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Determination of Antibacterial Activities of Spirulina *Plantensis* in Owo Local Government against Selected Pathogens

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Abstract: ***Spirulina plantensis is a filamentous, spiral-shaped alga used as a primary source of food over the years. They are suspected to have antioxidant and antimicrobial activities. Therefore, this study aimed at determining the antimicrobial activities of Spirulina plantensis extracts in Owo local government, Ondo-State, Nigeria. Spirulina platensiswas sampled from the Enete river and cultivated in the laboratory on synthetic media andriverwater. The biomass was harvested after ten days, and extraction of bioactive compounds was carried out using the maceration method. The ethanols, hot and cold-water extracts of S. plantensis were tested against pathogenic strains of Staphylococcus aureus and Pseudomonas aeruginosa using the agar well and disk diffusion methods. The extracted pigments were identified by thin-layer chromatography. The results showed that the tested microbes were resistant to thebioactive compounds in S. plantensis algal extract. Comparative antibiotic tests against the bacteria strains showed resistance to all antibiotic disks used. The growth pattern of the algae on the synthetic medium was poor as compared to that of river water used as control. There was a deterioration of the algae growth from day three of cultivation on the synthetic medium which could be attributed to nutrient depletion. Thin-layer chromatography revealed bands that confirmedthat biological components were present in the algae which were xanthophylls, chlorophyll b, chlorophyll a, Beta-carotene and phycobiliprotein.***

Keywords: Spirulina Plantensis, Thin layer chromatography, Antimicrobials, Biomass, Bioactive Components

1. Introduction

Infectious diseases are one of the major causes of high morbidity and mortality in the world especially in developing countries (Waldvogel, 2004). Increased resistance and decreased efficiency of pathogens to antimicrobials have necessitated the development of new alternatives (Smith et al. 1994). Aquatic organisms are a rich source of novel bioactive compounds. Oneof the major problems facing most communities in Nigeria is the lack of treated pump water. Hence, most communities resort to stream or river water. However, most of these freshwaters contain aquatic organisms. Hence the objective of this study is aimed to determine the antimicrobial activities of algae available in Owo, Ondo State, Nigeria.

Algae are important organisms in aquatic ecosystems and are the primary source of food. Algae are one of the major sources of bioactive compounds, these bioactive compounds have antimicrobial properties.There is increased attraction to microalgae as a natural source of bioactive molecules as they have the potential of producing bioactive compounds in culture, which are difficult to produce by chemical synthesis (Goud*et al.*, 2007; Kaushik and Chauhan, 2008). Most of those compounds are accumulated in the microalgal biomass; others are excreted during growth in the environment (Jaki *et al.*, 2001).

In addition, cyanobacterial secondary metabolites have been shown to have hypocholesterolemic properties, enzyme inhibiting, and other pharmacological effects. These metabolitesare used asnatural therapeutics but also as structural models in the production of synthetic molecules. Biologically active substances were extracted from cyanobacteria (Mundt *et al*., 2001; Volk *et al.*, 2006). Microalgae have meanwhile been found to produce antibiotics: manymicroalgal extracts and/or extracellular products have proven antibacterial, antifungal, antiprotozoal, and antiplasmodial properties. (Kellan and Walker, 1989).

Algae are a very large and diverse group of autotrophic organisms that ranges from unicellular to multicellular forms. Algae are ubiquitous in their habitat and may grow in waters of varying salinity, in freshwater, buildings, soils, and brackish water, also living mutualistic with other organisms.Algae can be divided into macroalgae (macroscopic algae) and microalgae (microscopic algae). The presence of different pigments is one of the characteristics used to classify the different types of algae.

Algae are a repertoire of bioactive compounds such as amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones, alkenes, and cyclic polysulphides. Marine algae are one of the major producers of biomass in marine environments. They produce chemically active metabolites in their surroundings, these bioactive

metabolites potentially aid in protecting them against the other settling organisms (Mo et al., 2009).

Spirulina is multicellular, freshwater photosynthetic, filamentous, spiral-shaped, blue-green microalga. The two major species are *Spirulina maxima and Spirulina platensis.* They contain carotenoids, chlorophyll, and major phycocyanin pigment. It is a cyanobacteriumcharacterized by spiral chains of cells enclosed in a thin sheath. *Spirulina* is rich in nutrients, such as proteins, vitamins, minerals, carbohydrates, and γ-linolenic acid (James *et al*., 2006). The antioxidant activity of Spirulina housesa variety of bioactive compounds such as carotene and xanthophyll phytopigments which, together with phycocyanin, these compounds seem to be related to its antioxidant activity (Pineiro*et al*., 2001).

