

Asparagopsis armata and *Sphaerococcus coronopifolius* as a natural source of antimicrobial compounds

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Abstract Methanol, *n*-hexane and dichloromethane extracts of twelve marine macro-algae (Rhodophyta, Chlorophyta and Heterokontophyta divisions) from Peniche coast (Portugal) were evaluated for their antibacterial and antifungal activity. The antibacterial activity was evaluated by disc diffusion method against *Bacillus subtilis* (gram positive bacteria) and *Escherichia coli* (gram negative bacteria). *Saccharomyces cerevisiae* was used as a model for the antifungal activity by evaluating the growth inhibitory activity of the extracts. The high antibacterial activity was obtained by the *Asparagopsis armata* methanolic extract (10 mm–0.1 mg/disc), followed by the *Sphaerococcus coronopifolius n*-hexane extract (8 mm–0.1 mg/disc), and the *Asparagopsis armata* dichloromethane extract (12 mm–0.3 mg/disc) against *Bacillus subtilis*. There were no positive results against *Escherichia coli*. *Sphaerococcus coronopifolius* revealed high antifungal potential for *n*-hexane (IC₅₀ = 40.2 µg/ml), dichloromethane (IC₅₀ = 78.9 µg/ml) and methanolic (IC₅₀ = 55.18 µg/ml) extracts against *Saccharomyces cerevisiae* growth. The antifungal potency of the *Sphaerococcus coronopifolius* extracts was similar with the standard amphotericin B. *Asparagopsis armata* and *Sphaerococcus coronopifolius* reveal to be interesting sources of natural compounds with antimicrobial properties.

Keywords Marine algae · Antibacterial activity · Antifungal activity · Seaweeds · Marine natural compounds · Natural antibiotics

Introduction

Natural compounds have been applied for the treatment of many diseases since ancient time all over the world. Resistance of the microorganisms to the commonly used antibiotic has enhanced morbidity and mortality and has triggered the search for new drugs. As a consequence of an increasing demand for biodiversity in the screening program seeking therapeutic drugs from natural products, there is greater interest particularly in the oceans throughout the world (Murray et al. 2013).

Several marine organisms produce bioactive metabolites in response to ecological pressures such as competition for space, prevention of predation and the ability to successfully reproduce (König et al. 1994; Bhakuni and Rawat 2005; Salvador et al. 2007). The exploration of this chemical diversity for pharmaceutical purposes has revealed important chemical prototypes for the discovery of new agents. Focusing on bioproducts, recent trends in drug research from natural sources suggest that algae are one of the major producers of bioactive secondary metabolites with high biomedical potential that have the ability to interfere with the pathogenesis of many human diseases. A variety of biological activities for these compounds have been reported including antibacterial, antifungal, antitumor, anticoagulant and antiviral (Mayer et al. 2007; Murray et al. 2013). Moreover, algae have the potential to provide not only novel biologically active substances, but also essential compounds for human nutrition (Pintéus et al. 2009; Gómez-Ordóñez et al. 2010).

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Our view as researchers is that some answers to the most challenging problems in the health and economic areas may lie underwater and from a biotechnological view, Portugal is a country that has an enormously and rich marine coast that is mainly unexplored. The coast of mainland Portugal is approximately 830 km long. With regard to seaweed biogeography, Portugal is situated in the warm temperate Mediterranean-Atlantic region: continental Portugal falls in the Lusitania Province; the Azores and Madeira Islands fall in the Canary Province (Lüning 1990).

It was recognized (Ardré 1970) the existence of 246 species of Rhodophytes, 98 Phaeophytes and 60 Chlorophytes and these numbers have not changed substantially to nowadays. In this work we studied the antimicrobial biotechnological potential of twelve macro-algae belonging to the three divisions, namely Rhodophyta: *Asparagopsis armata*, *Sphaerococcus coronopifolius*, *Plocamium cartilagineum* and *Ceramium ciliatum*; Heterokontophyta: *Halopteris filicina*, *Stypocaulon scoparium*, *Fucus spiralis* and *Sacchorhiza polyschides*; Chlorophyta: *Ulva compressa*, *Codium adherens*, *Codium vermilara* and *Codium tomentosum*. These are marine species found in the intertidal zone.

