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SCREENING OF MARINE ORGANISMS FOR ANTIMICROBIAL AND ANTIPROTOZOAL ACTIVITY

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ABSTRACT

Ethanol extracts from a group of 53 marine organisms, including a newly identified species, from Baja California Sur (México), were evaluated for their antimicrobial and antiparasitic activity. The activity against Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis *(Gram+),* Escherichia coli *(Gram−) and* Candida albicans *(yeast) was determined by the diffusion agar method.* Aplysina gerardogreeni *(Demospongiae) was found to be the most active sample. In addition,* A. gerardogreeni, Pacifigorgia media, *and* Pacifigorgia *sp. possess significant activity against* Mycobacterium tuberculosis $H_{37}Rv$ *and* Pacifigorgia media *and* Geodia *sp. against* Mycobacterium avium. *From this group, 15 ethanol extracts were tested* in vitro *against* Entamoeba histolytica *and* Giardia lamblia. Litotamnium crassiussculum, Geodia *sp.,* Pacifigorgia *sp. showed significant activity against* Entamoeba histolytica *while* Myxilla incrustans *and* Muricea appressa *were active against* Giardia lamblia. Litotamnium crassiussculum *showed activity against both trophozoites.*

INTRODUCTION

Infectious diseases remain leading causes of death among adults in much of the developing world. Of more than 50 million deaths worldwide in 1997, about one-third were caused by infections and parasitic diseases (WHO-Report, 1998). In Mexico, infectious diseases, including tuberculosis, have increased in recent years and are an important problem that requires resolution. In Baja California Sur, Mexico, these problems have also increased in recent years (Sistema Nacional de Epidemiología. SSA, 1999). Tuberculosis, magnified by increasing HIV infection and the emergence of multidrug resistant strains, continues to be the world's leading cause of death from a single infectious agent (Dye et al., 1999). Diseases caused by parasites occur in people and animals throughout the world. Little published data is available on the systematic evaluation of marine natural products for their antiprotozoal potential (Crews & Hunter, 1993). Marine natural products with activity against *Giardia lamblia* and *Entamoeba histolytica* are just emerging.

Marine organisms represent an enormous resource of natural products that remains unexplored with respect to the diversity of active substances. Evaluation of natural resources from Baja California Sur, particularly of their active natural compounds, is essential to develop new antibiotics and drugs for the treatment of infectious and parasitic diseases. Therefore, a group of marine organisms from this area were tested for their antimicrobial and anti-protozoan activities. The low cost and simple technique of the agar diffusion bioassay is advantageous in the determination of the antibacterial activities of crude extracts. This classic method requires that the substances tested be soluble in water, or can diffuse into agar. Accordingly, antibacterial activity was determined using the agar-diffusion method with filter paper discs (Encarnacion & Keer, 1991; Rios et al., 1988). Antimycobacterial bioassays were performed using the BACTEC 460 system

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(Cantrell, et al., 1998; Collins & Franzblau, 1997). *In vitro* antiprotozoal tests to determine IC_{50} values against *Entamoeba histolylica* HMI-IMSS and *Giardia lamblia* were carried out according to standard protocols (Cedillo-Rivera et al., 1992).

MATERIALS AND METHODS

Collection of Marine Organisms

The marine organisms were collected in the Complejo Insular La Partida-Espiritu Santo Island B.C.S. (México) by scuba diving. The collected samples were cleaned and one part of each sample was fixed in EtOH for identification. The remaining sample was kept frozen for extraction. Voucher specimens were retained at the Pharmacognosy Laboratory of the Marine Biological Department of Universidad Autónoma of Baja California Sur (U.A.B.C.S.), México. The identification of the samples was made by B. Carlos Sanchez and P.BM. Martín García Ramirez from the Marine Biological Department of UABCS, according to Brusca (1973), Verrill (1870), Matamoros (1984), Gomez and Bakus (1992), Vazquez (1994) and Prahl et al. (1986).

Preparation of Extracts

To prepare initial extracts for biological testing, one part of the material was macerated with about five parts of ethanol for 8 days. Since the purpose of this study was qualitative not quantitative, weight and volume of solvent were not recorded. The ethanolic extracts were evaporated at room temperature (no more than 40 °C) and the dry residue of each sample was again dissolved in the proper solvent for each bioassay.