2. Materials and Methods

2.1 Sample collection

Freshwater samples with algae were collected from flowing streams in sterile flasks from the Enetesawmill river in Owo local government, Ondo State, and transported to the laboratory.

2.2 Preparation of growth medium

The bold basal medium was prepared by dissolving the under-listed components in water as indicated and autoclaved at 121^{o} C for 15minutes to sterilize. Three modified basal media were prepared with some components removed to ascertain the best growth medium for the algae.

2.2.1Bold Basal Medium: Medium A was prepared using all the components of the bold basal medium listed below

Bold Basal Medium (BBM)

Stock:	per 400ml
$NANO_3$	10.0g
$MgSO_4$. $7H_2O$	3.0g
NaCL	1.0g
K_2HPO_4	3.0g
KH_2PO_4	7.0g
$CaCL_2.2H_2O$	1.0g
Trace elements solution (autoclave to dissolve)	per liter
$ZnSO_4$. $7H_2O$	8.82g
$MnCL_2$. $4H_2O$	1.44g
MoO_3	0.71g
$CuSO_4$. $5H_2O$	1.57g
$CO(NO_3)_2$. $6H_2O$	0.49g
H_3BO_3	11.42g
EDTA	50.0g
KOH	31.0g
$FeSO_4$. $7H_2O$	4.98g
H_2SO_4 (Conc)	1.0ml
Medium	perliter
Stock solution 1-6	10ml each
Stock solutions 7-10	1.0ml each
Makeup to 1 liter with glass distilled or deionized water	

2.2.2 Medium B: BBM -EDTA (Ethylenediaminetetraacetic Acid (EDTA)
The second medium was prepared as above (2.2.1) without the EDTA and coded as medium B

2.2.3 Medium C: BBM – KOH (Potassium hydroxide)
The third medium was prepared as above (2.2.1) but without the KOH and coded as medium C

2.2.4 Control (Enete river water).
The water sampled from the Eneteriver was sterilized and used as the control growth medium of the experimentas it had a large amount of algal growth.

2.2 Purification of algal cells

The isolated clump of algal cells was washed using the Centrifuge method ofJamie (2010). A volume of 12 ml microalga sample was taken aseptically into four centrifuge tubes. The samples were centrifuged at 5000 rpm for 5 minutes. The supernatant was removed, and the cells were suspended in fresh sterile water. The resuspended microalga sample was vortexed using a vortex mixer and rotated at 1000-1500 rpm. Centrifugation and washing were repeated six times to expel most of the microorganisms present in the algal sample. After washing individual algae cellswere viewed under the microscope and drop them inside sterile Bold Basal Medium and river water for cultivation and maintenance

2.3 Isolation of algae samples using the streak plating method

The purified algal cells were inoculated onto Petri plates on bold basal medium (1.5 % w/v agar) following the method of Yang et al. (2012). The Petri plates were incubated at 30 °C for seven days. The purities of the culture were ensured by repeated plating and observation under the microscope. A single colony was picked and inoculated into Bijou bottles with 5 ml of Bold basal medium and river water and left to grow for 10 days

2.4 Biomass production

After ten (10) days of cultivation, the culture was filtered, harvested, and weighed. Harvested algae were dried in the oven at 45^{O}C and weight was recorded every 24 hours until a constant weight was attained, and the biomass was calculated. The dried algae sample was ground into powder. 50g of the dried algal sample was steeped in 95% v/v ethanol, cold water, and hot water and allowed to stand for 3days under constant agitation. All extracts were preserved at 4^{O}C (Gonzalez *et al*., 2001) for later use.

2.5 Collection and Maintenance of Micro-organisms:

Bacteria (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) were collected from the Federal University of Technology Akure and maintained on nutrient agar slants and were kept under refrigeration at 4^{0}C until use.