Materials and methods

Algae identification

Macro-algae were collected in Peniche Coast (Portugal) and identified as *Asparagopsis armata*, *Sphaerococcus coronopifolius*, *Plocamium cartilagineum*, *Ceramium ciliatum*, *Halopteris filicina*, *Stypocaulon scoparium*, *Fucus spiralis*, *Sacchorhiza polyschides*, *Ulva compressa*, *Codium adherens*, *Codium vermilara* and *Codium tomentosum*.

Preparation of extracts

Immediately after collection (between April and June of 2012), algae were washed first in sea water and then in fresh water to remove the epiphytes, sand and other extraneous matter. The samples were frozen at -80°C and then ground with a mixer grinder to make a powder. Each algal samples were sequential extracted in 1:4 biomass:solvent ratio with methanol and dichloromethane (Fisher Scientific, U.K.) at constant stirring for 12 h. Liquid–liquid extraction was also performed for the methanolic fraction, using *n*-hexane (Lab-Scan analytical Sciences, Poland). The solvents were evaporated in a rotary evaporator (Laborota 4000, Heidolph), and the extracts were then solubilized in Dimethyl

Sulfoxide (DMSO) (Sigma, Steinheim, Germany) at 100 mg/ml and stored at -20°C until further use (Mayachiew and Devahastin 2008; Pintéus et al. 2009).

Test organisms

The test organisms used were *Bacillus subtilis* (ATCC 6633) a gram-positive bacteria, *Escherichia coli* (ATCC 10536) a gram-negative bacteria and *Saccharomyces cerevisiae* (ATCC 9763) a yeast. All microorganisms were obtained from the American Type Culture Collection.

Antibacterial assay

Single disc method as described by Vandepitte et al. (2003) was used. All bacteria were grown in Nutrient Broth (Merck, Germany) incubated at 37°C for 24 h. A liquid microorganism suspension corresponding to a 0.5 McFarland scale was applied onto Petri dishes containing Mueller–Hinton agar (Merck, Germany), using a sterile swab. Sterile paper discs of 6 mm diameter were embedded with 10 μl of the algae extracts (0.03–1 mg/disc)—experimental group—or with the solvent (DMSO)—control group—and added to the cultured dishes. Toxicity of the macro-algae extracts against microorganisms was determined after 24 h at 37°C by measuring the diameter of the halo around the discs, using a caliper. All experiments were performed in triplicate. Chloramphenicol (10 $\mu\text{g}/\text{disc}$) (Oxoid, United Kingdom) was used as positive control.

Antifungal assay

The antifungal activity was measured by following the *S. cerevisiae* growth (OD_{600 nm}) in YPD broth at 30°C (Guo et al. 2008) in the presence or in the absence of the extracts (100–1,000 $\mu\text{g}/\text{ml}$). Amphotericin B (Sigma, St. Louis, USA) was used as positive control.

Data analysis

One-way analysis of variance (ANOVA) was used followed by Dunnett test (Zar 2010) to discriminate significant differences between algae extracts and controls (or vehicle). These analyzes were performed with GraphPad InStat for Windows. Results are presented as mean \pm SEM. The significance level was inferred at $p < 0.05$ or $p < 0.01$ for all statistical tests. The IC₅₀ concentration was calculated from nonlinear regression analysis using the GraphPad Prism software with the equation: $Y = 100/[1 + 10^{(X - \text{LogIC}_{50})}]$.

