frequencies above 16 kHz [24]. It disrupts cell membranes through cavitation effects, generating pressure, shear, and localized temperature gradients [19, 25-28].Sonication's ultrasonic waves form, inside cells, cavitation bubbles that subsequently collapse, generating shock waves that rupture cell walls then contents are released [29, 30, 19, 25, 31]. Plaza et *al*. [19], found similar functional and antimicrobial activities of the extracts obtained from microalgae after UAE in comparison with pressurized liquid extraction. Kong et *al*. [32], compared the effects of UAE (200 W/78.7 min/61.4 _C) and conventional heat extraction on chlorophyll recovery from C. vulgaris. They observed a significant yield increase (59 %) when they used UAE. On the other hand, Kwang et *al*. [33], reported a higher yield of chlorophyll a and b compared to maceration and soxhlet extraction, but lower compared to pressurized liquid extraction. In addition, Macı´as-Sa´nchez et *al*. [34] and Pasquet et *al*. [35], observed an increased extraction of chlorophylls a and b from *Dunaliella salina*, *Dunaliella tertiolecta*, and *Cylindrotheca closterium* when they applied UAE in comparison with conventional treatments and other nonconventional treatments such as supercritical fluid extraction and microwave-assisted extraction.

PEF is largely used technology that induce cell membranes permeabilization by "electroporation" phenomenon [29, 32]. Pulsed electric field treatment involves applying short pulses of current at electric field strength varying from 20 to 80 kV/cm. At the critical intensity, electroporation occurs, and increasing the number of pulses can disrupt the cell envelopes with minimal heating [29-32]. The electric field applied create pore formation and loss of cell membrane, that can be reversible or irreversible depending on the intensity of to the cells, semi-permeability [36, 23]. Several authors have studied the effect of PEF to extract different valuable compounds from microalgae. In a study conducted by Foltz [37], it was concluded that PEF treatment can be a useful technology to extract lipids from microalgae species (*Chlorella vulgaris*, *Chlamydomonas reinhardtii* and *Dunaliella salina*). Zbinden et *al*. [38], evaluated the use of PEF as a pre-treatment for lipid recovery from *Ankistrodesmus falcatus*. They observed a decrease in extraction time and an increase of 130 % in lipid extraction when PEF was used compared to control sample. They attributed this phenomenon to cell disruption that occurs when the algae are exposed to the PEF pretreatment.

Coustets et *al*. [21], also observed an increase in the extraction of total cytoplasmic proteins from microalgae (*Nannochloropsis salina* and *C. vulgaris*) when they applied PEF treatment. They observed that PEF induced the formation of pores in cell membrane thus favoring the cytoplasmic content compared to control sample. This fact was confirmed by the authors by means of microscopic observation. They observed a loss of contrast after pulsed delivery. The objective of this study was to compare the effectiveness of ultrasound and pulsed electric field on the extraction of bioactive compounds from *Chlorella* and to assess the antibacterial, antifungal, and antioxidant properties of the resulting phytochemical extracts, trying to reduce the time of extraction, and decrease the yield. In another hand, add some informations of the algal specie, so it can be used as a neutraceutical.

MATERIEL AND METHODS

1. Algal strain

Chlorella (species undetermined) native to Lake El-Golea, Menea, Algeria, was used throughout this study. It was freshly obtained from the CNRDPA, Bousmail, Tipaza, Algeria. It was grown in the laboratory under the conditions described below. The alga was grown in Bold Basal Medium [48] under cool-white fluorescent tubes at 4,500 Lux (60.75µmol m^{-2} s^{-1}) with a light/dark cycle regime of 8/16. Agitation and aeration were maintained by bubbling air using an aquarium pump. The pH and salinity were measured using a pH meter (WTW, 315i/SET, Germany) and a conductivity meter (WTW, 197i, Germany). Cell morphology, culture purity, and biomass concentration were observed daily using a Malassez cell with an optical microscope (OPTIKA DC7 5V, France) at magnifications of 10X and 40X. At the end of the exponential growth phase (3 weeks), algal cells were harvested by centrifugation at 4,500 rpm for 15 min using a SIGMA 2-15 centrifuge (Germany), washed twice with distilled water and then treated directly. The growth conditions are presented in Table 1. All the chemicals used for the medium were of analytical grade obtained from the Biochem Chemopharma (France).

Table 1: *Chlorella* growth conditions

Temperature (°C)	Humidity (%)	Luminosity (Klx)	pH	Conductivity (mS/cm)
27.54 ± 0.87	8.43 ± 1.77	3.98125 ± 0.42	8.806 ± 0.58	0.932 ± 0.23

2. Preparation of algal extracts

About 1g of fresh algal biomass was suspended in 10 mL of solvent (chloroform, acetone, ethanol, methanol, or distilled water) and adjusted to a concentration of 0.1% (w/v). These suspensions were then subjected to one of the treatments described below. All the chemicals used to prepare the extracts and for analysis were of analytical grade obtained from the Biochem Chemopharma (France).

2.1. Soaking

The biomass suspension kept in vials was allowed to stand for 18 h, and then homogenized and centrifuged at 4,500 rpm for 15 min. The supernatant (primary extract) was kept at 4°C in the dark until analysis. The pellet was resuspended in solvent and centrifuged. This was repeated until the pellet became colorless (in the case of water, the pellet remained green). These supernatants were pooled, concentrated in a Rotavapor R-210 (BüchiLabortechnik AG, Flawil, Switzerland), cooled, and analyzed.

2.2. Ultrasound-assisted extraction

A Wiseclean WUC-D06H sonicator was used. It comprises a simple bath with dimensions of 29 cm x 15 cm x 15.6 cm, capable of operating at a maximal frequency of 40 kHz. After several preliminary trials at 10 to 40 kHz for 5 to 30 min, 40 kHz for 20 min was chosen according to data obtained, (data not shown), 20minutes was the optimum extraction time for the pretreatment giving the maximum yields, after 20minutes, the yields decreased.. Fresh *Chlorella* suspended in solvent at 0.1% w/v was sonicated in volumes of 10 mL immerse in the water bath, the extraction was then carried out as described above, under controlled temperature (30°C) as a maximum reaching temperature.

2.3. Pulsed-electric-field-assisted extraction

Electrical conductivity is a crucial parameter to consider when using this technology, since it determines the extent of electro-permeabilization and electrofusion of cells.

If the conductivity is too high, the peak electric field generated will be low because of the greater electrical current [49]. All *Chlorella* samples were therefore standardized using the same procedure [50] in which 10 g of fresh biomass was washed twice with distilled water, suspended in 1,000 mL of sodium phosphate buffer (0. 025M, pH 5.8) to obtain an initial conductivity of 1.877 mS/cm.

The pulsed electric field treatment was carried out in a system that delivers exponentially decaying pulses. A variable autotransformer was used to supply power to a high-voltage transformer, the output voltage of which is rectified by a diode and used to charge a 20 nF capacitor. A gas spark switch was used to discharge the energy stored in the capacitor to the treatment chamber.

Chlorella in buffer was treated continuously in a 0.05 mL cofield treatment chamber at a flow rate of 0.4 mL/s. The gap between the electrodes was 0.1 cm and the electric field strength was 42.2±2.5kV/cm. The total pulse number *n* was calculated using equation 1. $\boldsymbol{n = (f \cdot V)/Q}$ **(1)** where *Q*, *f*, and *V* are, respectively, the flow rate (mL/s), pulse frequency (Hz), and treatment chamber volume (mL). The pulse frequency was maintained at about 400± Hz and a constant pulse duration (τ) of 28.3 µs was verified along with the wave form using a digital storage oscilloscope (SIGLENT, SDS 1102 CHL, China). The total treatment time (t) was 1,220 seconds, calculated using Equation 2. $\boldsymbol{t = n \cdot \tau}$ **(2)**

A Tektronix P60151000:1 high-voltage probe (Beaverton, OR, USA) and a current monitor (model 410, PEARSON Electronics Inc., PaloAlto, CA, USA) were used. The energy per pulse (W_{pulse}) [kJ] and the total specific energy input (W_{spec}) [KJ/kg] were calculated using the following equation (3) and (4):

$\boldsymbol{W_{pulse} = \frac{1}{2} \times V^2 \times C}$ **(3) ,**

$\boldsymbol{W_{spec} = \frac{f}{\dot{m}} \times W_{pulse}}$ **(4).** Where: $\boldsymbol{V^2}$: Voltage (V) ; $\boldsymbol{C}$: Capacitance (F), $\boldsymbol{f}$: Frequency (s^{-1}), $\dot{m}$: Mass flow rate ($kg.s^{-1}$). The total energy input was 11.43 kJ/Kg.

