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Research Article

GC-MS Analysis and Antimicrobial Properties of Methanolic Extracts of the Marine Algae *Skeletonema costatum* and *Chaetoceros* spp.

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ABSTRACT: In this report, we describe the antimicrobial potential and phytochemical constituents of two Nigerian marine algae; *Skeletonema costatum* and *Chaetoceros* spp. The minimum inhibitory concentration (MIC) of the methanolic extract of the algae spp. was determined against 7 clinical isolates (*Enterococcus faecalis, Staphylococcus aureus* ATCC 25923*, Bacillus species, Klebsiella pneumonia, Salmonella typhi, Escherichia coli* ATCC 25922 *and Candida albicans*) using broth dilution method. The volatile constituents of the extracts were analyzed using the Gas Chromatography- Mass Spectrometry technique. The results showed that *S. costatum* inhibited all isolates except *Salmonella typhi* and ATCC *E.coli* while *Chaetoceros* spp. had no inhibitory effect on *Candida albicans*. The MIC values of the two algae extract was least; < 2.0 mg/ml and highest; 10 mg/ml for the test isolates. A total of 42 and 25 volatile compounds which includes 9- Octadecenamide, (Z) - as the major constituent were obtained in *S. costatum* and *Chaetoceros* spp. respectively. Other compounds identified were known antimicrobial compounds; 1-Hexacosanol, Eugenol, Benzenesulfonamide, 4-methyl-N-phenyl- and Thiazole, 4-(4-aminophenyl)-2-methylamino- as well as some phytochemicals such as flavonoids, tannins, terpenoids, steroids and phenols. These may account for the antibiotic property of the algae extracts. This study showed that *S. costatum* and *Chaetoceros* spp. possess antimicrobial compounds which may be explored for therapeutic purposes.

KEYWORDS: Antimicrobial, Marine algae, Clinical isolates, GC-MS, Phytochemicals.

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INTRODUCTION

There is growing concern for antibiotic drug resistance by pathogenic organisms (Pradhan *et al*., 2014). In recent times, attention has been focused on the search for novel natural antibiotic compounds (Okunowo *et al*., 2013). In this part of the world, antimicrobial agents are mostly sourced from medicinal plants of terrestrial origin. However, there are several reports of therapeutic antimicrobial agents from marine source (Ibañez *et al*., 2012; Pradhan *et al*., 2014).

Marine algae have received immense attention for the discovery of new bioactive molecules (Bhatnagar and Kim, 2010; Sithranga Boopathy and Kathiresan, 2011). It is estimated that about 30,000 distinct microalgae inhabit earth with over 15 000 isolated novel compounds (Cardozo *et al*., 2007; Rodríguez-Meizoso *et al*., 2010).

Reports have shown that bioactive compounds from microalgae posses antioxidant, antimicrobial and antiviral activities (Plaza *et al*., 2010; Rodríguez-Meizoso *et al*., 2010) and several studies have extensively investigated the antibacterial compounds from algae (Rajasulochana *et al*., 2009; Salvador Soler *et al*., 2007; Seenivasan *et al*., 2010). Some of these therapeutic compounds are produced as secondary metabolites and used by algae as defense mechanisms against microbial attacks.

However, little information is available on the antimicrobial properties of marine algae found in Nigeria waters. Hence, this research aims to determine the chemical composition and antimicrobial potential of two marine algae indigenous to Nigerian water.

MATERIALS AND METHODS

Microbial Isolates

Seven clinical isolates (*Enterococcus faecalis, Klebsiella pneumonia, Staphylococcus aureus* ATCC 25923*, Candida albicans, Salmonella typhi, Bacillus species and Escherichia coli* ATCC 25922*)* used in this study were obtained from the Department of Medical Microbiology and Parasitology, Faculty of Basic Medical Sciences, College of Medicine, Idi-Araba, Lagos State, Nigeria. The bacterial isolates were grown on Nutrient agar medium at 37 °C while the fungus was grown on Potato Dextrose Agar (Okunowo *et al*., 2013). The stock cultures were maintained on the nutrient medium at a temperature of 4 °C and subcultured at 37 °C for 24 hours on the Nutrient Agar and Potato Dextrose Agar (PDA) slant at 25 °C for 3 days.