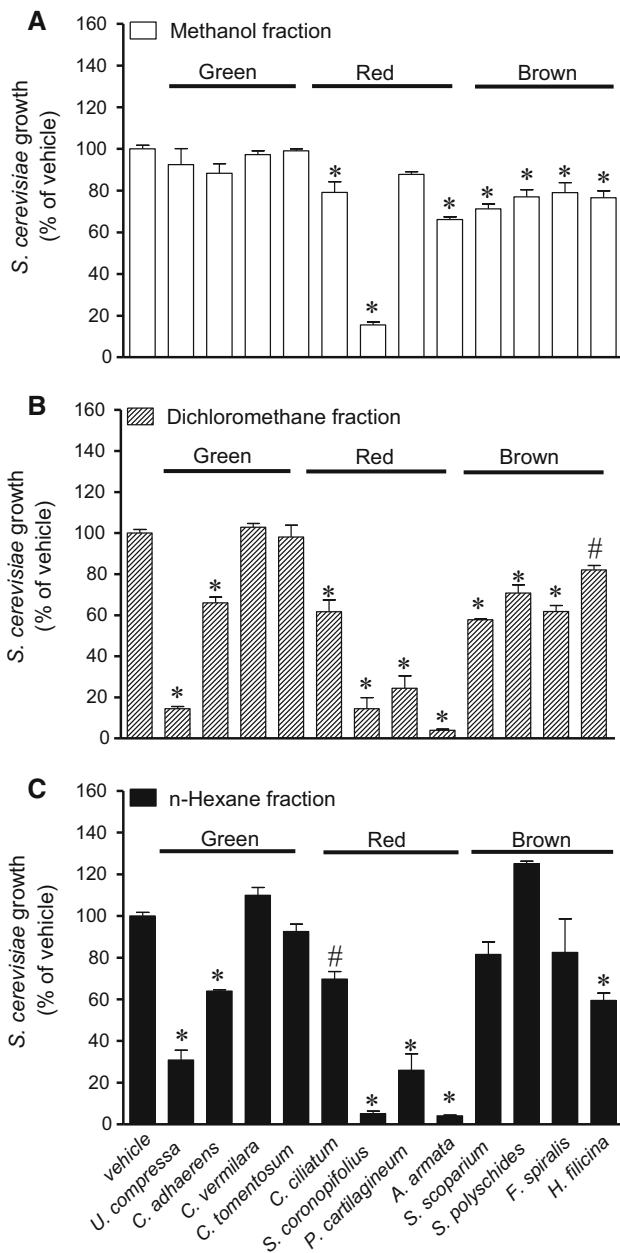


Fig. 1 Antifungal activity of the methanolic (a), dichloromethane (b) and n-hexane (c) fractions against *Saccharomyces cerevisiae* growth (1 mg/ml). Each column represents the mean of 8 experiments per group; vertical lines show SEM. #*p* < 0.01 and **p* < 0.05 compared with vehicle

Results

Antibacterial activity

The antibacterial results are summarized in Table 1. The twelve species of marine algae extracted by the three different solvents used were screened and positive results

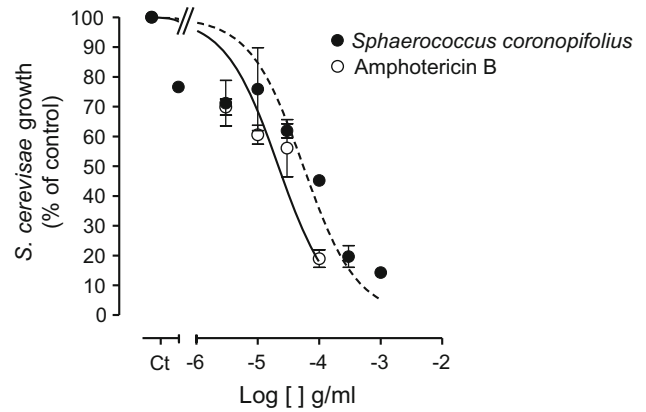


Fig. 2 *Saccharomyces cerevisiae* growth in the presence of different concentrations of amphotericin B or methanolic fractions (100–1,000 µg/ml). Symbols represent mean of 8 independent determinations; vertical lines show SEM

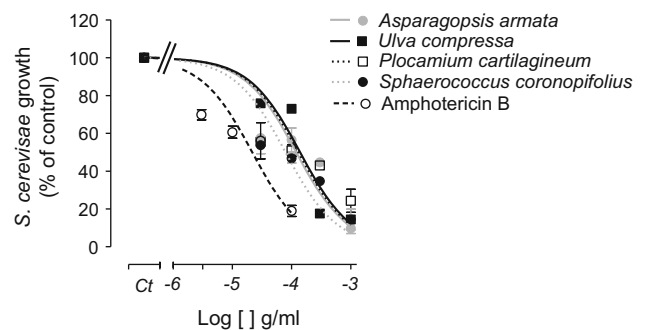


Fig. 3 *Saccharomyces cerevisiae* growth in the presence of different concentrations of amphotericin B or dichloromethane fractions (100–1,000 µg/ml). Symbols represent mean of 8 independent determinations; vertical lines show SEM

