

**THE ANTIBACTERIAL PROPERTIES OF
THE SOFT CORAL
*Sinularia polydactyla***

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BY

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Sinularia polydactyla.**

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ABSTRACT

Colonies of the soft coral *Sinularia polydactyla* collected from the vicinity of Panjang island, Jepara region, Central Java, Indonesia were tested to examine their antibacterial properties.

Chemical analysis and bioassay-directed purification by repetitive column chromatography using *Vibrio harveyi* as tester strain revealed that *S. polydactyla* possesses antibacterial properties against this test strain. Purification gives a mixture of esters, which inhibited the growth of the marine biofilm forming bacterium, *V. harveyi* with an EC₅₀ value of 0.075 mg/10 mL of culture medium.

Chemical synthesis of the esters Hexadecyl palmitate (1), hexadecyl stearate (2), octadecyl palmitate (3), and octadecyl stearate (4) was accomplished from the alcohols and fatty acid acyl halides. Each purified ester was characterized by NMR and MS. Comparison of the MS for these authentic synthetic samples with that for the biologically derived sample showed that the latter was composed predominantly of esters 1 and 3. The individual esters were tested against *V. harveyi*. Compound 1 was active and 4 was less active, while 2 and 3 were inactive. Hence, 1 is the major active compound against *V. harveyi* in the natural mixture.

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1. INTRODUCTION

1.1. Background

Soft corals are an important and diverse group of marine invertebrates that can dominate many Indo-Pacific reefs. Many of these corals belong to the order Alcyonacea, a highly diverse group of benthic colonial invertebrates, with hundreds of different species. Alcyonaceans vary widely in form from the soft and fleshy members of the Xenidiidae family to the very beautiful but prickly members of the genus *Dendronephthya*, and from the hard, leather like forms of the genus *Sinularia* (*Sinularia dura*) to the other erect, free-like form of the same genus (*Sinularia flexibilis*) (Coll and Sammarco, 1986).

Soft corals produce natural compounds, some of which are unique to one, or a few closely related species. The type, concentration and function of these secondary metabolites vary widely. It has been found that the secondary metabolites in soft corals play an important role in their ecology, including defense against predators, competition for space between species, reproduction and antifouling; a detailed discussion of secondary metabolites from soft coral is found in Chapter 2.2.

One of the most interesting aspects of the soft corals is that fouling organisms usually do not colonize their surfaces. According to Fenical (1982), the alcyonacean soft corals contain a wealth of secondary metabolites, some of which may inhibit fouling (Hadfield and Ciereszko, 1978).

Marine fouling causes huge economic losses to marine industries, since almost all types of structural materials exposed to seawater may become fouled. In seawater the microbial population on surfaces creates an additional problem by producing the primary biofilm, which is generally thought to be a prerequisite for the attachment and

metamorphosis of fouling organisms (Young and Mitchell, 1972). Biofilm accumulation has been linked with accelerated marine biofouling; a detailed discussion of the marine biofilm forming bacteria is presented in Chapter 3.

1.2. Study Objective

The objective of this study is to examine the antibacterial properties of marine natural products of Alcyonacean soft coral. The species of soft coral examined is *Simularia polydactyla*, while the bacterium used as a test strain is *Vibrio harveyi*, which is known to be one of the marine biofilm forming bacteria (Venugopalan *et al*, 1988). In the event that antibacterial activity was found, it was our aim to determine the chemical compound(s) responsible.

2. CHEMICAL ECOLOGY OF SOFT CORALS

Chemical ecology is the study of chemically mediated interaction between organisms, and between organisms and their environment (Gerhart, 1985). Recently, chemical ecology has been extended into the marine environment, and the study of soft corals.

According to Sammarco and Coll (1992), one of the reasons for the evolutionary success of the alcyonacean soft corals in the Indo-Pacific is considered to be the high level of secondary metabolites commonly found in their tissues. These compounds are known to play a role in the ecology of these organisms (Bakus *et al*, 1986).

2.1. Taxonomy of Soft Coral

Soft corals are a group of colonial invertebrates belonging to the Phylum Coelenterata (Cnidaria), Class Anthozoa, Subclass Octocorallia. Each autozooid polyp of an octocoral colony has eight pinnate tentacles which can be used to capture small food particles usually in the form of marine zooplankton. Soft corals are considered carnivorous .

One of the major groups within the Octocorallia is the Order Alcyonacea which has small colonies, and distinct polyps fused into a common mass of coenchyme (Tursch *et al*, 1978).

The shape of colonies within the genus *Simularia* varies widely: some colonies are low and flat, sometimes dish-shaped (Verseveldt, 1980). Other colonies are erect, tree-like forms (Coll and Sammarco, 1986). *Simularia polydactyla* has colonies with distinct stalks, sometimes encrusting, lobes crowded, arborescent, with fingerlike branches. The surface layer of the lobes and of the sterile stalk usually contain club-shaped sclerites.

Many, but not all, clubs have a central wart. The length of the clubs varies, on the average, from 0.09 to 0.19 mm. The maximum length of the coenenchymal spicules is 3 to 5.5 mm (Verseveldt, 1980).

2.2. Function of Secondary Metabolites

Alcyonacean soft corals are known to be rich in secondary metabolites, especially terpenes (Faulkner, 1987). Sammarco and Coll (1990) mentioned that terpenes have been found to play roles in predator defense, interspecific competition for space, reproduction and antifouling.

Chemical analysis suggests that soft corals are rich in nutritionally important substances such as fats, proteins and carbohydrates and could serve as a food source for predators. One type of protection from predation in soft corals is toxicity. Coll *et al* (1982) reported that soft corals exhibit a wide range of ichthyotoxicity. Responses range from death within a short period of time to almost no effect at all. Some corals cause narcotizing effects, ranging from acute to mild in the test fish *Gambusia affinis*. High concentrations of certain terpenoid compounds in many soft corals may serve as a defense mechanism.

One of the most important factors limiting populations of sessile marine invertebrates, including alcyonacean corals, is competition for space. The species within alcyonaceans possess numerous characteristics which contribute to the maintenance of living space, namely: allelopathy (Sammarco *et al*, 1982); simple overgrowth (Nishihira, 1982); whole-colony movement and defensive secretions (La Barre and Coll, 1982).

The presence of allelochemical defenses has been strongly implicated in interactions between alcyonaceans and scleractinians or among alcyonaceans themselves. Initial mortality that appeared in the scleractinian corals under non-contact conditions

with the alcyonacean corals may indicate that an increased amount of secondary metabolites is released from alcyonaceans into surrounding water (Coll *et al.*, 1982).

Kashman and Groweiss (1980) reported that the chemical composition of some soft corals varies throughout the year. This raises the possibility that terpenoid compounds may play a role in the annual life cycle of soft corals. There were differences between the chemical composition of the tissues of a soft coral colony and the eggs spawned by that colony. In certain soft corals, the secondary metabolites were found in high concentration in the eggs released from the colonies and were absent several months later after the peak of the reproductive season.

The presence of terpenes in the eggs of soft corals might play a defensive role. Alternatively, the compounds present in the eggs may be used as a chemotactic cue to attract sperm to the eggs. Sammarco and Coll (1990) demonstrated that sperm chemotaxis occurs in several species, but the chemical responsible for this does not appear to be any of the egg-specific terpenoids.

Alcyonacean soft corals are usually characterized by the absence of fouling epibiota. According to Sammarco and Coll (1990) antifouling represents another ecological role of terpenes in the alcyonacean. The gorgonian octocoral *Leptogorgia virgulata* is rarely overgrown by fouling organisms (Gerhart, 1988). Some diterpenes involved in antifouling are effective against both adult and larvae forms of marine organisms (Rittschof *et al.*, 1985).

Coll *et al.* (1987) reported that the mechanism by which soft corals maintain their clean surfaces is the secretion of mucous sheets that are sloughed off. These sheets often harbour microalgae. Slow release of terpenes from the colony may ultimately assist in microalgae removal.

Another function of secondary metabolites is as antibacterial agents. Many groups within the phylum Cnidaria possess antibiotic and toxic compounds. Burkholder and Burkholder (1958) were the first who reported the antimicrobial activity of alcyonarians

such as *Antillogorgia americana*, *Antillogorgia turgida*, *Rhipidogorgia flabellum*, *Briareum asbestinum*, *Plexaura dichotoma*, *Plexaura crassa*, and *Plexaura homomalla*.

According to Ciereszko *et al* (1960); Ciereszko (1962), a terpenoid lactone, crassin acetate obtained from two species *Pseudoplexaura wagnaari* and *Pseudoplexaura crassa*, may account for the antibiotic activity of the gorgonian extracts. A compound named eunicin, which was extracted from *Eunicea mammosa*, has been found to inhibit the growth of *Staphylococcus aureus* and *Clostridium feseri* (Weinheimer *et al*, 1968).

