

## Antibacterial activity of *Falkenbergia hillebrandii* (Born) from the Indian coast against human pathogens

### Actividad antibacteriana de *Falkenbergia hillebrandii* (Born) de la costa India contra patógenos humanos

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**Abstract.** The antibacterial property of the red algae, *Falkenbergia hillebrandii* (Born) collected from the southwest coast of India (Indian Ocean) was evaluated against three multidrug resistant human pathogens. Four different solvents: ethyl acetate, dichloromethane, methanol and phosphate buffer saline (PBS) were used with this purpose. Dried samples extracted with methanol showed broadest and highest antimicrobial activity when compared to other solvents. However, PBS extract showed no antibacterial activity. The highly active compounds red alga, *F. hillebrandii* were fractionated and purified using different chromatographic systems, including reverse phase HPLC and GC-MS. The analysis revealed that the most abundant metabolite was the oleic acid (51.33%) followed by *n*-hexadecanoic acid (42%).

**Key words:** Antimicrobial activity, *F. hillebrandii*, Multidrug resistant human pathogens, Methanolic extract, Bioactive compounds.

**Resumen.** La propiedad antibacteriana del alga roja, *Falkenbergia hillebrandii* (Born), recogida en la costa suroeste de la India (Océano Índico), fue evaluada contra tres cepas de patógenos humanos resistentes a varias drogas. Con este propósito se utilizaron cuatro solventes diferentes: acetato de etilo, diclorometano, metanol y un amortiguador (buffer) de fosfato salino (PBS). Las muestras secas extraídas con metanol mostraron una actividad antimicrobiana mayor y más amplia en comparación a otros disolventes. Sin embargo, los extractos de PBS no mostraron actividad antibacteriana. Los principios bioactivos del alga roja, *F. hillebrandii* fueron fraccionados y purificados utilizando diferentes sistemas de cromatografía de fase inversa incluyendo HPLC y GC-MS. El análisis reveló que el metabolito más abundante fue el ácido oleico (51,33%), seguido por el ácido *n*-hexadecanoico (42%).

**Palabras clave:** actividad antimicrobiana, *F. hillebrandii*, patógenos humanos resistentes a drogas, extracción metanólica, compuestos bioactivos.

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## INTRODUCTION

Marine environments are a wealthy source of biological and chemical diversity (Minh et al., 2005). Worldwide, there has been a renewed interest in marine natural products with enormous therapeutic potential to heal many infectious diseases. Historically, marine ecosystems have provided an inspiration source for novel drug compounds, as marine-derived medicines have made large contributions to human health and well-being. In recent years, marine natural product search has yielded a considerable number of drug candidates (Haefner, 2003). Nearly all forms of life in marine environments (e.g., algae, sponges, corals, tunicates, nudibranches) have been investigated for secondary metabolite contents (Faulkner, 2000). Several of these bioactive natural products provide vital starting materials for the rational generation of libraries of compounds against infectious diseases, cancer and neurological targets, prepared through semisynthesis and biocatalysis (Cooper, 2004).

Naturally occurring antimicrobial agents were reported more than a century ago (Maruzzella & Sicurella, 1960) mainly by botanists (Bérdy, 2005). Of the approximately 25,000 higher plant species described, only about 6% have been screened for biological activity (Fabricant & Farnsworth, 2001). According to the World Health Organization, botanics should be the best source to obtain a variety of drugs (Santos, 1995). The currently secondary metabolites identified in microbes display a great variety of biological effects, primarily antimicrobial activities. Plant-based antimicrobial compounds continue to play an essential role in primary health care of about 80% of the world's population (Cooper, 2004). Investigation of these compounds from seaweeds has been determined during several hundred years (Harder & Oppermann, 1953). Ever since, the development and use of these seaweed secondary metabolites have increased due to the appearance of new and re-emerging infectious diseases.