Microorganisms and Inoculum Preparation

Bacillus subtilis, Streptococcus faecalis (Gram-positive), *Escherichia coli* (ATCC 25922) (Gram-negative) and *Candida albicans* (yeast) were supplied by SCRIPPS Institute of Oceanography of the University of California, San Diego, California, U.S.A. *Staphylococcus aureus* was supplied by Q.BP Ana Ma. Ramirez from Laboratorio de Analisis Especiales, La Paz B.C.S. Microorganisms were cultured in 5 ml of nutrient broth (Gram-positive and Gram-negative microorganisms) or 5 ml of Sabouraud broth (yeast) for 24 h at 37 °C and then adjusted to match the turbidity of a McFarland #5 standard. The growth and purity of each suspension was verified by using a Gram stain.

Mycobacterium tuberculosis $H_{37}Rv$ (ATCC 27294, American Type Culture Collection, Rockville, MD) was cultured at 37 °C on a rotary shaker in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% v/v glycerol and 0.05% v/v Tween 80 until the culture turbidity achieved an optical density of 0.45–0.55 at 550 nm. Bacteria were centrifuged and the pellet washed twice and resuspended in one-fifth the original volume in Dulbecco's phosphate-buffered saline (PBS, Irvine Scientific, Santa Ana, CA). Large bacterial clumps were removed by passage through an 8 μm filter (Nalgene, Rochester, NY) and aliquots were frozen at –80 °C.

M. avium (ATCC 25291) was cultured in BACTEC 12B broth until a daily GI of 999 was reached. Cultures were then diluted 1:25 in BACTEC 12B broth and frozen at –80 °C until needed.

Growth Medium

Screening by the agar diffusion method (ADM) was performed on plates of peptone agar (15–20% agar, pH 7.0–7.4), sterilized for 15 min at 120 °C. Approximately 20 ml of this medium was added to each 100 mm sterile Petri dish and kept for 24 h to control sterility.

Antibacterial Testing by the Agar Diffusion Method

All tests were performed by placing the disks (7 mm of diameter) impregnated with the ethanolic crude extracts (2 mg/disk) on the agar surface previously inoculated with a sterile loop containing the microbial suspension. Standard disks of chloramphenicol (30 μ/disk), erythromycin (15 μ/disk), and nalidixic acid (30 μ/disk) were used as reference (positive) controls. Disks with evaporated ethanol used for the preparation of plant extracts served as negative controls. Zones of inhibition were measured following incubation of plates at 37 °C for 24 h. The tests were made in duplicate.

Anti-Mycobacterial Bioassay

Antimycobacterial testing was performed using the BACTEC 460 system (Cantrell et al., 1998). Stock solutions and subsequent dilutions were prepared at 80× the final desired concentration in dimethylsulfoxide (DMSO) and sterilized by passage through 0.22 μm PFTE filters (Millex-FG, Millipore, Bedford, MA). Fifty μl of test samples were added to 4 ml of BACTEC 12B medium (Becton Dickinson). Controls received 50 μl DMSO, producing a final concentration of 1.25% v/v. Rifampin (Sigma Chemical Co., St. Louis. MO) and clarithromycin (Abbott Labs, North Chicago, IL) were included as positive drug controls for *M. tuberculosis* and *M. avium*. They were

solubilized and diluted to achieve a range of concentrations for determination of minimum inhibitory concentrations. Cultures were inoculated with approximately 4×10^5 cfu in a volume of 100 μl. For determination of percent inhibition, cultures were incubated at 37 °C and the growth index (GI; one GI unit = 0.25 nCi CO_2) determined daily in a BACTEC 460 instrument until control cultures achieved a GI of 999. Additional control vials were included which received a further 1:100-diluted inoculum of *M. tuberculosis* and *M. avium* for use in calculating the MIC of rifampin and clarithromycin by established procedures (Inderlied & Nash, 1996). Assays were usually completed in 5–8 days for *M. tuberculosis* and 6 days for *M. avium*. Percent inhibition was defined as 1 − (GI of test sample/GI of control) × 100. For determination of MIC, the GI was determined daily until the GI of the 1:100 controls was at least 30. All vials were read the following day and the daily change in GI (ΔGI) recorded for each drug dilution. The MIC was defined as the lowest concentration for which the ΔGI was less than the ΔGI of the 1:100 control (Inderlied & Nash, 1996).

