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Seasonal variation in antifungal, antibacterial and acetylcholinesterase activity in seven South African seaweeds

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Abstract Seven seaweeds were collected from the intertidal zone at Rocky Bay on the east coast of South Africa. The species were *Caulerpa racemosa* var. *laetevirens*, *Codium capitatum*, *Halimeda cuneata*, *Ulva fasciata*, *Amphiroa bowerbankii*, *Amphiroa ephedraea* and *Dictyota humifusa*. Six bimonthly collections were made within a few days of the new moon to correspond with spring tide. Methanol extracts were tested for antifungal, antibacterial and acetylcholinesterase (AChE) inhibitory activity. No seasonal variation was observed in antifungal activity, with *D. humifusa* extracts being the most active. The seaweed extracts inhibited the growth of the Gram-positive bacteria, with *Bacillus subtilis* being more susceptible than *Staphylococcus aureus*. *Dictyota humifusa* was the only seaweed able to inhibit the Gram-negative *Escherichia coli*. Seasonal variation in antibacterial activity was observed, with the extracts generally having no activity in summer and having antibacterial activity in late winter (July collection) and early spring (September and November collections). *Dictyota humifusa* was the most effective seaweed species, having antibacterial activity throughout the year. All the extracts tested had AChE inhibitory activity, with no seasonal variation in the levels of activity. *Dictyota humifusa* extracts were the most effective at inhibiting AChE activity.

Key words acetylcholinesterase activity · antibacterial · antifungal · seasonal variation · seaweeds

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Seaweeds provide a rich source of structurally diverse secondary metabolites. These are mainly terpenes, acetogenins, alkaloids and polyphenolics, with many of these compounds being halogenated (Watson & Cruz-Rivera 2003). The functions of these secondary metabolites are defense against herbivores, fouling organisms and pathogens; they also play a role in reproduction, protection from UV radiation and as allelopathic agents (Hay 1996; Watson & Cruz-Rivera 2003). Variation in secondary metabolites is both genetically and environmentally controlled. Transplant experiments suggest that environmental conditions are able to alter the concentrations of secondary metabolites, although the types of compounds are genetically fixed (Hay 1996).

Chemical defense strategies against herbivory encompass both long-term defense (constitutive defense) as well as rapid activation induced by certain conditions; for example, grazer-induced mechanical damage triggers the production of certain chemicals that act as feeding deterrents or toxins in some seaweeds (Watson & Cruz-Rivera 2003). The

occurrence of bacterial biofilms on seaweed surfaces is ubiquitous, and has many negative effects on the seaweed, such as increased drag, competition for nutrients and blocking light and gaseous exchange. Chemical defense mechanisms that inhibit biofilm development are a common occurrence in seaweeds, with many secondary metabolites produced by seaweeds having bacteriocidal or bacteriostatic properties (Steinberg et al. 1997). Physical stress such as desiccation, UV and visible light and nutrient availability are able to alter the secondary metabolites in seaweeds (Watson & Cruz-Rivera 2003). Secondary metabolite production in seaweeds is a function of life history—with different stages having different physiological properties—and the age of the seaweed, e.g., higher concentrations of defense chemicals were found in areas of new growth in *Halimeda* species (Paul 1992).

Although terrestrial biodiversity is the foundation of the pharmaceutical industry, the oceans have enormous biodiversity and potential to provide novel compounds with commercial value (Hay 1996; Smit 2004). In the current study, we screened for antibacterial, antifungal and acetylcholinesterase activity. Overuse of antibiotics and the ability of bacteria to acquire resistance to the drugs means there is a constant search for new classes of antibiotics with novel structures that are effective against human pathogens. There has also been an increase in antifungal infections in people whose immune system has been compromised as a result of a disease such as AIDS or as a consequence of immunosuppressive drug therapy (Espinel-Ingroff & Pfaller 1995). Alzheimer's Disease is a chronic neurological disease that is becoming a major problem, especially in developed countries as the life-expectancy of the population increases. Acetylcholine is a neurotransmitter that is inhibited mainly by the enzyme acetylcholinesterase (AChE). Increasing acetylcholine concentrations by inhibiting AChE activity is considered the most effective treatment strategy against Alzheimer's Disease (Orhan et al. 2004). The present commercial drugs have some negative side effects, and are only effective against mild types of Alzheimer's Disease. Thus there is a great need to develop new drugs to combat Alzheimer's Disease (Elgorashi et al. 2004).