were obtained against *B. subtilis* for all Rhodophyta (*Sphaerococcus coronopifolius*, *Ceramium ciliatum*, *Plocamium cartilagineum* and *Asparagopsis armata*), one Heterokontophyta (*Halopteris filicina*) and one Chlorophyta (*Ulva compressa*). The most active species were *A. armata* and *P. cartilagineum* that belong to the Rhodophyta division. The minimum active concentration against *B. subtilis* of the n-hexane fractions was 100 µg/disc for *Plocamium cartilagineum*, *Sphaerococcus coronopifolius* and *Ulva compressa* with 7, 8 and 7 mm, respectively. Similarly, the minimum active concentration against *B. subtilis* of the methanolic fraction was 100 µg/disc for *Asparagopsis armata* and *Sphaerococcus coronopifolius* with 10 and 7 mm, respectively. By contrast, the minimum active concentration against *B. subtilis* of the dichloromethane fraction was 300 µg/disc for *Ceramium ciliatum*, *Plocamium cartilagineum*, *Asparagopsis armata*

Table 1 Antimicrobial activity of algae extracts against *Bacillus subtilis* (ATCC 6633)

Algae	Methanol (mg/disc)				Dichloromethane (mg/disc)				<i>n</i> -Hexane (mg/disc)			
	0.03	0.1	0.3	1	0.03	0.1	0.3	1	0.03	0.1	0.3	1
<i>Sphaerococcus coronopifolius</i>	–	7	7	10	–	–	7	7	–	8	9	11
<i>Asparagopsis armata</i>	–	10	11	17	–	–	12	Nt	–	–	8	Nt
<i>Plocamium cartilagineum</i>	–	–	11	13	–	–	7	Nt	–	7	9	10
<i>Ulva compressa</i>	–	–	–	–	–	–	–	–	–	7	7	8
<i>Ceramium ciliatum</i>	–	–	–	–	–	–	7	10	–	–	–	–
<i>Halopteris filicina</i>	–	–	–	11	–	–	–	6.5	–	–	–	–

^a Zone of inhibition, including the diameter of the filter paper disc (6 mm); mean value of three independent experiments; – no activity, Nt not tested. Chloramphenicol (10 µg/disc) was used as positive control (30 mm) and DMSO as negative control (0 mm)

and *Sphaerococcus coronopifolius* with 7, 7, 12 and 7 mm, respectively. There were no positive results against *E. coli*.

Antifungal activity

S. cerevisiae growth was monitored spectrophotometrically [optical density at 600 nm (OD₆₀₀)] during 48 h (data not shown). To determine the effects of algae extracts on the growth of *S. cerevisiae*, we studied the growing curve after several time points of exposure to extracts compared to the vehicle (1 % DMSO and YPD broth) (data not shown). After this experiment, growth inhibition was determined spectrophotometrically after 20 h of incubation, which is a point in the exponential growing phase. The growth inhibition was assessed as the percentage of change in turbidity in the presence or absence of the macro-algae extracts.

As shown in the Fig. 1, almost all the algae extracts (1 mg/ml) inhibited the *S. cerevisiae* growth. All brown algae, presented antifungal activity in the dichloromethane and the methanolic fractions, however, in the *n*-hexane fraction, only *Halopteris filicina* showed activity. In line with this observation, all the red algae (Rhodophyta) presented high antifungal activity in the three fractions, with the only exception of *Plocamium cartilagineum* in the methanolic fraction (Fig. 1a). The red algae *Sphaerococcus coronopifolius*, *Asparagopsis armata*, and *Plocamium cartilagineum* dichloromethane extracts (1 mg/ml) induced an inhibitory activity against *S. cerevisiae* of 86, 96, 76 and 86 %, respectively (Fig. 1b). The *n*-hexane fraction (1 mg/ml) of the same algae also showed strong inhibition of the *S. cerevisiae* growth (Fig. 1c). Two green algae, *Ulva compressa* and *Codium adhaerens*, presented high antifungal activity in the dichloromethane and *n*-hexane fractions with an inhibitory effect of 86, 35 and 70, 37 % respectively, however, no antifungal activity was achieved for green algae with the methanolic extraction.

