Antibacterial activities of polyunsaturated fatty acid extracts from
*Sardinella longiceps* and *Sardinella fimbriata*

Chitra Som R S* & C K Radhakrishnan.

Department of Marine Biology Microbiology and Biochemistry, Cochin University of Science and Technology (CUSAT)
Fine Art’s Avenue, Kochi, 682016, India
[Email: chitramarine@yahoo.com]

Received 20 April 2010; revised 7 January 2011

Polyunsaturated fatty acids (PUFA) content of two species of fishes - *Sardinella fimbriata* and *Sardinella longiceps* - was examined and their antibacterial activities were compared. PUFA isolated from the tissue extracts were used to conduct Antibacterial Sensitivity Tests on pathogenic bacterial strains like *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonieae*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Salmonella enterica* and *Pseudomonas aeruginosa*. These extracts were subjected to Gas Chromatography for the quantitative analysis of PUFA. Extracts from both species showed inhibitory effects on the strains of *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*. No inhibitory activities were observed on the strains of *K. pneumonieae*, *E. aerogenes*, *P. vulgaris* and *S. enterica*. *S. fimbriata* extract showed a higher inhibitory effect on all the inhibited bacterial strains. Gas Chromatography analysis revealed higher concentrations of docosahexaenoic acid (DHA) in the extracts from *S. fimbriata* as compared to *S. longiceps*. The differences between the antibacterial activities of the two extracts could be attributed to the disparity in DHA content.

[Keywords: PUFA, EPA, DHA, Antibacterial Activity, *Sardinella fimbriata*, *Sardinella longiceps*]

Introduction

Several bioassay reports have indicated the presence of antimicrobial compounds among marine flora and fauna1-7. The antibacterial activities of fatty acids in general have been noted in several pioneering studies 8-11. Significant antibacterial properties have been observed in fatty acids from marine sources such as algae11-14 and diatoms16-18. Moreover, there have been sporadic studies on the antibacterial activity of the products and by-products of marine fishes. Subsequently, antimicrobial characteristics have been reported in the epidermal mucus of fishes19-20 and fish skin gelatins as well21.

Sardines are a group of small, oily, pelagic fishes which comes under the family *Clupeidae*. *Sardinella longiceps* and *Sardinella fimbriata* are well-known to be rich in long chain polyunsaturated fatty acids (PUFA)22. Fish oil from a sardine species *Sardinops melanostica* has also been shown to inhibit microbial growth23. However, studies on antibacterial activity of PUFA extracts from fishes have been very few till date. Marine lipids, especially n-3 fatty acids such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), have been documented as a major source of PUFA24-25. EPA, one of the major constituent omega-3 fatty acids found in marine sources exhibits antibacterial activity against a wide range of bacteria18,26. Thompson et al. (1994) in their experiments on *Helicobacter pylori*, with an extensive range of fatty acids having different unsaturation levels, concluded that inhibitory effects increase with level of unsaturation; however they did not go beyond EPA in their unsaturation levels of fatty acids27. Later, DHA having a higher unsaturation level than EPA, was demonstrated to have an inhibitory effect on Gram negative bacteria that surpasses that of EPA28.

The objective of this study was to determine the antibacterial response of PUFA extracts from two different species of Sardines, viz. *S. fimbriata* and *S. longiceps*, found in the same area with in their ranges. Comparison of their respective activity profile is also attempted. Screening of such new organisms for antibacterial activity and searching for novel antibacterial drugs is important due to the constant generation of antibiotic-resistant strains of pathogenic bacteria.
Materials and Methods

Fish samples
Fishes, *S. longiceps* and *S. fimbriata*, were collected from the Vypin island, Kochi and prior to analysis were washed in sterile water.

Preparation of extracts
The internal organs were removed and the flesh sliced. Slices were blended and centrifuged at 10,000 rpm for 15 minutes. Post centrifugation, the oil phase was separated and subjected to saponification for converting the triglycerides to free fatty acids. This fatty acid mixture was then subjected to urea complexing, followed by low temperature fractional crystallization, to obtain a mixture of substantially pure PUFA.

