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Screening a Mediterranean Sponge *Axinella verrucosa* For Antibacterial Activity in Comparison to Some Antibiotics.

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The antibacterial effect of crude extract of marine sponge *Axinella verrucosa* at room temperature against seven nosocomial bacteria and one fungal isolates including, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter septicus* and *Proteus vulgaris*, *Acinetobacter meningitis*, *Klebsiella pneumonia*, *E. coli* and the fungal pathogen *Candida albicans* were studied by Kirby-Bauer disc diffusion assay and, the findings of antibacterial activity of crude methanolic extract sponge *A. verrucosa* were compared to the efficiency of some marketed antibiotics that were tested against the same bacteria at given concentrations. In result, it was found that MeOH crude extract of *Axinella verrucosa* is effective against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter septicus* and *Proteus vulgaris* and except for its ineffectiveness against *Acinetobacter meningitis*, *Klebsiella pneumonia*, *E. coli* and the fungal pathogen *Candida albicans*. It was more active than Azithromycin and Gentamicin Against Proteus vulgaris and more efficient than ciprofloxacin against *Acinetobacter septicus*. Hexane and Ethyl acetate crude extracts derived from *A. verrucosa* revealed no activity against all bacterial and fungal pathogen. Sponge *Axinella verrucosa* remains an interesting source of new antibacterial metabolites with better activity than some antibiotics

Keyword: Antimicrobial Activity, *Axinella verrucosa*, Nosocomial Pathogens

1. Introduction

The marine environment comprises of complex ecosystem with a plethora of organisms are known to possess bioactive metabolites compounds a common means of defense. Predominantly microorganisms and plants, have provided mankind with many of the therapeutic agents currently on the market. These natural products have been used directly as drugs, or have provided leads for the synthetic preparation of pharmaceutical products[1]. Members of the phylum *Porifera* are renowned for their medicinal applications, as important sources of chemicals used in traditional medicine[1][2]. The sponges represent a diverse and ancient group of multicellular animals. Basic sponge body plants consist of multiple cell layers, each with individual specialization, functioning in the place of discrete organs. Due to porous structure and filter feeding sponges produce such a wide array of bio-active chemicals may be to defend themselves against the effects of potentially pathogenic organisms inadvertently ingested by the sponge[3]. Many biologically active

substances have been successfully isolated from sponges especially demosponges.

It's been found that that antimicrobial activity is influenced by sponge morphology; branched and massive sponges showed higher bioactivity comparing to encrusting forms[4][5]. Marine sponges of genus *Axinella* are a well-known source of brominated pyrrole alkaloids ,the genus *Axinella* (class Demospongiae, order Halichondrida, family Axinellidae) contains approximately 20 species, distributed world-wide is known to be a source of a variety of secondary metabolites such as bromocompounds, cyclopeptides, polyethers, sterols, and terpenes[6][7]. Antimicrobial peptides were isolated from many marine sponges[6][8], the sponge genus Axinella is one of them due to the densely tiny pore- structure of *Axinella* on one hand it provides a unique environment for microorganisms which is supposed to play a role in producing proteins on the other hand[9][10]. Nosocomial infections are those that originate or occur in a hospital or hospital-like setting. They are responsible for high morbidity. Nosocomial infections are primarily caused by opportunists. The sites of nosocomial infections, in order from most to least common, are as follows: urinary tract, surgical wounds, respiratory tract, skin (especially burns), blood (bacteremia), gastrointestinal tract, central nervous system. Nosocomial pathogens may tend to be more resistant to antibiotics[11]. Members of the phylum *Porifera* are renowned for their medicinal applications, as important sources of chemicals used in traditional medicine[1][2]. The sponges represent a diverse and ancient group of multicellular animals. Basic sponge body plans consist of multiple cell layers, each with individual specialization, functioning in the place of discrete organs. Due to porous structure and filter feeding sponges produce such a wide array of bio-active chemicals may be to defend themselves against the effects of potentially pathogenic organisms inadvertently ingested by the sponge[3]. Many biologically active substances have been successfully isolated from sponges especially demosponges. It's been found that that antimicrobial activity is influenced by sponge morphology, branched and massive sponges showed higher bioactivity comparing to encrusting forms[4][5]. Considering all the above facts the present study has been undertaken to test the crud extracts derived from branched form sponge *Axinella verrucosa* against some nosocomial pathogens in comparison to some antibiotics that are in use, then, to identified some compounds found in MeOH extract.

