The *In-vitro* Antimicrobial Activity of *Chlorella vulgaris* Extracts on Selected Clinical Isolates

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Abstract: The antifungal and antibacterial activities of extracts *Chlorella vulgaris* was studied by using methanol, ethanol, chloroform, acetone, hot aqueous and cold aqueous extracts on; *Staphylococcus aureus, Klebsiella* sp., *Pseudomonas* sp., *Bacillus* sp., *Aspergillus niger, Aspergillus fumigatus, Candida* sp. and *Penicillium* sp. **The well-**in-agar and disc diffusion techniques adopted showed inhibitory effect with varying degree of susceptibility except cold aqueous extract which had no activity on any of the isolates used in the study. The highest zone of inhibition for bacterial isolates was observed in ethanol extract (6mm) for *Klebsiella* sp., while the highest zone of inhibition for fungal isolates was seen in methanol extract (4.5mm) for *Candida* sp. Hot aqueous extract showed the least zone of inhibition (0.5mm). The algal extract showed more activity on the bacterial isolates than commercial antibiotics used, while the antifungal drugs are more active on the fungal isolates than the extract. The natural derivatives from this algal species may be useful in treating certain specific infections and diseases as well as limit the proliferation of some clinical strains with little or no side effect.

Keywords: *Chlorella vulgaris,* isolates, antimicrobial activities, ethanol, chloroform, acetone, methanol, aqueous hot and aqueous cold.

Introduction

Increased resistance of microorganisms to drugs including antibiotics and antifungal agents has being a key factor responsible for increasing mortality from infections, and high cost of healthcare especially in developing countries.To this effect, effort has been made to investigate the phytochemicals and antimicrobial potency of different extracts of microalgae such as *Chlorella vulgaris.* There is a wide spread believe that green medicines are healthier or safer than synthetic ones because of their limited side effects (Pradhan *et al.*, 2014). In the drug production, natural products play an important role because of their limited or no side effect, thus necessitating the investigation of new algal compounds in phytomedicine. Most primary metabolites form the most important physical component of cell which is used for their maintenance and anabolic processes but their catabolic metabolites contain a lot of antioxidant properties (Annamalai and Nallamuthu, 2014). Various phytochemicals like flavonoids, saponins, glycosides, carotenoids, phycocayanine, phenolics, amino acids and polysaccharides can be found in different microalgae in enormous quantity. These compounds exhibits diverse biological activities as anti-allergy, anticancer, antioxidant, antihypertensive, antidiabetic, antimicrobial, antiviral, antitumor, immune stimulatory, detoxifying, and anti-inflammatory effects (Harsha *et al.*, 2012; Sanmukh *et al.*, 2014). Chlorellin the first antimicrobial substance obtained from *Chlorella* was extracted by Pratt *et al.* (1944). The substance is a mixture of fatty acids exhibits diverse inhibitory activity against both Gram positive and Gram negative bacteria. They normally act on multiple cellular targets such as cell membranes, resulting in damages leading to cell leakage, inhibition of cellular respiration and reduction of nutrient uptake (Pradhan *et al.*, 2014). Microalgae exhibit remarkable ecological plasticity, ability to adapt to changes in extreme environmental conditions such as light, pH, temperature, salinity, mineral uptake and stress, pressure, concentration of oxygen and carbon (IV) Oxide and moisture, which define their worldwide distribution (Falkowski and Raven, 1997). These organisms have developed adaptive and defence strategies to survive in a complex and competitive environment, which are related to the synthesis of various bioactive compounds, some of which are unique, compounds from different metabolic pathways. This study investigates the in-vitro antimicrobial activity of the microalga *Chlorella vulgaris* on selected clinical isolates.

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Materials and Methods

Sample Collection

Water samples containing microalgae were aseptically collected from the University of Port Harcourt pond using sterile screw capped container. These samples were transported to the laboratory in an ice packed cooler for immediate analysis.