Approximately one-half of deaths in tropical countries are affected severely by infectious diseases (Iwu et al., 1999). The World Health Organization (WHO, Geneva) estimates globally that about 1500 people die each hour from infectious diseases, half of these are children under five years of age (Meylears et al., 2002). In the last five decades, increased resistance of bacteria strains to drugs, including antibiotics, has been a major factor increasing morbidity, mortality and health care costs to bacterial infections. The economic worldwide crisis, high cost of industrialized medicines, inefficient public access to medical and pharmaceutical care, and side effects caused by synthetic drugs are some of the factors contributing to the central role that medicinal plants have in health care (Johann et al., 2007). It is urgent the discovery and development of new infection-fighting strategies that counteract the alarming increases in the indiscriminate and abusive use of synthetic antibiotics. Moreover, the accumulation of spontaneous, drug-resistant mutations over time within bacterial populations, and the horizontal spreading of these mutations

across major taxonomic units, dangerously accelerate the development of strains which become resistant to a wide range of conventional antibiotics (Meylears et al., 2002).

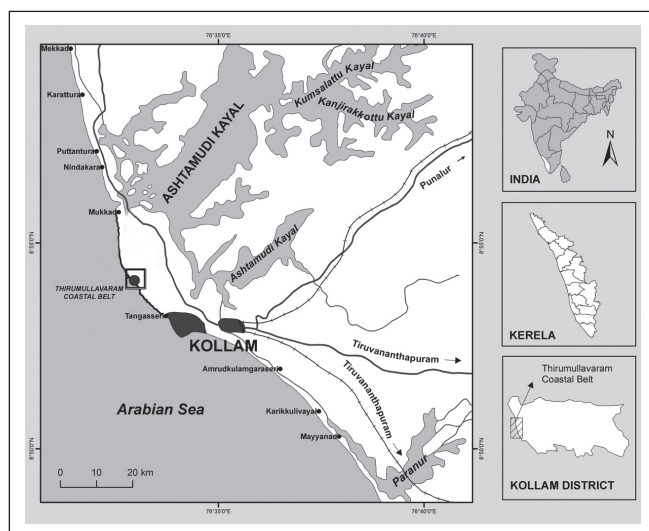
The seaweeds from the southwest coast of India (Kollam coast) are well known for its antibacterial, anticandidal (Shanmughapriya et al., 2008), cytotoxic, mosquito larvicidal, anti-feedant and nematocidal activities (Manilal et al., 2009). The objective of this work was to evaluate the potential antimicrobial activity of the red algae, *Falkenbergia hillebrandii* against several multidrug resistant clinical pathogens, and purification of bioactive compounds.

## MATERIALS AND METHODS

**Collection of algae.** *Falkenbergia hillebrandii* was collected (December to April 2008) during the lowest ebb tide of chart datum from the seaweed infested locations along the southwest coast of India, Kollam (08° 54' N, 76° 38' E) (Fig.1). The intertidal, subtidal region of Kollam coast is characterized with vertical rock walls to horizontal ledges, sloping or flat bed rock, broken rock, boulder fields, and aggregations of cobbles is rife with different marine algal species. Robust turf of *F. hillebrandii* was found during the winter season. The red algae, which infested exclusively on the intertidal rocky and other substratum, were selected for collection to avoid other algal contamination. Immediately after collection, they were washed in fresh seawater to eliminate the epiphytes, coarse sand and other calcareous impurities. The collected samples were transported in ice bags to avoid decomposition and loss of metabolites. Morphology and anatomical features of collected samples were analyzed, and reconfirmed with the help of Dr. M.V.N Panikkar, Algologist, Sree Narayana College, Kollam, Kerala, India.

**Fig. 1.** Map showing the study area Kollam coast (southwest coast of India).

**Fig. 1.** Mapa que muestra el área de estudio Kollam (costa suroeste de la India)



**Extraction of fresh plant material.** Ten gram of tissue was pounded with 100 ml solvent of increasing polarity, including ethyl acetate, dichloromethane, methanol and phosphate buffer saline. This material was vortexed and filtered through double folded muslin cloth and the filtrate was centrifuged at 8000 xg during 10 min (Eppendorf). The supernatant was evaporated (Yamato) to dryness at 40°C, and it was used for microbicidal assays.

**Extraction of shade dried plant material.** A definite quantity (20.0 g) of dried algal powder was submerged in Scott Duran flasks containing 200 ml of solvents of increasing polarity. They were placed at 35°C on a shaker at 120 rpm for two weeks to allow full extraction of the active compounds. After two weeks, algal material was filtered using Whatman filter paper No 1 fitted with a Buchner funnel using suction pressure followed by centrifugation (Eppendorf) at 8000 xg during 5 min at 20°C. The supernatant was poured in a round-bottomed flask, and the solvent was concentrated up to 5-10 ml in a rotary vacuum evaporator (Yamato). The gummy extract was collected in air-tight plastic vials and stored in the refrigerator for further studies.