Algae collection and propagation

Two marine microalgae (*S. costatum* and *Chaetoceros* spp) used in the study were obtained from and identified at the Nigerian Institute of Oceanography and Marine Research (NIOMR), Lagos State in the month of October 2014. The algae were grown in Guillard F medium at a temperature of about 22 ^{o}C with continuous illumination (with a 30 W fluorescence bulb) and agitation for one week. The algae species were subcultured weekly (Guillard 1975). The algae samples were harvested by centrifugation at 4000 rpm for 15 minutes. The algae pellet obtained were dried in a vacuum and weighed.

Preparation of Algae extract

A 20 g dried algae sample was dissolved in 100 ml of methanol for 24 hours. The extract was obtained by centrifugation at 4000 rpm for 15 minutes. The supernatant (extract) was concentrated to dryness under reduced pressure using rotary evaporator (40-50°C) and pellet obtained was weighed and stored in an airtight sterile 10 mL tube at room temperature until analysis (Zakaria *et al*., 2011). A sample of the dried extract (2 g) was dissolved in 1 ml methanol to obtain a stock solution of 2g/ml. The stock solution was further serially diluted with methanol to 10%, 1%, 0.5%, 0.2% and 0.1% w/v corresponding to 200, 20, 10, 4, and 2 mg/ml respectively.

Antibacterial and Antifungal Screening of Extract

The antimicrobial activities of the extract were determined by agar well diffusion method as described in our earlier studies (Okunowo *et al*., 2013). Microorganisms showing inhibitions to the extract were further assayed for MIC values.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the algal extract was determined by the tube dilution method (Singh *et al*., 2011). Stock algae extract was made by dissolving 200 mg algae extract in 2 ml methanol. To a sterile tube contaning the nutrient broth (NB), the stock algae extract was added at room temperature to concentrations ranging from 1.0 – 0.1% (v/v). A 100 µl sample of the innoculate (McFarland: 1.5 x 10^{8} cells ml^{-1}) was also added and tubes were incubated at 37 ^{o}C for 24 hours. The tubes were prepared in triplicates for each test concentration, the tubes were incubated for 18-24 h at 37 °C (bacteria) and 28 °C (fungi) respectively. The MIC was determined as the lowest concentration of algae extract inhibiting bacterial growth in a minimum of two test tubes. The MIC value for the positive control drugs 2 mg/ml Ciprofloxacin infusion and 0.05% w/v Nystatin suspension were obtained for bacteria and fungi isolates respectively following the above procedures.

Table 1: MIC Values of Methanolic Extract of *Skeletonema* and *Chaetoceros spp.* on Microorganisms.

Organisms	Control (mg/ml)		Concentration (mg/ml)	
	Ciprofloxacin	Methanol	*Skeletonema costatum*	*Chaetoceros spp*
Gram positive				
Enterococcus faecalis	0.63	NI	10.0	< 2.0
Staphylococcus aureus ATCC 25923	0.63	NI	4.0	10.0
Bacillus spp	0.63	NI	10.0	< 2.0
Klebsiella pneumonia	1.25	NI	< 2.0	< 2.0
Gram negative				
Salmonella typhi	< 0.31	NI	NI	< 2.0
Escherichia coli ATCC 25922	0.63	NI	NI	< 2.0
	Nystatin	**Methanol**		
Fungus				
Candida albicans	< 0.31	NI	< 2.0	NI

GC-MS chromatography of volatile compounds of algae extract

This was done by dissolving the algae samples in hexane and left for extraction for 24 hours, after which anhydrous sodium sulphate and cotton wool was placed in a Pasteur pipette and the sample was filtered through the cotton wool to absorb the water present in the sample. The GC-MS was performed using an Agilent 7890 gas chromatograph (Agilent Technologies, U.S.A) system equipped with Agilent 5975C mass spectrometer detector operating in electron impact mode (ionization voltage, 70 eV). A Chrompack HP-5MS capillary column (30 m length × 0.32 mm internal diameter, film thickness 0.25 µm) was used for the separation of the sample components. A 1.0 µL of the extract was injected, using splitless mode, into the chromatograph at injector temperature of 250 °C and Helium was used as the carrier gas at a flow rate of 5 ml/min with inlet pressure of 12.936 psi. The column oven temperature was programmed to increase from 80 °C to 240 °C at a rate of 5 °C/min, with a final hold time of 5 minutes. The compounds were identified using standard reference compounds and also by matching the mass spectra fragmentation pattern with National Institute of Standards and Technology (NIST) Mass Spectra Library stored in the GC-MS database.