An antimicrobial sesterterpene, called palauolide has been isolated from a mixture of at least three Palauan sponges that had been inadvertently combined by the ship's crew when being transferred from one freezer to another. Palauolide shows activity against *Bacillus subtilis* and *Staphylococcus aureus* (Sullivan and Faulkner, 1982). Pama *et al* (1992) reported that some of the active compounds from the sponge *Dysidea herbacea* which belong to a family of polybrominated diphenyl ethers show antimicrobial activities against the pathological microbes *Staphylococcus aureus* and *Bacillus subtilis*.

According to Zhong *et al* (1994) active compounds from the marine sponge *Dysidea figalis*, Dysamide A and 2,3-dihydrodysamide C exhibit antibiotic activities toward *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. Suwanborirux and Plubrukarn (1994) reported that a Thai marine sponge, *Reniera* sp., yielded four new isoquinoline alkaloids which had antimicrobial activities.

3. MARINE BIOFILM FORMING BACTERIA

Microbial cells attach firmly to almost any surface submerged in an aquatic environment. These cells grow, reproduce, and produce extracellular polymer, which extends from the cell forming a matrix of fibres that provide structure for the assembly of the biofilm (Characklis and Escher, 1988).

Biofilms can consist of a monolayer of cells covering only a fraction of the substratum or can be as thick 300–400 mm, as in algal mats. The biofilm is generally heterogeneous, providing a variety of microenvironments for microbial growth (Characklis and Marshall, 1990).

In biofilm formation, zymogenous chemoorganotrophs, primarily pseudomonads, that exhibit a rapid response to a high level of nutrients, and are characterized by possessing relatively high maximal growth rate and low substrate affinities, are the first species to become attached (Marshall, 1980). Such bacteria firmly adsorb onto solid surfaces within a matter of hours following immersion of the solid in a natural aqueous system. Corpe (1972) reported that the primary colonizers are replaced by oligotrophic types, (*Caulobacter*, *Hyphomicrobium*, and *Saprospira*) after 48 to 72 hours. This formation of initial bacterial film is then followed by the attachment of larger organisms, such as cyanobacteria, diatoms and ciliated and stalked protozoa, other unicellular algae, protozoa, and eventually invertebrates (Colwell, 1988).

As many as five compartments can be defined in a biofilm system (Figure 1). Each compartment is characterized by at least one phase (gas, liquid, or solid). The substratum plays a major role in the biofilm formation process during the early stages of biofilm accumulation and may influence the rate of cell accumulation as well as the initial cell population distribution. In addition, the substratum can also serve as substrate (the rate limiting nutrient for growth), as illustrated by microbial biofilm attack on wood structures (Characklis and Marshall, 1990).

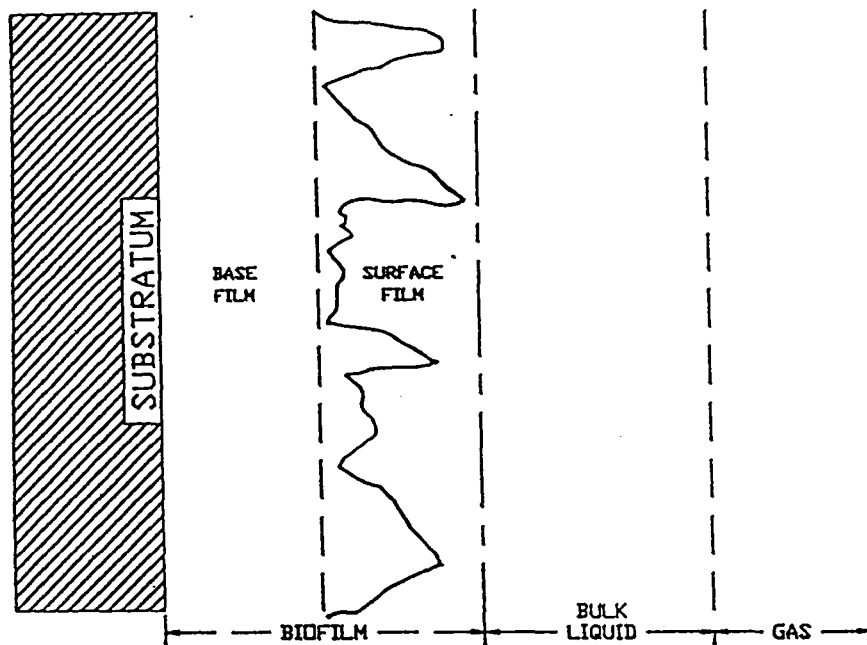


Figure 1. The biofilm system includes the following five compartments: (1) substratum, (2) base film, (3) surface film, (4) bulk liquid, and (5) gas. The base and surface film constitute the biofilm (Characklis and Marshall, 1990)

3.1. Role of Biofilm in Marine Biofouling

Biofouling is defined as the attachment and metabolism of microorganisms (microbial fouling) and macroorganisms (macrofouling) to solid surfaces and is usually

considered detrimental. Biofouling marine bacteria are the first type of organism to populate a surface placed in the marine environment and normally constitute the predominant organisms in most biofilms, thereby comprising the first stage of a progression that leads to a complex macrofouling community (Smit, 1988). According to McFeters *et al* (1990), the biofilm consists of two major components: (1) microorganisms that embed within the substratum, and (2) a matrix of extra polymeric substances (EPS) produced by bacteria that are important in adsorption.

Surface films of marine fouling bacteria appear to play a role in the settlement, attachment and metamorphosis of some marine invertebrate larvae. There is a complex relationship between microbial films and settling larvae. Bacteria not only appear to possess factors that may stimulate larval settlement and metamorphosis, but factors that may inhibit them (Maki and Mitchell, 1988). In addition, Rittschof (1985) reported that external biochemical cues are important in the metamorphosis of larval stages of fouling and other sessile marine invertebrates that may function by stimulating or inhibiting the steps in the morphosis process.

Surface films, which form rapidly as a result of the activity of microorganisms, may influence settlement through their effect on the micro-roughness of the surface. They also change its firmness and its suitability as a base for adhesion. The majority of larvae studied so far settle more readily on filmed surfaces (Crisp, 1974). Attached bacteria modify the chemistry of a surface through the production and hydrolysis of extracellular polymers, making available a vast array of molecules for recognition by competent larvae examining the suitability of the surface for attachment (White, 1984).

Under favorable conditions, permanent attachment and metamorphosis follow. If the the surface is unfavorable, larvae return to the water column (Rittschof *et al*, 1984).

3.2. Taxonomy of *Vibrio harveyi*

According to Austin *et al.* (1979) marine *Vibrio*, as part of the mixed microbial flora in the marine environment, form a primary film that attracts fouling and boring organisms. The *Vibrio* are very common in marine and estuarine environments, and on the surfaces and in the intestinal contents of marine animals. Some marine *Vibrio* are heterotrophic; others are pathogenic to marine animals and humans (Venugopalan *et al*, 1988).

The genus *Vibrio* are straight or curved Gram negative rods, motile by one or more polar flagella which are enclosed in a sheath continuous with the outer membrane of the cell wall. They are chemoorganotrophic, having both respiratory and fermentative types of metabolism. Most grow at 30°C. D-Glucose and other carbohydrates are catabolized with production of acid, but not gas. They are oxidase positive. Carbohydrates fermented by most species include maltose, D-Mannose and Trehalose.

Vibrio harveyi can be found in aquatic habitats with a wide range of salinities, within the marine environment, including planktonic (free-living), saprophytic, and gut symbiotic forms (Nealson and Hastings, 1992). *Vibrio harveyi* is a halophilic *Vibrio* species that has been isolated from many geographical locations, including coastal and open ocean seawater, as well as on the surfaces and in the feces of fish and squids

(Farmer and Hickman-Brenner, 1992). The taxonomic characteristics of *Vibrio harveyi* are shown in Table 1.

Table 1. Taxonomic characteristics of the bacterium *Vibrio harveyi*

(Nealson and Hastings, 1992).

Tests	Results
Preliminary tests:	
Salt requirement	+
Acid on glucose	+
Acid on lactose	-
Diagnostic tests:	
Growth at 4°C	-
Growth at 35°C	+
Amylase	+
Lipase	+
Gelatinase	+
Growth on:^a	
Maltose (0.2%)	+
Cellobiose (0.2%)	+
Gluconate (0.1%)	+
Glucuronate (0.1%)	+
Mannitol (0.1%)	+
Proline (0.1%)	+
Lactate (0.2%)	+
Pyruvate (0.1%)	+
Acetate (0.2%)	+
Propionate (0.05%)	+
Heptanoate (0.05%)	+
L-tyrosine (0.4%)	+
Flagellation type^b	SP Pr
Polar flagella	1
PHB accumulated^c	-
Gas from glucose	-

^aNumbers indicate amount of carbon source used

^bS, sheated flagella; P, polar flagella; Pr, peritrichous flagella; SP Pr, either type possible

^cPHB=poly-beta-hydroxybutyric acid formed when grown on glucose

4. EXPERIMENTAL METHODS

4.1. Collection of Samples

The colonies of *Simularia polydactyla* were collected using SCUBA at a depth of approximately 5 meters in the vicinity of Panjang island, Jepara region, Central Java, Indonesia in the month of July 1993. The colonies of *S. polydactyla* were placed into a container which contained seawater immediately after they had been collected. The colonies were then taken into the laboratory for preparation.

