BIOACTIVITY AND CHEMICAL ECOLOGY OF MARINE ORGANISMS FROM GOA WATERS

Thesis submitted to the Goa University for the Degree of Doctor of Philosophy in Marine Sciences

By

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STATEMENT REQUIRED TO BE SUBMITTED UNDER ORDINANCE 0.19.8(vi) OF THE GOA UNIVERSITY

This thesis is based entirely on the experimental work carried out by me under the guidance of Dr. C.T. Achuthankutty. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature concerning to the problem investigated has been surveyed and list of references is appended. Due acknowledgements have been made wherever outside facilities have been availed of.

Cynthia O.L. Gonsalves

CERTIFICATE

This is to certify that all corrections and modifications suggested by the referees and Viva-Voce Board have been incorporated in the thesis.

14/12/2000

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14/12/2000

CERTIFICATE

This is to certify that the thesis entitled **"BIOACTIVITY AND CHEMICAL ECOLOGY OF MARINE ORGANISMS FROM GOA WATERS'** submitted by Cynthia O.L. Gonsalves for the award of the Degree of Doctor of Philosophy in Marine Sciences is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any Universities or Institutions.

Place: Dona Paula, Goa

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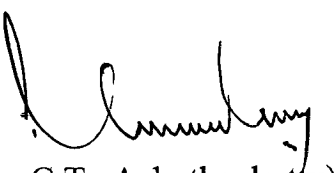

(Dr. C.T. Achuthankutty)

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CYNTHIA

CONTENTS

1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Natural products have long served as a rich source of drugs for mankind. Ancient medical records reveal that most remedies included either powders or extracts of plants, animals and minerals. As the knowledge and skill in chemistry evolved, these crude preparations slowly began to be substituted with their pure chemical substances leading to the introduction of antibiotics, tranquilizers and other medical formulations into medical practice. In recent years, with increasing advancements in ocean exploration, a great deal of attention is being paid to the vast and diverse marine resources as potential sources for natural drugs and other commercial products. For centuries, the Chinese and Japanese have been using seaweeds for various purposes. The liver oil of some fish has been used as sources of vitamins A and D. Insulin, a peptide, responsible for reducing blood sugar level is extracted from whales and tuna. The red alga, *Digenia simplex* has been serving as an anthelminthic for more than 1000 years. Chitosan, the mucopolysaccharide derived from chitin is used in various biomedical applications such as in haemodialysis membranes, artificial skin, haemoperfusion columns, haemostatic agent and so forth. Selected red and brown algae are commercially harvested for manufacturing agar-agar, carageenans and alginic acids that are widely used in medicine, food additives, microbiology, etc. The orange pigment, β-carotene, abundant in the microalga, *Dunaliella salina* a brackish water alga is widely used as an eco-friendly food colouring agent.

Research on marine organisms over the past few years have yielded several structurally unique secondary metabolites, possessing many useful biological properties. These compounds fall into diverse organic chemical classes, such as terpenoids, halogenated phenols, polyphenolics, fatty acids, amino acids, peptides, alkaloids, saponins, prostaglandins, etc. Many of them were found to exhibit promising antimicrobial, cytotoxic, cardiovascular, immuno-stimulatory, anti-inflammatory and other activities. These compounds and therefore the organisms that produce them are of great interest to natural product chemists, looking for novel bioactive molecules from marine flora and fauna because of their potential applications in pharmacology and other drugs. Several well co-ordinated studies have already been undertaken by marine biologists, chemists and pharmacologists in their search for new biomedical compounds from these sources. The presence of unusual quantities of prostaglandins in the Caribbean gorgonian, *Plexaura homomalla* (Weinheimer & Spraggins,1969), was perhaps one of the most important discovery that initiated the exploration of the sea for new drugs by the University of Oklahoma Marine Research Group. Cephalothin (Newton & Abraham,1954) is an antibiotic that is active against a number of penicillin resistant staphylococci and some gram negative species of bacteria. It is a modified form of cephalosporin C, a metabolic product of the marine fungus, *Cephalosporium acremonium*. The anthelminthic, kainic acid from the marine red algae, *Digenea simplex* in combination with santonin is marketed as Digesan (Morimoto& Nakamori,1957). *Cryptotethia crypta* has become an useful anticancer agent and is one of

the primary agents presently employed in cancer chemotherapy (Bergman & Burke,1956) and more recently also in patients suffering from *Herpes encephalitis*.

It has been known since historical times that a variety of reactions are produced by marine organisms that may range from skin infections to food poisoning. To cite a few are the tetrodotoxin, paralytic shellfish poisonings, ciguatera poisoning, brevetoxins, surugatoxins, stings of sea urchin, jelly fish, stingrays, octopus and so forth. Effects of these toxins are well described in the literature (Hashimoto,1979). Many of the toxins isolated from these organisms have found use in variety of fields such as research tools in the study of biological membranes in the field of biology and medicine, anticancer compounds, anti-inflammatory agents, etc. For example, tetrodotoxin, the chemical responsible for puffer fish poisoning, was found to inhibit specifically the sodium permeability of nerve membranes. The main action of tetrodotoxin is paralysis of peripheral nerves. As paralysis is a reversible reaction of excitation, tetrodotoxin has become a valuable tool for the elucidation of the excitation mechanism. (Faulkner,1992). Tetrodotoxin is being used clinically as a muscle relaxant and pain killer in cases of neurogenic leprosy and terminal cancer and as a local anaesthetic in Japan. Similarly, the insecticide, nereistoxin has been isolated from the marine worm, *Lumbriconeries heteropoda* based on its activity against the rice stem borer and other insect pests. A number of derivatives of the latter compound have been synthesized and Cartap hydrochloride being the most active and widely marketed

compound (Hashimoto and Okaichi,1960). So also, Quinn et al (1974) have reported that palytoxin, the most potent of all marine toxins, isolated from *Palythoa* spp, possesses anticarcinogenic activitiy.

While considering the biological properties of secondary metabolites from marine organisms for pharmacological and other drug application, it has to be realized that many ecological interactions in the marine environment are mediated by these metabolites. For example, secondary metabolites play important roles in the different life activities of these animals like catching prey, protection against settling of fouling organisms, production of itchthyotoxins, anti-predation, act as settlement cues for larvae and oviposition by insects, or serve as pheromones in mate-searching behaviour (Bakus et al,1986). It is the exploitation of these secondary metabolites that has led to the development of the vast and emerging discipline of marine natural product chemistry. However, the ecological significance of the secondary metabolites produced by marine organisms has become a subject of investigation only recently and although different types of natural products have been isolated from different groups of marine organisms, very little is known about their role in their natural environment. Ecological studies not only help in understanding the mechanism of metabolite production but also provides lead for generation of bioactivity. Research on these compounds may result in the finding of vast marine biochemical resources, useful to mankind.

1.2. OBJECTIVES AND HISTORICAL BACKGROUND

The present work was designed to study the bioactivity and ecological interactions of two species of marine invertebrates belonging to the Phylum Coelenterata. One of the objectives of the study was to screen the organic extracts of these organisms for pharmacological and other biological activities with the aim to finding new compounds suitable in medical and other drug applications. The other objective was to understand the chemical ecological responses of these organisms.

The animals belonging to the Phylum Coelenterata are predominantly marine invertebrates that include gorgonians, soft corals, hard corals, jelly fish, hydroids, zoanthids and many other lesser known animals. This phylum includes 3 classes, viz. Hydrozoa, Scyphozoa and Anthozoa. Two species of the Class Anthozoa, Order Zoanthidea, are the subjects of the present investigation. The history of research on zoanthids for pharmacological properties is summarized below.

Limu–make–0–Hana, meaning deadly seaweed of Hana was the name given to the organism that was used by the ancient Hawaiian natives to tip their weapons as a defensive advantage against invaders from the island of Hawaii. Taxonomic examination of the toxic organism showed that it was not a limu, but an animal of

the Phylum Coelenterata, Order Zoanthidea, Family Zoanthidae. Palytoxin, an extremely poisonous, water soluble substance from the zoanthid (genus *Palythoa*) was finally isolated and its structure was elucidated (Moore & Scheuer,1971). This compound was found to be highly lethal to mice (LD_{50} in mice = 0.15 μg/kg) being extremely active in cardiovascular systems, particularly the coronary arteries. Quinn et al (1974) have reported on the anticarcinogenic activity of palytoxin. The fascinating aspect of palytoxin is that it is synthesized by a marine bacterium (*Vibrio* sp) growing symbiotically with the coelenterate *Palythoa* and is apparently related to *Vibrio cholerae*. Since then, Attaway (1968) has reported the occurrence of toxic *Palythoa caribaeorum* and *P. mammilosa* from Jamaica and the Bahamas and Hashimoto,1979 has reported the occurrence of the toxic *P. tuberculosa* at Ishigaki island in the Ryukyus. Subsequently, four minor toxins, viz. homopalytoxin, bishomopalytoxin, neopalytoxin and deoxypalytoxin have also been isolated from *Palythoa tuberculosa* and from another unclassified species of *Palythoa* from Ishigaki island (Quinn,1988). Palythazine and isopalythazine are two pyrazine metabolites of *Palythoa tuberculosa*. The same zoanthid also contained mycosporine derivative mycosporine–Gly. The polyps of *Palythoa* are symbiotic with a blue-green alga and its possibility that mycosporine-Gly stimulates reproduction of the alga has been reported (Sims et al,1978). Two additional zwitterionic mycosporine derivatives, palythinol and palythene, have also been isolated from *P. tuberculosa*. A group of nitrogen pigments, paragracine–I to paragracine–VII that vary only in the number and position of N–methyl substituents have been isolated

from the anthozoan *Parazoanthus gracilis* (Quinn,1988). It has been found that paragracine displays papaverine-like pharmacological properties (Chevolot,1981). 4α-methyldehydrocholest-22E-en-3β-ol, 23-methylcholesta-5,22-dien-3β-ol and 4-dimethylsterol have been isolated from the cultured zooxanthellae of *Zoanthus sociatus* (Nancy,1983). Zoanthoxanthins, a series of novel yellow pigments which are highly fluorescent under ordinary light have been isolated from *Parazoanthus axinellae*, besides seven additional related pigments parazoanthoxanthins A-G have also been obtained from the same species. Other zoanthoxanthins isolated are epizoanthoxanthins A and B also from *Parazoanthus axinellae*, Palyzoanthoxanthins A,B and C from *Palythoa mammilosa* and *P.tuberculosa.* Pseudozoanthoxanthin and 3-norpseudozoanthoxanthin have also been isolated from *Parazoanthus axinellae.* It has been found that zoanthoxanthins and 3-norpseudozoanthoxanthin are DNA intercalating agents and both compounds selectively inhibit DNA synthesis (Chevolot,1981). So also, Rao et al (1985) have reported the presence of a new class of alkaloids zoanthamine, zoanthenamine and zoanthamide from the colonial zoanthid, *Zoanthus* sp from the Vishakapatnam coast of India. The zoanthid *Gerardia savaglia* was found to contain enormous quantities of the crustacean moulting hormone, ecdysterone (Sturaro et al,1982). The related compounds, palythoalones A and B (ecdysteroids) have recently been isolated from the marine zoanthid, *Palythoa australiae* (Shigemori et al,1999).

As part of the National Institute of Oceanography's on-going research project entitled, "Development of Potential Drugs from the Indian Ocean", a large number of organisms collected from the Indian coasts are being screened regularly for their pharmacological and toxic properties. It has been reported by Kamat et al (1981) that *Gemmaria* sp I (zoanthid) collected from Baga (Goa) possessed hypotensive activity and the extracts of *Gemmaria* sp II collected from Malvan (Maharashtra) were extremely toxic to mice and the LD_{50} was only 20 μg/kg animal by the intraperitoneal route. An interesting feature observed with *Gemmaria* sp I was that the hypotensive activity showed distinct seasonal variations in the activity. While the animals collected in October displayed the activity, those collected in June did not show the activity thereby clearly indicating that the secondary metabolites produced by zoanthids vary in activity depending on the season. Therefore, a study was conducted on two least studied species of zoanthids inhabiting the rocky intertidal area of the Goa coast with the aim to examine some of their bioactive and ecological properties. The results of the study are described in the following chapters.

1.3. PHYSIOGRAPHY OF THE STUDY AREA

Anjuna beach in Goa was chosen for the present work. Goa along India's West Coast has an area of about 3,701 sq. km. Its geographical position extends from $14^0 54'$ to $15^0 48'$N latitude and $73^0 40'$ to $74^0 12'$ E longitude and is flanked by the Arabian Sea on the west and the western ghats (Sahyadri) on the east. The coastline of Goa is about 110 km long. The coast trends in a north-north east and south-south east direction. The coast is sandy and the sandy beaches are separated from one another by rocky headlands and rivermouths.

Anjuna is a rocky beach and is situated north of the confluence of the two important rivers, the Mandovi and Zuari with the Arabian sea. It is 20 km north of Panaji, the capital city of Goa. The beach is about 1500m long. Many rocks get exposed during low tides thus exposing the attached flora and fauna for considerable length of time. The location of the beach on the map of Goa is shown in Figure 1.

The year can broadly be divided into 3 seasons based on the prevalence of the southwest monsoon (summer monsoon), viz. pre-monsoon (February–May), monsoon (June– September) and the post-monsoon (October–January). Some of the important meteorological parameters that influence Goa and in turn the Anjuna beach are as follows (Ramesh Kumar & Sathe,1996).

Temperature: The mean daily atmospheric temperature is highest in May (29.1^0C) and lowest in February (25.5^0C). Therefore in Goa, the hottest month of the year is May and the coolest month is February. The annual maximum range of temperature is 3.6^0C.

Humidity: The lowest relative humidity (61%) occurs in December. During the monsoon months (June–September) the relative humidity is maximum and the mean value is around 85%. The highest humidity touches at ≈ 98% during heavy rainy days. The vapour pressure is minimum in December and maximum in June.

Surface winds: Easterlies prevail at the surface in the morning (0830 IST) during the post monsoon and winter months. With the advance of summer, the wind direction shifts progressively to south westerlies and westerlies. The winds are mainly westerlies in the afternoon (1730 IST). The wind speed generally lie in the range 1-19 km/hr almost throughout the year except in July when most of the days the wind speeds experienced is in the range 20-61 km/hr. This makes the monsoon in Goa very windy.

Clouds: The cloudiness is very low (about 2 octas) during the winter months of January and February with minimum rainfall. The cloudiness increases as the year progresses and reaches a maximum during southwest monsoon months of June and July. These are the months of maximum rainfall. Thereafter, the cloudiness gradually decreases with retreating southwest monsoon.

Rainfall: The monthly rainfall distribution shows that the highest rainfall occurs in the month of July when the summer monsoon activity peaks over the

subcontinent. February is the driest month of the year. July has the maximum number of rainy days (26 days on an average).

Southwest monsoon/Summer monsoon: In Goa, the southwest monsoon normally sets in by the first week of June. The period June–September contributes to about 89% of the annual rainfall. The mean rainfall for the season is 2233 mm. The month of July contributes the maximum rainfall.

Northeast monsoon: The northeast monsoon, (October–November) which plays a significant role along the east coast of India, is generally very weak in Goa. The mean seasonal rainfall recorded during this season is only 182 mm and contributes to about 7% of the mean annual rainfall. The month of October contributes maximum rainfall of the season.

Tides: The tidal amplitude varied from -0.13 to 2.51 m during the period May 1996 to April 1997. The Anjuna beach is protected with rocky cliffs. Many of the rocks get exposed during low tide.

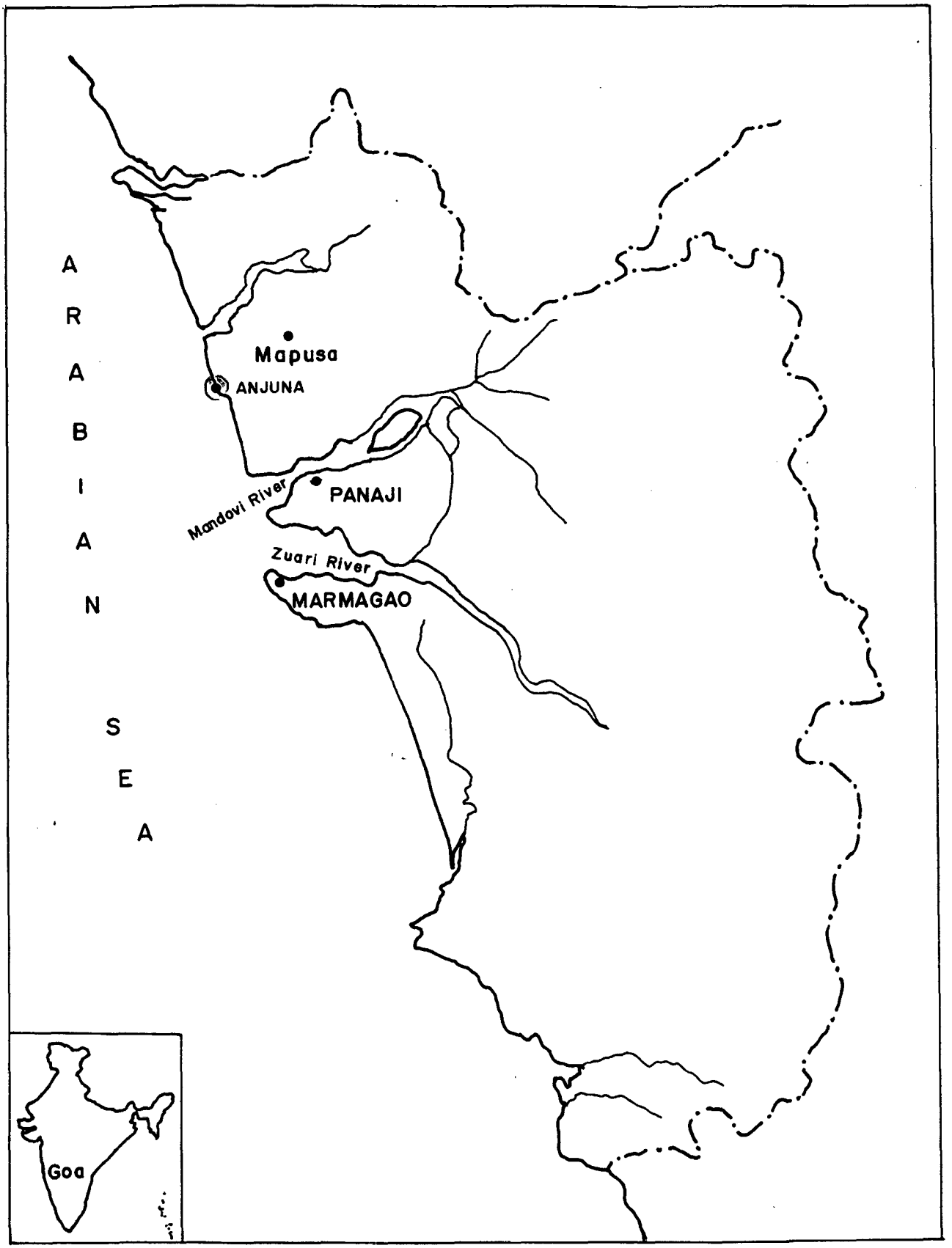


FIG. 1 MAP SHOWING STUDY AREA

1.3.1. HYDROLOGICAL PARAMETERS OF THE STUDY AREA (MAY 1996-APRIL 1997)

The various hydrological parameters mentioned below (Table 1) were measured once a month between May 1996 and April 1997, following standard procedures. Temperature was recorded using a thermometer. Salinity was determined by the Mohr-Knudsen titration method as described in Strickland & Parsons (1968). Dissolved oxygen estimation was done based on the method proposed by Winkler and modified by Carritt and Carpenter (1966). Estimation of pH was done using pH meter. Nitrate content in water was measured based on the method of Morris & Riley (1963) as modified by Grasshoff & Wood (1967). Nitrite was measured according to the method described by Bendschneider & Robinson (1952). Phosphate content in water was estimated according to the method described by Murphy & Riley (1962). Chlorophyll *a* was measured by the acetone extraction method as described by Parsons et al (1984). Procedures for estimation of the above parameters viz. dissolved oxygen, nitrates, nitrites, phosphates and chlorophyll *a* are given in Parsons et al (1984).

Table 1: Hydrological parameters of Anjuna beach during the study period

Month & Year	*Temperature (^{0}C)*	*Salinity (0/00)*	*D.O (ml/l)*	*pH*	*Nitrate (μg at l^{-1})*	*Nitrite (μg at l^{-1})*	*Phosphate (μg at l^{-1})*	*Chlorophyll a (μg at l^{-1})*
May'96	29.0	29.48	6.29	8.13	(-)	1.38	0.78	0.62
June'96	29.5	25.57	5.24	7.27	2.93	0.91	0.25	1.30
July'96	27.0	23.98	4.72	8.28	2.10	0.55	1.27	3.74
Aug'96	26.0	21.29	4.89	7.96	8.13	1.52	0.70	7.29
Oct'96	27.5	21.72	5.77	7.98	6.62	1.01	0.27	(-)
Nov'96	27.0	25.01	6.63	8.22	0.98	0.04	0.39	1.86
Jan'97	27.0	25.82	4.88	8.17	2.13	0.28	0.25	2.52
Feb'97	28.0	25.99	5.06	8.12	9.37	0.65	0.35	(-)
Mar'97	27.5	27.01	5.40	8.09	1.49	0.16	0.17	4.48
April'97	26.0	27.01	(-)	8.21	3.42	0.38	0.25	0.74

D.O = dissolved oxygen
(-) = no data

1.4. COLLECTION OF SPECIMENS AND PREPARATION OF EXTRACTS

The two species of zoanthids, viz. *Zoanthus* sp and *Protopalythoa* sp (Plate 1) were collected during low tides from the rocky intertidal expanse of the Anjuna beach, Goa (Plate 2). The zoanthids were removed from the rock surface using a large metal spatula. Specimens collected were washed with fresh sea water and brought to the laboratory. They were left in methanol for 8 days for crude extract preparation. Extracts were obtained from the whole body tissues. Solvent extracts obtained were then decanted, filtered (Whatman I) and vacuum evaporated at 40^0C to crude residue. Each batch of animal material was extracted 3 times with the solvent and all 3 extracts were pooled inorder to ensure maximum extraction of compounds from the organisms. Crude extracts thus obtained were used for the different bioactivity tests.

