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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF MICROORGANISMS ASSOCIATED WITH MARINE INVERTEBRATES FROM THE MOROCCAN ATLANTIC COAST.

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Abstract

In vitro antibacterial screening of bacterial strains isolated from marine invertebrates, collected from the Moroccan Atlantic coast, against human pathogenic bacteria was conducted in this study. However fourteen marine microorganisms were tested against three Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus sp.* and *Enterococcus faecalis*) and three Gram negative bacteria (*Escherichia coli*, *Pseudomonas fluorescens* and *Pseudomonas sp.*) using the agar disk-diffusion and the agar cylinders methods. The identification of the isolates shows that are belong to the genera *Enterobacter*, *Morganella*, *Aeromonas*, *Pantoea*, *Kluyvera*, *Raoultella*, *Stenotrophomonas*, *Pseudomonas*, *Sphingomonas* and *Staphylococcus* which indicates a diversification in the marine microflora. The evaluation of antibacterial activity of these isolates on human pathogenic bacteria shows that all strains are active against at least one pathogen studied for the two used methods. For the agar cylinders method, six isolates are showing a significant antibacterial activity with inhibition zone diameters greater or equal to 15 mm (*Aeromonas sobria*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia*, *Staphylococcus capitis*, *Pantoea sp3* and *Raoultella ornithinolytica*). For the agar disk-diffusion method, among 28 extracts of the marine isolates, five extracts which are active against two pathogenic bacteria (*Pseudomonas sp* and *Enterococcus faecalis*) with inhibition zone diameters greater or equal to 15 mm (two ethanol extract of *Enterobacter cloacae*, ethanol extract of *Raoultella ornithinolytica*, ethanol extract of *Morganella morganii* and ethanol extract of *Aeromonas sobria*). By comparing the two used methods we can conclude that the agar cylinders method gave better results compared to the agar disk-diffusion method.

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Introduction:-

Among benthic animals, soft-bodied, sessile animals have concentrated most of the interest in pharmaceutical studies. The toxic chemicals are crucial for invertebrates lacking morphological defence structures such as shells or spines (De Rosa, 2002; Blunt et al., 2008). Indeed, sponges are the most prolific marine producers of novel compounds in terms of new metabolites reported annually (Taylor et al., 2007; Menna, 2009). Furthermore, more sponge-derived compounds are in clinical and preclinical trials (e.g., as anticancer or anti-inflammatory agents) than compounds from any other marine taxa (Taylor et al., 2007; Menna, 2009). A wide variety of novel secondary bioactive metabolites have been isolated from various species of marine sponges world-wide, including powerful antiviral, antimalarial, antitumour and anti-inflammatory, as well as antimicrobial (antibiotic) compounds (Faulkner, 2002). Marine-based microorganisms are also a potential source of new medicines. However, the successes to date are based upon a very limited investigation of these microorganisms in few areas of the world oceans (Pushparaj et al., 1998), suggesting a high potential for continued discovery of new drugs from these microbes (Board, 2002). Accumulated evidence also suggests that microorganisms living in the body of sponges could well be the true source of at least some of these metabolites found in Mediterranean sponges (Thiel et Imhoff, 2003) and in other species from other oceans (Anand et al., 2006; Taylor et al., 2007). Marine sponges often contain diverse and abundant microbial communities, including bacteria, microalgae, and fungi. In some cases, these microbial associates comprise as much as 40% of the sponge volume and can contribute significantly to host metabolism via e.g. photosynthesis or nitrogen fixation (Taylor et al., 2007). Many antimicrobial, antifouling substances have been found among these kinds of bacteria due to the specialized role they play in their respective hosts (Burgess et al., 1999; Holmstrom et al., 2002). It is suggested that the primary role of these antibiotic substances could be related to ecological competition (Zheng et al., 2005). In the present work, we have studied antibacterial activity of isolated marine bacteria associated with same invertebrates belonging to the Moroccan Atlantic coast.

Material and Methods:-**Biological materials:-**

The marine sponges (*Haliclona viscosa*, *Cliona viridis*, *Ircinia spinulosa* and *Paraleucilla magna*) and the sea cucumber were collected from the littoral Atlantic of El-Jadida (Morocco) at a depth ranging from 3 to 10 m. After sampling, the samples were cleaned, photographed, washed with sea water and immediately transported to the laboratory. The sponges *Haliclona viscosa*, *Cliona viridis* and *Ircinia spinulosa* were identified by Dr. Maria-Jesús Uriz, Research Professor at the Centro de Estudios Avanzados (El Amraoui et al., 2010), while the sponge *Paraleucilla magna* was identified by Dr. Paco Cardenas, Researcher at Evolutionary Biology Centre, Uppsala University, Department of Systematic Biology, Norbyvägen 18D 752 36 UPPSALA, SWEDEN and Dr. Michelle Klatau from Universidade Federal do Rio de Janeiro, Brazil.