IC₅₀ values were determined for algae that presented high activity at 1 mg/ml and ranged from 40.2 (32.4–49.9)

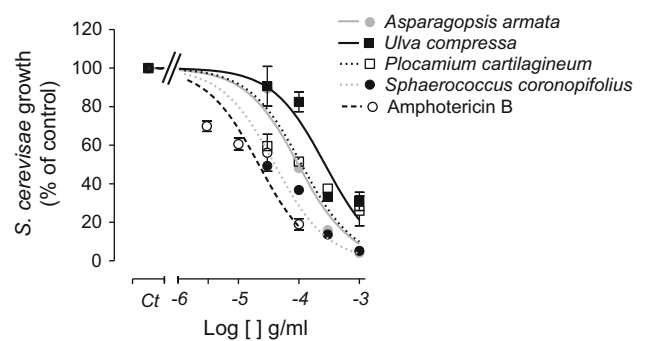


Fig. 4 *Saccharomyces cerevisiae* growth in the presence of different concentrations of amphotericin B or *n*-hexane fractions (100–1,000 µg/ml). Symbols represent mean of 8 independent determinations; vertical lines show SEM

exhibited by *Sphaerococcus coronopifolius* to 270.5 (179.7–407.2) exhibited by *Ulva compressa*, both in the *n*-hexane fraction (Figs. 2–4; Table 2).

Discussion

Antimicrobial activity was evaluated by the disc diffusion method, which is the most widely used method for susceptibility testing and is simple, economical and reproducible. Moreover, this standardized procedure is accepted for determining antimicrobial susceptibility by the National Committee for Clinical Laboratory Standards (NCCLS) (Salvador et al. 2007). In this test, filter paper disc impregnated with algae extracts showed clear inhibition zones against *B. subtilis*. For *E. coli* there was no inhibition zones around discs impregnated either with methanol, *n*-hexane or dichloromethane extracts of all the tested algae. The generally low activity of the extracts against the gram negative organisms may be due to the fact that gram negative bacteria possess an outer membrane and a periplasmic space, both of which are absent in gram positive bacteria. The outer membrane of gram negative bacteria is known to

Table 2 IC₅₀ values for antifungal activity of the dichloromethane, *n*-hexane and methanolic extracts

Algae	Dichloromethane IC ₅₀ (µg/ml)	<i>n</i> -Hexane IC ₅₀ (µg/ml)	Methanol IC ₅₀ (µg/ml)
<i>Asparagopsis armata</i>	119.8 (79.7–180.1)	97.6 (75.5–126.3)	–
<i>Ulva compressa</i>	139.3 (102.4–189.4)	270.5 (179.7–407.2)	–
<i>Plocamium cartilagineum</i>	130.3 (76.7–221.1)	111.1 (70.3–175.4)	–
<i>Sphaerococcus coronopifolius</i>	78.9 (51.3–121.5)	40.2 (32.4–49.9)	55.2 (32.6–93.5)

The results are the mean of eight independent determinations

Amphotericin B—IC₅₀ = 21.6 µg/ml (13.89–33.71)

present a barrier to the penetration of numerous antibiotic molecules. In addition, the periplasmic space contains enzymes which are capable of breaking down foreign molecules introduced from outside (Sofidiya et al. 2009). Of the twelve species of marine algae screened, all Rhodophyta (*Sphaerococcus coronopifolius*, *Ceramium ciliatum*, *Plocamium cartilagineum* and *Asparagopsis armata*), one Heterokontophyta (*Halopteris filicina*) and one Chlorophyta (*Ulva compressa*) showed activity against *B. subtilis*. The most active species were *A. armata* and *P. cartilagineum* that belong to the Rhodophyta division. The high efficiency of algae belonging to the Rhodophyta agrees with the results of previous studies using other test microorganisms (Mahasneh et al. 1995; Padmakumar and Ayyakkannu 1997). The inhibitory zones of the extracts are much smaller than those of the positive control, chloramphenicol. This is not surprisingly because extracts are complex mixtures of many compounds and the portion of active compounds is very low.