The experiments were performed in triplicate . All samples were centrifuged after pretreatment and washed twice with distilled water to eliminate buffer for solvent extraction as described above.

3. Phytochemical screening

The extracts were tested qualitatively to identify phytochemical constituents such as tannins, terpenes, carotenoids, steroids, saponins, flavonoids, alkaloids, glycosides and reducing sugars, according to standard procedures described elsewhere [51].

4. Determination of total phenols

The total phenolic content was determined previously [52]. An aliquot (50 µL) of each extract or standard solution was mixed with 1 mL of H_2O and 500 µL of Folin– Ciocalteu's phenol reagent. After that, 2.5 mL of 20% Na_2CO_3solutionwere added to the mixture, which was incubated at ambient temperature in the dark for 2 hours. The absorbance against a blank was measured at 735 nm ((JENWAY Genova Plus, UK)**.** Gallic acid was used to prepare a standard curve (0.05–0.3 mg/mL) using equation 5: $\mathbf{y = 0.008x + 0.084}$ **(5),** R^2= 0.957. The values were expressed as mg of gallic acid equivalents per g of dry extract.

5. Determination of total flavonoids

Total flavonoid content was determined as described previously [53]. An aliquot (250 µL) of each extract or standard solution was mixed with 1.25 mL of H_2O and 75 µL of 5% $NaNO_2$ solution. After 6 min, 150 µL of 10% $AlCl_3H_2O$ solution were added. 5 min later, 0.5 mL of 1 M NaOH solution was added and then the total volume was made up to 2.5 mL with H_2O. Following the thorough mixing of the solution, the absorbance against a blank was determined at 510 nm.Catechin(+) was used to plot the standard curve (0.05–0.5 mg/mL) using equation 6: $\mathbf{y = 0.001x + 0.03}$ (6), R^2 = 0.977). The values were expressed as mg of catechin equivalents per g of dry extract.

6. Determination of chlorophyll a and b and of total carotenoid contents

The absorbance of each extract at 470 nm, 653 nm, and 666 nm against the corresponding pure solvent (in a JENWAY (UK) Genova Plus spectrophotometer) was used to calculate the concentrations of respectively chlorophyll *a* and *b* and total carotenoids using equations 7,8 and 9 [54].

$\boldsymbol{Chla = 15.65 \times (A_{666}) - 7.34(A_{653})}$ **(7)**

$\boldsymbol{Chlb = 27.05 \times (A_{653}) - 11.21(A_{666})}$**.(8)**

$\mathbf{Car\ T = \frac{(1000x\,(A_{470}) - 2.86xChl\ a - 129.2xChl\ b)}{221}}$ **(9)**

7. Microbial susceptibility studies

7.1. Test microorganisms

The microbial strains were provided by the microbiology laboratory culture collection at Mhamed Bougara University in Boumerdes, Algeria.

These were: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Listeria innocua* ATCC 74915, *Bacillus subtilis* ATCC 6633, *Eschericia coli* ATCC 25922, *Saccaromyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 6275.

7.2. Disc diffusion method

Sterile paper discs (6 mm diameter) were wetted with extract (25 µL) or ampicillin solution as a control and dried in a laminar flow hood for 1 h. Pure solvent was used as a mock treatment. The discs were then placed on Muller-Hinton agar seeded with log-phase microbial suspension culture (10µL). The plates were incubated for 18 h at 37°C and the inhibition zone diameters were measured using calipers.

8. Antioxidant activity

8.1. Free-radical scavenging activity

The free-radical scavenging power of the extracts was assessed first by measuring the color loss in 1,1-diphenil-2-picrylhydazyl (DPPH) solution. According to Kitada et al, [15]. Briefly, 0.2 mL of extract was mixed with 0.4 mL of 0.05 mol L^{-1} DPPH in methanol. The reaction mixture was shaken vigorously and then kept at room temperature in the dark for 30 min. Absorbance at 517 nm was measured before (A_0) and after (A_t) mixing using a JENWAY Genova Plus spectrophotometer. Analytical grade methanol, ethanol, acetone, chloroform, and water were used as negative controls.BHT, quercetin, and catechin solutions (0.1 mg/ml) were used as positive controls. Equation 10 was used to calculate DPPH decolorizing.

$$\textbf{DPPH decolorized}\ (\%) = \left(\frac{(\mathbf{A0 - At})}{\mathbf{A0}}\right) \times \mathbf{100} \quad (10)$$

8.2. ABTS$^{\bullet+}$radical cation scavenging assay

A modified radical cation scavenging assay was used [55]. The 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)solution diluted 50-fold in96% ethanol (absorbance at 734 nm =0.65–0.75) was mixed with half volume of sample. After 15 minutes at room temperature, A_{734} was read. ABTS neutralization was calculated using equation 11.

$$\textbf{Neutralization(\%)} = \frac{\textbf{Initial absorbance – final absorbance}}{\textbf{Initial absorbance}} \times \mathbf{100} \quad (11)$$

The positive controls were 0.1 mg/ml BHT, quercetin, and catechin. Analytical grade methanol, ethanol, acetone, chloroform, and water were used as negative controls.

9. Statistical analysis

Values presented are means ± standard deviation of three determinations. Statistical analyses were performed using one-way analysis of variance and Tukey's test. Differences with a probability<0.05 were considered significant. All computations were carried out using statistical software R version 3.0.2 (R Core Team, 2013).

RESULTS

1. Phytochemical screening

The color of the *Chlorella* extracts was generally greenish to dark green, suggesting the presence of phytochemical compounds. The ability of the different solvents to extract tannins, terpenoids, carotenoids, steroids, saponins, flavonoids, alkaloids, glycosides and reducing sugars from the wet biomass is summarized in Table 2.

Table 2: Screening of *Chlorella* extracts based on phytochemical content

	Solvents				
Chemical group	**Water**	**Methanol**	**Ethanol**	**Acetone**	**Chloroform**
Tannins	+	+	+	+	+
Terpenes	-	-	-	-	-
Carotenoids	-	+	+	+	+
Steroids	-	-	-	-	-
Saponins	-	-	-	-	-
Flavonoids	+	+	+	+	+
Alkaloids	+	+	+	+	+
Glycosides	+	+	+	+	+
Reducing sugar	+	+	+	+	+

2. Determination of phenol content

Total phenolics extracted by soaking in the solvent without or with ultrasound or pulsed electric fields are shown in Figure 1. Water was a significantly less effective solvent, whereas the physical treatments had no significant effect on extraction. Pulsed electric field might have had some effect in methanol, but clearly none in water. It was expected to induce electroporation and thereby disrupt *Chlorella* cell walls and membranes and ultimately cause cell lysis [61], which could only facilitate penetration by the solvent and extraction of bioactive compounds soluble therein [23].

