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On the GRAS status of seaweeds. I. Observations on the association between antibacterial activity of ethanolic extracts and metal levels present in selected seaweeds.

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Abstract: The relationship between antibacterial activity and levels of 24 metals in 19 selected southern African seaweeds was examined. The antibacterial activity of ethanolic extracts of these seaweeds was assessed by agar diffusion against selected Gram positive and Gram negative test bacteria. Metal levels associated with seaweeds were determined using X-ray fluorescence (XRF) analysis. Extracts of the Phaeophyta exhibited the highest level of antibacterial activity, followed by the Rhodophyta and then the Chlorophyta, in decreasing order. Metal levels, however, were highest in the examples studied from the Rhodophyta and then the Phaeophyta and Chlorophyta, in decreasing order. There was no relationship between the observed antibacterial activity of the crude extracts tested and the levels of metals occurring in their tissues. Thus the antimicrobial activity of extracts from the seaweeds tested should also be *g*enerally *re*cognised *as* safe (GRAS).

Key words: antibacterial activity, southern African seaweeds, macroalgae, metals.

Introduction

Natural products, which are not harmful to humans, have been used in food preservation. These natural products have been termed "GRAS" (generally recognised *as* safe ; Holzapfel et al., 1995). For centuries, seaweeds have been a source of food and medicine for the people of the Far East (South and Whittick, 1987). These seaweeds have not posed any known health risks to humans and can thus also be regarded as "GRAS". Secondary metabolites responsible for antibacterial activity in seaweeds are, by definition, also likely to be "GRAS" and therefore crude extracts have potential use in food preservation and medicinal applications.

Notwithstanding the above, it is well known that certain marine macroalgae accumulate heavy metals to a level higher than those in the surrounding seawater (Seeliger and Edwards, 1977; Chung and Lee, 1989). Metals may occur naturally in the marine environment by mineral erosion or weathering, but with a rapid increase in industrialisation in the form of mining, smelting and fossil fuel combustion, levels of certain metals (particularly heavy metals) in the marine environment have escalated (Chung and Lee, 1989). Metals accumulated in seaweeds can be transferred to higher trophic levels through food chains. High levels of some of these metals can become toxic to humans, thus if the antibacterial properties of seaweed extracts are to be used in the food and pharmaceutical industries, one must speculate as to whether the antibacterial activity of seaweed extracts is due solely to antibacterial compounds (secondary metabolites) produced by the algae, or whether accumulated metal concentrations associated with macroalgal tissues also contribute to the antibacterial activity of their crude extracts.

Many compounds in nature have the ability to inhibit the growth of microorganisms. However, microorganisms have developed resistance to these natural compounds and microbiologists are forced to continue their search for naturally occurring antimicrobial compounds. The marine environment provides a unique and still largely unexploited source of novel bioactive compounds in the form of secondary metabolites (Tariq, 1991; König et al., 1994). Surprisingly, it was only relatively recently that the potential of seaweeds as sources of antimicrobial compounds was recognised (Pesando and Caram, 1984; Pesando, 1990).

The presence of antibacterial activity has been documented from seaweeds collected worldwide, including southern Africa (Hornsey and Hide, 1974; Campos-Takaki et al., 1988; Niang and Hung, 1984; Moreau et al., 1988; Caccamese et al., 1985; Rao, 1991; Vlachos et al., 1997). Compounds reportedly responsible for these antimicrobial properties range from acrylic acid and diterpenoids in the green seaweeds (Glombitza, 1970) and halogenated terpenoids in the red seaweeds, to metabolites of mixed terpenoid-aromatic origin in the brown seaweeds (Pesando, 1990).

This study investigates the association between antibacterial activity of crude seaweed extracts and levels of selected metals in southern African seaweeds, known to exhibit antibacterial activity (Vlachos et al., 1997) and which also contain elevated levels of selected metals (Vlachos et al. 1998).

Materials and Methods

Seaweed sample collection

Seasonally and locally abundant seaweeds were collected from the intertidal zone of Swakopmund (Namibia), Palm Beach, Rocky Bay, Isipingo Beach and Mission Rocks (South Africa) on the southern African coast.

Swakopmund samples (Namibia):

Phaeophyta- Bifurcariopsis capensis, Laminaria schinzii
Rhodophyta- Mazzaella capensis, Plocamium rigidum.