Antibacterial activity has been the most widely investigated in seaweeds, with less attention paid to antifungal activity. To our knowledge, no seaweeds have been screened for AChE activity. As the production of secondary metabolites by seaweeds is influenced by the interaction of many biotic factors (herbivory, pathogens and physiology of the seaweed) and abiotic factors (seasonality and geographic location), numerous collections need to be made to cover all these variables before suitable species can be targeted for compound isolation. The aims of this study were restricted to determining seasonal variation in some biological activities for several seaweeds.

To decrease any geographical variation, seven seaweeds were collected from the intertidal zone within a 50 m^2 area at Rocky Bay (30°23′S; 30°43′E), situated on the east coast of South Africa. Maximum seawater temperatures during the summer months (December to February) are 24°C, and minimum temperatures during the winter months (June and July) are 18°C (De Clerck et al. 2005). The species were *Caulerpa racemosa* (Forsskål) J. Ag. var. *laetevirens* (Mont.) Weber-van Bosse, *Codium capitatum* Silva, *Halimeda cuneata* Hering and *Ulva fasciata* Del. from the Chlorophyta, *Amphiroa bowerbankii* Harv. and *Amphiroa ephedraea* (Lamarck) Decne from the Rhodophyta and *Dictyota humifusa* Hörnig, Schnetter and Coppenjans from the Phaeophyta. Six bimonthly collections were made within a few days of the new moon to correspond with spring tide during 2004 (23/01, 22/03, 23/05, 17/07, 12/09 and 14/11). *Ulva fasciata* and *H. cuneata* were not collected on the 23/01 collection, and *C. racemosa* was not found on the shore during the 23/05 and 17/07 collections. The samples were transported to the laboratory in a cooler box. Epiphytes were removed, and the samples freeze-dried and ground to powders using liquid nitrogen. Samples were stored at −70°C until analyzed.

Each sample (2 g) was extracted with 20 mL distilled methanol. After 1 h in an ultrasound bath, the extracts were left overnight at 10°C, sonnicated for a further 30 min and then vacuum filtered using Whatman No. 1 filter paper. The extracts were dried under vacuum and then redissolved in 5 ml distilled methanol. The extracts were partially purified by filtration through an activated Sep-Pak †C_{18} cartridge (Waters). The extracts were dried under vacuum, and the yields of the dried extracts recorded. It was necessary to partially purify the extracts prior to testing in the assays to remove some of the photosynthetic pigments, as they caused interference in the assays, which are based on colour reactions or absorbance readings at set wavelengths.

The extracts were dissolved in 100 mg mL^{-1} DMSO, and tested against *Candida albicans* (ATCC 10231) obtained from the South African Bureau of Standards. Minimum inhibitory concentration (MIC) values were obtained using a microdilution method (Espinel-Ingroff and Pfaller 1995) modified as described in Buwa and van Staden (2006) where two-fold serial dilutions were done in microplates to give a concentration range of 6.25 – 0.05 mg mL^{-1} extract. A set of standards was included on each microplate, with Amphotericin B used as a reference standard. The absorbance was read at 630 nm using a Microplate Reader (Opsys MR) before and after the 24 h incubation period at 37°C. A difference greater than 0.3 indicated growth of *C. albicans*, showing that the extract had no antifungal inhibition. The MIC value was taken to be the lowest concentration where growth was less than 0.3. There were three replicates per extract, each tested on separate microplates.

