Antimicrobial, Antioxidant Potential and Chemical Composition of the Methanolic Extracts of *Spirogyra setiformis* and *Navicula* species

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Abstract

The present study investigated the antimicrobial and antioxidant activities as well as the chemical composition of the methanolic extracts of *Spirogyra setiformis and Navicula spp.* found in Nigerian waters. The minimum inhibitory concentration (MIC) of the methanolic extracts of the algae were determined against 7 clinical isolates (*Enterococcus faecalis, Staphylococcus aureus ATCC 25923, Bacillus cereus, Klebsiella pneumoniae, Salmonella typhi, Escherichia coli ATCC 25922 and Candida albicans*) using the broth dilution method. The antioxidant activity of the algae extract was assessed using 1,1-diphenyl-1-picrylhydrazyl, nitric oxide and reducing power assays. The volatile constituents of the extracts were also analysed using GC–MS. The results revealed that the *S. setiformis* extract inhibited all the clinical isolates except *S. typhi* and *C. albicans* while *Navicula spp.* had inhibitory effects on all the test isolates. The MIC values of the algae extracts varied between 2.0 mg/L and 10.0 mg/L. Both extracts showed potent free radical scavenging activities evidenced in DPPH, nitric oxide and reducing power assays. A total of 16 and 11 volatile compounds were obtained from *S. setiformis* and *Navicula spp.*, respectively. The major component in *S. setiformis* was 11,14,17-methyl ester eicosatrienoic and *trans*-geranyl geraniol in *Navicula spp.* These findings suggest that these indigenous algae species could be good sources of antimicrobial and antioxidant agents.

Keywords: antimicrobial, antioxidant, GC–MS, *Navicula spp., Spirogyra setiformis*

Introduction

Algae are ubiquitous organisms that are found throughout the biosphere. They have been known as rich sources of structurally and biologically active metabolites (Ely *et al*., 2004). It is estimated that over 15,000 compounds have been isolated from about 30,000 algal species, which exist on the surface of the earth (Cardozo *et al*., 2007; Rodríguez-Meizoso *et al*., 2010). Some of these isolated bioactive compounds have been reported to possess anti-inflammatory, antioxidant, antiviral, antibiotic and cytotoxic activities (Salvador *et al*., 2007; Khanavi *et al*., 2010; Liu *et al*., 2011; Ibañez *et al*., 2012).

In recent times, attention has been geared towards the use of botanicals for antimicrobial purposes against the use of synthetic antibiotics (Mbosso *et al*., 2010; Pradhan *et al*., 2014). Unlike single active-agent antibiotics, microbial resistance to botanicals or whole plant extracts is rare because they contain many bioactive compounds, which exert antimicrobial effects via numerous modes of action.

The ability of algae to produce bioactive compounds enables them to resist microbial attack and photo-oxidative stress. This trait makes them excellent sources of antimicrobial and antioxidant agents, respectively (Salvador *et al*., 2007; Rodríguez-Meizoso *et al*., 2010; Okunowo *et al*., 2016). There have been several studies on the antioxidant and antibacterial compounds from algae in different parts of the world (Cornish and Garbary, 2010; Seenivasan *et al*., 2010; Sushanth and Rajashekhar, 2013).

However, little or no report is available on the antioxidant and antimicrobial properties of macro- and micro-algae from Nigeria. Hence, this research aims to determine the antimicrobial, antioxidant and chemical composition of two algal species found in Nigerian waters.

Materials and Methods

Microbial Isolates

The clinical isolates used in this study were obtained from the Department of Medical Microbiology and Parasitology, University of Lagos, Lagos, Nigeria and they included *Bacillus cereus, Escherichia coli ATCC 25922, Enterococcus faecalis, Klebsiella pneumoniae, Staphylococcus aureus ATCC 25923,*

Salmonella typhi and *Candida albicans*. The isolates were grown in nutrient agar media (Oxoid Ltd., Basingstoke, UK), slant at 37 °C (bacteria) and potato dextrose agar (*C. albicans*) slant, and stored at 4 °C. The isolates were reactivated before use by subculturing them at 37 °C for 24 hrs on the nutrient and potato dextrose agars (PDA) at 25 °C for 3 days.

