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Antimicrobial and antioxidant activities of ethyl acetate and methanol extracts of *Littorina littorea* and *Galatea paradoxa*

Lawrence Sheringham Borquaye^{1*}, Godfred Darko¹, Novisi Oklu¹, Constance Anson-Yevu¹ and Annette Ababio¹

Abstract: The aquatic environment is a vital resource for bioprospecting pharmacologically important natural products. Molluscs are known to harbour compounds with antimicrobial, antitumor and antioxidant activities. This study evaluated the antimicrobial and antioxidant properties of ethyl acetate and methanol tissue extracts of two molluscs, *Littorina littorea* (*L. littorea*) and *Galatea paradoxa* (*G. paradoxa*). Agar diffusion and broth dilution assays were used to test for antimicrobial activity against nine microbes. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) method was used to determine antioxidant activity of the extracts. Extracts of both molluscs showed significant activity against all the bacteria strains tested but were inactive towards the fungus. The best antibacterial activity was recorded by methanol extract of *L. littorea* towards *Pseudomonas aeruginosa*. In comparison to ethyl acetate extracts, methanol extracts were more efficient in scavenging the DPPH radical. Methanol extracts of *L. littorea* had an IC_{50} of 0.37 mg/mL which was closer to that of the standard ascorbic acid drug (0.0048 mg/mL) than any of the other extracts. The findings of this work indicate that the tissue extracts of *L. littorea* and *G. paradoxa* are promising sources of antimicrobial and antioxidant agents that can be utilized for pharmaceutical and nutraceutical purposes.

Subjects: Chemistry; Medicinal & Pharmaceutical Chemistry; Microbiology; Organic Chemistry; Pharmaceutical Science

Keywords: DPPH; agar diffusion; broth dilution; antimicrobial index; molluscs

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PUBLIC INTEREST STATEMENT

The aquatic ecosystem, especially the sea, has proven to be a rich hub of pharmacologically important molecules with diverse therapeutic applications. Currently, numerous marine natural products are either on the market as approved drugs or in various stages of clinical trials. Ghana's waters, however, have received little to no attention with only an estimated 5% of non-fish species having been so far properly classified. In this work, two Ghanaian water molluscs, *Galatea paradoxa* and *Littorina littorea*, were collected, extracted and tested for their antimicrobial and antioxidant activities. The extracts proved to be very potent against bacteria but inactive against the only fungus tested. The results indicate that these molluscs could potentially be sources of new compounds for pharmaceutical, nutraceutical and cosmetic purposes.

1. Introduction

Infectious diseases are a major threat to human health due to the unavailability of vaccines or limited chemotherapy. They are responsible for approximately one half of all deaths recorded in tropical countries (Iwu, Duncan, & Okunji, 1999). Available antimicrobial regimes have substantial limitations in terms of antimicrobial spectrum and side effects. In addition, their promiscuous use has led to increasing trends of resistance among emerging and re-emerging microbial pathogens (Franklin & Snow, 2013; Prescott, Harley, & Klein, 2002). This, in turn, has led to a need to find new therapeutic compounds with preferably novel modes of action. Natural products have led the way in this respect and provided various success stories. Crude natural product extracts have played important roles in the discovery of modern drugs and drug scaffolds for the treatment of various ailments (Sneader, 2005).

Aquatic organisms have evolved many different survival mechanisms to thrive in various harsh conditions. These conditions include extreme temperatures, changes in salinity and pressure and actions of pathogenic microbes (Skropeta, 2008). The ability of aquatic organisms to adapt and survive in different environments depends both on their physical and chemical adaptive features. Organisms with no apparent physical defence, like sessile organisms, have evolved chemical defences to protect themselves (Thakur, Thakur, & Müller, 2005). Aquatic invertebrates such as bryozoans, molluscs, sponges and others have soft bodies and lead a sedentary lifestyle, making a chemical system of defence absolutely essential for survival. These chemicals, when released into their aqueous habitat, are rapidly diluted. To be effective, the chemicals must be very potent (Jimeno, Faircloth, Sousa-Faro, Scheuer, & Rinehart, 2004; Newman & Cragg, 2004). The high potency of chemicals used in aquatic defence systems and the requirement for them to be water soluble have attracted many researchers to prospect for biologically active compounds from these ecosystems.