2.6 Evaluation of antimicrobial activity

2.6.1 Agar well diffusion method

Antimicrobial screening of algal extracts was carried out by the agar well diffusion methods of Bibhishan and Milind (2015). Broth cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were prepared and standardized with barium sulfate to attain McFarland's constant. 20ml of molten nutrient agar was poured aseptically and allowed to set. A loopful of 18 hours of a broth culture of the test bacteria was used to streak the surface of the set agar. Wells of 6mm diameter were punched on the surface of the inoculated agar. Crude extract from algae samples was used to fill the holes and the plates were allowed to sit for 15mins to allow the extract to diffuse into the agar. Streptomycin was used as the positive control. The plates were incubated for 24hours at 37^{o}C. The diameter of the inhibition zone was measured with calipers and the results were recorded e (CLSI, 2010).

2.6.2 Minimum Inhibitory Concentration

The MIC was carried out following the method ofFan et al. (2013). Microtiter plates were filled with broth composed of *Pseudomonas aeruginosa* and *Staphylococcus aureus* with varying concentrations of the extract (15%, 25%, and 50%) and were observed to check for turbidity. The plates were put into an incubator at 37^{0}C for 24 hours. After 24 hours the plates were removed and checked for bacteria growths. The minimum inhibitory concentration (MIC) or lowest concentration of antibiotics in which no growth was observed was recorded as the MIC.

2.6.3 Antibiotics sensitivity test

The antimicrobial activity of the ethanol extract of *Spirulina plantensis* was tested against *Pseudomonas aeruginosa and Staphylococcus aureus* usingthe disk diffusion method of Muthulakshmi et al., (2012). The activity of the extract was compared to a control of standard antibiotics, using standardized broth cultures of *Pseudomonas aeruginosa and Staphylococcusaureus*. Antibiotics discs were placed on the surface of the inoculated plates and pressed gently on the plates using sterile forceps to ensure that the antibiotics discs were in firm contact with the surface of the inoculated plates. The plate was kept in an inverted position for 5 minutes. The plates were incubated upright for 24hours at 37^{o}C. After incubation, the diameter of the inhibition zone was measured around each disc in mm. The tests were carried out in triplicates and mean values were recorded. Antibiotic disk used as control include Pefloxacin (10mg), Gentamycin (10mg), Ampiclox (30mg), Zinnacef (20mg), Amoxacillin (30mg), Rocephin (25mg), Ciprofloxacin (10mg), Streptomycin (30mg), Septrin (30mg), Erythromycin (10mg), Chloramphenicol (30mg), Sparfloxacin (10mg), Augmentin (30mg) and Tarivid (30mg).

2.7 Thin-layer chromatography (TLC) of algae extract

The TLC analysis was carried out by modifying the method of Sathya (2017). The stationary phase was made by weighing 14g of Silica into 25ml of water and mixing till a slightly thick suspension was formed. With the glass slides cleaned and dried, the slides were coated with the suspension. The thickness of the layer of suspension was not more than 1mm in thickness. The plates were left to air dry to allow the plaster to set until they were white and smooth. The TLC plates were activated by oven drying at 100^{o}C for 1 hour. They were cooled and the plates were ready for use. Acetone, chloroform, and methanol were used as mobile phases in the following proportion to separate the filtrate into different compounds.

Chloroform		Acetone		Methanol
1	:	1	:	1
1	:	2	:	1
1	:	1	:	2
2	:	1	:	1
2	:	1	:	2
1	:	2	:	2
0	:	3	:	0

One drop of each extract was spotted on the two edges of the TLC and allowed to dry. The dried spotted plates were then put into the developing chamber containing the mobile phase and then covered to separate the different fractions based on their relativemobility insolvent system. The spots were stained with iodine pellets for clear observation identification. The Rf values were measured and recorded. The Rf was calculated using the formula:

$$Rf = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solute}}$$

3. Results

3.1 Physical identification of algae.

The Algae sample appeared green in color during washing, centrifugation at 5000rpm distorted the algae while centrifugation at 800rpm and 1000rpm did not.

3.2 Cultivation of algae and identification of algae

Spirulina platensis was cultured in a selective liquid medium viz. Bold Basal medium. However, the growth in freshwater (i.e., Enete river water) was more luxurious than the growth observed in other synthetic media used. Algae were identified through literature and texts.Spirulina was observed to be photosynthetic, filamentous, spiral-shaped, multicellular blue-green microalgae.

3.3. Growth presentation of isolated algae

Spirulina plantensis showed a color change from the first day of cultivation from green which was the original color of the algae to brownish green and finally brown from about the second day of cultivation in different growth mediums. However, the algae maintained its green color in the river water which serves as control until about the 7th day of cultivation. The color change is presented in table 1.

