Antimicrobial Activity of Extracts from Six Green Algae from Tanzania

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ABSTRACT

The antimicrobial activity of the extracts of six marine green algae from Tanzania was screened against three bacterial species Viz.; *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS), *Escherichia coli* (EC), and a yeast, *Candida albicans* (CA) using a disk assay method. A brine shrimp bioassay using newly hatched *Artemia salina* larvae was used for cytotoxicity studies of extracts from three algal species. Of the six species tested, *Valonia aegrophila* extract was most active against all the tested organisms, and its extract was even more active against CA, SA and EC than Penicillin G at a concentration of 2.5 flg hole-I. The extracts of *Halimeda opuntia* and H. *tuna* showed mild activity against all organisms. The extract of *Ulva pertusa* was more active against SA and BS but less active against EC and was not active against CA. The extract of *Caulerpa mexicana* was inactive against all the tested organisms. Occasional development of antimicrobial resistance colonies within the inhibition zones were seen in H. *opuntia* and H. *tuna* extracts when they were assayed against CA and EC. After 24 months storage, the extract of *Valonia aegrophila* exhibited halved antifungal activity and a slight decrease against EC, but increased activity against SA and BS. The crude extract of *U. pertusa* had lethal effects and killed 50% of the shrimp larvae when LC_{50} was 116 µg ml^{-1}, whereas extracts of *Call1erpa raccl1losa* and *Valoniaa aegrophila* LC_{50} occurred far above 1000 µg ml^{1}

INTRODUCTION

Many algae species have been shown to have bactericidal or bacteriostatic substances (Glombitza, I979; Michaneck, 1979; Caccamese *et al.,* 1980; Fenical & Paul, 1984; Niang & Hung, 1984). The antibacterial agents found in the algae include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, halogenated ketones and alkanes, cyclic polysulphides and fatty acids. In a large number of marine algae antimicrobial activities are attributed to the presence of acrylic acid. Other species have been found to contain antifungal activities such as in *Laurencia* sp. (Glombitza, 1979), *Caulerpa* sp. (Amicao *et al.,* 1979). The active antifungal agents so far reported include acrylic acid, phlorotannins, terpenoids and steroids. Marine algae have also been reported to contain antiviral substances (Faulkner, 1988), antitumour (Maruyama & Yamamoto, 1984), hypocholesterolemic (Yamoto *et al.,*

1984) and anthelminthic substances (Michanek, 1979; Mukarami *et al.,* 1953). Although many isolated compounds from marine algae show activity in-vitro, few are active in vivo (McConnel & Fenical, 1979) due to inactivation by serum protein binding, poor absorption at the site of infection (Reichelt &: Borowitzika, 1984) or easily metabolized to inactive compounds (McConnell & Fenical, 1979). However, many of these compounds can be used as agro-chemicals, in soaps, lotions, shampoos, creams, antiseptics and preservative agents.

In Eastern Africa no studies have been reported on the antimicrobial activities of extracts from seaweed of the region except that of three G*racilaria* spp. (Mtolera & Semesi, 1992). Thus far studies on the chemicals present in the seaweed have concentrated on the Phycocolloids (Semesi, 1987*;* Semesi & Mshigeni, 1977; Oyieke, 1993), and currently two *Euchema* species are being cultivated as a source of κ- and *i*- carrageenan. As such, this study was initiated to find a seaweed useful in Tanzania as a source of antimicrobial substances.

MATERIALS AND **METHODS**

Valonia acgrophila V. Ag., H*alimeda Opuntia* (L) L*amouroux* and *Halimedatuna* (Ellis & Sol.) Lamouroux were collected at Mbudya Island while *Caulepa racemosa* (forsk.) J. Ag. var. *clavifera f macrophysa* Web. V. Bosse, and *CauIerpa mexicana* (sonder.) J. Ag. were collected from Oyster Bay and *Ulva pertl/sa* Kjellman from Kunduchi, all allocations near the city of Dar es Salaam, Tanzania (Fig J). The algae were collected from the intertidal zone at spring low tides, taken to the laboratory, cleaned of epiphytes and rock debris and then given a quick freshwater rinse to remove surface salts. The algae were then air dried on blotting paper on laboratory benches. To hasten drying in order to avoid decomposition, forced air current was employed. Samples with 3-8% moisture content were milled before extraction.

15 g of the milled algal samples were left in 150 ml diethyl ether for 24 hours at room temperature. The cxtract's were filtered and the filtrate concentrated under reduced pressure at 37-40^{0}C. The crude extracts each dissolved in 5 ml solvent were assayed for antimicrobial activity.