In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from aquatic organisms. Many varieties of bioactive substances are being isolated and characterized with great promise for the treatment of many diseases (Benkendorff, 2010; Faulkner, 2001). Spongouridine and spongothymidine, from the Caribbean sponge, *Cryptotheca crypta*, were isolated in the early 1950s and approved 15 years later as anticancer and antiviral drugs (Jimeno et al., 2004). So far, over 10,000 bioactive compounds have been discovered from aquatic sources, with hundreds of new compounds being discovered every year (Newman & Cragg, 2004).

Most natural products' research programmes in Ghana and Africa have focused on terrestrial plants, with a number of bioactive compounds isolated so far (Addae-Mensah & Achenbach, 1985; Dadson & Minta, 1976; Ekuadzi et al., 2014). Ghana's aquatic biodiversity has to this point been explored to only a limited extent. In this study, methanol and ethyl acetate extracts of the tissues of a marine mollusc, *Littorina littorea* (*L. littorea*) and the freshwater clam, *Galatea paradoxa* (*G. paradoxa*) were assayed against nine test micro-organisms to evaluate their antimicrobial activity. Agar well diffusion and broth dilution assays were used to investigate antimicrobial activities of these extracts. In addition, the antioxidant capacity of these extracts was investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. *L. littorea* is a marine gastropod that belongs to the family Littorinidae and inhabits rocky shores. *G. paradoxa*, on the other hand, belongs to the family Donacidae. It is a freshwater, bivalve mollusc that can be found in many rivers in West Africa (Adjei-Boateng, Agbo, Agbeko, Obirikorang, & Amisah, 2012; Obirikorang, Amisah, & Adjei-Boateng, 2013).

2. Materials and method

2.1. Sample collection and extraction

The two molluscs, *L. littorea* and *G. paradoxa*, were collected from the Labadi Beach in Accra and Sogakope, on the Volta River, respectively, in February 2015. The samples were transported on ice to the laboratory at the Department of Chemistry, Kwame Nkrumah University of Science and Technology, (KNUST) Kumasi, Ghana. Samples of the two molluscs were sent to the Department of Fisheries and Marine Sciences, KNUST, where there were correctly identified by a marine taxonomist.

For each mollusc, the shells were removed to get the fresh tissues which were thoroughly washed with distilled water and then stored at 0°C until further use. The fresh tissues were cut into small pieces, washed with distilled water and homogenized by blending. The homogenates were separately extracted with ethyl acetate and methanol. The extracts were filtered (Whatman filter paper No. 41) and concentrated *in vacuo* on a rotary evaporator (Buchi RE-200, Postfach, Switzerland). To make sure no residual solvent remained, extracts were freeze dried (Labconco, Kansas City) and reconstituted in water.

2.2. Antimicrobial assay

2.2.1. Microbial cultures

To evaluate the antimicrobial activities of the extracts, nine micro-organisms were used. Eight of the microbes were bacteria and one was a fungus. Of the nine test bacteria, four were Gram negative: *Escherichia coli* (*E. coli*), *Salmonella typhi* (*S. typhi*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and the other four were Gram positive: *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Enterococcus faecalis* (*E. faecalis*) and *Streptococcus pneumoniae* (*S. pneumoniae*). The fungal strain used was *Candida albicans* (*C. Albicans*). All microbial strains were obtained from the Department of Pharmaceutical Microbiology, College of Health Science, KNUST. Sabouraud growth media was prepared and sterilized in an autoclave at 121°C for 15 min. All nine micro-organisms were individually incubated in the media at 37°C for 18 h and 72 h in the case of *C. albicans*.

2.2.2. Agar well diffusion assay

The antimicrobial potency of all the extracts was evaluated by agar well diffusion method. The microbial isolates used were grown in Mueller Hinton agar. The media was poured into plates and allowed to solidify. The plates were swabbed with cotton wool impregnated with the organisms prepared at 0.5 McFarland standards. Wells in the agar were made using a sterile 6-mm cork-borer. Three wells were bored on each plate; two were filled with extracts and the other with a standard drug (ciprofloxacin). The plates were allowed to stand for 30 min then incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the zones of inhibition against the test organisms. The distance from the border of the disc to the edge of the clear zone represented the zone of inhibition (ZI).