Parasites

Entamoeba histolytica was maintained in TYI-S-33 medium, supplemented with 10% bovine serum (Diamond & Bartgis, 1971), and *Giardia lamblia* was cultured in TYI-S-33 modified medium, supplemented with 10% calf serum (Cedillo et al., 1991). Both strains were maintained in axenic conditions and for the assays were employed in the log phase of growth.

Antiprotozoal Assay

In vitro testing against *E. histolytica* and *G. lamblia* was done using a method previously described (Cedillo-Rivera et al., 1992; Cedillo-Rivera & Muñoz, 1992). Each test extract (100 mg) was dissolved in 1 ml of DMSO and 19 ml of culture medium and incorporated in disposable tubes with 4 ml of medium to obtain the required range of concentration, 2.5–200 μg/ml. The tubes containing the extract-incorporated medium were inoculated with *E. histolytica* HMI MSS to achieve an inoculum of 6×10^3 trophozoites/ml or with *G. lamblia* IMSS:0989:1 to achieve 5×10^4 trophozoites/ml. Each test included metronidazole (Sigma) as standard amoebicidal and giardicidal drug, a control (culture medium plus trophozoites and DMSO) and a blank (culture medium). After incubation for 48 h at 37 °C, trophozoites were detached by chilling. Fifty μl from each culture tube was subcultured in fresh medium and inoculated by another 48 h before counting. The final number of parasites was determined with a haemocytometer and the percentages of trophozoite growth inhibition were calculated by comparison with the control culture. The results were confirmed by a colorimetric method. For this, the trophozoites were washed by centrifugation and incubated for 45 min at 37 °C in phosphate-buffered saline with 0.075% of MMT (3-[4,5-dimethylthiazol-2-i1]-2, 5-diphenyl tetrazolium bromide) and 250 mg of phenazine methosulfate. The dye produced (formazan) was extracted with HCl/I-PrOH and the absorbance was determined at 570 nm. In both cases, the percentage of inhibition calculated for each concentration was transformed into probit units.

The plot of probit against log concentration was made. The best straight line was determined by regression analysis. The 50% inhibitory concentration (IC_{50}) values were calculated. The experiments were performed in duplicate and repeated at least three times.

RESULTS AND DISCUSSION

The antimicrobial activities of 53 marine organism extracts are reported in Table 1. From this group, 34 samples were identified to species and 10 samples were identified to the genus level. *Muricea* sp. (RED 9630) is a new species. Two samples (RED 9625 and RED 9626) belong to the Palaemonidae. Two samples are corals from the class Anthozoa (RED 9612 and RED 9639A). Four more samples were difficult to identify and the activity of these samples is reported in the group of species not identified.

The most active samples against Gram-positive bacteria belong to the Desmospongiae (Table 1). *Aplysina gerardogreeni* (RED 9660) had the highest activity against *S. aureus, B. subtilis, S. faecalis*. *E. coli* as well as being inhibitory for *M. tuberculosis* at 300 μg/ml. Within this group were three other samples also identified as *A. gerardogreeni* (RED 9601, RED 9613, RED 9616). Sample RED 9601 was active against *M. tuberculosis* (99% inhibition at 300 μg/ml) but not against the other bacteria. Although both were identified as *A. gerardogreeni*, samples RED 9613 and RED 9616 displayed different antimicrobial activities. Since wet samples were used for extraction, it is possible that the differences in activity might be explained by differences in water content which could in turn influence the extraction and consequently the concentration of the active components in the ethanol extracts. Another difference among the samples was the specific sites within

Table 1. Antimicrobial activity of ethanol extracts from marine organisms from Baja California Sur (México).

