Antimicrobial potential of *Chlorella* algae isolated from stacked waters of the Andean Region of Ecuador

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Abstract. In this study, the focus will be primarily on antimicrobials extracted from Microalgae *Chlorella* from stacked water from three different places. The results provide dates for possible applications in the pharmaceutical industry for developing antiseptic and antimicrobial products to guarantee the health of the people. The study initially outlines the isolation of microalgae and secondly was the extraction of chlorellin as an antimicrobial metabolite. To replicate the environmental conditions which are useful for growth colonies of *Chlorella* it was maintained at room temperature, oscillating between 10 °C to 18 °C in Bold Basal medium enriched with NH4Cl. After that, chlorellin was extracted from aqueous supernatant and sediment with ethanol, isopropyl alcohol, and water. The results of this study show that the chlorellin extracted from *Chlorella* present an essential antimicrobial capacity against bacteria isolated from the hand. The antimicrobial capacity was equal with ampicillin and oxacillin to inhibit *Staphylococcus* spp.

1. Introduction

The increasing development of resistance of microorganisms to drugs and treatments, to combat specific diseases, has made scientists look for new sources of antimicrobial substances. Nowadays, several of those natural sources are being studying because they have antimicrobial activity, such as exotic Plantae, plant root and microalgae [1, 2, 3, 4]. Microalgae are microorganisms which are represented by a wide variety of species, living in diverse ecological habitats [5, 6, 7]. Likewise, these organisms are a rich source of structurally novel and biologically active metabolites. Primary and secondary metabolites which are produced by microalgae could be unusual for chemical and pharmaceutical industry [8, 9, 10, 11]. The industrial applications of macro and microalgae have been focused on the farming of edible species, or on the production of agar, carrageenan, and alginate [12, 13, 14]. Microalgae can support extreme environments for that reason they have developed defensive and adaptive strategies, including the synthesis of a diversity of compounds from different metabolic pathways [15]. Many of their metabolites have been shown to possess antibacterial, antiviral, antifungal, enzyme inhibiting, immunostimulant, cytotoxic and antiplasmodial activities [16, 17, 18, 19]. Although the potential that microalgae could have, the attention on it has recently been focused on its biotechnological potential for obtaining antimicrobial metabolites [20]. One of the antibacterial substances, named “chlorellin”, extracted from the microalgae *Chlorella* was found to exhibit inhibitory activity against both Gram-positive and Gram-negative bacteria [21, 22, 23, 24]. In this sense, this research aimed to evaluate the antimicrobial potential of the *chlorella* algae extracted from the stacked waters from three different irrigation ditch in Ambato-Ecuador.
2. Materials and Methods

2.1. Sample Collection
Stacked water was collected from three different irrigations ditches of Ambato-Ecuador. The places for collecting the samples were Atocha, Tilulum and El Socavón. Taking account the green color which indicated the presence of algae, 100 ml was collected from each point in sterile plastic containers; the samples were collected in triplicate. Once the samples arrived at our facilities, they were kept at a temperature of 18 °C in sterile plastic flasks without covering them.

2.2. Selective Isolation of Chlorella
Before the particular isolation, a drop of each sample was placed on a slide and observed under the microscope. Identification of Chlorella was based on its spherical morphology, about 2-10 microns in diameter, due to its absence of flagella so it is immobile, due to its lack of hairs, to be unicellular, and in its pigmentation, since at containing high levels of chlorophyll a and b the medium in which it grew was green. In a Bold Basal medium which is a nutrient medium for multiplication of microalgae that contained 1ml of trace salts solution (MgSO4,7H2O, FeSO4.4H2O, CuSO4.4H2O, each at a concentration of 0,1g/100mL), K2HPO4 (4 g/L), KNO3 (2 g/L) and agar (15 g/L). The dilution 10-3 were prepared, and 100 uL of this dilution were spread over the surface of plates. To replicate the environmental conditions of Ambato, the colonies growing at room temperature, oscillating between 10 °C to 18 °C. Finally, the microalgae were isolated several times to obtain pure cultures.

2.3. Growth and Purification in Liquid Medium
For growth and purification, 250ml of Bold Basal medium enriched with NH4Cl for the massification microalgal was prepared. Samples were placed in sterilized bottles, aerated each of them and exposed to sunlight to help to the cells to be adapted to the medium. Each bottle had continuous aeration because the microalgae are aerobic, avoiding their sedimentation and allowing the homogenization of the nutrients significantly. This process improves the assimilation of nutrients because the movement of the water decreases the layer laminar around the microalga [25, 26, 27]. This procedure was repeated until obtaining pure cultures of Chlorella based mainly on its microscopic round morphology. Finally, the samples were allowed to stand for seven days for their overcrowding microalgal.

2.4. Cell Counting
Using 1 liter of bold basal medium and 10% of pure cultures per liter samples from Atocha, Tilulum and El Socavón were prepared. Formula (1) was used to calculate the concentration of the microalga by the hemocytometer method.