Palm Beach samples (South Africa):

Phaeophyta- Anthophycus longifolius, Sargassum incisifolium, Stypopodium zonale, Zonaria tournefortii;
Rhodophyta- Amphiroa ephedraea, Beckerella pinnatifida, Gelidium abbottiorum, Osmundaria serrata;
Chlorophyta- Halimeda cuneata.

Rocky Bay samples (South Africa):

Rhodophyta- Arthrocardia carinata.

Isipingo Beach sample (South Africa):

Rhodophyta- Cheilosporum sagittatum.

Mission Rocks samples (South Africa):

Phaeophyta- Dictyopteris longifolia, Ecklonia radiata, Zonaria subarticulata;

Chlorophyta- Codium duthieae.

Several individual algae, comprising 200g wet weight, were collected in the field and pooled to provide a single sample. Three such replicate samples were collected, air-dried and cleaned of sand, crustaceans and epiphytes as described by Vlachos et al. (1996).

Determination of metal levels

Metal levels in the southern African seaweeds listed above were determined using X-ray fluorescence (XRF) analysis. Samples were prepared according to standard methods described in Vlachos *et al.* 1998. Three replicate pellets of each sample were prepared and subjected to XRF analysis (Philips PW 1400, Holland). Metal levels were then scored according to a key based on concentration ranges (Table 1).

Table 1. Inhibition zone diameters and metal concentrations in selected southern African seaweeds

SEAWEED	INHIBITION ZONE DIAMETER				METAL CONCENTRATIONS							
	Bacillus subtilis	*Staphylococcus aureus*	*Acinetobacter lwoffii*	*Escherichia coli*	As	Co	Cr	Cu	Mo	Pb	Sr	Al_2O_3
Zonaria subarticulata	++++	+++++	++++	+++	+	+	+	+	+	+	+++	++
Zonaria tournefortii	+++	+++	+++	++	+	+	+	+	+	+	+++++	+++
Plocamium rigidum	++++	+++	++	+	+++	+	+	+	+	+	++	++++
Gelidium abbottiorum	++++	+++	++	+	+	+	+	+	+	+	++	+++++
Osmundaria serrata	++	++	++	+	++++	+	+	+	+	+	+	++++++
Codium duthieae	+++++	++++	++	+	+	+	+	+	+	+	++	++
Halimeda cuneata	+++++	++	++	+	+	+	+	+	+	+	++++++	++

Determination of antibacterial activity

Preparation of Crude extracts and selection of test bacteria

Milled seaweed material was extracted in 80% ethanol (in water) according to the method described by Vlachos *et al.* (1996).

The 4 test bacteria used in the study were isolates of common food-borne pathogens or spoilage organisms, *viz. Bacillus subtilis, Staphylococcus aureus, Acinetobacter lwoffii* and *Escherichia coli.*

Antibacterial activity assay

Crude extracts were tested for antibacterial activity by agar diffusion (6.1mm wells in overlay agar ; Vlachos *et al.* 1996). A control of 80% ethanol (in water) was used. Plates were incubated for 24 hours at 25℃, and inhibition zone diameters were measured and means calculated. For each plate, the inhibition zone diameters around the control well were subtracted from those around the well of the seaweed extracts. Inhibition zone diameters were then scored according to a key based on size (Table 1).

Analysis of results

A multi-factorial analysis of variance of the means, at the 95% confidence level, was used to compare metal levels between seaweeds, as well as to compare the inhibition zone diameters produced by ethanol extracts from seaweeds.

Results and Discussion

Table 1 shows the inhibition zone diameters and the metal levels of the seaweeds showing the highest level of antibacterial activity in each of the divisions Phaeophyta and Rhodophyta (*Zonaria subarticulata* and *Plocamium rigidum*, respectively), as well as the seaweed(s) showing the highest level of metals in each division (the phaeophyte *Zonaria tournefortii* and the rhodophytes *Osmundaria serrata* and *Gelidium abbottiorum*). The metals levels and inhibition zone diameters of the 2 Chlorophyta used in this study are also shown in Table 1. According to Trevors et al. (1985) of all the metals tested for in this study, only As, Co, Cr, Cu, Mo, Pb and Sr, and the metal oxides Al_2O_3 and Fe_2O_3 are toxic to microorganisms when present in high concentrations and are thus reported in Table 1.

Antibacterial activity of selected southern African seaweed crude extracts

Extracts of the Phaeophyta showed antibacterial activity against all the test bacteria. Extracts of the brown alga *Zonaria subarticulata* showed the broadest spectrum of antibacterial activity of all the seaweeds used in this study (Table 1).