2. Material and Methods

2.1 Sampling

At depths between 25-35 m, namely*: Axinella verrucosa, was* collected by scuba diving during the first half of February 2007 from Ibn- Hani (35°35'37"N 35°45'20"E) of Latakia coast. The sponges were sorted, cleaned from associated biota, placed in zipper freezer bags, and then frozen at -20 °C before extraction. The sponge *Axinella verrucosa* was classified depending on (shape, color and type of spicules).

2.2 Preparation of Pathogens

The pathogens were obtained from university hospital lab, nosocomial pathogens taken from hospitalized patients were identified using API20E. The test microorganisms were *Staphylococcus aureus, Escherichia coli, Acinetobacter meningitis, Klebsiella pneumonia, Acinetobacter septicus, Proteus vulgaris, Pseudomonas aeruginosa and one fungal pathogen Candida albicans,* they were from different clinical specimens (table 1).

Table 1: The Test Microorganisms and Their Sources

Clinical specimen	Test microorganism
Pharyngeal swab(neonates)	*Candida albicans*
Umbilicus swab	*Staphylococcus aureus*
CSF	*Acinetobacter meningitis*
CSF	*Klebsiella pneumonia*
Urea	*Escherichia coli*
Gastric secretion(neonate)	*Pseudomonas aeruginosa*
Umbilicus swab	*Acinetobacter septicus*
Blood	*Proteus vulgaris*

2.3 Obtaining of Crude Extract

2.3.1 Preparation of Sponge

Frozen sample of two Sponge species were left to thaw and gently washed with distilled water to remove salts and epibionts. Wet samples of both species were weighed, and placed in a fume hood for 48 hours to be dried, in order to remove as much water as possible. The wet sponges were found to weigh approximately 150 g. The final weight of the dry sponges was approximately 130 g. The dry sponges were 1 cm cube chopped using sterile scissors

2.3.2 Extraction Procedure

The extraction procedure was based on Larsen's method[3] with minor modifications. The dry sponge pieces were successively processed using solvents with different polarities namely: methanol, ethyl acetate, and hexane. The amount of each solvent was approximately 130 ml. The sponges were soaked in methanol three times overnight, and filtered, and then they were immersed in ethyl acetate three times overnight and filtered .In addition the sponges were soaked once in hexane for 24 hours and filtered. Following each soak the sponges were allowed to dry in fume hood to remove any remaining solvent. As a final step, the sponge pieces were homogenized with 130 ml of ethyl acetate using a macerator and left at room temperature for 24 hours, then filtered. The filtrate was collected and combined with ethyl acetate extract collected from the initial extractions. In the end, three sets of immersion extracts one each of methanol, ethyl acetate and hexane were obtained. Individually, the extract mixtures were rotavapped under vacuum. The temperature of the water bath was set at -30 °C and the rotation rate was medium. The crude extracts of both species were placed in small vials and kept at – 4 °C for the susceptibility testing using Kirby-Bauer disc diffusion assay[8].

2.4 Seeding the Plates

Petri dishes were filled with approximately 4 mm thickness of previously sterilized Mueller-Hinton agar for bacteria and YPD for the yeast. Using sterilized swab, both media were inoculated with the pure isolates of bacteria and the yeast. The inoculum density was standardized for a susceptibility test, a $BaSO_4$ turbidity standard, was equivalent to a 0.5 McFarland standard**,** suspension contained approximately 1 - 2 x 10^8 CFU/ml

2.5 Kirby-Bauer Antimicrobial Assay

Punched sterilized 6mm disks (Whatman, no,1) were impregnated with 20 µl of the obtained crude extracts from each sponge species, left to dry and then placed on the inoculated media. A sterilized blank disk was used as a control[8]. The bacterial cultures were incubated at 37 °C for 24 hours and the yeast cultures were incubated at 27 °C for 24 hours.

2.6 Collection of Data

The day after, inhibition zone diameters were measured and the results were scored as follow:

1. - (no zone of activity),
2. + (8-10 mm diameter zone of activity),
3. ++ (11-15 mm diameter zone of activity)
4. +++ (>15 mm diameter zone of activity).

On the other hand, the results were compared to reference values currently used antibiotics that belong to different groups

- Ceftriaxone (30 micro grams per disk) β-lactam group, Cephalosporin 3rd generation.
- Azithromycin (15 micro grams per disk) a macrolide
- Gentamicin (10 micro grams per disk) an aminoglycoside.
- Ciprofloxacin (5micrograms per disk) an Fluoroquinolone

The antibiotics were chosen according to the pathogen, disease and age.

