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Original Article

Characterizations of the Secondary Metabolites of the Sea Squirt *Diplosoma listerianum* Milne-Edwards, 1841) (Ascidiacea, Enterogona) and its Associated Micro-organisms

Naif Al Qurashi

Department of Basic Science, Deanship of Preparatory Year and Supporting Studies, Imam Abdulrahman Bin Faisal University, Dammam, SAUDI ARABIA.

ABSTRACT

Background: The biochemistry of secondary metabolites of marine origin has been identified as a promising area for obtaining new and potent pharmaceutical agents. These secondary metabolism molecules may have important functions such as anti-biotic and anti-parasitic activity, mediate symbiotic relationships between organisms and even have reproductive functions, representing a great ecological advantage for the survival of marine organisms. **Aim:** This study aimed to characterize the secondary metabolites of the sea squirt *Diplosoma listerianum* and its associated micro-organisms. **Materials and Methods:** The squirts were frozen, dried, weighed, roughly crushed and extracted using methanol and dichloromethane (1:1). The crude extract was dissolved in methanol and fractionated in four solvents of increasing polarity: saturated hexane, acetic ester, butyl alcohol and water. The active substances were isolated by liquid chromatography, purified and their structures determined. Bacteriostatic action against the crude extract was assessed using longitudinal *in vitro* growth in Müller-Hinton broth. **Results:** *D. listerianum's* crude extract showed antibiotic activity against various pathogenic microorganisms. Analysis of variance and Tukey's multiple comparison tests indicated significant differences among exposure times. Mouse fibroblasts exposed to the crude extract showed fragmented and sickle-shaped nuclei, visible centrosomes, abnormal multipolar mitotic spindles and endoplasmic reticulum vacuolization. The phytagel assay showed a correlation between the gel's algal density and associated organism numbers. The protein tyrosine kinase assay showed that the crude extract had enzyme-inhibiting properties. Preparative high-performance liquid chromatography, Sephadex column chromatography, high-resolution electrospray ionization mass spectrometry and electron impact mass spectra identified zeaxanthin, tubastrine (an epidermal growth factor receptor inhibitor), inosine, guanosine, oxazolidin-2-one, LL-PAA216 and 2-aminododecan-3-ol. **Conclusion:** Marine biotechnology generates technological products from the diversity of marine organisms and their strategic adaptations to the extreme conditions of the seas and, in turn, presents itself as a promising source of bioproducts and processes, being able to solve issues in areas such as health, food safety, cosmetics, agriculture, pollution control, climate and industry, among others.

Keywords: *Diplosoma listerianum*, Bacteriostatic action, Mouse fibroblasts, Field study, Aromatic compounds.

Correspondence:
Associate Professor. Naif Al Qurashi
Department of Basic Science, Deanship of Preparatory Year and Supporting Studies, Imam Abdulrahman Bin Faisal University, P.O. Box 1982, Dammam, SAUDI ARABIA.
Email: nalqurashi@iau.edu.sa
ORCID: 00 00000344883506

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INTRODUCTION

Many intuitive compounds of marine origin have many miscellaneous biologic activities, including antibacterial, anticoagulant, anti-inflammatory, anti-fungal, anti-malarial, anti-tuberculosis and anti-viral.[1,2] Most were isolated from poriferans, cnidarians, hexapods, mollusks, tunicates and their associated micro-organisms.[3,4] Sessile invertebrates are a very profitable venture for natural bioactive substances.[5,6] For many centuries, plants represented the only source of remedial compounds for humans, with many still in use today. Opium prepared from *Papaver somniferum* bulbs (Linnaeus, 1753) is known for its soporific and analgesic properties.[7,8] Alkaloids obtained from opium include morphine, the main analgesic in use today, codeine (an antitussive), thebaine (a morphine antagonist), narcotine (an antitussive and spasmolytic) and papaverine (a spasmolytic). Studies have discovered alkaloids

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from marine invertebrates and concluded that they are important candidates for anticancer drugs.[9] The most important milestone in remedial progress from intuitive compounds was the discovery of acetylcholinesterase inhibitors such as the pulmonarin families and synoxazolidinones A and C. Obtained from the sub-arctic sea squirt Synoicum pulmonaria, they have analgesic and antipyretic properties. Salicylic acid and acetylsalicylic acid are glycoside salicin {2-(hydroxymethyl) phenyl β-d-(glucopyranoside)} derivatives.

Sessile marine organism evolution and survival have led to organisms producing and accumulating incidental breakdown products with diverse biological activities.[10,11] These metabolites are used for communication, defense, inhibiting competitor development, reproduction, or simply as metabolism by-products.[12,13] Studying synthetic analogs of these nucleosides led to the development of the antiviral agent Ara-A[14] and anticancer agent Ara-C.[15] These compounds were the first natural marine product derivatives to reach the clinical market.[16,17] Many of these substances are undergoing preclinical and clinical testing. For example, bryostatin 1 is a heteroaromatic quinone isolated from the sea moss *Bugula neritina* currently undergoing clinical testing.[18] Other examples of intuitive compounds of marine origin undergoing clinical testing include ecteinascidin 743, a tetrahydroisoquinoline alkaloid isolated from the *Ecteinascidia turbinata* that showed anti-cancer activity.[19,20] Kahalalide F is a cyclic depsipeptide with anticancer activity found in the marine mollusk *Elysia rufescens*. Aplidine isolated from the ascidian *Aplidium albicans* also has anticancer properties. The hydroxylated quinone discodermolide isolated from the poriferan *Discodermia dissoluta* has potent immunosuppressive and anticancer activity.[21,22]

Nitrogen compounds, alkaloids, peptides and amino acid derivatives comprise 85% of the secondary metabolism products isolated from sea squirts. The remaining 15% are compounds produced via the acetate and acetate-mevalonate pathways.[23,24] Cytotoxic substances allow the producer to fight off predators and defend themselves. Like antimicrobial activity, cytotoxic effects protect against epibiont colonization, provided they occur on the animal's surface,[24] such as the marine fouling organisms.