Phytochemical screening

Phytochemical screening of the methanolic extracts of the algae samples were carried out using standard procedures (Edeoga *et al*., 2005; Sofowora, 1993).

Tannins: A 0.5 g sample of dried algae was boiled in 20 ml of water in a test tube and it was filtered. Few drops of 0.1% Ferric Chloride was added and observed for a brownish green or blue black colouration.

Phlobatannins: Few drops of the methanolic extract of the algal sample was boiled in 1% HCl in a test tube and the sample was observed for red precipitate deposition.

Alkaloids: A 5 mg algae extract dissolved in 3 ml of acidified ethanol was warmed slightly and then filtered. Few drops of Mayer's reagent and 1 ml of Dragendroff's reagent were added to 1 ml of the filtrate and turbidity was observed.

Saponins: The algae sample was boiled in 20 ml of distilled water in a water bath and then filtered. A 10 ml sample of the filtrate was mixed with 5 ml of distilled water and shaken vigorously to a stable persistent froth. The Froth was mixed with 3 drops of olive oil, shaken vigorously, and then observed for the formation of emulsion.

Table 2: Volatile components of methanolic extract of *Skeletonema costatum*

Peak No	Compounds	Retention time (min.)	Proportion (%)
1	Nonadecane[a]	7.067	1.91
2	5-methyl Undecane[a]	8.005	1.06
3	3,6-Dimethyl-2-nitrobenzaldehyde[f]	8.789	1.01
4	1-iodo- octadecane[b]	12.193	2.53
5	Dodecanal[f]	12.560	2.05
6	2,4-bis (1,1-dimethylethyl) phenol[e]	12.674	2.58
7	Heptadecane[a]	13.304	1.52
8	9-Octadecene, (E)- [a]	14.568	0.88
9	Tetradecane[a]	14.751	0.82
10	n-Tetracosanol-1[c]	16.004	0.68
11	Hexatriacontane[a]	17.109	1.30
12	Tetratetracontane [a]	17.320	2.25
13	1-chloro Tetradecane[b]	17.698	0.82
14	Heptadecane[a]	18.310	1.83
15	Oxalic acid, allyl octadecylester[h]	19.197	1.12
16	Nonadecane[a]	19.340	1.04
17	Hexadecane[a]	21.274	1.56
18	1-chloro- Heptacosane[b]	21.469	1.33
19	2-Piperidinone, N-[4-bromo-n-butyl)[m]	21.578	0.80
20	Oxalic acid, allyl hexadecyl ester[h]	21.864	1.01
21	Heneicosane[a]	21.944	2.48
22	Hexadecanoic acid, methyl ester[h]	22.030	1.66
23	Oxalic acid, 6-ethyloct-3-yl heptyl ester[h]	22.356	0.71
24	2H-1,3-Benzimidazol-2-one, 5-amino -1,3 dihydro[m]	22.751	1.55
25	2-methyltetracosane[a]	22.808	3.15
26	decyl Oxirane[j]	23.185	0.84
27	tetradecyl- Oxirane[j]	23.208	0.52
28	2-Benzyloxy-4-bromobutane-1,3 diol[m]	23.643	3.10
29	Carbonic acid, 2-ethoxyethyl 2,2,2 trichloroethyl ester[j]	25.062	6.21
30	2-Piperidinone, N-[4-bromo-n butyl[m]	25.188	0.34
31	Tetratriacontyl pentafluoropropionate[g]	25.457	2.33
32	14-chloro -1-Tetradecanol,[d]	25.709	3.17
33	Sulfurous acid, 2-propyl tetradecyl ester[k]	26.115	1.33
34	Toluene-4-sulfonic acid, 2-benzyloxy-3-hydroxy-1-hydroxymethyl-propyl ester[k]	26.258	2.55
35	Tetradecanamide[h]	26.899	4.94
36	9- Octadecenamide, (Z)- [l]	29.308	9.59
37	Oxalic acid, allyl octadecyl ester[h]	29.869	5.19
38	1 -Octadecanesulphonyl chloride[k]	29.960	0.82
39	9-Octadecenamide, (Z)- [l]	30.098	12.02
40	9-Octadecenamide ,(Z)- [l]	30.550	4.74
41	2-octyl-1-Decanol [c]	30.761	1.30
42	1,54 dibromoTetrapentacontane[b]	32.003	0.01
43	1-chloro Octadecane[b]	32.409	0.57
44	1-chloro Octadecane[b]	32.833	1.71
45	Phthalic acid, isohexyl 3-methylbutyl ester[h]	33.205	1.06
		Total	**99.99**