Determination of Fatty acid composition
The composition of PUFAs in the above mixture was directly analysed by Gas Chromatography (GC) adopting the fatty acid methyl ester (FAME) method. The fatty acids were separated by gas liquid chromatography (Thermo Trace GC Ultra) equipped with a capillary column (30 m long and 0.54 mm diameter) and a flame ionization detector in the presence of hydrogen and air. Nitrogen was used as the carrier gas at a flow rate of 0.8 mL/min. Initial temperature was set at 70°C and was increased at a rate of 3°C/min until peak temperature of 250°C was reached. Injector and detector temperatures were maintained at 260°C and 275°C respectively. Fatty acids thus separated were identified by comparing their retention times with those obtained by the separation of a mixture of standard fatty acids. Measurement of peak areas and the processing of data were carried out by Thermo Chrom card software. Individual fatty acids were expressed as a percentage of total fatty acids.

Antibacterial Assay
The following bacteria were used for antibacterial study - *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pnemoniae*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Salmonella enterica* and *Pseudomonas aeruginosa*. These bacteria were cultured in nutrient agar at 37°C and maintained on nutrient agar slants. Each bacterial strain was then transferred into a separate test tube containing nutrient broth to reanimate them by culturing overnight at 37°C. Agar well diffusion method was used to screen the antibacterial activity of fish extracts. In vitro antibacterial activity was screened using Mueller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were prepared by pouring 20 mL of molten media into sterile petri plates. The plates were allowed to solidify for 5 min and three wells of 10 mm diameter were cut in all the plates. The bacterial strains were inoculated separately in peptone water. It was incubated at 37°C for 4 hrs (till it became turbid) and then the inoculum suspension was swabbed uniformly on the medium. To these plates, 100 µl of different dilutions of fatty acid extracts from the two fishes in acetone were introduced. For each bacterial strain, controls were maintained where acetone alone was used. The plates were incubated at 37°C for 24 h. Zones of inhibition were measured at the end of the incubation period. The experiment was repeated four times for each variant and the mean and SD calculated. Photographs were also taken showing the peak activity of fish extracts on bacterial cultures.

The obtained results were analyzed using 1-way ANOVA against the control and p<0.01 was considered as significant. The Minimum Inhibitory Concentration (MIC), which is the lowest dilution of the test agent at which no visible growth occurred, was calculated for the inhibited bacteria keeping p<0.01 as significant.

Results
Of the eight bacterial cultures, four of them showed a positive activity towards the fish-oil extracts in comparison with pure acetone control. Two of them showed no activity while two others showed a negative activity in comparison with the acetone extract. Results are summarized in Table 1. It may be noted that both gram-positive bacteria used in the study showed positive activity.

Both extracts showed maximum inhibitory effect on *P. aeruginosa* while *S. fimbriata* extract also showed remarkable inhibition of *E. feacalis*. Both *S. aureus* and *E. coli* showed marginal response with both extracts though *S. fimbriata* extract showed a higher inhibitory effect among the two. *K. pneumoniae* and *E. aerogenes* showed an inhibitory response to pure acetone control solution but also demonstrated significant negative activity with increased concentrations of fish-oil extracts. *P. vulgaris* and *S. enterica* showed no response either to acetone or to various concentrations of fish-oil extracts. Details of the values for both species are
shown in Table 2 and Table 3. Values of high significance (p < 0.01) are marked.

A comparison of the activity profile at high (80%) and mid (50%) concentrations indicate that S. fimbriata extracts are significantly better than S. longiceps ones for P. aeruginosa and E. faecalis. At high concentrations, this is also true for E. coli. At mid-concentration S. fimbriata extracts demonstrated a higher activity of borderline significance (p<0.05) for S. aureus. The trend of activity for both species based on high and mid concentration along with their significance values are illustrated in Figures 1 & 2. It is clear that S. fimbriata extracts had an overall higher inhibitory action on all the test species as compared to S. longiceps.

Images 1-4 illustrate the peak activity detected in each of the four bacterial strains.

MIC, calculated with a significance of (p<0.01), is summarized in Table 4. S. fimbriata showed remarkable lower levels of MIC on inhibited strains. MIC was lowest for P. aeruginosa among the four inhibited bacterial strains.

The PUFA extracts were analyzed by GC to identify the fatty acids present in the extract. The major compounds identified were unsaturated fatty acids ranging from C20 to C24 with a preponderance of C20:5 (EPA) and C22:6 (DHA) PUFA. GC analyses of the PUFA from the fish S. longiceps showed an EPA presence of 55.54% and a DHA presence of 32.52%. The GC analyses of the PUFA from the fish S. fimbriata gave a much lower EPA.
Fig. 1—Comparison of antibacterial activity at highest concentration

Fig. 2—Comparison of antibacterial activity at mid-concentration

Image 1—*Staphylococcus aureus*. 0% solution showed an activity of 14 mm, while *S. fimбриata* solution at 80% concentration showed an activity of 22 mm and *S. longiceps* showed an activity of 19 mm at same concentration.

