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Biological and Chemical Study of Some Soft Corals and Sponges Collected in Mauritian Waters

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***Abstract*—**Thirty-seven samples of soft corals and sponges collected in July 2004 between the north and west coasts of Mauritius at depths varying from 6-26 metres have been biologically screened against inhibition of urchin egg cell division, brine shrimp lethality test, anti-acetylcholinesterase test and against two bacteria *Escherichia coli* and *Micrococcus luteus*. The sponges *Rhabdastrella* sp. aff. *providentiae* (Dendy) **(M03 SP3 FF)** and *Pericharax heterorhaphis* **(M03 SP4 FF)** were found to be active against *Escherichia coli* and *Micrococcus luteus* respectively at a concentration of 1 µg/mL and the sponge *Liosina paradoxa* **(M03 SP3 BC)** exhibited anti-acetylcholinesterase activity. Fractionation and purification of the crude extract of the soft coral **M03 SC1 GB** using flash, open column and high performance liquid chromatography resulted in the isolation of one secondary metabolite (Guaianediol). Seventeen sponges have also been taxonomically identified.

INTRODUCTION

The greatest biodiversity of plants and animals is found in the oceans. To date, there are more than 300,000 plant and animal species identified in the oceans. They thus constitute a unique resource for provision of a diverse array of natural products, primarily from invertebrates such as sponges, soft corals, tunicates, bryozoans and mollusks, and from bacteria and cyanobacteria (Donia & Hamann, 2003). Among all these groups of organisms, soft corals and sponges are the most abundant and diverse in the Indo-Pacific oceans. Due to their soft bodies and sedentary life styles, these marine invertebrates have developed chemical means of defence against predators and colonisers by biosynthesising secondary metabolites. During the last 30 years, more than 15,000 novel secondary metabolites have been characterised in marine organisms. Some of these secondary metabolites may exhibit interesting pharmacological activities such as anti-cancer, anti-microbial, anti-fungal or anti-inflammatory (Carte, 1996; Mayer & Lehman, 2000; Banaigs, 2001; Venkateswarlu *et al.*, 2001; Proksch *et al.*, 2002; Haefner, 2003; Ely *et al.*, 2004; Jha & Xu, 2004). A few of them or their derivatives are presently in the stages of clinical trials for the treatment of cancer (Cragg & Newman, 2005). The search for new molecules having interesting pharmacological properties is of paramount importance in the fight against cancer,

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cardiovascular diseases and such infectious diseases as the HIV.

Mauritius, a small island found in the Indian Ocean (20°S, 57°E) about 1,700 km east of Madagascar and of an area of 1,865 km^2, possesses an Exclusive Economic Zone (EEZ) of about 1.9 million km^2, which has not presently been fully exploited. The general objectives of this work were to evaluate the biodiversity of the marine environment around the seas of Mauritius, to isolate and characterise secondary metabolites from sponges and soft corals and screen them for potential anti-bacterial and other pharmacological properties. This paper reports the taxonomic identification of some sponges, and the preliminary biological and chemical screening of some sponges and soft corals and isolation of one secondary metabolite from a soft coral.

MATERIALS AND METHODS

General

The Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Spectrospin, AVANCE DPX 250 MHz. Deuterated chloroform ($CDCl_3$) was used as solvent and tetramethylsilane was the internal standard. The chemical shifts were given in δ (ppm) and coupling constants in Hertz. Silica gel 60 (Merck, 230-400 mesh) was used for column chromatography and pre-coated silica gel plates (Merck, Kieselgel 60, 0.25 mm) were used for Thin Layer Chromatographic (TLC) analysis. Chemicals and solvents were purchased from Aldrich and Carlo Erba. High Performance Liquid Chromatography (HPLC) was conducted with a thermoseparations system fitted with a Microsorb 100 C18 column and a Waters 410 refractive index detector.

Collection of samples

Samples of soft corals (n = 30) and sponges (n = 30) were collected in July 2004 in the Mauritian waters from four sites, namely Grand Baie, Trou aux Biches, Albion and Flic en Flac at depths varying from 6-26 metres. The samples were frozen at the Chemistry Laboratory of the University of Mauritius and a small part was cut and stored under 10% formaldehyde solution for identification. Some samples were collected only in small quantities as they were not abundant at the site of collection.