Isolation and identification of microorganisms:-

1 cm³ of each marine organism was sampled using a sterile scalpel, ground in a sterile mortar in the presence of sterile physiological water, and inoculated on the culture media. After inoculation, the dishes are incubated in an oven at 35 ± 2 ° C for 24 hours. Several subcultures were conducted to obtain pure culture.

The identification of marine microorganisms was carried out at the Maghreb laboratory, Rabat (Morocco) to the following protocol: the Gram-positive or Gram-negative bacterial differentiation was carried out by the Gram staining. The Gram-positive bacteria are tested for catalase whereas Gram-negative bacteria undergo the oxidase test, and there-after, according to the results of biochemical tests, a type of gallery is used. Galleries “ID32 STAPH” are used for Gram (+), catalase (+), galleries “ID32 STREP” for Gram (+), catalase (-). Whereas galleries “ID32 GN” for Gram (-), oxidase (+) and galleries “ID32 E” are used for the Gram (-), oxidase (-). Galleries and results are analyzed using an identification device “ATB Expression 2000 SYSTEMS bioMérieux VITEK” linked to the computer.

Antibacterial activity of isolated microorganisms:-

Antibacterial activity of marine microorganisms was estimated by the agar cylinders and the agar disk diffusion methods against three Gram-positive bacteria (*Staphylococcus aureus* ATCC25923, *Bacillus sp.* CIP104717 and *Enterococcus faecalis* ATCC19433) and three Gram-negative bacteria (*Escherichia coli* CIP54127, *Pseudomonas fluorescens* CAN 228-1 and *Pseudomonas sp.*) using Mueller-Hinton agar medium [bioMérieux® SA]. These

reference strains were obtained from the Collection of Pasteur Institute (CIP), and from the American Type Culture Collection (ATCC). The tests pathogens inoculate were prepared by suspending in 10 mL of sterile water the colonies from 18 h culture on Luria Bertani medium. The cell density was determined by a haemocytometer and adjusted to 10^6 UFC/mL.

Agar disk diffusion method:-

The isolated bacteria were cultured in nutrient broth for 7 days then each culture was extracted with ethyl acetate (3 × 100 ml). The ethyl acetate extracts were combined, dried on anhydrous sodium sulphate (Na_2SO_4), filtered and concentrated at reduced pressure to give an ethyl acetate extract (extract C). The aqueous phases were lyophilised and twice dissolved in absolute ethanol, then filtered and concentrated at reduced pressure to give an ethanol extract (extract B). The extracts (B and C) were tested for their antibacterial activity using agar disk diffusion methods.

6 mm diameter cellulose discs were saturated with 300 µg of extract (B or C) of isolated bacteria then applied on the test media which were previously inoculated with each pathogen strain. Plates were first kept at 4 °C for at least 2 h to allow the diffusion of chemicals, and then incubated at 37 °C. Inhibition zones were measured after 24 h of incubation. Standard disks of the antibiotic tetracycline (30 µg) and penicillin (10 µg) served as the positive antibacterial controls. All tests were performed in triplicate.

Agar cylinders method:-

Isolates were grown on marine agar plates for four days at 37 °C, and then a calibrated cylinder (6 mm in diameter) was cut out and placed on the surface of the test medium (Muller Hinton) previously inoculated with each test strain. Plates were kept first at 4 °C for at least 2 h to allow the diffusion of active metabolites, and then incubated at 37°C. Inhibition zones were measured after 24 h of incubation.

Results and Discussion:-

Six microorganisms were identified from the sponge *Ircinia spinulosa* (Sarcotragus), four microorganisms from the sponge *Paraleucilla magna*, one microorganism from the sponge *Cliona viridis*, one microorganism from the sponge *Haliclona viscosa* and two microorganisms from the sea cucumber. The results of identification of microorganisms are summarized in table 1. Identified isolates belong to the genera Enterobacter, Morganella, Aeromonas, Pantoea, Kluyvera, Raoultella, Stenotrophomonas, Pseudomonas, Sphingomonas and Staphylococcus. This shows a diversification in the marine microflora. Isolates species and their antibacterial activity against six pathogen bacteria were reported in tables 2 and 3.