After preliminary screening of the crude extracts, these were fractionated into different fractions with solvents of increasing polarity. The crude extracts were thus fractionated into petroleum ether, chloroform, n-butanol and aqueous fractions. This was done by extracting the crude with the respective solvent repeatedly until extraction was complete. All the four fractions were again screened for bioactivity and the active fractions were further purified by column chromatography over silica gel or gel permeation chromatography over Sephadex. The former was carried out with petroleum ether:acetone gradient systems, while

LATE 1 – *Zoanthus* sp *Protopalythoa* sp

LATE 2 – Rocky intertidal expanse of the Anjuna beach (Goa) with zoanthid cover at low tide.

acetone or chloroform:methanol (1:1) were used as the mobile phase for gel chromatography. The chromatographed fractions that were collected by column chromatography were pooled together on the basis of TLC (thin layer chromatography) and purified further in a similar way wherever necessary. Subfractions were once again tested for the different bioassays. Column chromatography and the testing was repeated until isolation of the active principle. Column chromatography for isolation of active principle was followed for *Zoanthus* sp only while activity testing of *Protopalythoa* sp was done upto the crude fraction level. The chemical studies of the active compound and fractions were done by spectral analysis viz. IR, ^{1}H NMR, ^{13}C NMR, SEFT and COSY.

2. BIOACTIVITY

2.1. ANTISPASMODIC ACTIVITY

2.1.1. MATERIALS AND METHODS

The guinea pig ileum was used for testing the inhibitory effect of the extracts on spasmogen-induced contractions. The spasmogens used were histamine, acetyl choline (Ach), 5-hydroxy tryptamine (5-HT, serotonin), barium chloride and nicotine.

A guinea pig weighing about 400-500 gms was starved for 24-48 hrs, water being allowed *ad libitum*. The animal was killed by a stunning blow on the head. The abdomen was immediately cut open. The lower 10 cm of the ileum was discarded and 2-3 pieces measuring around 10 cm were cut and immersed in a petri plate containing aerated Tyrode solution (Ghosh,1984). The mesenteries and blood vessels were trimmed with a fine pair of scissors and the lumen of the ileum was rinsed gently with the Tyrode solution using a 5 ml capacity syringe. Care was taken not to damage the tissue while rinsing. The ileum was later cut into small pieces of 3-4 cm length in fully relaxed state. Two small loops of thread were made on either side of the ileum. One end of the ileum was tied to the frontal simple lever, which was balanced to provide a tension of 0.5 g with plasticene. The lower end was tied to the tissue holder of the organ bath of 10 ml capacity. The tissue was immersed in

aerated Tyrode solution and the organ bath was immersed in a water bath maintained at 32^0C. The tissue in the bath was left to stabilise for 1/2 hr. The Tyrode solution from the organ tube was changed every 10 minutes to ensure constant pH conditions (pH = 7.4) and also to supply nutrient salts and oxygen to the tissue which otherwise gets depleted.

After the tissue was stabilised for 1/2 hr, 2-3 doses of a spasmogen were added to the bath to obtain uniform amplitude of contraction (7-10 cm). The dose was adjusted according to the amplitude of contraction which was recorded on a smoked kymograph by a frontal writing lever with 5-6 fold magnification. The spasmogen was allowed to act for 30 seconds. After uniform contractions were obtained, the extract/fraction/pure compound as the case may be was added to the Tyrode solution (50 & 250 μg/ml) and allowed to act for 1.0 minute. After 1.0 minute, the spasmogen was added and left for another 30 seconds. Effect of the extract/fraction/pure compound on the spasmogen induced contraction was observed by comparing with the respective spasmogen-induced contraction. The tissue was immediately washed 2-3 times with the physiological solution (Tyrode solution) and allowed to rest for 5-10 minutes. If the extract caused a contraction during the 1.0-minute period, the spasmogen was not added and the extract was treated as inactive. The above procedure was followed using all the spasmogens. Before proceeding with the next spasmogen, the tissue was again stabilised with the respective spasmogen and the procedure was repeated as described

above. Each experiment was repeated 4 times and the average values are recorded.

2.1.2. RESULTS

2.1.2.1. *Protopalythoa* sp

Methanol crude extract of the above species induced a dose dependent decrease of spasmogen-induced contraction in all the 5 spasmogens tested. Percentage inhibition observed at concentrations of 50 and 250 μg/ml was 3.2 and 15.3% for histamine, 18.0 and 30.0 % for acetylcholine, 20.5 and 68.8% for 5-hydroxy tryptamine (5-HT), 14.1 and 26.6% for barium chloride and 19.1 and 38.3% for nicotine (Table 2).

Upon fractionation of the crude extract into the petroleum ether, chloroform, n-butanol and the aqueous fractions, the following results were obtained (Table 3). The petroleum ether and chloroform fractions induced contractions by self at 250 μg/ml. At 50 μg/ml, in the petroleum ether fraction, decrease was most marked for Ach (78.0 %), followed by nicotine (64.3 %), 5-HT (60.0 %) and histamine (29.4 %). There was no effect on barium chloride induced contraction (0% inhibition).

In the case of chloroform fraction, no decrease was observed at 50 μg/ml for histamine and acetylcholine. Nicotine, 5-HT, and barium chloride showed

TABLE 2: *Protopalythoa* sp - Crude methanol extract

Percentage inhibition of spasmogens

Concentration	Histamine	Acetyl choline	5-hydroxy tryptamine	Barium chloride	Nicotine
50 µg/ml	3.2	18.0	20.5	14.1	19.1
250 µg/ml	15.3	30.0	68.8	26.6	38.3

TABLE 3: *Protopalythoa* sp - Fractions

Petroleum ether

Percentage inhibition of spasmogens

Concentration	Histamine	Acetyl choline	5-hydroxy tryptamine	Barium chloride	Nicotine
50 µg/ml	29.4	78.0	60.0	0.0	64.3
250 µg/ml	C	C	C	C	C

Chloroform

Percentage inhibition of spasmogens

Concentration	Histamine	Acetyl choline	5-hydroxy tryptamine	Barium chloride	Nicotine
50 µg/ml	0.0	0.0	37.0	16.0	78.0
250 µg/ml	C	C	C	C	C

n-Butanol

Percentage inhibition of spasmogens

Concentration	Histamine	Acetyl choline	5-hydroxy tryptamine	Barium chloride	Nicotine
50 µg/ml	0.0	0.0	15.0	18.0	0.0
250 µg/ml	0.0	0.0	40.0	36.4	0.0

C = contraction by self

78.0%, 37.0% and 16.0% respectively. The n-butanol fraction showed a dose-dependent decrease only for 5-HT (15 and 40%) and barium chloride (18.0 and 36.4%). The fraction was inactive upon histamine, acetylcholine and nicotine induced contractions.

The aqueous fraction was inactive on all the spasmogens tested.

2.1.2.2. *Zoanthus* sp

Inhibitory action of the crude methanol extract on guinea-pig ileum is shown in Table 4. A dose-dependent inhibition was observed only for 5-HT and nicotine-induced contractions. Percentage inhibition observed was 15.1 and 81.4 and 16.5 and 51.1%, respectively at 50 and 250 µg/ml. Potentiation of Ach and barium chloride induced contractions was observed at both test concentrations. A percentage decrease of 7.2 at 50 µg/ml and potentiation at 250 µg/ml were observed for histamine.

The active crude extract was fractionated with solvents of increasing polarity, viz. petroleum ether, chloroform, n-butanol and aqueous fractions and screened for the antispasmodic activity observed in the crude extract. Among these, the petroleum ether fraction was found to be the most active one and this fraction was followed up by a bioassay-guided fractionation to identify the active component. For this, the petroleum ether fraction was separated over silica gel. In all, 10 fractions were obtained and the activity was located

TABLE 4: *Zoanthus* sp - Crude methanol extract

Percentage inhibition of spasmogens

Concentration	Histamine	Acetyl choline	5-hydroxy tryptamine	Barium chloride	Nicotine
50 µg/ml	7.2	Potentiation	15.1	potentiation	16.5
250 µg/ml	potentiation	Potentiation	81.4	potentiation	51.1

TABLE 5: Active sub-fractions of the petroleum ether fraction.

Percentage inhibition of spasmogens

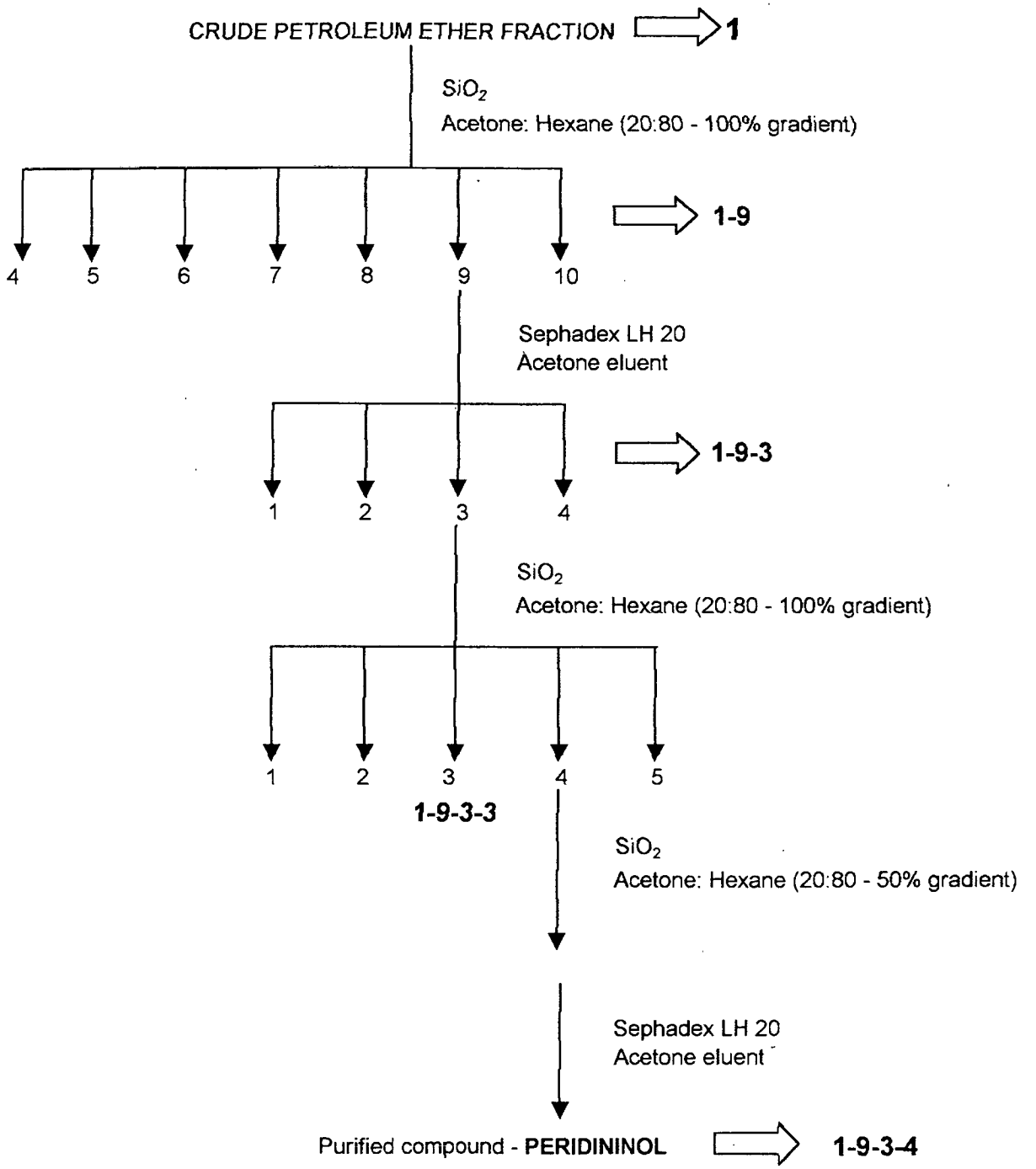
Concentration	Histamine	Acetyl choline	5-hydroxy tryptamine	Barium chloride	Nicotine
Fraction 4					
50 µg/ml	0.0	18.6	20.0	37.3	7.6
250 µg/ml	C	C	C	C	C
Fraction 5					
50 µg/ml	0.0	0.0	20.0	0.0	0.0
250 µg/ml	0.0	0.0	35.0	0.0	36.0
Fraction 7					
50 µg/ml	0.0	29.5	19.5	16.1	0.0
250 µg/ml	C	C	C	C	C
Fraction 9					
50 µg/ml	0.0	10.0	34.5	0.0	15.0
250 µg/ml	36.5	28.7	67.8	0.0	46.7
Fraction 10					
50 µg/ml	26.3	0.0	30.9	0.0	0.0
250 µg/ml	40.9	0.0	55.6	0.0	52.1

C = contraction by self

only in fractions 4,5,7,9 and 10. Results of the activity tests with active fractions are shown in Table 5. It can be seen that fraction 9 was the most active one and this fraction was therefore chosen for follow-up studies, i.e. the bioassay-guided fractionation for identification of active principle. The bioassay-guided fractionation of fraction 9 (column chromatography) is shown in Figure 2.

Fractions 1-9-3-3 and 1-9-3-4 were identified to be the active ones (Table Nos. 6 and 7). Fraction 1-9-3-3 which was collected as a mixture inhibiting nicotine (57.2%) and Ach (40.6%) induced contractions at 50 μg/ml. Infra Red (IR) spectrum (Fig. 3) of fraction 1-9-3-3 exhibited absorption at 3402 and 1020 cm^{-1}, indicating the presence of hydroxyl groups. The absorption at 2930 and 2851 cm^{-1} are characteristic of C-H stretch, indicating presence of aliphatic methylene, methine and methyl groups. At the same time, almost equal intensities of the peaks at 1456 cm^{-1} (CH_2 group) and 1373 cm^{-1} (CH_3 group) indicated more branchings in the molecule (i.e. the compound is not a straight chain molecule like fatty acids). The carbonyl absorption at 1728 could be due to acetate groups. This is also supported by the absorption at 1256 cm^{-1}. The absorptions in the range 1714– 1700 cm^{-1} could be from carboxylic acids or ketones. The former possibility is ruled out as there are no matching absorption in the region 3500–3100 cm^{-1}. The large number of

FIG. 2



Bioassay-guided fractionation of active component peridininol (fraction 1-9-3-4) and fraction 1-9-3-3.

TABLE 6: Fraction 1-9-3-3

Percentage inhibition of spasmogens

Concentration	Acetyl choline	Nicotine
50 µg/ml	40.6	57.2

TABLE 7: Pure compound – Peridininol (fraction 1-9-3-4)

Percentage inhibition of spasmogens

Concentration	5-hydroxytryptamine	Nicotine
50 µg/ml	38.0	60.0
100 µg/ml	63.0	60.0

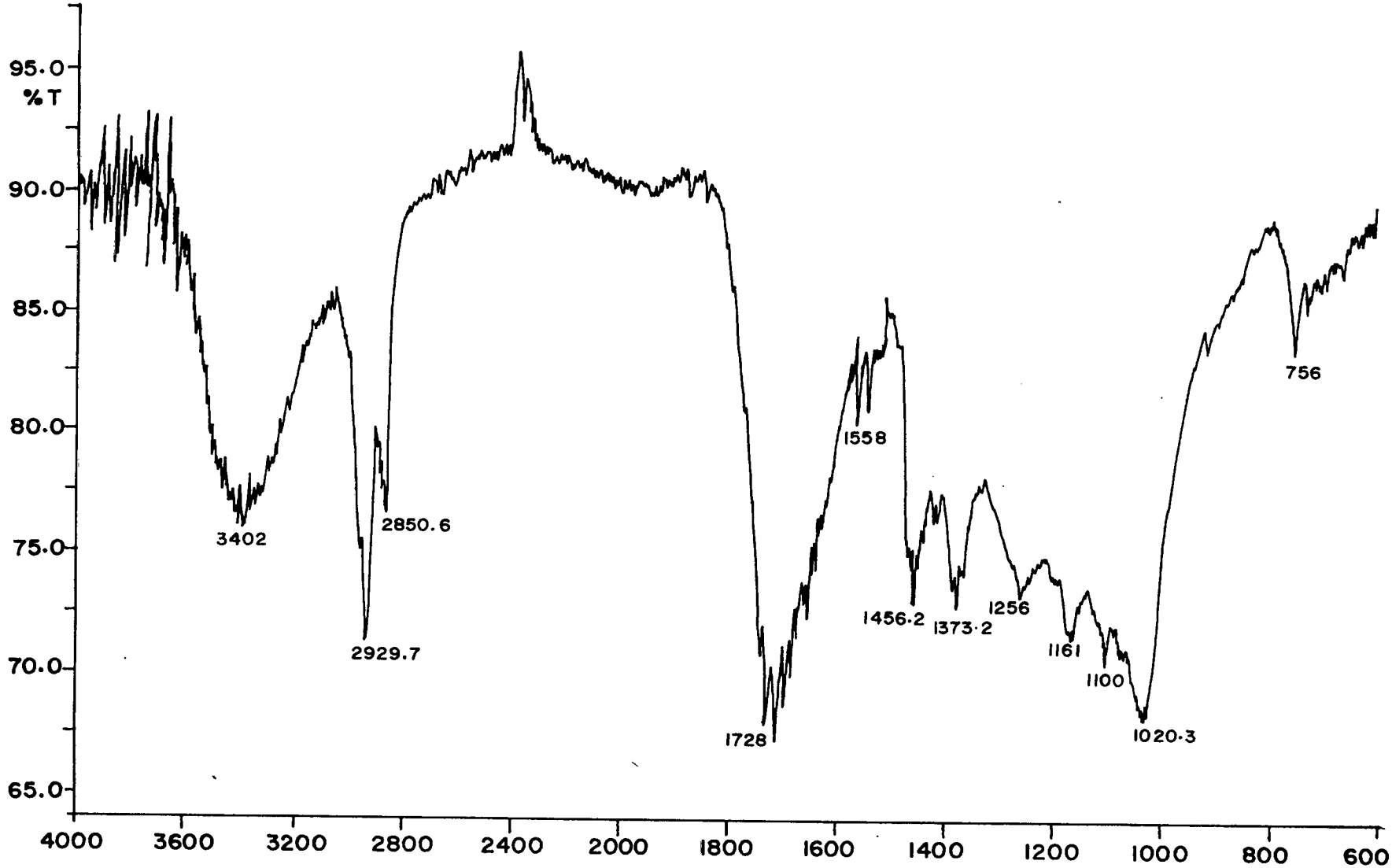


FIG. 3 IR spectrum of fraction 1-9-3-3

peaks in the range 1600–1650 cm^{-1} are indicative of multiple unsaturations. The above studies, as well as the TLC (thin layer chromatography) characteristics and light orange-yellow colour of the compound lead to the conclusion that fraction 1-9-3-3 is a mixture of carotenoids. However, absence of any peaks in the region 2400–1900 cm^{-1} rules out the presence of acetylenic or allenic groups, which are commonly associated with most of the carotenoids (eg. fucoxanthin, peridinin, etc.).