Preparation of crude extracts

The frozen samples were diced and lyophilised. Afterwards, the dried organisms (10 g) were exhaustively macerated in a 1:1 mixture of methanol and dichloromethane. The extracts were then filtered and the organic portions were evaporated before being partitioned between ethyl acetate and water. Thirty-seven extracts including twenty-two of sponges were thus prepared. The crude organic fractions of the different samples were then subjected to biological and chemical screening.

Biological screening

Inhibition of urchin egg cell division - The sea urchins *Pseudopneustes variolaris* were collected at the Grand Fond beach, in the west coast in Reunion Island. Potassium chloride solution (0.5 M) was injected into the freshly collected sea urchins to release the male and female gametes. The excreted male and female gametes were collected separately and then brought together to induce fertilisation. In a vial was added 90 µL of the gametes solution followed by 10 µL of the crude extract at a concentration of 1 mg/mL. The final concentration of the crude extract in the vial was 100 µg/mL. After one and a half hours, the number of divided cells was counted. Water and ethanol were used as controls. All the tests were performed in duplicate.

Brine shrimp lethality test - *Eggs of Artemia salina* (JBL GmbH & Co, Germany) were hatched in a small tank filled with distilled seawater. After 48 hours, the phototropic nauplii were collected, and 10 hatched larvae were transferred using a pipette to small sample vials. Seawater was added to make 180 µL, followed by 20 µL of the crude extract (1 mg/mL). Distilled water and 10% ethanol were used as controls. The experiments were carried out in duplicate and the mean percentage death was determined after 24 hours.

Acetylcholinesterase inhibition test - Acetylcholinesterase (Sigma, St Louis, USA; 1000 U) was dissolved in 150 mL of 0.05 M tris-(hydroxymethyl) aminomethane hydrochloride buffer at pH 7.8, and 150 mg of bovine serum albumin (Merck, Darmstadt, Germany). After deposition of 100 µg of each sample on TLC and migration in a 1:1 mixture of hexane and ethyl acetate, the TLC plate was dried to remove solvent, sprayed with the enzyme solution and dried again. The plate was then placed in an incubator at 90% humidity and 37°C for 20 min. For detection of the enzyme, 10 mL of a 1-naphtylacetate solution (250 mg in 100 mL of ethanol) and 40 mL of a Fast Blue Salt solution (400 mg in 160 mL of distilled water) were mixed and sprayed onto the plate to give a purple coloration after 1-2 min. Inhibitors of acetylcholinesterase appear as white spots. Galanthamine was used as the control.

Anti-bacterial assay - The technique used was based on a method published by the National Committee of Laboratory Safety and Standards (NCLSS) in 1997. The crude products were dissolved in dimethylsulphoxide (not exceeding 5% total volume) and were incubated with two bacterial strains (Institut Pasteur, Paris): a Gram-positive (*Micrococcus luteus*) and a Gram-negative (*Escherichia coli*) in 96 well-plates (MERCK) in PB medium, at 37°C for 24 hours, under stirring. Assays were carried out in triplicate and the results were averaged. Growth was evaluated by reading the optical density (630 nm). When an activity was detected (absence of growth), a sample of media was taken on rich solid medium (Petri dishes) to establish the effect (bacteriostatic or bactericidal).

Chemical screening

A minute portion of the crude extract dissolved in dichloromethane was placed on a thin layer chromatographic plate and eluted using mixtures of hexane and ethyl acetate in a saturated chromatographic cell. The plate was then sprayed with each of the following locating agents: (i) vanillin solution (1% vanillin dissolved in 50% phosphoric acid); (ii) ninhydrin solution (0.4% of ninhydrin in ethanol); and (iii) Dragendorff reagent (ten-fold dilution of a 1:1 mixture of solution A (850 mg of bismuth nitrate dissolved in acetic acid (10 mL) and distilled water (40 mL)) and solution B (8 g of potassium iodide dissolved in distilled water (20 mL)) and saturated with tartaric acid. Cholesterol was used as control when vanillin and Dragendorff reagents were used as locating agents whereas alanine was used in the case of ninhydrin.

Fractionation and purification

Deactivated silica was obtained by refluxing silica (40-63 µm) in methanol for 24 hrs using soxlet method and then evaporating the excess methanol. The crude extract of the soft coral **M03 SC1 GB** (583 mg) was flash chromatographed over deactivated silica (40-63 µm) using solvents of increasing polarity (hexane, ethyl acetate and methanol). The three fractions were evaporated and the ethyl acetate fraction (288 mg) was fractionated on a silica gel column using a mixture of ethyl acetate and hexane as eluant to yield 100 fractions. After further purification using sephadex LH-20, fractions 59-69 were subjected to reverse phase HPLC with CH_3CN/H_2O 1:1 as eluant. A pure product, Guaianediol (5.6 mg), was isolated. It was characterised by proton (^1H) and carbon (^{13}C) NMR spectroscopy and the data obtained are given below.

