SUPPLEMENTARY MATERIAL

Antibacterial effect of the red sea soft coral *Sarcophyton trocheliophorum*

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The marine soft corals *Sarcophyton trocheliophorum* crude extracts possessed antimicrobial activity towards pathogenic bacterial strains i.e. *Bacillus cereus*, *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Bioassay-guided fractionation indicated that the antimicrobial effect was due to the presence of terpenoid bioactive derivatives. Further biological assays of the *n*-hexane fractions were carried out using turbidity assay, inhibition zone assay and minimum inhibitory concentration for investigating the growth-inhibition effect towards the gram-positive and gram-negative bacteria. The fractions were screened and the structure of the isolated compound was justified by interpretation of the spectroscopic data, mainly mass spectrometry (GC-MS). The structure was assigned as 5S)-3-[(3E,5S)-5-Hydroxy-3-hepten-6-yn-1-yl]-5-methyl-2(5H)-furanone and was effective at concentrations as low as 0.20 mg/mL. The above findings, in the course of our ongoing research on marine products, may implicate that the profound anti-microbial activity of the *Sarcophyton trocheliophorum* soft corals, inhabiting the red sea reefs, is attributed to the presence of growth-inhibiting secondary metabolites mainly terpenoids.

Keywords: soft coral, *Sarcophyton trocheliophorum*, terpenoid, *in vitro*, antimicrobial assays.

Experimental

3.1. General Experimental Procedures

EI-MS, HR-FAB-MS, and positive-ion FAB-MS (with glycerol as matrix) spectra were recorded with a JEOL JMS SX/ SX102A instrument. HR-FAB-MS: JEOL JMS-SX102 spectrometer in positive-ion mode. UV spectra were recorded with a Perkin-Elmer Lambda 2 UV/vis spectrophotometer. ^1H and ^{13}C NMR spectra: Bruker DRX-400 spectrometer at 400 at 100.1 MHz., and all chemical shifts are expressed with reference to TMS. TLC was performed on precoated aluminum sheets [silica 60 F₂₅₄, 0.25 mm (Merck, Darmstadt, Germany)] and preparative TLC on precoated glass sheets [silica 60 F₂₅₄, 0.5 mm (Merck)], with the detection provided by UV light (254 and 366 nm) and by spraying with vanillin-sulfuric acid reagent followed by heating (120°C) (El-Seedi et al. 2013).

Accelerating gradient chromatography (AGC) was performed using variable-length MPLC glass columns (BaeckströmSeparo AB, Lidingö, Sweden) with inner diameters of 4.0, 2.5, and 1.5 cm, packed with silica gel 60, 40-63 μm (Merck), and an FMI Lab pump, model QD (Fluid Metering Inc., Oyster Bay, NY), delivering a flow rate of 15-18 ml/min. Fractions of 20 ml were collected manually. Analytes were eluted from the columns by continuous gradient elution running from hexane, through CH_2Cl_2 , EtOAc, and MeOH, to H_2O generated by a Separo constant-volume mixing chamber combined with an open reservoir. The mixing chamber initially contained 100 ml of hexane and the reservoir contained the first of 15-20 premixed binary gradient mixtures of gradually increasing polarity, each of 15-30 ml, which were successively fed to the reservoir during the separation (El-Seedi, 2007).

3.2. Collection of *Sarcophyton trocheliophorum*

Soft coral (*S. trocheliophorum*) colonies were collected by trained divers working in the harbor areas of Hurghada on the Red Sea in Egypt, within an area ranging from 27°00'N to 27°45'N and from 33°30' E to 34°00' E. Voucher specimen Str100 was kept at National Research Center, Cairo, Egypt.

3.3. Extraction and Isolation

The collected samples were immediately transported to the laboratories of the Hurghada Marine Station, National Institute of Oceanography and Fisheries. Samples were washed twice with filtered sea-water then briefly with distilled water. The washed samples were blended with absolute ethanol (1:1 w/v) and centrifuged (2570 g for 10 min). The material was extracted with *n*-hexane at room temperature three times with occasional stirring and filtered to give (265.1 g).