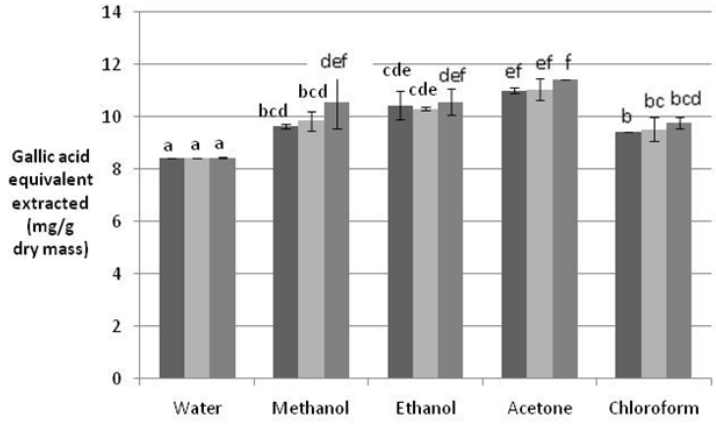

Figure 1: Phenolics extracted from Chlorella biomass by a single solvent combined witha physical treatment: soaking only (black),ultrasound (light grey), pulsed electric field (dark grey)

a, *b*, *c*, *d*, *e*, *f*, *g*, *h* – the same letters mean no statistical differences between samples ($p \leq 0.05$).

3. Determination of flavonoid content

In the case of flavonoids, the solvent and physical treatment had more conspicuous effects (Figure 2). Ethanol appeared to be the best solvent, and in acetone at least, physical treatment improved extraction. Chloroform appeared to be a poor solvent for flavonoids, although the extraction may have been improved slightly by physical treatment.

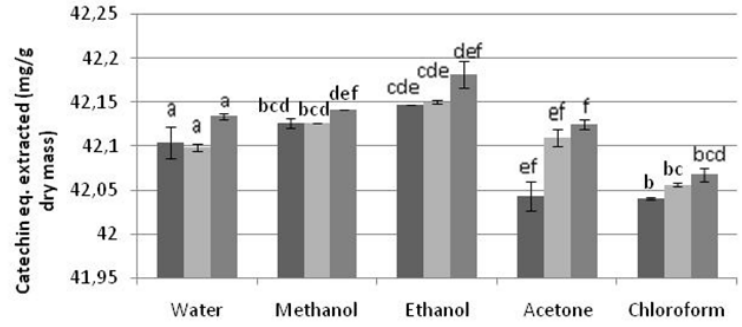

Figure 2: Flavonoids extracted from Chlorella biomass by a single solvent combined with a physical treatment: soaking only (black),ultrasound (light grey), pulsed electric field (dark grey)

a, *b*, *c*, *d*, *e*, *f*, *g*, *h* – the same letters mean no statistical differences between samples ($p \leq 0.05$).

4. Pigment content

4.1. Chlorophyll a and b

Chlorophyll is a highly useful bioactive compound that can be extracted from microalgal biomass. It is both a natural food coloring agent and an antioxidant [76]. Figures 3 and 4 show the total chlorophylls *a* and *b* extracted from *Chlorella*. The yield of both types of chlorophyll was generally increased when a physical treatment was applied, especially of type *b* with pulsed electric field. Electroporation thus could be enhancing the recovery of these pigments. Ethanol appeared to be the better solvent for chlorophyll *a*, while methanol and acetone were better for extracting chlorophyll *b*.

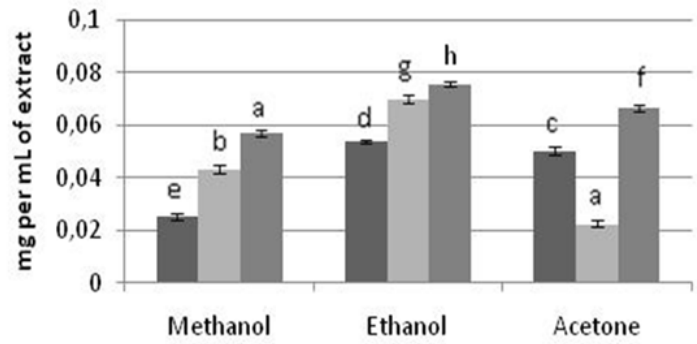

Figure 3: Chlorophyll a extracted from Chlorella biomass by a single solvent combined with a physical treatment: soaking only (black),ultrasound (light grey), pulsed electric field (dark grey)

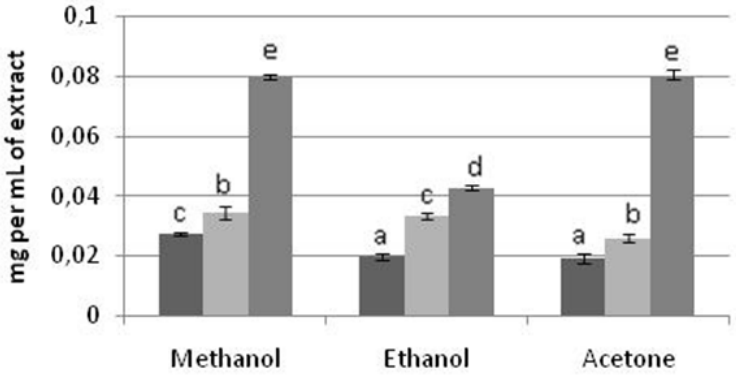

Figure 4: Chlorophyll b extracted from Chlorella biomass by a single solvent combined witha physical treatment: soaking only (black),ultrasound (light grey), pulsed electric field (dark grey)

4.2. Total Carotenoids

In recent decades, evidence has accumulated in support of a role for carotenoids as antioxidants with beneficial effects on human health, especially with regard to the prevention of chronic illnesses, particularly certain cancers, cardiovascular diseases and loss of visual acuity [82-84]. The amounts of carotenoids extracted from *Chlorella* are shown in Figure 5.

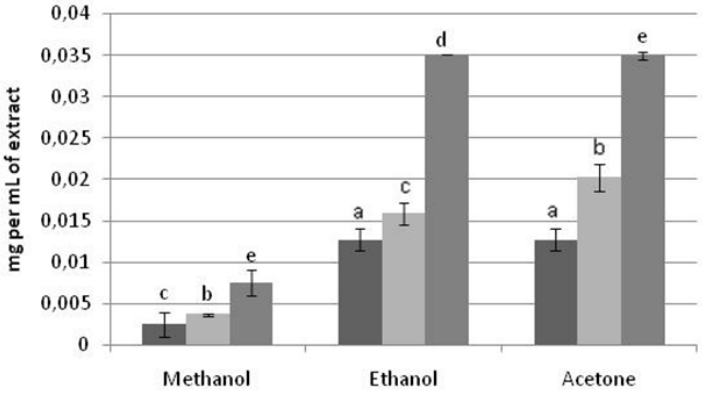

Figure 5: Total carotenoids extracted from Chlorella biomass by a single solvent combined with a physical treatment: soaking only (black),ultrasound (light grey), pulsed electric field (dark grey)

5. Antibacterial activity

The antibacterial activities of the *Chlorella* extracts are summarized in Table 3. The inhibition zone diameters obtained with *Staphylococcus aureus* were larger in association with the physical treatments (p <0.05). The effect of pulsed electric field was generally greater.

Table 3: Antibacterial activity of *Chlorella* extraction terms of inhibition zone diameter (mm) on agar

