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Antimicrobial Screening of Marine Endophytes and Epiphytes Isolated from Marine Algae of Kenyan Indian Ocean

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Abstract Marine algae have been known to produce secondary metabolites used in the pharmaceutical industry to treat various human diseases. This study embarked on the isolation of microbes associated with the marine algae and explores their potential as sources of antimicrobial agents that can be used in the production of drugs to treat the emerging and re-merging resistant human pathogens. The associated microbes that is, endophytes and epiphytes were isolated from different red, green and brown marine algae species which were collected from Mkomani Island in Mombasa Kenya. Eight hundred and thirty isolates were isolated of which three hundred and six were endophytes while five hundred and twenty four were epiphytes. Antimicrobial screening showed that two hundred and thirty isolates (28%) exhibited antimicrobial activity against *Staphylococcus aureus* ATCC 25922, *Escherichia Coli* ATCC 25923 and *Candida albicans* ATCC 90028. Most isolates showed inhibition activity against *Escherichia Coli* (47%). Inhibition against *Candida albicans* was 33% whereas for *Staphylococcus aureus* was 21%. The results reveal that marine algae harbor microbes that are potential producers of antimicrobial compounds that need to be investigated further for their pharmaceutical and biotechnological potential.

Keywords: *marine microbes, antimicrobial screening, pathogens, marine algae*

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1. Introduction

Nature has always been a source of medicinal and pharmaceutical agents for thousands of years and a huge number of currently used modern drugs have been isolated from microorganisms. In the past century, an increasing role has been played by microorganisms in the production of antibiotics and other drugs for the treatment of microbial infections. Microorganisms have been the source of many valuable compounds in medicine, industry and agriculture; most are derived from terrestrial habitats. Previously the marine environment was considered devoid of life but it's now clear that the ocean is thriving with tremendous diversity [1,2]. In addition, this environment is becoming increasingly appreciated as an excellent reservoir of bioactive natural compounds that exhibit structural and chemical features not found in terrestrial natural products [3].

Since the ocean covers 70% of the earth s surface and harbors most of the planet s biodiversity [4], it contains a variety of species, many of which have no terrestrial counterparts [5]. Hence, marine microorganisms have become an important target of study in developing for novel antibiotics since it's a largely untapped resource. In addition, the huge microbial diversity in the ocean is assumed to translate into metabolic diversity resulting in the potential for new bioactives. The marine species live in a stressful habitat, under cold, lightless and high pressure conditions. These factors have resulted in the development of unique metabolisms, which provide the opportunity to produce metabolites that differ from the terrestrial ones.

Marine algae or seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities [6] with antiviral, antibacterial and antifungal activities [7] which acts as potential bioactive compounds of interest for pharmaceutical applications [8]. Current emerging and reemerging infectious diseases are a major problem in public health causing high mortality rates. These infections are mainly caused by drug resistant microbes, mostly involving bacteria and fungi, both of which are becoming progressively more resistant to conventional antibiotic compounds. However, much of the microbial diversity in marine ecosystems with its potential for uncovering new antimicrobial compounds remains to be discovered. Hence, the present study was undertaken to isolate marine

endophytes and epiphytes associated with marine algae and to evaluate their antibiotic producing efficacy against known human pathogens.

2. Materials and Methods

2.1. Sample collection

Different classes and species of marine algae were collected. They included: Red algae (*Gracilaria salicornia, Hypnea musciformis, Acanthophora spicifera, Chrondrophycus papillosus, Gracilaria corticata, Phacelocarpus tristichus, Hypnea pannosa, Laurencia sp.*); green algae (*Codium geppiorum, Ulva fasciata, Caulerpa Mexicana, Caulerpa sertulariodes, Ulva reticulata*) and brown algae (*Turbinaria decurrens, Sargassum cristaefolium, Spatoglossum asperum, Padina tetrastromatica, Dictyota cervicornis, Colpomenia sinuosa, Labophora variegata*). These marine algae samples were collected from the intertidal zone of Mkomani (North Coast) Mombasa Kenya (Latitude 4° 31' S and Longitude 39° 41' E) during the low tide, in the months of February 2014 and March 2014 (Northeast monsoon season). The algal samples were washed with ocean water to remove the loosely attached debris and put in sterile plastic bags filled with ocean water to avoid desiccation, stored in a cooler box with ice packs and transported aseptically to the laboratory for further processing.