The microplate method of Eloff (1998) was used to determine the MIC values of the extracts tested against the Gram-negative bacteria *Escherichia coli* (ATCC 11775) and the Gram-positive bacteria *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600). Two-fold serial dilutions were done to give a concentration range of 12.50 – 0.10 mg mL^{-1}. A set of standards was included on each microplate, with neomycin used as a reference standard. The microplates were incubated at 37°C for 24 h, after which 50 μL p-iodonitrotetrazolium violet (INT) solution was added to each well, and the microplates incubated for 1 h. The colourless tetrazolium salt acts as an electron acceptor, and is reduced to a red-coloured formazan product by biologically active organisms (Eloff 1998). Where bacterial growth was inhibited by the extracts, the solution in the wells remained clear after incubation with INT. The plates were visually evaluated, and the MIC was taken to be concentration in the last well with no observable colour change. There were two replicates per extract.

The microplate assay for AChE inhibition activity was based on the modified Ellman's method as described by Elgorashi et al. (2004). Each microplate contained a set of standards, including galanthamine as a reference standard and a water blank. Extracts were two-fold serially diluted. After preparation and 1-min incubation, the microplates were read six times at 1-min intervals at 405 nm with a Microplate Reader. The AChE solution was added and the absorbance read again eight times at 1-min intervals to detect the developing yellow colour. AChE cleaves acetylthiocholine codide to give thiocholine, which reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) and produces a yellow product 5-thio-2-nitrobenzoate (Risa et al. 2004). From these readings, the reaction velocity was calculated by Multiskan EX software (version 1.0) and Microsoft Excel. This was used to determine the % of AChE inhibition, and the concentration of the extracts causing 50% inhibition (IC_{50}) was calculated. There were three replicates per extract.

Fingerprinting of the *D. humifusa* extracts was performed where the TLC plate was developed in hexane:ethyl acetate (3:2 v/v) and stained with anisaldehyde.

The dry weights of the extracts after partial purification using Sep-Pak cartridges did vary between species, although no seasonal variation was recorded. The two calcified red seaweeds, *A. bowbankii* and *A. ephedraea*, had the lowest yield (60–100 mg extract 2 g^{-1} freeze-dried seaweed). The other five species had similar yields (140–270 mg extract 2 g^{-1} freeze-dried seaweed).

No seasonal variation was observed in antifungal activity tested against *C. albicans*. *Dictyota humifusa* was the most active seaweed tested, with a MIC of 3.125 mg mL^{-1} for all of the collections except the sample collected on 12/09/2004, which had a MIC of 6.26 mg mL^{-1}. All the other species had a consistent MIC of 6.25 mg mL^{-1}, with the two exceptions being *C. capitatum* collected on 22/03/2004 and *H. cuneata* collected on 23/05/2004, which had MIC values of 3.125 mg mL^{-1}. Amphotericin B standards showed consistent inhibition of fungal growth at 0.001 mg mL^{-1}.

The seaweed extracts inhibited the growth of the Gram-positive bacteria, with *B. subtilis* being more susceptible than *S. aureus*. *Dictyota humifusa* was the only species able to inhibit the Gram-negative *E. coli* in the concentration range tested. Seasonal variation in antibacterial activity was observed, with the extracts generally having no activity in summer and having antibacterial activity in late winter (July collection) and early spring (September and November collections). *Dictyota humifusa* was the most effective seaweed species, having antibacterial activity throughout the year, the lowest MICs and being the only species to inhibit *E. coli*. *Codium capitatum* was the least effective species, having no inhibitory

Table 1 MIC values (mg mL^{-1}) of seaweeds collected bimonthly tested for antibacterial activity against three bacteria. Omitted from the table are *C. capitatum*, which had no inhibitory activity against any of the bacteria, *U. fasciata* and *A. ephedraea*, which had no activity against *S. aureus,* and the species which did not inhibit *E. coli* (all except *D. humifusa*)