Microorganisms	Physical treatment	Solvents used for extractions					STD
		Water	Methanol	Ethanol	Acetone	Chloroform	
S. aureus	Soaking	6 ± 0^{a}	10.5 ± 0.7^{ab}	6 ± 0^{a}	6 ± 0^{ab}	6 ± 0^{a}	23.5 ± 0.7
	US	11.5 ± 0.54^{ab}	15.33 ± 0.57	20 ± 0	13 ± 0^{abc}	10 ± 0^{ab}	
	PEF	15.33 ± 0.57^{abc}	19.66 ± 0.57	24 ± 1.41	21.5 ± 0.71	24.66 ± 0.57	
E. coli	Soaking	6 ± 0^{a}	19.25 ± 0.95^{cd}	21 ± 0^{d}	17.66 ± 1.25^{c}	6 ± 0^{a}	20.2 ± 0.57
	US	11.5 ± 0.54^{b}	19 ± 1^{cd}	20.25 ± 0.5^{d}	19 ± 1.09^{cd}	6 ± 0^{a}	
	PEF	11 ± 0^{b}	20.2 ± 0.44^{cd}	20 ± 1.41^{cd}	22.17^{d}	6 ± 0^{a}	
P. aeruginosa	Soaking	9.5 ± 0.7^{a}	16.5 ± 0.57^{def}	15.33 ± 0.57^{cdef}	13 ± 0^{bcde}	11.66 ± 057^{abc}	9 ± 0
	US	10.5 ± 0.7^{ab}	16 ± 1^{efg}	17.5 ± 0.7^{efg}	12.33 ± 0.57^{abcd}	8.5 ± 07^{a}	
	PEF	11 ± 0^{ab}	19.66 ± 0.57^{gh}	20.5 ± 0.57^{fgh}	16.33 ± 057^{defg}	22 ± 0^{h}	
L.innocua	Soaking	6.75 ± 0.95^{a}	11.33 ± 0.57	10.75 ± 0.5^{bc}	10.2 ± 0.44^{b}	30 ± 0^{f}	20.5 ± 0.7
	US	7.8 ± 0.44^{a}	11 ± 0.81^{bcd}	11.25 ± 0.95^{bcd}	12.5 ± 0.7^{bcde}	36 ± 0^{g}	
	PEF	13.75 ± 0.95^{e}	13.66 ± 0.57^{de}	11.5 ± 0.57^{bcde}	12.66 ± 0.57^{cde}	40 ± 0^{h}	
; *B. cereus*	Soaking	6 ± 0^{a}	16 ± 0^{bc}	12.66 ± 0.57^{b}	15.5 ± 0.7^{cd}	15.5 ± 07^{bc}	23 ± 0
	US	8 ± 1^{a}	17 ± 0^{cde}	20.5 ± 0.7^{efg}	18 ± 0^{cde}	19 ± 1.41^{cdef}	
	PEF	8 ± 1^{a}	20 ± 0^{efg}	23 ± 1.41^{g}	20 ± 0^{defg}	22 ± 0^{fg}	

US : ultrasound; PEF :pulsed electric field; STD: Standard Error, *a, b, c, d, e, f, g, h* – the same letters mean no statistical differences between samples ($p \leq 0.05$).

The associated solvent effect ranked chloroform and ethanol (24mm) followed by acetone (21.5mm)and methanol (15.33mm). In conjunction with ultrasound, the ethanol extract gave the inhibition zone up to 20mm in diameter. These results suggest that medium-to-high polarity solvents are better for extracting antibacterial compounds from microalgae. The inhibition zone associated with aqueous extract was comparatively small. For *Escherichia coli*, the inhibition zone diameters ranged from 6 mm to 22 mm and the largest zones were obtained for ethanol/ultrasound, soaking/ethanol and PEF/acetone extracts. Chloroform appears to have extracted, independently of physical treatment, some substance that is an effective inhibitor of *Listeria innocua*, while chloroform/PEF extract inhibited *Pseudomonas aeruginosa* and ethanol/PEF extract inhibited *Bacillus cereus* to greater degrees than any others did.

6. Anti-fungal activity

Inhibition of the growth of *Candida albicans*, *Saccaromyces cerevesiae*, and *Aspergillus niger* on agar by *Chlorella* extract is summarized in Table 4. A strong fungal inhibitor appears to have been extracted by chloroform, whereas water apparently did not extract any antifungal substance. Methanol and ethanol enhanced by PEF extracted a relatively strong inhibitor of *Candida albicans*. In the case of *Saccaromyces cerevesiae*, the physical enhancer did not have a significant effect on the resulting inhibitory activity of the extract. Ethanol and acetone appeared to be better than methanol for extracting inhibitors of this yeast. For *Aspergillus niger*, the strongest inhibitor was obtained using acetone in conjunction with PEF.

Table 4: Antifungal activity of *Chlorella* extractsin terms of inhibition zone diameter (mm) on agar

Microorganisms	Physical treatment	Solvents used for extractions					STD
		Water	Methanol	Ethanol	Acetone	Chloroform	
C. albicans	Soaking	6 ± 0^{ab}	20.2 ± 0.44^{cd}	25.5 ± 0.7^{cd}	20.33 ± 1.03^{c}	No growth	21.5 ± 0.7
	US	8 ± 0^{ab}	21.66 ± 1.15^{c}	22.33 ± 1.15^{c}	20.25 ± 0.5^{b}	No growth	
	PEF	8 ± 0^{ab}	29 ± 0^{cd}	29 ± 0^{d}	23.33 ± 0.57^{cd}	No growth	
S. cerevisiae	Soaking	6 ± 0^{b}	20 ± 1^{c}	26 ± 1.41^{d}	23.25 ± 0.5^{cd}	No growth	20.5 ± 0.7
	US	8 ± 0^{b}	20.5 ± 0.57^{c}	25.5 ± 0.7^{d}	23.25 ± 0.57^{cd}	No growth	
	PEF	8 ± 0^{b}	23.6 ± 1.5^{c}	26.5 ± 0.7^{d}	24.75 ± 0.95^{cd}	No growth	
A.niger	Soaking	8 ± 0^{a}	19 ± 1.15^{de}	21 ± 1.41^{de}	12 ± 0^{c}	No growth	20.66 ± 0.57
	US	8 ± 0^{a}	18.5 ± 1^{d}	18.66 ± 1.15^{d}	13 ± 0^{c}	No growth	
	PEF	8 ± 0^{a}	21 ± 0^{de}	14 ± 0^{c}	24.75 ± 0.95^{f}	No growth	

US : ultrasound; PEF :pulsed electric field; STD: Standard Error, *a, b, c, d, e, f, g, h* – the same letters mean no statistical differences between samples ($p \leq 0.05$).

7. Antioxidant activity

In general, the extracts of *Chlorella* biomass subjected to ultrasound or pulsed electric field treatment had considerable antioxidant activity compared to those obtained without such treatment ($p<0.05$). Ethanol and acetone extracts enhanced by PEF were the most potent (Table 5).

Table 5: Antioxydant activity expressed in % of inhibition

	DPPH			ABTS		
	Soaking	USAE	PEFAE	Soaking	USAE	PEFAE
Water	35,85±1,56	44,06±0,66	67,02±0,67	44,44±0	44,72±1,02	52,92±1,3
Methanol	38,70±0,29	33,33±0	48,26±0,01	37,29±0,29	48,35±0,75	50,65±0,92
Ethanol	41,46±0,39	66,43±0,32	**84,41±0,28**	47,32±1,31	71,44±0,02	80,60±0,34
Acetone	78,01±0,9	79,45±0	82,01±0,83	91,37±0,81	95,41±0,88	**96,95±1,29**
Chloroform	6,59±0,1	8,09±1,4	10,00±1,37	6,59±0,2	8,07±1,6	10,35±0,7
Quercetin	26,44±1,4			43,49±1,33		
Catechin	29,66±0,03			57,27±1,22		
BHT	47,23±0,02			37,61±1,13		

DISCUSSION

For the phytochemical screnning, water could not extract carotenoids, terpenoids or steroids, and saponins appeared to absent in all extracts. Similar results have been reported previously, except that only glycosides were not extracted [56]. In other studies, saponins and sterols were extracted from *Chlorella vulgaris*, whereas tannins were not [57, 11]; saponins, flavonoids, and alkaloids were extracted from *Chlorella* spp. [36] and tannins and alkaloids were not [5]. The presence or absence of phytochemicals in the extracts also depends on solvent polarity, pH and extraction time, temperature, and method. The phytochemical content of the algal biomass might also vary with growth conditions and growth phase [50, 5, 52].