^1H NMR ($CDCl_3$): δ (ppm) 1.86 (m, 1H), 1.74 (m, 2H), 1.61 (m, 2H), 2.16 (dd, 1H), 5.49 (m, 1H), 1.92 (m, 1H), 2.18 (m, 1H), 1.81 (m, 1H), 1.46 (m, 1H), 2.24 (m, 1H), 0.97 (d, 3H, J=7 Hz), 0.96 (d, 3H, J=7 Hz), 1.26 (s, 3H), 1.20 (s, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 21.2, 21.3, 21.4, 21.5, 22.6, 25.1, 37.3, 40.5, 42.6, 50.3, 50.7, 75.3, 80.3, 121.3 and 149.7.

RESULTS AND DISCUSSION

Thirty samples each of soft corals and sponges were collected from four sites found between the north and west coasts of Mauritius, namely Grand Baie, Trou aux Biches, Albion and Flic en Flac (Figure 1) at depths varying from 6-26 metres.

Seventeen (17) samples of sponges were identified by Dr R Van Soest of the University of Amsterdam (Table 1). According to our knowledge, no studies on taxonomic identification of sponges in Mauritius have been reported in the literature. For soft corals, a pre-identification of

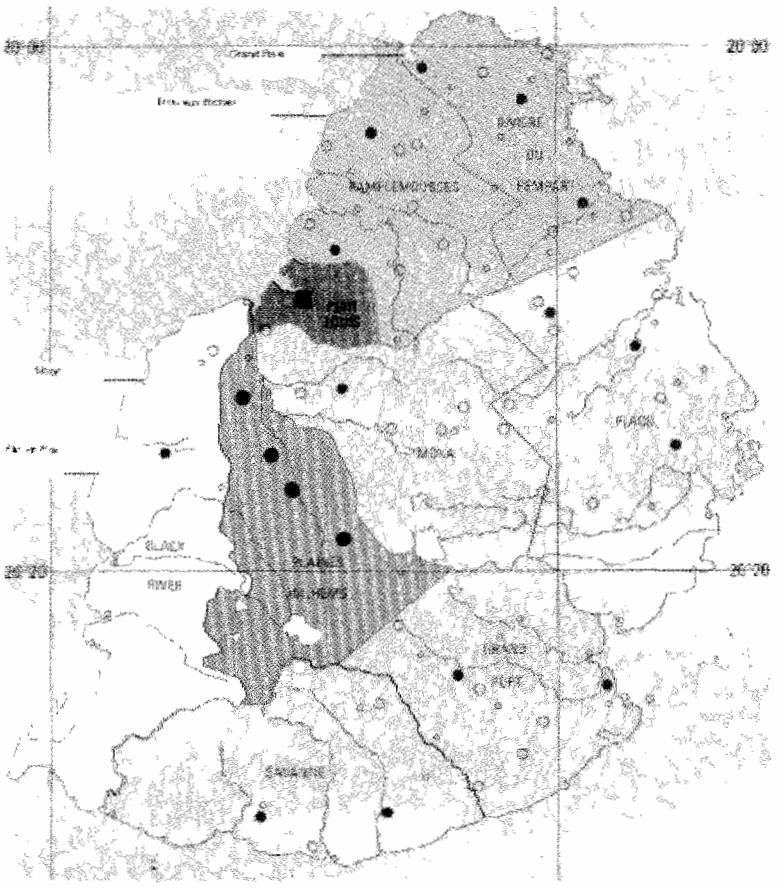

Fig. 1. Map of Mauritius showing the collection sites

Table 1. List of identified sponges

Sample No.	Serial No.	Location	Colour	Species
1	**M03 SP3 AL**	Albion	Orange-red	*Myrmekioderma granulata* (Esper)
2	**M03 SP4 AL**	Albion	Orange	*Ptilocaulis spiculifer* (Lamarck)
3	**M03 SP1 BC**	Trou aux Biches	Yellow-brown	*Pericharax heterorhapsis* (Poléjaeff)
4	**M03 SP2 BC**	Trou aux Biches	Dark brown	*Plakortis* aff. *simplex*
5	**M03 SP3 BC**	Trou aux Biches	Dark-brown	*Liosina paradoxa* (Thiele)
6	**M03 SP4 BC**	Trou aux Biches	Dark-brown	*Spheciospongia inconstans* (Dendy)
7	**M03 SP5 BC**	Trou aux Biches	Green	*Halichondria cartilaginea* (Esper)
8	**M03 SP3 FF**	Flic en Flac	Yellow-brown	*Rhabdastrella* sp. aff. *providentiae* (Dendy)
9	**M03 SP4 FF**	Flic en Flac	Yellow-brown	*Pericharax* heterorhapsis (Poléjaeff)
10	**M03 SP5 FF**	Flic en Flac	Dark green	*Scalarispongia* sp.
11	**M03 SP6 FF**	Flic en Flac	Dark green	*Dysidea* sp.
12	**M03 SP1 GB**	Grand Baie	Dark green	*Petrosia (Strongylophora) mauritiana (Carter)*
13	**M03 SP3 GB**	Grand Baie	Orange	*Axinella donnani* (Bowerbank)
14	**M03 SP1 TB**	Trou aux Biches	Red	*Stylissa* sp.
15	**M03 SP3 TB**	Trou aux Biches	Brown	*Dysidea* sp.
16	**M03 SP5 TB**	Trou aux Biches	Orange red	*Stylissa carteri* (Dendy)
17	**M03 SP9 TB**	Trou aux Biches	Reddish violet	*Spirastrella* sp.

AL = Albion, BC = TB = Trou aux Biches, FF = Flic en Flac, GB = Grand Baie

the genus for some samples was effected by comparison with known ones from the literature (Gosliner *et al.*, 1996; Fabricius & Alderslade, 2001).