2.2. Isolation of Marine Algae Associated Endophytes and Epiphytes and Antimicrobial Assay

2.2.1. Isolation of Marine Endophytes

Portions of marine algae samples were thoroughly washed with sterile ocean water to remove the loosely attached epiphytes for one minute, followed by two minutes wash in 70% ethanol and washing in 2% sodium hypochlorite for one minute. The samples were rinsed with sterile ocean water for five minutes with shaking and dried with sterile paper towels. Some algal samples were cut into sections of 2-3 cm using a sterilized scalpel. The cut sections were then placed in contact with Nutrient Agar, Tryptic Soy Agar and Potato Dextrose Agar media. The media were prepared using Ocean water to maintain the natural environment of the microbes.

2.2.2. Isolation of Marine Epiphytes

The marine algae were washed with sterile ocean water for a few seconds. The surface of the algae was swabbed with a wet sterile cotton swab to obtain the epiphytes. The swabs were spread plated on Nutrient Agar, Tryptic Soy Agar, and Potato Dextrose Agar media and were all prepared using Ocean water.

All the inoculated media plates were incubated for 24-48 hours at 30°C until colonies appeared. After incubation the colonies were sub- cultured to obtain pure isolates and stored at 4°C for further study.

2.3. Antimicrobial Assay

All the obtained pure endophytic and epiphytic isolates were screened for their antimicrobial activity against a Gram positive and Gram negative bacteria and yeast. This was performed using the disc diffusion method as described in [9] on Mueller Hinton agar (MHA) using 24 hour culture of the pathogens *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923) and fungi *Candida albicans* (ATCC 90028).

The pure colonies were cultured overnight in Mueller Hinton broth prepared using ocean water, to mimic the natural environment of the isolates and to maximize the production of their antimicrobial substance. The pathogens were also cultured overnight in Mueller Hinton agar plates. For overnight cultures of the pathogens, a 0.5 MacFarland standard was prepared which was uniformly spread on sterile Mueller Hinton agar plates using sterile cotton swabs. A volume of 20µl of the marine broth overnight culture was then used to saturate sterilized paper discs (Whatman 6mm), and placed on the surface of the Mueller Hinton agar plates which had been freshly swabbed with the test pathogens. The loaded plates were incubated for 24 hours at 37°C. The zones of inhibition were checked and recorded as positive if the diameter was more than 7mm.

3. Result and Discussion

3.1. Isolation of Marine Algae Associated Microbes and Antimicrobial Assay

In this study 830 strains of microbes were isolated from 20 species of different marine algae, with the endophytic isolates being 306 and the epiphytic isolates being 524 (Table 1). The isolation of endophytes was based on the algae morphology, since not all algae have stems that can be cut. For the antimicrobial screening assay, all the isolates were tested for the production of antimicrobial metabolites using the disc diffusion assay against *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923) and *Candida albicans* (ATCC 90028). The antimicrobial assay showed that 230 marine strains showed activity against one or more test organisms. Active endophytic strains were 97 while active epiphytic strains were 133 (Table 1). These results are a proof that marine algae associated microbes does produce metabolites that have antimicrobial activity just like the host plant. In the recent years, the bioactive properties of marine algae and marine microorganisms have been studied and analyzed and in both cases positive results have been reported. A study carried out by [10] reported that a number of marine plants exhibited antibacterial activity. [11], also reported antibacterial and/or antifungal activities related to marine algae against several pathogens. Many of the marine algae species are often accompanied by several microbial strains. The mutually beneficial interaction between the host and the microorganisms presents a chemically driven interaction .This cross-relationships between microbes and their eukaryotic hosts, in which organisms producing antimicrobial compounds (antimicrobials) may protect the host surface against over colonization in return for a nutrient rich environment [12]. Some seaweed species are known to need vitamins for their growth and possibly the bacteria living in association with them are partially responsible for the production of these substances; some of them produce antibiotics [13,14] hence the bioactivity demonstrated by the screened microbes.