Collection and Identification of Clinical Isolates

The clinical isolates (*Staphylococcus aureus, Klebsiella* sp., *Pseudomonas* sp., *Bacillus* sp., *Aspergillus niger, Aspergillus fumigatus, Candida* sp. and *Penicillium* sp.) were collected from the Microbiology and Parasitology unit of the Medical Laboratory of the University of Port Harcourt Teaching Hospital. The organisms were cultured on nutrient agar, incubated at 37^{0}C for 24h for bacteria, while the fungi were cultured on potato dextrose agar and incubated at room temperature for 5-7 days. They were then sub-

cultured on appropriate media and kept until when required. Their identity was confirmed using morphological and biochemical tests as described by Agwa and Wokoma (2011).

Sample Preparation

Pond water (30ml) was added to 70ml of the synthetic medium in a 250 ml conical flask, the mixture was kept under natural illumination for blooming of algae and shake intermittently every 2h (Agwa and Abu, 2014). The control experiment was also set up following the same conditions but using the synthetic medium (0.132 g/l Potassium nitrate, 0.066 g/l Sodium silicate, 0.066 g/l Monosodium phosphate and 0.066 g/l EDTA. The pH was adjusted to 7.5 prior to autoclaving at 121°C for 15 mins) only (Agwa *et al*., 2016).

Identification of *Chlorella valgaris*

An aliquot of the pond water was collected and viewed under the X40 objective of the microscope to identify the organism, using morphological characteristics (Agwa *et al*., 2011a). Purity of the culture was periodically checked under the microscope using X10 objective and by streaking onto agar plates. Subculture was done after every four week of inoculation. The organism was identified on the basis of its phenotypic and molecular characterization by the Polymerase Chain Reaction (PCR) technique following the slightly modified protocol of Burja *et al*. (2011).

Proximate Analysis

Moisture and ash were determined by the air oven method (AOAC, 1990). Crude protein was determined by the micro- Kjeldahl method (AOAC, 1990) and the conversion factor from nitrogen to protein was 6.25. Crude lipids were determined by the soxhlet extraction method of Egan *et al*., (1981).Total carbohydrate content was determined by using the Anthrone method (Osborne and Voogt, 1978). The crude fibre content was calculated by difference using the formula: Crude fibre = 100 – (% protein + % TAC + % moisture + % fat + % ash).

Preparation of Inoculum

Colonies of the isolates were picked from the slants and suspended in about 9ml of sterile potato dextrose broth and nutrient broth for fungal and bacterial isolates respectively. The turbidity of the isolates suspension was adjusted to correspond to 0.5 McFarland standards before use.

Preparation of Algal Extract

Algal cells of 2.5g (Vishnu and Sumathi. 2014) each were put into six (6) different conical flasks. To the solvents (50ml) were added into each algal cell in different conical flask, kept at room temperature for 48h and stirred rapidly every 8 h and subsequently filtered after 48h. The filtrates were taken in separate beaker and kept in a water bath at 45°C until the solvents evaporate. The greasy final material (crude extract) obtained was transferred to sterile screw capped bottles and stored until when required.

Preparation of antibiotics and antifungal Agents

Ampicillin, ampiclox, amoxicillin, chloramphenicol, griseofulvin, nystatin, itraconazole and ketoconazole were purchased from a drug store in Port Harcourt. Stock solutions were prepared by dissolving500mg of the powder in 10ml of sterile distilled water to give a concentration of 50mg/ml (Mukhtar and Huda, 2005). Varying concentrations of the antibiotics were obtained by serial dilution of the antifungal agents from their stock solutions. The concentrations obtained were 25, 12.5, 6.25 and 3.12mg/ml respectively.