The soft corals and sponges were lyophilised. Crude organic extracts were obtained after maceration in a 1:1 mixture of dichloromethane and methanol, filtration and evaporation. Thirty-seven extracts were thus prepared.

Biological screening

Inhibition of urchin egg cell division

Two samples of soft corals, **M03 SC2 FF** and **M03 SC3 FF**, belonging most probably to the *Lobophytum* genus, showed significant activity by inhibiting nearly completely the first cell division of the sea urchin eggs at a concentration of 100 µg/mL.

Brine shrimp (*Artemia salina*) lethality test

Seven samples were found to be weakly toxic against *Artemia salina* at a concentration of 100 µg/mL. Two sponges, **M03 SP3 BC** (*Liosina paradoxa*) and **M03 SP4 BC** (*Spheciospongia inconstans*) exhibited a higher activity by recording a mortality rate of 35 and 30% respectively.

Acetylcholinesterase inhibition test

This test measures the inhibiting activity of the extracts at 100 µg against the acetylcholinesterase enzyme. Only the sponge **M03 SP3 BC** (*Liosina paradoxa*) was found to exhibit anti-acetylcholinesterase activity. In the literature, very few secondary metabolites have been found to inhibit acetylcholinesterase (Husain *et al.*, 1996; Sepcic *et al.*, 1998, 2001).

Antibacterial assay

The crude extracts were tested for their antibacterial activities against two bacteria, namely *Escherichia coli* and *Micrococcus luteus* at concentrations ranging from 1-10 µg/mL. Five sponges, **M03 SP3 AL** (*Myrmekioderma granulata*), **M03 SP2 BC** (*Plakortis* aff. *simplex*), **M03 SP4 FF** (*Pericharax heterorhaphis*), **M03 SP5 FF** (*Scalarispongia* sp.) and **M03 SP3 TB** (*Dysidea* sp.) were found to be active at a concentration of 10 µg/mL against *Escherichia coli* and one sponge, namely **M03 SP3 FF** (*Rhabdastrella* sp. aff. *providentiae* (Dendy)) was found to be active at a concentration of 1 µg/mL.

On the other hand, two sponges, **M03 SP2 BC** (*Plakortis* aff. *simplex*) and **M03 SP3 TB** (*Dysidea* sp.) were found to be active against the bacterium *Micrococcus luteus* at a concentration of 10 µg/mL and one sponge, namely **M03 SP4 FF** (*Pericharax heterorhaphis*) at 1 µg/mL.

Chemical screening

The different crude extracts were chemically screened using thin layer chromatography. Three different locating reagents, namely vanillin, ninhydrin and Dragendorff were used to evaluate the abundance of secondary metabolites in the crude extracts. Vanillin was used to detect the presence of organic compounds such as terpenes, alkaloids, peptides and sterols; ninhydrin to detect the presence of amino acids and peptides, and Dragendorff to detect the presence of alkaloids.