2.7 Identification of Chemical Structures of Some Compounds

Spectral analyses were carried out and three compounds tentatively identified as being present in the crude extract of the sponge *Axinella verrucosa*. The crude methanol extract was partitioned by Dr. Inman at UCSC between methylene chloride (coded DCM) and water. The solvent from DCM layer was rotary evaporated.

A portion of the DCM dried material was dissolved in methanol, concentration approx. 1 mg/mL This crude solution was analyzed by LC-ESI-MS (in positive mode, equipped with a 150 x 4.6 mm, 5 micron, C18 column (Luna, Phenomenex Inc.) with a solvent gradient consisting of solvent A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile. The gradient began with 10% of the 0.1% formic acid in acetonitrile to 100% over 20 minutes, 10 µl injection. The molecular ions of several of the most important peaks were obtained from a full mass spectrum scan and this information was dereplicated using standard databases at UC Santa Cruz including the Dictionary of Natural Products and Marine Lit**.**

3. Results

3.1 Sponge Description

Mediterranean sponge *Axinella verrucosa*, belongs to the class of demosponges. It is medium sized, branching, ophidian like shape. Dark brown in color, the branches are regular in diameter. The sponge has no stem, the surface is rough textured and elastic, pores are numerous and obvious. Table 2 Fig 1

Fig. 1**:** Morphological Description of Sponge *Axinella verrucosa*

Table. 2: The Classification and Description of the Sponge Samples

Sponge species	**Sub Class**	**Order**	**Family**	**Description**
Axinella verrucosa (Esper,1794)	Tetractinomorpha	Halichondrida	Axinellidae	Medium sized ,branching, ophidian like shape. Dark brown in color. The branches are regular in diameter. the sponge has no stem, the surface is rough textured and elastic, pores are numerous and obvious

3.2 The Antimicrobial Activity

The antimicrobial activity of crude methanolic, ethyl acetate, and hexane extracts of sponge *Axinella verrucosa* was evaluated against some hospital-acquired pathogens: *Staphylococcus aureus, Escherichia coli, Acinetobacter meningitis, Klebsiella pneumonia, Acinetobacter septicus, Proteus vulgaris, Pseudomonas aeruginosa and fungal pathogen Candida albicans*. Different concentrations of various commercial antibiotic discs, (Ceftriaxone, Gentamicin, Azithromycin, and Ciprofloxacin) were used to assay their antimicrobial activity against the test pathogens. Hexane and Ethyl acetate crude extracts derived from *A. verrucosa* revealed no activity against all bacterial and

fungal pathogens. Crude methanolic extract of *Axinella verrucosa* revealed strong activity against Gram positive *Staphylococcus aureus*, the zone of inhibition was found more than 21 mm Table 3 Fig 2. Gram negative *Acinetobacter septicus*, was extremely sensitive to *Axinella verrucosa*, the zone formed was more than 21 mm Table 4 fig 4. Crude methanolic extract of *Axinella verrucosa* revealed strong activity against Gram negative *Pseudomonas aeruginosa* the zone of inhibition was found more than 20 mm Table 5 Fig 6 Gram negative *Proteus vulgaris* revealed high susceptibility against MeOH extract of *Axinella verrucosa* the zone of inhibition was found more than 20 mm Table 6 Fig8. Comparatively ,as shown in Table3 Fig 3, the antibiotics Ceftriaxone 30 µg , Azithromycin 15 µg showed maximum activity against *S. aureus* (20 mm) ,Gentamicin 10 µg exhibited potent activity against *S. aureus*(16 mm).
Ceftriaxone 30 µg, Azithromycin 15 µg showed maximum activity against *A. septicus* (20 mm) , Ciprofloxacin 5 µg exhibited medium activity against *A. septicus* (13mm) Table 4 Fig 5. Ceftriaxone 30 µg, Azithromycin 15 µg were strongly active against *P. aeruginosa* (20 mm), Ciprofloxacin 5 µg exhibited high degree of antibacterial activity against *P. aeruginosa* (17 mm) Table5 Fig7. While, Ceftriaxone30 µg, Azithromycin 15 µg showed maximum activity against *P. vulgaris* (20 mm), Ciprofloxacin 5µg and Gentamicin 10µg exhibited moderate activity against *P. vulgaris* (12 mm and 13 mm respectively) Table 6 fig 9.