Antimicrobial Activity Assay

The disc diffusion (Bauer, 1966) and well in agar diffusion (Osadebe and Ukwueze, 2004) were adopted. The disc diffusion was carried out using filter paper of about 6mm in diameter. The sterilized paper discs were dipped into the *Chlorella* extracts and placed on sterile aluminium foils and allowed to dry slowly. About 0.1ml of the test organism was inoculated onto the plates from the broth culture and the discs impregnated with the algal extracts were placed on the culture plates aseptically. For the well-in-agar diffusion method, after spreading the organisms on the plates, about four holes were bored using a sterile cork borer. Equal volumes of the *Chlorella* extract and the antimicrobial agents (antibiotics and antifungal) were transferred into the holes, allowed to stand for about one hour for pre diffusion of the extracts and antimicrobial agents to occur. The experiments were carried out in duplicates. Control experiment was carried out using the test organism with distilled water instead of the solvent extracts. Incubation was carried out at 37°C for 48h for *Staphylococcus aureus*, *Klebsiella* sp., *Pseudomonas* sp., *Bacillus* sp. and *Candida* sp. and at room temperature for 72h for *Aspergillus niger*, *Aspergillus fumigatus* and *Penicillium* sp. Zones of inhibition were measured in millimeters (mm).

Minimum Inhibitory Concentration (MIC)

To determine the MIC, the broth dilution method is used Lowest concentration of any antimicrobial agent needed to prevent the growth of a given organism is regarded as the MIC. Nutrient broth(2ml) was poured into test tubes and sterilized appropriately and 2ml of different concentrations (25, 12.5, 6.25 and 3.12mg/ml) of each antimicrobial agent was added to test tubes. Test organism(0.1ml) was then added and incubated at 37°C for 48h for *Staphylococcus aureus*, *Klebsiello* sp., *Pseudomonas* sp., *Bacillus* sp. and *Candida* sp. and at room temperature for 72 hours for *Aspergillus niger*, *Aspergillus fumigatus* and *Penicillium* sp. After incubation, the lowest

concentration that prevents the growth of the organism is the minimum inhibitory concentration.

Results

The agarose gel reveals no DNA from sample in lanes 1 and 4 which served as control, but DNA was found in lanes 2 and 3. The electrophoresis of PCR products revealed band sizes of 550bp, and corresponded to nuclear small subunits (SSU). Lanes 5-8 was the replicate of lanes 1-4 (Fig 1). Table 1 shows the proximate analysis of *Chlorella vulgaris* cells. Fig. 2 illustrates the average zone of inhibition of *Chlorella vulgaris* extracts on the isolates using well-in-agar diffusion method. Average zone of inhibition of *Chlorella vulgaris* on the isolates using disc diffusion was shown in Fig 3. Fig. 4 shows the inhibitory effect of some antifungal agents on the growth of some fungal isolates using well-in-agar diffusion method. The inhibitory effect of some antibiotics on the growth of some bacterial isolates using well-in-agar diffusion method as can be seen from fig 5. Table 2 and 3 indicates the minimum inhibitory concentration (MIC) of the antifungal and antibacterial agents respectively.

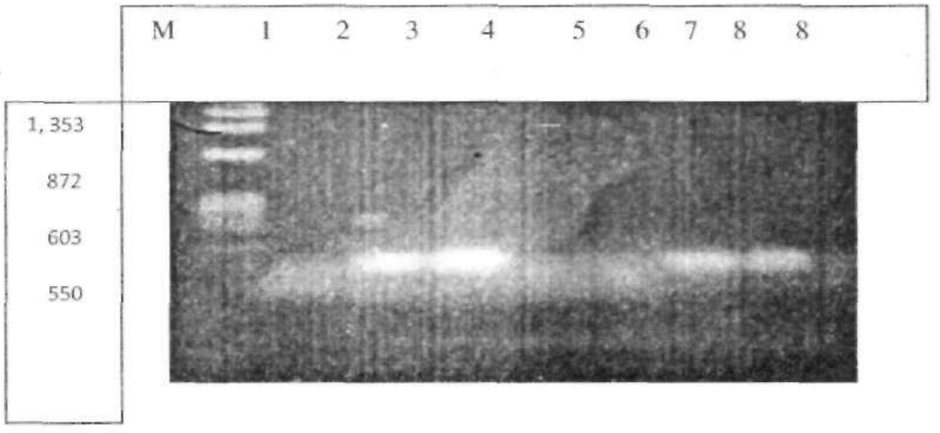

Fig 1. Gel electrophoresis of the amplified PCR products for the detection of *Chlorella vulgaris* genes.