This study characterized the incidental breakdown products of the sea squirt *Diplosoma listerianum* and its associated microorganisms. It uses their extracts in four assays with different structures and contents to study their biological-ecological and cell-biological effects. It provides evidence of interactions between sea squirts and their environment and isolates and characterizes the structures of their active substances based on biological activity tests.

MATERIALS AND METHODS

Experimental Procedure

The sea squirt *D. listerianum* was collected from the Arabian Gulf in 2019 (Figure 1). Identification of the squirt was done according to Ma, *et al.* (2019).[25] The squirts collected were frozen, dried, weighed, roughly crushed and extracted with methanol and dichloromethane (1:1 v/v). The crude extract was dissolved in methanol and fractionated in four solvents with increasing polarity: saturated hexane, acetic ester, butyl alcohol and water. Saturated hexane was used to separate non-polar substances and the inorganic substances and salts were isolated from the water phase. The acetic ester and butyl alcohol phases were enriched for polar incidental breakdown products. High-Performance Liquid Chromatography (HPLC) was performed using a diol column. Acetic ester or butyl alcohol phase separation was achieved using size exclusion chromatography with a Sephadex LH-20 column (PerkinElmer®, Waltham, Massachusetts). Small amounts of the pre-separated extract were purified by subjecting polar fractions to vacuum and low-pressure liquid chromatography. HPLC served as the preparative mixture separation and qualitative and quantitative analysis.

Nuclear Magnetic Resonance (NMR)

NMR spectra were obtained using a Bruker AC 400 (Nanalysis Corp.®, Tokyo, Japan) operating at 400 MHz at the Hydrogen frequency (^{1}H) and 100.10 MHz at the Carbon frequency (^{13}C) on the FFCLRP to ascertain the chemical structure of the isolated extract. Samples were prepared for analysis using methyl alcohol, Dimethyl Sulfoxide (DMSO-d_6), Deuterated Chloroform (CDC_{l3}), deuterated acetonitrile (MeCN-d_3) and deuterated benzene (C_6D_6). The standard internal reference was tetramethylsilane ≥99.0% (gas chromatography).[26,27]

Figure 1: The northern coast of Arabian Gulf in Saudi Arabia. The red pen indicates the study area.

Ultraviolet-visible (UV-vis) and Infrared (IV) Spectra

UV-vis spectra were obtained with a Hitachi U-3210 spectrophotometer in the 200-500 nm range (Hitachi®, USA). The samples were prepared in spectroscopic ACS-grade 99.9% methanol (Sigma-Aldrich Merck). IV spectra were obtained using a Bomem MB-102 Fourier-transform infrared spectrophotometer (Sigma-Aldrich Merck). The samples were dissolved in a small amount of appropriate volatile solvent and applied as a film on a silicon disc, evaporating the solvent under a vacuum in a desiccator.[28,29]

Mass Spectrometry (MS) and Optical Rotation $(\alpha)_D$

Mass spectra were obtained using fast atom bombardment MS, Electron Impact MS (EIMS), chemical ionization MS and Electron Spray MS (ESI-MS). Low-resolution mass spectra were obtained using a VG-7060 manometer (Trade India®, New Delhi) working at 75 eV. High-resolution mass spectra were obtained at a nominal resolution of 5000-10000. Perfluorquerosene, 3-nitrobenzyl alcohol, or polyethylene glycols were used as the internal standard. High-resolution mass spectra were collected using a Bruker-Hewlett Packard 1100 Esquire Spectrometer (ESI quadrupole ion trap MS) and a Bruker Daltonics Ultra-TOF spectrometer (collision gas N_2). Optical rotation measurements were obtained using a Perkin-Elmer 241 Polarimeter (DQ-UFSCar) or a Perkin-Elmer Model 341 polarimeter at 589 nm (UNICAMP). HPLC-mass spectrometry was used to spectrometrically characterize the mixture.

HPLC

The system used comprised a Waters system with two pumps (model 600), an in-line Degasser degasser, a UV-vis spectrophotometric detector reading at two wavelengths (model 2487), a recorder (model 746) and a Controller 600 control system. The Waters chromatographic system comprised the system control panel (model 2695) coupled to a UV-vis spectrophotometric detector (model 2996) with a photodiode array detector (observing 200-800 nm wavelengths), a light scattering detector (model 2424) and a Micromass ZQ MS detector[30] (Shimadzu Europa GmbH®, Germany).