2.2.3. Minimum inhibitory concentration

The broth dilution method was employed to determine the minimum inhibitory concentration MIC of the two crude extracts. To a row on a 96-well micro titer plate were added serial dilutions of extract or standard drug, representing different concentrations. Hundred-microliter nutrient broth and 10 µL of a suspension of test micro-organism were then added. Sterile water was added to top each well up to the 200-µL mark. Control wells were prepared using standard ciprofloxacin drug. Plates were incubated at 37°C for 24 h after which a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. Microbial growth was indicated by wells that changed colour to violet. Wells that were unchanged (in colour) indicated inhibition of microbial growth by the extracts.

2.2.4. Antimicrobial activity index

The antimicrobial index (AI) of each extract was calculated as the average of the antimicrobial activity obtained against all test micro-organisms. To do this, the activity of extracts against each test organism was assigned weightages. For a ZI of 1–10 mm, a weightage of one (1) was assigned. A weightage of two (2) was assigned for ZI of 11–20 mm, three (3) if the ZI was greater than 20 mm and zero (0) for no antimicrobial activity. The AI was obtained by dividing the sum total of weightages obtained for each individual extract by the total number of test micro-organisms. Separate AI was calculated for Gram-positive bacteria, Gram-negative bacteria and fungi to compare the activity of the various extracts (Ghosh, Subudhi, & Nayak, 2008; Sathyan, Chaithanya, Anil Kumar, Sruthy, & Philip, 2014).

2.3. Determination of antioxidant activity

The antioxidant activities of the extracts were assessed by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay (Blois, 1958). Two milliliters of 0.1 mM DPPH solution in methanol was added to 1 mL of different concentrations of each extract. Mixture was vigorously shaken and incubated in the dark at room temperature for 30 min. The absorbance of the resultant solution was measured at 517 nm with a UV-Visible Spectrophotometer (Perkin Elmer Lambda 35). Methanol was used as blank. The standard drug, ascorbic acid, was used as positive control.

The antioxidant activity was evaluated as:

$$\text{Activity (\%)} = [(A_0 - A)/A_0] \times 100\%$$

where A_0 is the absorbance value of DPPH only and A is the absorbance value of the mixture of DPPH and sample.

IC_{50} values were obtained from a dose-response curve obtained by plotting % antioxidant activity against extract concentration.

3. Results

Antimicrobial activity of methanol and ethyl acetate extracts of two molluscs was evaluated against eight strains of bacteria and one fungus. All extracts tested were inactive towards the fungus tested, *C. albicans*. The zones of inhibition recorded against *C. albicans* were either zero or negligible (0.5 mm for *G. paradoxa*, ethyl acetate extract). In general, ethyl acetate extracts seemed to be more active than methanol extracts (Table 1). Ethyl acetate extracts of *G. paradoxa* (Gal EtOAc) recorded their highest ZI as 19 mm towards the Gram-negative bacteria *P. aeruginosa*. Methanol extracts of *G. paradoxa* (Gal MeOH) recorded appreciable activity only against *S. aureus*, *B. subtilis*, *S. typhi* and *P. aeruginosa*. Ethyl acetate extracts of *L. littorea* (Lit EtOAc) showed considerable activity towards all test microbes except for *C. albicans* and *B. subtilis*, while methanol extracts (Lit MeOH) exhibited activity against only *E. faecalis*, *S. pneumoniae* and *P. aeruginosa*. Remarkably, neither extracts were active towards the fungus. When active, high zones of inhibition were recorded by the Lit MeOH (17–23 mm). Similarly, Lit EtOAc had zones of inhibitions between 11.13 and 12.63 mm when active.