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Table 3: Chemical classes of the volatile components of methanolic extract of *Skeletonema costatum*

	Compound class	Proportion (%)
A	Alkanes, alkenes	19.80
B	Halogenated hydrocarbon	6.97
C	Alcohol	1.98
D	Halogenated alcohol	3.17
E	Aromatic compound	2.58
F	Aldehydes	3.06
G	Halogenated monoterpene	2.33
H	Fatty acids and fatty acids esters	10.75
I	Halogenated fatty acids and fatty acids esters	6.21
J	Ether compound	1.36
K	Sulphur containing compound	4.70
L	Amide containing compound	31.29
M	Others	5.79
	Total	**99.99**

Flavonoids: A portion of the sample was heated in 1 ml of dilute ammonia solution in test tube. The development of a yellow colouration indicate the presence of flavonoids.

Steroids: Acetic anhydride was added to the extract of each sample, followed by 2 ml of H_2SO_4. The colour change from voilet to blue observed in the sample indicates the presence of steroids.

Terpenoids: A 2 ml sample of the methanolic algae extract was mixed with 2 ml chloroform and 3 ml concentrated H_2SO_4. A reddish brown colouration at the interface shows the presence of terpenoid.

Cardiac Glycosides: The algae extracts were treated with 2 ml of glacial acetic acid containing one drop of Ferric Chloride, after which 1 ml of concentrated H_2SO_4 was added. A brown ring formed at the interface indicates the presence of cardaic glycoside. A violet ring may appear below the ring while in the acetic acid layer a greenish ring may appear below the brown ring.

Free hydroxyl anthroquinone: A 0.5 g sample was mixed with 1 ml of Diethylether and shaken properly before filtering, after which 0.5 ml of 10% NH_4OH was added to the filtrate. The mixture was shaken and the presence of pink colouration in ammonical phase indicates the presence of free hydroxyl anthroquinone.

Combined anthroquinone: A sample of the dried algae (0.5 g) was boiled with 10 ml concentrated H_2SO_4 and filtered when hot. The filtrate was shaken with 5 ml chloroform. The chloroform layer was pipetted into another test tube and 1ml of diluted NH_4OH was also added. The tube was observed for any colour change which indicate the test is positive.

Phenol: A 0.5 g of algae extract was mixed with 5 ml Ferric Chloride solution and the development of a blue colouration indicates a positive result.

Reducing sugar: Water (1 ml) was added to 1 ml of the extract. Few drops of Fehling Solution was added to the mixture and boiled. The formation of a red brick precipitate indicates the presence of reducing sugars.

RESULTS

Minimum inhibitory concentration

The methanolic extract of *Chaetoceros* spp. had inhibitory activity on all bacterial isolates at varying concentration except C*andida albicans* while *Skeletonema costatum* inhibited all organisms except *Salmonella typhi* and *E.coli* ATCC 25922. The extract of *Chaetoceros* spp. had a more inhibitory effect on the isolates with MIC values between < 2 and 10 mg/ml (Table 1).

Gas chromatography-mass spectrometry analysis

A total of 42 compounds were identified in *S. costatum* extract, while 25 compounds were identified in *Chaetoceros* spp. The major compounds detected in *S. costatum* includes: 9- Octadecenamide, (Z)- (26.35%), Carbonic acid (6.21%), and Tetradecanamide (4.94%). The compounds in *Chaetoceros* spp are 9-Octadecenamide, (Z) - (34.05%), 3-Tetradecanol (7.6%) and Eugenol (7.16%); (Tables 2,3,4, and 5). The major class of compounds in the extracts were amide and hydrocarbon for *S. costatum* and amide and alcohol for *Chaetoceros* spp. Some of the identified components in the extract of *Chaetoceros* spp have been previously shown to possess antimicrobial property (Table 6).

