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Antimicrobial activity of the sea hare (*Aplysia fasciata*) collected from the Egyptian Mediterranean Sea, Alexandria

Hassan A. H. Ibrahim[1], Mohamed S. Amer[1*], Hamdy O. Ahmed[2] and Nahed A. Hassan[3]
[1]Microbiology Department, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt.
[2]Marine invertebrates Department, NIOF, Alexandria, Egypt.
[3]Zoology Department, Faculty of science, Mansoura University, Egypt.

[*]Corresponding Author: mohammed_saleh74@yahoo.com

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ABSTRACT

A species of sea hare was collected from the Mediterranean Sea, Alexandria, Egypt. It was identified based on general morphological and anatomical features as *Aplysia fasciata*. The antibacterial and antifungal activities were investigated via the standard techniques. Data obtained revealed that the highest antibacterial activity was detected against *P. aeruginosa* (AU = 3.4), followed by *E. coli* (AU = 2.9), then by *B. subtlis* (AU = 2.7). The other bacterial pathogens were not affected at all. Likewise, the maximum fungal suppression, via the pouring method, was observed against *P. crustosum* (50%). AUs against both *F. solani* and *A. niger* were 20 and 10%, respectively, while there was no activity recorded against the others. Also, the antifungal activity via the well-cut diffusion method conducted that the highest AU (6.8) was recorded against *A. flavus*, followed by AU = 4.8 against *F. solani*, then 1.8 against *P. crustosum*. Moreover, the antifungal AU against reference yeast strains ranged between 3.1 and 6.8. The highest one was recorded against *C. tropicalis*, followed by AU (4.8) against *R. mucilaginosa*. Regarding investigating the efficacy of some commercial antibiotics (mm), data confirmed that the Gram-negative bacteria were more resistant than Gram-positive bacteria. On the other side, the result of GC-MS/MS of crude extract observed the presence of several bioactive constituents, most of which had antimicrobial activities.

INTRODUCTION

Recently, drug discovery programs have directed their attention to unusual sources like marine invertebrates (mollusks, sponges, sea cucumber, etc.) hoping to identify more efficacious therapeutic tools with novel chemical structures and unique modes of action (Nocchi *et al.*, 2017).

Sea hares are a group of mollusk, Gastropoda; shell-free marine Opisthobranchs, which includes several genera and many species (Derby, 2007). Definitely, *Aplysia* is a genus of sea hares that belongs to the family Aplysiidae, to which *Aplysia fasciata* Poiret, 1789 belongs. It is common in Mediterranean habitats. It is herbivorous organism, which feeds on several green, brown, or red algae (Susswein *et al*, 1984). In general, sea hares

within the *Aplysia* clade possess a planktonic larval stage, but have limited dispersal as adults, having only short-range crawling and swimming capabilities. Most species inhabit tidal and subtidal zones of most tropical seas (Mander and Liu, 2010).

On the other hand, all sea hares use a chemical mixture for defense and communication (Palaniveloo *et al.,* 2020). Specifically, *Aplysia fasciata* has multiple chemical defenses to deter predators. The passive chemical defenses of *A. fasciata* are found in the skin, thus producing a distasteful surface to predators, and many of them have been identified (Mona *et al.,* 2016). The active chemical defenses are released from *A. fasciata* only upon predatory attack; secreting from two separate glands, which secret a bright purple fluid, known as ink.. In addition, there are 250 and 320 kDa glycoproteins, which have been purified from the eggs and the albumen gland of *A. kurodai*, respectively. Both glycoproteins showed potent anticancer activities towards some human tumors in mice either *in vitro* and *in vivo* (Takamatsu *et al*., 1995). Moreover, anti-cancer dolastatins has been isolated from the sea hare *Dolabella auricularia*, Lightfoot, 1786; girolline from the New Caledonian sponge *Pseudaxinyssa cantharella* Levi, 1983 that may represent a lead compound for new drugs to fight malaria (Nocchi *et al*., 2017).