Bioassays

Four different bioassays were performed in the laboratory to provide insight into the ecological function of incidental breakdown products and information about the possible pharmacological effects of the sea squirt incidental breakdown products.[31]

Evaluation of Antimicrobial Potential

The following microbial samples were used: *Cryptococcus neoformans* (ICC 107 - sorotipo B), *Candida albicans* (American Type Culture Collection [ATCC] 10231), *Micrococcus luteus* (ATCC 9341), *Herpetomonas samuelpessoai* (ATCC 30252), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 11778), *Enterobacter aerogenes* (ATCC 13048), *Escherichia coli* (ATCC 8739), *Proteus mirabilis* (ATCC 25933), *Klebsiella pneumonia* (ATCC 13883), *Saccharomyces cerevisiae* (ATCC 2601), *Pseudomonas aeruginosa* (ATCC 25619), *Staphylococcus aureus* (ATCC 6538), *Salmonella typhimurium* (ATCC 14028), *Streptococcus pyogenes* (ATCC 19615) and *Staphylococcus epidermidis* (ATCC 12228). Müller-Hinton broth agar (Sigma-Aldrich Merck®) containing 20 mg/L β-nicotinamide adenine dinucleotide (MH-F) was used. The MH-F plates were dried before inoculation (20-25°C overnight) with the cap removed for 15 min to reduce veil (swarming). Microplates were incubated at 37°C for 24 hr and microbial growth was evaluated by reading the optical density (620 nm) in an Enzyme-linked Immunosorbent Assay (ELISA) reader.[32,33]

Antimicrobial Assay

The antimicrobial activity of sea squirt extracts was determined using agar diffusion assays. This assay is an easy method for assessing the effects of different extracts on various bacterial strains. Sixteen bacterial strains were used in this experiment, which came from different habitats and differed in morphology and physiology. Extracts were examined for their antimicrobial activity, which was assessed based on inhibition zone characteristics. The sea squirt extracts were applied to the filter sheets and incubated with the plate. The sea squirt tunic was quickly covered by a biofilm, whose physical and chemical properties influenced settlement. The capacity of this biofilm composition with antimicrobial substances represents an opportunity for sea squirts to control the tunic to a limited extent.

Phytagel Assay

The sea squirt extracts from eight gel plates were tested on 12 phytagel plates (Gel Press®, USA) to investigate the settlement-inhibiting ingredients sea squirts release into their ecosystem. In addition, five other gel plates mixed with methanol were used as the control. Water movement guarantees slight sedimentation and a good supply of the sessile fauna with nutrients and extensive dispersion of their pelagic larvae. The phytagel plates were placed on seven ladders embedded in the jetty wall and mounted at a depth of 5-6 m. The plates were examined after nine, 12 and 18 days of incubation to increase the substance's diffusion rate from the gel substrate. The possible anti-fouling effects of the incidental breakdown products were documented.

The sea squirt extracts were tested for their colonization-inhibiting effects by pouring them into Phytagel plates (Sigma-Aldrich) and inserting them into the estuary in their natural ecosystem. The vegetation grew after a defined time. Eight plates (20×28 cm) were fitted with twelve phygels. The gels were poured into plastic Petri dishes (volume 28 mL) that were then placed between two

pinched plastic sheets. The lower plastic plate was placed below each petri dish (~1 mm depth, ∅ 26 mm). Through a hole in the bottom of the Petri dishes (∅ 1 cm), a Phytag anchor could be used to fix the gel in place. For three parallel extracts, 90 mg of extract was weighed and dissolved in 1.5 mL of methanol in an Eppendorf reaction vessel.[34]

Cytotoxic Assay

The samples were tested for their cytotoxic effects using an antimicrobial assay. The effects of crude extract on mouse subcutaneous fat tissue fibroblasts (cell line L929/A) (Catalent®, USA) from C_3H/An mice (connective tissue, adriamycin, doxorubicin, drug-resistant; stored at -196ºC) were examined. The cytotoxic activity was assessed using the extract's Minimum Inhibitory Concentration (MIC), with a MIC <20 µg/mL considered a relevant cytotoxic effect. Like its antimicrobial activity, the cytotoxic activity of the crude extract isolated from the sea squirt is a protective mechanism. Target organisms are neighboring sessile invertebrates competing for space and food and predators such as starfish and slugs. Intuitive compounds with cytotoxic activity are of pharmacological significance since they represent potential new agents for tumor therapy.

Protein Tyrosine Kinase (PTK) Assay

The extract's PTK-inhibiting activity was assessed using ELISA. This test was performed using a commercial PTK assay (PTK-101; Sigma-Aldrich®). The microtiter plate wells were washed overnight with a synthetic Poly-Glu-Tyr substrate. This substrate contains multiple phosphorylated tyrosine residues and can be replaced by one PTK type. After incubation, excess substrate was removed by washing the wells and drying the plate. The Epidermal Growth Factor Receptor (EGFR) enzyme was diluted in tyrosine kinase buffer containing magnesium, manganese and adenosine triphosphate. EGFR, buffer and the substance being tested were added to the wells and incubated according to the manufacturer's instructions. After incubating and washing the wells, the activity was assessed by adding a phosphotyrosine-specific monoclonal antibody that binds to the phosphorylated tyrosine residues. The antibody was conjugated with peroxidase. When the o-phenylenediamine dye was added, the resulting staining was measured using an ELISA reader (Anthos Lucy 1).

Scanning Electron Microscopy (Hitachi High-Tech® America, Inc.)

The treated mouse fibroblasts were fixed with Karnovsky's solution (4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer [pH 7.4]) for 3 hr at 4ºC. Next, they were washed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 1% osmium tetroxide and 0.8% potassium ferricyanide for 2 hr at room temperature. Then, the samples were dehydrated using increasing ethanol concentrations (30%-100%) and dried at a critical point in CO_2.

Transmission Electron Microscopy

The treated mouse fibroblasts were fixed in Karnovsky's solution for 3 hr at 4ºC. Next, they were washed in 0.1M sodium cacodylate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide for 2 hr at room temperature. Increased serial alcohol dehydration and embedded in Epon 812. Semi-thin sections were stained with 1% toluidine blue. The ultrafine cuts were contrasted with uranyl acetate and lead citrate and subsequently analyzed and imaged using a Zeiss 900 transmission electron microscope (Thermo Fisher® Scientific Inc.).