4.2. Preparation of Samples

After collection, the tissues of *S. polydactyla* were cleaned using distilled water, cut using a knife into small pieces, weighed and placed into a beaker ready for extraction.

4.3. Extraction

The tissues of *S. polydactyla* were homogenized in hexane (750 mL/500 g fresh weight) in a Waring blender. The mixture was filtered using vacuum filtration. The filtrate was reduced to a dried crude extract on the rotary evaporator and then was weighed.

4.4. Preparation of Medium

The luminous broth medium as recommended by ATTC was used to grow the bacterium *Vibrio harveyi* ATTC 33868 as the test organism.

The luminous medium consists of the following chemicals:

NaCl	30.0 g
K ₂ HPO ₄	3.9 g
KH ₂ PO ₄	2.1 g
NH ₄ Cl	5.0 g
Yeast extracts	5.0 g
Tryptone	5.0 g
1 M Tris buffer pH 7.5	50.0 mL
Glycerol	3.0 mL
MgSO ₄ .7H ₂ O	1.0 g
CaCO ₃	1.0 g
KCl	0.75 g
Distilled water	1.0 L

All the chemicals were weighed into an Erlenmeyer flask. Distilled water was added up to 1 liter. The medium was heated on a hot plate and was homogenized using a magnetic stirrer. The pH of the medium was adjusted to 7.1 with NaOH. The medium was then sterilized in the autoclave.

4.5. Preparation of *Vibrio harveyi* Culture

The bacterium *Vibrio harveyi* strain ATCC 33868 was obtained from American Type Culture Collection (ATCC). A test tube which contained 10 mL luminous broth medium was inoculated with *V. harveyi*, and then was incubated over night at 30°C.

4.6. Preliminary Test for Determining Antibacterial Properties

A preliminary test was performed on the crude hexane extract to determine whether the extract possessed antibacterial properties against *Vibrio harveyi*. The antibacterial bioassay was used as a standard screen to provide a profile of the antibacterial properties of the extract. A range of concentrations of crude extract (0, 2, 4, 6, 8, 10 mg/tube) of *S. polydactyla* were prepared by dilution into chloroform and addition into a number of test tubes, which then were evaporated. To each tube was then

added 10 mL luminous broth medium . The medium without crude extract was used as a control. Each tube was inoculated with test strain *V. harveyi* (0.2% v/v). The tubes were then incubated for 3 days at 30°C. Antibacterial activity was determined by measuring growth inhibition. Growth was measured daily using a Perkin-Elmer lambda 9 UV Spectrophotometer. Absorption of the inoculated tubes versus uninoculated tube was measured at 324.9 nm in 2 cm plastic cuvettes. These data were used to generate growth curves, to determine percent inhibition, and thus to determine the EC₅₀ value for growth inhibition. The percent inhibition was determined according to the following formula:

$$\% \text{ Inhibition} = (100 - (A_n/A_0) \cdot 100)$$

where A_n = Absorbance at n concentration of extract

A_0 = Absorbance at 0 concentration of extract

4.7. Bioassay-Directed Isolation and Purification of Antibacterial Compounds

The inhibitory compounds were isolated from *S. polydactyla* crude extract by using the antibacterial assay to guide purification (Scheme 1).

The hexane crude extract was first separated by flash column chromatography on silica gel using three different solvents : hexane, then chloroform, then 10% methanol in chloroform. Two fractions of 150 ml fractions were collected from each different solvent and then were evaporated to dryness on the rotary evaporator. Each dried extract was weighed. These extracts were then employed in antibacterial assays against *V. harveyi* to guide further purification.

A range of concentrations of the active extract (0, 0.05, 0.25, 0.50, 0.75 and 1.0 mg/tube) were prepared by dilution in chloroform and evaporation into a number of test tubes to which then were added 10 ml luminous broth medium. The medium without

extract was used as a control. Each tube was inoculated with the test strain *V. harveyi* (0.2% v/v). The tubes were then incubated for 3 days at 30°C. The growth curves were measured daily using Perkin-Elmer lambda 9 UV spectrophotometer. These data were used to generate percent inhibition curves and to determine the EC₅₀ for growth inhibition.

The active fraction from the work described above that inhibited the growth of *Vibrio harveyi* was then analyzed by TLC (Thin Layer Chromatography) using Whatman silica gel plates to determine an appropriate solvent to be used for further separation of the compounds in the active fraction. The plates were visualized using short-wave ultraviolet light. The active fraction was rechromatographed on silica gel using hexane:ethyl acetate as eluent. The percentages of ethyl acetate were increased as the elution proceeded (0, 5, 10, 15, 20, 30 and 50%). Fractions were collected from each different percentage of ethyl acetate and evaporated on the rotary evaporator. These dried fractions were weighed and were employed in the antibacterial assay against *V. harveyi* to guide further purification.

A range of concentrations of the active dried fraction (0, 0.045, 0.09, 0.18 and 0.225 mg/tube) were prepared by dilution in chloroform and evaporation into a number of test tubes to which were added 10 mL luminous broth medium. The medium without dried fraction was used as a control. Each tube was inoculated with the test strain *V. harveyi* (0.2% v/v). The tubes were incubated for 3 days at 30°C. The growth curves were measured daily using a Perkin-Elmer lambda 9 UV spectrophotometer. These data were used to generate percent inhibition curves and to determine EC₅₀ for growth inhibition.

The active dried fraction was analyzed using TLC and rechromatographed on silica gel using hexane:ethyl acetate as eluent. The percentages of ethyl acetate were increased as the elution proceeded (0, 1, 2, 5, 7 %). Each fraction was collected from each different percentage of ethyl acetate, evaporated and weighed. These dried fractions were employed in antibacterial assays against *V. harveyi* to guide further purification.

Further purification of the active fraction from the work described above was accomplished on preparative HPLC (High Performance Liquid Chromatography) using hexane:ethyl acetate (99:1) as eluent. HPLC was performed on a Beckman 110B Solvent Delivery Module using a Beckman 4.6 mm x 15 cm column of silica gel. The active fraction was injected into the HPLC, and eluting fractions were collected using a Gilson microfraction collector. Each fraction was evaporated and tested against the bacterium *V. harveyi*. All the active fractions were combined, evaporated, weighed and prepared for structural analysis.

4.8. Structural analysis

Structure elucidation of the inhibitory compound isolated from soft coral *Sinularia polydactyla* was accomplished by ^1H NMR spectroscopy in CDCl_3 at 500 MHz and mass spectrometry (MS). Since the mass spectra indicated the presence of a mixture of esters within the inhibitory compound, a synthesis of the esters was required to elucidate the structure of the inhibitory compound.

4.8.1. Synthesis of Esters

Two acid chlorides: palmitoyl chloride and stearoyl chloride, and two alcohols: 1-hexadecanol and 1-octadecanol were used to synthesize 4 esters: hexadecyl palmitate, hexadecyl stearate, octadecyl palmitate and octadecyl stearate.

1. Synthesis of hexadecyl palmitate.

To a round bottom flask with stirbar containing 1 g 1-hexadecanol (0.0041 mol) in ether (10 mL) was added 1.492 mL (0.00492 mol) palmitoyl chloride. After the reaction finished, the mixture was added to water and the *pH* was adjusted to 7 using NaOH. The aqueous phase was extracted with ether. The ether layer was collected, and magnesium sulphate was added to remove water, and the organic phase was evaporated to dryness. Crystallization from hexane and methanol was carried out to give the pure product.

2. Synthesis of hexadecyl stearate

To a round bottom flask with stirbar containing 1 g 1-hexadecanol (0.0041 mol) in ether (10 mL) was added 1.661 mL (0.0041 mol) stearoyl chloride. After the reaction finished, the mixture was added to water and the *pH* was adjusted to 7 using NaOH. The aqueous phase was extracted with ether. The ether layer was collected, magnesium sulphate was added to remove water, and the organic phase was evaporated to dryness. Crystallization from hexane and methanol was carried out to give the pure product.