Furthermore, the bacterial resistance to antibiotics has a significant threat to public health (Morens *et al.,* 2004) due to their threating to patients care with infection, besides, the viability of the current health care system (Diaz-Granados *et al.,* 2008). Despite the scientists give all efforts recently to synthesize more potent antibiotics, bacterial resistance still continues to largely rise up because of the overuse of antibiotics. The treatment of several pathogens, including methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and vancomycin-resistant *enterococci*, is problematic (Lieberman, 2003). Antibiotic resistance is usually associated with significant morbidity and high cost, which may be due to higher antibiotic acquisition costs and/or longer duration of hospitalization, mortality and osteomyelitis (Cosgrove, 2006) or due to delayed appropriate antibiotic therapy or a necessity to perform surgery (Sipahi, 2008).

Thus, this work aimed to access the antimicrobial activity of sea hare crude extract, *Aplysia fasciata*, habiting in the Mediterranean Sea, Alexandria, Egypt, to obtain alternative source of antibiotics. In addition, this study extended to detect the bioactive substances in the crude extract.

MATERIALS AND METHODS

Collection of sea hare samples

Sea hare samples were collected from the Eastern Harbor (29885 E longitude and 31205 N latitude) located at Alexandria, Egypt (Fig. 1). The sea hare samples were collected manually, including the holdfast from the Scout club located in Eastern Harbor in the intertidal zone at the depth of (5-100 m). The fresh samples were then washed with seawater at the sampling site to remove the adhered sediments and impurities, and then put in polyethylene bags. Quick rinsing of the sea hare with tap water was done in the laboratory on the same day to get rid of the remaining impurities and epiphytes.

Fig. 1. A map off Alexandria City, Egypt, showing sample collection station.

Reference microbes and culture media

During this work, there five Gram positive bacterial pathogens (*Bacillus subtlis* ATCC 6633, *B. cerues, Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermids, Enterococcus faecalis* ATCC 29219) besides three Gram negative bacterial ones (*Pseudomonas aeruginosa* ATCC 9027, *Klebsilla pneumoniae,* and *Escherichia coli* ATCC 8739) were used as reference strains. Also, there five yeast species (*Candida albicans, C. krusei, C. tropicalis, C. glabrata,* and *Rhodotorula mucilaginosa*) were used as reference strains. As well as, there seven fungal pathogens (*Penicillum crustosum, Penicillum notatum, Aspergillus terreus, Aspergillus niger,* and *Fusarum solani*). Some of these strains were kindly provided from Microbiology Laboratory (National Institute of Oceanography and Fisheries, Alexandria, Egypt). Some others purchased from the Center of Fungi, Asuit University, Egypt.

On the other side, five common media were used to culture the reference strains and determine the antimicrobial activity of sea star extracts (Atlas, 1997; Guinea *et al*., 2005). They were: nutrient broth (NB), nutrient agar (NA), Sabouraud dextrose agar (SDA) to cultivate dermatophytes and other types of fungi, potato dextrose (PDB), and potato dextrose agar (PDA) to culture on are yeasts such as *C. albicans* and *Saccharomyces cerevisiae* and molds such as *A. niger*.

Preparation of the crude extracts

Sea hare samples were cut into very small pieces of about 2 cm size. The extraction was carried out with aqueous ethanol (70%), by soaking the material in the respective solvents (1:10, w/v) on a rotary shaker at 150 rev min^{-1} at ambient temperature for 96 h. The extract from consecutive soaking was pooled and filtered using filter paper (Whatman no 4). After evaporation of the solvent, the crude extract was re-suspended in 5 ml of dimethyl sulphoxide (DMSO). The antibacterial efficiency of the tested extract dissolved in DMSO was screened against different microbial pathogens (Ibrahim et al., 2020).

Antibacterial and anti-yeast bioassay

All reference strains of bacteria and yeasts were examined as pathogens. A volume of 15 ml of the sterilized nutrient agar for bacteria and Sabouraud dextrose agar for yeast were poured into sterile caped test tubes and were allowed to cool to 50°C in a water

bath. A half of ml of inocula (10^8 CFU for bacteria and yeast) were added. The tubes were mixed using a vortex for 15-30 s. Thereafter, each test tube contents were poured onto a sterile 100 mm diameter Petri dish for solidification (Khan *et al*., 2019). The activity was evaluated using well-cut diffusion technique. Wells were punched out using a sterile 0.7 cm cork-porer in nutrient agar plates containing the tested microorganisms. About 100 µL of crude extract was transferred into each well. They were subjected to 4°C incubation for 2 h, and then were later incubated at 37°C for 24 h. The results were obtained by measuring the diameter of inhibition zone three times for each well and expressed in millimeter. Furthermore, the absolute activity (AU) was calculated according to Ibrahim (2012) as: $AU=Y^2/x^2$, where Y is the diameter of inhibition zone around the well, which its diameter is represented by X.