RESULTS

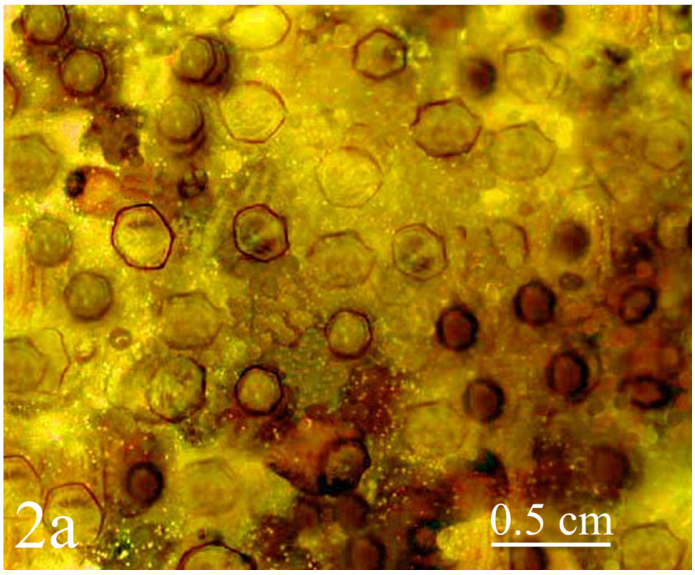
The *D. listerianum* colony measured 3-6 cm in diameter and between 1-2 mm in thickness (Figure 2a). Zooids were distributed randomly without forming organized systems. The colony can grow extensively, forming soft, gelatinous sheets that measure 1-3 mm in thickness and up to several cm across. The tunic lacks calcareous spicules, but often possesses white pigment flecks. This ascidian is often translucent and either colorless or with a gray, light green, or tan hue and forms transparent gelatinous colonies on algae. The small zooids are scattered densely throughout the sheet. It was first described from the English Channel in 1841 by Milne-Edwards, but similar species were subsequently found and given many different names around the world, most of which were eventually reduced to synonyms. *D. listerianum* is almost certainly a global complex of an unknown number of different species, to which some of these submerged names may apply. Because of this confusion and the fact that it was already established in much of its range when researchers began surveying for tunicates. The crude *D. listerianum* extract aliquot showed antibiotic activity against all microbial samples used in this study. Bacteriostatic action was established based on longitudinal *in vitro* growth of strains exposed to the crude extract during four exposure times. The cumulative distribution of average bacteriostatic activities in Müller-Hinton broth agar was estimated for exposure times of 1, 3, 5 and 15 min (Figure 2b). One-way Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) tests showed significant differences among exposure times at a critical value of 0.001.

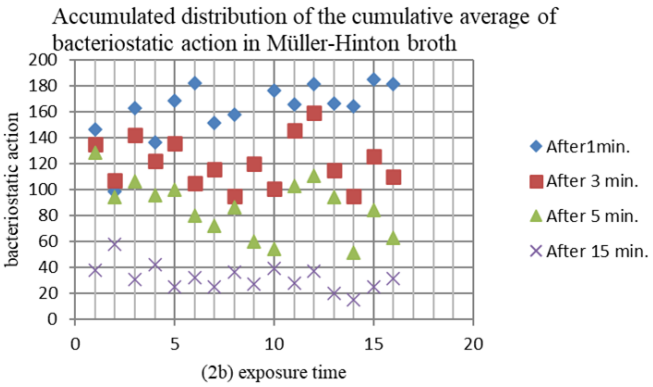
The sea squirt crude extract was assessed for its cytotoxic potential by incubation with mouse fibroblasts. The extracts were used in a series of dilutions and living fibroblast cells were differentiated from dead cells using the MT test. A MIC (in µg/mL) was determined for each extract dilution. The saturated hexane phase showed a MIC of 0.5 µg/mL, indicating cytotoxic potential. The acetic ester phase showed a significantly lower MIC of 36 µg/mL. The crude extract's saturated hexane phase was separated using preparative HPLC. Fractions obtained between 45 and 46 min

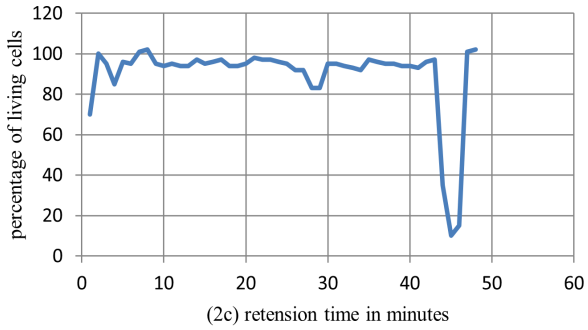
showed cytotoxic activity (Figure 2c). All 17 factions influenced mouse fibroblast growth. Fraction L5 had a MIC of 2 µg/mL and fractions L1 and L11 had a MIC of 185 µg/mL.