3. Synthesis of octadecyl palmitate

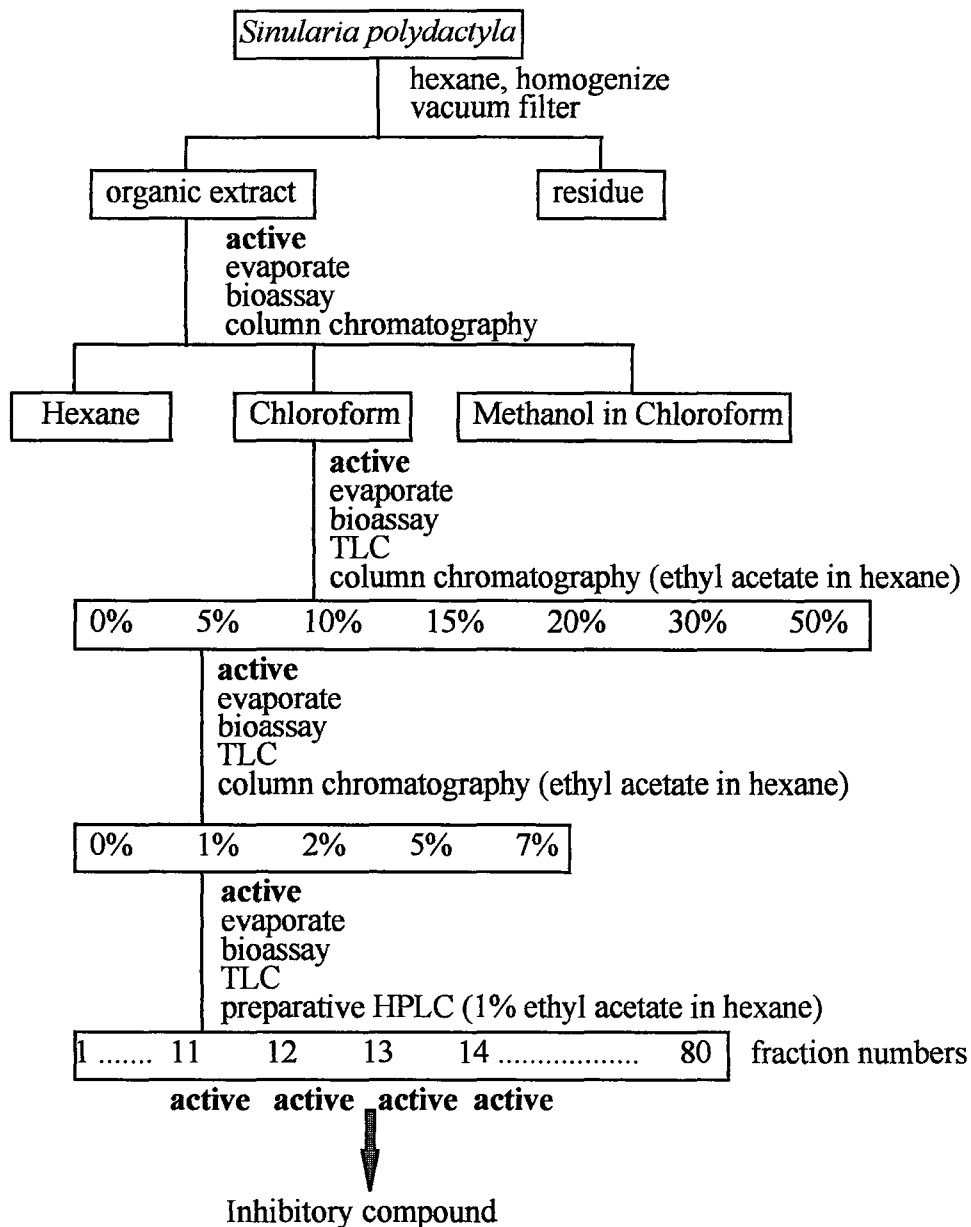
To a round bottom flask with stirbar containing 1 g 1-octadecanol (0.00365 mol) in ether (10 mL) was added 1.328 mL (0.00438 mol) palmitoyl chloride. After the reaction finished, the mixture was added to water and the pH was adjusted to 7 using NaOH. The aqueous phase was extracted with ether. The ether layer was collected, and magnesium sulphate was added to remove water, and the organic phase was evaporated to dryness. Crystallization from hexane and methanol was carried out to give the pure product.

4. Synthesis of octadecyl stearate

To a round bottom flask with stirbar containing 1 g 1-octadecanol (0.00365 mol) in ether (10mL) was added 1.478 mL (0.00438 mol) stearoyl chloride. After the reaction finished, the mixture was added to water, and pH was adjusted to 7 using NaOH. The aqueous phase was extracted with ether. The etherlayer was collected, and magnesium sulphate was added to remove water, and the organic phase was evaporated to dryness. Crystallization from hexane and methanol was carried out to give the pure product.

The structure of the four synthetic esters was confirmed using NMR and mass spectrometry; the results were compared to the NMR and mass spectrum of the inhibitory ester mixture. The four synthetic esters were then employed against the *Vibrio*

harveyi at the appropriate inhibitory concentration to determine which of the compounds inhibit the growth of *Vibrio harveyi*.



Scheme 1. Schematic diagram representing the bioassay-directed isolation and purification of the inhibitory compound

5. RESULTS AND DISCUSSION

Marine organisms, including soft corals, have become sources of great interest to natural product chemistry, since they produce metabolites with different biological activities. Several studies have focussed on the potential of metabolites within soft corals as antimicrobial agents. Alcyonacean soft corals, which are plentiful in the Indo-Pacific regions, are rich in secondary metabolites and are becoming targets of the continuing search for antibacterial agents. However, several phases must be undertaken in any study, such as collection of soft corals, extraction, preliminary screening using bioassays, isolation of active compounds, and structure elucidation, before the metabolites within soft corals are used for a particular purpose.

An antibacterial agent is defined as a chemical that kills (bactericidal) or inhibits (bacteriostatic) the growth of bacteria (Brock, 1979). Such a substance may be either a synthetic chemical or a natural product. One of the ways of observing the effects of the chemical is to add it at an inhibitory concentration to an exponentially growing bacterial culture. For experimental work, measurement of the turbidity is a practical way to follow the bacterial growth within the liquid medium (Tortora *et al*, 1982). In liquid medium, as cells grow and divide, the concentration of cells increases, the progeny are dispersed throughout the medium, and as a result the medium becomes turbid (cloudy) with cells. To estimate turbidity, a beam of light is transmitted through a bacterial suspension to a photoelectric cell. The loss of light intensity will be registered on the scale of the

instrument as the absorbance, a value derived from the percentage of transmission. By plotting the number of cells expressed as absorbance against time, a growth curve, which will show the effect of the agent upon the bacterial population over a period of time, can be obtained, which for a given species growing under given conditions has a characteristic shape.

A preliminary test using antibacterial bioassay is used to identify crude extracts as a potential source of antibacterial agent. The preliminary test revealed that the extract of *Sinularia polydactyla* possessed antibacterial properties. The hexane crude extract showed growth inhibition (Fig. 1a) of test strain *Vibrio harveyi* with an EC_{50} value of 5.5 mg/10 ml of the culture medium (Fig. 1b).

The crude extract which passed the preliminary test was then prepared for further separation. The bioassay-directed separation of crude extract of *S. polydactyla* using three different solvents (hexane, then chloroform, and then 10% methanol in chloroform) showed the active compound that inhibited *V. harveyi* came from the chloroform fraction with an EC_{50} value of 0.715 mg/10 mL of the culture medium (Fig. 2).

Further separation of the chloroform active fraction in column chromatography using increasing proportion of ethyl acetate in hexane afforded 5% ethyl acetate fraction from which active compound came out. The fraction possessed an EC_{50} value of 0.669 mg/10 mL of the culture medium (Fig. 3).

Further separation of the active fraction of 5% ethyl acetate in hexane showed the active compound was obtained from the eluted fraction with 1% ethyl acetate in hexane.

The fraction showed growth inhibition when employed in the antibacterial bioassay and was found to have an EC_{50} value of 0.129 mg/10 mL of the culture medium (Fig.4).

The separation of the active fraction of 1% ethyl acetate in hexane using preparative HPLC (hexane:ethyl acetate; 99:1) yielded active fractions that inhibited the growth of *V. harveyi* and possessed an EC_{50} value of 0.075mg/10 mL of the culture medium (Fig.5). The separation of the crude organic extract through column chromatography on silica gel and HPLC has led to the isolation and purification of the inhibitory compound(s). The results have shown that for each step of further purification of the crude organic extract, the EC_{50} of the active fraction decreased. For hexane crude extract, EC_{50} was 5.5 mg/10 mL; chloroform fraction was 0.715 mg/10 mL; 5% ethyl acetate in hexane was 0.669 mg/10 mL; 1% ethyl acetate in hexane was 0.129 mg/10 mL and HPLC fraction was 0.075 mg/10 mL. These results show that the purity of the inhibitory compound increased for each further separation of the soft coral extract.