Table 1: Color change in the medium used for cultivation of *Spirulina plantensis*

Medium	Days									
	1	2	3	4	5	6	7	8	9	10
Bold Basal Medium	G	G	GB	B	B	B	B	B	B	B
BBM- EDTA	G	G	GB	B	B	B	B	B	B	B
BBM-KOH	G	GB	B	B	B	B	B	B	B	B
Enete river	G	G	G	G	G	G	G	B	B	B

KEY: G = Green, B = Brown, GB = Greenish Brown

3.4. Biomass production by *Spirulina plantensis* in different media.

It was observed during this study that the biomass decreased over time in all media used including the Enete river water that served as the control. This result is presented in table 2.

Table 2: Biomass production of *Spirulina plantensis* after cultivation

Medium	Time in hours			
	48	72	96	120
BBM	10g	6g	4g	4g
BBM –EDTA	8g	4g	2g	2g
BBM- KOH	6g	4g	2g	2g
Enete river	40g	20g	18g	18g

3.5. Antibacterial activity of *Spirulina plantensis*

The antibacterial test carried out on two bacteria*P. aeruginous* and *S.aureus* showed that the hot water, cold water, and ethanol extracts of *Spirulina plantensis* using the disk diffusion and agar well diffusion methodhad no antimicrobial activity. Similarly, the antimicrobial activity using the microtiter plate did not show any activity even at concentrations ranging from 12.5mg/ml to 100mg/ml.

3.6. Comparative antimicrobial activity using standard antibiotics.

Pefloxacin was effective against both *S. aureus* and *P. aeruginosa* with zones of inhibition ranging from 8mm and 5mm respectively. In the same vein, ciprofloxacin showed a zone of inhibition of 6mm against *P. aeruginosa* while septrin had a zone of inhibition of 7mm against *S aureus.* However, Zones of inhibition less than 12mm are considered resistant. Therefore, the test organisms are classified as resistant to the antibiotics tested. This is presented in table 3.

Table 3: Antibacterial activity of standard antibiotic disc against the test organism

Antibiotics	Zone of inhibition	
	S. aureus	*p. aeruginosa*
Pefloxacin	8mm	5mm
Gentamycin	-	-
Ampliclox	-	-
Zinnacef	-	-
Amoxicillin	-	-
Rocephin	-	-
Ciprofloxacin	-	6mm
Streptomycin	-	-
Septrin	7mm	-
Erythromycin	-	-

3.7 Chemical components of *S. plantensis*

Extracts of *S. plantensis* were subjected to thin-layer chromatography analysis to ascertain the chemical components of the algae. Several fractions were observed. In the cold-water extract, only one band was observed and the Rf value was 0.42 which was brown.The ethanol extract presented two bands with Rf values of 0.54 and 0.63. The two bands were green in color. Similarly, the hot water extract produced two bands with Rf values of 0.76 and 0.84. Both bands were brown.

4. Discussion

The use of freshwater algae in the production of antimicrobials is of interest as the menace of antibiotic resistance is threatening to take the world back to the pre- penicillin era.

In recent times, there has been much interest in the use of freshwater algae in the production of valuable molecules ranging from therapeutic proteins to biofuel. Algae are known for producing several biologically active compounds with antiviral, antibacterial, antifungal, and anticancer activities. The extraction of bioactive compounds differs according to the nature of the solvent used (Prarthana and Maruthi, 2018).

Several authors recorded the antibacterial activities of freshwater algae. Thomas *et al.,* (2021) observed activities of spirogyra against multi-drug resistant *S. aureus and S.xylosus strains*. Das *et al*., 2005 also discovered that the organic solvent extracts of *Euglenaviridis* were active against virulent strains *of Pseudomonas putida, P. fluorescens, Aeromonashydrophila, Edwardsiellatarda, Vibrio alginolyticus, V. anguillarum, V. parahemolyticus,* and *Escherichia coli.*

All these observations were contrary to the result obtained in this study. Both cold and hot aqueous extract did not show any antibacterial activities against the bacterial strains used. Similarly, the ethanol extract was not active against the test bacteria. Several reasons may be responsible for this. *S. platensis* may not contain any bioactive compound, and the extraction procedure may not have extracted the active components. The lack of antibacterial activity could also be a result of the consistency of the extract in the agar well diffusion method. Literature has shown that thick extract may not diffuse easily into agar medium and therefore reduce the activity of this extract (Cavenaghi *et al*., 1992). Furthermore, it could be that the test organisms used have a high level of resistance. Studies have shown that *Staphylococcus aureus* and *Pseudomonas aeruginosa* have a high level of resistance to antimicrobials (Avestiyan *et al*., 2015).