TABLE 1: PROXIMATE ANALYSIS OF *Chlorella* CELLS

Parameters	Results (%)
Crude Protein	56.0
Crude Fat	10.3
Carbohydrate	13.7
Ash	1.5
Moisture content	6.6
Others	11.9

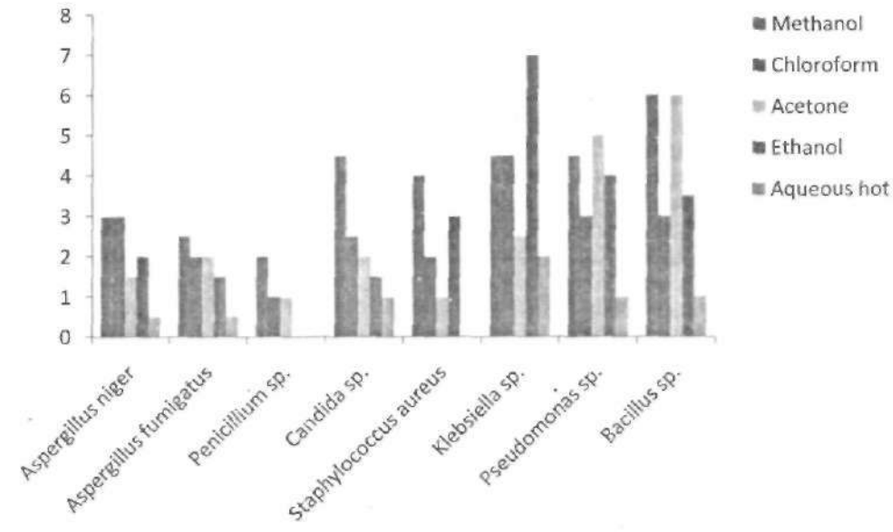

Fig 2: Average zone of inhibition of *Chlorella vulgaris* extracts on the isolates using well-in-agar diffusion method

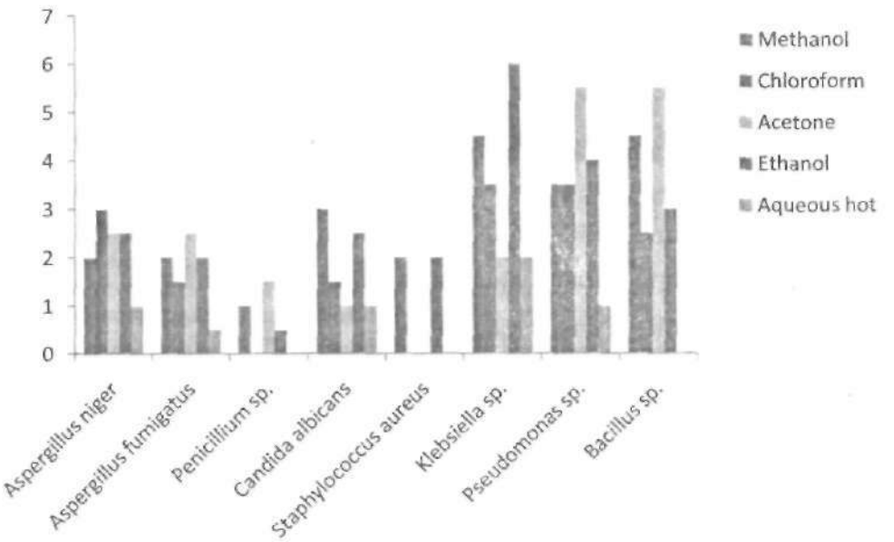

Fig 3: Average zone of inhibition of *Chlorella vulgaris* extracts on the isolates using disc diffusion method.