Structural analysis of the active fraction by 1H NMR could not determine the structure of the inhibitory compound; however, the presence of long chain fatty acid ester(s) with some impurities was indicated (Fig.6). Mass spectrometry was required to determine the structure of the inhibitory compound(s), which revealed a mixture of some esters (Fig.7) that led to the questions about of which ester(s) is/are present, and which are within the mixture. To provide the profile of the inhibitory compound, synthesis of expected esters, using two alcohols: 1-hexadecanol and 1-octadecanol, and two acid chlorides: palmitoyl chloride and stearoyl chloride, that were present in the mixture were undertaken and the structure of the synthetic esters was determined using

NMR and mass spectrometry. The results were compared to the NMR and mass spectra of the inhibitory compound (Fig.8).

The comparison of spectra of synthetic esters and inhibitory compound, therefore, showed that 536 presents as small peak with fragmentation signal of 285 indicated the presence of octadecyl stearate as minor compound. 480 indicated the presence of hexadecyl palmitate with strong fragmentation signal of 257. 508 with two different fragmentation signals: 285 indicated the presence of hexadecyl stearate and 257 indicated the presence of octadecyl palmitate. The results showed that hexadecyl palmitate and octadecyl palmitate were the predominant compounds within the natural inhibitory compound.

The four synthetic esters: hexadecyl palmitate; hexadecyl stearate; octadecyl palmitate and octadecyl stearate were then tested against *Vibrio harveyi* at inhibitory concentration to provide the profile of the inhibitory compound. The results revealed that hexadecyl palmitate inhibits efficiently the growth of *V. harveyi* while octadecyl stearate is less active, and hexadecyl stearate and octadecyl palmitate are inactive (Table 2). This result has confirmed hexadecyl palmitate as the inhibitory compound which has also been shown to be the major compound in the natural mixture based on the mass spectra of the inhibitory compound (Fig.7).

The results show that *Simularia polydactyla* possesses antibacterial properties as a result of mixture of esters, with hexadecyl palmitate as the major inhibitory compound. Additional field research needs to be performed, however, to demonstrate whether or not

the inhibitory compound can actually provide antifouling protection in the marine environment by inhibiting the settlement of fouling organisms.

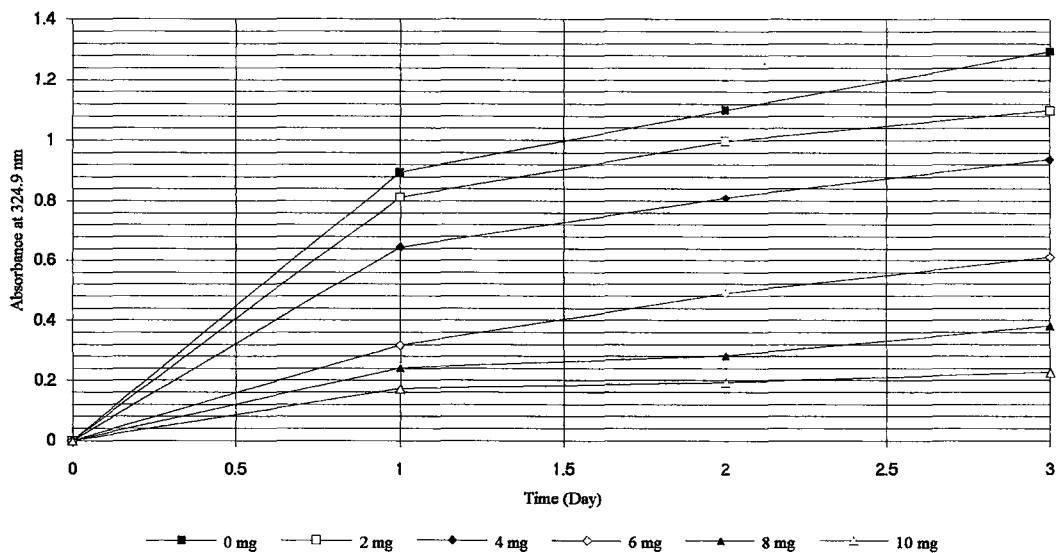


Fig. 1a. The growth inhibition of *Vibrio harveyi* by soft coral hexane crude extract

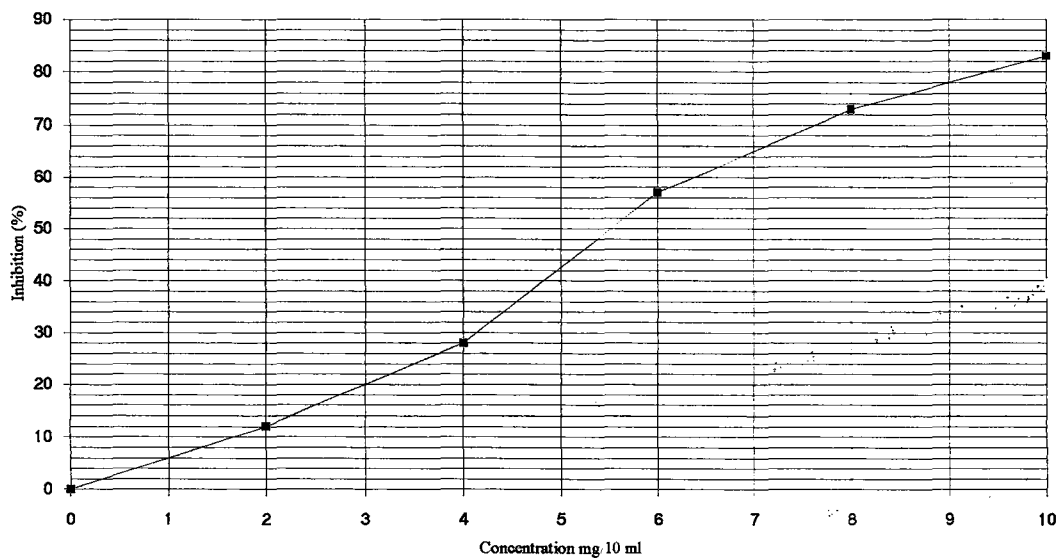


Fig. 1b. Percent inhibition of *Vibrio harveyi* by soft coral hexane crude extract

- * The absorbance at each concentration from Fig.1a was compared with that for zero concentration to obtain % inhibition for each data point. The average inhibition from days 1, 2, and 3 was then calculated for each concentration and replotted to give Fig.1b