Algae Collection and Propagation

Marine microalgae (*Navicula spp.*) used in the study were obtained from the Nigerian Institute for Oceanography and Marine Research, Lagos, Nigeria while the *Spirogyra setiformis* was obtained from the pond behind the Faculty of Science, University of Lagos. It was identified and authenticated by Dr. Charles Onyema (Department of Marine Sciences, University of Lagos, Nigeria). The algae were grown in Guillard F medium (Okunowo *et al*., 2016) and the samples were harvested by centrifugation at 4000 rpm for 15 mins, dried in a vacuum and weighed.

Algae Extract Preparation

The extracts were prepared as described by Okunowo *et al*. (2016). Briefly, 20 g of dried algae was added to 100 mL of methanol and allowed to stand for 24 hrs at room temperature. This was centrifuged at 4000 rpm for 15 mins to obtain the supernatant, which was concentrated to dry pellets *in vacuo* using a rotary evaporator (40–50 °C), weighed and stored at room temperature in a sterile airtight tube.

Minimum Inhibitory Concentration (MIC)

The MIC of the extracts were determined by the tube dilution method with slight modifications. The stock extract was made by dissolving the algae extract (200 mg) in methanol (2 mL). To a sterile tube containing the nutrient broth, the stock extract was added in concentrations ranging from 1–10% (v/v), at room temperature. 100 µL of the isolate (McFarland: 1.5 x 10^8 cells mL^{-1}) was added to each tube and incubated for 24 hrs at 37 °C (bacteria) and 28 °C (*C. albicans*). The experiment was conducted in triplicates for each isolate. The MIC values were determined as the lowest concentration of algae extracts inhibiting the growth of the isolates (no turbidity) in at least 2 test tubes. The MIC values for the positive control drugs: Ciprofloxacin infusion (2 mg/mL) and Nystatin suspension (0.05% w/v) were also obtained for the bacteria and fungi isolates, respectively.

DPPH Radical Scavenging Activity Assay

The ability of the algae extracts to scavenge stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was estimated as described (Cuendet *et al*., 1997; Burits and Bucar, 2000). An aliquot of extract (0.5 mL) in ethanol (95%) at different concentrations (25, 50, 75, 100 µg/mL) was mixed with 2.0 mL of DPPH solution (0.004 g in 100 mL methanol). The mixture was shaken vigorously and left to stand at room temperature. The decrease in absorbance of the test mixture (due to DPPH free radicals quenching) was read at 517 nm after 30 mins. Ascorbic acid was used as reference standard while methanol was used as the blank. The scavenging effect was calculated using the expression:

$$\% \text{ Inhibition } = \frac{A_o - A_1}{A_o} \, x \, 100 \qquad (1)$$

where A_o is the absorbance of the blank sample and A_1 is the absorbance of the extract or control.

Reducing Power Assay

The reductive ability of the algae extracts to convert Fe^{3+} to Fe^{2+} was assayed, with slight modifications, from the method of Kuda *et al*. (2005). Different concentrations (25, 50, 75 and 100 µg/mL) of the extracts in deionised water (1.0 mL) were added to the phosphate buffer (2.5 mL) and potassium ferricyanide (2.5 mL). The mixture was incubated at 50 °C for 20 mins. An aliquot of 10% w/v trichloro-acetic acid (2.5 mL) was added to the mixture and centrifuged at 3000 rpm for 10 mins. The supernatant (2.5 mL) was then mixed with distilled water (2.5 mL) and freshly prepared ferric chloride solution (0.1% w/v; 0.5 mL). The absorbance of the mixture was measured at 700 nm. The above procedure was repeated with the ascorbic acid standard.

Nitric Oxide Scavenging Activity Assay

4 mL of the algae extracts at different concentrations (25, 50, 75, 100 µg/mL) was taken in different test tubes; into which 1 mL of sodium nitroprusside (25 mM in phosphate buffered saline) solution were added and incubated for 2 hrs at 30 °C. 2 mL of the incubated mixture was then added to 1.2 mL of Griess reagent and measured at 550 nm (Alisi *et al.*, 2008). Ascorbic acid was used as the standard. The % inhibition was calculated using equation (1).

GC–MS Analysis of Volatile Compounds

The algae extract was dissolved in hexane and left for 24 hrs. This was then filtered in a Pasteur pipette containing anhydrous sodium sulphate and cotton wool to absorb moisture. The volatile compounds were identified using standard reference compounds stored in the mass spectra library of the GC–MS database and also by matching the mass spectra fragmentation patterns with those of the National Institute of Standards and Technology (NIST).