**Test organisms.** Extracts were tested against a panel of clinical isolates such as *Enterococcus faecalis*, *Salmonella typhi* and *Shigella* sp. These isolates were established as multidrug resistant pathogens and deposited in the Biomedical Diagnostic Laboratory, Bharathidasan University. The resistance patterns of these isolates were confirmed using selective antibiotics. Preliminary experiments confirmed that the isolates showed resistance against chloramphenicol, streptomycin, oxytetracycline, ampicillin and erythromycin.

**Antimicrobial assays.** The antibacterial assay was carried out following Selvin & Lipton (2004). Briefly, the base layer was prepared with 10 ml (1.5%, w/v) of Muller Hinton agar (Himedia). Five numbers of sterile porcelain beads were placed on the base layer at 60° angle apart. The overlaid seed layer was prepared by pouring 15 ml of media containing 0.2 ml of prepared inoculum (~ 0.2 OD at 630 nm). The porcelain beads were removed carefully with sterile forceps. The resultant wells in triplicate were filled with 100 µl of algal extract. The well with solvent used for dissolution was taken as negative control. After 24 h of incubation at 37°C, the diameter of inhibition around the wells was determined as average of triplicates.

**Fractionation and purification of *F. hillebrandii*.** The methanolic extracts of the seaweed (10 gm) were applied in a silica gel (60-120 mesh) (Merck) column packed with petroleum ether and eluted with petroleum ether and ethyl acetate (9:1 to 1:9 and 100% ethyl acetate) followed by ethyl acetate and methanol (9:1 to 1:9 and 100% methanol). This yielded

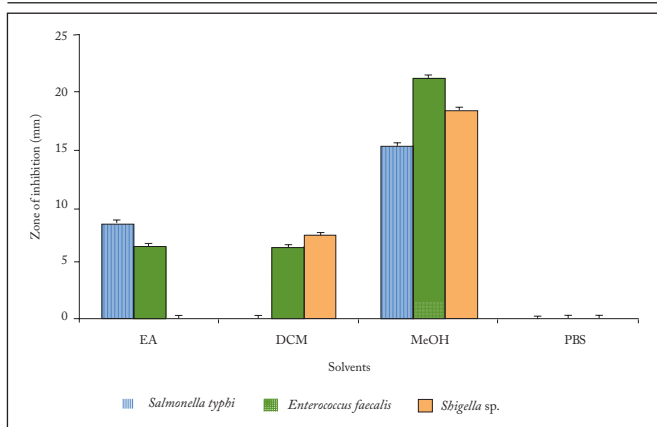
seven fractions. Individual fractions were collected and tested in the antimicrobial assay.

The fraction that was collected using petroleum ether: ethyl acetate (1:1), exhibiting antimicrobial activity, was further purified by preparative TLC using silica gel G as stationary phase with 1% methanol in dichloromethane as mobile phase. After the development of the chromatogram, the resolved spots were sprayed with 50% sulphuric acid to obtain a single spot with a *R<sub>f</sub>* value of 0.641. The TLC resolved spot were recovered by scrapping off and eluted with methanol and finally centrifuged at 10000 xg for 5 min. The active TLC resolved spot was again purified with reverse phase HPLC (Shimadzu Chromatographic System Kyoto, Japan) at 254 nm absorbance with methanol at a flow rate of 1 ml/min; head pressure at 25 kgf/cm<sup>2</sup>. The whole setup was maintained at room temperature (25°C). It showed two major peaks with retention times (min) of 2.69 and 3.00, respectively, at a wavelength of 254 nm (Fig. 3). The eluted HPLC peak that retained antimicrobial activity (data not shown) was chosen for GC-MS analysis using a Hewlett Packard 5890 Series II gas chromatographic system (Hewlett Packard, Waldbronn, Germany). It was equipped with a HP-5971 mass selective detector (MSD, Hewlett Packard, Palo Alto, CA, USA) and a capillary column (30m × 0.25mm × 0.25mm). The temperature was programmed from 110°C to 280°C at a rate of 5°C/min. Helium was used as a carrier gas. The GC-MS analysis of phyco-constituents was based on the interpretation of the mass spectral fragmentation followed by the comparison of the spectra that was available in the NIST library Ver. 2.0, 2005.