Table 1. Summary of marine microbe isolation and their antimicrobial assay profiles

HOST ALGAE SPECIES	ENDOPHYTES	BIOACTIVE ENDOPHYTES	EPIPHYTES	BIOACTIVE EPIPHYTES
A) RED ALGAE				
1.*Gracilaria salicornia*	49	22	24	8
2. *Hypnea musciformis*	-	-	26	14
3. *Acanthophora spicifera*	38	18	18	5
4. *Chrondrophycus papillosus*	52	16	24	5
5. *Gracilaria corticata*	-	-	32	6
6. *Phacelocarpus tristichus*	-	-	32	4
7. *Hypnea pannosa*	-	-	32	7
8. *Laurencia sp*	40	3	33	7
B) GREEN ALGAE				
9. *Codium geppiorum*	19	5	29	6
10. *Ulva fasciata*	-	-	31	3
11. *Caulerpa mexicana*	13	7	27	3
12. *Caulerpa sertulariodes*	-	-	19	4
13. *Ulva reticulata*	-	-	18	5
C) BROWN ALGAE				
14. *Turbinaria decurrens*	44	10	28	7
15. *Sargassum cristaefolium*	51	16	23	7
16. *Spatoglossum asperum*	-	-	37	9
17. *Padina tetrastromatica*	-	-	22	6
18. *Dictyota cervicornis*	-	-	33	18
19. *Colpomenia sinuosa*	-	-	16	6
20. *Labophora variegata*	-	-	20	3
TOTAL	**306**	**97**	**524**	**133**

From the antimicrobial screening assay, most isolates were bioactive against the Gram negative bacteria compared to Gram positive bacteria and fungi. Activity against was *Escherichia coli* (47%), *Candida albicans* (33%) and *Staphylococcus aureus* (21%) respectively. This indicates the presence of bioactive metabolites produced by the marine microbes. The susceptibility of Gram positive bacteria to the marine isolates extract was less compared to the Gram negative bacteria, this concurs with a study carried out by [15]. However, many authors have made reverse observations indicating that Gram positive bacteria are more susceptible to marine algal compounds compared to Gram negative bacteria [16, 17, 18, 19]. This means that the marine isolates posses' compounds that can act against the Gram negative outer membrane that inhibits most substances including antibiotics [20].The presence of thick murine layer in the Gram negative cell wall also prevents the entry of the inhibitors [21]. Antimicrobial activity of the red, green and brown algae against both Gram positive and Gram negative bacteria has been established by several scientists [22]. Considerable activity against *Candida albicans* was also noted from the study (Figure 1).