Statistical Analyses

6 replicates per treatment were used. Linear increases or decreases in the bioactivity of the toxins were

compared separately between treatments using the general linear model (GLM) with concentration set as the continuous predictor. The mean differences in phytotoxin production by various treatments (effects of varying pH, light, media, and carbon and nitrogen sources) were compared using the one-way analysis of variance (ANOVA). When significant differences existed among treatments, post-hoc Tukey tests were employed to compare the treatments. Effects of heat and light on the phytotoxicity of preparations were analysed using the two-way analysis of variance and the Bonferroni post-test of significant pairwise comparisons. Analyses were made with GraphPad Prism (5.00) for Windows (GraphPad Software, San Diego, USA), defining significance as $P < 0.05$.

Results

The MIC of the methanolic extract of *S. setiformis* showed good inhibitory activity on all test isolates at varying concentrations except on *S. typhi* and *C. albicans* while the *Navicula spp.* extract inhibited all the bacterial isolates. The extracts of *S. setiformis* and *Navicula spp.* had MIC values from < 2.0 mg/mL and 10.0 mg/mL, respectively (Table 1).

All the 3 antioxidant activity assays used showed significant increases ($P < 0.05$), with increasing concentration. Ascorbic acid was used as a positive control and exhibited the highest antioxidant activity. Both extracts showed dose-dependent scavenging activities.

The results of the free radical scavenging potentials of the 2 algae extracts at varying concentrations of 25, 50, 75 and 100 µg/mL were tested with the DPPH assay as shown in Figure 1. The scavenging effects of the 2 methanolic algae extracts and ascorbic acid standard on the DPPH radical increased in the order: *Navicula spp.* < *S. setiformis* < ascorbic acid, at 25, 75 and 100 µg/mL. There was a significant difference ($P < 0.05$) in the scavenging activity of the methanolic extract of *Navicula spp.* at 75 and 100 µg/mL compared to the standard. No significant difference ($P > 0.05$) between the *S. setiformis* extract and standard was observed.

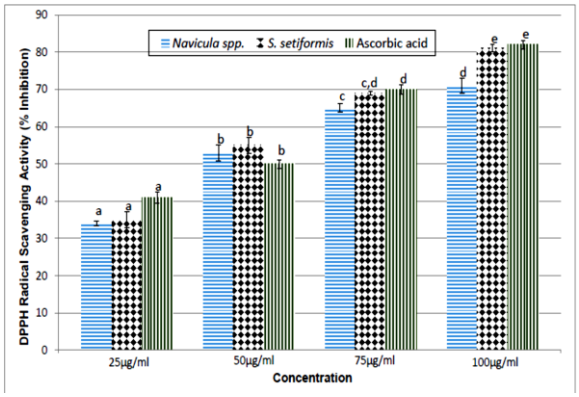

Figure 1: DPPH Radical Scavenging Activity

The methanolic extract of both algae inhibited nitric oxide in a dose-dependent manner (Figure 2). The inhibitory effect of ascorbic acid on nitric oxide was significantly ($P < 0.05$) higher than that of the methanolic extracts of *S. setiformis* at 25 and 100 µg/mL and *Navicula spp.* at test concentrations. The NO scanvanging effect of the algae extracts increased significantly ($P < 0.05$) with concentration increase.

The reducing power assay of ascorbic acid was significantly ($P < 0.05$) higher than that of *Navicula spp.* at 100 µg/mL and *S. setiformis* at 50, 75 and 100 µg/mL (Figure 3). Higher absorbances, relative to the standard, typically indicate a higher reducing power.

In the GC–MS, 16 and 11 volatile compounds were identified in the methanolic extracts of *S. setiformis* and *Navicula spp.*, respectively. The main compounds found in *S. setiformis* included 11,14,17-methyl ester eicosatrienoic acid (14.60%), bicyclo[4.2.0]oct-7-ene (8.47%) and 2,6,10-trimethyl dodecane (8.27%) while the major compounds identified in *Navicula spp.* included *trans*-geranyl geraniol (35.40%), 2-octyne (25.82%) and *n*-hexa-decanoic acid (23.37%) (see Tables 2 and 3).