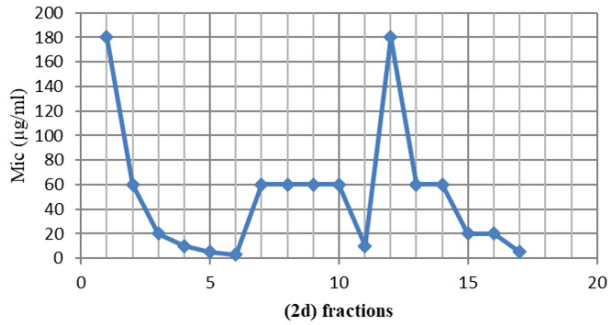
Six cell nucleus fractions were identified (Figure 2d). The field study shows that green algae slightly affected gels with sea squirt extracts. The settlement of sessile epibionts was attributed to heavy algae overgrowth. It was shown that the gels represent a soft and resistant matrix that serves as a suitable substrate for various invertebrate larvae to settle. The rapid and massive colonization of the phytagel with algae spores was observable after five days. The gels were examined at 3-6 day intervals to check growth and incubation progress. The experiment's incubation ended after 30 days because algal growth increased significantly. The controls were the least algally overgrown. Average hydrozoan, copepod, amphipod, polychaete and sessile organism (e.g., mussels) numbers were significantly higher than controls, with 25±34 per gel, mainly due to increased epibiont settlement. The colonization of control gels was significantly lower ($p<0.05$, one-way ANOVA and Tukey's HSD).

The increase in the epibiont population and the appearance of new species showed that the main invertebrate larvae distribution is only in July and August. This assumption is supported by the fact that gels with a degree of fouling were settled by significantly more sessile organisms than equally overgrown un-fouled gels (Figure 2e). The associated epibionts were counted by placing the polyvinyl chloride plates in a running water basin and removing the gels one by one. The 793 epibionts on 25 gels (28±34 per gel) were significantly larger in the second than in the first experiment (482 epibionts on 25 gels; 18±7 per gel). Bacteria are the first organisms to colonize a free surface in a marine habitat. The resulting biofilm colonizes fouling organisms such as bryozoans, hydrozoans and polychaetes.









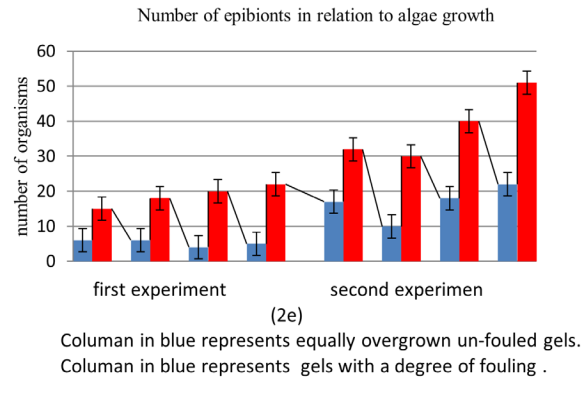


Figure 2: 2a. Photomacrograph of the colony of the jelly crust sea squirt Diplosoma listerianum. 2b. Shows the accumulated distribution of bacteria in Müller-Hinton broth. 2c. Shows the cytotoxicity of the fractionated n-hexane phase. 2d. Shows the six fractions of the cell nucleus in ethyl acetate phase. 2e. Shows the number of epibionts settlement on agar plates during field study.

Mouse fibroblasts exposed to the crude extract showed atypical cell nuclei, endoplasmic reticulum and microtubule characteristics in the cytotoxic test. Healthy fibroblasts predominantly contain mitochondria, rough endoplasmic reticulum, free or rosette ribosomes, lysosomes and Golgi apparatus. After extract exposure, they showed fragmented and sickle-shaped cell nuclei (mostly crescent in shape), visible centrosomes, abnormal multipolar mitotic spindles and endoplasmic reticulum vacuolization, indicative of apoptosis. Classic apoptosis morphological characteristics include genomic DNA condensation into crescent shapes. Programmed cell death is an important prerequisite for regulating tissue regression and eliminating defective cells. The exposed fibroblasts were rich in the rough endoplasmic reticulum with dilated cisterns and abundant ribosomes. There were free ribosomes and globular mitochondria with irregularly arranged ridges. The fibroblasts' ultrastructure indicated active protein synthesis with marked smooth endoplasmic reticulum hyperplasia and visible peroxisomes. Some fibroblasts showed mitochondrial changes and the smooth endoplasmic reticulum was undergoing involution, suggesting necrosis. Fibrillar material accumulation was observed in some fibroblasts. Some fibroblasts showed autophagic activity in the supra-nuclear cytoplasm. Endosymbiotic, possibly gram-negative, bacteria were observed in the culture (Figure 3).

Fraction 4, collected after purification by preparative HPLC and Sephadex column chromatography, contained a yellow-orange compound as a pure substance. Its molecular formula was $C_{40}H_{56}OA$ and its molecular weight was 568.871 Da based on EIMS. The range of aromatic-olefinic protons in the ^{1}H NMR spectrum (Figure 4a) included three multiplets (δ=6.65-6.57, 6.25 and 6.15-6.08) with 4H, 2H and 6H intensities and one doublet (δ=6.36) with a 2H intensity. A multiplet of 2H intensity was present at δ=3.98, representing the chemical shift according to an adjacent oxygen atom. A doublet with 2H intensity was present δ=2.36. Furthermore, two multiplets (δ=2.05-1.99 and 1.78-1.72) and one triplet (δ=1.45) were present with one 2H intensity each. Three singlets with 12H and 6H intensities and again methyl groups were assigned to 12H at δ=1.96, 1.73 and 1.05.

The ^{13}C NMR spectrum (Figure 4b) indicated the area of sp2-hybridized carbon atoms on eleven signals. There was a signal shifted up-field for a carbon atom oxygen binding at δ=66.2, which had already been identified in the ^{1}H-NMR spectrum. High-resolution ESI-MS (HR-ESI-MS) of substance EL10X3 determined its empirical formula to be $C_9H_{11}N_3O_2$ (m/z=194.0922 [M]+Δm 1.1 ppm), from which six double bond equivalents were derived. EI-MS determined its molecular weight to be 193.202 Da. The ^{1}H chemical shifts (Figure 4c).