The results of phenolic content obtained corroborate the values reported previously: 25mg [62], 39.4 mg [63], 29.1 mg [64], and 19.15mg [65] of gallic acid equivalents per gram of *Chlorella* in ethanol. In aqueous extracts of *Chlorella vulgaris*, phenolic contents range from 3.45 mg/g [52] to 108.66 mg/g [66], whereas methanol extract 220 mg of gallic acid equivalent per g of *Chlorella vulgaris* and 150 mg of *Chlorella reinhardtii* [67]. Total phenolic content may differ among algal species, for example, from 22.94 to 39.34 mg (gallic acid equivalent) per g of *Nannochloropsis gaditana, Phaedactylum tricornutum, Nannochloris* spp. and *Tetraselmis suecica* [68], 11.15 mg per g of *Euglena tuba* [69] and 0.287 mg per g of *Spirulina platensis* [70]. Although the concentration of antioxidant compounds varies among algal species, the amount extracted depends largely on the solvent type and extraction method [71]

Flavonoid concentrations close to the range obtained (up to 33 mg of catechin equivalent per g) have been reported for extracts of *Chlorella vulgaris* [62,76]. Smaller amounts have been extracted from *Amphora* spp. using ethanol (17.69 mg/g) or water (4.27 mg/g) in another study.

Since flavonoids are polyphenolic compounds, they are potential antioxidants and free radical scavengers [73, 74]. Different flavonoid classes such as isoflavones, flavanones, and flavonols are found in microalgae [75], showing that complex secondary metabolism is not limited to land plants [5].

In other work which studied pigment content, two-phase solvent extraction (organic/aqueous) in conjunction with pulsed electric field treatment allowed the recovery of pigments from *Nannochloropsis* spp. in high yield without degrading proteins [77]. Pulsed electric field treatment is widely reported to improve the extraction of pigments from microalgae suspensions [78-80]. This is attributed to the enhancement of solvent permeation by electroporation [77].

An increase in the electrical conductivity (ionic strength) of the suspension medium leads to faster permeabilization of the cells. Indeed, conductivity increases the electrical current flow through the suspension and therefore the amount of energy applied to the cells [81].

For the carotenoids: In this case, pulsed electric field treatment improved the extraction considerably regardless of the solvent used, and it is noted that methanol was a poor solvent in comparison with ethanol or acetone. Similar patterns have been reported previously [85] for carotenoids (0.057 to 0.062 mg/g) extracted from *Chlorella protothecoides*. In another study, acetone was found superior to ethanol for extraction from *Chlorella vulgaris* [19].

Several studies have shown that pulsed electric fields can increase the yields of other valuable compounds such as colorants without resorting to the use of organic solvents. The cell membrane permeabilization afforded by this treatment undoubtedly facilitates the permeation of the eluting solvent into the cytoplasm and hence dissolving of pigments [23]. We found pulsed electric field treatment to be the most effective method of enhancing carotenoid extraction, which corroborates several previous studies. Extraction of chlorophyll and carotenoids from *Spirulina* and *Chlorella* may thus be improved [86], by up to 200% in conjunction with ethanol and4-fold in the case of lutein [23].

Antibacterial activity: Previous reports of the inhibitory effects of *Chlorella* extracts against these bacterial species mention inhibition zone diameters in the same range, namely, 8 to 38 mm [11, 87, 88, 5].

The compounds that are likely responsible for the direct effects on bacterial growth are believed to be phenolics, polyphenols, fatty acids, lipids, pigments, and carbohydrates [89, 12]. In addition, flavonoids, triterpenoids, amides, and alkaloids are also thought to affect bacterial growth and metabolism [90, 91].

Both chlorophylls *a* and *b* and carotenoids have been reported to inhibit bacteria [92,93]. The results of the present study show clearly that both physical treatments enhanced the extraction of antibacterial substances from *Chlorella*.

Cell wall structure and composition influences the effectiveness of the cell disruption method [94] and hence the extraction of all compounds from microalgal cells. Cell walls of green microalgae can be divided into two groups: low and high resistance. *Chlorella* cell walls are of the latter type [95]. Sonication is suitable for species with less resistant cell walls [96]. In the present study, PEF appears to have been more effective than sonication at disrupting *Chlorella* cells. In addition, mass transfer is increased by heat at the expense of degrading valuable compounds and denaturing proteins. Innovative technologies such as high hydrostatic pressure (HHP), ultrasonics, and PEF have been devised to improve mass transfer while avoiding these undesired thermal effects [97]. It therefore comes as no surprise that the physical treatment applied affects the antibacterial activity of microalgae extracts to a considerable degree, leading to different responses according to the bacterial strain.

For the antifungal activity; *Chlorella* extract has been found previously to be a strong inhibitor of *Candida albicans*, with zone diameters similar to our results [66].*Chlorella vulgaris* extracts have been found to inhibit *A. niger* and *C. albicans* [90]. In one study, antifungal activity was found only in methanolic extracts [16], whereas another study [98] concluded that methanol extract of *Chlorella* did not inhibit *Aspergillus fumigatus* (RCMB 02564),*Candida albicans* (RCMB 05035), *Geotricum candidum* (RCMB 05096) or *Trichophyton mentagrophytes* (RCMB0925). These contradictory results may be due to differences in algal species. We obtained strong inhibitory effects, compared to inhibition zone diameters of less than 6 mm observed previously for *Chlorella vulgaris* extract against *Candida albicans* and *Aspergillus niger* [11].

Several researchers have studied the inhibition of fungal growth by extracts of microalgae, including of at least one pathogen [99, 100, 98, 16, 101]. The bioactive compounds in these extracts may collectively be antibacterial, antifungal, antiviral, and antioxidant [102].

From these results, it can be concluded again that pulsed electric field was the most effective treatment for enhancing solvent extraction of antimicrobial substances from *Chlorella*.

The results obtained for the antioxydant activity are in agreement with a recent report [62] in which DPPH decolorizing reached 85.62% with *Chlorella vulgaris* extract. However, values for *Chlorella* spp. had been found previously to range from 78% for acetone to only 18%, 10%, and 3%, respectively, for ethanol, chloroform, and methanol [51]. ADPPH decolorizing of 50% has been reported recently for sonicated *Chlorella* spp. aqueous methanol extract [103]. The antioxidant activity of *Chlorella vulgaris* has been found to reach 68.5% in aqueous extract [52] and as low as 0.74% in ultrasound-assisted ethanol extract [4]. This variability is attributable to algal species, to the chosen solvent, and to the extraction method. In addition, the strong activities found in the present study may be due also to the concentrations of phenols and flavonoids in the extracts [104, 66]. Their presence could contribute to free radical scavenging individually or by synergistic action [105].

The results obtained in this study show overall that solvent extraction assisted by ultrasound or pulsed electric field, particularly the latter, increases the yield of bioactive compounds from microalgal wet biomass. Several researchers have investigated both physical treatments as a means of increasing the yield of valuable compounds such as antioxidants from algae [105, 40, 105, 22, 103].

CONCLUSION

The present study confirms that the green alga *Chlorella* spp. is very rich in compounds that have potential uses in pharmacology and in the nutraceutical field. These compounds include inhibitors of microorganisms and antioxidants. Solvent extraction of bioactive molecules from *Chlorella* is enhanced by physical treatments such as ultrasound and pulsed electric fields. Acetone and ethanol are suitable solvents for obtaining extracts that give high scores in different biological activity tests. Nevertheless, ethanol would be the most appropriate solvent since it also has generally recognized as safe (GRAS) status. Future work should focus on identifying the algal compounds that are directly responsible for the antimicrobial and antioxidant activities.

References

[1] . **Wijesekara I. Kim SK.,** (2010). Angiotensin-I-converting enzyme (ACE) inhibitors from marine resources: Prospects in the pharmaceutical industry. Marine Drugs :8:1080-1093.

[2] . **Uma R., Sivasubramanian V. , Niranjali Devaraj S**., (2011). Evaluation of in vitro antioxidant activities and antiproliferative activity of green microalgae, Desmococcus olivaceous and Chlorococcum humicola. Journal of algal biomass utilisation..2 (3): 82– 93

[3] . **Manchu N., Melpha Y., Jeeva S., Edwin James J**.. (2015). Phytochemical analysis and antibacterial activity of chaetomorpha antennina from rasthacaud coast, tamil nadu, india.world Journal of Pharmaceutical Research. Volume 4, Issue 3, 1337-1351.