Fraction 1-9-3-4, on the other hand, was isolated in almost pure form with minor impurities. But, repeated column chromatography yielded the pure compound identified to be peridininol (Fig. 4). About 50 mg of the pure compound was isolated from initial ≈2 kg wet weight of animal. Peridininol was found effective in decreasing the nicotine and 5-HT induced contractions (Fig. 5). It decreased the nicotine contractions of guinea-pig upto 60.0% at a concentration of 50 and 100 μg/ml. However, its effect on the 5-HT induced contractions was found to be dose dependent as 38.0% decrease occurred at concentration of 50 μg/ml and 63.0% at 100 μg/ml (Table 7). Fraction 1-9-3-4 gave a bright orange spot on TLC plates (Rf= 0.8, mobile phase acetone:petroleum ether, 40:60) and the optical rotation was $[\alpha]^{25}_{D} = +2.27$ (c=2.0,$CHCl_3$). Its IR spectrum (Fig. 6) had peaks at 3410 (OH), 2926, 2860, (Aliphatic C-H), 1928 (allene); 1748 (α:β unsaturated γ-lactone); 1520 (C=C); 1454 (CH_2); 1370 (CH_3); 1180, 1148, 1128 and 1022 (C-O); 986, 952, 910 and 818 (C=C); 758 and 640 cm^{-1} . The methanolic solution of this

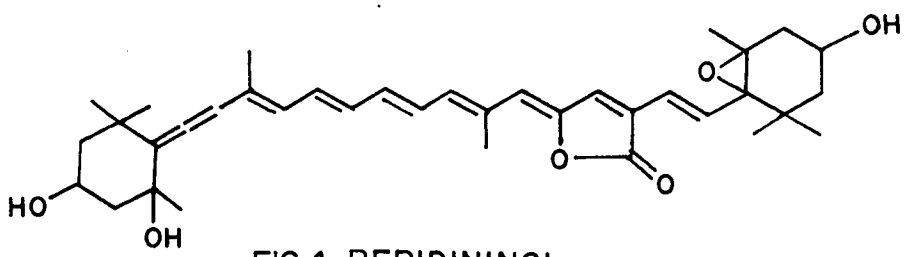
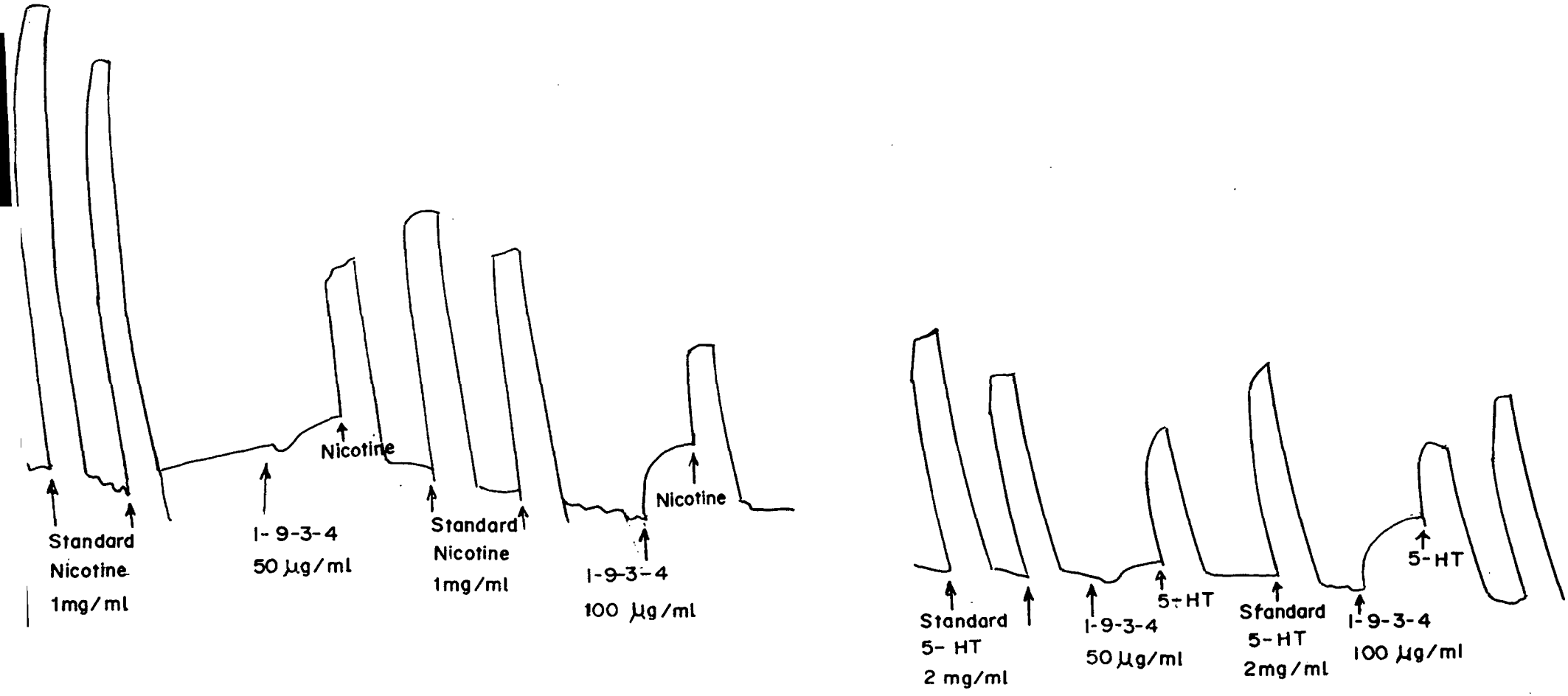


FIG.4. PERIDININOL

Fig. 5 Anti-nicotine and anti-serotonin activity of Peridininol from *Zoanthus sp*



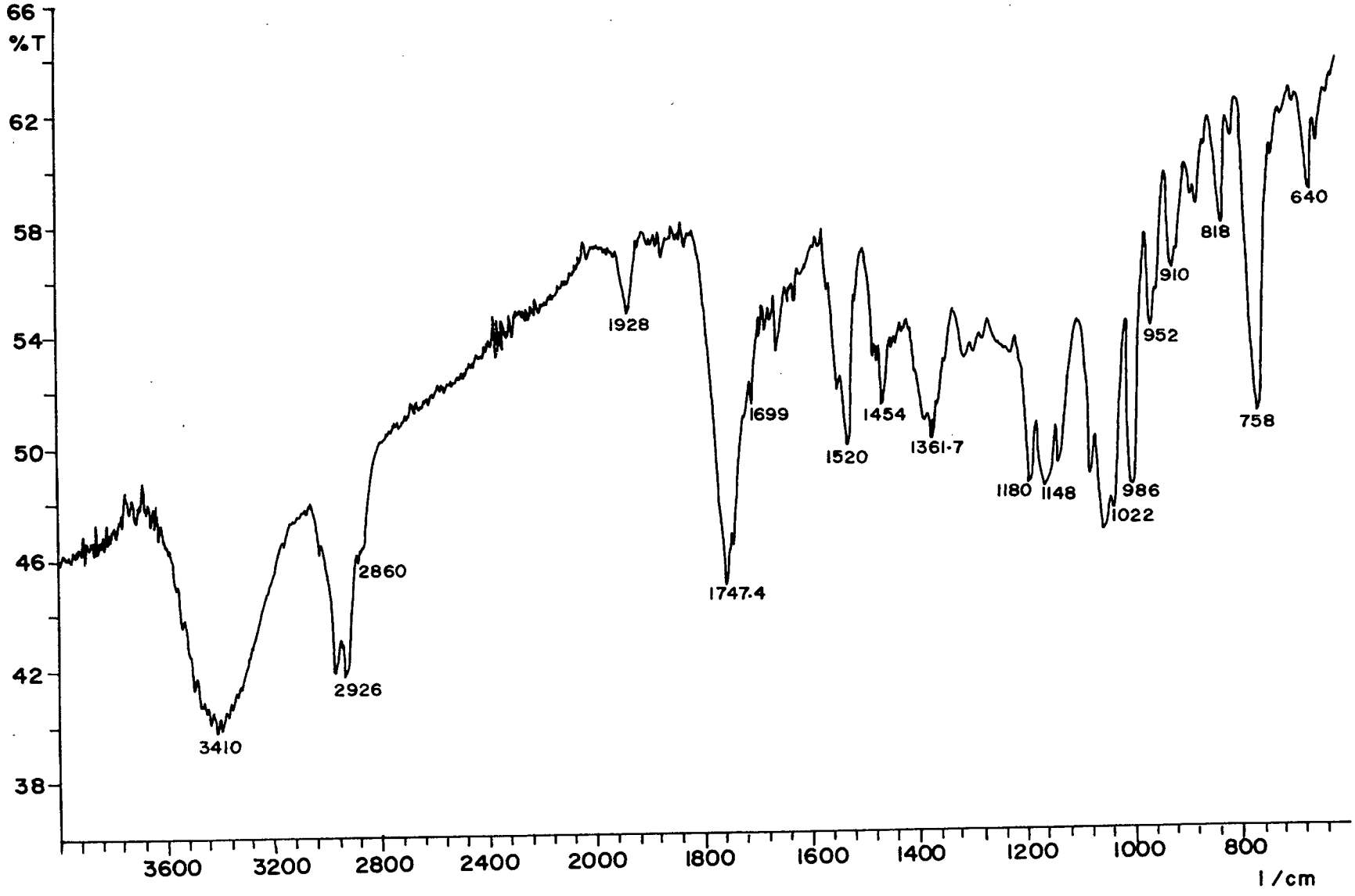


FIG. 6 IR spectrum of peridininol (fraction 1-9-3-4)

compound absorbed at 450 nm during UV-Vis absorption measurements. These preliminary results indicated that the compound is a carotenoid.

Its ^{1}H NMR spectrum showed the presence of 11 vinyl protons and 8 methyl groups in the molecule. The ^{13}C NMR and SEFT spectra had 36 carbon signals between δ 169.35 and 14.57. The allenic middle carbon which normally appears around 200 ppm was not seen in these spectra, indicating that the compound is a C_{37} carotenoid. Molecular weight of the compound was found to be 588 by FABMS, corresponding to the molecular formula $C_{37}H_{48}O_6$, the same as peridininol, the truncated C_{37} carotenoid endemic to Dinoflagellates. The proton-proton (or ^{1}H - ^{1}H) COSY, HMQC and HMBC experiments as well as comparison of its NMR spectral values with those reported in literature helped in establishing the structure as peridininol. Furthermore, these studies also helped in the unambiguous assignment of almost all the carbon and proton signals.

2.1.3. DISCUSSION

Sir Henry Dale, in 1914, distinguished between the muscarinic and nicotinic Ach receptors (Palmer,1996). While muscarinic receptors are activated selectively by muscarine, nicotinic receptors are activated by nicotine. Muscarinic receptors are blocked by atropine and nicotine receptors are blocked by curare. These two receptors belong to two separate gene superfamilies, but share the same characteristic of being activated by the same ligand, acetyl choline (Ach). Nicotinic receptors are ligand-gated ion channels and their activation always causes a rapid increase (millisecond) in cellular permeability to Na^+ and Ca^{2+} depolarisation and excitation. On the other hand, muscarinic receptors belong to G protein-coupled type receptors and not necessarily linked to changes in ion-permeability (Robert et al,1996). Multiple variants exist for both the muscarinic and nicotinic receptors. M2 and M3 type of muscarinic receptors are present in the intestine, M3 subtype being the predominant receptors that mediate the contractile effect of Ach on the gut (Palmer,1996). The effects of nicotine on the gastro-intestinal tract are due largely to parasympathetic stimulation. The combined activation of parasympathetic ganglia and cholinergic nerve endings results in increased tone and motor activity of the bowel. Systemic absorption of nicotine in individuals not exposed to nicotine previously leads to nausea, vomiting and occasionally diarrhea.

5-HT (5-hydroxy tryptamine, serotonin) causes contraction of the gastro-intestinal smooth muscle, increasing tone and facilitating peristalsis. This action is due to the direct action of 5-HT on smooth muscle receptors plus a stimulating action on ganglion cells located in the enteric nervous system (Alan et al,1995). Observations made on the small intestine led to the suggestion that two main subtypes of 5-HT receptors are present, the D and M receptors. The D receptors are present on the intestinal smooth muscle whereas M receptors are on enteric nerves (Alan et al,1990). Many types of receptors are present but the D receptor is now considered to be the $5\text{-}HT_2$ receptor/class (Engel et al,1984). It has been proposed that 5-HT plays an important physiological role as a local hormone controlling gastrointestinal motility. Overproduction of 5-HT in carcinoid tumour is associated with severe diarrhea (Alan et al,1995).

Histamine causes stimulation of smooth muscle, especially of the bronchioles and causes reduction of blood pressure by arterioles and capillary dilation (Parfitt,1999). Intradermal injection of histamine produces a classic reaction known as the "triple response" which consists of an immediately developing red spot around the site of injection followed by large reddened area and finally the appearance of a wheal (Lewis,1927). Histamine acts on 3 types of receptors, H_1, H_2 and H_3. Activation of H_1 receptors produces bronchoconstriction, contraction of gastrointestinal smooth muscle and

increased microvascular permeability (Ash & Schild,1964). These responses can be blocked by specific H_1 receptor antagonists (antihistamines) like diphenhydramine or chlorpheniamine (Babe & Serafin,1996). Stimulation of H_2 receptor increases gastric acid secretion. Drugs like cimetidine, renitidine and famotidine block these responses (Black et al,1972). H_3 receptors are associated mainly, if not entirely, with neural tissue, predominantly at presynaptic sites. Their activation results in inhibition of the release of a variety of neurotransmitters (Rang et.al,1995).

Barium salts are toxic and not used in therapeutics. Barium chloride causes contraction of all muscles and a compound causing inhibition of this response can be useful as a muscle relaxant.

The present study relates to the anti-spasmodic activity of *Protopalythoa* sp and the bioassay-guided isolation of peridininol, a C_{37} carotenoid pigment and fraction number 1-9-3-3 from *Zoanthus* sp collected from the Anjuna beach along the Goa coast. The anti-spasmodic activity of the animal extracts, fractions and the compound peridininol was tested on isolated guinea pig ileum after inducing their contractions using the five standard spasmogens, viz, histamine, acetyl choline, 5-HT, barium chloride and nicotine. The tissues were spiked with the above standard spasmogens which induced contraction of the tissues. The efficacy of the testing agent was measured based on its ability in reducing the contractions which is expressed as

percentage reduction in the contraction. The effect of the compound peridininol and fraction 1-9-3-3 isolated from *Zoanthus* sp exhibited antagonistic effects on acetyl choline, 5-HT and nicotine induced spasms. This is for the first time that the compound peridininol was isolated from *Zoanthus* sp and its novel use for reducing serotonin and nicotine induced spasms is demonstrated in the guinea pig ileum. The compound may find its use in abdominal colics, diarrhea and other gastro-intestinal disorders.

In marine environment, carotenoids are present in all algae, bacteria, yeasts and fungi. They are also encountered in several invertebrates and vertebrates. *De novo* carotenoid synthesis does not occur in animals because these do not photosynthesise, and the carotenoids present in these organisms therefore originate from their dietary sources, sometimes being metabolic modifications of the dietary carotenoids (Synnove,1978).

In the present study, the carotenoid, peridininol, a degradation product of peridinin may be ascribed to the zooxanthellae symbionts of *Zoanthus* sp. Symbiotic dinoflagellates (zooxanthellae) produce structurally more complex carotenoids and peridinin seems to be synthesised only by members belonging to the Dinophyceae (Sheuer,1978). Carotenoids have many functions in nature, some of them yet to be known (Burnett,1976). In those organisms that are capable of *de novo* carotenoid synthesis, carotenoids are used as chemosystematic markers. β-β-carotene is used in patients suffering

from light sensitivity. Carotenoids function as accessory pigments of photosynthesis. Light energy trapped by carotenoids is transferred to chlorophyll, thereby allowing the organism to utilise a broader part of spectrum for photosynthesis (Sheuer,1978). Carotenoids initiate phototropism and phototaxis in plants, animals and bacteria. Epoxidic carotenoids participate in oxygen transport. Indirect evidences suggest that trisporic acid, a metabolite of β-β-carotene, plays a role in reproduction. The biological effects of other metabolic products of carotenoids such as abscissic acid, the allenic grasshopper ketone and vitamin A are well known. In addition to the role of retinal in vision, studies have revealed the cancer-preventing effect of certain retinoids (Sporn et al,1976). Synthetic retinoids can prevent the development of epithelial cancer of the skin, respiratory tract, mammary gland and urinary bladder in experimental animals (Sporn et al, 1976). Carotenoids are also known to stabilise the proteins with which they are associated (Cheesman et.al,1967).

The extraction, isolation and purification of the carotenoid pigment peridininol, from *Zoanthus* sp adds another function to this class of organic compound. The present study derives its importance from the fact that this is the first of its kind of isolation of this pigment from this invertebrate source and has potential application in pharmacology, particularly in treatment of abdominal colics, diarrhea and many other gastrointestinal disorders.

2.2. OXYTOCIC ACTIVITY

2.2.1. MATERIALS AND METHODS

The guinea-pig uterus was used for testing the oxytocic activity. The experimental set up was the same as that mentioned for the antispasmodic activity. A female virgin guinea-pig that was in estrous and weighing around 400-500 g was used for the test. The animal was killed by a stunning blow on the head. The abdomen was immediately cut open. The uterus was dissected out and placed in a petri plate containing aerated De Jalon's solution (Ghosh,1984). The uterus was freed from fat and the two uterine horns were separated by cutting the lower end. Only one horn was used for the experiment. Two small loops of thread were made at each end of the uterus and kept in tissue bath of 10 ml capacity containing aerated De Jalon's solution. The lower end was tied to the tissue holder and the upper end to the writing lever. The lever was balanced to provide a tension of 1 g with plasticene. The tissue was left in the bath for 1/2 hr for stabilisation before starting the experiment and the physiological solution (De Jalon's solution) was renewed every 10 minutes. After stabilisation, 2-3 doses of oxytocin (Parke Davis Ltd, Hyderabad) were added as standard to the

physiological solution, the dose depending on the amplitude of contraction (6-14 cm) to obtain uniform contractions. The extract was then added to the bath (50 & 250 μg/ml) and allowed to act for 1 minute. If a contraction was observed during that 1-minute period, extract was considered to be oxytocin-like and having oxytocic property. After every contraction, the tissue was immediately washed twice with the physiological solution and relaxed for 5-10 minutes. The same procedure was followed with all extracts/fractions and the pure compound. Each experiment was repeated 4 times and average values are presented.

2.2.2. RESULTS

Crude methanol extracts of both *Zoanthus* sp and *Protopalythoa* sp when subjected to oxytocic activity caused contraction of the tissue during the first 1-minute period after addition of the extract. It was seen that at the two test concentrations (50 and 250 μg/ml), both species showed a dose dependent increase in contraction. The percentage response of *Zoanthus* sp extract to the guinea pig uterus at 50 and 250 μg/ml, was 13.3 and 126.3% respectively compared to the standard contraction of oxytocin. Similarly, *Protopalythoa* sp, caused contractions that amounted to 15.8 and 112.5% compared to standard contraction of oxytocin at 50 and 250 μg/ml, respectively (Table 8).

Crude extracts of both the species were partitioned into petroleum ether, chloroform, n-butanol and the aqueous fractions in that order. Each individual fraction was again subjected to the bioactivity test. It was seen that the petroleum ether, chloroform and n-butanol fractions from the extracts of both the species were active (Tables 9 & 10). At concentrations of 50 and 250 μg/ml, percentage response observed for the petroleum ether fraction of *Zoanthus* sp, was 57.4 and 102.5% respectively. Chloroform and n-butanol fractions exhibited 47.1 and 104.6% and 83.8 and 100.0% response, respectively at the above concentrations. On the other hand, *Protopalythoa* sp displayed a slightly different response. (Table 10). Although the first 3 fractions were found to be active, the petroleum ether fraction remained inactive at 50 μg/ml, but caused contraction of the tissue at 250 μg/ml which corresponded to 156.3% compared to the standard contraction of oxytocin. Similarly, the n-butanol fraction was also inactive at 50 μg/ml concentration, but exhibited a 100.0% response at 250 μg/ml. Nonetheless, a dose dependent contraction of the uterine muscle was observed with the chloroform fraction with activity being 56.3 and 150.0% at doses of 50 and 250 μg/ml respectively.

The chloroform fraction of *Zoanthus* sp was chosen for further purification and isolation of the active compound responsible for the activity. This fraction was purified by chromatography over silica gel (mesh 60-120, acetone-hexane gradient system) and sephadex LH-20 (acetone eluent) columns. This led to the

TABLE 8: Percentage response of the crude extract of *Zoanthus* sp and *Protopalythoa* sp on the isolated guinea pig uterus.

% Response

Crude extract	50 µg/ml	250 µg/ml
Zoanthus sp	13.3	126.3
Protopalythoa sp	15.8	112.5

TABLE 9: Percentage response of the fractions of *Zoanthus* sp on the isolated guinea pig uterus.

% Response

Fractions	50 µg/ml	250 µg/ml
Petroleum ether	57.4	102.5
Chloroform	47.1	104.6
n-Butanol	83.8	100.0
Aqueous	inactive	inactive

TABLE 10: Percentage response of the fractions of *Protopalythoa* sp on the isolated guinea pig uterus.

% Response

Fractions	**50 µg/ml**	**250 µg/ml**
Petroleum ether	*inactive*	*156.3*
Chloroform	*56.3*	*150.0*
n-Butanol	inactive	100.0
Aqueous	inactive	inactive

TABLE 11: Percentage response of the active compound on isolated guinea pig uterus.

Concentrations	50 µg/ml	100 µg/ml	200 µg/ml
% response compared to standard oxytocin	*80.8*	92.3	118.0
% response compared to standard $PGF_{2\alpha}$	69.4	82.3	114.1

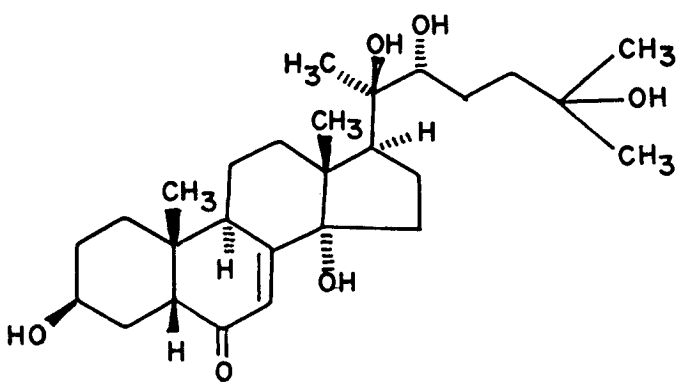


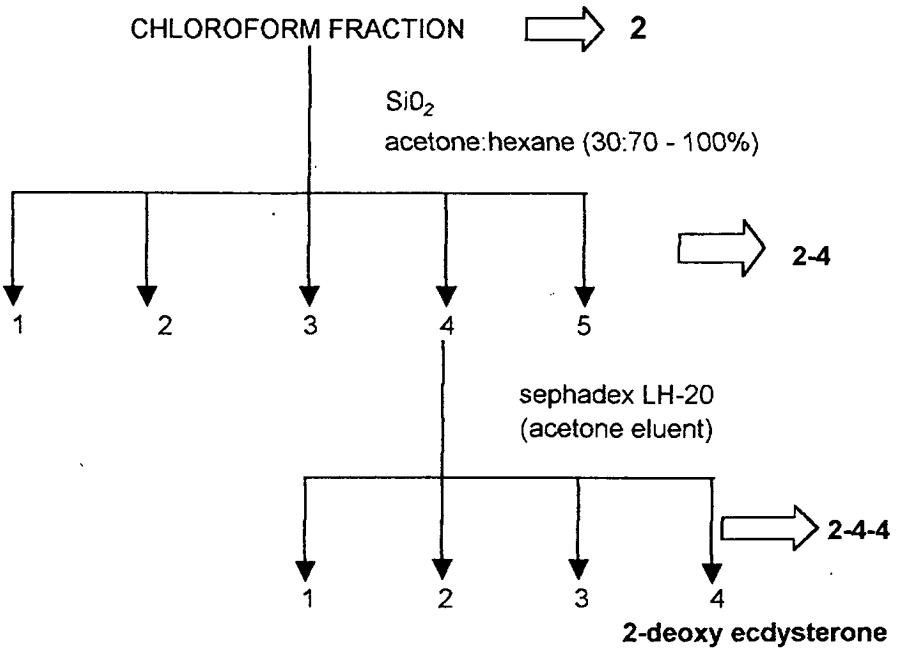
FIG.7 2 - deoxy ecdysterone

isolation of the active component (fraction 2-4-4) as a white solid in pure form, later identified as 2-deoxy ecdysterone (Fig. 7). The bioassay-guided fractionation of the active compound is shown in Figure 8. The compound had an Rf value of 0.72 (mobile phase–acetone:petroleum ether 1:1) during TLC analysis and an optical rotation of $[\alpha]^{25}_{D}$ = +75.11 (c=1.2,MeOH). From the crude extract approximately weighing 30 gm, about 50 mg of the pure compound was isolated. IR spectrum showed peaks at 3344, 2933, 2874, 1639 and 1447 (Fig. 9). FABMS indicated that the molecular weight of this compound was 464. The fragment ions at m/z 447 $(M+H-H_20)^+$; 429 $(M+H-2H_20)^+$; 411 $(M+H-3H_20)^+$; were indicative of successive losses of elements of water. HRMS revealed its molecular formula to be $C_{27}H_{44}O_6$ (observed m/z 465.3229) as against those of ecdysone, ecdysterone and related compounds and revealed its structure to be 2-deoxy ecdysterone.