Antifungal bioassay

By pouring technique

Sea hare crude extract was tested against the indicator fungi by adding aliquots of it to PDA medium at a concentration of 10% (v/v). One disc of the seven fungal growths was separately placed on the center of a plate containing crude extract-PDA medium. All plates were incubated at 28ºC until the control was completely covered with fungal growth. The radius-growth of each indicator fungus was measured to estimate the suppressive effect (%) of crude extract against the indicator fungi (Amer and Ibrahim, 2019).

By well-cut diffusion technique

One disc of the five fungal growths was separately put on the top of a plate containing PDA medium. About 100 µL of sea hare crude extract was transferred into each well. All plates were incubated at 28ºC until the control was completely covered with fungal growth. The results were obtained by measuring the inhibition zone diameter three times for each well and expressed in millimeter. Furthermore, the absolute activity (AU) was calculated according to Amer and Ibrahim (2019) as previously mentioned.

Susceptibility test of commercial antibiotics

Five commercial antibiotics: Cephalexin (CL, 30 µg), Rifampicin (RF, 30 µg) Piperacillin (TZP, 10 µg), Metronidazole (MTZ, 20 µg), and Amikacin (AMK, 30 µg) were selected to test their inhibition capacity against the bacterial strains besides the yeast strain *C. albicans*. The microbial strains were inoculated in the sterilized prepared medium. Instead of the crude extract of sea hare, small discs of the five antibiotics were put associated with each microbial strain. All plates were subjected to 4°C incubation for 2 h, and then later incubated at 37°C for 24 h (Khan *et al*., 2019; Shaaban *et al*., 2020). The results were estimated through measuring the diameter of inhibition zone three times for each well and expressed in millimeter.

GC-MS/MS analysis of sea hare extract

The filtrate was subjected to gas chromatography-mass spectrometry (GC-MS) analysis (Perkin Elmer, Waltham, MA, USA) according to (Muller *et al*., 2002). The analyses were performed in Agilent 7693 series GC system equipped with an OV-5 capillary column (length 30 m 9 diameter 025 mm 9 film thickness 025 lm; Ohio Valley Specialty Chemical, Inc., Marietta, OH, USA) and an Agilent 5975C network selective mass detector, with initial temperature 90°C for 1 min, reaching to 300°C for 30 min, the splitless mode with injection volume 1 µL (total run time 6187 min). The mass spectrometer was operated in the electron impact (El) mode at 70 eV in the scan range

60-600 m/z. The helium was used as the carrier gas pressurized to 2223 psi, whereas the gas flow was 122 ml/min. The chemical constituents of the extract were identified by comparing the GC-MS peaks with retention times of standards, and the mass spectra obtained were compared with those available in the Mass Spectral Library NIST 2015. The percentage of each component was estimated as the ratio of the peak area to the total chromatographic area.

RESULTS AND DISCUSSION

Characterization and identification of collected sea hare

During the current study, a common species of sea hare was collected from its normal habitate in the Egyptian Mediterranean**,** Alexandria. It was characterized and then identified according to Susswein *et al*. (1984) and Mander and Liu (2010) as *Aplysia fasciata*. However, data in Fig. 2 confirmed such characteristcs, while data in Table 1 show the modern classification position of this sea hare.