**Determination of the mechanism of antibiosis (bacteriostatic or bactericidal).** The minimal inhibitory concentration (MIC) was determined by the broth dilution method. The multidrug resistant *S. typhi*, *E. faecalis* and *Shigella* sp. were used for the determination of MIC. The 96-well microtiter plates were filled with 0.1 ml active fractions prepared in Mueller-Hinton broth (MHB). The microtiter plates were incubated in a 10% CO<sub>2</sub> atmosphere at 37°C for 48 h. In every microtiter plate, one row was set for control (without column fractions) and standard (nystatin and nalidixic acid). After incubation, the OD was read at 610 nm (Spectronic 20 UV-Vis spectrophotometer). MICs were recorded as the lowest concentrations inhibiting visible growth. To measure the minimal bactericidal concentrations (MBC), the MIC cultures were plated on Mueller-Hinton agar with 5% lysed horse blood and incubated them for 24 h at 37°C in a 10% CO<sub>2</sub> atmosphere. A reduction of at least 90% of the colonies, compared with the culture of the initial inoculum of the strain, was regarded as evidence of bactericidal activity. When the ratio of MBC/MIC was ≤ 2, the active fractions were considered as bactericidal. Otherwise they were considered as bacteriostatic. If the ratio was ≥ 16 the fractions were considered as ineffective.

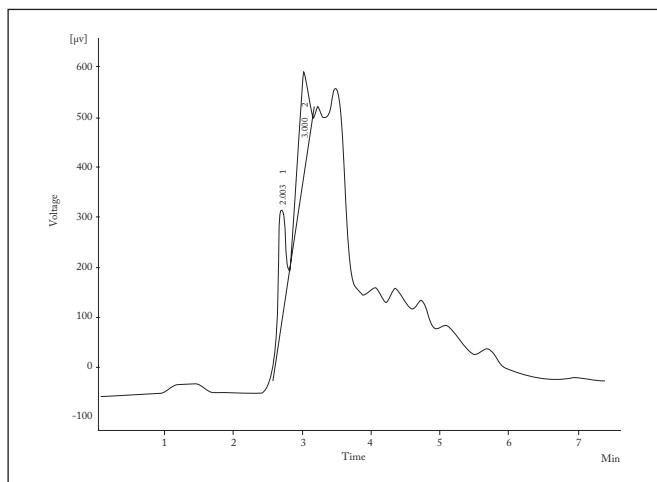
**Fig. 2.** Overall antibacterial activity of *F. hillebrandii* against multidrug resistant human pathogens. (EA-Ethyl acetate, DCM-Dichloromethane, MeOH-Methanol, PBS-Phosphate buffered saline).

**Fig. 2.** Actividad antibacteriana de *F. hillebrandii* contra patógenos humanos de resistencia múltiples. (EA-acetato de etilo, DCM-Diclorometano, MeOH-metanol, PBS-amortiguador de fosfato salino).



**Fig. 3.** HPLC profile of active TLC fraction of *F. hillebrandii*.

**Fig. 3.** Perfil de HPLC de la fracción activa TLC de *F. hillebrandii*.



## RESULTS AND DISCUSSION

**Study area.** Kollam coast lies at the lower portion of the southwest coast of India, and is approximately 45 km long. Kollam coast possesses a tropical wet climate produced by its location in the peninsula in Southeast Asia, and the influence of oceanic and continental weather systems. The monsoon is the characteristic climate feature and represents a seasonal shift in the prevailing winds from the northeast (continental influence) to the southwest (oceanic influence). Temperature and humidity are high and relatively consistent throughout the year. The intertidal and subtidal rock out crops and pools contain a large roster of marine algal species. Extensive beds of *F. hillebrandii* can be found during the winter season.