Scientific Name (Col. Number)	Agar Diffusion Method[ab]					BACTEC 460[bc]			
						300	100	300	100
						μg/ml			
	A	B	C	D	E	F		G	
Class demospongia									
Aplysina aff. *Aztecus* (RED-9640)	++	–	–	–	–	7	7	0	0
Aplysina fistularis (RED-9658)	–	–	–	–	–	31	0	0	0
Aplysina gerardogreeni (RED-9601)	–	–	–	–	–	99	89	16	0
Aplysina gerardogreeni (RED-9613)	–	+	–	–	–	13	13	0	0
Aplysina gerardogreeni (RED-(9616)	+	+	–	+	–	55	8	0	0
Aplysina gerardogreeni (RED-9660)	++++	+++	+	+++	–	92	9	0	9
Callyspongia sp. (RED-9614)	–	–	–	–	–	8	0	0	0
Clathria microjoanna (RED-9650)	–	–	–	–	–	90	63	0	0
Geodia sp. (RED-9644)	–	+	–	–	–	32	0	79	5
Myxilla incrustans (RED-9649)	–	–	–	–	–	96	75	0	0
Plocamia mannarensis (RED-9654)	–	–	–	–	–	96	76	0	0
Tethya aurantia (RED-9604)	–	–	–	–	–	52	19	0	0
Class anthozoa									
Antipathes galapagensis (RED-9633)	–	–	–	–	–	n.t[d].	n.t.	n.t.	n.t.
Antipathes galapagensis (RED-9646)	–	–	–	–	–	80	45	0	0
Antipathes sp. (RED-9632)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Antipathes sp. (RED-9655)	–	–	–	–	–	54	34	0	0
Epizoanthus sp. (RED-9602)	+	–	–	–	–	64	31	0	0
Epizoanthus sp. (RED-9606)	++	–	–	–	–	17	3	0	0
Eugorgia daniana (RED-9656)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Eugorgia multifida (RED-9637)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Eugorgia multifida (RED-9645)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Coral (sp. n.i.) (RED-9612)	–	–	–	–	++	n.t.	n.t.	n.t.	n.t.
Coral (sp. n.i.) (RED-9639A)	++	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Muricea appressa (RED-9634)	+	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Muricea appressa (RED-9648)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Muricea appressa (RED-9657)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Muricea appressa (RED-9659)	–	–	–	–	–	74	42	0	0
Muricea austera (RED-9628)	+	–	–	–	–	50	36	0	0
Muricea fructicosa var. *misser* (RED-9631)	–	–	–	–	–	75	48	1	0
Muricea sp. *(*new sp.) (RED-9630)	+	–	–	–	–	87	52	0	32
Muricea sp. (RED-9638)	–	–	–	–	–	73	37	0	0
Pacifigorgia media (RED-9642)	–	–	–	–	–	99	98	80	39
Pacifigorgia sp. *1* (RED-9635)	–	–	–	–	–	96	83	9	0
Pacifigorgia sp. *2* (RED-9636)	–	–	–	–	–	97	97	7	0
Pocillopora elegans (RED-9618)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Pocillopora elegans (RED-9622)	–	–	–	–	–	53	0	12	0
Zoanthus danae (RED-9621)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Class ascidaceae									
Archidistoma pachecae (RED-9603)	–	–	–	–	–	0	0	0	0
Class asteroidea									
Leiaster teres (RED-9609)	++	–	–	–	–	82	8	21	0
Mithrodia enriquetacasoni (RED-9652)	–	–	–	–	–	10	0	0	0
Narcissia gracilis (RED-9610)	++	++	–	–	–	0	0	13	0
Pharia pyramidata (RED-9607)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Phataria unifascialis (RED-9608)	–	–	–	–	–	0	0	17	0
Class malacostraca									
Alpheus sp. (RED-9624)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Manucuplanus varians (RED-9639)	++	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Family: Palaemonidae. sp.1 (RED-9625)	+	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Family: Palaemonidae. sp.2 (RED-9626)	+	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Trapezia ferruginea (RED-9623)	–	–	–	–	–	n.t.	n.t.	n.t.	n.t.
Class floridophyceae									
Litotamnium crassiussculum (RED-9641)	–	+	–	–	–	23	11	0	0
Not identified species									
Bryozoan (RED-9605)	++	+	–	–	–	n.t.	n.t.	n.t.	n.t.

Table 1 continues

Table 1. (continued).