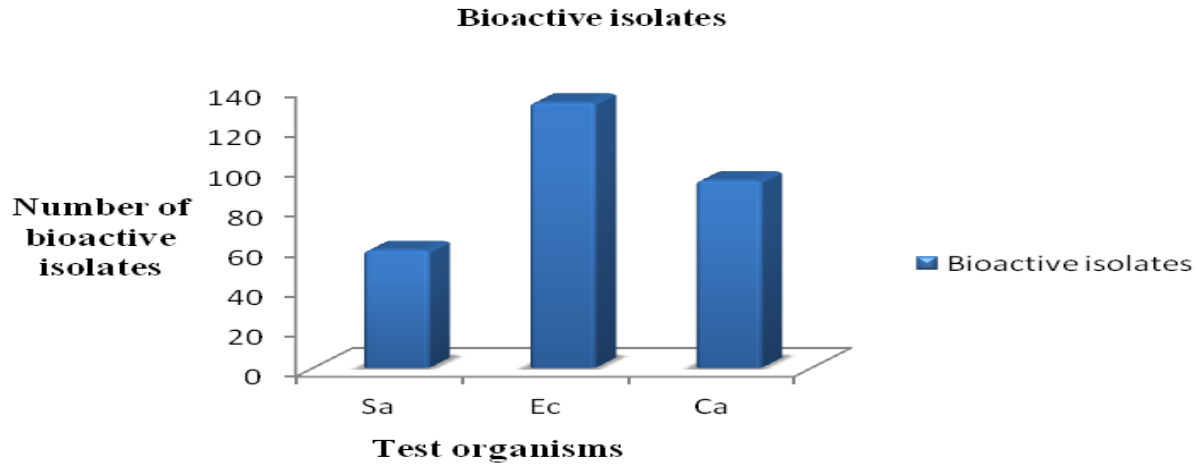

Figure 1. Inhibition activity against the test organisms

This is quite encouraging since *Candida albicans* is the most common cause of nosocomial infections, and especially in immune compromised individuals. The antimicrobial susceptibility assay gives a promising indication of developing a potent drug from these marine microbes to be used in combating the infections due to such pathogens.

The marine algae are classified into three classes: red algae (Rhodophyta), brown algae (Phaeophyta) and green algae (Chlorophyta). These different classes of algae are characterized by different chemical composition. Many types of seaweed are known to synthesize a wide variety of bioactive secondary metabolites which have antimicrobial, antifeedant, antihelmintic and cytotoxic

properties. This study showed different levels of biological activity against the different test organisms (Figure 2). The red algae produced more active isolates compared to the green and brown algae (Figure 3 and Figure 4). This difference in activity could be attributed to the difference in chemical composition of the algae. These chemical compounds range from acyclic entities with a linear chain to complex polycyclic molecules and included bioactive terpenes, phenolics compounds, alkaloids, polysaccharides and fatty acids. Many of these secondary metabolites are halogenated [23].

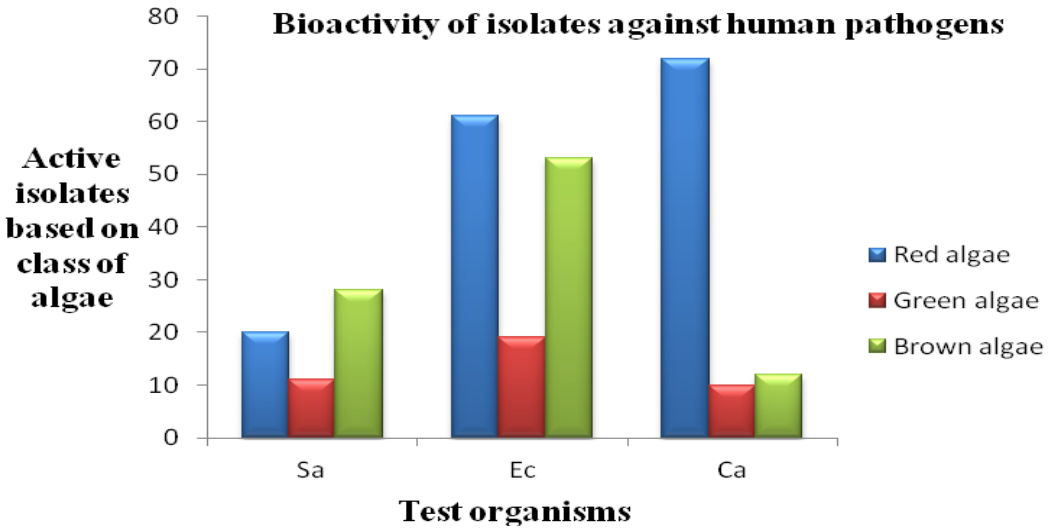

Figure 2. Bioactivity of marine isolates against the test organisms based on class of algae