Image 2—*Escherichia coli*. 0% solution showed an activity of 14 mm, while *S. fimбриata* solution at 80% concentration showed an activity of 18 mm and *S. longiceps* showed an activity of 16 mm.
The results showed that the DHA-rich *S. fimbriata* extracts have an overall higher activity against all the four bacterial strains as compared to *S. longiceps*. It tallies with the generalization by Thompson *et al.* (1994) that inhibitory effects on certain bacterial strains increase with levels of unsaturation. Higher activity shown by DHA-rich extracts on Gram negative bacterial strains in this study also matches with contemporary studies on DHA. However, PUFA extracts from both species showed inhibitory activity against both gram-positive and gram-negative bacterial strains. This is congruent with previous results on EPA and DHA showing activity against a range of both gram-positive and gram-negative bacteria. However, it is noteworthy that bacterial strains that showed negative or no activity were all gram-negative (Table 1).

Long chain fatty acids are well-known to be inhibitory on gram positive bacteria even at low concentrations. However, gram negative bacteria are known for their complex lipopolysaccharide layer as compared to the former. But, PUFA are known to have inhibitory effect on these strains as compared to saturated fatty acids as they are readily incorporated into the outer cell membranes of these organisms, where they significantly increase membrane fluidity. It is possible that by opening up permeability channels, the concentration gradients necessary between the organism and its environment may be dissipated resulting in fatality of the organism.

Shin *et al.* (2006) demonstrated that EPA can reduce the viability of *P. aeruginosa*. In their experiment, scanning electron microscopy (SEM) study of bacterial cells clearly exhibited the antibacterial effect of EPA evidenced by the damages found in the outer membrane of the cells when treated with EPA. Shin *et al.* (2007) later found out that DHA is even more potent against this bacterium. High positive results in this study for inhibiting cultures of *P. aeruginosa* could also be due to high DHA and EPA concentrations in both the extracts. Higher DHA concentration of *S. fimbriata* correlates with greater inhibitory effect on this bacterium.

Algal extracts with high EPA concentrations are known to show high levels of inhibitory effects on *S. aureus*. This is fairly in line with the results of the current study where both extracts in mid-
concentrations (50%) itself showed significant (p<0.01) activity. Studies by Shin et al. (2006) also showed inhibitory actions of EPA on this bacterium. It was also found that EPA and DHA have similar antibacterial action on this bacteria.\(^{28}\) Lipids extracted from cultured fishes like sea bass (Dicentrarchus labrax) and gilthead sea bream (Sparus aurata) are also known for their strong activity against S. aureus.\(^1\)

Algal extracts\(^{36-37}\) from marine sources is known to show moderate activity on E. coli as also apparent in this study. Only extracts from S. fimbriata showed significant activity on E. coli and the activity even at high concentration is not that high. DHA is known to have a better inhibitory effect as compared to EPA against this bacterium.\(^{28}\) Lipids extracted from cultured fishes like sea bass (Dicentrarchus labrax) and gilthead sea bream (Sparus aurata) are also known for their strong activity against E. coli.\(^1\)

PUFA\(^{26}\) and marine algal extracts\(^{18}\) are also known to show moderate activity on E. fecalis. No comparative studies have happened specifically on this Gram positive bacterium and hence it is not clear if DHA or EPA has a higher inhibitory effect. In the current study, DHA-rich S. fimbriata extract demonstrated a significantly greater action on E. fecalis.

It may be worth highlighting that the growth media also seem to play an important role in the determination of the antibacterial activity. Muller-Hinton agar appears to be the best medium to explicate the antibacterial activity\(^{38}\) and the same was used in the present study.

In conclusion, widely available marine fishes like Sardines are rich sources of DHA and EPA and have the potential to be an excellent source of pharmaceuticals that target microbes which mar human and animal life. Fish oil extracts from Sardinella fimbriata have higher concentrations of DHA than EPA and hence seem to have greater potential in inhibiting the growth of several strains of pathogenic bacteria.

**Acknowledgement**

Authors are thankful to the authorities of Cochin University of Science and Technology for facilities and financial help.

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