Table 1. Zones of inhibition (in mm) of ethyl acetate and methanol extracts of *L. littorea* and *G. paradoxa* against test micro-organisms

Test micro-organisms	<i>G. paradoxa</i>		<i>L. littorea</i>		Positive control
	Ethyl acetate	Methanol	Ethyl acetate	Methanol	
<i>E. coli</i> *	17.17 ± 0.76	0.00 ± 0.00	11.53 ± 0.76	0.00 ± 0.00	36.3 ± 1.25
<i>S. typhi</i> *	13.00 ± 0.50	11.03 ± 0.21	12.00 ± 0.50	0.00 ± 0.00	35.3 ± 0.69
<i>K. pneumoniae</i> *	11.00 ± 1.00	0.57 ± 0.21	12.63 ± 0.40	0.00 ± 0.00	35.0 ± 0.09
<i>P. aeruginosa</i> *	19.00 ± 1.73	11.67 ± 1.15	11.53 ± 0.45	23.17 ± 0.76	35.3 ± 1.22
<i>S. aureus</i> **	15.83 ± 0.70	9.67 ± 0.58	12.13 ± 0.32	0.00 ± 0.00	24.7 ± 0.64
<i>B. subtilis</i> **	17.43 ± 0.51	12.43 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	19.0 ± 1.06
<i>S. pneumoniae</i> **	0.90 ± 0.26	1.13 ± 0.32	11.13 ± 0.40	17.50 ± 0.50	20.0 ± 1.74
<i>E. faecalis</i> **	9.83 ± 0.76	0.00 ± 0.00	12.13 ± 0.45	21.10 ± 0.66	34.7 ± 0.61
<i>C. albicans</i> ***	0.50 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	41.0 ± 2.00

Notes: Values reported as mean ± standard deviation. Mean of three experiments.

Zone in mm indicates the distance from the border of the disc to the edge of the clear zone.

*Gram-negative bacteria.

**Gram-positive bacteria.

***Fungi.

The broth dilution test was used to determine the MIC of the extracts against the test microbes. The results are presented in Table 2. The least MIC of 1 mg/mL was recorded by the Gal EtOAc for *E. coli* and *P. aeruginosa*. Gal MeOH also recorded low MIC (1 mg/mL) towards *E. faecalis* and *P. aeruginosa*. The MICs of all extracts towards *C. albicans* were beyond the concentrations tested in this work (0–50 mg/mL).

A high AI suggests better inhibitory capacity. Gal MeOH (AI of 0.9) had lower activity than Gal EtOAc (AI of 1.4). In the same vein, Lit MeOH (AI of 0.9) was lower in activity when compared with Lit EtOAc (AI of 1.6). Interestingly, Lit EtOAc was slightly more active than Gal EtOAc. Methanol extracts from both *L. littorea* and *G. paradoxa* had about the same overall activity on the microbes tested. Lit EtOAc had greater overall activity against all test microbes as depicted in Figure 1.

Figure 2 represents the antimicrobial activity index of the various extracts towards the different classes of microbes tested (fungi, Gram-positive and Gram-negative bacteria). None of the extracts had any activity towards *C. albicans* and hence fungus had an AI of 0. With respect to Gram-negative bacteria, ethyl acetate extracts were twice as active as their corresponding methanol extracts. The same trend is observed in Gram-positive bacteria, but the difference between ethyl acetate and

Table 2. MIC of ethyl acetate and methanol extracts of *L. littorea* and *G. paradoxa* against test micro-organisms

Test micro-organisms	Minimum inhibitory concentrations (mg/mL)			
	<i>G. paradoxa</i>		<i>L. littorea</i>	
	Ethyl acetate	Methanol	Ethyl acetate	Methanol
<i>E. coli</i> *	1.0	ND	5.0	ND
<i>S. typhi</i> *	5.0	5.0	5.0	15.0
<i>K. pneumoniae</i> *	15.0	15.0	5.0	25.0
<i>Paeruginosa</i> *	1.0	5.0	5.0	1.0
<i>S. aureus</i> **	5.0	5.0	5.0	15.0
<i>B. subtilis</i> **	5.0	5.0	ND	15.0
<i>S. pneumoniae</i> **	5.0	15.0	5.0	5.0
<i>E. faecalis</i> **	5.0	ND	5.0	1.0
<i>C. albicans</i> ***	ND	ND	ND	ND

Note: ND—no microbial growth inhibition within the range of concentrations tested ($n = 2$).

*Gram-negative bacteria.