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\text{Concentration (cell/ml) = Cells number/Volume (ml)}
\]  

One drop of each sample was placed in the Hemocytometer to be observed; each sample was covered with a coverslip to avoid air bubbles that could affect the visualization. Formula (1) was used taking account the four external squares were counted, the volume was calculated by multiplying the height, width and length of the hemocytometer (0.1cmx0.1cmx0.01cm). The data were recorded during ten days; each day cell counts were made to obtain the growth curve with the number of Chlorella cells.

2.5. Chlorellin Extraction
Taking into account that cell growth did not vary significantly (cells of the tests are in an exponential state) for three days, 10 ml of each sample was collected in test tubes, which were centrifuged at 5000 rpm for 20 minutes. To determine the antimicrobial compound the measurements were developed in aqueous supernatant and sediment after centrifugation. This procedure was repeated three times to obtain three tubes of sediment and three tubes of supernatant for each test; then different solvents were placed in each one; 5 ml of ethanol, 5 ml of isopropyl alcohol and 5 ml of water to extract the antimicrobial compound.
2.6. Antimicrobial Capacity

2.6.1. Bacterial Identification. With the purpose to establish the antimicrobial potential, bacteria which are in hands were isolated. Tests were carried out on blood agar (Gram-positive) and on Mac Conkey agar (Gram-negative) to identify the type of bacteria[28]. Likewise, the catalase test [29, 30] and gram test [31] were performed. When it was observed growth on blood agar, the coagulase test [31, 32] was performed to establish the presence of Staphylococcus spp.

2.6.2. Antimicrobial Potential. The antimicrobial potential of chlorellin as a metabolite of Chlorella was evaluated on hands bacteria because chlorellin could be used as an antiseptic gel for hands. An antimicrobial sensitivity test with five discs, four at the ends and one in the center, was placed with 300 μl of chlorellin. The discs were placed manually with sterile tweezers ensuring that they had perfect contact with the surface of the agar, so the discs were pressed lightly on the surface. The discs were placed less than 15 mm from the edge of the plate, and they were distributed so that no overlapping of the inhibition halos occurred. After this process, the plates were incubated in groups of no more than five at 37 °C for 24 hours. The inhibition halos were measured with a ruler in a light-illuminated box on a black background to facilitate measurement.

2.7. Positive Control

The positive control to establish the susceptibility of microorganisms to a variety of microbial agents was developed. The inoculum was adjusted to the MacFarlane turbidity pattern No. 0.5, antibiotic sensitives such as 10 μg ampicillin, 10 μg oxacillin were used for having a predominant gram-positive spectrum including Staphylococcus spp, and the inhibition halos were measured after 24 hours of incubation.

2.8. Statistical Analysis

Statistical analysis was performed with the GraphPad Prism 5.0 program (GraphPad Software, San Diego, California, USA) with a bi-directional analysis of variance. The test of comparisons was carried out with the Tukey test with a significance level of P ≤ 0.05.

3. Results and Discussion

3.1. Identification of Chlorella

The samples collected from the stacked water from three different irrigations ditches (Atocha, Tilulum and El Socavón) of Ambato-Ecuador were observed under a microscope at 40 x. Taking into account that the size of colonies were 2 to 10 microns in diameter, green color attributable to the presence of chlorophyll, irregularly shaped colonies retaining their spherical shape and mainly the absence of flagellum allowed to establish the presence of the Chlorella microalgae in all samples [25, 35].

3.2. Growth and Purification

The average initial population of each sample was calculated using equation 1. Socavón sample had an initial population of 2.60±0.57×10^5 cell×ml^{-1} lower than Atocha sample (4.8±0.54×10^7 cell×ml^{-1}) and Tilulum sample (13.60±0.51×10^6 cell×ml^{-1}). From the growth curve (Figure. 1) can be concluded that induction phase lasted for 24 hours, for this reason color of the culture was almost transparent. In this phase cells were treating to adapt to the new environment and there was not cellular replication. After 24 hours, the exponential phase appears and cell density increased as a function of time according to an exponential function (r^2 = 0.99) until 72 hours. The color of the culture was light green and significant difference in specific growth rate was showed between Atocha and Tilulum ditches (p≤0.05) (Table 1). The specific growth rate in this phase mainly depend on algal species, light intensity, and temperature [36]. Next, of this phase, a linear phase was showed since 72 hours until 192 hours (r^2
The color of the cultures changed to dark green without significant differences in specific growth rate between ditches. Finally, in the stationary phase cell division slows down as a function of time according to an exponential function \( r^2 = 0.98 \) until 240 hours, without significant differences in specific growth rate between ditches. At this point, there was no production of new cells because nutrients, light, pH, carbon dioxide or others physical and chemical factors limit growth, and there were not significant change in coloration.

The extraction process of the chlorellin was performed at 240 hours, in this point the final population was \( 54.23 \pm 0.57 \times 10^5 \) cell \( \times \) ml \(^{-1} \) in Tilulum sample followed by Atocha sample \( (45.76 \pm 0.57 \times 10^5 \) cell \( \times \) ml \(^{-1} \)) and Socavón sample \( (42.86 \pm 0.57 \times 10^5 \) cell \( \times \) ml \(^{-1} \)).