It was observed that for the active compound, the percentage of active component of oxytocin contained in 50 µg/ml of compound amounts to 80.8%, that at 100 µg/ml amounts to 92.3% which further increases to 118.0% at concentration of 200 µg/ml (Table 11, Fig. 10). Uterine contractions produced by the compound were also compared with the standard contraction produced by prostaglandin ($PGF_{2\alpha}$). It was observed that for the active compound, the percentage of active component $PGF_{2\alpha}$ contained in 50 µg/ml of compound amounts to 69.4%, that at

100 µg/ml to 82.3% which further increases to 114.1% at concentration of 200µg/ml (Table 11, Figure 10).

FIG. 8



Isolation of the active component, 2-deoxy ecdysterone from the chloroform fraction of *Zoanthus* sp during the bioassay-guided fractionation for oxytocic activity.

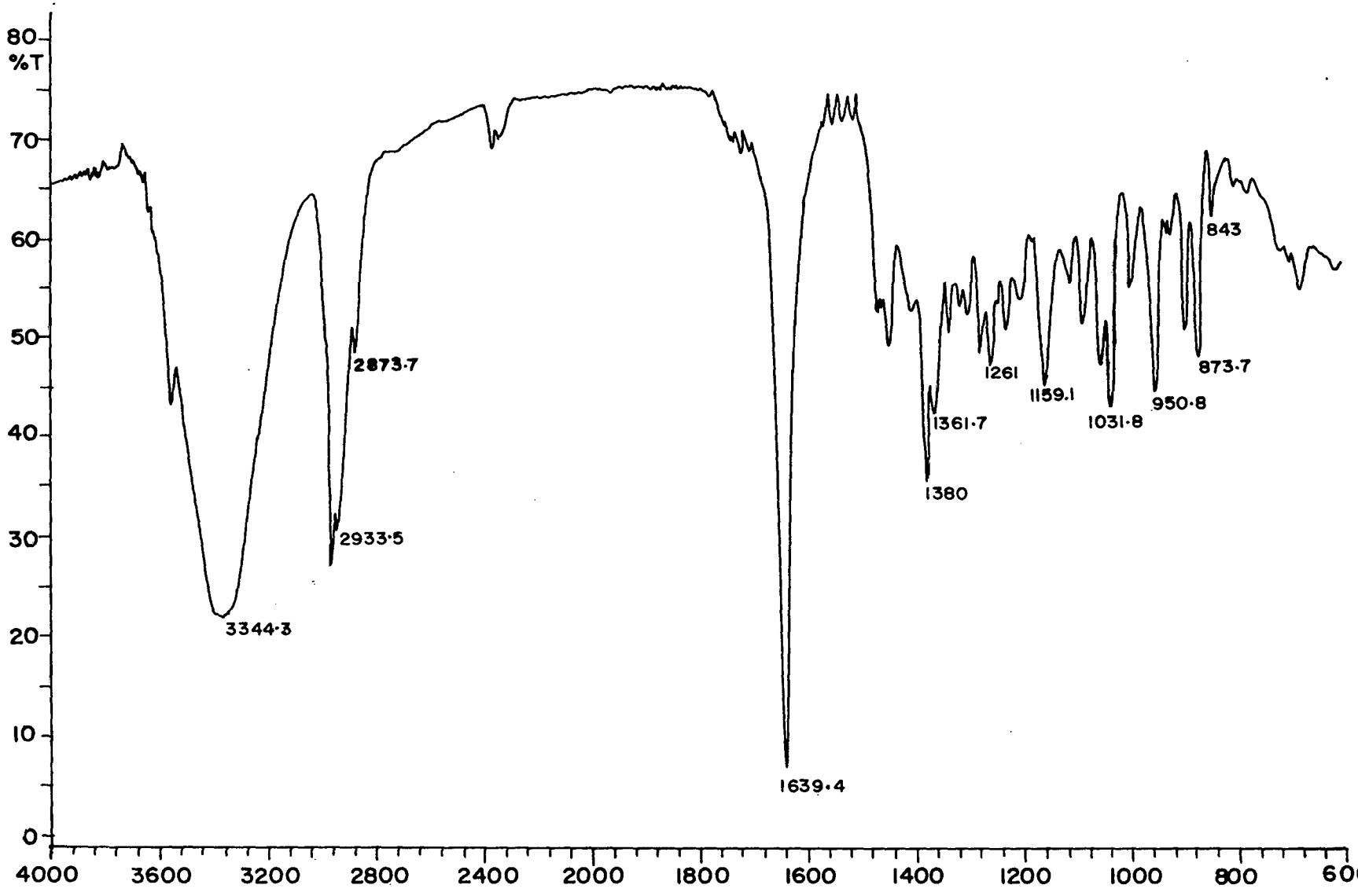


FIG. 9 IR spectrum of 2-deoxy ecdysterone (fraction 2-4-4)

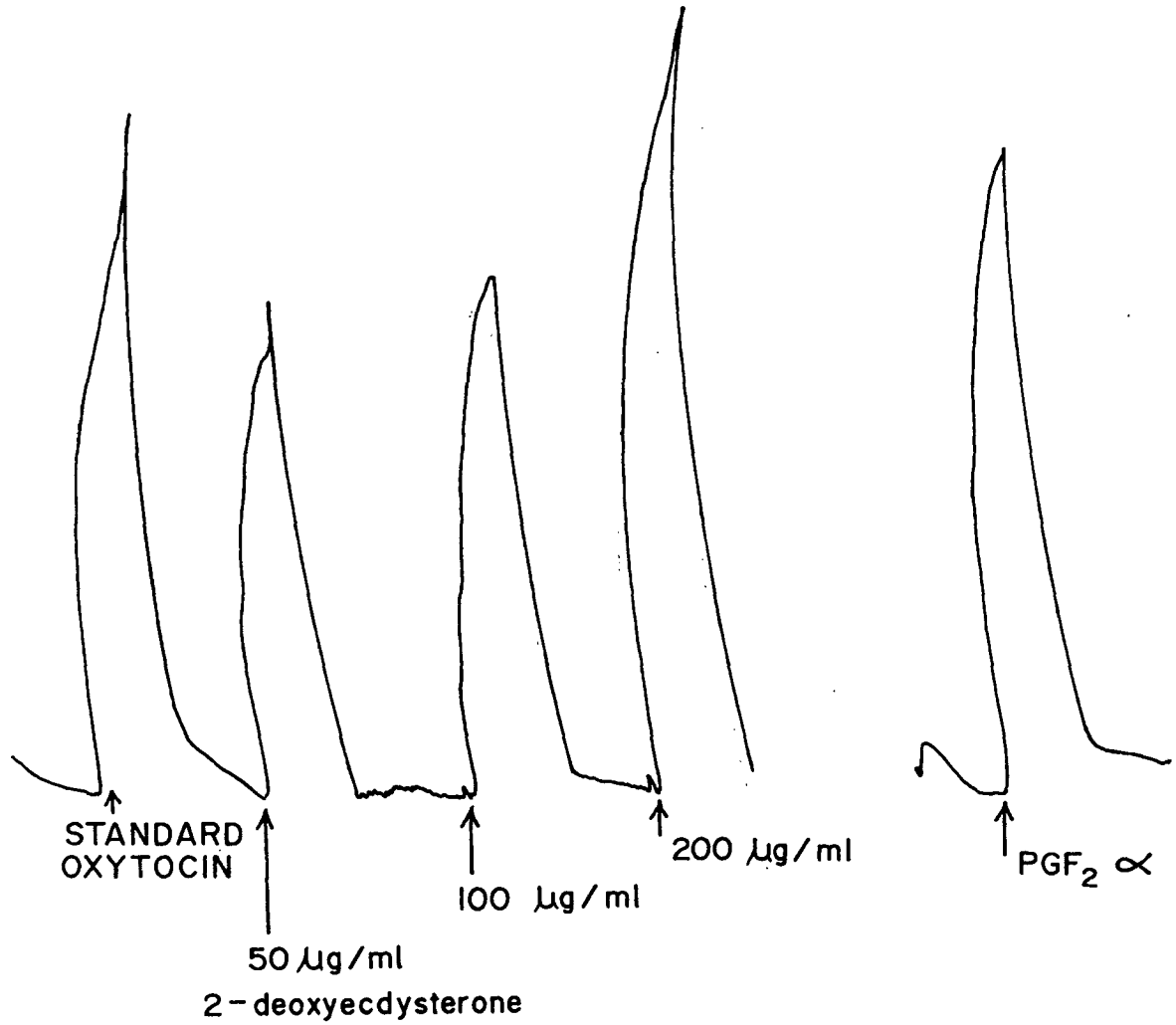


FIG. 10 OXYTOCIC ACTIVITY OF 2-DEOXY ECDYSTERONE FROM *Zoanthus* sp.

2.2.3. DISCUSSION

Agents that stimulate the pregnant uterus and are of importance in obstetrics are: (1) – oxytocics: oxytocin and ergometrine and (2) – E and F type prostaglandins Oxytocin causes regular co-ordinated uterine contractions each followed by relaxation. It is the drug of choice used to induce or augment labour when the uterine muscle is not functioning efficiently (Rang et al,1995). It is particularly used in cases such as diabetes, isoimmunisation, hypertensive states, intrauterine growth retardation, placental insufficiency, etc. in which continuation of pregnancy is considered to be more harmful to the mother and/or fetus than the risks of delivery or pharmacological induction (Andrew & Robert,1995). Oxytocin may also be used in the treatment of postpartum hemorrhage resulting from uterine atony (Andrew & Robert,1995). Historically, the ergot alkaloids were the first agents used to initiate or accelerate parturition. In modern obstetrics, oxytocin is used for this function and ergot alkaloids are most often used for treatment of postpartum hemorrhage (Cornelia,1996). Specific receptors for oxytocin in human myometrium have been identified and differences in receptor density at various stages of labour have also been noted (Bosmar et al,1994). Oxytocin has dual effects on the uterus. It regulates the contractile properties of myometrial cells and elicits prostaglandin production by endometrial/ decidual cells.

Prostaglandins currently used in obstetrical practice include PGE_2, $PGF_{2\alpha}$ and the synthetic derivative 15-methyl $PGF_{2\alpha}$. More recently, the PGE_1 analog, misoprostol, has been under clinical investigation for use as an abortifacient and cervical ripening. The major use of PGE_2 (dinoprostone, Prostin E_2) and 15-methyl $PGF_{2\alpha}$ (Carboprost, Hemabate) that is currently approved in U.S.A, is for the performance of mid-trimester abortions. 15-methyl $PGF_{2\alpha}$ also may be used as an alternative to ergonovine or oxytocin in the treatment of postpartum hemorrhage. In addition, numerous studies have supported the beneficial effect of locally applied PGE_2 as a cervical ripening agent (Buchanam et al,1984).

In the present study, the extracts of both, *Zoanthus* sp and *Protopalythoa* sp was evaluated for oxytocic property and was found to be capable of inducing contractions in the guinea pig uterus. The finding of the oxytocic property of the pure compound, 2-deoxy ecdysterone isolated from *Zoanthus* sp relates to its novel use in obstetrics and could have the following potential uses. (1) - to induce or augment labour, (2) - to control postpartum uterine atony and hemorrhage, (3) - to cause uterine contraction after cesarean section or during uterine surgery and (4) – to induce therapeutic abortion.

2-deoxy ecdysterone falls under the chemical class of ecdysones, i.e. steroids with moulting hormone activities. Ecdysones have been identified primarily from insect and crustacean sources (Horn et al,1968; Butenandt & Karlson,1954;

Tighe-Ford,1977; Galbraith et al,1968). However, ecdysones have also been isolated from plant sources (Chong et al, 1970). Besides, the zoanthid, *Gerardia savaglia* was also found to contain large quantities of the crustacean moulting hormone, ecdysterone (Sturaro et al,1982) and recently, palythoalones A and B (ecdysteroids) have been isolated from the marine zoanthid *Palythoa australiae* (Shigemori et al,1999). Isolation of 2-deoxy ecdysterone from *Zoanthus* sp in the present study adds to yet another source of ecdysteroids and the pharmacological investigation of this compound revealed its oxytocic property. This study relates to a novel use of 2-deoxy ecdysterone in obstetrics. The study on the effect of 2-deoxy ecdysterone on the isolated uterine tissue has established that this compound is relatively more potent than the standard oxytocin and $PGF_{2\alpha}$ at higher concentration (200 μg/ml). In addition, 2-deoxy ecdysterone is also a very stable compound and soluble in aqueous solvents and hence may become the compound of choice for similar therapies in the future.

2.3. MOSQUITOCIDAL ACTIVITY

2.3.1. MATERIALS AND METHODS

Larvae of *Anopheles stephensi* and *Culex quinquiefasciatus* at 3rd instar stage were used for the test. The larvae used were procured from the Malaria Research Centre, Goa. Stock solutions of the extracts (1cc=50mg) of both species of zoanthids were prepared in distilled water and desired concentrations were prepared by diluting the stock using dechlorinated tap water. Concentrations used ranged from 200-1000 ppm. Those fractions that were not soluble in distilled water, were dissolved by adding 0.1 ml of acetone. Ten larvae of the respective species were released in 25 ml of test solution in 50 ml beakers. Two sets were run every time for each concentration. Controls were also maintained in duplicate for each concentration. In case of those sets where acetone was added to dissolve the fraction, control beakers were also maintained with acetone.Experiments were conducted at room temperature ($\cong 28^0$ C). Mortality counts were taken after 24 hr of the treatment. Activity of crude extracts were tested only against larvae of *C. quinquiefasciatus*, but fractions were tested against both species. After testing the crude extracts of both species for activity, they were fractionated into 4 different fractions, viz; petroleum ether, chloroform, n-butanol and aqueous fraction and

were tested for activity following the above procedure. LC_{50} values for crude extracts and fractions were calculated using the Probit Regression method (Fisher & Yates, 1963).

2.3.2. RESULTS

Crude extracts of both species of zoanthids were active against larvae of *C. quinquiefasciatus*. LC_{50} of *Zoanthus* sp was 643.7 ppm (Table 12) while that of *Protopalythoa* sp extract was 549.8 ppm (Table 13). Fractions of the two extracts when tested for activity, the petroleum ether fraction of *Protopalythoa* sp (LC_{50} = 441.8 ppm) and the chloroform fraction of *Zoanthus* sp (LC_{50} = 826.3 ppm) were found to be most active against *A. stephensi* larvae (Table 14). In case of *C. quinquiefasciatus* larvae, the active fractions were found to be the petroleum ether (LC_{50} = 790 ppm) for *Protopalythoa* sp and n-butanol fraction for *Zoanthus* sp (LC_{50} = 715.8 ppm) (Table 14).

TABLE 12:Percentage mortality of *Culex quinquiefasciatus* with crude extract of *Zoanthus* sp in the different test concentrations.

Test concentration (ppm)	% mortality
200	10
400	10
600	50
800	70
1000	90

Table 13: Percentage mortality of *Culex quinquiefasciatus* with crude extractof *Protopalythoa* sp in the different test concentrations.

Test concentration (ppm)	% mortality
500	50
1000	80
2000	80
3000	90
4000	100

TABLE 14:Percentage mortality of *Culex quinquiefasciatus* and *Anopheles stephensi* with the active fractions of *Zoanthus* sp and *Protopalythoa* sp.

Concentration (ppm)	*Culex quinquiefasciatus*		*Anopheles stephensi*	
	n-Butanol	**Petroleum ether**	**Chloroform**	**Petroleum ether**
	Zoanthus sp	*Protopalythoa* sp	*Zoanthus* sp	*Protopalythoa* sp
200	20	10	10	10
400	30	20	20	30
600	50	30	30	50
800	60	50	40	100
1000	60	70	70	100

2.3.3. DISCUSSION

Extracts of both species of zoanthids are capable of inducing mortality in larvae of *C. quinquiefasciatus* and *A. stephensi*. Petroleum ether fraction of *Protopalythoa* sp was active for both species of mosquito larvae, the activity probably associated with same component(s) in the fraction. For *Zoanthus* sp, the activity was located in two different fractions, viz. chloroform and n-butanol, suggesting that compounds are species specific.

Resistance of insects to conventional synthetic insecticides like DDT, BHC and malathion, because of their role in pollution, has initiated the search for active non-polluting compounds of natural origin. Besides, synthetic insecticides are

also toxic to non-target organisms (Saxena & Yadav,1983). Natural products being degradable, reduce the severity of pollution (Thangam & Kathiresan,1991a). Many workers have studied the larvicidal action of terrestrial plant extracts, marine plants, mangroves, cyanobacteria (Perich et al,1995; Kiviranta et al,1993; Jalees et al,1993; Achary et al,1993; Thangam & Kathiresan,1991b) and in a few cases the active compounds have been isolated (Watanabe et al,1989; 1990). In the present study, the larvicidal action of *Zoanthus* sp and *Protopalythoa* sp against larvae of *C. quinquiefasciatus* and *A. stephensi*, the vectors of filaria and malaria parasites have been evaluated. Results of the toxicity test revealed their efficiency as a larvicidal agent against larvae of the two mosquito species suggesting its possible use in mosquito control programmes. Natural insecticides would form an alternative to overcome the various problems posed by synthetic insecticides.

3.1. GENERÁL INTRODUCTION

Evolutionary success of marine organisms may be attributed to the production of toxic secondary metabolites or their accumulation via food chain in their tissues that protect against predation, help in competiting for space, enable communication, reproduction, antifouling defense, pheromone production, synchronisation of release of sexual products, etc. Many interactions of attack, defense and behavioural response involve not only physical force but in most cases chemical agents. Perhaps, the secretion of defensive chemical metabolites by organisms seem to be of major significance in the adaptation of species and organisation of communities. Chemical defense is a protective measure employed by living organisms that involves use of chemical substances. Marine secondary metabolites play mainly three natural functions, (1) anti-predatory - those which protect marine organisms against predators which would otherwise prey upon them, (2) anti-fouling - that prevent marine larvae, algal spores and other forms from settling on the bodies of organisms and (3) competition for space. In addition to the above three major functional categories, secondary metabolites also serve important roles in reproduction, communication, symbiosis and other activities (Bakus et al,1986).

It has long been recognized that many soft-bodied and exposed organisms are not preyed upon and often are rejected by aquarium fish when offered to them as

food. These organisms have characteristics that render them either inaccessible or inedible. Studies have shown that many marine organisms accomplish this by making themselves distasteful to predators by the secretion of diffusible repellant substances (Lucas et al,1979; Ireland & Faulkner,1978; Faulkner & Ghiselin,1983). In the absence of any protective devices, many of the soft-bodied animals would easily succumb to predation. Chemical defense constitutes one of the efficient means of protection for these organisms (Wylie & Paul,1989; Thompson,1960; Rice,1985). Recent studies have revealed the presence of antifeedant substances in several soft bodied marine organisms (Rice,1985; Hay et al,1987; Lucas et al,1979). For example, many soft corals and nudibranchs possess several unique terpenoids which render them inedible to predators (Coll et al,1982; Pawlik et al, 1987; Tursch et al,1978; Sammarco & Coll,1988). The nudibranchs are also known to bioaccumulate in their bodies these terpenoids, primarily obtained from food sources such as algae, sponges etc. Recently, several antifeedant polypropionates (C_3 units), apparently biosynthesized *de novo* in the body have also been reported from many nudibranchs (Davies-Coleman & Garson,1998). In the case of some ascidians, low intracellular pH in the tunic fluids as well as high vanadium content within their bodies have been implicated as antipredatory measures (Stoecker 1980a, 1980b, 1980c).

Several marine organisms are also known to contain toxins (Hashimoto,1979). Toxicity, whereby the predator ingests the prey and becomes ill, experiences physiological stress or dies, may yet be another defensive strategy of these

organisms. For organisms that are otherwise defenseless against predation, toxicity offers an important defensive adaptation. It has been suggested that toxicity in sedentary or sessile marine invertebrates may have probably evolved via natural selection due to high intensities of fish predation (Bakus,1976; Cameron,1974; Cameron,1976). According to Bakus, (1969) competition for food in the tropical waters might be the result of increased species diversity of fishes which was reflected in a natural selective force on the prey and grazed organisms favouring individuals with chemical defenses against predation and grazing fishes. This was supported by Green's observation (1977) that among the sponges which are relatively vulnerable organisms with sessile habits and soft bodies, evolved toxicity presumably as a defensive mechanism. Studies in sponges and holothurians have shown that fish predation and grazing play an important role in selecting for toxicity in coral reef invertebrates (Bakus & Green,1974; Bakus,1964; 1970). Palytoxin, isolated from *Palythoa* spp of the family Zoanthidae is known to be highly toxic among marine toxins (Moore & Scheuer,1971). It's LD_{50} value in mice by intravenous injection is only 0.15 μg/kg. In addition, the toxin was found to be highly active in cardiovascular systems (Kaul et al,1974; Kulkarni et al,1978), particularly the coronary arteries, which accounts for most of its lethality to animals. It contracts the peripherovascular system of dogs as revealed by electrocardiograms (Kaul et al,1974). Quinn et al (1974) has also reported on the anti-carcinogenic activity of this compound.