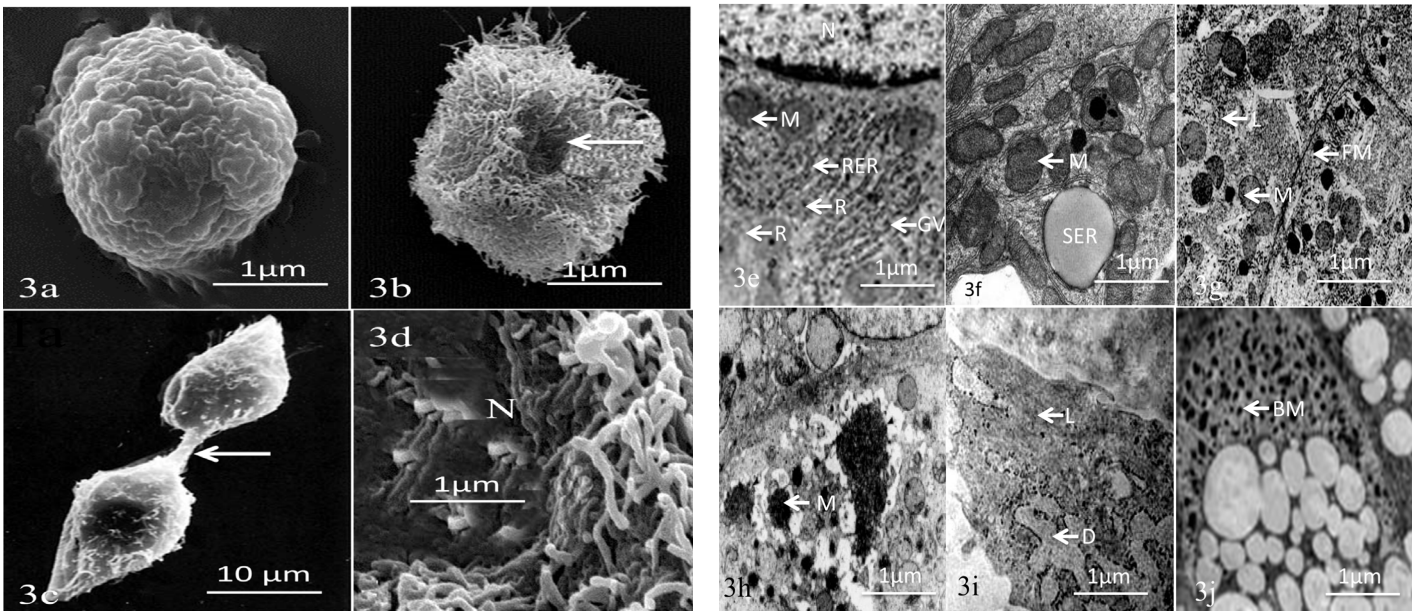


Figure 3: 3a. Photomacrograph of the colony of the jelly crust sea squirt Diplosoma listerianum. SEM and TEM of mouse fibroblast showing, 2a. SEM of a normal healthy fibroblast, before incubation in the crude extract. 3b. SEM of a fibroblast exposed to acetic ester phase 57 µg/mL extract shows sickle-shaped nucleus, mostly crescent in shape, indicated by an arrow. 3c. SEM of a fibroblast exposed to n-hexan phase 0.5 µg/mL extract shows active mitotic division, indicated by an arrow. 3d. SEM of a fibroblast exposed to acetic ester phase 57 µg/mL extract shows nuclear fragmentation. 3e. TEM of a fibroblast exposed to n-hexan phase 0.5 µg/mL extract shows rough endoplasmic reticulum, free and rosette ribosomes, lysosomes and Golgi vesicles. 3f. TEM of a fibroblast exposed to acetic ester phase 57 µg/mL extract shows mitochondrial deformities. 3g. TEM of a fibroblast exposed to acetic ester phase 57 µg/mL extract shows numerous lysosomes and defected mitochondria. 3h. TEM of a fibroblast exposed to acetic ester phase 57 µg/mL extract shows apoptosis of organelles. 2i. TEM of a fibroblast exposed to n-hexan phase 0.5 µg/mL extract shows advanced apoptosis. 3j. TEM of a fibroblast exposed to acetic ester phase 57 µg/mL extract shows intracellular debris and huge amount of lysosomes. BM: Bacteria; D: Debris; DC: Dilated Cisterns; FM: Fibrillar Material; GV: Golgi Vesicle; L: Lysosome; M: Mitochondrion; N: Nucleus; R: Ribosome; RER: Rough Endoplasmic Reticulum; SER: Smooth Endoplasmic Reticulum.