Only two soft corals, namely **M03 SC1 GB** and **M03 SC7 TB** belonging most probably to the *Sarcophytum* sp. showed positive responses with the three reagents implying that they may contain compounds such as terpenes, amino acids and/or alkaloids.

The soft coral **M03 SC1 GB** was selected for further study. Three fractions were obtained from flash chromatography of the crude organic extract using solvents of increasing polarity: hexane, ethyl acetate and methanol. Purification of the ethyl acetate extract by open column chromatography followed by high performance liquid chromatography yielded one pure product. The latter was characterised by ^{1}H and ^{13}C NMR spectroscopy and was found to be Guaianediol (Figure 2). Fifteen (15) distinct carbon signals were observed in its ^{13}C NMR spectrum. The assignments for the carbon atoms are given in Table 2. The ^{13}C NMR signals at δ 149.7 and 121.3 ppm were attributed to the olefinic carbons C-7 and C-6. The signals of the methine carbons C-4 and C-

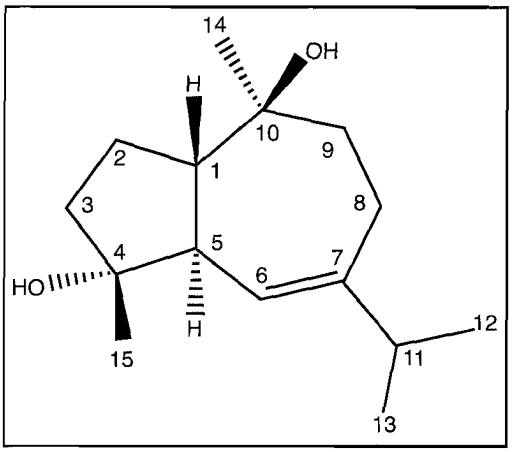

Fig. 2. Structure of Guaianediol

Table 2. ^{13}C NMR data of Guaianediol

Carbon No.	δ C*, ppm	δ C**, ppm
1	50.7	50.7
2	21.5	21.5
3	40.5	40.5
4	80.3	80.2
5	50.3	50.3
6	121.3	121.3
7	149.7	149.6
8	25.1	25.1
9	42.6	42.6
10	75.3	75.3
11	37.3	37.3
12	21.4	21.4
13	21.3	21.3
14	21.2	21.1
15	22.6	22.5

* Our work
** El Sayed & Hamann (1996)

10 were downfield implying that they are attached to electron-withdrawing groups.

In the ^1H NMR spectrum the two doublets found at δ 0.96 and 0.97 ppm were correlated to the methyl groups at C-12 and C-13. The two singlets at δ 1.26 and 1.20 ppm were attributed to the two methyls at C-14 and C-15. The olefinic proton at C-6 appeared at δ 5.49 ppm while the methine proton at C-11 appeared as a multiplet at δ 2.24 ppm. The other protons appeared in the region 2.16-1.46 ppm. Based on these spectral data and by comparison with literature values, it was concluded that the product has been isolated previously from the soft coral *Sinularia gardeneri* (El Sayed & Hamann, 1996).

CONCLUSION

This study has enabled the taxonomic identification of 17 samples of sponges. Biological screening of the crude extracts has revealed some interesting activity in some soft corals and sponges. The soft corals, **M03 SC2 FF** and **M03 SC3 FF**, belonging most probably to the *Lobophytum* genus, showed significant activity by inhibiting nearly completely the first cell division of the sea urchin eggs. Two sponges **M03 SP3 BC** (*Liosina paradoxa*) and **M03 SP4 BC** (*Spheciospongia inconstans*) induced a mortality rate of 35 and 30% respectively in the brine shrimp (*Artemia salina*) lethality test. The sponges *Rhabdastrella* sp. aff. *providentiae* (Dendy) **(M03 SP3 FF)** and *Pericharax heterorhaphis* **(M03 SP4 FF)** were found to be active against *Escherichia coli* and *Micrococcus luteus* respectively at a concentration of 1 μg/mL and the sponge *Liosina paradoxa* **(M03 SP3 BC)** exhibited anti-acetylcholinesterase activity. Fractionation and purification of the crude extract of the soft coral **M03 SC1 GB** using flash, open column and high performance liquid chromatography resulted in the isolation of one secondary metabolite (Guaianediol). Future work will be oriented towards isolation of other secondary metabolites and their biological screening.

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