Table 4: Volatile components of methanolic extract of *Chaetoceros spp.*

Peak No	Compounds	Retention Time	Proportion (%)
1	Sulfurous acid, 2-propyl tetradecyl ester[g]	7.067	2.28
2	Eugenol[e]	8.789	7.16
3	Sulfurous acid, pentadecyl 2-propyl ester[g]	12.182	2.14
4	2-methyl Decane,[a]	13.298	2.08
5	Oxalic acid, isobutyl nonyl ester[f]	14.751	2.83
6	Oxalic acid, isobutyl nonyl ester[f]	17.109	2.28
7	Nonadecane[a]	17.320	2.55
8	Hexadecane[a]	18.299	2.07
9	Pentadecane[a]	19.346	1.66
10	1-chloro- Tetradecane[b]	21.320	2.33
11	Heptadecanoic acid, heptadecyl ester[f]	22.356	2.22
12	Tetratetracontane[a]	22.802	3.24
13	Thiazole, 4-(4-aminophenyl)-2-methylamino-[i]	23.643	2.99
14	1-Hexacosanol[c]	25.051	2.13
15	Benzenesulfonamide, 4-methyl-N-phenyl-[g]	26.247	5.24
16	9-Octadecenamide, (Z)-[h]	26.893	12.02
17	4-methoxy 2-Butyn-1-ol[c]	28.484	2.53
18	9-Octadecenamide, (Z)-[h]	30.098	22.03
19	3-Tetradecanol[c]	30.555	7.60
20	1-Decanol, 2-hexyl-[c]	32.049	2.06
21	Oxalic acid, cyclobutyl pentadecyl ester[f]	32.140	2.20
22	dl-Isopulegol[c]	33.205	3.68
23	n-Tetracosanol-1[c]	37.239	0.43
24	Hexadecanal[d]	37.284	0.71
25	Ethyl Cyclododecane[a]	37.416	2.29
26	1,54-dibromo Tetrapentacontane[b]	37.450	1.24
		Total	**99.99**

Table 5: Chemical classes of the colatile component of methanolic extract of *Chaetoceros spp.*

	Compound class	Proportion (%)
a	Alkanes, alkenes	13.89
b	Halogenated hydrocarbon	3.57
c	Alcohol	18.43
d	Aldehydes	0.71
e	Phenylpropanoids	7.16
f	Fatty acids and fatty acids esters	9.53
g	Sulphur containing compound	9.66
h	Amide containing compound	34.05
i	Thiazole containing compound	2.99
	Total	**99.99**

Table 6: Referenced antimicrobial activity of some identified bioactive components in the methanolic extract of *Chaetoceros spp.*

Compound name	Activity	Reference
1-Hexacosanol	*Staphylococcus aureus, Streptococcus faecalis, Bacillus cereus, Pseudomonas aeruginosa, Shigella dysenteriae, Shigella flexneri, Candida albicans, Candida krusei.*	(Kavitha *et al*., 2009; Mbosso *et al*., 2010)
Eugenol	*Listeria monocytogene,, Lactobacillus sakei*	(Gill & Holley, 2004)
Benzenesulfonamide, 4-methyl-N-phenyl-	*Nocardia species*	(Isik and Özdemir-Kocak, 2009)
Thiazole, 4-(4-aminophenyl)-2-methylamino-	*Pseudomonas aeruginosa, E. coli B. Subtilis, S. aureus MRSA, C. albicans*	(Abdellatif *et al*., 2015; Bondock *et al*., 2007)

Phytochemicals in algae extracts

Phytochemical analysis was done on *Skeletonema costatum* and *Chaetoceros* spp. The result showed that the two algal spp. contains the same phytochemical constituents (flavonoids, tannins, terpenoid, steroid and phenol) (Table 7).