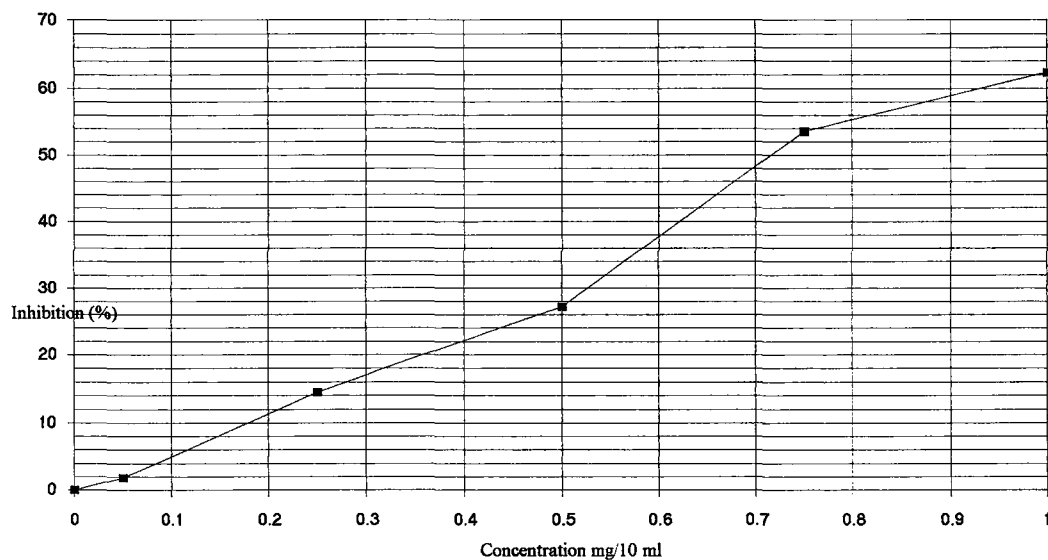


Fig.2. Percent inhibition of *Vibrio harveyi* by chloroform fraction

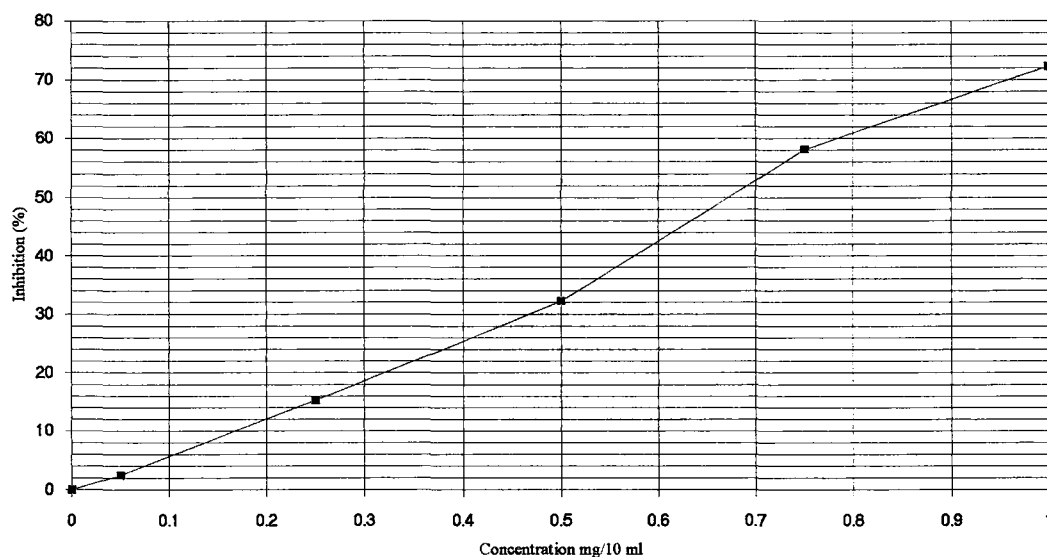


Fig.3. Percent inhibition of *Vibrio harveyi* by fraction of 5% ethyl acetate in hexane

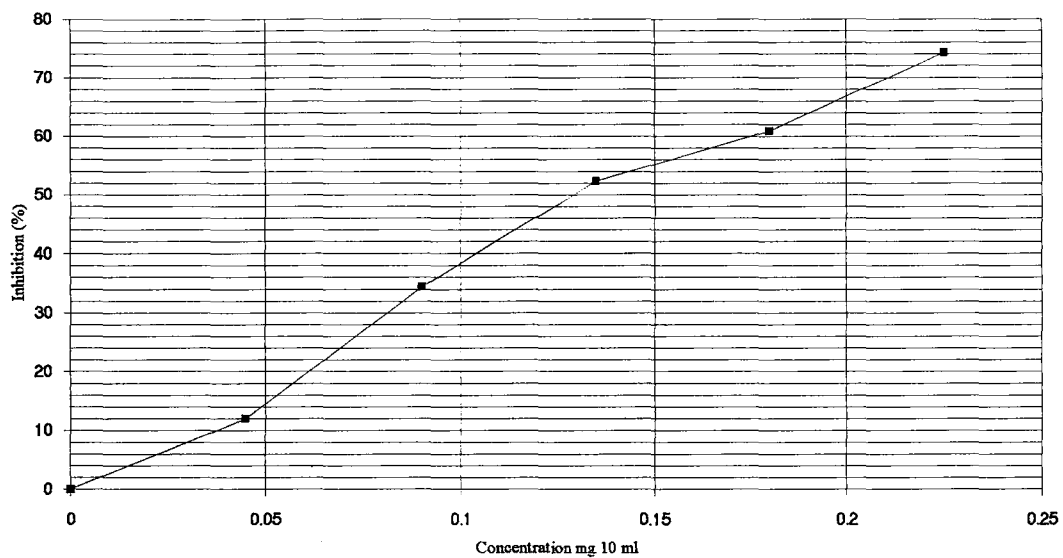


Fig.4. Percent inhibition of *Vibrio harveyi* by fraction of 1% ethyl acetate in hexane