![Figure 1. The growth of algae culture.](image)

### Table 1. Specific growth rate.

| Ditches | Exponential phase \( \times 10^5 \) cell \( \times \) ml \(^{-1} \) | \( r^2 \) | Linear phase \( \times 10^5 \) cell \( \times \) ml \(^{-1} \) | \( r^2 \) | Stationary phase \( \times 10^5 \) cell \( \times \) ml \(^{-1} \) | \( r^2 \) |
|---------|-------------------------------------------------|--------|--------------------------------|--------|--------------------------------|--------|
| Atocha  | 0.328\( \pm \)0.06\(^c\) 0.997 5.560\( \pm \)0.137\(^a\) | 0.992 | 0.024\( \pm \)0.013\(^a\) 0.999 |        |                                |        |
| Tilulum | 0.197\( \pm \)0.023\(^b\) 0.989 5.524\( \pm \)0.508\(^a\) | 0.998 | 0.033\( \pm \)0.006\(^a\) 0.966 |        |                                |        |
| Socavón | 0.276\( \pm \)0.033\(^b\) 0.999 5.815\( \pm \)0.036\(^a\) | 0.982 | 0.035\( \pm \)0.009\(^a\) 0.981 |        |                                |        |

Results are the mean ± standard deviation. One-way ANOVA: different letters (\(a,b\)) in the same column indicate significant differences between samples \( (p \leq 0.05) \).

#### 3.3. Antimicrobial Capacity

##### 3.3.1. Chlorellin extraction

Tubes with sediment and supernatant were used to determine the antimicrobial compound of Chlorella. Different solvents such as 5 ml of ethanol, 5 ml of isopropyl alcohol and 5 ml of water to extract chlorellin were placed in each one. It was observed that the tubes with sediment-solvent were dark green, which indicated the predominance of microalgae, while the tubes with supernatant-solvent were almost transparent.

##### 3.3.2. Bacterial identification tests

The bacterial identification was performed 24 hours after the samples were inoculated on blood agar with cultures obtained from the hands. The results showed growth of whitish colonies of circular shape and smooth surface with yellow and orange coloration. The catalase test to establish the presence of Enterococcus was performed, the presence of bubbles was observed, it indicates a splitting of the peroxide in water and gaseous oxygen, which allowed discarding the presence of Enterococcus. The gram test was positive. The results showed the presence...
of gram-positive bacteria. The growth of microorganisms on blood agar, which is a standard medium where Staphylococcus spp grows. Likewise, the coagulase test was positive; the results indicate the presence of Staphylococcus aureus because it is the only species that produces the coagulase enzyme and yellow pigmentation [37, 38, 39]. This isolated microorganism was used for the halo inhibition test.

3.3.3. Antimicrobial Potential. The sample from Atocha in which isopropyl alcohol was applied to extract chlorellin had an inhibition halo of 0.22 ± 0.01 mm, higher value than the Atocha sample that was obtained with ethanol whose inhibition halo value was 0.17 ± 0.02 mm. In the Tilulum sample whose extraction solvent was isopropyl alcohol, the inhibition halo was 0.28 ± 0.01 mm while the halo of inhibition of the sample in which ethanol was used was 0.19 ± 0.01 mm. In the sample from El Socavón in which Isopropyl Alcohol was used shown an inhibition halo of 0.19 ± 0.01 mm, whereas the sample of El Socavón in which ethanol was used shown an inhibition halo of 0.12 ± 0.01 mm. The samples from Atocha, Tilulum, and Socavón with isopropyl alcohol had higher values of inhibition compared to the treatments in which ethanol was used for extraction; however, the difference was not significant p <0.05, which indicated that both solvents could be used to extract Chlorellin. The results show that the extract of Chlorella microalgae can inhibit bacterial growth when compared to the positive control [1, 2]. The positive control showed that the gram-positive bacterium was sensitive to ampicillin with an inhibition halo of 5 ± 0.01 mm and the oxacillin with a halo of 1 ± 0.01 mm, and these values are within the sensitivity range according to the Minimum Inhibitory Concentration table. The samples from Tilulum had greater inhibition, followed by the Atocha sample and finally the El Socavón samples. In this study it was possible to determine antibacterial activity against the bacteria Staphylococcus aureus using the active principle of Chlorella, thus proving that the Chlorella microalga extract has activity against gram-positive bacteria. The results were similar to other obtained by other researchers [9, 17, 20].

4. Conclusion
The study was carried out with the purpose of determining the antibacterial power of the Chlorella microalgae. The growth kinetics was established for the identification of the exponential phase in which the samples were taken for extraction with the different solvents. The results show the antibacterial potential of the Chlorella microalgae extracted from the stacked waters of the ditches of various sectors of the city of Ambato-Ecuador. The results obtained from the antibiogram method show that the best treatment corresponds to the samples from the Tilulum area in which isopropyl alcohol was used for its extraction, however, with ethanol the inhibition halos did not have significant differences, whereas with the water solvent no inhibition was observed with both the sediment and the supernatant. The results of the antibacterial activity of the isolated microalgae show promising possibilities for obtaining secondary metabolites that could be exploited by the pharmaceutical industry.

5. References
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