**Antibacterial activity.** Table 1 shows the antibacterial activity of crude organic extracts of *F. hillebrandii* against three multidrug resistant pathogens. Of the four solvent tested, methanol was determined to be the best solvent for isolation of bioactive secondary metabolites from dried red algae followed by ethyl acetate and dichloromethane (Fig 2). However, PBS extract of *F. hillebrandii* showed no antibacterial activity. This may be due to the high lipophilic nature of bioactive metabolites. These results indicate that the extraction method had definite effects on the isolation of bioactive principles. The effectiveness of extraction methods reported by many authors showed that methanol extraction yielded higher antimicrobial activity than n-hexane and ethyl acetate (Rosell & Srivastava, 1987; Sastry & Rao, 1994; Fables et al., 1995; Paul & Puglisi, 2004). There are few reports that chloroform is a better solvent than methanol and benzene (Takaki-Campos et al., 1988). Dried seaweed extracts had higher activity on bacteria in comparison to the fresh seaweed extracts. Earlier research reported the lower activity in extracts from fresh tissues than that from dried materials (Rao et al., 1986; Khotimchenko & Vaskovsky, 1990).

**Table 1.** Antibacterial activity against three human pathogens of fresh and dried *F. hillebrandii* extracted in different solvents.

Data shown are the mean zone of inhibition  $\pm$  1 S.D. (mm) of n=3.

**Tabla 1.** Actividad antibacteriana contra tres patógenos humanos de *F. hillebrandii* desde tejido fresco y seco extraído en diversos solventes. Los datos son la zona promedio de inhibición  $\pm$  1 D.S. (mm) de n=3.

Solvents	Tested Microorganisms		
	<i>Salmonella typhi</i>	<i>Enterococcus faecalis</i>	<i>Shigella sp.</i>
<b>Dried <i>F. hillebrandii</i></b>			
Ethyl acetate	6.32 $\pm$ 2.05	9.21 $\pm$ 2.89	-
Dichloromethane	-	6.26 $\pm$ 1.21	7.31 $\pm$ 2.1
Methanol	15.33 $\pm$ 1.6	21.53 $\pm$ 2.1	18.76 $\pm$ 2.31
Phosphate buffer saline	-	-	-
<b>Fresh <i>F. hillebrandii</i></b>			
Ethyl acetate	4.32 $\pm$ 2.05	6.32 $\pm$ 2.75	-
Dichloromethane	-	4.21 $\pm$ 1.28	7.31 $\pm$ 2.1
Methanol	10.6 $\pm$ 2.16	13.61 $\pm$ 2.4	12.33 $\pm$ 2.9
Phosphate buffer saline	-	-	-

In the present study, methanolic extract from dried *F. hillebrandii* exhibited the broadest and highest activity, which successfully inhibited *Enterococcus faecalis* and *Shigella sp.* to the extent of 21.53 mm and 18.76 mm, respectively, at 37°C. Inhibition zones for those pathogens were 13.61 mm and 12.33 mm, respectively, using the fresh methanolic extracts.

A gram-negative bacterium, *Salmonella* sp. was moderately sensitive to *Falkenbergia hillebrandii*, which produced a zone of inhibition of 15.33 mm and 10.6 mm against dried and fresh methanolic extracts, respectively. Our results agree with the antimicrobial activity of genus *Falkenbergia* reported from different geographic regions (Olessen et al., 1963; Bansemir et al., 2006; Salvador et al., 2007).

**Spectrum of active fraction.** The GC-MS chromatogram of the *F. hillebrandii* active fraction is shown in Fig.4, and the relative percentage of identified compounds is summarized in Table 3. It was found that the main phycoconstituent of the active fraction was oleic acid (51.33%) followed by n-hexadecanoic acid (42%). This result agrees with the report of Khotimchenko & Vaskovsky (1990), who suggested that C<sub>16</sub>, C<sub>18</sub>, C<sub>20</sub> fatty acids are dominant in red algae. We also found a mixture of fatty acids on the basis of spectral data by GC-MS (Table 2).