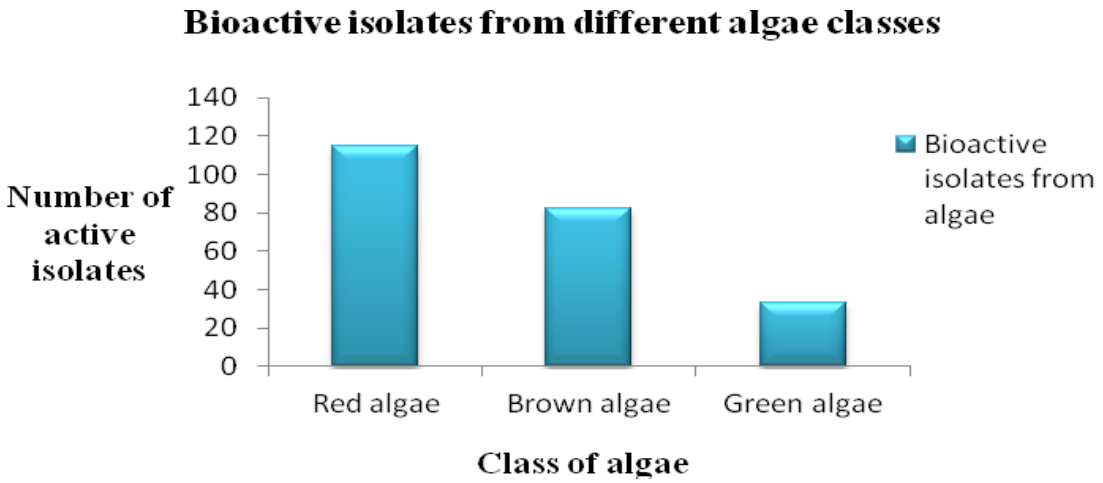

Figure 3. Bioactive isolates from Red, Brown and Green algae

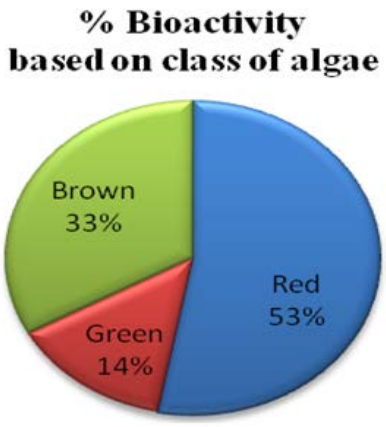

Figure 4. Bioactivity of isolates based on class of algae

In a study carried out by [24] they found that the red macro algae (Rhodophyta) stands out as the major producer of halogenated compounds above the green and brown macro algae groups. This could be the contributing factor to the high level of antimicrobial activity of the red algae demonstrated in this study. The marine algae and associated micro-organisms posses an inexhaustible source of useful chemical substances for the development of new drugs. These epiphytic and endophytic microorganisms found on and in marine algae respectively, are capable of biosynthesizing a broad variety of secondary metabolites .Hence; ocean thriving microbes are crucial organisms that can be used in biotechnology in the discovery of new compounds from marine origin. Numerous studies on seaweeds and other living plants show that they evolve a wide range of allelochemicals. Microbes living in close association with their host were later been found to also be producers of the same compounds. Therefore, bioactive producing marine microorganisms can be easily cultured and manipulated and represent the best renewable source of biologically active compounds and this would also contribute to environmental conservation.

In summary, the results indicate that the different classes and species of the marine algae collected from Mkomani Island at the Kenyan North coast are a host to many microbes that present significant antimicrobial and antifungal activity. Based on the results we suggest that the isolated microbes be investigated further for potential production of bioactive compounds that can be harnessed for the development of drugs for use in treatment and management of human pathogens, cancer and other reemerging human diseases and identified by morphological, biochemical methods and molecular characterization of the species responsible for the bioactive compound production.

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