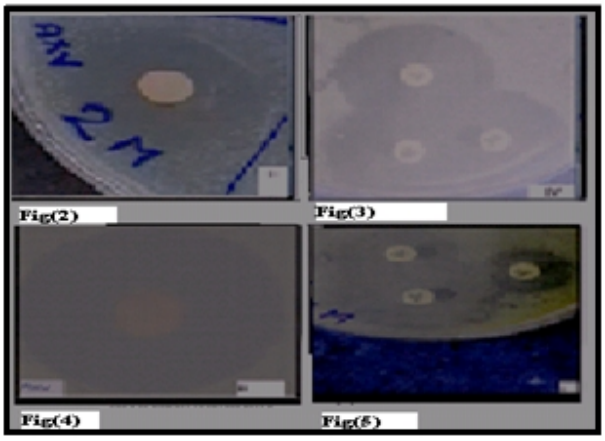

Fig (2): *S. aureus* susceptibility to crude MeOH extract of *A. verrucosa*
Fig (3): *S. aureus* susceptibility to Ceftriaxone, Azithromycin and Gentamicin
Fig (4): *A. septicus* susceptibility to crude MeOH extract of *A. verrucosa*
Fig (5): *A. septicus* susceptibility to Ceftriaxone, Azithromycin and Ciprofloxacin

Table 3: In vitro antimicrobial activity of MeOH crude extracts of *Axinella verrucosa* against *S. aureus* in comparison to Ceftriaxone, Azithromycin and Gentamicin.

	A. verrucosa	**Ceftriaxone 30 µg**	**Azithromycin 15 µg**	**Gentamicin 10µg**
S. aureus	>21 +++	20 +++	20 +++	16 +++

Table 4: *In vitro* antimicrobial activity of MeOH crude extracts of *Axinella verrucosa* against *A. septicus* in comparison to Ceftriaxone, Azithromycin and Ciprofloxacin.

	A. verrucosa	**Ceftriaxone 30 µg**	**Azithromycin 15 µg**	**Ciprofloxacin 5µg**
A. septicus	**>21** +++	**20** +++	**20** +++	**13** ++

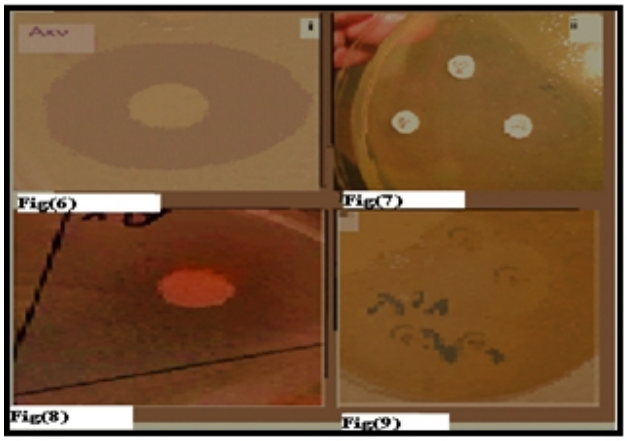

Fig (6): *P. aeruginosa* susceptibility to crude MeOH extract of *A. verrucosa*
Fig (7): *P. aeruginosa* susceptibility to Ceftriaxone, Azithromycin and Gentamicin
Fig (8): *P. vulgaris* susceptibility to crude MeOH extract of *A. verrucosa*
Fig (9): *P. vulgaris* susceptibility to Ceftriaxone, Ciprofloxacin, Azithromycin and Gentamicin

Table 5: In vitro antimicrobial activity of MeOH crude extracts of *Axinella verrucosa* against *P. aeruginosa* in comparison to Ceftriaxone, Azithromycin and Ciprofloxacin.

	A. verrucosa	**Ceftriaxone 30 µg**	**Azithromycin 15 µg**	**Ciprofloxacin 5µg**
P. aeruginosa	20 +++	20 +++	20 +++	17 +++

Table 6: In vitro antimicrobial activity of MeOH crude extracts of *Axinella verrucosa* against *P. vulgaris* in comparison to Ceftriaxone, Azithromycin, Gentamicin and Ciprofloxacin.