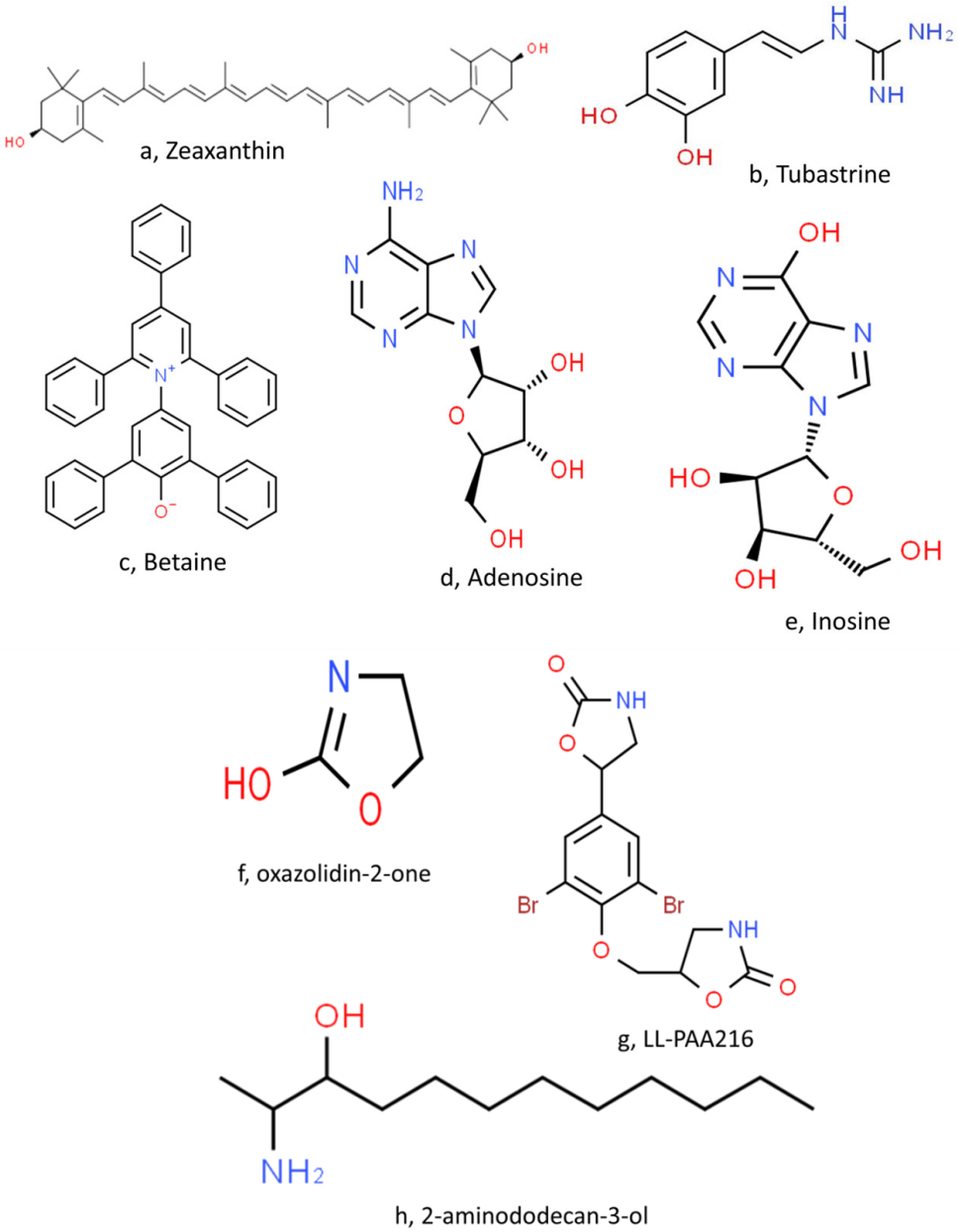


Figure 4: Different bioactive metabolites identified through NMR spectra: a. Zeaxanthin, b. tubastrine, c. betaine, d. adenosine, e. inosine, f. oxazolidin-2-one, g. LL-PAA216 and h. 2-aminododecan-3-ol.

The HPLC chromatogram for the acetic ester phase showed one striking UV signal with a retention time of 21 min. It was subjected to NMR and MS analyses due to its small amount and impurities. A comparison of retention times with known substances suggested the methylated amino acid N,N,N-trimethylglycine (betaine; Figure 4d). Oxazolidin-2-one was isolated in the AfAR-4B4B fraction and identified using the sample's ^{1}H NMR spectrum (DMSO-d_6, MHz; Figure 4e). The AfAR-4B4B fraction's ^{1}H NMR spectrum showed the following chemical shifts: a-singlet at δ=156.2 due to oxygen linked to aromatic ring sp2 carbons, a-singlet at δ=3.65 due to a hydroxide group bonded to sp3 carbon functionalized with oxygen and multiplets at δ=3.18 due to nitrogen linked to aromatic ring sp4 carbons. It can be naturally formed by combing ethanolamine with urea (Figure 4f). The obtained fraction's chemical shifts were compared with the literature. It was possible to suggest the relative stereochemistry of the chiral carbons in oxazolidin-2-one (Figure 4g) isolated in this study as being (7S, 11R) based on the measured values of [α] D (+9.4o, c=0.8) with those in the literature (-9.2o, c=0.25), where the isolated compound had the configuration (7R, 11S). The hydrolysis and standard compounds were treated separately with 1-fluorine-2,4-dinitrophenyl-5-L-alaninamide (Marfey Reagent). The absolute configuration of the compound LL-PAA216 was concluded (Figure 4h).

DISCUSSION

Incidental breakdown products, also called intuitive compounds, comprise bioactive molecules with diverse specific functions and structural groups.[35] Several incidental breakdown products have functions, including hormones, pigments, pheromones, signalers and chemical defenses.[36] Incidental breakdown products such as alkaloids, polyphenols, terpenes and acetogenins are found among marine organisms.[35-37] Sea squirts use incidental breakdown products to defend themselves from predators and bio-encrustation; for intraspecific and interspecific chemical host-epibiont and host-pathogen communication; reproduction; and for substrate, nutrients and light competition, making them essential to the marine ecosystem.[36,37] The production of incidental breakdown products can be altered by abiotic and biotic conditions such as light, temperature, nutrient availability, competition, the presence of predators and symbiosis.[38] The physical conditions and chemical interactions between marine invertebrates and microorganisms influence the settlement of latter on dead surfaces.[39]