Species	Collection (2004)					
	23/01	22/03	23/05	17/07	12/09	14/11
	S. aureus					
C. racemosa	–	6.25	nc	nc	6.25	3.125
H. cuneata	nc	–	6.25	–	6.25	–
A. bowerbankii	–	–	6.25	–	–	6.25
D. humifusa	6.25	3.125	3.125	0.781	1.563	1.563
Neomycin	1.56 μg mL^{-1}					
	B. subtilis					
C. racemosa	–	1.563	nc	nc	3.125	3.125
U. fasciata	nc	–	1.563	3.125	3.125	3.125
H. cuneata	nc	–	6.25	6.25	6.25	–
A. ephedraea	–	–	–	–	6.25	6.25
A. bowerbankii	–	–	3.125	–	6.25	6.25
D. humifusa	6.25	0.781	3.125	1.563	0.781	0.781
Neomycin	0.20 μg mL^{-1}					
	E. coli					
D. humifusa	–	6.25	6.25	–	–	–
Neomycin	0.78 μg mL^{-1}					

– = no inhibitory activity at the concentration range tested (12.5 – 0.10 mg mL^{-1})

nc = not collected

activity against any of the bacteria in the concentration range tested (Table 1). There have been many papers on the screening of seaweeds for antibacterial activity. All report that the extracts were generally more effective against Gram-positive bacteria than against Gram-negative bacteria, probably due to the more complex structure of the cell wall of Gram-negative bacteria (Caccamese & Azzolina 1979; Pesando and Caram 1984; Vlachos et al. 1997).

All the extracts tested had similar AChE inhibitory activity, with no seasonal variation in activity (Table 2). The most active extracts were made from *D. humifusa* collected on 23/05 (IC_{50} 4.75 mg mL^{-1}) and *U. fasciata* collected on 12/09 (IC_{50}=4.82 mg mL^{-1}). Galanthamine, the pure compound used as a reference had an IC_{50} of 0.0007 mg mL^{-1}.

Dictyota humifusa was consistently the most biologically active species tested in all three assays used in the current study. There appeared to be very little seasonal variation in the compounds present in the extracts, with similar bands being observed on the TLC plate for all collections (Fig. 1). The genus

Table 2 IC_{50} values (mg mL^{-1}) of seaweeds collected bimonthly tested for AChE inhibitory activity. Results are given as mean ± SD (n=3)

Species	Collection (2004)					
	23/01	22/03	23/05	17/07	12/09	14/11
C. racemosa	5.5±0.5	8.4±4.2	nc	nc	7.2±1.1	5.6±0.9
C. capitatum	7.8±1.3	8.8±1.0	8.4±4.7	8.8±1.3	8.6±1.8	8.3±2.0
U. fasciata	nc	8.7±2.5	8.4±5.0	6.4±0.9	4.8±1.2	6.0±0.6
H. cuneata	nc	7.1±0.5	5.9±1.9	8.2±1.6	5.7±0.4	5.8±0.9
A. ephedraea	6.9±1.6	7.9±7.0	6.6±0.8	6.6±2.0	5.1±0.6	6.1±0.5
A. bowerbankii	5.5±1.6	7.6±2.4	5.7±1.6	6.1±0.7	6.6±1.0	5.3±1.9
D. humifusa	5.7±1.2	5.9±2.1	4.8±0.6	8.3±0.1	7.3±2.4	5.3±2.5