**Gram-positive bacteria.

***Fungi.

Figure 1. Overall antimicrobial activity indices of *G. paradoxa* and *Litorina littorea* extracts against test micro-organisms.

Notes: Gal EtOAc—*G. paradoxa* ethyl acetate extract, Gal MeOH—*G. paradoxa* methanol extract, Lit EtOAc—*L. littorea* ethyl acetate extract and Lit MeOH—*L. littorea* methanol extract.

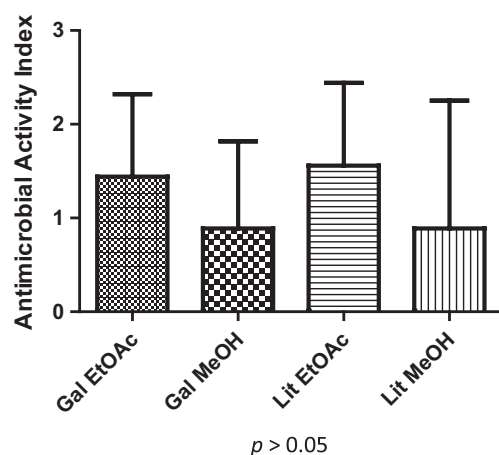


Figure 2. Antimicrobial activity indices of *G. paradoxa* and *Litorina littorea* extracts against Gram-positive bacteria, Gram-negative bacteria and fungi.

Notes: Gal EtOAc—*G. paradoxa* ethyl acetate extract, Gal MeOH—*G. paradoxa* methanol extract, Lit EtOAc—*L. littorea* ethyl acetate extract and Lit MeOH—*L. littorea* methanol extract.

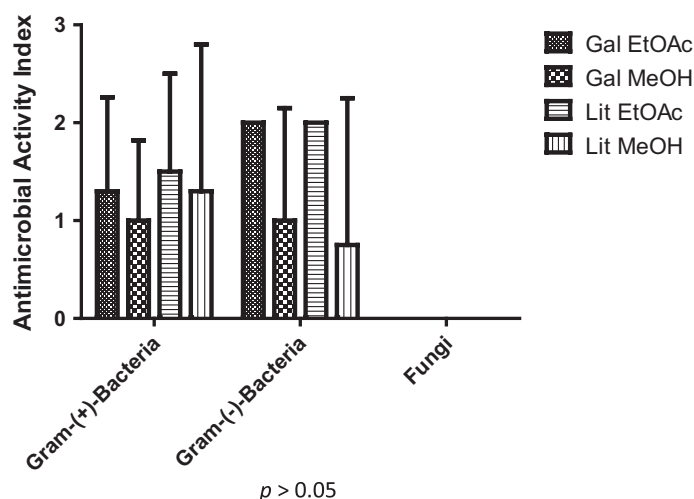


Table 3. IC₅₀ values for free radical scavenging activity by extracts

Extract	IC ₅₀ (mg/mL)
Ascorbic acid	0.0048
Gal EtOAc	1.2590
Gal MeOH	0.3700
Lit EtOAc	1.0650
Lit MeOH	0.7800

Notes: Gal EtOAc—*G. paradoxa* ethyl acetate extract.
 Gal MeOH—*G. paradoxa* methanol extract.
 Lit EtOAc—*L. littorea* ethyl acetate extract.
 Lit MeOH—*L. littorea* methanol extract.

methanol extracts is not as pronounced as in Gram-negative bacteria. Statistically, however, there were no significant differences ($p > 0.05$) between the activity indices of all four extracts.

The IC₅₀ values obtained from the antioxidant assays (Table 3) showed methanol extracts to possess better DPPH radical scavenging capacities than ethyl acetate extracts. Gal MeOH had an IC₅₀ value (5.7 µg/mL) very close to standard drug, ascorbic acid (4.8 µg/mL), and thus indicates it is a potential source of good antioxidant compounds. Gal MeOH, however, had the highest IC₅₀ value.

4. Discussion

Antimicrobial inhibitory studies for a fungus, Gram-negative and Gram-positive bacteria were investigated in this work using four different extracts: two from *G. paradoxa* and another two from *L. littorea*.