One of the perennial problems in the marine environment is fouling, which occurs on all offshore structures leading to serious consequences. Similarly, undefended, exposed, sessile and sedentary marine organisms also get fouled by other settling organisms. However, most of the marine organisms are relatively free from this menace. The consequences of biofouling being fatal, most marine organisms resort to different strategies for preventing the menace. Some organisms may overcome fouling pressure to a considerable extent while some others maintain their body surfaces clean of epibionts. The production and exudation of unfavourable or toxic chemical compounds by vulnerable organisms is a widespread antifouling defense strategy in the marine environment.

The purpose of this study was to provide information on the incidence of chemical defenses of the 2 species of zoanthids that are the candidates for the present work. Zoanthids are benthic, sessile, colony forming organisms, comprising of thousands of individual polyps. They normally occur as dense mats and colonize large intertidal areas. It appears that their ability to produce the chemical substances plays major defensive mechanism in preventing other organisms to over populate in the surrounding area. A large number of chemical compounds have been isolated from zoanthids, possessing various biological properties (Moore & Scheuer,1971; Quinn,1988; Bakus et al,1986; Fenical & McConnell,1975; Cimino et al,1973; Rao et al,1985; Bergman et al,1951; Gupta & Scheuer,1969). However, very little is known about the functions of these metabolites in its natural environment. Focus of this section is to

understand the probable chemical defense strategies employed by the two species of zoanthids with respect to their activity against the microalgal foulers, bacteria and predator defenses using toxicity and feeding deterrency measurements. The microalgal foulers used are the diatoms, *Navicula subinflata* and *Nitzchia closterium,* while the antibacterial assay was carried out using fouling bacteria belonging to genus *Bacillus* and *Pseudomonas*. Toxicity was studied on the fish, *Oreochromis mosambica* (revised nomenclature of *Tilapia mosambica)* and *Rhodeus sericeus,* larvae of *Artemia salina* (instar I) and stage III nauplii of *Penaeus monodon,* while feeding deterrency was tested using three different groups of consumers, viz, (1) fish - *Oreochromis mosambica* and *Carassius auratus,* (2) the rock crab belonging to *Cancer* sp and (3) juveniles of penaeid prawns, *Penaeus monodon* and *P. merguiensis*.

3.2. ANTI-FOULING ACTIVITY

3.2.1. MATERIALS AND METHODS

All the anti-fouling bioassays were carried out at quarterly intervals and the anti-bacterial and anti-diatom aspects were evaluated. The two test animals were collected on a quarterly interval and crude extractions and fractionations were done as mentioned in earlier chapters. The four quarterly samplings were,

Quarter I - May,1996

Quarter II - August,1996

Quarter III - November,1996

Quarter IV - February,1997.

3.2.1.1. ANTI-BACTERIAL ASSAY

Five bacterial isolates, all belonging to the fouling community were used for the assay. The bacterial assays were carried out by the agar plate diffusion method (paper disc method) (Casida,1986) using Zobell marine agar (Himedia, Mumbai) as the medium. Bacterial strains used for the assay are *Bacillus cereus*, *B. circulans*, *B. pumilus*, *Pseudomonas vesicularis* and *P. putida*. Concentrations of 0.5 mg/6mm disc of the extract prepared in methanol was introduced on sterilized filter paper discs. After solvent evaporation, the discs were laid on agar plates seeded with the test species. Controls (in duplicate) contained only the solvent.

Four replicates of each concentration were used for the assay and average values are presented. Plates were incubated overnight at room temperature (≈28^{0}C). Growth inhibition zone was measured in mm (Berquist & Bedford,1978) and scored as ++++ = 4-5mm, +++ = 3-4mm, ++ = 2-3mm, + = 1-2 mm. Inhibition zones measuring less than 1mm were not scored. The above procedure was repeated for all the extracts prepared during the different quarterly collections. The bacterial assay results were compared with those obtained using standard antibiotic discs of penicillin-G (10 units/disc) and chloramphenicol (30 mcg/disc).

3.2.1.2. ANTI-DIATOM ASSAY

CRUDE EXTRACT: The diatoms, *Navicula subinflata* and *Nitzchia closterium* both belonging to the fouling community were maintained in enriched sea water for algal culture maintenance (Gentile & Johnsor,1974). Actively growing cultures with an initial suspension of around 500-1000 cells ml^{-1} were used for the bioactivity tests. Concentration series ranging from 200 to 1000 ppm were used for the assay. Aliquots of each extract stock solution (1cc=50mg) prepared in distilled water was taken corresponding to the desired experimental concentration and the volume was adjusted to 50ml in 125ml Erlenmeyer flasks. All assays were carried out in quadruplicate. Control flasks contained only the cells in medium. Flasks were incubated at room temperature (≈28^{0}C) with a 12 hr light and 12 hr dark (12L:12D) exposure. Growth inhibition was determined after 6 days (i.e. during the exponential growth phase) in terms of the number of

attached algal cells using a Sedgwick rafter (Guillard, 1978) and is expressed as % growth inhibition. Results of all the four quarters were compared. Extracts that were able to produce 50% growth inhibition were considered as active ones.

FRACTIONATION: Crude extract of both the species obtained from one of the four quarters (quarter III) were fractionated into four different fractions, viz. petroleum ether, chloroform, n-butanol and aqueous. All fractions were tested for activity as mentioned above. The non-polar fractions that were insoluble in distilled water during the stock solution preparation, were dissolved by adding acetone (0.005-0.01ml). Control flasks in these sets had cells plus acetone (equal volume added to make the stock solution) in the medium.

Only the most active fraction (petroleum ether) of *Zoanthus* sp was selected for the bioassay-guided isolation of active principle by fractionation using *N. closterium* as the test organism. Fractions active against *N. closterium* were also tested against, *N. subinflata*. While following bioassay-guided fractionation, concentration series used were adjusted based on the % growth inhibition of individual fractions.

3.2.2. RESULTS

3.2.2.1. ANTI-BACTERIAL ASSAY

The results of the antibacterial screening with extracts of *Zoanthus* sp and *Protopalythoa* sp in all quarterly collection periods are presented in Tables 15 & 16. Activity was exhibited by the extracts of first 3 quarters. Efficacy with respect to zones of inhibition varied for all the bacterial isolates in all the sets and the number of bacteria inhibited also varied. The same set of bacteria inhibited by the extract of one quarter, was not inhibited with that of another quarter, For example, the extract of May 1996 of *Zoanthus* sp caused inhibition zones for *Bacillus cereus*, *B. circulans* and *B. pumilus*. In the next collection (August, 1996), a different set of bacteria were inhibited, viz. *Bacillus circulans*, *Pseudomonas vesicularis* and *P. putida*. But, all bacterial isolates were inhibited by extracts of the first 3 quarters at one time or the other. Bacterial inhibition with the standard antibiotic discs proved more effective than the extracts showing maximum inhibition zones of 17 and 26 mm respectively, for Penicillin-G and Chloramphenicol (Table 17).

TABLE 15: Results of the antibacterial screening with extract of *Zoanthus* sp of the four quarterly collection.

	Quarter I	Quarter II	Quarter III	Quarter IV
Bacillus cereus	+	-	+	-
Bacillus circulans	+++	++++	+	-
Bacillus pumilus	+	-	-	-
Pseudomonas vesicularis	-	++	+	-
Pseudomonas putida	-	++	+	-

+ = 1-2 mm diameter, ++ = 2-3 mm diameter, +++ = 3-4 mm diameter,

++++ = 4-5 mm diameter, - = no inhibition

TABLE 16: Results of the antibacterial screening with extract of *Protopalythoa* sp of the four quarterly collection.

	Quarter I	Quarter II	Quarter III	Quarter IV
Bacillus cereus	-	+++	+	-
Bacillus circulans	+++	+++	++	-
Bacillus pumilus	+	-	-	-
Pseudomonas vesicularis	-	-	-	-
Pseudomonas putida	-	+	+	-

+ = 1-2 mm diameter, ++ = 2-3 mm diameter, +++ = 3-4 mm diameter,

++++ = 4-5 mm diameter, - = no inhibition

TABLE 17: Growth inhibition (mm) of bacteria using the standard antibiotic discs of Penicillin-G (10 units/disc) and Chloramphenicol (30 mcg/disc).

	Penicillin-G	Chloramphenicol
Bacillus cereus	nil	19
Bacillus circulans	7	26
Bacillus pumilus	nil	14
Pseudomonas vesicularis	nil	nil
Pseudomonas putida	17	18

nil= no inhibition

TABLE 18: Percentage inhibition in growth of diatoms, *Navicula subinflata* and *Nitzschia closterium* by extracts of *Zoanthus* sp of the four quarterly collection.

	Concentration (ppm)	Quarter I	Quarter II	Quarter III	Quarter IV
Navicula subinflata	200	stimulation	stimulation	49.7±0.8	28.2±18.9
	400	5.13 ±7.5	stimulation	96.3±0.4	85.1±1.1
	600	16.3±18.0	39.7±6.1	100.0±0.0	99.6±0.3
	800	58.0±6.7	49.8±3.5	100.0±0.0	100±0.0
	1000	93.8±1.8	91.2±2.7	100.0±0.0	100±0.0
Nitzschia closterium	200	stimulation	68.6±14.1	5.5±8.3	78.1±3.9
	400	67.6±32.9	99.7±0.0	100.0±0.0	100.0±0.0
	600	100±0.0	100.0±0.0	100.0±0.0	100.0±0.0
	800	100±0.0	100.0±0.0	100.0±0.0	100.0±0.0
	1000	100±0.0	100.0±0.0	100.0±0.0	100.0±0.0

3.2.2.2. ANTI-DIATOM ASSAY

The extracts of both zoanthids on the growth of diatoms, *N. subinflata* and *N. closterium* showed two different types of effects, i.e. growth inducement and growth inhibiting effects. Compared to the controls, inducement of growth was observed only at low concentrations, but inhibiting effects were seen at higher concentrations. However, the extent of inducement and inhibition occurred varied with all the four quarterly collections. Inhibitory effects were, however noted in all the quarters.

Growth stimulation of *N. subinflata* with *Zoanthus* sp extract was present only in the I and II quarter extracts. In the I quarter, stimulation was seen only at 200 ppm but in the II quarter, stimulation was effective at 400 ppm concentration also. Other higher concentrations had only inhibitory effects. The III and IV quarter extracts, however, showed only the inhibitory effects at all test concentrations (Table 18). With regard to *N. closterium*, *Zoanthus* sp extract caused growth stimulation only in the I quarter extract at 200 ppm. All other higher concentrations and collections had inhibitory effects (Table 18).

Stimulatory metabolites of *Protopalythoa* sp were more active and occurred more frequently than that of *Zoanthus* sp. For *N. subinflata*, stimulatory effect was seen in the I, II and IV quarter collections upto concentrations 600, 800 and 400 ppm respectively. Growth stimulation in *N. closterium* was observed in the II and IV quarter extracts at 400 and 200 ppm, respectively. At all the test concentrations, extracts of I and III quarters, showed only inhibitory effect on *N. closterium* (Table 19).

Among the 4 fractions of the crude extracts tested for the anti-diatom assay, the petroleum ether fraction of *Zoanthus* sp was found to be the most active against both the diatom species (Table 20). While the chloroform fraction exhibited stimulatory effect at lower concentrations in both diatoms, the aqueous fraction was stimulatory only to *N. subinflata* at 40 ppm. But, all the 4 fractions had inhibitory metabolites and were capable of inhibiting more than 50% of diatom growth. Fractions of *Protopalythoa* sp were tested only against *Navicula subinflata*. Here, only the aqueous fraction exhibited stimulatory effects at all test concentrations, while the other fractions exhibited inhibitory effects (Table 21).

Bioassay-guided purification of the crude petroleum ether fraction of *Zoanthus* sp for activity location is shown in Figure 11. Fractions 1-4-3-1-1, 1-4-3-1-2 and 1-4-3-2-5 were found to be the active ones. Percentage growth inhibition of *N.*

TABLE 19: Percentage inhibition in growth of diatoms, *Navicula subinflata* and *Nitzschia closterium* by extracts of *Protopalythoa* sp of the four quarterly collection.

	Concentration (ppm)	Quarter I	Quarter II	Quarter III	Quarter IV
Navicula subinflata	200	stimulation	stimulation	9.9±5.1	stimulation
	400	stimulation	stimulation	23.1±0.8	stimulation
	600	stimulation	stimulation	34.4±9.7	23.6±7.0
	800	52.6±12.8	stimulation	38.4±6.9	88.2±3.5
	1000	87.3±5.1	58.0±18.5	62.6±8.8	98.8±0.8
Nitzschia closterium	200	0.2±14.8	stimulation	6.0±5.2	stimulation
	400	5 2.4±6.2	stimulation	8.5±0.7	21.5±4.7
	600	100.0±0.0	. 74.5±8.4	10.9±1.0	75.5±9.4
	800	100.0±0.0	81.6±3.3	17.7±7.0	88.3±1.2
	1000	100.0±0.0	100.0±0.0	71.3±1.8	100.0±0.0

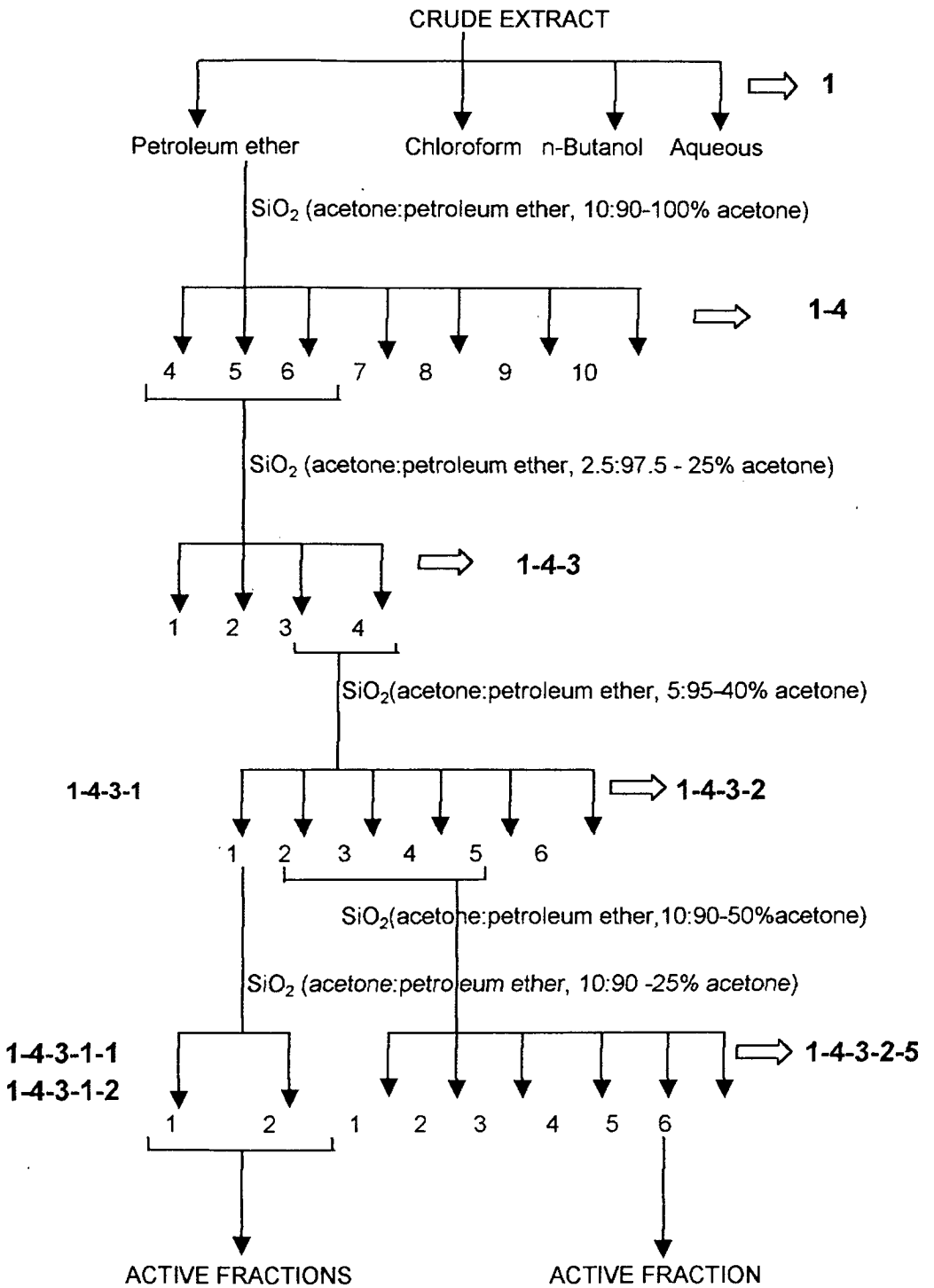
TABLE 20: Percentage inhibition in growth of diatoms, *Navicula subinflata* and *Nitzschia closterium* in presence of different fractions of *Zoanthus* sp extract.

	Concentration (ppm)	Petroleum ether	Chloroform	n-butanol	Aqueous
Navicula subinflata	40	22.8±3.0	stimulation	2.8±1.6	stimulatio n
	80	30.5±3.0	stimulation	6.7±1.6	11.33±4.2
	120	99.7±0.1	stimulation	15.0±1.2	42.2±9.3
	160	100.0±0.0	14.8±35.4	64.6±0.9	44.7±21.4
	200	100.0±0.0	89.4±11.7	74.5±1.2	69.0±7.1
Nitzschia closterium	40	81.0±5.2	stimulation	5.2±6.8	23.3±11.3
	80	100.0±0.0	44.1±8.9	14.8±1.6	30.7±3.0
	120	100.0±0.0	50.4±5.9	59.9±7.2	48.7±13.7
	160	100.0±0.0	77.7±14.8	100.0±0.0	67.2±6.3
	200	100.0±0.0	100.0±0.0	100.0±0.0	77.8±8.7

TABLE 21: Percentage inhibition in growth of the diatom, *Navicula subinflata* in presence of different fractions of *Protopalythoa* sp extract.

Concentration (ppm)	Petroleum ether	Chloroform	n-butanol	Aqueous
200	39.7±17.9	10.8±5.6	28.8±6.5	stimulation
400	43.1±16.1	57.2±2.8	53.0±13.7	stimulation
600	59.4±12.2	70.9±8.5	98.2±0.9	stimulation
800	95.8±1.6	78.0±4.8	99.1±0.3	stimulation
1000	100.0±0.0	88.8±3.3	99.7±0.0	stimulation

FIG. 11



Bioassay-guided fractionation for location of active fraction in the anti-diatom assay.

closterium in the presence of the above fractions is shown in Table 22. Among the three active fractions, fraction 1-4-3-2-5 was the most active one with 100% growth inhibition at 40 ppm followed by fractions 1-4-3-1-1 and 1-4-3-1-2 respectively inhibiting the diatom growth upto 99.5 and 74% at 50 ppm concentration. The above 3 active fractions were also tested for their inhibitory effect against the other test diatom, *N. subinflata* (Table 22). Here, only one active fraction i.e. 1-4-3-1-1, inhibited more than 50% diatom growth at concentration of 100 ppm. The other two fractions though had inhibitory effects, were active only upto 48.0% and 43.2% respectively at 100 ppm concentration .

IR spectrum of fraction 1-4-3-1-1 (Fig. 12) showed absorption at 3400, 1716 & 1022 cm^{-1}, indicating the presence of hydroxyl (-OH) and carbonyl (>C=O) groups. The aliphatic nature of the compound was evident from the absorption at 2928, 2853, 1456 and 1377 cm^{-1}. The relatively stronger peak at 1377 cm^{-1} compared to the absorption at 1456 cm^{-1} suggested that the molecule is not a straight chain compound but branched as it should contain more methyl groups. The IR absorption at 887 cm^{-1} is due to the presence of unsaturation in the form of an exomethylene group in the molecule. It gave a positive Liebermann Burchard test indicating it to be a 3β-hydroxy-keto steroid. The IR spectrum of 1-4-3-1-2 showed (Fig. 13) absorption at 3400 and 1047 cm^{-1}, indicating the presence of hydroxyl group. It also exhibited a number of peaks of lower intensity in the region 1600-1800 cm^{-1} indicating it to be a mixture of carbonyl compounds with some traces of the above keto steroid. Thin layer

some traces of the above keto steroid. Thin layer chromatography of the active fraction, 1-4-3-2-5 indicated that it was a mixture of several compounds.

TABLE 22: Percentage inhibition in growth of diatoms, *Navicula subinflata* and *Nitzschia closterium* by the active fractions of the petroleum ether fraction of *Zoanthus* sp.