Similarly, Bibhishan and Milind (2015) found that cold water, hot water, ethanol, and methanol extracts did not show any inhibitory effect against all bacteria while acetone,

chloroform, petroleum ether, and toluene showed notable antibacterial activity against selected bacterial strains. All algal extracts did not show any antibacterial activity against *Pseudomonas aeruginosa*.

The comparative antibiotic test against the organisms using standard antibiotics also showed to a greater degree a high level of resistance which could also be responsible for the inactivity of the extracts of *Spirulina plantensis* against these notoriously resistant bacteria. Although there were slight antibacterial activities of the antibiotic disk against the test organisms (Table 2). However, Adebolu and Oladimeji (2005) in their work suggested that any zone of inhibition less than 10mm is not considered to be effective.

The extracting medium viz-a-viz water may also not be able to extract active components. Many authors have established the fact that the efficacy of plant components depends on the polarity of the extracting medium. Furthermore, the efficacy of compounds from plant materials is influenced by the extraction technique, the extraction time, and the temperature. Organic solvents are known to extract more bioactive components than water. Abdullah *et al*., (2014) noted that water was not a good extraction medium.

The cultivation of algae using different media did not produce appreciable biomass, this could be that the method of cultivation was not conducive to optimal growth of the algae. Llavarsasi*et al.,* (2011) in their work discovered that a bold basal medium supported the growth of *Spirulinaplantensis* at 12^0C, the temperature difference could also be a factor in the inability of algae to grow in a bold basal medium because the experiment was carried out at room temperature. Soni *et al.,* (2019) suggested an open well-aerated medium with a pH of between 9.5 -9.8 for maximum biomass yield. He further opined that a closed reactor system produces a greater yield than the open realtor system. Most of the parameters highlighted by Soni *et al*., (2019) were not strictly adhered to. This could explain the low growth rate and poor biomass production by the algae during cultivation. However, since *Spirulina plantensis* grew exceedingly well in the river water medium, it is safe to assume that open cultivation especially in the natural habitat of the organism is better than synthetic medium. Bark (2012) reported that the open pond system in the cultivation of algae is better and more durable than the closed synthetic medium.

Biofuel production has been reported as first and second-generation based on the manufacture of biofuels using biomass as sustainable biological resources (Abdeshahian *et al*.2010). The extraction of bioactive compounds differs according to the nature of the solvent used (Prarthana and Maruthi, 2018).

The color change observed in the culture from the second to the third day of cultivation of alga could be because of the depletion of nutrients in the medium. This is evidenced by the decrease in biomass production over time.TLC analysis showed the presence of pigments with corresponding Rf values. That presupposes that the content of the river water stimulated the production of pigments which are suggested based on the Rf values of 0.42, 0.54, 0.63, 0.84, and 0.77 to be xanthophylls, chlorophyll b, chlorophyll a, Beta-carotene and phycobiliprotein respectively. The Rf value was compared with literature data (Jeffrey *et al*., 2011 Sathya 2017). However, some of these pigments have been reported to have antimicrobial properties. Prakash et al. (2007), Bhagavathy et al. (2011), Muthulakshmi et al., (2012),and Fan et al. (2013) reported biological activities of pigments found*in S. platensis. Anabaenacylindrical Chlorococcum, humicola, Nostoc* against *Escherichia coli, S. typhimurium, K. pneumoniae, V. cholerae, S. aureus, B. subtilis, Streptococcus sp., Pseudomonas sp., Bacillus sp., Staphylococcus sp., E. coli,and Enterobacteriaaerogenes.*

5. Conclusion

The study showed the tested bacteria wereresistant to the cold water, hot water, and ethanol extracts of *Spirulina plantensis.* However, it confirmed the presence of bioactive compounds such as xanthophylls, chlorophyll b, chlorophyll a, Beta-carotene and phycobiliprotein. The study showed that the natural growth medium (river water) increased the growth of the algae as compared to the synthetic medium which reduced the growth and biomass of the algae

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