Scientific Name (Col. Number)	Agar Diffusion Method[ab]					BACTEC 460[bc]			
						300	100	300	100
						μg/ml			
	A	B	C	D	E	F		G	
Bryozoan (RED-9611)	–	+	–	–	–	98	78	46	0
Bryozoan (c.f.) (RED-9615)	–	–	–	–	–	88	40	0	0
Bryozoan (c.f.) (RED-9620)	+++	++	+	–	–	n.t.	n.t.	n.t.	n.t.

a) Grading of results: –, no inhibition; +, zone of inhibition less of 10 mm in diameter; ++ zone of inhibition of 10 to 15 mm in diameter; +++, zone of inhibition 15 to 20 mm in diameter; ++++, more than 20 mm.
b) Test Organisms: A, *Staphylococcus aureus*; B, *Bacillus subtilis*; C, *Streptococcus faecalis*; D, *Escherichia coli*; E, *Candida albicans*; F, *Mycobacterium tuberculosis* H_{37}Rv(ATCC 27294); G, *Mycobacterium avium* (ATCC 25291)
c) Percent of inhibition determined at 300 μg/ml and 100 μg/ml. Rifampin 0.25 μg/ml, showed 99% inhibition of *M. tuberculosis. Clarithromycin* 0.5 μg/ml, showed 99% inhibition of *M. avium*
d) n.t.: not tested.

Table 2. *In vitro* antiprotozoal activity of ethanol extracts from marine organisms from Baja California Sur (México).

Scientific name	IC_{50} μg/ml (95% confidence limits) *E. histolytica*	*G. lamblia*
Class Demospongia		
Aplysina gerardogreeni (RED 9613)	265 (261–269)	400 (399–401)
Clathria microjoanna (RED 9650)	209 (205–213)	139 (134–139)
Geodia (RED 9644)	47 (46–47)	218 (217–219)
Myxilla incrustans (RED 9649)	253 (249–258)	53 (53–53)
Plocamia mannarensis (RED 9654)	157 (155–159)	206 (205–206)
Class Anthozoa		
Anthipates galapagensis (RED 9646)	159 (157–160)	173 (173–173)
Epizoanthus (RED 9602)	95 (95–96)	143 (143–144)
Epizoanthus sp (RED 9606)	340 (331–349)	174 (174–175)
Muricea appressa (RED 9659)	178 (176–181)	70 (70–71)
Muricea austera (RED 9628)	317 (311–324)	100 (100–100)
Muricea sp (new sp.) (RED 9630)	105 (104–105)	376 (374–378)
Pacifigorgia sp. 2 (RED 9636)	72 (71–72)	213 (212–214)
Class Ascidaceae		
Archidostoma pachecae (RED 9603)	387 (379–395)	130 (130–131)
Class Floridophyceae		
Litotamnium crassiusculum (RED 9641)	42 (42–43)	92 (92–93)
Not identified species		
Bryozoa (RED 9611)	181 (179–183)	191 (190–191)
Metronidazole	0.04	0.21

the Espiritu Santo Islands where they were collected. The samples identified as *Geodia* sp. (RED 9644) showed slight activity against *B. subtilis* but had significant activity against *M. avium* (79% of inhibition at 300 μg/ml).

Two other sponges, *Myxilla incrustans* (RED 9649) and *Plocamia mannarensis* (RED 9654), showed significant activity against *M. tuberculosis*.

Among the Anthozoa, the amount of the ethanol extract available from 13 samples was insufficient for screening against the mycobacteria. The extract of the coral (RED 9612) was active against *C. albicans.* The coral (RED 9639A) and *Epizoanthus* sp. (RED 9606) were active against *S. aureus.* Slight activity was shown against *S. aureus* by *Epizoanthus* sp. (RED 9602), *Muricea appressa* (RED 9634), *Muricea austera* (RED 9628) and *Muricea* sp. (new sp.) (RED 9630). High activity against *M. tuberculosis* was observed with *Muricea* sp. (RED 9630), *Pacifigorgia* sp. 1 (RED 9642) and *Pacifigorgia* sp. 2 (RED 9683). *PacifIgorgia* sp. (RED 9642) showed significant activity against both *M. tuberculosis* and *M. avium.*

Fifteen ethanol extracts were tested against axenically grown trophozoites of *E. histolytica* and *G. lamblia*. The

results are summarized in Table 2. The extracts from *Pacifigorgia* sp. 2 (RED 9636*), Litotamnium crassiusculum* (RED-9641), *Geodia* (RED-9644) and *Epizoanthus* (RED 9602), were the most active samples against *E.histolytica* with IC_{50} values from 42 to 95 μg/ml. *Muricea appressa* (RED-9659), and *Myxilla incrustans* (RED-9649) were the most active samples against *G. lamblia* with IC_{50s} between 53 to 70 μg/ml. From this group, *L. crassiussculum* had significant activity against both trophozoites.