[4] . **Wang H,** et al. (2010). Identification of anti-lung cancer extract from Chlorella vulgaris C-C by antioxidant property using supercritical carbon dioxide extraction. Proc Biochem. 45:1865–1872

[5] .**Annamalai J. , Nallamuthu T.** (2014). Antioxidant potential phytochemicals from methanol extract of Chlorella vulgaris and Chlamydomonas reinhardtii. Journal of algal biomass utilisation. 5 (4). 60-67.

[6] . **Brown M.R. and Jeffrey S.W..** (1992) .Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. J. Exp. Mar. Biol. Ecol. 161: 91-113.

[7] . **Fuentes M.M.R., Fernandez G.G.A., Perez J.A.S. and Guerrero J.L.G** (2000) Biomass nutrient profiles of the microalga Porphyridium cruentum. Food Chem. 70: 345- 353.

[8] . **González del Val A, Platas G, Basilio A, Cabello A, Gorrochategui J, Suay I, et al.** (2001) Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain). Int Microbiol; 4: 35-40.

[9] . **Diker KS, Akan M, Hascelik G, Yurdakök M**. (1991) The bactericidal activity of tea against Campylobacter jejuni and Campylobacter coli. Lett Appl Microbiol ; 12: 34-5.

[10] .**Salvador N., Garreta A.G., Lavelli L. and Ribera M**.. (2007). Antimcrobial activity of Iberian macro algae. Sci. Mar. 71: 101-113.

[11] . **Adhoni S.A., Thimmappa S.C,. Kaliwal B.B**. . (2016), Phytochemical analysis and antimicrobial activity of Chorella vulgaris isolated from Unkal Lake. Journal of Coastal Life Medicine4(5): 368-373

[12] **. Pradhan J., Das S., Das B.K.** Antibacterial activity of fresh water microalgae: A review. African Journal of Pharmacy and Pharmacology. 2014.Vol 8 (32).p.p. 809-819.

[13] . **Guzma´n S., Gato A., and Calleja J. M**.,. (2001) Antiinflammatory, analgesic and free radical scavenging activities of the marine microalgae Chlorella stigmatophora and Phaeodactylum tricornutum. Phytotherapy research.. 15, 224–230 .

[14] . **Vijayavel K, et al**. (2007) Antioxidant effect of the marine algae Chlorella vulgaris against naphthalene-induced oxidative stress in the albino rats. Molecular Cellular Biochemistry.;303:39–44.

[15] . **Kitada K. et al,** . (2009) Antioxidant and Antibacterial Activity of Nutraceutical Compounds from Chlorella vulgaris Extracted in Hydrothermal Condition. Separation Science and Technology.44: 1228–1239

[16] .**Amaro H. M., Guedes A. C., Malcata F. X.** (2011) Antimicrobial activities of microalgae: an invited review. Science against microbial pathogens: communicating current research and technological advances..A. Mendez vilas ed

[17] . **Grosso C., Valentão P., Ferreres F., Andrade P. B**. (2015) Alternative and Efficient Extraction Methods for Marine-Derived Compounds. Marine . Drugs, 13, 3182-3230;

[18] . **Ruen-ngam D., Shotipruk A., Pavasant P,**. (2011) Comparison of extraction methods for recovery of astaxanthin from Haematococcus pluvialis. Sep Science Technology. 46:64–707

[19] . **Plaza M., Santoyo S., Jaime L. et al,.** (2012) Comprehensive characterization of the functional activities of pressurized liquid and ultrasound-assisted extracts from Chlorella vulgaris. LWT Food Science Technology.46:245–253

[20] **.Zou T-B, Jia Q, Li H-W et al** . (2013) Response surface methodology for ultrasound-assisted extraction of astaxanthin from Haemotococcus pluvialis. Mar Drugs 11:1644–1655.

[21] .**Coustets M., Tiessié J..** (2013) the use of pulsed electric fields for protein extraction from nanochloropsis and chlorella. IFMBE Proceedings.Vol. 53. p.p. 405-408.

[22] .**Grimi N, Dubois A, Marchal L et al** (2014) .Selective extraction from microalgae Nannochloropsis sp. using different methods of cell disruption. Bioresource Technologie ..153:254 259

[23] . **Luengo E., Martínez J. M. , Bordetas A. , Álvarez I., Raso J.,** (2015) Influence of the treatment medium temperature on lutein extraction assisted by pulsed electric field.

[24] . **Buchmann, L., Brändle, I., Haberkorn, I., Hiestand, M., Mathys, A.,** (2019). Pulsed electric field based cyclic protein extraction of microalgae towards closed-loop biorefinery concepts. Bioresour. Technol. 291, 121870.https://doi.org/10.1016/J.BIORTECH.121870 .

[25] **. Bernaerts, T.M.M., Gheysen, L., Kyomugasho, C., Kermani, Z.J., Vandionant, S., Foubert, I., Hendrickx, M.E., Van Loey, A.M.,** (2018). Comparison of microalgal biomasses as functional food ingredients: focus on the composition of cell wall related polysaccharides. Algal Res. 32, 150-161. https://doi.org/10.1016/j.algal.2018.03.017.

[26] . **Kim, D-Y., Vijayan, D., Praveenkumar, R., Han, J-I., Lee, K., Park, J-Y., Chang, W-S., Lee, J-S., Oh, Y-K.,** (2016). Cell-wall disruption and lipid/astaxanthin extraction from microalgae: *Chlorella* and *Haematococcus*. Bioresour. Technol. 199, 300-310.http://dx.doi.org/10.1016/j.biortech.2015.08.107

[27] . **Lee, A.K., Lewis, D.M., Ashman, P.J.,** (2012). Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements. Biomass Bioenergy 46, 89-101. http://dx.doi.org/10.1016/j.bionbioe.2012.06.03

[28] . **Show, P.L., Tang, M.S.Y., Nagarajan, D., Ling, T.C., OOi, C-W., Chang, J-S.,** (2017). A holistic approach to managing microalgae for biofuel applications. Int. J. Mol. Sci. 18, 215. Doi:10.3390/ijms18010215.

[29] . **Geciova J, Bury D, Jelen P**. (2002). Methods for disruption of microbial cells for potential use in the dairy industry-a review. Int Dairy J 2002;12:541e53

[30] .**Ranjan A, Patil C, Moholkar VS** (2010) Mechanistic assessment of microalgal lipid extraction. Ind Eng Chem Res 49:2979–2985

[31] . **Cesaro A, Velten S, Belgiorno V, Kuchta K.** (2014). Enhanced anaerobic digestion by ultrasonic pretreatment of organic residues for energy production. J Clean Prod 2014;74:119e24

[32] .**Kong W, Liu N, Zhang J et al** (2014) Optimization of ultrasound- assisted extraction parameters of chlorophyll from Chlorella vulgaris residue after lipid separation using response surface methodology. J Food Sci Technol 51(9):2006–2013

[33] . **Kwang HC, Lee HJ, Koo SY et al** (2010) Optimization of pressurized liquid extraction of carotenoids and chlorophylls from Chlorella vulgaris. J Agric Food Chem 58:793–797

[34] . **Macı´as-Sa´nchez MD, Mantell C, Rodrı´guez M et al** (2009) Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from Dunaliella salina. Talanta 77:948–952

[35] . **Pasquet V, Che´rouvrier J-R, Farhat F et al** (2011) Study on the microalgal pigments extraction process: performance of microwave assisted extraction. Process Biochem 46:59–67

[36] **. Zimmermann, U**. (1986). Electrical breakdown, electropermeabilization electrofusion. *Reviews of Physiology Biochemistry and Pharmacology, 105*, 176−257.