	A. verrucosa	**Gentamicin 10µg**	**Ceftriaxone 30 µg**	**Azithromycin 15 µg**	**Ciprofloxacin 5µg**
P. vulgaris	>21 +++	13 ++	20 +++	20 ++++	12 ++

3.3 Identification of Bioactive Compound Present in Sponge

The MS m/z data and UV data were consistent with that of three compounds: Hymenialdisine, and two derivatives, 10- E-Hymenialdisine and Spongiacidine B, whose structures are shown below. However none of these compounds were isolated in pure form; consequently additional data such as NMR properties were not obtained. Their positive antibacterial actions are synergistic, and so more powerful, as compared with those from terrestrial origins.

a) Hymenialdisine:

4-(2-Amino-4-oxo-2-imidazolidin-5-ylidene)-2-bromo-4, 5, 6, 7-tetrahydropyrrolo [2, 3-c] azepin-8-one.molecular formula $C_{11}H_{10}BrN_5O_2$ molecular weight 324.136 melting point160-164ºC rates of elements C 40.76%; H 3.11%; Br; 24.65%; N 21.61%; O 9.87% this compound belongs to Pyrrole Imidazole alkaloids .Fig 10 shows LCMS chromatogram that illustrates molecular ions {M^+H} m/z 324.08, 326.07, 327.10 at retention times (4.26-4.27-4.29 mins) ,peaks of UV absorbance : max λ nm 238.00, nm 260.00, 345.00 nm and chemical structure .

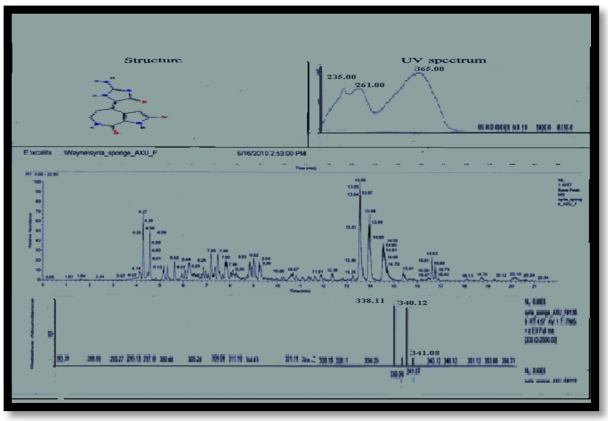

Fig 10: UV, ESI_MS spectra and chemical structure of the elucidated compound Hymenialdisine : peaks of UV absorbance **:** max λ nm 238.00, nm 260.00, 345.00 molecular ions {M^+H} m/z 324.08, 326.07, 327.10 at retention times (4.26-4.27-4.29 mins)

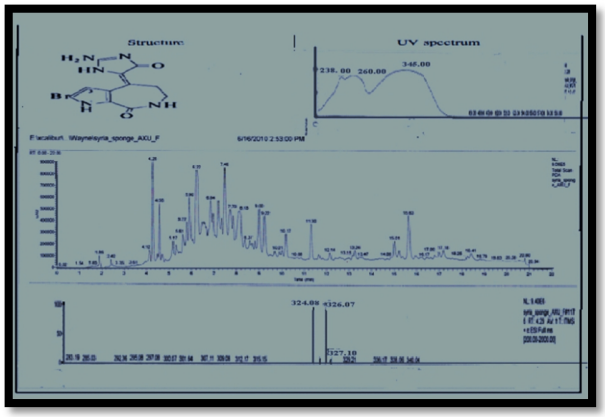

Figure 11: UV, ESI_MS spectra and chemical structure of the elucidated compound Hymenialdisine 10E*:* peaks of UV absorbance**:** max λ nm235.00, nm261.00, nm 355.00 molecular ions {M^+H} m/z 338. 11, 340.12, 341.08 at retention times (4.57-4.58-4.59 mins)

b) E Hymenialdisine

Molecular formula $C_{11}H_{10}BrN_5O_2$ molecular weight 324.136 melting point160-164 ºC rates of elements C 40.76%; H 3.11%; Br; 24.65%; N 21.61%; O 9.87% this compound belongs to Pyrrole Imidazole alkaloids.Fig11 reveals LCMS chromatogram that shows molecular ions {M^+H} m/z 338. 11, 340.12, 341.08 at retention times (4.57-4.58-4.59 mins), peaks of UV absorbance: max λ nm235.00, nm261.00, nm 355.00 and the chemical structure.

c) Spongiacidine B:

Molecular formula $C_{11}H_{10}BrN_5O_2$ Molecular weight 324.136 Melting point160-164 ºC rates of elements C 40.76%; H 3.11%; Br; 24.65%; N 21.61%; O 9.87% This compound belongs to Pyrrole Imidazole alkaloids. Fig 12 represents LCMS Chromatogram that shows molecular ion{M^+H} m/z 339. 09, 341.07, 342.13 at retention times (7.46-7.48-7.49 mins), peaks of UV absorbance: max λ nm239.00, nm265.00, nm 359.00 and the chemical structure.