For the agar cylinders method (Table 2), the results show that all strains (14 isolates) have antibacterial activity against at least one out of six test pathogens studied.

Four microorganisms of the genus Enterobacter are of the same species (*Enterobacter cloacae*). Three of them, Cv, Is₄ and Pm₄, were isolated from marine sponges (*Cliona viridis*, *Ircinia spinulosa*, *Paraleucilla magna*) while Sc₁ was isolated from Sea cucumber. The four isolates show significant antibacterial activity but only Sc₁ and Pm₄ haven't antibacterial activity against *Pseudomonas fluorescens*. Two microorganisms of the genus Aeromonas are of the same species (*Aeromonas sobria*). One of them, Sc₂, was isolated from Sea cucumber while Is₁ was isolated from marine sponge *Ircinia spinulosa*. The two isolates show significant antibacterial activity but only Sc₂ haven't antibacterial activity against *Pseudomonas fluorescens*. This strain has a very high potential resistance against the antibacterial action of the majority of microorganisms.

Table. 1:- Identification of microorganisms isolated from the marine invertebrates

Marine invertebrate	Reference of isolate bacteria	Identification of isolate
<i>Cliona viridis</i> sp.	Cv	<i>Enterobacter cloacae</i>
<i>Haliclona viscosa</i>	Hv	<i>Morganella morganii</i>
<i>Sea cucumber</i>	Sc ₁	<i>Enterobacter cloacae</i>
	Sc ₂	<i>Aeromonas sobria</i>
<i>Ircinia spinulosa</i> (Sarcotragus)	Is ₁	<i>Aeromonas sobria</i>
	Is ₂	<i>Pantoea sp3</i>
	Is ₃	<i>Kluyvera cryocrescens</i>
	Is ₄	<i>Enterobacter cloacae</i>
	Is ₅	<i>Raoultella ornithinolytica</i>
	Is ₆	<i>Stenotrophomonas maltophilia</i>
<i>Paraleucilla magna</i>	Pm ₁	<i>Pseudomonas putida</i>
	Pm ₂	<i>Sphingomonas paucimobilis</i>
	Pm ₃	<i>Staphylococcus capitis</i>
	Pm ₄	<i>Enterobacter cloacae</i>

Table. 2:- Antibacterial activity of marine microorganisms using Agar cylinders method

Isolate microorganisms	Bacterial growth inhibition diameter (mm)					
	Gram-negative bacterium			Gram-positive bacterium		
	<i>E. coli</i> CIP 54127	<i>P. fluorescens</i> CAN 228-1	<i>Pseudo-</i> <i>monas. sp.</i>	<i>Bacillus. sp.</i> CIP104717	<i>S. aureus</i> ATCC25923	<i>E. faecalis</i> ATCC19433
Is ₁	8	7	18	17	9	10
Is ₂	8	-	10	16	8	7
Is ₃	8	-	9	-	8	-
Is ₄	10	15	19	12	14	9
Is ₅	12	11	14	13	15	13
Is ₆	11	-	18	10	8	8
Pm ₁	8	8	8	14	9	7
Pm ₂	-	-	8	10	8	7
Pm ₃	9	10	20	14	15	11
Pm ₄	+	-	12	14	10	7
Sc ₁	8	-	9	12	9	7
Sc ₂	8	-	9	8	9	8
Hv	8	-	10	10	10	10
Cv	8	+	9	22	15	10

- : no activity; +: inhibition diameter <7 mm.

Table 3: Antibacterial activity of marine microorganisms using Agar disk-diffusion method

Isolate microorganism	Extract	Bacterial growth inhibition diameter (mm)					
		Gram-negative bacterium			Gram-positive bacterium		
		<i>E. coli</i> CIP 54127	<i>Pseudo-</i> <i>monas.</i> <i>sp.</i>	<i>P. fluorescens</i> CAN 228-1	<i>Bacillus.</i> <i>sp.</i> CIP 104717	<i>S. aureus</i> ATCC 25923	<i>E. faecalis</i> ATCC19433
Is ₁	B	8	9	-	-	+	20
	C	9	8	-	+	-	-
Is ₂	B	8	11	-	+	-	11
	C	9	ND	-	+	8	-
Is ₃	B	-	9	-	+	+	-
	C	-	ND	-	ND	-	-
Is ₄	B	+	16	-	+	+	25
	C	+	ND	-	-	7	-
Is ₅	B	11	15	-	+	+	18
	C	+	ND	8	-	-	-
Is ₆	B	12	11	-	8	-	13
	C	-	ND	8	+	-	-
Pm ₁	B	8	8	-	-	-	-
	C	9	ND	-	+	-	+
Pm ₂	B	+	-	-	-	-	-
	C	ND	ND	-	-	-	ND
Pm ₃	B	9	12	-	8	-	11
	C	+	ND	-	-	-	+
Pm ₄	B	8	+	-	-	-	-
	C	+	10	-	-	-	+
Sc ₁	B	+	+	-	-	-	-
	C	+	+	-	-	-	-
Sc ₂	B	8	8	-	-	-	-
	C	+	+	-	-	-	-
Hv	B	9	15	-	-	-	-
	C	-	8	-	-	8	+
Cv	B	10	20	-	-	-	22
	C	ND	ND	-	-	-	+
Tetracycline 30 µg		24	34	22	25	16	32
Penicillin 10 µg		20	17	20	12	-	16