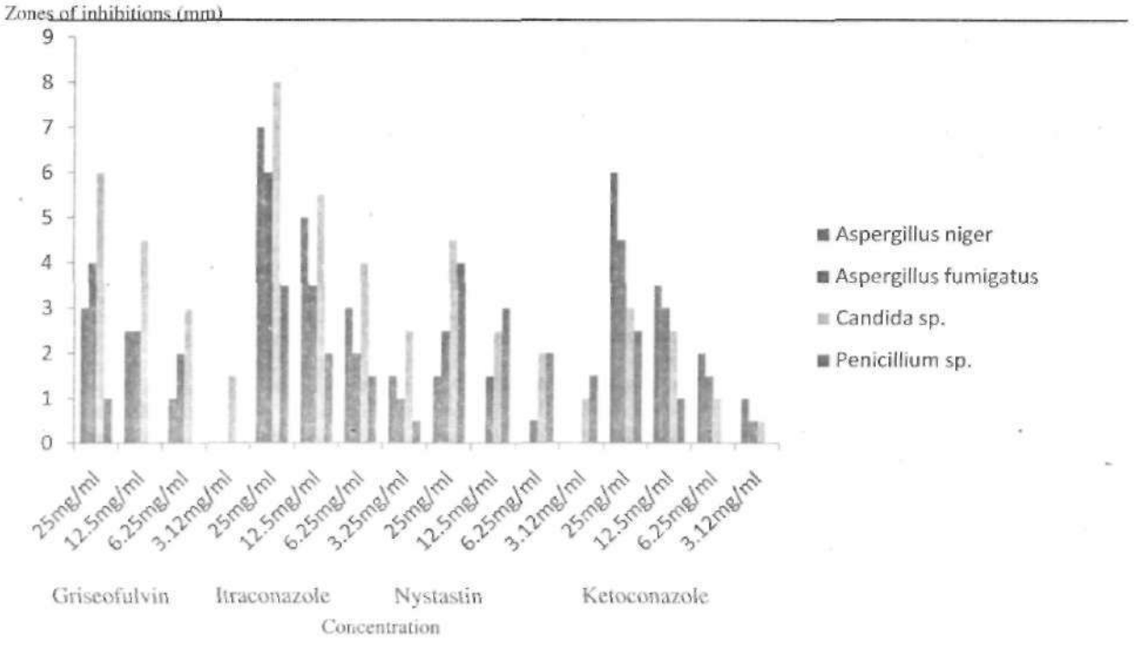

Fig 4: The inhibitory effect of some antifungal agents on the growth of some fungal isolates

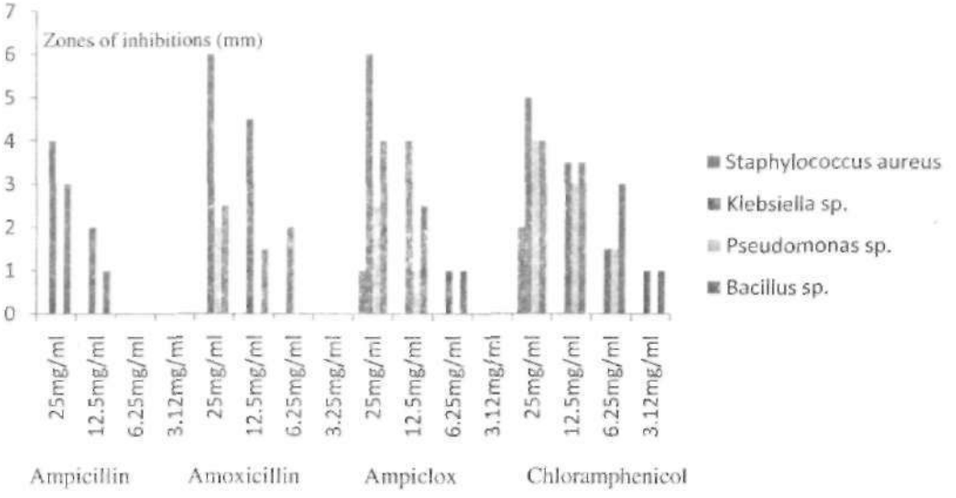

Fig 5: The inhibitory effect of some antibiotics on the growth of some bacterial isolates