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REFERENCES

Brusca RC (1973): *Common Intertidal Invertebrates of the Gulf of California*. p. 513, University, USA: Arizona Press.

Cantrell CL, Fischer NH, Orbatch L, McGuire MS, Franzblau SG (1998): Antimycobacterial crude plant extracts from South, Central, and North America. *Phytomedicine 5:* 139–147.

Cedillo-Rivera R, Enceso-Moreno JA, Martinez-Palomo A, Orega Pierres G (1991): Isolation and axenization of *Giardia lamblia* isolates from symptomatic and asymptomatic patients in Mexico. *Arch Invest Med (Mex) 22:* 79–85.

Cedillo-Rivera R, Ramirez A, Muñoz O (1992): A rapid colorimetric assay with the tetrazolium salt MTT and phenazine methosulfate (PMS) for viability of *Entamoeba histolytica*. *Arch Med Res 23:* 59–61.

Cedillo-Rivera R, Muñoz O (1992): *In vitro* susceptibility of *Giardia lamblia* to albendazol, mebendazol and other chemotherapeutics agents, *J Med Microbiol 37:* 221–224.

Collins L, Franzblau SG (1997): Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob Agents Chemother 41:* 1004–1009.

Crews P, Hunter LM (1993): The search for antiparasitic Agents from Marine Animals in Marine Biotechnology. Vol. I. In: *Pharmaceutical and Bioactive Natural Products* DH Attaway, OR ans Zaborsky, (eds), NY, London: Plenum Press.

Diamond LS, Bartgis IL (1971): Axeninic cultures *in vitro* testing of drugs against *Entamoeba histolytica*. *Arch Invest Med (Mex)2 (Suppl. 1):* 339–343.

Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC (1999): Consensus statement. Global burden of tuberculosis: Estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA 282:* 677–686.

Encarnación DR, Keer S (1991): Antimocrobial screening of medicinal plants from Baja California Sur (Mexico). *J Ethnopharmacol 31:* 181–192.

Gomez P, Bakus GJ (1992): *Aplysina gerardogreeni* and *Aplysina aztecus* (Porifera; Desmospongiae). New species from the Mexican pacific. *Anales del Instituto de Ciencias del mar y Limnologia. UNAM 19(2):* 175–180.

Inderlied CB, Nash KA (1996): Antimycobacterial agents: *In vitro* susceptibility testing, spectra of activity, mechanisms of action and resistance, and assay for activity in biological fluids. pp. 127–175. In: *Antibiotics in Laboratory Medicine*. V. Lorian (ed.), Baltimore: Williams and Wilkins.

Matamoros R (1984): Sistematica y Distribución de Corales blandos de la Bahía de Mazatlan, Sin (México). p. 112. Tesis Profesional. México.

Prahl HV, Escobar D, Molina YG (1986): Octocorales de aguas someras del Pacifico Colombiano. *Revista Biol Trop 34 (1):* 13–33.

Rios JL, Recio MC, Villar A (1988): Screening methods for natural products with antimicrobial activity: A review of the literature, *J Ethnopharmacol 23:* 127–149.

Sistema Nacional de Epidemiologia (1999): Secretaria de Salud y Asistencia Pública. México. *EPI 16 (1).*

Vazquez L (1994): Estudio Taxónomico y Distribución de las esponjas del Pacífico Mexicano de los Estados de Nayarit Michoacan y Guerrero, México. Tesis. UABCS B.C.S. (México)

Verrill AE (1870): *Notes on Radiata in the Museum of Yale College*. p. 590. New Haven, USA.

World Health Organization. World Health Report. Executive Summary (1998): pp. 2–27.

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