[37] .**Foltz G** (2012) Algae lysis with pulsed electric fields. California Polytechnic State University, San Luis Obispo

[38] . **Zbinden MDA, Sturm BSM, Nord RD et al** (2013) Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae. Biotechnol Bioeng 110:1605–1615

[39] . **Jayasooriya et al.** (2004). Effect of high power ultrasoundwaves on properties of meat: a review. International Journal of Food Properties. 7, 2, 301-319

[40] . **Barba F.J., Grimi N., Vorobiev E**.. (2014). New Approaches for the Use of Non-conventional Cell Disruption Technologies to Extract Potential Food Additives and Nutraceuticals from Microalgae. Food Eng Rev. 7: p.p.45–62

[41] . **Silve A., Kian C. , Papachristou I., Kubisch C., Nazarova N., Wustner R., Leber K., Strassner R., Frey W.** (2018).,. Incubation time after pulsed electric field treatment of microalgae enhances the efficiency of extraction processes and enables the reduction of specific treatment energy. Bioresource Technology 269. p.p.179-187

[42] .**Scherer D., Damaris Krust D., Frey W., Mueller G., Nick P. , Gusbeth C**., (2019). Pulsed electric field (PEF)-assisted protein recovery from Chlorella vulgaris is mediated by an enzymatic process after cell death. Algal Research..41.101536

[43] . **Chittapun S., Jonjaroen V., Khumrangsee K., Charoenrat T.**. (2020). C-phycocyanin extraction from two freshwater cyanobacteria by freeze thaw and pulsed electric field techniques to improve extraction efficiency and purity. Algal Research. 46. 101789 electric fields from Chlorella vulgaris. Innovative Food Science and Emerging Technologies.

[44] .**Asavasanti et al**. (2011).. Permeabilization of plant tissues by monopolar pulsed electric fields: effect of frequency. Journal of Food Science, 76, p.p.98–11

[45] . **Amiali M. and Ngadi M.O**. (2012) Microbial decontamination of food by Pulsed Electric Fields (PEFs), in Microbial decontamination in food industry: Novel Methods and Applications, Chap. 14, pp 407- 449, Woodhead Publishing Ltd. U.K.

[46] .**De Boer K.,. Moheimani N. R,. Borowitzka M. A, and Bahri P. A**., (2012). Extraction and conversion pathways for microalgae to biodiesel: A review focused on energy consumption, J. Appl. Phycol., vol. 24, no. 6, pp. 1681–1698

[47] .**Bot F., Verkerk R., Mastwijk H., Anese M., Fogliano V., Capuano E.** (2018)The effect of pulsed electric fields on carotenoids bioaccessibility: the role of tomato matrix. Food Chemistry.

[48] . **Connon R**. (2007) Culturing of Chlorella vulgaris - Standard Operating Procedure. Daphnia.

[49] .**Amiali M; Ngadi M.O ;Raghavan V.G.S; Nguyen D-H.** (2006). Electrical Conductivities of Liquid Egg Products and Fruit Juices Exposed toHigh Pulsed Electric Fields. Int. J. Food Properties. 9: 533–540.

[50] . **Aouir A., , Amiali M, Kirilova-Gachovska T, Benchabane A and Bitam A.** (2015)The Effect of Pulsed Electric Field (PEF) and Ultrasoud (US) Technologies on the Extraction of Phycopiliproteins from *Arthrospira Platensis.* IEEE Canada International Humanitarian Technology Conference (IHTC)

[51] . **Rajendran N., Karpanai Selvan K., Piriya S., Logeswari V., Kathiresan E., Tamilselvi A., Vennison J.** (2014). Phytochemicals, antimicrobial and antioxidant screening from five different marine microalgae. Journal of Chemical and Pharmaceutical Sciences ISSN: 0974-2115

[52] . **Dantas D.M.M, Costa R., Carneiro-da-Cunha M.G., Galvez A. O., Drummond A. R., Bezerra R.S..** (2015). Bioproduction, Antimicrobial and Antioxidant Activities of Compounds from Chlorella vulgaris. Research & Reviews: Journal of Botanical Sciences. Volume 4 | Issue 2 |

[53] . **Zhishen J., Mengcheng T., Jianming W.,** (1999)**..** The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food chemistry. Vol 64. Issue

[54] .**Wellburn A.R**. .(1994). The Spectral Determination of Chlorophylls a and b, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. Journal of Plant PhysiologyVolume 144, Issue 3, , Pages 307-313

[55] .**Re R., Pellegrini N., Proteggente A., Pannala A.,.Yang M, Rice-Evans C.** (1997) Antioxidant Activity Applying An Improved Abts Radical Cation Decolorization Assay. Free Radical Biology & Medicine, Vol. 26, Nos. 9/10, Pp. 1231–1237,

[56] .**Geetha BV, Navasakthi R, Padmini E**.(2010) Investigation of Antioxidant Capacity and Phytochemical Composition of Sun Chlorella , An Invitro Study. J Aquac Res Development.; 1:104

[57] . **Anchang K.A., Lewi D., Nji C..** (2016). Toxicological, phytochemical, and antibacterial assessment of Chlorella vulgaris and Spirulina platensis powder in albino rats. a preliminary study. Revista peruana de medicina integrativa. 1(3):5-117

[58] . **Drummond A. R., . Bezerra R.S**., (2015). Bioproduction, Antimicrobial and Antioxidant Activities of Compounds from Chlorella vulgaris. Research & Reviews: Journal of Botanical Sciences. Volume 4 | Issue 2 |

[59] . **Wustner R., Leber K., Strassner R., Frey W.,** (2018). Incubation time after pulsed electric field treatment of microalgae enhances the efficiency of extraction processes and enables the reduction of specific treatment energy. Bioresource Technology. 269. p.p.179-187

[60] **. Ekpo MA, Etim PC**. (2009). Antimicrobial activity of ethanolic and aqueous extracts of Sida acuta on microorganisms from skin infections.. Journal of Medicinal Plants Research; 3(9), 621-624.

[61] . **Qin S, Timoshkin I V, Maclean M, Wilson M P, MacGregor S J, Given M J, Anderson J G and Wang T.,**. (2014).Pulsed electric field treatment of microalgae: Inactivation tendencies and energy consumption IEEE Transactions on Plasma Science. 1-6

[62] . **El-fayoumy E.A., Shanab S.M. M., Shalaby E.A**.(2020) Metabolomics and Biological activities of Chlorella vulgaris grown under modified growth medium (BG11) composition. Journal of Natural Sciences.vol 19.1.

[63] . **El makawy A.I., Abdel-Aziem S. H., Ibrahim F. M., Sharaf H. A., O. M. Abd-Elmoneim, A.M Darwish,**. (2016) Potential Modulator Role of Chlorella vulgaris and Spirulina platensis On Monosodium Glutamate Oxidative Stress, Genotoxicity, Apoptotic Gene Expression and Histopathological Alterations. International Journal of PharmTech Research .. Vol.9, No.11, pp 161-177,

[64] . **Choochote W. , Suklampoo L. , Ochaikul D..** (2013) Evaluation of antioxidant capacities of green microalgae. Journal of applied phycology,

[65] . **Hajimahmoodi M., Faramarzi M.A., Mohammadi N., Soltani N., .Oveisi M.R., Nafissi-Varcheh N.,.** (2010) Evaluation of antioxidant properties and total phenolic

[66] . **Ahmed E.A.** (2016). Antimicrobial Activity of Microalgal Extracts Isolated From Baharia Oasis, Egypt. Global. Advanced. Research. Journal of . Microbiology. Vol. 5(3) pp.. 033-041,

[67] .**Jayshree A., Jayashree S., Thangaraju N.,.** (2016). Chlorella vulgaris and Chlamydomonas reinhardtii: Effective Antioxidant, Antibacterial and Anticancer Mediators. Indian Journal of Pharmaceutical Sciences. 8(5):575-581

[68] . **Haoujar I. et al.,** (2019) The Contribution of Carotenoids, Phenolic Compounds, and Flavonoids to the Antioxidative Properties of Marine Microalgae Isolated from Mediterranean Morocco. Molecules.. 24, 4037.

[69] . **Chaudhuri D., Ghate N., Deb S., Panja S., Sarkar R., Rout J., Mandal N.,** (2014) Assessment of the phytochemical constituents and antioxidant activity of a bloom forming microalgae Euglena tuba. Biological Research.. 47:24

[70] . **Seghiri R., Kharbach M., Essamri A**.,. (2019) Functional Composition, Nutritional Properties, and Biological Activities of Moroccan Spirulina Microalga. Journal of Food Quality.Hindawi.