Table 1: MIC values of the methanolic extracts of *Spirogyra setiformis* and *Navicula spp.*

Organisms	Control (mg/mL)		Concentration (mg/mL)	
	Ciprofloxacin	Methanol	*S. setiformis*	*Navicula spp.*
Enterococcus faecalis	0.63 ± 0.02	NI	10.0 ± 1.00	4.0 ± 0.58
Staphylococcus aureus ATCC 25923	0.63 ± 0.02	NI	10.0 ± 1.00	10.0 ± 1.00
Bacillus cereus	0.63 ± 0.01	NI	< 2.0 ± 0.58	10.0 ± 1.00
Klebsiellia pneumoniae	1.25 ± 0.03	NI	10.0 ± 1.00	10.0 ± 1.00
Salmonella typhi	< 0.31 ± 0.01	NI	NI	< 2.0 ± 0.58
Escherichia coli ATCC 25922	0.63 ± 0.02	NI	10.0 ± 1.00	< 2.0 ± 1.00
Nystatin				
Candida albicans	< 0.31 ± 0.01	NI	NI	< 2.0 ± 0.58

Values are expressed as mean ± SEM, NI = No inhibition, (n = 3)

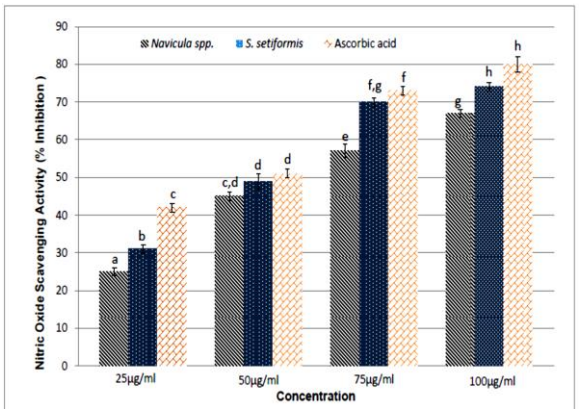

Figure 2: Nitric oxide scavenging activity

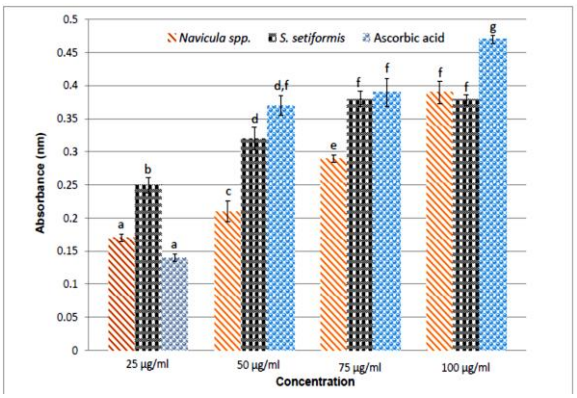

Figure 3: Reducing power assay

Discussion

Algae have been found to possess many bioactive compounds; some of which are antibiotic in nature. The resistance of *S. typhi* and *C. albicans* to the methanolic extract of *S. setiformis* has also been documented for some *Spirogyra spp.* (Kamenarska *et al.*, 2000; Chowdhury *et al.*, 2015). Similarly, the inhibitory effect of *Navicula spp.* on test bacterial isolates is in conformity with the previous claim on its broad spectrum antibacterial activity by Findlay and Patil (1984), who showed that the algae extract inhibited the growth of some Gram positive and Gram negative bacteria including *S. aureus, E. coli* and *S. typhi.*

Junthip *et al.* (2013) reported that the ability of the algae extracts to scavenge free radicals indicated that algae are electron donors, which can react with free radicals to convert them to stable products thereby terminating radical chain reactions. The potent free radical scavenging effects of the algal species studied suggest that algae are a good source of antioxidants. Reports have also shown that *Spirogyra spp.* such as *S. neglecta* and *S. porticalis* possess good antioxidant properties (Thumvijit *et al.*, 2013; Kumar *et al.*, 2015). Also, studies have shown that *Navicula spp.* such as *N. incerta* and *N. clavata* contain antioxidants (Affan *et al.*, 2007; Hemalatha *et al.*, 2013). *Spirogyra* species with antioxidant activities have been shown to possess vasorelaxative properties, which have been explored for cardiovascular diseases (Kang *et al.*, 2015).

Algae with antioxidant abilities have health benefits, which include: anti-inflammatory, anticoagulation, anti-ageing and hypocholesterolemic effects as well as protection against cancer and neurodegenerative disorders and the inhibition of H_2O_2-mediated DNA damage (Munir *et al.*, 2013). The algae in this study may, therefore, possess some of these health benefits owing to their high antioxidant properties.