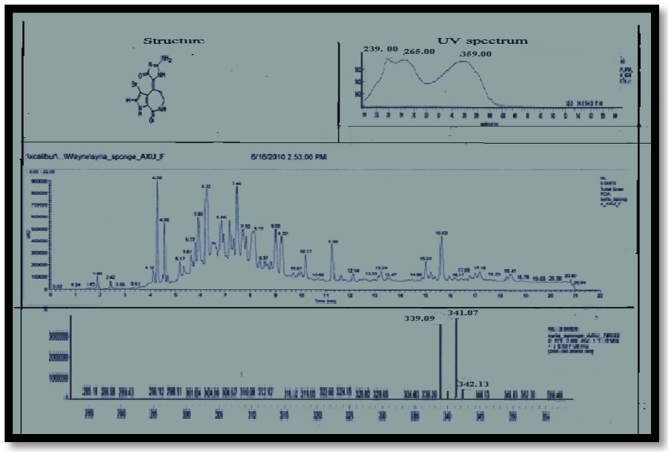

Fig 12: UV,ESI_MS spectra and chemical structure of the elucidated compound spongiacidin B :peaks of UV absorbance **:** max λ nm 238.00, nm 260.00, 345.00 nm molecular ions {M^+H} m/z 324.08, 326.07, 327.10 at retention times (4.26-4.27-4.29 mins)

4. Discussion

In this study, sponge *Axinella verrucosa* was selected to be evaluated for its antimicrobial activity against some human pathogens due to its erected branch shape known to have potent antimicrobial activity[4][5]. This could be attributed wide exhibition to water currents and to predators in comparison to massive shaped sponges. We specifically focused on Methanol extracts since (MeOH) has the potential to penetrate cell membranes on one hand[12] **.**Previous studies indicate to the surface origin of polar metabolites which are easily extracted and first used in sponge defense before non-polar metabolites or even metabolites with moderate polarity on the other hand[13]. Pyrrole Imidazole alkaloids in sponges are of a wider variety.

Majority of them show antimicrobial, antitumor and anticancer properties[14][15][16]. References show that sponge antimicrobial extract attacks some bacteria and retain its activity against resistant bacteria to conventional antibiotics[6]. This activity encourages us to compare the antimicrobial capacity of the crude extracts to that for Antibiotics such as β-lactam group that comes first. Some antimicrobial activities can be lost in the process of fractionation of sponge crude extract the activity of the crude extract, and chromatographic procedure could affect the activity of the compounds by reducing the

solubility of the bioactive compounds, this leads us to postulate that an extract may contain a number of pharmacologically active components acting simultaneously or synergistically to affect bacterial pathogens[8]. MeOH crude extract of *Axinella verrucosa* is effective against *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter septicus* and *Proteus vulgaris* and except for its ineffectiveness against *Acinetobacter meningitis*, *Klebsiella pneumonia*, *E. coli* and the fungal pathogen *Candida albicans*.
Comparatively, MeOH extract of *Axinella verrucosa* had stronger activity than (Ceftriaxone Azithromycin and gentamicin respectively) against *S. aureus*.

MeOH crude extract of *Axinella verrucosa* was as efficient as Azithromycin, ceftriaxone and Ciprofloxacin against *P. aeruginosa*.

While, crude MeOH extract, Azithromycin and ceftriaxone showed the same degree of activity, they were more active than ciprofloxacin against Gram negative Acinetobacter septicus. In addition, Gram negative *Proteus vulgaris* revealed high susceptibility against MeOH extract of *Axinella verrucosa*. Comparatively, Ceftriaxone and Ciprofloxacin had stronger activity than Azithromycin and gentamicin against *P. vulgaris*.

The compounds present in the extract as well as three compounds that were identified: Hymenialdisine, and two derivatives, 10-E-Hymenialdisine and Spongiacidine B of Pyrrole Imidazole alkaloids and others of various chemical classes presumably showed synergistic antibacterial activity.

5. Conclusion

Based on the findings, promising methanolic extract from Mediterranean sponge *Axinella verrucosa*, hence this information may help to develop potential purified bioactive compounds in the pharmaceutical industry for the development of drugs. In future, this may lead the way towards large scale profitable production of antimicrobials from *Axinella verrucosa*.

6. Acknowledgments

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