DISCUSSION

In this study, we showed that the methanolic extract of *S. costatum* had inhibitory effect on the test isolates except *E. coli* ATCC 25922 and *S. typhi.* However, *S. costatum* extract have been shown to inhibit the growth of *E. coli* and *S. typhi* (Shanmugapriya and Ramanathan, 2011). The difference in the inhibition of *E. coli* and *S. typhi* (Table 1) could be due to the difference in the extraction solvent used as well as the difference in the origin of algae spp. Organic solvents used in medicinal plant extraction and the origin of the medicinal plant may affect their antimicrobial activity (Ibañez *et al*., 2012; Tuney *et al*., 2006). The inhibition of *K. pneumonia* and *S. aureus* by the algal extract is corroborated by previous study (Shanmugapriya and Ramanathan, 2011).

The result showed that the extract of *S. costatum* inhibited only the gram positive bacteria; *E. faecalis, K. pneumonia, S. aureus* ATCC 25923 and *Bacillus* spp. This result is consistent with the previous reports that showed that gram positive bacteria are more sensitive to extracts of marine algae (Ghasemi *et al*., 2007; Ghasemi *et al*., 2004).

The methanolic extract of *Chaetoceros* spp. also had inhibitory effect on all bacterial isolates except the fungus; *C. albicans.* Similar studies have also shown that the extract of *Chaetoceros* spp. inhibited the growth of *S. aureus, Bacillus species and E. coli* except *C. albicans* (Seraspe *et al*., 2012; Sushanth and Rajashekhar, 2013).

The GC-MS analysis of the volatile compounds of the extract of *S. costatum* and *Chaetoceros* spp. showed the presence of amide containing compounds, long chain hydrocarbons, aldehyde, alcohols, fatty acids esters, amines and sulphur containing compounds.

To the best of our knowledge, there is no published data on the volatile constituents of *S. costatum and Chaetoceros* spp. However, some of the bioactive compounds identified in *Chaetoceros* spp. in this study such as 1-Hexacosanol, Eugenol, Benzenesulfonamide, 4-methyl-N-phenyl-, Thiazole,

4-(4 aminophenyl)-2-methylamino- have been documented to have antimicrobial activity against some of the isolates tested (Abdellatif *et al*., 2015; Bondock *et al*., 2007; Gill and Holley, 2004; Isik and Özdemir-Kocak, 2009; Kavitha *et al*., 2009; Mbosso *et al*., 2010). In this study, the extract of *Chaetoceros* spp. was shown to contain 1-Hexacosanol and Thiazole, 4-(4 aminophenyl)-2-methylamino- which are *C. albicans* growth inhibitors (Table 6) however, the inability of the extract to inhibit the growth of the fungus may be due to the difference in the source of the isolate. Tannins, terpenoids, phenols and saponins were the phytoconstituents identified from the extract of the *S. costatum* and *Chaetoceros* spp. Several previous reports have also identified similar phytoconstituents in marine algae (Li *et al*., 2007; Scholz and Liebezeit, 2006).

These phytoconstituents may contribute to the antimicrobial properties of the extracts. Phytochemicals similar to those reported in this study have been documented to possess antimicrobial activities (Ali *et al*., 2002; Chakraborty *et al*., 2010; Khaliq-uz-Zaman *et al*., 2001). Moreso, most test isolates appeared to be more sensitive to the extract of *Chaetoceros* spp. with lower MIC values (Table 1), this may be due to the presence compounds such as 1-Hexacosanol, Eugenol, Benzenesulfonamide, 4-methyl-N-phenyl-, Thiazole,

4-(4 aminophenyl)-2-methylamino- (Table 3) which have been previously reported as antibiotic against the test isolates (Table 6).

Table 7: Phytochemicals of methanolic extract of the marine algae.

Phytochemicals	Marine Algae extract	
	Skeletonema costatum	*Chaetoceros spp.*
Flavonoid	+	+
Tannin	+	+
Phlobatanin	-	-
Alkaloids	-	-
Saponin	-	-
Terpenoid	+	+
Steroid	+	+
Cardiac glycosides	-	-
Free hydroxyl anthraquinone	-	-
Combined anthraquinone	-	-
Reducing sugar	-	-
Phenol	+	+

Note: + = present, - = absent

In conclusion, this study has shown that the methanolic extract of *S. costatum* and *Chaetoceros* spp contains useful bioactive principles, with strong antimicrobial activities. Thus, they may be explored for the development of therapeutic agents. However, further research is needed to identify the effect of each bioactive component on test isolates.

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