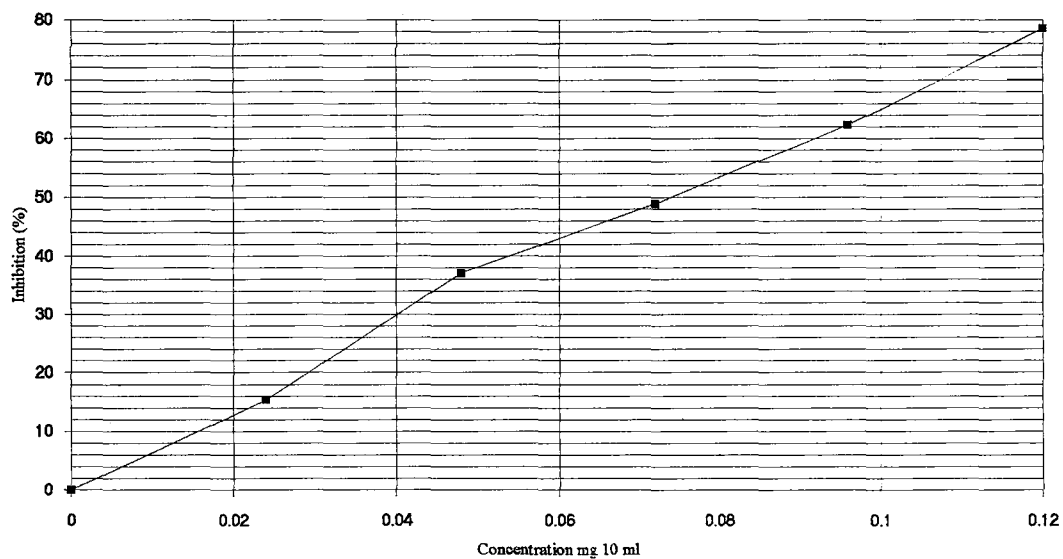


Fig.5. Percent inhibition of *Vibrio harveyi* by fraction collected from HPLC

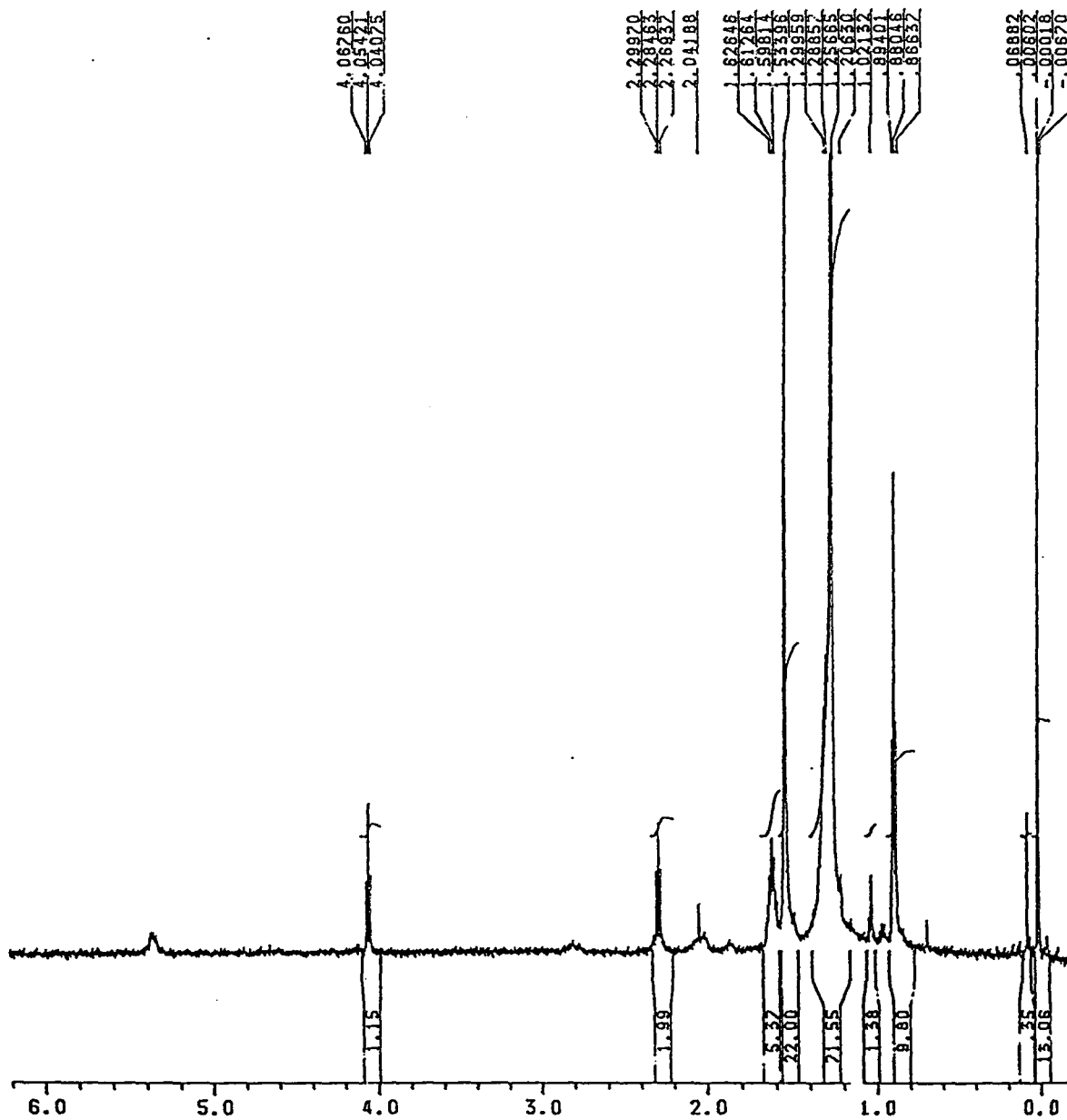


Fig.6. ^1H NMR spectrum of inhibitory compound in CDCl_3 at 500 MHz

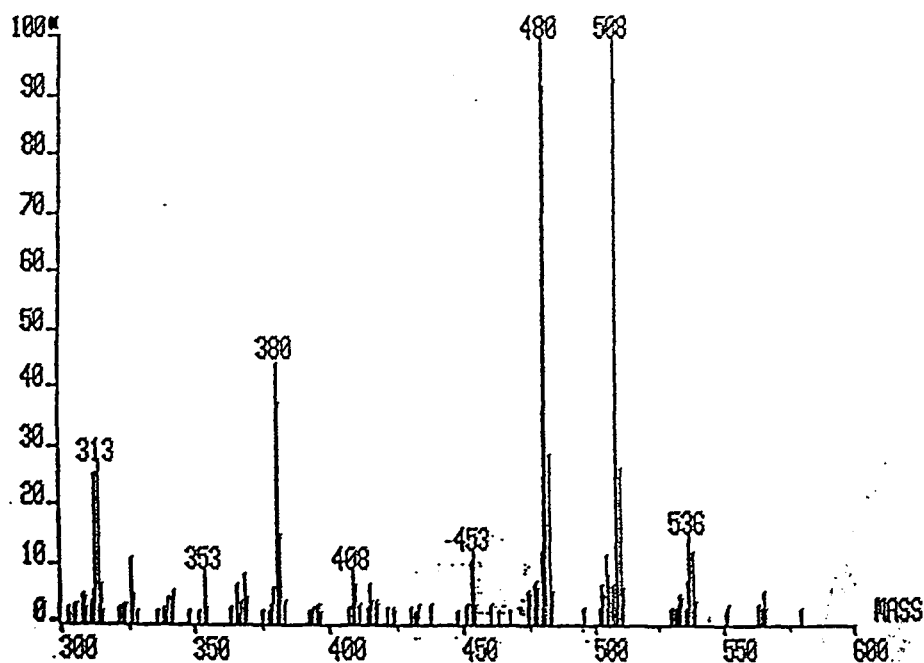
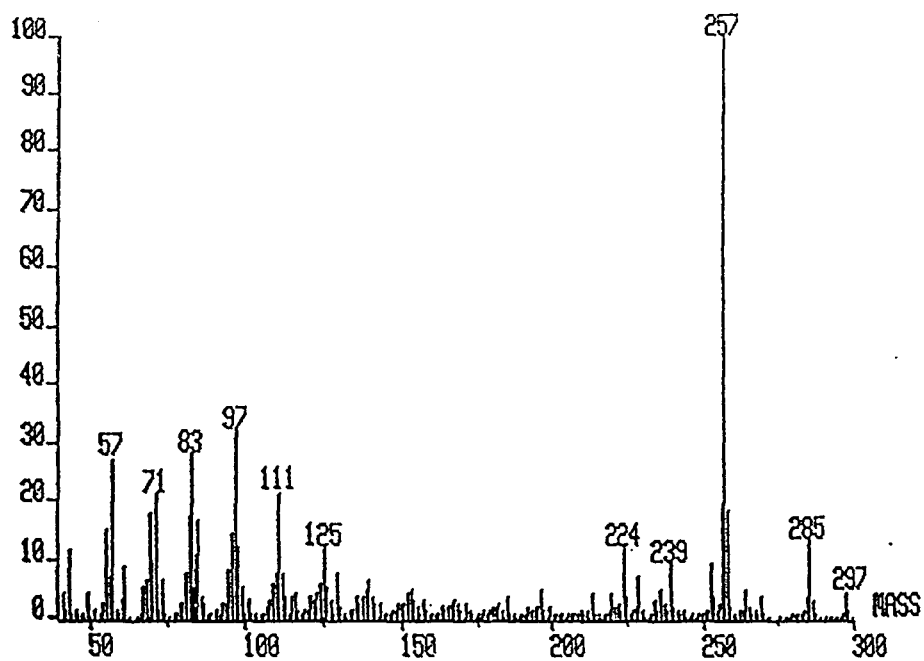
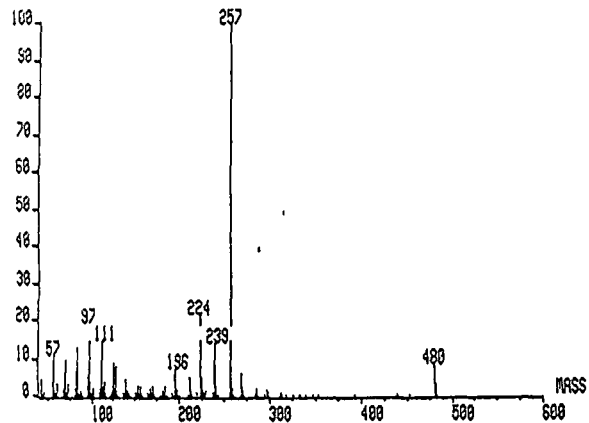
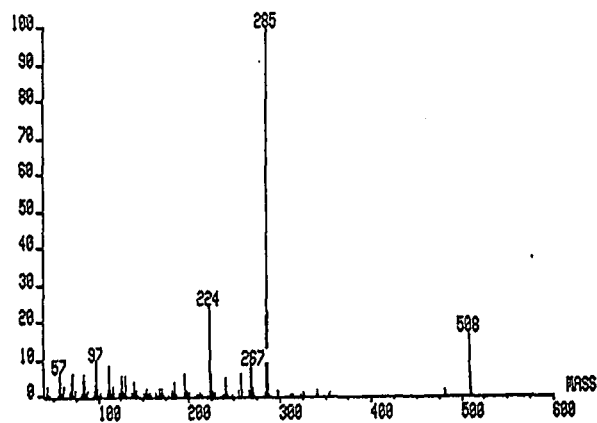


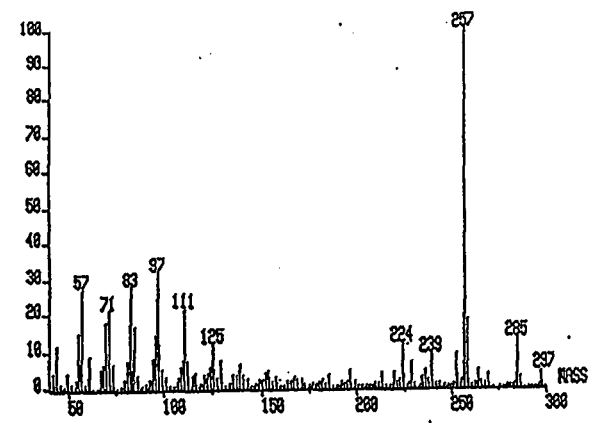
Fig. 7. Mass spectrum of inhibitory compound in DEI system