Table 2: Minimum Inhibitory Concentration (MIC) of the antifungal agents on the fungal isolates

Antifungal agents	Concentration (mg/ml)	*Aspergillus niger*	*Aspergillus fumigatus*	*Candida* sp.	*Penicillium* sp.
Griseofulvin	25	-	-	-	-
	12.5	-	-	-	+
	6.25	+	+	-	+
	3.12	+	+	+	+
Itraconazole	25	-	-	-	-
	12.5	-	-	-	-
	6.25	-	+	+	-
	3.12	+	+	+	+
Nystatin	25	-	-	-	-
	12.5	+	-	-	+
	6.25	+	+	-	+
	3.12	+	+	+	+
Ketoconazole	25	-	-	-	-
	12.5	-	-	-	+
	6.25	+	+	+	+
	3.12	+	+	+	+

Key: += growth; = no growth

Table 3: Minimum Inhibitory Concentration (MIC) of the antibiotics on the bacterial isolates

Antibiotics	Concentration (mg/ml)	*Staphylococcus aureus*	*Klebsiella* sp.	*Pseudomonas* sp.	*Bacillus* sp.
Ampicillin	25	+	-	+	-
	12.5	+	-	+	+
	6.25	+	+	+	+
	3.12	+	+	+	+
Amoxicillin	25	+	-	-	-
	12.5	+	-	+	-
	6.25	+	-	+	+
	3.12	+	+	+	+
Ampiclox	25	+	-	-	-
	12.5	+	-	+	-
	6.25	+	+	+	+
	3.12	+	+	+	+
Chloramphenicol	25	-	-	-	-
	12.5	+	-	-	-
	6.25	+	+	+	-
	3.12	+	+	+	+