ND: not determined; —: no activity; +: inhibition diameter <8 mm.

Among the marine microorganisms, six isolates which are showing a significant antibacterial activity with inhibition zone diameters greater or equal to 15 mm:

- Four isolates were active against *Pseudomonas sp*, *Aeromonas sobria*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia* and *Staphylococcus capitis*;
- one isolate (*Enterobacter cloacae*) was active against *Pseudomonas fluorescens*;
- three isolates (*Aeromonas sobria*, *Pantoea sp3*, and *Enterobacter cloacae*) were active against *Bacillus sp.* ;
- three isolates (*Raoultella ornithinolytica*, *Staphylococcus capitis* and *Enterobacter cloacae*) were active against *Staphylococcus aureus*.

For the agar disk-diffusion method (Table 3), the results show that all isolate have antibacterial activity against at least one out of six test pathogens studied.

All B extracts (14 extracts) of studied marine microorganisms have displayed some activity against at least one out of six test pathogens studied.

Except two C extracts of isolates (*Kluyvera cryocrescens* and *Sphingomonas paucimobilis*) were not active against any pathogens studied.

Among 28 extracts of the marine microorganisms, five extracts which are showing a significant antibacterial activity with inhibition zone diameters greater or equal to 15 mm:

- four extracts were active against *Pseudomonas sp.*, two B extract of *Enterobacter cloacae* (Is₄ and Cv), B extract of *Raoultella ornithinolytica* (Is₅) and B extract of *Morganella morganii* (Hv);
- four extracts (B extracts of *Aeromonas sobria* (Is₁), *Enterobacter cloacae* (Is₄ and Cv) and *Raoultella ornithinolytica* (Is₅)) were active against *Enterococcus faecalis*.

By comparing the two methods used to determine the antibacterial activity of the studied microorganisms against the six test pathogens studied, we can conclude that the agar cylinders method gave better results compared to the agar disk-diffusion method.

It is evident then that marine macro- and microorganisms, living in an environment where competition and predation are the maximum without physical defence structure, defends themselves by producing chemicals to survive. It is found that the sea surface or cavum of marine organisms such as seaweeds and invertebrates are more nutritious than inanimate material and seawater, and a large number of bacteria could live on it (Sponga et al., 1999). These bacteria species are not generally real symbiotic to the host, but they can instead be regarded as associated bacteria (Ponce et al., 1999), and are forced to develop resistance to antibiotics secreted by its host. In fact, they can produce antibiotics and antifungal to inhibit or limit the development and growth of other competitive microorganisms. Several products have been isolated from marine microorganisms with antimicrobial (Romanenko et al., 2007; Charyulu et al., 2009), antifungal (Barsby et al., 2002), antibacterial (Li et al., 2006; Asha Devi et al., 2011), antiviral (Yasuhara-Bell et al., 2010) and cytotoxic (Maskey et al., 2002; Shaaban et al., 2011) activities.

It is evident then that the antibacterial spectra of the active marine bacteria are different (Zheng et al., 2005). This difference comes from the variation of the ecological conditions of the environment of the microorganisms (El-Wahidi et al., 2011). However, some isolates of the same species show a difference in their antibacterial activity. Moreover, molecular studies of these species could provide the answer.

Conclusion:-

Preliminary results show that marine microorganisms associated to different marine invertebrates have an antibacterial potential. They can also constitute a potential source of new compounds to be used in the field of health. *Aeromonas sobria* (Is₁), *Enterobacter cloacae* (Is₄ and Cv), *Stenotrophomonas maltophilia* (Is₆), *Staphylococcus capitis* (Pm₃), *Pantoea sp3* (Is₂) and *Raoultella ornithinolytica* (Is₅) bacteria that exhibit a significant antibacterial activity should be investigated for isolation and identification of natural antibacterials.

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