	Active fraction	Concentration (ppm)	% inhibition
Nitzschia closterium	1-4-3-1-1	25	23.6±14.3
		40	84.3±6.7
		50	99.5±0.2
	1-4-3-1-2	25	4.1±4.5
		40	7.0±0.7
		50	74.0±7.5
	1-4-3-2-5	25	99.5±0.1
		40	100.4±0.0
		50	100.0±0.0
Navicula subinflata	1-4-3-1-1	50	45.6±0.3
		100	62.9±6.5
	1-4-3-1-2	50	13.8±7.2
		100	48.0±13.5
	1-4-3-2-5	50	21.6±2.0
		100	43.2±5.4

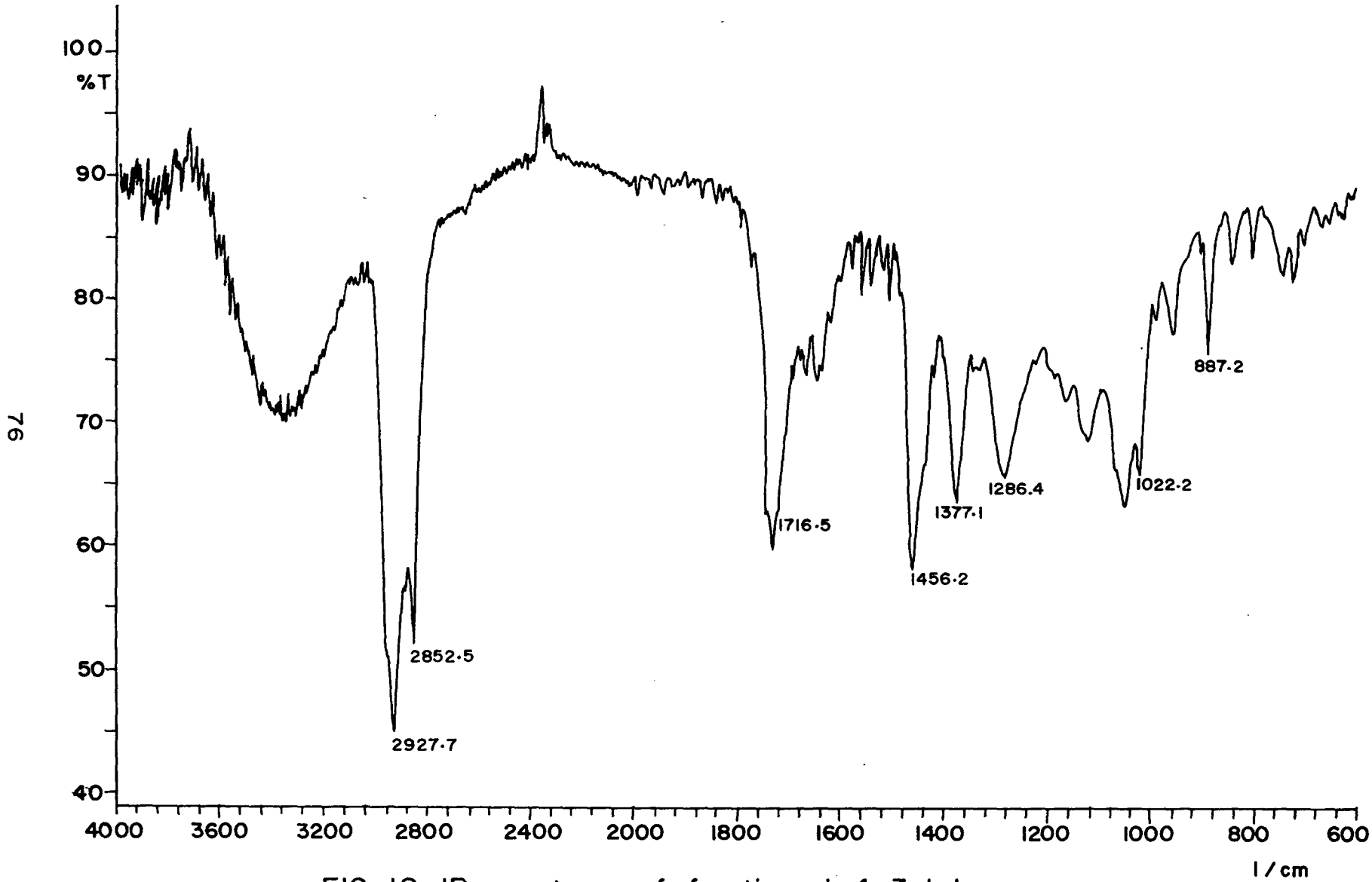


FIG. 12 IR spectrum of fraction 1-4-3-1-1

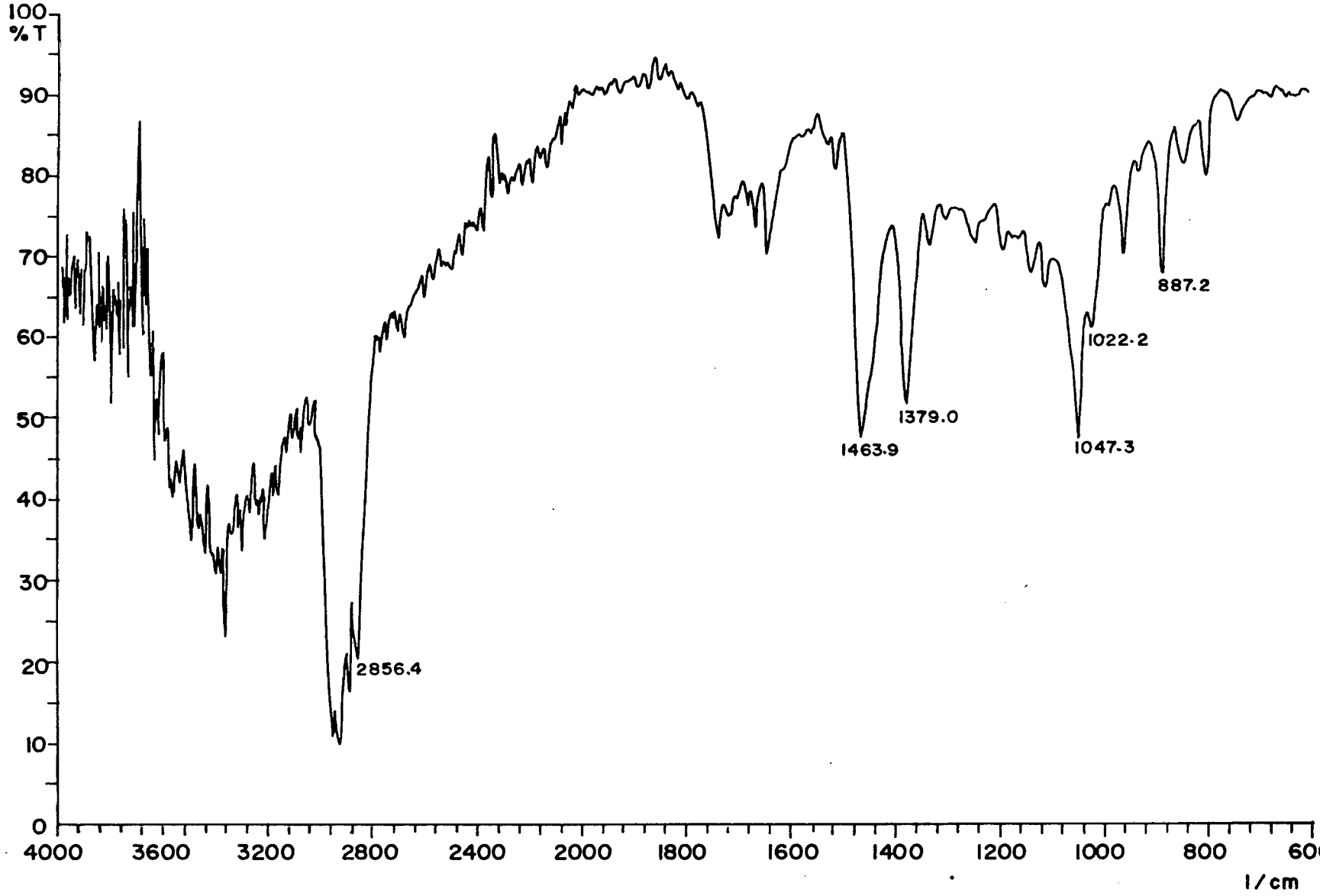


FIG. 13 IR spectrum of fraction 1-4-3-1-2

3.2.3. DISCUSSION

The present study confirms that the zoanthids, *Zoanthus* sp and *Protopalythoa* sp possess secondary metabolites that are capable of inhibiting the growth of diatoms and bacteria. In the study reported herein, variation in the anti-fouling property (anti-bacterial and anti-diatom) was examined at quarterly intervals for a one-year period. It was thus possible to determine whether the extracts had the same effect throughout the year or varied during certain periods of the year. Chemical defense against the diatoms, *N. subinflata* and *N. closterium* was effective year round whereas, with bacteria, these forms did not seem to rely on secondary metabolites all through the year. From the anti-algal assay, it was obvious that the extracts exhibited two different types of effects, a growth stimulatory effect at lower concentrations and growth inhibitory effect at higher concentrations. While in the case of anti-bacterial assay, extracts of only 3 quarters displayed the activity. Further, the efficacy with respect to the zones of inhibition varied for all the bacterial isolates in all three extracts and the number of bacteria inhibited also varied.

Stimulatory and inhibitory effect of extracts on algal growth have also been reported earlier by several other workers (Davis et al,1991; Sr. Vitalina et al,1991; Szewzyk et al,1991; Standing et al,1984; Targett,1988). According to Standing et al, (1984), inducer and inhibitory effects in larval settlement was due

to presence of both high and low molecular weight components respectively in gorgonian crude extracts. Based on the observations that non-polar fractions of *Crella incrustans* were attractive to ascidian larvae while the polar end had strong repellant activity, Davis et al, (1991), stated that attractive substances are the results of basic biochemical processes and pressures from fouling lead to evolution of secondary metabolites which overrules the attractive effect of molecules.

In the present study, growth inhibitory effects were observed with crude extracts of all four quarters at higher concentrations, but, stimulatory metabolites were encountered only during certain periods of the year and that too at lower concentrations. So also, the concentration at which minimum inhibition was observed, varied with the extract for both species. This observed temporal variability in bioactivity has been ascribed to environmental factors, the presence and abundance of microsymbionts, ecological characteristics, variation in biology or intraindividual variability (Thompson et al,1987; Berquist & Wells,1983; Thompson et al,1985; Coll et al,1987; Paul & Van Alstyne,1988; 1992).

Recurrence of antibacterial activity studied over a one year period revealed that crude extracts of *Zoanthus* sp and *Protopalythoa* sp were specific with respect to the bacteria it inhibited and activity though persisted with extracts of the 3 quarters, the species inhibited varied with the extracts of the different quarters. This could either be due to the presence of several active substances having

species specific activity and their occurrence varying with different seasons, or may be due to a single or a few compounds responsible for the activity that vary in concentration during different periods of the year and inhibition of bacterial growth requiring a certain concentration of the metabolite. Non-polar components in the crude extract may also be responsible for the observed antibacterial activity. However, since the non-polar components diffuse less through agar, it is more likely that the varying concentrations of the active component(s) in different collections may have played a role in its effectiveness in displaying the activity.

In many instances, organisms have been found to employ a combination of chemical and physical defenses (Tursch et al,1978; Sullivan et al,1983; Targett,1988; Davis et al,1989). In addition to chemical anti-fouling defenses, non-chemical defenses like mucus sloughing have been reported in many invertebrates (Patton,1972; Barthel & Wolfrath,1989; Rublee et al,1980; Davis et al,1989; Thorp et al,1991). The sloughing of mucus may play an important role in maintaining the body surface free of epibionts. It is believed that population levels of bacteria in coral mucus including *Palythoa* sp (Zoanthidea) is regulated by the self cleaning behaviour of the host (Ducklow & Mitchell,1979a, 1979b; Lewis,1973), which periodically accumulate and release web like mucus from their surface. It is likely that mucus production prevalent in *Zoanthus* sp and *Protopalythoa* sp may also be playing a role in regulating surface bacterial population. This could probably explain for the low level of chemical deterrency

exhibited by these animals towards fouling bacteria. In majority of cases, microbial film is a pre-requisite for successional stages of development of fouling community (Keough & Raimondi,1995; Kirchman et al,1982; Maki et al,1988; 1989). Marine bacteria being the primary organisms to colonize on any surface (microfilm), are important in larval settlement. Consequently, the ability to inhibit bacterial growth will form an important means of limiting later successional fouling stages. Bacteria can also produce surface-bound and soluble chemical cues that either stimulate or inhibit larval settlement (Maki et al,1990; 1992; Kirchman et al,1982).

A negative correlation between anti-fouling activity and the intensity of fouling in organisms has been observed in a majority of cases (Clare,1996; Amade et al,1987; Al-Ogily & Knight-Jones,1977; Kazumi et al,1994). Several compounds from marine organisms have been isolated possessing anti-fouling activity (Shimidzu et al,1993; Kawamata et al,1994; Mizobuchi et al,1996; Targett et al,1983). However, absence of foulers on the body surfaces of organisms may not in itself indicate the presence of secondary chemical anti-fouling metabolites (Davis,1998; Henrickson & Pawlik,1998) because marine organisms employ a suite of other defenses in addition to chemical compounds. Nonetheless, extracts from species that were observed to be fouled heavily were found lacking in secondary metabolites (Davis,1998; Mc Caffrey & Endean,1985).

The two species of zoanthids of the present study though not totally unfouled, were found in the field with epibionts in a comparatively lesser degree. During monthly field observations, for a period of one year (May 1996 to April 1997), it was observed that the percentage cover of the zoanthids was the same throughout the year, and constituted the most abundant fauna of the study area. *Zoanthus* sp, however, was more dominant than *Protopalythoa* sp. Gastropods belonging to Genus *Trochus*, *Conus*, *Bursa* and *Thais* were found growing in between them although not in big numbers. It was observed that the numbers of gastropods growing were comparatively very less among the zoanthids as compared to the rocks having no zoanthid cover. One species of sea anemone was found growing among *Zoanthus* sp (<10 nos in 60 sq m area). Sponges also were present and occasionally seen in small patches overgrowing *Zoanthus* sp. Polychaete worms and amphipods were the other associated fauna. During the period October to January, algae growing among the zoanthids were very little, but were more during February to April and found overgrowing in many patches in April. During May to August, no algal growth was seen among the zoanthids.

Clare (1996) opined that non-polar metabolites are more useful inhibitory compounds compared to the polar metabolites and are active at very low concentrations. Jennings & Steinberg (1997) also support these claims. Contrary to this, Walters et al, (1996), have reported that some tropical algae rely on water soluble compounds to deter foulers. It has been reported by Targett (1988) that the non-polar fraction and the fraction of intermediate polarity of

Pseudopterogorgia americana, were the active ones while the most polar fraction of *P. acerosa* was the active fraction. Steinberg et al (1998) explain that polar compounds due to strong boundary layer effects, readily dissolve and dilute away. Therefore, they either have to be effective at very low concentrations or produce in large quantities inorder to display any effect. Such limitations on production restrict the use of polar metabolites as inhibitors. On the other hand, non-polar metabolites, on account of adsorption or slower dissolution rates, are likely to prevail on or in the vicinity of the host (Jennings & Steinberg 1997; Walker et al,1985; Schmitt et al,1995). Nevertheless, majority of the anti-fouling compounds isolated are non-polar metabolites and are more common as deterrents of epibiota (Steinberg et al, 1998).

Fractionation studies for the anti-algal test in the present assay revealed that the activity lay amongst all the fractions of *Zoanthus* sp, i.e. from non-polar to the polar fractions. Of all the tested fractions, the petroleum ether fraction (non-polar) was the most active one while the aqueous fraction was least active though all fractions were capable of inhibiting more than 50% of the algal growth compared to the controls. Aqueous fraction of *Protopalythoa* sp displayed only stimulatory effects while other fractions had inhibitory action.

As mentioned, the anti-bacterial and anti-algal tests were carried out at quarterly intervals. Such studies are important because they provide a better picture of the secondary metabolite production, i.e. the defensive properties of the animals over

a range of time and frames out the time of highest and lowest activity. Knowing that secondary metabolite production is dependent on various factors and varies accordingly, long term studies are useful because a certain activity that is present may not be encountered at one time collection. Variations in toxicity are not unusual even among specimens of the same species, because these toxic compounds are secondary metabolites and are affected by changes in the metabolism of the species (Becerro et al, 1994). Bioassay-guided fractions proved activity to be restricted to more than one fraction of the crude extract as well as that of the different petroleum ether fractions. It can be concluded that atleast with regards to the anti-algal assay, many compounds are responsible for the activity which may act individually against target species or synergistically, to enhance effect of each other. A comparison of the inhibitory action of the active fractions to both diatom species reveals that compounds though inhibitory to one diatom species need not elicit the same response to another species. The amount of the inhibitory constituent required for producing the effect also varies with species. This is also applicable in case of stimulatory metabolites. The same chloroform fraction of *Zoanthus* sp, differed in extent upto which it could stimulate growth in the two species of diatoms.

Targett et al (1983) found that homarine from gorgonians, a simple 2-carboxy N-methyl pyridine, acts as an anti-foulant against the diatom *Navicula salinicola*. Bandurraga & Fenical (1985) found that some muricins from *Muricea fructosa* (gorgonacea) possess anti-fouling characteristics against diatoms. Brominated

compounds, terpenoids, (Walls et al,1993; Anderson et al,1974; Hadfield & Ciereszko,1978; Scheuer,1973) are the compounds most often quoted to be responsible for chemical defenses in marine organisms. The active compounds isolated as part of this study appear to be sterols including some ketosterols. Laboratory assays conducted presently revealed that the animals *Zoanthus* sp and *Protopalythoa* sp possess secondary metabolites that have anti-algal and anti-bacterial properties and that these metabolites may play an ecological role as anti-foulants in the natural environment. As rightly pointed out by Vrolijk et al (1990), biofouling is a complex phenomenon with several anti-fouling strategies operating synergistically in order to enable the organisms to successfully deter fouling.

3.3. FEEDING DETERRENCE ACTIVITY

3.3.1. MATERIALS AND METHODS

TEST ANIMALS

1. Fish–*Oreochromis mosambica* and goldfish, *Carassius auratus*
2. Rock crab - *Cancer* sp
3. Juvenile prawns - *Penaeus monodon* and *Penaeus merguiensis*

3.3.1.1. PRAWN/GOLD FISH

Preparation of feed

A stock solution of the extracts (1cc=100mg) was prepared in methanol. Commercially available prawn feed (Higashi grower) pellets (≈0.5 cm length) were impregnated with the extracts. Concentrations used were 1, 2, 3 and 4 mg per pellet for prawns and 2, 3, and 5 mg per pellet for goldfish. Control pellets were prepared using only methanol. Control and experimental pellets after incorporation of extracts or solvent as the case may be were kept open in the laboratory for natural drying before using for the bioassay.

Experimental procedure

PRAWNS

Juveniles of *Penaeus monodon* and *P. merguiensis* reared in the Prawn Hatchery of the National Institute of Oceanography (Goa) were used for the test. Ten

juvenile prawns (≈0.1 g) of each species were kept in separate glass aquarium (15″x8.5″x 9″) and two tanks were maintained for each species. Before start of the experiment, prawns were fed with the pellets (without extract or methanol) for about 5-6 days so that they were attracted towards the food once it was dropped. Feeding tests were performed for 1-minute period. Control pellets were offered first and if animals consumed the pellets, the experimental pellets were given. Only one pellet was dropped each time. Feeding response of the organisms towards the pellet was observed and then the next pellet was dropped. If the pellet was not eaten during the first 1-minute, it was discarded. Experimental and control pellets were given at random during the feeding session. Initially, the feeding test was conducted with crude extracts. Later they were fractionated and each fraction was tested separately in different feeding sessions. The four fractions tested were, petroleum ether, chloroform, n-butanol and the aqueous fractions numbered 1, 2, 3,and 4 respectively.

GOLDFISH- *Carassius auratus*

Goldfish for the experiment were procured from a local aquarium fish dealer. The experimental procedure followed was the same as described for the penaied prawns, except that 3 fishes were kept in each glass aquarium. Test was carried out in duplicate.

3.3.1.2 CRAB/OREOCHROMIS

Preparation of feed

Agar powder (3%, Himedia, Mumbai) was mixed with freshly ground sergestid shrimp, *Acetes* sp (≈5g/10ml agar). Concentrations used were 4,5 and 10 mg of extract per ml of agar. A stock solution of the extract was prepared in distilled water (1cc=100 mg) and added to the agar-*Acetes* mixture, corresponding to the desired experimental concentrations. The extract was added to the molten agar only in luke warm condition so as to avoid denaturing of any compound. After addition of the extract, the agar-*Acetes* mixture was mixed thoroughly for uniform distribution of the extract. The mixture was left for cooling in a petri plate and after solidifying, was cut into small pellets (1.5x0.5 cm). Control pellets were prepared without the extract.

Experimental Procedure

CRAB

Small sized rock crabs (*Cancer* sp) were collected from the rocky shores of the Dias beach (Dona Paula, Goa) and brought alive to the laboratory. Nine crabs were kept in a fibreglass tank (size, 31″x18″x11″) containing 10 litres of seawater (salinity 32 ppt). To simulate the natural environment, pebbles were placed at the bottom of the tank. The tank was covered with a nylon netting to prevent escape of crabs. Before start of the experiment, the animals were fed with the control feed for 5 days so that they were attracted towards the food when

offered. During the experimental procedure, control and experimental pellets were given at a time, but by keeping the two feed at opposite ends of the tank. Ten pellets each (control and experiment) were given at one time. Experimental feed containing only one concentration of the extract was given during one feeding session. The experiment was run for 10 minutes and the number of control and experimental pellets consumed were compared. The palatability of different concentrations of the extracts was observed during the different feeding sessions.

Oreochromis mosambica

Juvenile *Oreochromis* (≈ 6cm in length) were brought alive from Dauji fish farm of the Directorate of Fisheries, Goa. Ten fishes were used for the test and five were kept in each glass aquarium (size, 5″x8.5″x9″). Before start of the experiment, the animals were fed the control feed for 5 days so that they were attracted towards the food when offered. Experiment was run for 1-minute period. One pellet was given at one time and if not eaten during the 1-minute period, it was discarded. The feeding response of the fish towards the pellet was observed and then another pellet was offered. Experimental and control pellets were given at random.

3.3.2. RESULTS

The response to the experimental food was classified as follows according to that followed by La Barre et al (1986).

1. Acceptance: the animal detected the food particle, seized it and ingested it - an indication of lack of feeding deterrence.

2. Rejection: the animal detected the food particle, seized it and expelled it immediately - an indication of feeding deterrence via taste.

3. Avoidance: the animal detected the food and moved towards it, but turned away upon approaching and failed to ingest it - an indication of the presence of an olfactory deterrent.