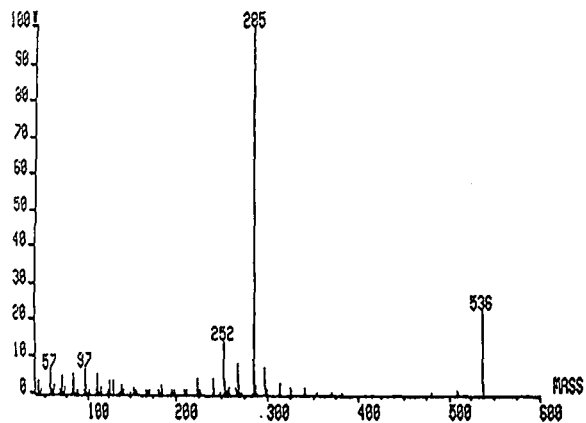
Hexadecyl palmitate



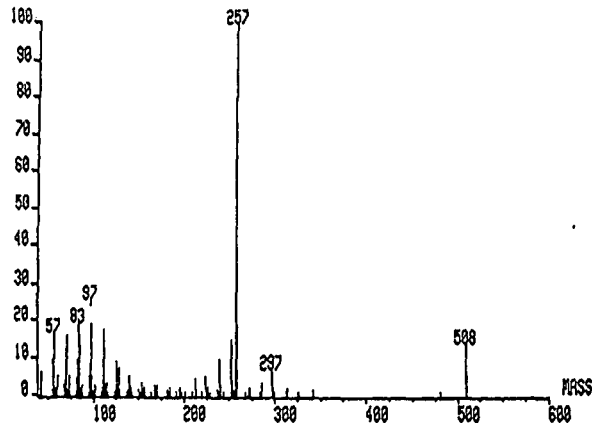
Hexadecyl stearate



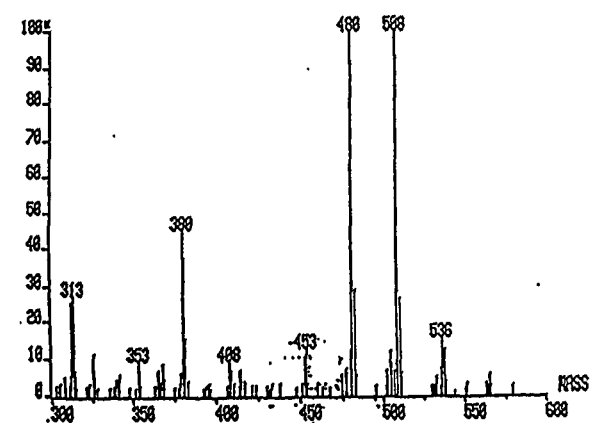
Inhibitory compound



Octadecyl stearate



Octadecyl palmitate



Inhibitory compound

Fig. 8. Mass spectra of the four synthetic esters and the inhibitory compound

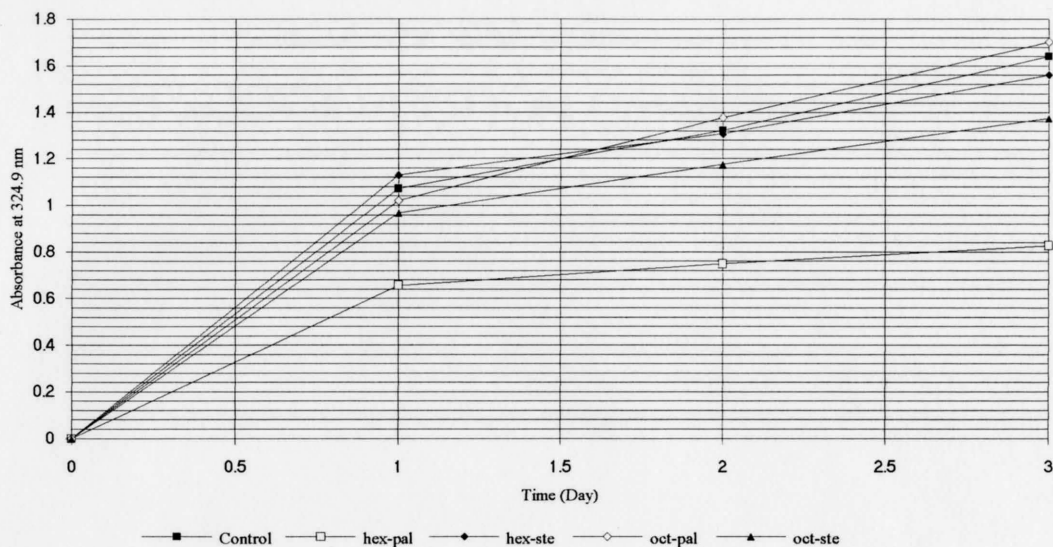


Fig.9. Growth inhibition of *Vibrio harveyi* by synthetic esters

Table 2. Growth inhibition of *Vibrio harveyi* by synthetic esters

Name of ester	Presence in natural mixture	Inhibition vs <i>Vibrio harveyi</i>	Inhibition (%)	EC ₅₀ (mg/10 mL)
hexadecyl palmitate	major	++	47.8	0.0879
octadecyl stearate	minor	+	12.6	-
hexadecyl stearate	major	-	0	-
octadecyl palmitate	minor	-	0	-

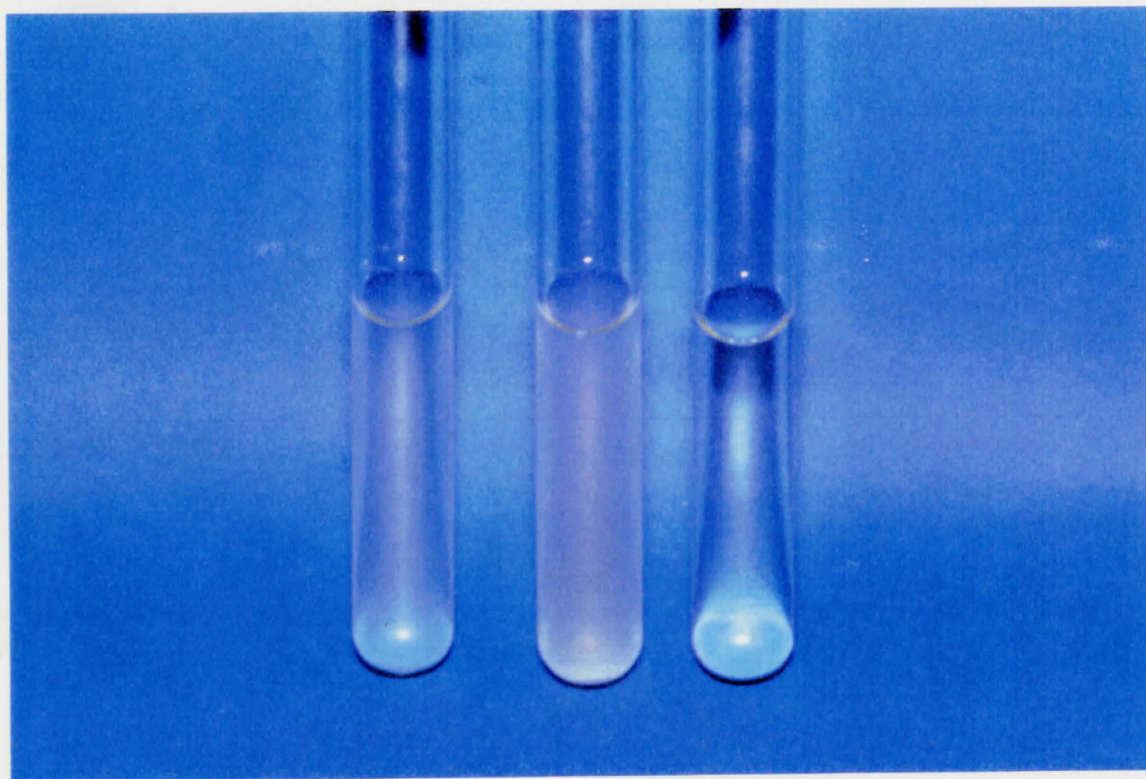


Fig.10. *V. harveyi* growth medium: (a) inoculated and grown 1 day in presence of 0.075 mg of mixture of bioactive esters; (b) inoculated and grown 1 day; (c) uninoculated

6. CONCLUSION

There are progressive attempts that are being made to exploit biologically active compounds already known to occur in the soft corals. One of the potentials that exists within the soft corals is that of marine antimicrobial agents. As part of a continuing search for new antimicrobial agents from the soft corals, a study of the antibacterial properties of the soft coral *Simularia polydactyla* was undertaken.

The results reveal that the soft coral *Simularia polydactyla* possesses an antibacterial properties against the growth of the marine biofilm forming bacterium , *Vibrio harveyi*. The major inhibitory compound present was shown to be the ester hexadecyl palmitate , with an EC₅₀ value of 0.075 mg/10 ml of culture medium.

6.1. Future work.

Future work that could be done in this study includes the following :

1. Isolation of antibacterial compounds from other species within the genus *Simularia* and within the family of Alcyonaceae using various marine biofilm forming bacteria as the tester strains.
2. Combining the antibacterial assay with barnacle settlement assay to guide isolation and purification of the inhibitory compounds from the soft corals.

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Appendix

1. Standard Deviation of cuvettes.

A standard deviation was accomplished to the cuvettes in order to determine the deviation of the cuvettes. 30 cuvettes were measured their absorbances at 324.9 nm. The data were then used to generate the standard deviation. The results showed that standard deviation among the cuvettes measured was 0.00889 AU (Absorbance Unit).

2. Standard deviation of the assay.

A standard deviation was carried out to two rows of inoculated tubes with addition of a range of concentration of inhibitory compounds, which then were incubated for 3 days. The growth curves expressed as absorbance at 324.9 nm were measured, and the data were used to generate standard deviation of the assay. The results showed that standard deviation of the assay was 0.0763 AU (Absorbance Unit).