Key: +, growth, -, no growth

Discussion

The phenotypic characteristics of *Chlorella* sp investigated revealed that they belong to the phylum Chlorophyta. *Chlorella* can be found in soil, fresh and marine water and even in polluted environment, their sizes ranges from 2-12µm (Crofcheck *et al.*, 2012). It is a single, unicellular, spherical, ellipsoidal, cup-shaped, gregarious, coccal green alga that is non-motile, which accumulates enormous amounts of protein and lipid (Agwa *et al.*, 2016). The molecular identification reveals a polyphyletic organism with diverse ancestors. The nuclear chloroplast DNA of *Chlorella* sp. revealed a band size of 550bp fragment corresponding to nuclear SSU (Fig.1). The primer sequence for *Chlorella vulgaris* showed that Nuclear SSU primers typical of *Chlorella vulgaris* are amplified in lanes 2 and 3.This agrees with the findings of Burja *et al.* (2011) who showed a genetic sequence of about 578bp fragment of the 16S rRNA gene present within the chloroplast genome and suggested that the isolate be identified as *Chlorella vulgaris*. Microalgae can produce several valuable metabolites such as vitamins, proteins, mineral elements, lipids and carbohydrate amongst others (Annamalai and Nallamuthu, 2014). The proximate analysis reveals the presence of high percentage of 56%Protein, 10.3% fat, 13.7% carbohydrate and other components such as ash1.5% and a moisture content of 6.6%. These components are very important in nutrition and can be refined into useful biological products for use in food, pharmaceutical, nutraceutical and bioenergy industrie (Iwamoto, 2004; Agwa *et al.*, 2012). This agrees with the result of Warren (1997) who reported about 45% protein, 20% fat, 20% carbohydrate. Bertoldi *et al.* (2008) who reported 52.4% protein in BBM and Chia *et al.*(2013) who reported about 50% protein and 40%carbohydrate in Chu medium. But the reports of Illman *et al.* (2000) 29% of protein and 50% carbohydrate in watambe medium and Dineshkumar *et al.* (2017) 34.56%protein and 41.09% carbohydrate are in contrast with our findings. Primary metabolites form the most important physical components of cell which is used for their maintenance and anabolic processes but their catabolic metabolites contain a lot of antioxidant properties (Annamalai and Nallamuthu, 2014). *Chlorella* is a useful precursor for the biosynthesis of various bioactive compounds (Shabudeen *et al.*, 2015). Precursor metabolites are carbon skeleton that serve as starting substrates for the synthesis of monomers and other building blocks needed for the synthesis of macromolecules. These macromolecules are produced through the biosynthetic pathways which are organised to optimize efficiency by conserving raw materials and energy (Willey *et al.*, 2008). Resistance of microorganisms to antimicrobial agents is one major problem facing healthcare delivery. Thus, the search for natural products with antimicrobial activity is now considered because they have limited side effects. In response to this, the antimicrobial activities of six solvent extracts (methanol, ethanol, acetone, chloroform, hot aqueous and cold aqueous) extracts of *Chlorella vulgaris* was studied on clinical isolates using well-in-agar and disc diffusion methods. The well-in-agar diffusion method showed greater antimicrobial effect than disc diffusion method (Agwa *et al.*, 2011; 2013). The methanol extract proved to be most effective extract in extracting the major phytoconstituents that have antimicrobial capabilities, especially on fungal isolates while the ethanol extract showed more activity on the bacterial isolates. Cold aqueous extract showed no inhibitory activity on both fungal and bacterial isolates (Agwa *et al.*, 2011; 2013). The well-in-agar method showed that ethanol extracts had the highest observed effectiveness on *Klebsiella* sp., followed by methanol and acetone extracts on *Bacillus* sp. The disc diffusion method followed similar pattern with the ethanol extract, followed by acetone on *Bacillus* and *Pseudomonas* sp. Of the two methods employed in the study, the least effectiveness was observed on *Staph. aureus*. The fungal isolates revealed the same results with the two methods employed, thus the highest effectiveness was shown by methanol extract on *Candida* sp and the least on *Penicillum* sp. The finding of this study correspond with the findings of Dineshkumar *et al.* (2017), Shabudeen et al. (2015), Vishnu and Sumathi, (2014) and Sanmukh *et al.* (2014) who found the ethanolic extracts more effective against these isolates especially on *Klebsiella* sp. with strong potential and promising applications encompassing antifugal, antibacterial and antiviral activities for the production of effective biological products. Thus, the effectiveness or potency of the alga extract as an antimicrobial agent depends on the method used to obtain the extract and the concentration of extracts used (Priya, 2012). Comparing the activity of commercial antibiotics on the isolates, ampiclox was the most active with the highest effect on *Klebsiella* sp. but least effect on *Staph. aureus* Itraconazole exerted the highest activity on *Candida* sp. followed by *Aspergillus niger* and least effect by ketoconazole. But this is in contrast with the result of Shabudeen *et al.* (2015), who reported that, the antibiotic tetracycline with the highest effect on *Klebsiella* sp. The high antimicrobial effect of some of these solvents may be due to the interactions of these solvents with the phytochemical components with inhibitory potential and the synergistic activity of the bioactive compounds. The results of the minimum inhibitory concentration (MIC) of the antimicrobial agents were shown in table 1 and 2. The lowest MIC of 12.5mg/ml was observed to be sensitive for both antibacterial and antifungal agents. The algal extract proved to be more effective on the isolates than some antimicrobial agents used. This may be due to the growing resistance of these bacteria to commercial antimicrobial agents. Although the isolates were also susceptible to the commercial antimicrobial agents, the side effect of these agents, unavailability, adulteration and high cost make drug users more comfortable with natural products (Naveen-Prasad *et al.*,2008). In conclusion, result suggests that the extract of *Chlorella vulgaris* has good antimicrobial activity, and could be explored as a source of natural product for controlling some bacterial and fungal species.

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