3.3.2.1. GOLDFISH / CRAB / OREOCHROMIS

Extracts of both the zoanthid species tested were unable to deter feeding in the above test animals. Control and experimental extract treatments were consumed in the same length of time, indicating lack of feeding deterrency. The response therefore is classified as acceptance.

3.3.2.2. PRAWNS

Feeding was strongly deterred in the two species of prawns tested. The animals were seen seizing the pellet and immediately releasing it, an indication of rejection response. In case of *P. monodon*, *Zoanthus* sp deterred feeding in the chloroform (60%) and n-butanol (90%) fractions at an extract concentration of 1 mg/pellet (Table 23) while in petroleum ether and aqueous fractions, no rejection was observed at all concentrations. With *Protopalythoa* sp, however, deterrence was observed in all 4 fractions at concentrations ranging from 1-3 mg/pellet depending on the fraction tested and the rejection rate was 100%, except for n-butanol (80%) (Table 24). *P. merguiensis* deterred its feeding in all except the aqueous fraction of *Zoanthus* sp and the petroleum ether fraction of *Protopalythoa* sp. Rejection was observed at an extract concentration of 4 mg/pellet for both species in all other fractions. Deterrence was 100% in n-butanol fraction of *Zoanthus* sp and chloroform and aqueous fractions of *Protopalythoa* sp (Tables 23 and 24).

TABLE 23: Palatability of *Zoanthus* sp extracts to juvenile penaied prawns, *Penaeus monodon* and *P. merguiensis.*

	P. monodon		*P. merguiensis*	
FRACTION	EXPERIMENTAL CONCENTRATION	% REJECTION	EXPERIMENTAL CONCENTRATION	% REJECTED
Petroleum ether	all consumed	0	4.0 mg/pellet	75
Chloroform	1.0 mg/pellet	60	4.0 mg/pellet	60
n-butanol	1.0 mg/pellet	90	4.0 mg/pellet	100
Aqueous	all consumed	0	4.0 mg/pellet	0

TABLE 24: Palatability of *Protopalythoa* sp extracts to juvenile penaied prawns, *Penaeus monodon* and *P. merguiensis.*

	P. monodon		*P. merguiensis*	
FRACTION	EXPERIMENTAL CONCENTRATION	% REJECTION	EXPERIMENTAL CONCENTRATION	% REJECTION
Petroleum ether	2.0 mg/pellet	100	4.0 mg/pellet	0
Chloroform	3.0 mg/pellet	100	4.0 mg/pellet	100
n-butanol	1.0 mg/pellet	80	4.0 mg/pellet	83
Aqueous	3.0 mg/pellet	100	4.0 mg/pellet	100

3.3.3. DISCUSSION

Except for the penaied prawn juveniles that were tested for feeding deterrency, chemical response that deter feeding and thereby protect the animal against predation was found to be absent in both species of the zoanthids. It was found that for the fish and crab assay, pellets containing the zoanthid extracts were consumed within the same length of time as they did for the control pellets. No significant differences between the control and experimental extract treatments were observed. In case of prawns however, the observations were different. Chloroform and n-butanol fractions of *Zoanthus* sp deterred feeding of *P. monodon* at a concentration of 1 mg/pellet. In the case of *Protopalythoa* sp, at an extract concentration between 1-3 mg/pellet, all the four fractions, viz. petroleum ether, chloroform, n-butanol and aqueous fractions deterred feeding of *P. monodon*. However, in the case of *P. merguiensis*, only the petroleum ether and aqueous fractions were inactive even at a concentration of 4 mg/pellet, for *Protopalythoa* sp and *Zoanthus* sp respectively. This study provides evidence that among the fish and crabs that were used as consumers, the zoanthids are very poorly defended against their predators because both the experimental and control pellets were ingested in the same degree. While in the case of penaied prawns where the feeding was strongly deterred, more than one fraction of *Zoanthus* sp and *Protopalythoa* sp were found to be active

It has been observed by Gleibs & Mebs (1998) during field studies on Caribbean zoanthid species that several fish species feed on *Palythoa* colonies (*P. caribaeorum*, *P. mammillosa*), particularly more intensely when the colonies were dissected or removed from the substrate, providing access to the mesenteries and ovaries. The two species of zoanthids *P. caribaeorum* and *P. mammilosa* are well known for the production of palytoxin. The effects of palytoxin as seen on terrestrial vertebrates are also well known. It was also observed that *Palythoa mammilosa* elicited avoidance from several species of sharks under simulation of natural conditions. Palytoxin inhibited olfactory electroencephalogram patterns and elicited aversive behaviour and head shaking by sharks (Hodgson,1981). The shark *Fundulus*, when offered paired choices between food pellets and toxin pellets, initially recorded high numbers of contacts with potential food that quickly was followed by a falloff in contacts with the toxin pellets (Hodgson,1981).

The present study adds to yet another observation on the feeding deterrent properties of two more species of zoanthids, *Zoanthus* sp and *Protopalythoa* sp occurring along the Indian coast. Extracts of the two species of zoanthids that were strong deterrents for the prawn juveniles suggests that zoanthids are feeding deterrents for only some groups of animals. It has been reported that associations occur between shrimps, prawns and a wide range of other marine invertebrates. Relationships involved represent mutualism, removing sand, sediment as well as organic and inorganic debris, often referred to as fish cleaners or protection from

predators (Herrnkind et al, 1976). It has also been observed that among the animal phyla, the Coelenterata attract a wider variety of shrimp associates than any other phylum (Bruce,1976). Although several genera and numerous species of prawns and shrimps are associated, mostly with anthozoan coelenterates, no shrimps have yet been described in association with the Zoanthidea (Bruce,1976). It could probably be assumed that the feeding deterrent properties prevailing in this group may account for the lack of association between the Zoanthidea and prawns. The present observation have shown that these two species of zoanthids are strong feeding deterrents to the two prawn species tested, and the results may form clue to the lack of any association between shrimps and the Zoanthidea.

3.4. TOXICITY

3.4.1. MATERIALS AND METHODS

The organisms used for determining the toxicity of *Zoanthus* sp and *Protopalythoa* sp are,

1 - Fish (*Oreochromis mosambica* and *Rhodeus sericeus*)

2 - larvae of the brine shrimp, *Artemia salina* (instar I) and prawn, *Penaeus monodon* (nauplius III).

3.4.1.1. FISH TOXICITY

Twenty grams of the fresh,whole body tissues of *Zoanthus* sp and *Protopalythoa* sp were weighed and ground separately in a mixer with 20 ml dechlorinated tap water. The ground tissue was later centrifuged at 10,000 rpm for 10 minutes. Supernatant obtained was decanted and further diluted to 400 ml with dechlorinated tap water. The above diluted aqueous solution of both species was divided into 2 equal parts in 500 ml glass beakers to which 3 fish (3-4 cm in length) of the respective species were introduced. A control set was also run simultaneously that contained only the fish in dechlorinated tap water. The experiments and controls were maintained in duplicate for both species of zoanthids and all beakers were continuously aerated. Mortality in the test fish was checked after 20, 45 and 90 minutes and 3, 6, 12 and 24 hours. Toxicity

was measured as, 1-2 fish dead - mildly toxic, 3-4 fish dead - moderately toxic, 5-6 fish dead - highly toxic (McClintock,1989).

3.4.1.2. LARVAL TOXICITY

***Peneaus monodon*, nauplius III**

Crude extracts of *Zoanthus* sp and *Protopalythoa* sp were weighed and dissolved in filtered sea water to prepare a stock solution (1cc= 50mg) of both species. Desired quantity from the stock solution was pipetted out to prepare a concentration series. Concentrations used ranged from 40-200 ppm. Ten healthy larvae (reared at NIO prawn hatchery) were picked with a Pasteur pipette and released in 15 ml of each of the test solutions prepared in filtered sea water (salinity 30 ppt). Care was taken to minimize further dilution of the test solutions while transferring the larvae. The control set contained only sea water and larvae. Experiments were carried out in duplicate and average values were taken. Larvae were incubated for 24 hours and the numbers of larvae dead were counted at the end of 24 hours. LC_{50} was calculated (Fisher & Yates,1963) to determine the toxicity.

***Artemia salina* (instar I)**

Mortality (LC_{50}) of the I instar stage of the brine shrimp, *Artemia salina* was used as the criterion for the toxicity assay. Salinity of sea water was made to 40 ppt by addition of common salt (NaCl). A pinch of *Artemia* cysts was left overnight into aerated seawater for hatching in a test tube. First instar stage that emerged the following day were used for the bioassay.

Toxicity was checked with both the zoanthid species. *Zoanthus* sp extract was fractionated and column chromatographed for isolation of active component. Stock solutions of the extracts and fractions were prepared by weighing the required amount and dissolving in sea water (1cc=50mg). In case of those fractions that did not dissolve in sea water, were made soluble by adding ≅ 0.05-0.1 ml of acetone to which the sea water was later added to make up to the required concentration. Concentrations used for the bioassay ranged from 500 – 5000 ppm for crude extracts and crude fractions and gradually lowering the concentration from 50–250 ppm during the process of fractionation.

After preparation of the desired test solution, it was added into cellwell plates (volume 3.5 ml/well). Five nauplii were transferred into each well using a Pasteur pipette. For each concentration, 4 replicates were maintained and the average was taken. Care was taken while transferring the nauplii so as to avoid addition of water into the test solution. Control sets had only seawater and nauplii. In case of those sets wherein acetone was added to dissolve the compounds, control sets were also maintained with equal quantity of acetone that was used to dissolve the extract. After the 24 hour treatment, the numbers of dead nauplii in each well were counted. Nauplii were considered dead when no swimming was observed. The LC_{50} values were calculated using the probit regression method (Fisher & Yates,1963).

3.4.2. RESULTS

Both species of zoanthids were non-toxic to *Oreochromis mosambica*. With respect to *Rhodeus sericeus*, *Zoanthus* sp was moderately toxic, killing 4/6 of the test fish while *Protopalythoa* sp was mildly toxic killing 2/6 of the test fish at the end of 24 hr. Lethal concentration at 50% mortality with *Zoanthus* sp and *Protopalythoa* sp extracts to the prawn larvae, was found to be 65.3 and 148.3 ppm respectively. Against brine shrimp larvae, extracts of *Zoanthus* sp only was found to be active (LC_{50}=3162.2 ppm). Subsequently, when the crude extract of *Zoanthus* sp was fractionated into petroleum ether, ethyl acetate and aqueous fractions, all 3 fractions were found to be active against the brine shrimp instar. The highest activity was located in the ethyl acetate fraction (100% mortality at 500 ppm), followed by aqueous fraction (LC_{50}=2583.33 ppm) and petroleum ether fraction (LC_{50}= 3321.73 ppm), respectively. The ethyl acetate and aqueous fractions were further purified by chromatography with the fractions thus obtained also being screened for the activity.

Details of this purification are given in Figures 14 and 15. While following bioassay–guided chromatography for activity location, only the most active fractions were chosen for further purification. LC_{50} for active fractions 2-4-1 and 2-4-2 were found to be 349.2 and 267.8 ppm, respectively. These two fractions were a mixture of several compounds as seen from the thin layer chromatography (TLC). Similarly, the LC_{50} values for active fractions 3-6-3-3-5-1 and 3-6-3-3-5-2

FIG. 14

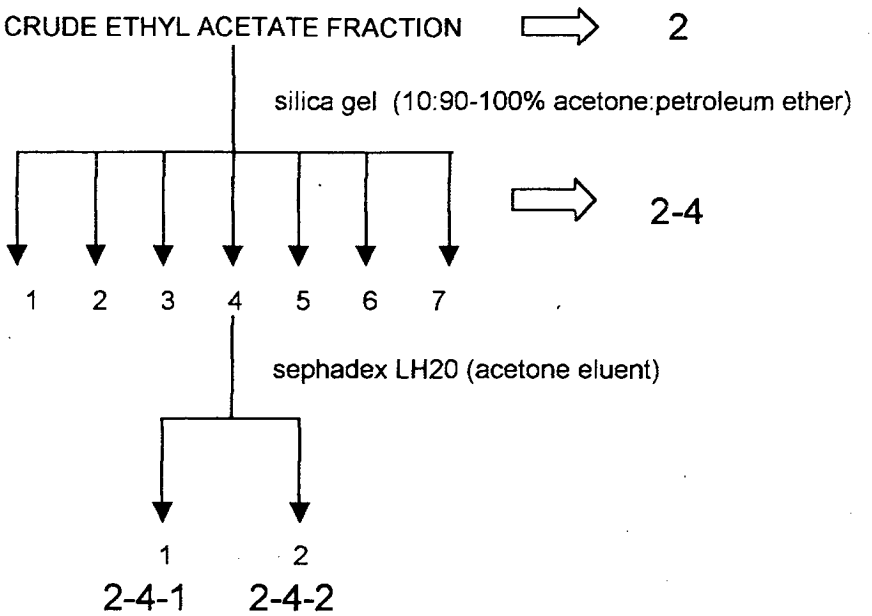


Figure showing the bioassay - guided fractionation of the ethyl acetate fraction in the brine shrimp toxicity assay.

FIG. I5

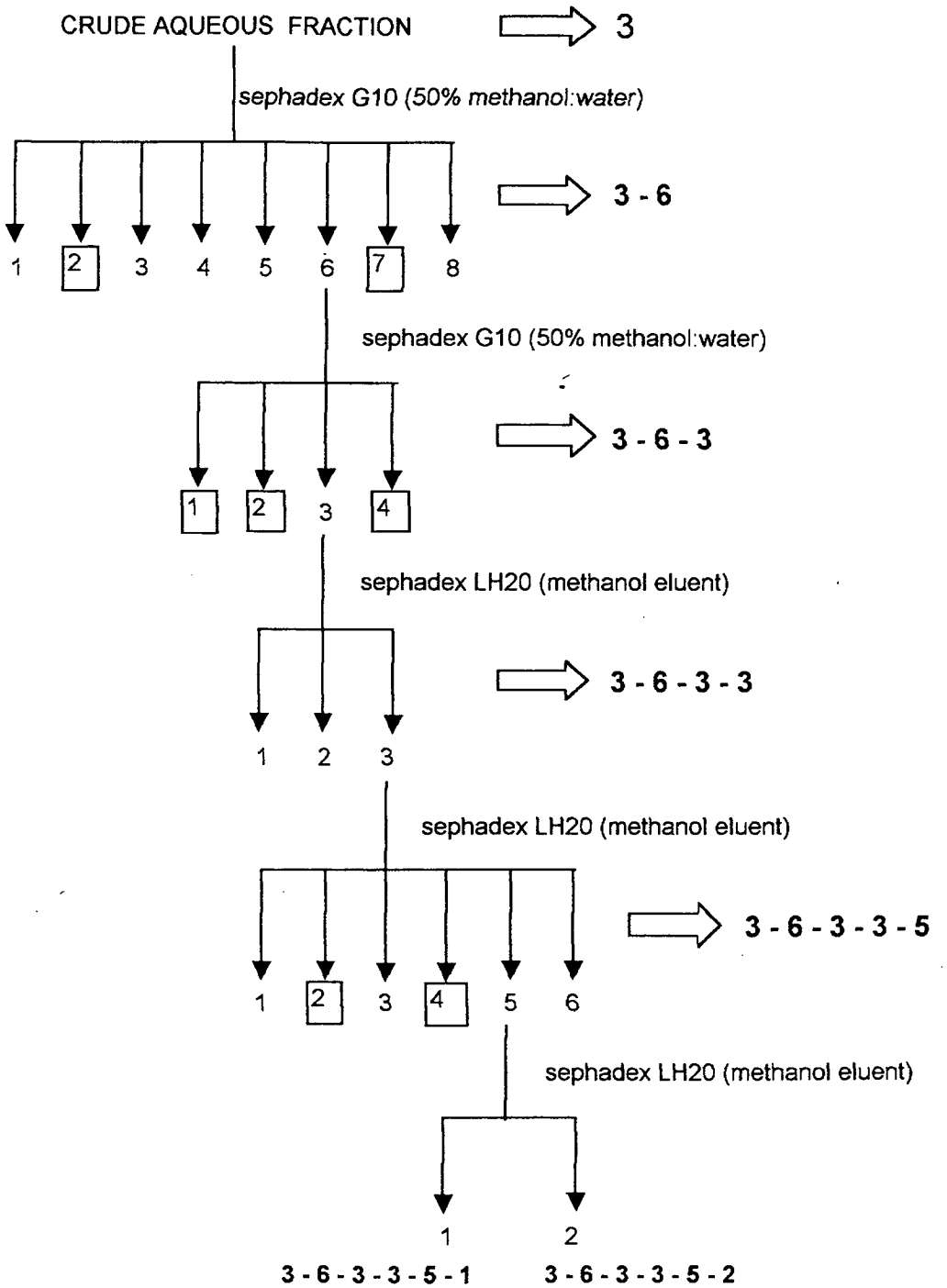


Figure showing the bioassay - guided fractionation of the crude aqueous fraction in the brine shrimp toxicity assay.

☐ = other active fractions

were 184.9 and 158.8 ppm, respectively. Rf value of fraction 3-6-3-3-5-1 was 0.16 (mobile phase 50% ethyl acetate:methanol). Fraction 3-6-3-3-5-2 showed 2 spots on TLC with Rf values of 0.16 and 0.35 (mobile phase 50% ethyl acetate:methanol). Fraction 3-6-3-3-5-1 (Fig. 16) isolated by gel filtration chromatography over sephadex LH 20 (methanol eluent) had strong absorption at 3400 cm^{-1} in the IR spectrum. The peak at 1634 cm^{-1} indicated amide functionality (CO-NH) similar to those found in peptides. The possibility of the compound being a long chain molecule or of terpenoid origin is ruled out as it did not have strong peaks in the region of 1460 and 1375 cm^{-1}. Fraction 3-6-3-3-5-2 (Fig. 17) also had strong absorption at 3400 cm^{-1}. Together with the peak at 1020 cm^{-1}, it indicated the presence of polyhydroxy system. The multiple absorptions in the region 1610–1660 cm^{-1} is indicative of unsaturation and amide groups. But the finger print region in the IR spectrum was different from that of 3-6-3-3-5-1. However, absorptions due to C-C unsaturation, hydroxyl and amide groups were common to both, thus indicating that these compounds may be similar in structure.