Three different solvents were used for the extraction of compounds, and positive results were obtained with them all. The *n*-hexane fraction presented antibacterial results with the highest activity for *Plocamium cartilagineum*, *Sphaerococcus coronopifolius* and *Ulva compressa*. On the other hand the methanolic fraction presented the highest activity for *Asparagopsis armata* and *Sphaerococcus coronopifolius* and the dichloromethane fraction presented the highest activity for *Ceramium ciliatum*, *Plocamium cartilagineum*, *Asparagopsis armata* and *Sphaerococcus coronopifolius*. These results indicate that the bioactive molecules involved in the antibacterial activity have different characteristics. The fractions obtained with methanol, *n*-hexane and dichloromethane contain compounds distributed according to their polarity. In this way, water-soluble organic material represented mainly by alkaloid salts, amino acids, polyhydroxysteroids, and saponins is found in the methanolic fraction. The dichloromethane fraction affords compounds of medium polarity such as peptides and depsipeptides. On the other hand with the *n*-hexane extraction only low polarity metabolites are obtained, such as hydrocarbons, fatty acids, acetogenins,

terpenes, etc. (Riguera 1997). In line with our results, several works also reported antimicrobial activity in the three fractions (Val et al. 2001; Lima-Filho et al. 2002; Bansemir et al. 2006; Taskin et al. 2007) which indicates the vast array of metabolites that can be involved in the antimicrobial activity.

When we looked for antifungal activity, the crude extracts were tested against *S. cerevisiae*. *S. cerevisiae* growth was monitored spectrophotometrically and the growth inhibition was determined after 20 h of incubation, which is a point in the exponential growing phase. Several macroalgae extracts inhibited the *S. cerevisiae* growth, however only those which revealed more than 60 % of inhibitory activity at the concentration of 1 mg/ml (IC₅₀ < 1 mg/ml) were evaluated for dose–response analysis. All the effects were obtained in a concentration dependent manner and IC₅₀ values were calculated. The *Sphaerococcus coronopifolius* presented the highest inhibitory effect off all algae in all the three extracts tested. Several halogenated metabolites have been isolated from *Sphaerococcus coronopifolius* and have been suggested to function as chemical defense against marine herbivores, and some of them have been proven to possess antibacterial, insecticidal, antifungal, and antiviral activities (Rhimou et al. 2010; Smyrniotopoulos et al. 2010). The results showed that highly potent antifungal activities could be detected in almost all red algae (with the exception of *Ceramium ciliatum*). Among the species investigated, as mentioned previously, *S. coronopifolius* was the most promising species tested by providing very active *n*-hexane (IC₅₀ = 40.2 µg/ml), dichloromethane (IC₅₀ = 78.9 µg/ml) and methanolic (IC₅₀ = 55.18 µg/ml) extracts against *S. cerevisiae* growth. Moreover, the potency obtained on the *n*-hexane and methanol fractions were similar to those obtained for the standard amphotericin B, which is one of the most potent antifungals, particularly used as therapeutically agent in the management of serious systemic fungal infections. This would agree with the finding that *S. coronopifolius* fractions are very promising for further efforts on purification and isolation of single bioactive compounds for future drug development. In fact, several works have

been done for screening antimicrobial activities in algae, and red algae have revealed interesting activities in vitro (Mayer et al. 2007; Felício et al. 2010; Allmendinger et al. 2010). Despite not having a marked effect as the red algae, *Ulva compressa* also inhibit *S. cerevisiae* growth substantially in the dichloromethane and *n*-hexane fractions with an $IC_{50} = 139.3$ and $IC_{50} = 270.5$ $\mu\text{g/ml}$, respectively. This result is in accordance with those of Kandhasamy and Arunachalam (2008), Osman et al. (2010) and Spavieri et al. (2010), who reported that green algae (Chlorophyceae) have great antimicrobial activity.

There are many factors that have to be noted in these kind of studies that can explain different results obtained by different authors, such as the variability in the production of secondary metabolites related to seasonal variations (Moreau et al. 1988; Lima-Filho et al. 2002), the differences in the stage of active growth or sexual maturity of the studied algae (Hornsey and Hide 1976) and the differences in the extraction protocols to recover the active metabolites (Val et al. 2001).

In summary our results indicate that algae collected from the Peniche coast produce a high variability of compounds, some of them with high antimicrobial activity which makes them interesting for programs screening natural products. This ability is not restricted to one division within macroalgae since all of them, Chlorophyta, Heterokontophyta and Rodophyta showed positive results. In addition, their capacities could be altered depending on the physiological state of algae and seasonal variations. All fractions in study revealed interesting results and given that these are multi-component fractions, there is a potential for discovering multiple active compounds in a single fraction, making these organisms a valuable source of novel substances for future drug development. The excellent activities observed in some of the fractions could stem from low-abundant substances, signifying a high potency for these compounds. Work is currently being carried out for the isolation and structural elucidation of the active compounds.

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