[71] **. Manivannan K, Anantharaman P, Balasubramanian T.** (2012). Evaluation of antioxidant properties of marine microalga Chlorella marina. Asian Pacific Journal of Tropical Biomedicine, p.p.342-346.

[72] . **Boukhris S. et al.** (2017) The Potential of a Brown Microalga Cultivated in High Salt Medium for the Production of High-Value Compounds. Hindawi . Biomedical Research International.

[73] . **Kahkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S.,** et al.. (1999). Antioxidant activity of plant extracts containing phenolic compounds. J. Agr. Food Chem 47: 3954- 3962.

[74] . **Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA.** (2001), Flavonoids: a review of probable mechanisms of action and potential applications. The American Journal of Clinical Nutrition, 74(4): 418-425.

[75] . **Klejdus B, Lojková L, Plaza M, Snóblová M, Stěrbová D.** (2010)**.** Hyphenated technique for the extraction and determination of isoflavones in algae: Ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry.

[76] . **Hosikian A., Lim S., Halim R., Danquah M. K.,** (2010)**..** Chlorophyll Extraction fromMicroalgae: A Review on the Process Engineering Aspects. International Journal of Chemical Engineering. Hindawi.

[77] .**Parniakov O., Barba F.J., Grimi N., Marchal L., Jubeau S., Lebovka N., Vorobiev E**.,. (2015) Pulsed electric field and pH assisted selective extraction of intracellular components from microalgae nannochloropsis, Algal Research.. 8 128–134,

[78] . **Raso V. and Heinz, J.** (2006). Pulsed Electric Field Technology for the Food Industry. Fundamentals and Applications, Springer, New York,. (Eds.),

[79] .**Vorobiev, E., & Lebovka, N**., (2008).. Pulsed-Electric-Fields-Induced Effects in Plant Tissues: Fundamental Aspects and Perspectives of Applications. Food Engineering Series, 39–81

[80] .**Lebovka, N., Vorobiev, E., Chemat, F**., (2011),. Enhancing Extraction Processes in the Food Industry. Series: Contemporary Food Engineering. CRC Press, Taylor & Francis LLC. p. 592.

[81] . **Rego D., Costa L., Pereira M. T., Redondo L. M**., (2012). Cell Membrane Permeabilization Studies of Chlorella sp. by Pulsed Electric Fields. Ieee transactions on plasma science.

[82] .**Johnson, E.J**. (2002), The role of carotenoids in human health. Nutritional. Clinic. Care, 5, 56–65. [CrossRef] [PubMed]

[83] . **Krinsky, N.I.; Mayne, S.T.; Sies, H**. , (2004).Carotenoids in Health and Disease; Marcel Dekker: New York, NY, USA,

[84] . **Fiedor, J.; Burda, K.,** (2014) Potential role of carotenoids as antioxidants in human health and disease. Nutrients, 6, 466–488. [CrossRef] [PubMed]

[85] . **Campenni L., Nobre B. P., Santos C. A., Oliveira A. C., Aires-Barros M. R., Palavra A. M. F., Gouveia L.** (2012)Carotenoid and lipid production by the autotrophic microalga Chlorella protothecoides under nutritional, salinity, and luminosity stress conditions. Bioenergy and biofuels.

[86] .**Toepfl S, Mathys A, Heinz V, Knorr D.** (2006) Review: potential of high hydrostatic pressure and pulsed electric fields for energy efficient and environmentally friendly food processing. Food Rev Int.22:405–423

[87] . **Syed S., Arasu A., Ponnuswamy I**. (2016). The Uses of Chlorella Vulgaris as Antimicrobial Agent and as a Diet: the Presence of Bio-active Compounds which caters the Vitamins, Minerals in General. International Journal of Bio-Science and Bio-Technology Vol.7, No.1 , pp.185-190

[88] .**Fadoul H.E**. (2014). Antimicrobial Activity of Extracts from Aquatic Algae Isolated From Salt Soil and Fresh Water in Thailand. International Journal of Research Studies in Biosciences (IJRSB) Volume 2, Issue 11 . PP 149-152

[89] . **El-Baky HHA, El Baz FK, El-Baroty GS**. (2008) Characterization of nutraceutical molecules in blue green alga Spirulina maxima. J Med Plants Res; 2(10): 292-300.

[90] .**Ghasemi Y, Yazdi TM, Shafiee A, Amini M, Shokravi S, Zarrini G.**. (2004) Parsiguine a novel antimicrobial substance from Fischerella ambigua. Pharmaceutical Biology,.42(4-5):318-322

[91] .**Yu H, Jia S, Dai Y**. (2009) Growth characteristics of the cyanobacterium Nostoc flagelliforme in photoautotrophic, mixotrophic and heterotrophic cultivation. Journal of Applied Phycology, 21:127-133.

[92] . **Mahanom Z., Azizah A.H., Dzulkifly M.H**., (1990). Effect of different drying methods on concentration of several phytochemicals in herbal preparation 8medecinal plant leaves. Malaysian Journal of Nutrition.5: 47-54

[93] . **Jaya Prakash goud M., Seshikala D., Singara Charya M.A**. (2007) Antibacterial activity and biomolecular composition of certain fresh water micro-algae from River Godavari (India). International Journal on Algae .9(4):350-358

[94] **.Günerken E., D'Hondt E., Eppink M.H.M. , Garcia-Gonzalez L.,. Elst K , Wijffels R.H.,.** (2015). Cell disruption for microalgae biorefineries. Biotechnology Advances 33. 243-260

[95] . **Dunker, S., Wilhelm, C..** (2018) Cell wall structure of coccoid green algae as an important.

[96] .**Alhattab, M., Kermanshahi-Pour, A., Brooks, MS-L.** (2019) Microalgae disruption techniques for product recovery: influence of cell wall composition. . J. Appl. Phycol. 31, 61-88.

[97] .**Weltin-Chanes, J., Vélez-Ruiz, J. F., & Barbosa-Canovas, G. V..** (2003)Transport phenomena in food processing. Florida: CRC Press.

[98] .Salem **Olfat MA, Hoballah EM, Ghazi Safia M, Hanna Suzy N**. (2014) Antimicrobial activity of microalgal extracts with special emphasize on Nostoc sp. Life Science Journal,.11(12): 752-758

[99] .**Vehapi M., Yilmaz A., Özçimen D**. (2018), Antifungal activities of Chlorella vulgaris and Chlorella minutissima Microalgae cultivated in bold basal medium, wastewater and extract water against Aspergillus niger and Fusarium oxysporum. Rom Biotechnol Lett 1:1

[100] . **De Morais MG, Vaz Bda S, de Morais EG, Costa JA.**. (2015) Biologically active metabolites synthesized by microalgae. Biomed Research International.

[101] . **Abo-State MAM, Shanab SMM, Ali HEA, Abdullah MA.** (2015), Screening of antimicrobial activity of selected Egyptian cyanobacterial species. J Ecol Health Environ 3:7–13

[102] . **Pérez MJ, Falqué E, Domínguez H.,**. (2016) Antimicrobial action of compounds from marine seaweed. Marine Drugs, 14:1–38

[103] .**Stirk W.A., B 'alint P., Vambe M., Lov´asz C., Molna´ r Z., van Staden J., O¨ rdo¨g V.,.** (2020). Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass. Journal of Biotechnology.

[104] . **Dash P, Padhi SB**. (2012); Natural Antioxidant Production by Oscillatoria Sp. and Microcystis Sp. From Ponds of Puri District, Odisha. International Journal of Microbiology Research. 4(6):249-252.

[105] . **Gerde JA, Montalbo-Lomboy M, Yao L et al.** (2012)**.**Evaluation of microalgae cell disruption by ultrasonic treatment. Bioresource Technology, 125 (0): 175-181

[106] . **Halim, R., Harun, R., Danquah, M.K., Webley, P.A.** (2012) Microalgal cell disruption for biofuel development. Appl. Energy. 91, 116-121