Research on isolating biologically active compounds and exploring their mechanisms of action, syntheses and economically viable means for producing drugs, antibiotics, insecticides and herbicides has become increasingly necessary.[35,40-42] A negative correlation was found between the antimicrobial activity of extracts and epibiotic colonization in tropical and subtropical sponges and sea squirts. Sessile invertebrates do not generally ward off colonization by microorganisms but prevent it using chemical attractants or antibodies that regulate bacterial lawn composition.[43] Some bacteria strains induce increased growth or discoloration of bacterial colonies.[44]

While the number of studies on intuitive compounds isolated from microorganisms, mainly from fungi associated with tunicates, is few, the isolated metabolites have high biological activity indices.[45] For example, oxepinamides A-C were extracted from the fungus Acremonium sp. isolated from the sea squirt *Ecteinascidia turbinata*. Oxepinamide A showed considerable anti-inflammatory activity in a bioassay using the ears of rats with edema.[46] Didemnidae is considered one of the best sources of intuitive compounds with chemical diversity and potent biological activity. Several species of the Didemnum genus have already been chemically investigated, with a predominance of alkaloids observed.[47]

In vitro preclinical studies have shown the cytotoxicity of sea squirt extracts against several tumor cell lines and freshly explanted human tumors at concentrations <0.01 μM. Subsequent *in vivo* experiments in clinical phase I and II studies confirmed these results, showing tumor reductions in breast, skin, lung and uterine cancer. Ongoing phase III studies are intended to investigate the possibility of using Ecteinascidin 743 to determine combinatorial and sequential therapy.[48]

The cytotoxic activity of sea squirts extracts has long been known. *Didemnum molle, Botryllus schlosseri, Eudistoma toealensis*,[49] *Ascidia mentula* and *Styela plicata* are tunicates with cytotoxic and anti-proliferative activity. Didemnines are well-known cytotoxic substances from *Tridemnum solidum* and *Ecteinascidia turbinates*, including ecteinascidine, grossularine 1 and 2 and dendrodoin. From *Dendrodoa grossularia*, the alkaloid plakinidine D from *Didemnum rubem* and thiazole cyclodedimnamida B from *Didemnum molle* showed modest activity against human colon cancer cells. Moreover, two polypeptides (anteaters A and B) isolated from an unknown species of sea squirts showed potent cytotoxic activity.[50] Two aromatic alkaloids, granulatimide and isogranulatimide, were isolated from *Didemnum granulatum*.

Two compounds were also isolated from the crude extract of sea squirt Clavelina oblong: 5-[3,5-dibromo-4 ([2-oxo-5-oxazonidinyl)] methoxyphenyl]-2-oxazolidinone and (2S, 3R)-2-amino-3-dodecanol. These two compounds were isolated in this study from the colonial sea squirt *D. listerianum*. The latter showed potent antifungal activity against *Candida albicans*. This study showed that sea squirts of the genus Diplosoma are rich in biologically active incidental breakdown products. Their crude extract contains oxazolidin-2-one, LL-PAA216 and 2-aminododecan-3-ol diastereomers. Oxazolidinones are a new class of synthetic antimicrobial agents against multidrug-resistant Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus, Staphylococcus epidermidis*, penicillin-resistant *Streptococcus pneunomiae* and vancomycin-resistant enterococci.[51-54] (2S, 3R)-2-amino-3-dodecanol has been isolated from the sea squirt *Clavelina oblongata*. This compound shows potent antifungal activity against *Candida albicans* and moderate activity against *Candida glabrata*. The methanolic extract of *Phallusia nigra* (Savigny, 1816) contained eleven chemical constituents, of which the main components were 3-bromo-1-adamantane acetate, n-hexadecanoic acid, hexadecen-1-of acid and 2,6-dimethyl-6-trifluoroacetoxyoctane. However, studies on this genus's antimicrobial potential have not been performed.[55-58]

CONCLUSION

The crude extract of *D. listerianum* showed antibiotic activity against various pathogenic microorganisms. Its bacteriostatic action differed significantly among exposure times at a critical value of 0.001. Mouse fibroblasts exposed to it showed fragmented and sickle-shaped cell nuclei, visible centrosomes, abnormal multipolar mitotic spindles and endoplasmic reticulum vacuolization. Preparative HPLC, Sephadex column chromatography, HR-ESI-MS and EI-MS identified zeaxanthin, tubastrine (an EGFR inhibitor), inosine, guanosine, oxazolidin-2-one, LL-PAA216 and 2-aminododecan-3-ol.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ETHICAL STATEMENT

Ethical clearance for this study was obtained from Imam Abdulrahman Bin Faisal University ethics committee. The author of this study respected the value of the sea squirts. He considered sea squirts sentient creatures with the capacity to feel pain. His treatment of animals, including their use in research, expresses his attitudes and influences him as a moral actor. The experiments used the minimum number of sea squirts possible.

SUMMARY

The crude extract of *Diplosoma listerianum* showed antibiotic activity against a series of pathogenic microorganisms. The bacteriostatic action of the aliquot with respect to the exposure time shows that all critical values present a value of 0.001 which would indicate that there are significant differences among exposure times. The effect of the aliquot on mouse fibroblasts cell line showed nuclear fragmentation, sickle-shaped cell nuclei, visible centrosomes, abnormal multipolar mitotic spindles and vacuolization of the endoplasmic reticulum. Preparative HPLC, Sephadex column chromatography, HR-ESI-MS and EI mass spectra were applied to identify zeaxanthin, the EGFR inhibitor tubastrine, Inosine, guanosine, oxazolidin-2-one, LL-PAA216 and 2-aminododecan-3-ol.

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