Screening using the agar plate diffusion technique involved the bacteria: *Bacillus subtilis* (V5007), *Staphylococcus aureus* (NCTC 6571) and *Esherichia coli* (NCTC 10418) and a yeast *Candida albicans* (LIMMC). The bacteria or yeast used were obtained from siants which were less than 30 days old. Loopfull samples taken from the slants were grown in sterile 50 1111 broth which had been autoclaved at 121°C under a pressure of 15 atmos-
pheres for 15 min. and left to grow for 16 hr at 37°C in a shaking incubator (TFL type 3022) agitating at 140 rpm. The nutrient broth for bacteria growth was composed of NaCI (5 g), pep ton (5 g), yeast extract (2 g), Leb Lanco

powder (1 g) and distilled water added to 1 litre. The yeast was grown in a media containing soluble starch (15 g), Difco yeast extract (4 g), K_2HPO_4 (lg), $MgSO_4.7H_2O$ (0.5 g) and distilled water to 1 litre. Agar plates for the Jiffusion tests against bacteria were prepared as follows: Agar (15 g), NaCl (5 g), pepton (5 g), yeast extract (2 g), Leb Lanco powder (1 g) and distilled water added to 1 litre. The yeast was grown in a media containing agar (20 g), soluble starch (15 g), Difco yeast extract (4 g), K_2HPO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g) and distilled water to 1 litre. The media were sterilised as for the nutrient broth media, and 15 ml of the media were poured into sterile caped test tubes. Test tubes were allowed to cool to *50°C* in a water bath and O.5 ml of uniform mixture of an inocula was introduced to each tube. The tubes were mixed using a vortex mixer (Vibrofix type VF 1) vibrating at I 500-2000 rounds min^{-1} for 15-30 seconds. Each test tube content was then poured onto a sterile 80 mm diameter petri dish for solidification. To a solidified media, four holes, each measuring 7 mm diameter were evenly dug using a sterile cork borer. 50 µl of a solvent, a solution containing a known antibiotic (Penicillin G for bacteria and nystatin for yeast), and of the extract sample were added to the holes dug in the plate. The plates were .1l1owed 1 hour for the solvents to evaporate from the wells before they were subjected to 4°C incubation for 24 hours. To prevent drying the plates were covered with sterile plastic bags. The plates were later incubated at 37°C for 24 hours. In positive reactions, clear zones of inhibition appeared around the holes. Measurement of the width of the zones extending from the edge of the hole were taken.

The toxicity study was carried out by using newly hatched larvae (nauplii) of a shrimp, *Artemia salina.* The larvae were produced as follows: a dish 135 cm x 7.5 cm) was divided into unequal compartments (11 cm x *7.5 cm* and 2.5 cm x 7.5 cm) using a plastic divider with several 2 mm holes. To such a dish 200 ml artificial sea water prepared with commercial salt mixture (Instant Oceans Aquarium Systems, Inc.) and a double distilled water was added. Eggs of *Artemia salina* about 10 mg in weight were scattered into the larger compartment which remained under darkness, while the smaller compartment received illumination. Twelve hours later, phototropic 1arvae separated from their shells were collected from the lighted side. Within 1-4 hours following collection from the hatching dish, the larvae were subjected to the toxicity assay.

Diethyl ether solutions of the crude extracts of concentrations 1 000 µg ml^{-1}, 100 µg ml^{-1} and 10 µg ml^{-1} were made. These were then transferred to 13 mm diameter filter paper (Whatman, No 1) discs. The discs were dried by air current at 25°C for an hour and placed in dry vials. Control discs were prepared with/without diethyl ether. Ten larvae per 5 ml artificial sea water were put in lighted transparent Vials and allowed one hour adaptation before subjection to all antimicrobial dose. One paper disc containing

antimicrobial substances was added in each tube. Survivors were counted after 24 hours of antimicrobial subjection and the death percent determined. The larvae were counted with the aid of a magnifying glass. Ten replicates were prepared for assays of each extract.

RESU LTS

In the preliminary studies carried out to evaluate suitable handling of the seaweeds, it was found that extracts prepared from the sea weeds dried at 60°C showed least or no activity against the test microorganisms used, while those prepared from the deep frozen (-18^{0}C) materials were the most active, fol. lowed by those prepared from air dried, and oven dried at 37°c.