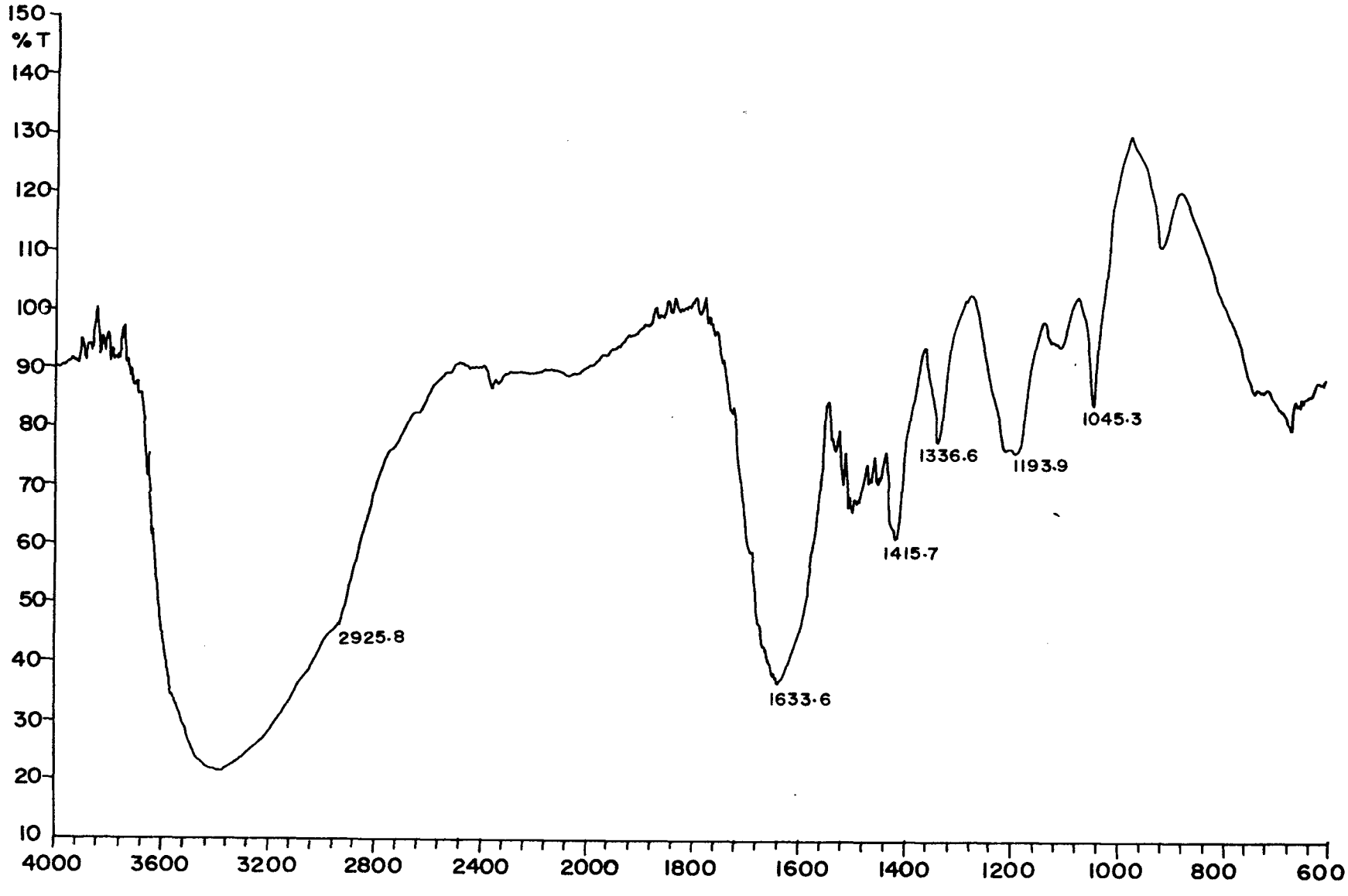


FIG. 16 IR spectrum of fraction 3-6-3-3-5-1

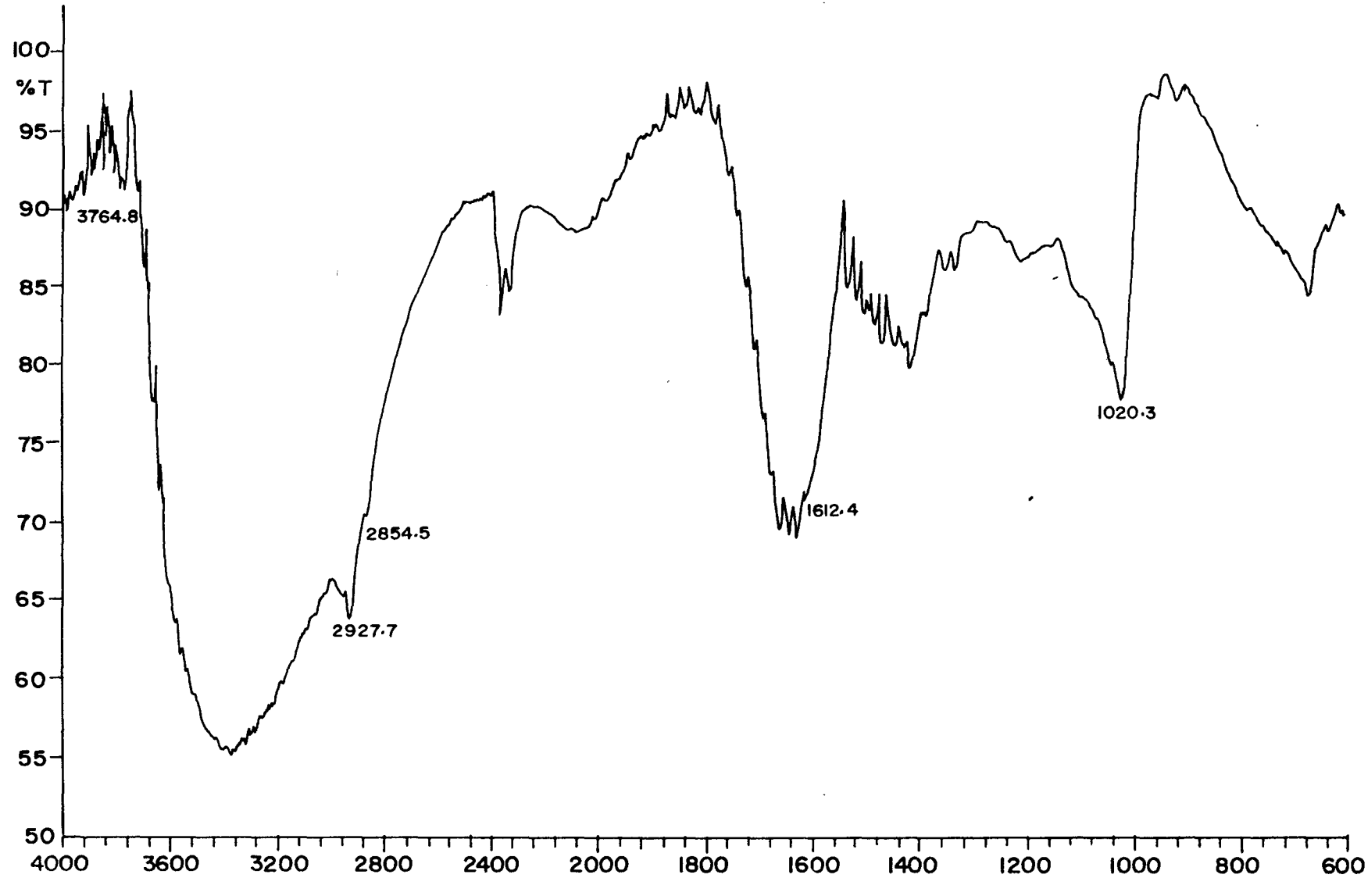


FIG. 17 IR spectrum of fraction 3-6-3-3-5-2

3.4.3. DISCUSSION

Toxicity studies revealed that *Zoanthus* sp is more toxic to the test animals than *Protopalythoa* sp. *Zoanthus* sp was toxic to all the test organisms except *O. mosambica*. *Protopalythoa* sp was toxic only to the prawn larvae and the fish, *Rhodeus sericeus*. The activity varied from mildly toxic to moderately toxic and non-toxic, to the different test species. Toxicity is indicative of an anti-predatory role and offers an important adaptation for organisms otherwise defenseless against predation (Whittaker & Fenny,1971; Gerhart,1984). The presence of toxic metabolites in the two zoanthids might be a contributory factor for the abundance of these animals in the intertidal expanse of the study area. Bakus (1974) and Cameron (1976) hypothesized that organisms with a sessile or slow moving habit use defenses such as toxicity which deliver no energy to the predator. Among the soft corals, toxicity is related in some way to the amount of physical protection that they possess, the more heavily armoured a species is, the less likely it is to be toxic to fish (Sammarco & Coll,1988). The present test organism, *Protopalythoa* sp, is a sand encrusted form and this character probably serves as a physical deterrent to predators. A comparative study of toxicity between the 2 zoanthids have shown that *Zoanthus* sp was more toxic to the test species than *Protopalythoa* sp. For example, in the case of *Rhodeus sericeus*, *Protopalythoa* sp was mildly toxic (2/6 fish dead) and *Zoanthus* sp was moderately toxic (4/6 fish dead). So also was the case with penaeid nauplii where LC_{50} values were 148.3 ppm and 65.3 respectively for *Protopalythoa* sp and

Zoanthus sp. *Zoanthus* sp has no visible physical protection to combat predation and seems to rely more on toxic components compared to *Protopalythoa* sp.

The tolerance of *Artemia* instars gives a toxicity measure for the two zoanthid species at 24 hour exposure. Of these, crude extract of *Zoanthus* sp only was capable of inducing mortality in the brine shrimp larvae (LC_{50}=3162.2 ppm). When the crude extract was fractionated into the petroleum ether, ethyl acetate and aqueous fractions, all three fractions had toxic effects upon the test larvae. The ethyl acetate fraction was most toxic one followed by aqueous and the petroleum ether fraction.

Sensitivity is a necessary attribute of toxicity assay (Sasikumar et al,1995) and the brine shrimp instar assay offers a quick, convenient, reliable and inexpensive assay in toxicity tests. The bioassay method proves to be the most promising and simple technique for toxicity determinations. Mortality parameter of the brine shrimp is widely used in experiments conducted by pharmaceutical, agricultural and petrochemical industries. Toxicity is a useful indicator for biological activity and provides important leads to other pharmaceutical properties of value (Sr. Vitalina et.al,1991). It appears that a positive brine shrimp lethality test (BSLT) is indicative of a broad spectrum of bioactivity (Meyer et al,1982), especially of cytotoxicity and pesticidal activity (Emilio,1993). Thus, the bioassay is recommended as a rapid, preliminary test for the evaluation of fractions during their purification.

Research on marine organisms over the past few years have illustrated that they produce varieties of structurally unique secondary metabolites that possess different types of biological activities. These metabolites and therefore the organisms that produce them interest natural product chemists because of their potential applications in pharmacology and other drugs. As a result, a systematic worldwide search for biomedical uses of marine flora and fauna has been undertaken. Biological activity of organic compounds extracted from marine organisms though has generated considerable pharmacological interest, ecological roles, if any, of most of these compounds remain unclear. Findings indicate that biologically active natural products may serve specific ecological roles.

The present work was designed to study the bioactivity and ecological interactions of two species of sedentary marine invertebrates, viz. *Zoanthus* sp and *Protopalythoa* sp belonging to Family Zoanthidea of the Phylum Coelenterata which have received very little attention. The different bioactivities looked for in this section include, antispasmodic activity, oxytocic activity and mosquitocidal activity. The ecological roles looked for are antifouling, feeding deterrency and toxicity assays.

The two species of zoanthids were removed from the rocks of the intertidal belt of Anjuna (Goa) and kept in solvent (methanol) for 8 days for extraction. Solvent extracts were vacuum evaporated to crude residue. Crude extracts obtained were used for the different bioactivity tests following procedures as mentioned in

earlier chapters. After preliminary screening of the crude extracts, these were fractionated into different fractions with solvents of increasing polarity. Individual fractions obtained were again subjected to bioactivity tests. A bioassay-guided fractionation via column chromatography was followed to locate the active component.

Secondary metabolites of both species of zoanthids, viz. *Zoanthus* sp and *Protopalythoa* sp, tested for different pharmacological activities revealed that these species possess organic compounds of pharmaceutical interest. The secondary metabolites also possess toxic compounds which may have effective use in mosquito control and/or eradication programmes.

The pure compound, peridininol, a C_{37} carotenoid pigment, derived from the petroleum ether fraction of *Zoanthus* sp had anti-nicotine and anti-serotonin (5-hydroxy tryptamine, 5-HT) activities. It decreased the nicotine contractions of guinea-pig upto 60.0% at a concentration of 50 and 100 µg/ml. However, its effect on the 5-HT induced contractions was found to be dose dependent as 38.0% decrease occurred at concentration of 50 µg/ml and 63.0% at 100 µg/ml Isolation of peridininol from *Zoanthus* sp and demonstration of its anti-nicotine and anti-serotonin activity have been done for the first time. Activity testing of the compound indicates its potential use in treatment of abdominal colics, diarrhea and other gastrointestinal disorders.

Isolation of the moulting hormone, 2-deoxy ecdysterone, from *Zoanthus* sp and demonstration of its oxytocic property have been achieved for the first time. It was observed that for the active compound, the percentage of active component of oxytocin contained in 50 μg/ml of compound amounts to 80.8%, that at 100 μg/ml amounts to 92.3% which further increased to 118.0% at concentration of 200 μg/ml. Uterine contractions produced by the compound were also compared with the standard contraction produced by prostaglandin ($PGF_{2\alpha}$). It was observed that for the active compound, the percentage of active component $PGF_{2\alpha}$ contained in 50 μg/ml of compound amounted to 69.4%, that at 100 μg/ml to 82.3% which further increased to 114.1% at concentration of 200μg/ml Therefore, the compound may have potential uses in several obstetrics related treatment such as (1) - to induce or augment labour, (2) - to control postpartum uterine atony and hemorrhage, (3) - to cause uterine contraction after cesarean section or during uterine surgery and (4) – to induce therapeutic abortion.

The larvicidal action of *Zoanthus* sp and *Protopalythoa* sp against larvae of *C. quinquiefasciatus* and *A. stephensi*, the vectors of filaria and malaria parasites revealed their efficiency as a larvicidal agent against larvae of the two mosquito species suggesting its possible use in mosquito control programmes.

Chemical defense studies provided evidences that the secondary metabolites from these two organisms may serve an ecological role against microfoulers and also have compounds that may dissuade predators.

Variation in the anti-fouling property (anti-bacterial and anti-diatom) examined at quarterly intervals for a one-year period demonstrated that chemical defense against diatoms, *Navicula subinflata* and *Nitzschia closterium* was effective year round whereas with bacteria, these animals did not seem to rely always on secondary metabolites. From the anti-algal assay, it was obvious that the extracts exhibited two different types of effects, viz. a growth stimulatory effect at lower concentrations and growth inhibitory effect at higher concentrations. While in the case of anti-bacterial assay, extracts of only 3 quarters displayed the activity. Further, the efficacy with respect to the zones of inhibition varied for all the bacterial isolates in all three quarterly extracts and the number of bacteria inhibited also varied. But, all the bacterial isolates were inhibited at one time or the other.

Feeding deterrency was tested against penaeid prawn juveniles, fish and crabs. It was found that in the fish and crab assay, pellets containing the zoanthid extracts were consumed within the same length of time as they did for the control pellets and no significant differences between the control and experimental extract treatments were observed. While in the case of penaied prawns where the feeding was strongly deterred and more than one fraction of *Zoanthus* sp and *Protopalythoa* sp extracts were found to be active suggested that feeding deterrency in these animals are species specific.

Toxicity studies revealed that *Zoanthus* sp is more toxic to the test animals than *Protopalythoa* sp. *Zoanthus* sp was toxic to all the test organisms except *Tilapia mosambica*. *Protopalythoa* sp was toxic only to the prawn larvae and the fish, *Rhodeus sericeus*. The activity varied from mildly toxic to moderately toxic and non-toxic, to the different test species.

The dominant position occupied by these two species in the intertidal belt at Anjuna and their large coverage may be attributable to their defensive mechanism played by the secondary metabolites, thus performing an ecological role.

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Short Communications

Effect of holothurian and zoanthid extracts on growth of some bacterial and diatom species

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The antifouling properties of the extracts from two zoanthids, viz. *Zoanthus* sp, *Protopalythoa* sp and one holothurian species, viz. *Holothuria leucospilota* occurring in the coastal waters off Goa were tested against 5 bacteria and 2 diatom species. All the three extracts possessed antifouling activity but their efficacy was found to be species specific. The highest bacterial inhibition zone ranged between 3-4 mm with both the zoanthid extracts. In case of diatoms, inducement of growth was observed in lower concentrations (200-400 ppm) and inhibition at higher concentrations (400 ppm and above).The results of the present study suggest that these marine natural compounds can possibly be used as antifouling agents.

Fouling is a widespread phenomenon causing serious maintainence problems of ship hulls, cooling systems and several other marine engineering facilities. To combat this menace, copper and organotin compounds are being used as chemicals in antifouling paints. These paints being deleterious to non-target life, necessitates the search for natural compounds of biological origin which are relatively free of chemical toxins[1,2] and are more species specific. It is known[2] that some marine invertebrates especially sessile forms are seldom covered by other organisms suggesting that they possess some bioactive compounds which prevent the settlement of the larvae of the later arriving species. In the search for newer approaches to antifouling, it is possible to mimic such organism's defensive faculties. The present work was therefore carried out to examine the antifouling properties of the compounds extracted from holothurians and zoanthids. Selected bacteria and diatoms were used as the test species in these bioassays.

Three species namely, *Holothuria leucospilota* Brandt (sea cucumber), *Zoanthus* sp and *Protopalythoa* sp (zoanthids) were collected from the rocky intertidal expanse of the Anjuna beach, Goa. The zoanthid colonies were removed from the rock surface using pincers and spatula while the holothurians were collected using big forceps. Specimens collected were rinsed well with fresh seawater and brought to the laboratory. Methanol extracts were obtained from the whole body tissues and concentrated by vacuum evaporation. Crude extracts obtained were tested for their inhibitory effects on 5 species of marine bacterial isolates (*Bacillus cereus, B. circulans, B. pumilus, Pseudomonas vesicularis* and *P. putida*) and 2 species of diatoms (*Navicula subinflata* and *Nitzschia closterium*). The test bacterial cultures were obtained from the stock cultures maintained in the Microbiology Laboratory of National Institute of Oceanography, Goa. For confirmation of their adhesive nature, sterilised filtered seawater in 50 ml beakers were inoculated with the bacteria culture. Glass coverslips were kept floating on the seawater inoculated with the bacteria. After 6 hours the coverslips were removed and stained with gention violet to check the adherence of bacteria. Only those cultures which formed a slime layer on the coverslips were chosen for the bioassay tests. The species identity of the chosen cultures were later confirmed using the Microbial Identification System (Hewlett Packard, USA).

The bacterial assays were carried out by the agar plate diffusion method[3]. Concentrations of 0.5 mg/6 mm disc of the extract prepared in methanol was introduced on sterilised filter paper discs (6mm). After solvent evaporation, the discs were laid on agar plates seeded with the test species. Controls were maintained in duplicate with solvent

alone. Four replicates of each concentrations were used in the assay and average values were recorded. Plates were incubated overnight at room temperature (~28°C). Growth inhibition zone was measured[4] in millimeters and scored as +++ = 3-4 mm, ++ = 2-3 mm, + = 1-2 mm. Inhibition zones measuring less than 1 mm were not scored. The bacterial assay results were compared with those obtained using standard antibiotic discs of penicillin-G (10 units/disc) and chloramphenicol (30 mcg/disc).

In the case of diatoms, seawater (~30 liters) collected from Dona Paula was filtered through 20μ nylon mesh. Phytoplankton samples thus collected were suspended in f/2 medium[5] in a 250 ml beaker. After 2 days, the medium holding the suspended cells was decanted and a fresh growth medium was added. The cells attached to the walls were then gently scraped with a fine brush and *Nitzschia closterium* and *Navicula subinflata* cells from these were picked up under microscope using fine capillary tubes. Subsequent subculturing and re-picking of cells was done until pure cultures were obtained. The method followed by Slattery *et al.*[6] was slightly modified for the bioassay, in that, instead of the tissue level concentrations of extracts, concentrations in terms of ppm are used in the present assay. Aliquots of each extract stock solution prepared in distilled water (1cc=50 mg) was taken corresponding to the desired concentrations to be used (200 - 1000 ppm). Actively growing cultures with an initial suspension (~1000 cells/ml) were used and the final volume adjusted to 50 ml using the growth medium in 125 ml Erlenmeyer flasks. All assays were carried out in quadruplets. Control flasks were without the extracts and contained only the cells in f/2 medium. Flasks were incubated at room temperature (~28°C, 12L:12D). Growth inhibition was monitored after 6 days in duplicate (i.e. during the exponential growth phase) in terms of the number of attached algal cells in a Sedgwick rafter[7] and expressed as cells/ml.

All the three extracts showed antibacterial properties in varying degrees and specificity (Table 1). The highest efficacy in terms of zone of inhibition was observed with *Zoanthus* sp and *Protopalythoa* sp extracts against *B. circulans* with inhibition zones of > 3 mm in diameter. Inhibition in other bacterial isolates ranged from 1-2 mm. Bacterial inhibition with the standard antibiotic discs was found to be more effective than the extracts and produced greater inhibition zones (17 and 26 mm respectively of penicillin-G and chloramphenicol, Table 2).

In case of diatoms, the extracts induced growth at lower concentrations and caused inhibition at higher concentrations. Extracts of *H. leucospilota* and *Zoanthus* sp stimulated growth of *N. subinflata* at 200 ppm concentration, whereas with *Protopalythoa* sp extract, growth stimulation was observed at 400 ppm, which decreased at enhanced concentration of 600 ppm (Fig. 1). In *Nitzschia closterium*, growth stimulation was observed at 200 ppm with the extract of *Zoanthus* sp, whereas extracts of *H. leucospilota* and *Protopalythoa* sp showed only the inhibitory effects.

Saponins from holothurians are well known toxic components possessing itchthyotoxic, hemolytic, neurotoxic, cytotoxic, antitumor

Table 1—Results on inhibition of growth of the test bacteria with crude extracts of *H. leucospilota, Zoanthus* sp and *Protopalythoa* sp against film forming marine bacteria in terms of inhibition of growth (mm) at concentration of 0.5 mg/6mm disc.

Test organism	Whole body extracts		
	H.leucospilota	*Zoanthus* sp	*Protopalythoa* sp
Bacillus cereus	–	+	–
Bacillus circulans	+	+++	+++
Bacillus pumilus	+	+	+
Pseudomonas vesicularis	–	–	–
Pseudomonas putida	+	–	–

+ = 1-2 mm diameter; ++ = 2-3 mm diameter; +++ = 3-4 mm diameter, – = no inhibition

Table 2—Growth inhibition (mm) of bacteria using the standard antibiotic discs of penicillin-G (10 units/disc) and chloramphenicol (30 mcg/disc).

Test organism	Antibiotics	
	Penicillin-G	Chloramphenicol
Bacillus cereus	-	19
Bacillus circulans	7	26
Bacillus pumilus	-	14
Pseudomonas vesicularis	-	-
Pseudomonas putida	17	18

– = no inhibition

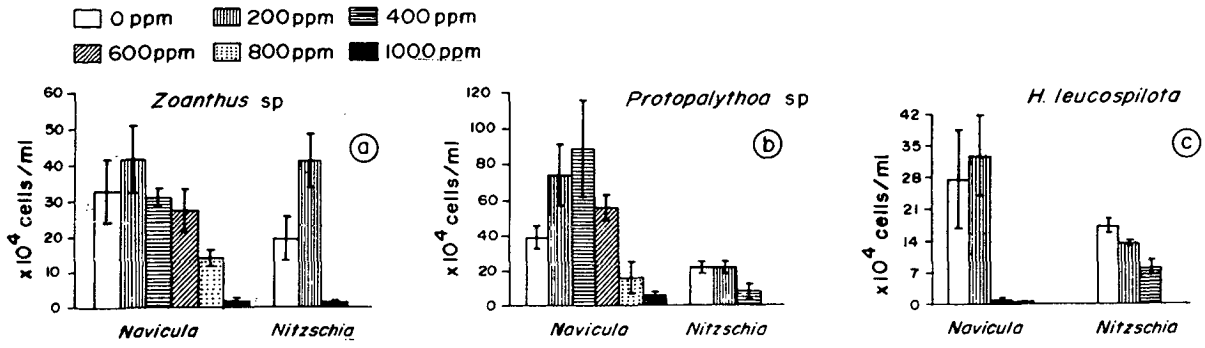

Fig. 1—Growth inhibition of the diatoms, *N. subinflata* and *N. closterium* in presence of the crude extracts of (a) *Zoanthus* sp (b) *Protopalythoa* sp and (c) *H. leucospilota*.

activities[8]. Similarly, Palytoxin[9] isolated from the zoanthids of *Palythoa* spp has cytotoxic properties and extremely active in cardiovascular systems. Paragracine from the zoanthid, *Parazoanthus gracilis* possesses antibradykinin, antihistamine and antiacetylcholine activities[9]. The present work adds to our knowledge on the antimicrofouling properties of crude extracts of *H. leucospilota, Zoanthus* sp and *Protopalythoa* sp and could be deduced that the bioactive compounds responsible for these activities can possibly be used as antifouling agents.

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