Generally, extracts of the Rhodophyta were inhibitory only to the Gram positive bacteria and *Acinetobacter lwoffii* of the Gram negative bacteria. *Plocamium rigidum* showed the highest level of antibacterial activity of all the rhodophyte extracts (Table 1).

The extracts of both the Chlorophyta, *Codium duthieae* and *Halimeda cuneata*, showed high levels of antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*, respectively. However, the level of antibacterial activity of these seaweed extracts was low against the remaining Gram positive and all the Gram negative bacteria (Table 1).

Metal levels in selected southern African seaweed crude extracts

Generally the highest levels of metals were found in the Rhodophyta, followed by the Phaeophyta and then the Chlorophyta in decreasing order. The rhodophyte, Osmundaria serrata contained the highest levels of As, Pb, Al_2O_3 and K_2O, and Gelidium abbottiorum, also a rhodophyte, contained the highest levels of V, Fe_2O_3, SiO_2 and TiO_2. Generally, *Zonaria tournefortii* and *Halimeda cuneata* contained the highest metal levels of the Phaeophyta and Chlorophyta, respectively.

Relationship between antibacterial activity and metal levels

The decreasing order of antibacterial activity of extracts of the Phaeophyta, Rhodophyta and Chlorophyta did not correspond to the observed order of metal levels in these algal groups. For example, extracts of the rhodophyte, *Plocamium rigidum*, showed significantly greater ($P < 0.05$) antibacterial activity than extracts of *Osmundaria serrata* against *Bacillus subtilis* and *Staphylococcus aureus*. By contrast, *Osmundaria serrata* showed higher levels ($P < 0.05$) of As and Al_2O_3 than *Plocamium rigidum* (Table 1). The levels of As, Co, Cr, Cu, Mo, Pb and Sr measured in these seaweeds was probably too low to inhibit the growth of the test microorganisms.

The antibacterial activity of extracts of the phaeophyte, *Zonaria subarticulata*, was greater than that of *Zonaria tournefortii* ($P < 0.05$) against the 4 test bacteria, yet the levels of Sr and Al_2O_3 were significantly higher ($P < 0.05$) in *Zonaria tournefortii* (Table 1). As with the Rhodophyta, the levels of Co, Cr, Cu, Mo, Pb and Sr levels measured here are probably too low to inhibit the growth of the test microorganisms. *Codium duthieae* extracts showed greater antibacterial activity than *Halimeda cuneata* against *Staphylococcus aureus* ($P < 0.05$). *Halimeda cuneata*, however, showed significantly greater ($P < 0.05$) levels of Sr than *Codium duthieae*.

The apparently high As level associated with *Osmundaria serrata* does not call for concern because As is accumulated in the non-toxic organic (pentavalent) form by seaweeds; it is the inorganic (trivalent) form which is toxic (Kesava Rao et al., 1991).

The high metal levels in the Rhodophyta are not likely to have an effect on the antibacterial activity of the seaweed crude extracts as the highest level of metals in the Rhodophyta were in *Osmundaria serrata* and *Gelidium abbottiorum*, and the greatest antibacterial activity was reported for *Plocamium rigidum*. Furthermore, the antibacterial activity of the Rhodophyta is reportedly due to halogenated terpenoids (Pesando, 1990), yet metals bind to alginic acid (Ramachandran *et al.*, 1994) and other polysaccharides (eg. agar and carrageenan) in seaweeds (Chung and Lee, 1989). *Gelidium abbottiorum* produces large quantities of agar (polysaccharide) (Aingworth and Critchley, 1997) which may bind to metals and thus be an explanation for the high levels of metals reported in this seaweed.

From the results obtained in this study, the levels of metals associated with seaweeds from southern Africa did not have a measurable effect on the antibacterial activity of ethanol crude extracts. It is suggested that the antibacterial activity exhibited by these southern African seaweed extracts can be attributed to naturally occurring secondary metabolites which are free of metals. These secondary metabolites, once purified, may have a commercial application in pharmaceuticals and/or food biopreservation, thus maintaining the concept that seaweeds are recognised as "GRAS".

Conclusion

It can be concluded that there is no relationship between the metal levels associated with seaweed tissues and the corresponding antibacterial activity of their ethanol crude extracts against the test bacteria used in this study. This finding implies that the antibacterial activity displayed by the seaweed extracts tested in this study are due to secondary metabolites which are free of metals. Ethanolic extracts, with antibacterial activity, made from the selected seaweeds tested here, should be considered as "GRAS" and would be suitable candidates for further testing in commercial applications.

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