It is well known that fatty acids are a vital constituent of both terrestrial and marine plants (Evans, 1996; Saravanakumar et al., 2008). Antimicrobial properties of fatty acids were reported as early as 1960 (Katayama, 1960). In recent years, mi-

**Table 2.** GC-MS data of active fraction of *F. hillebrandii*.

**Tabla 2.** Análisis de la fracción activa de *F. hillebrandii* obtenidos por GC-MS.

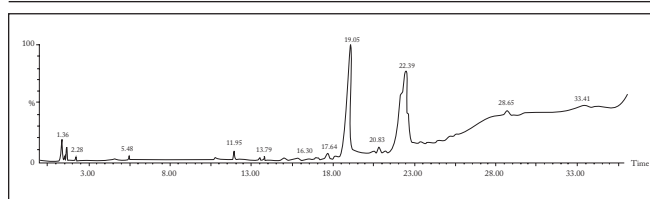
Sample #	RT	Compound Name	Molecular Formula	Molecular weight	Peak Area %
1	1.36	Nitroacetone	C <sub>2</sub> H <sub>2</sub> N <sub>2</sub> O <sub>2</sub>	86	1.02
2	1.68	Ethanamine,2-(methylthio)-	C <sub>3</sub> H <sub>9</sub> NS	91	0.39
3	2.28	Pentane,3-methyl-	C <sub>6</sub> H <sub>14</sub>	86	0.17
4	4.16	1-Hexane,2,5-dimethyl-	C <sub>8</sub> H <sub>16</sub>	112	0.11
5	4.60	3-Pentanol,3-ethyl	C <sub>7</sub> H <sub>16</sub> O	116	0.17
6	5.48	1-Hexene,4-Methyl-	C <sub>7</sub> H <sub>14</sub>	98	0.17
7	6.91	1-Heptyn-4-ol	C <sub>7</sub> H <sub>12</sub> O	112	0.11
8	9.25	(2S,3S)-(-)-3-Pro-pyloxiranemethanol	C <sub>6</sub> H <sub>12</sub> O <sub>2</sub>	116	0.11
9	10.22	1-Cyclopentyl-2,2-dimethyl-1-propanol	C <sub>10</sub> H <sub>20</sub> O	156	0.11
10	11.95	N-Decanoic acid	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>	172	0.85
11	13.54	Oxirane, (butocemethyl)	C <sub>7</sub> H <sub>14</sub> O <sub>2</sub>	130	0.17
12	17.64	2-Nonen-1-ol, (E)-	C <sub>9</sub> H <sub>18</sub> O	142	1.02
13	19.05	N-Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	42.87
14	20.83	Dodecyl acrylate	C <sub>15</sub> H <sub>28</sub> O <sub>2</sub>	240	1.47
15	22.39	Oleic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	282	51.33

**Table 3.** MIC and MBC values of the active principles against three pathogenic bacteria.

**Tabla 3.** Valores de MIC y MBC de los principios activos contra tres bacterias patógenas.

Test pathogens	Seaweed	MIC (µg)	MBC (µg)	MBC/MIC
<i>Salmonella typhi</i>		1300	380	0.2
<i>Enterococcus faecalis</i>	<i>F. hillebrandii</i>	800	180	0.2
<i>Shigella</i> sp.		800	170	0.2

**Fig. 4.** GC-MS chromatogram of the active fraction of *F. hillebrandii*.  
**Fig. 4.** GC-MS cromatograma de la fracción activa de *F. hillebrandii*.



crobiological properties of oleic acid from red algae are extensively reported in the literature (Saravanakumar et al., 2008). Synthesis of fatty acids in seaweed is controlled by both biotic and abiotic factors (Nichols, 1965). Evidence supporting bioactivity of fatty acids was earlier demonstrated in certain microalgae and mangrove plants (Findlay & Patil, 1984; Viso et al., 1987; Kellam et al., 1988; Russel, 1991; Agoramorthy et al., 2007). Microbicidal activity exhibited by seaweed fatty acids may be useful in developing alternative approaches to control different human pathogens, such as those examined in this study.

**Mechanism of antibiosis.** The MBC/MIC ratio was determined to identify whether the active principle was a bactericidal or a bacteriostatic compound. The results of MIC and MBC of the active principles from *F. hillebrandii* are presented in Table 3. Since the MBC/MIC ratio is less than 1, the active principles can be considered to be a bactericidal agent. (Shanmughapriya et al., 2008).

## CONCLUSION

The microbicidal activities observed in the crude methanolic extracts of *F. hillebrandii* from the southwest coast of India provide good evidence that algae maintain effective antimicrobial chemical resistance, and this antibacterial property is due to the presence of oleic (51.33%) and n-hexadecanoic acids (42%). From the present study, it can be concluded that the red alga *F. hillebrandii* is a potential source of bioactive compounds. These compounds can be utilized for the development of natural antibiotic against multidrug resistant bacteria.

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