Of the six species tested, *Valonia aegrophila* extract was most active against all the tested organisms, and its extract was even more active against CA, SA and EC than Penicillin G at a concentration of 2.5 μg hole^{-1} (Table 1). The extracts of *Halimeda opuntia* and H. *tuna* showed mild activity against all organisms. The extract of *Ulva pertusa* was more active against SA and BS but was less active against EC and not active against CA. The extract of *Caulerpa mexicana* was inactive against all the tested organisms. Occasional development of antimicrobial resistance colonies within the in-

Table 1. Activity of crude extracts of six green marine algae against selected bacteria and a yeast

Marine algae	Activities against test microorganisms			
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*
Valonia aegrophila	++	+	+++	++++
Ulva pertusa	++	++	+	–
Halimeda opuntia	++	+	+	+
Halimeda tuna	+	+	+	+
Caulerpa racemosa	+	+	++	–
Caulerpa mexicana	–	–	–	–
Reference antibiotic, Penicillin G (2.5 μg/hole)	++	+	+	+

Inhibition zones:

> 30 mm	++++
21–30 mm	+++
11–20 mm	++
2.5–10 mm	+
< 2.5 mm	–

hibition zones was seen in H. *opuntia* and H. *tuna* extracts when they were assayed against CA and EC. After 24 months storage, the extract of *Valonia aegrophila* exhibited halved antifungal activity and a slight decrease against EC but increased activity against SA and BS.

The crude extract of U. *pertusa* had lethal effects and killed 50% of the shrimp larvae when their LC_{50} was 116 µg ml^{-1}, whereas extracts of *Caulerpa racemosa* and *Valonia aegrophila* LC_{50} occurred above 1 000 µg ml^{-1}.

DISCUSSION

The preliminary results showed that the antimicrobial substances from the algae are heat labile. Since air dried samples at 25°C retained activity, tropical room temperatures appear suitable for handling the algae for large scale antimicrobial extraction.

Storage of crude extracts at -18°C for 24 months resulted in some extracts reducing or losing their activity, while storage improved the activity of others. It is not possible to explain thoroughly the reasons for this behaviour, but it can be assumed that the interaction of substances changed during storage due to molecular stereochemistry modifications. Nevertheless, a substance could have multi-active sites, thus molecular modification may favour activities of different sites unequally. Pre-incubation at 4°C of the inoculated plates slows growth and gives ample time for the antimicrobial agent to diffuse, improving sensitivity of the plate diffusion method as a result. However, if the pre-diffusion period is too long, test organisms might he weakened and as a consequence killed. A period of 12 hours preincubation storage at 4°C has been found sufficient.

The presence of active substances in *Halimeda tuna* and *Halimeda opumtia* is in agreement with what has been observed in other *Ha/imeda* spp. by Almodovar (1964), Rao and Pareksh (1981) and Paul and Fenica1 (1983a). Pesando and and Caram (1984), however, observed that *Halimeda opuntia* did not produce active substances. These differences in activity may be due to different developmental stages, locality, ete. It is most likely that the activities of *H. opuntia* and *H. tuna* may be due to halimedatrial or/and 2,9diacetoxyudoteal compounds both of which have antimicrobial and cytotoxic activities. The highly bioactive deterpenoid halimedatrial has been isolated from H. *opuntia,* H. *tuna,* H. *cylindracea, H. gigas,* H. *incrassata,* H. copiosa, H. *mantle, H. scabra,* and *H. sinulans* and 4,9-diacetoxyudoteal irom *H. Opuntia* (Paul & Fenical, 1983a). Halimedatrial has been shown to have minimum inhibition concentration of 4 µg ml^{-1} and 8 µg ml^{-1} against *Staphylococcus aureus* and *Bacillus subtilis,* respectively. Apart from activity against the two microbes, it is active against *Candida albicans* and proved cytotoxic at 1 µg ml^{-1}, as it inhibits first cleavage in fertilized sea urchin egg (Paul & Fenical, 1983a).

The antimicrobial activity shown by *Caulerpa racemosa* in this study may be attributed to caulerpin or caulerpein (Doty & Santos, 1970; Paul, *et al.,* 1987), or flexin and trifarin (Blackman & Wells, 1978) or by caulerpanyene (Amico *et al.,* 1978). The inactivity shown by an extract of C. *mexicana* suggests that the species might be lacking the substances mentioned.

Although U*lva pertusa* in this study has been shown to produce antibacterial and cytotoxic substances, it was reported by Jing-Wen and Wei-ci (1984) not to produce active substances. The antimicrobial activity shown by Ul*va pertusa* of Tanzania might be due to acrylic acid commonly found in the genus (Glombitza, 1979). *Ulva pertusa,* commonly known as sea lettuce, has long been used as food and as a traditional medical agent to treat helminthic infections, fever, urinary diseases, dropsy, etc. (Chengkui & Junfu, 1984). Some sterols have been isolated in *V. pertusa* and were shown to be hypocholesterolemic in rats and depress blood pressure in human atherocIerosis (Chapman, 1979).

The wide occurrence of antimicrobials and toxins in the seaweed must he important to the algae, as they might deter predators and epiphytes (Paul & Fenical, J 983b; Faulkner, J 984).

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