nc=not collected

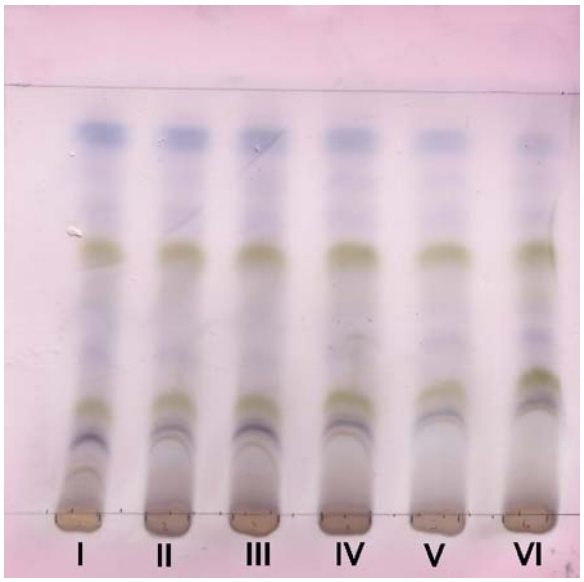

Fig. 1 TLC fingerprint of *Dictyota humifusa* extracts developed in hexane: ethyl acetate (3:2 v/v) and stained with anisaldehyde. Collection times are: *I*; 23/01, *II*; 22/03, *III*; 23/05, *IV*; 17/07, *V*; 12/09, and *VI*; 14/11

Dictyota has been extensively studied with regard to antiherbivory secondary metabolites. This genus produces a broad-spectrum of chemicals, with most being structurally similar prenylated guainane carbon skeleton diterpenes called dictyols (Barbosa et al. 2004). The composition and concentration of these show inherent genetic variation as well as biogeographic variation. These compounds can be active against not only macroherbivores such as fish and sea urchins but also mesograzers such as amphipods, crabs and mollusks (Pereira et al. 2000; Barbosa et al. 2004). The active compounds in *D. humifusa* need to be identified to determine if this antifungal, antibacterial and AChE inhibitory activity was due to the presence of diterpenes.

This was also the first report of seaweeds having AChE inhibitory activity. Some crude ethanol extracts of South African higher plants used in traditional medicine to increase memory and treat Alzheimer's Disease were previously tested in the same bioassay as used in the current study. The highest extract concentration tested was 0.1 mg mL^{-1}, with results ranging from 8% to 67% inhibition of acetylcholinesterase (Risa et al. 2004). Alkaloids can inhibit AChE activity (Elgorashi et al. 2004), and some higher plants are able to inhibit AChE activity, mainly due to their rich alkaloid content (Orhan et al. 2004). The AChE inhibitory activity found in the seaweed extracts looks promising, and requires further investigation to determine if this activity is due to alkaloids, as in higher plants.

Seasonal activity in the levels of inhibitory activity in the three assays varied for the different seaweed species tested. In another study on three South African seaweeds where five collections were made over a 5-year period, *Osmundia serrata* had the highest antibacterial activity in winter, while *Galaxaura diessingiana* and *Codium suthieae* showed little variation in antibacterial activity (Vlachos et al. 2001). Definite trends were found in the 14 species collected in India, where highest antibacterial activity was detected in samples collected during the post-monsoon season, and lower activity in the samples collected during the summer and monsoon season (Vidyavathi & Sridhar 1991). It would be interesting to see if there is a general trend of distinct seasonal variation in biological activity in tropical regions such as India, where there are greater fluctuations in environmental conditions, compared to temperate regions such as South Africa where environmental fluctuations are less. More such studies on seasonal variation of active compounds are needed before such generalizations can be made.

Activity in a number of assays and against a number of bacterial strains may be due to a single chemical entity with a broad spectrum of activity or many different chemical entities. Seasonal change in activity may be due to different quantities of a single compound, or the synthesis of different compounds due to different growth conditions. For example, when male and female gametophytes and tetrasporophytes of *Spyridia filamentosa* were grown at different irradiances, the various reproductive stages were able to inhibit different bacterial strains, with each stage also having optimal light conditions for maximum inhibitory activity (Robles Centeno & Ballantine 1999). *Dictyota humifusa* appears to the most promising species for further investigation, as it has a broad spectrum of biological activity and so is likely to yield more than one active compound. The compounds present in *D. humifusa* do not appear to vary seasonally.

Due to high rates of herbivory, pathogens and intense competition for space, seaweeds have developed an extensive chemical defense system, thus

providing an untapped resource of bioactive natural products. There appear to be no common trends in the peaks of biological activity with regard to season, and this study emphasizes the importance, when running a screening programme, to do multiple collections. *Dictyota humifusa* was identified as a good candidate for compound isolation, due to its broad spectrum of biological activity. This was also the first report of seaweeds inhibiting AChE activity.

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