The macerate was extracted three times with MeOH for 7 days to give (311.6 g). The extract was partitioned between EtOAc and H_2O to give (165.6 g) of an EtOAc extract and (121.2 g) of a H_2O extract after freeze-drying. An interfacial residue (24.8 g) was also produced. The EtOAc and *n*-hexane extracts (60 g) were adsorbed onto silica gel (120 g) and chromatographed on a silica gel (280 g) column eluted with continuous hexane- CH_2Cl_2 , CH_2Cl_2 -MeOH, to MeOH gradients. The eluted tubes, from AGC columns, were evaluated by TLC in order to get 12 main fractions. The most active fractions (H2, H3) were subjected for further purification over repeated AGC eluted with CHCl_3 -MeOH (1:1), which afforded 15 mg of pure compound.

3.4. Antibacterial Activity

3.4.1 Turbidity Assay

The Gram-positive bacteria *Bacillus cereus* EMCC 1080 and *Staphylococcus aureus* ATCC 13565 were obtained from Dairy Dept. of the Egyptian National Research Centre, together with the Gram-negative bacteria *Salmonella typhi* ATCC 25566, *Escherichia coli* 0157 H₇ ATCC 51659 and *Pseudomonas aeruginosa* NRRL B-272. The turbidity assay was used for determining the antibacterial activity of *Sarcophyton* extracts.

A single colony of the selected bacterial strain was grown on agar media for 24 h at 37 °C. The organisms were then transferred to an appropriate broth solution (tryptic soy broth, TSB) and incubated for 24 h at 37 °C to create pre-inoculum cultures.

Solutions of the hexane and EtOAc of *Sarcophyton* extracts were prepared to final concentrations of 0.5, 1.0, 2.5 and 5.0 mg/ml and filtered through Millipore 0.22 µm membranes. Pre-inoculum cultures of each organism (10⁵ cfu/ml, 24 h old) were inoculated into separate batches of tryptic soy broth (10 ml), supplemented with the extract to be tested, and incubated at 37°C for 24 h. Bacterial growth was monitored by measuring the culture's turbidity at 595 nm (Devienne & Raddi 2002).

Turbidity Assay, Inhibition Zone Assay, Minimum Inhibitory Concentration

3.4.2. Inhibition Zone Assay

Kirby-Bauer's Disc diffusion Technique (Bauer et al. 1966) was used as a complementary assay for antibacterial activity. Petri dishes containing 20 ml of sterile Muller Hinton Agar (MHA) were inoculated with a suspension of one of the microorganisms (10⁸ cfu/ml) at a density equivalent to the 0.5 McFarland standards in sterile distilled water.

Stock solutions with a concentration of 1.0 mg/ml were prepared for each fraction to be studied (H₁-H₇ and E₁-E₅). For each fraction, a sterile disc was impregnated with 10 µL of the stock solution, with DMSO-impregnated discs used as negative controls and discs impregnated with a cefoperazone solution (100 µg/ml) used as positive controls. Each disc was placed onto the dry surface of an inoculated plate and incubated for 24 h at 37°C. The radius of the inhibition zone for each fraction was then compared to that of the positive control (cefoperazone, 100 µg/ml).

3.4.3. Minimum Inhibitory Concentration (MIC)

A 24 h culture of the tested microorganism was diluted in 10 ml of TSB to prepare inoculate of approximately 10⁸ cfu/ml, corresponding to the 0.5 McFarland standard. Serial dilutions of the isolated fractions were established, giving concentrations of 2.5, 2.25, 2.0, 1.75, 1.5, 1.25, 1.0, 0.75, 0.5, 0.25, 0.2, and 0.1 mg/ml. Each concentration was inoculated with 0.1 ml of the standardized bacterial cell suspension and incubated at 37 °C for 24 h. The growth of the inoculum in the broth was measured based on its turbidity, and the lowest concentration of the extract that inhibited the growth of the test organism was recorded as MIC.

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