Because of their benthic nature, many aquatic invertebrates are potentially susceptible to microbial attack. In order to defend themselves, these organisms have evolved a very efficient chemical defence system. Due to the unique environment in which they are produced, secondary metabolites from aquatic sources are particularly well adapted for use in drug discovery programmes. Chemicals released by these organisms are invariably diluted following release into the water and must therefore be very potent to have any significant effect (Jimeno et al., 2004; Newman & Cragg, 2004;

Thakur et al., 2005). Many classes of natural products have been isolated from aquatic sources. These include lipids, amino acids, macrolides, nucleosides and terpenoids, among others. The activity of these compounds ranges from antimicrobial to antitumor to anti-inflammatory to antioxidant (Thakur et al., 2005).

Ethyl acetate extracts showed much greater activity towards the microbes tested. This can be seen in Figure 1 where ethyl acetate extracts had higher antimicrobial activity index than their methanol counterparts. Interestingly, Lit EtOAc was slightly higher in activity (AI of 1.6) when compared with Gal EtOAc (AI of 1.4). Ethyl acetate extracts of various marine molluscs have been shown to possess significant antimicrobial activity.

Anand and Edward(2002) investigated the antimicrobial activities of various marine cowries and ascidians against some pathogenic micro-organisms. The tests showed both methanol and ethyl acetate extracts to possess some degree of antimicrobial activity, with ethyl acetate fractions slightly more potent (Anand & Edward, 2002). In another test of antimicrobial activity, ethyl acetate extracts of the Sydney rock oyster, *Saccostrea glomerata*, proved to be much more potent than hexane or methanol extracts. Ethyl acetate extracts proved to be rich sources of fatty acids which the authors speculated to be responsible for the observed antimicrobial activity (Karthikeyan, Velmurugan, Donio, Michaelbabu, & Citarasu, 2014). Because of their lipophilic nature, fatty acids and lipids will most likely be found in the less polar ethyl acetate extracts than polar methanol extracts and probably account for the high AI of ethyl acetate fractions observed in this work.

No activity was recorded by any of the extracts towards the fungus, *C. albicans*. Overall, there was slightly higher activity by ethyl acetate extracts (Gal EtOAc and Lit EtOAc) towards Gram-negative bacteria than Gram-positive bacteria. The reverse was true for methanol extracts, where much more activity was observed for Gram-positive bacteria than the Gram-negative ones. *P. aeruginosa* was inhibited the most by the extracts. The highest inhibition zone was recorded by Lit MeOH towards *P. aeruginosa*. In a similar study, methanol extracts of *Perna viridis*, *Nerita albicilla* and *Ozium rugulosus* yielded the best results towards a set of test micro-organisms, with *P. aeruginosa* being the most susceptible micro-organisms towards the extracts (Kiran, Siddiqui, Khan, Ibrar, & Tushar, 2014).

In order to assess the antioxidant potential of the extracts, their ability to scavenge DPPH radicals was evaluated. Ascorbic acid was used as the standard. Methanol extracts were better at radical scavenging than ethyl acetate extracts. Gal MeOH had the lowest IC_{50} concentration of 0.37 mg/mL. One class of secondary metabolites known to possess antioxidant activity is polyphenols. Methanol extracts of *G. paradoxa* and *L. littorea* potentially contain these compounds in high concentrations. Many biological reactions proceed with the generation of free radicals. These free radicals have been implicated in many neurodegenerative diseases and chronic ulcers (Garcia-Estrada et al., 2003; Houghton, Hylands, Mensah, Hensel, & Deters, 2005). The results indicate that the extracts could possibly be useful sources of compounds for various pharmaceutical and nutraceutical preparations.

5. Conclusion

In the present study, the antimicrobial and antioxidant activities of ethyl acetate and methanol extracts of two molluscs, *G. paradoxa* and *L. littorea*, were investigated. The results from the study showed that all the extracts were incapable of inhibiting the fungus but more active towards the bacterial strains. The extracts showed impressive DPPH radical scavenging capacity in comparison to the standard ascorbic acid drug. These extracts could be further purified to isolate and characterize the compounds responsible for the observed activities. This work further provides credence to the notion that the aquatic environment is a rich source of bioactive compounds that could be potentially useful in drug discovery programmes.

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