Table 2: Volatile components of the methanolic extract of *Spirogyra setiformis*

Peak No.	Compounds	Retention Time (mins.)	Proportion (%)
1	2,6,10,15-Tetramethylheptadecane[a]	7.067	7.78
2	Tetradecane[a]	7.999	3.51
3	2-Methoxy-4-(1-propenyl)phenol[d]	8.795	2.98
4	Heptadecane[a]	12.182	7.96
5	2,4-bis(1,1-Dimethylethyl) phenol[d]	12.674	7.01
6	2,6,10-Trimethyl-dodecane[a]	13.296	4.06
7	1-Dodecene[a]	14.568	4.05
8	Heneicosane[a]	17.320	7.47
9	1-Iodo-octadecane[b]	18.305	4.65
10	2-Dodecanol[c]	19.191	4.73
11	Bicyclo[4.2.0]oct-7-ene[a]	21.440	8.47
12	Heptacosane[a]	21.944	5.57
13	10-Methyl undecanoic acid[e]	22.024	3.47
14	2,6,10-Trimethyl dodecane[a]	22.808	4.21
15	11,14,17-Methyl ester eicosatrienoic acid[e]	25.446	14.06
16	*N*-(4-Bromo-*n*-butyl)-2-piperidinone[f]	25.703	4.27
17	Hexatriacontane[a]	26.109	3.15
18	Trichlorooctadecyl silane[b]	26.887	2.58
		Total	**99.98**
	Compound class		
a	Alkanes and Alkenes		56.23
b	Halogenated hydrocarbon		7.23
c	Alcohol		4.73
d	Phenol		9.99
e	Fatty acids and Esters		17.53
f	Others		4.27
		Total	**99.98**

The presence of volatile compounds, such as long-chain hydrocarbons, fatty acids, esters and alcohols in the methanolic extracts of *S. setiformis* and *Navicula spp.* has also been reported for some *Spirogyra spp.* (Kamenarska *et al.*, 2000; Abdel-Aal *et al.*, 2015;

Kumar *et al*., 2015). Geranyl geraniol, which is the main component of *Navicula spp.* in this study, has been reported to exhibit antibacterial activity against *Staphylococcus aureus* (Inoue *et al*., 2005; Togashi *et al*., 2008). This and other volatile components from *Navicula spp.* may have contributed to the observed antimicrobial activities of the extract.

Table 3: Volatile components of the methanolic extract of *Navicula spp.*

Peak No.	Compounds	Retention Time (mins.)	Proportion (%)
1	1,2,3,4,5,6,7,8-Octahydro-1,4,9,9-tetramethyl-,4,7-methanoazulene[b]	14.602	0.89
2	Copaene[b]	15.329	4.77
3	*n*-Hexadecanoic acid[d]	23.780	5.45
4	*n*-Hexadecanoic acid[d]	23.952	4.23
5	*n*-Hexadecanoic acid[d]	24.072	4.57
6	*n*-Hexadecanoic acid[d]	24.107	1.92
7	*n*-Hexadecanoic acid[d]	24.238	7.20
8	*cis*-7-Dodecen-1-yl acetate[d]	26.241	2.26
9	*trans*-Geranyl geraniol[b]	27.156	35.40
10	2-Octyne[a]	27.311	17.20
11	2-Octyne[a]	27.339	8.62
12	2,2-Dimethyl-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl ester propanoic acid[d]	27.500	0.81
13	6-Methyl-3,4-pyridine dimethanol[e]	27.557	0.99
14	1-Ethenyl-1-methyl-2,4-bis(1-methylethenyl) cyclohexane[a]	27.980	0.75
15	1-Methyl-5-(1-methylethenyl) cyclohexene[a]	28.472	1.69
16	1-Ethenyl-1-methyl-2,4-bis(1-methylethenyl) cyclohexane[a]	28.645	1.76
17	*cis*-*Z*-*α*-Bisabolene epoxide[c]	29.645	1.46
		Total	**99.97**
	Compound class		
a	Alkanes and Alkenes		30.02
b	Terpenes		41.06
c	Oxygenated sesquiterpenes		1.46
d	Fatty acids and Esters		26.44
e	Others		0.99
		Total	**99.97**

Conclusion

In conclusion, this study has shown that the methanolic extracts of *S. setiformis* and *Navicula spp.* possess good antimicrobial and antioxidant properties and could be explored for the development of antibiotics against infectious diseases as well as in the development of dietary antioxidant supplements to relieve oxidative stress and/or associated disease conditions.

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