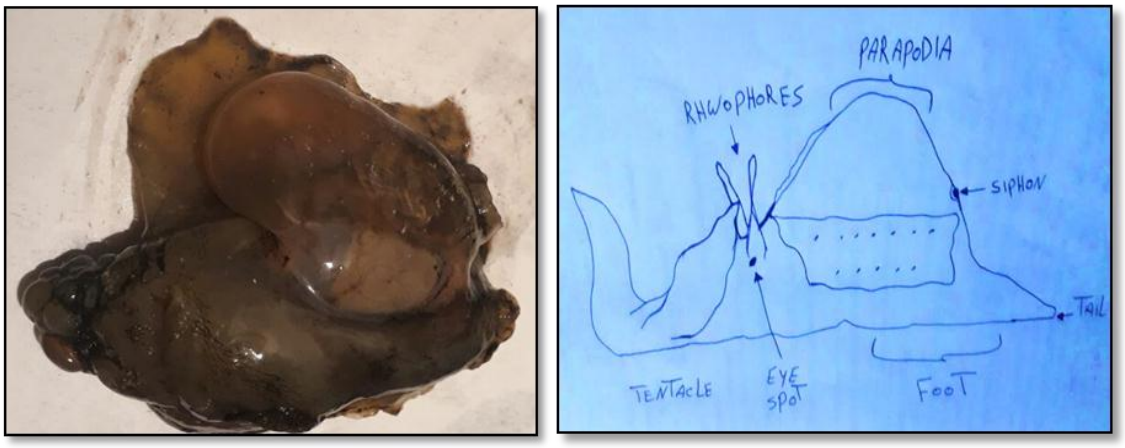

Fig. 2. General features of sample **(**Left) and external anatomy (Right) of sea hare; *A. fasciata* collected from Eastern Harbor, Alexandria, Egypt.

The characterization of the current species of sea hare was done based on general morphological and anatomical features. It was heavy bodied, thick, and highly contractile. The body was consisted of head, foot, and visceral mass. Its length was 30-40 cm. Its color was very dark brown, with a thin red border to the parapodia, foot, and tentacles. Also, it had mottled spots which span across its body. In addition, it had two oral tentacles and two smaller rhinopores in front on their neck. Its eyes are positioned in front of the rhinopores, and rounded tails are fixed to its hindside. A mantle covers its gills and internal organs. Inside the mantle, a thin, delicate inner shell lays, which is concave and a slightly hooked apex. The ink gland is found inside the mantle. Moreover, its secreted ink takes on a purple hue. Furthermore, its egg masses appeared as a long, pale cream mass. They were somewhat noodle-like in appearance .It was seen before collecting that it flapped its paradopia during swimming, showing its flapping wings. Therefore, that was satisfied to be identified as; *Aplysia fasciata*.

Table 1. Modern classification position of *A. fasciata* within Kingdom Animalia.

Item	Position
Kingdom	Animalia
Phylum	Mollusca
Class	Gastropoda
Subclass	Heterobranchia
Infraclass	Euthyneura
Order	Euopisthobranchia
Clade	Anaspidea
Superfamily	Aplysioidea
Family	Aplysiidae
Genus	*Aplysia*
Species	*Aplysia fasciata*

Aplysia fasciata, common name the "mottled sea hare", or the "sooty sea hare", is an species of sea hare or sea slug, a marine opisthobranch gastropod mollusk in the family Aplysiidae (Kamiya *et al,* 2006). This species was observed to be habited in the Egyptian Mediterranean Sea, Alexandria. Mona *et al.* (2016) collected *A. fasciata* (ranging in weight from 200 g to 450 g) from the same coast of Alexandria, Egypt in the summer during the spawning season. Much more sea hares had been collected from other habitats all over the world. For instance, *A. punctata* lives in the Western Atlantic from New Jersey to Brazil, and in the Eastern Atlantic including the Mediterranean and the West African coast. They have also been sighted along the Atlantic coast of France. It is a rare visitor to the seas off the southern British Isles. Some authors consider the species *A. brasiliana*, present in the Atlantic coast of the Americas, to be a synonym of *A. fasciata* with just a different regional color pattern (Bebbington and Hughes, 1973). Early, Barash and Danin (1982) reported the isolation of four species of Aplysia from the Mediterranean coast of Israel: *A. fasciata* Poiret, 1789, *A. juliana* Quoy and Guimard, *A. parvula* Morch, and *A. punctuta* Cuvier. Another species, *A. depilans* Gmelin, is known from other parts of the Mediterranean (Bebbington, 1975), but is not reported by Barash and Danin (1971). *Aplysia juliana* and *A. parvula* are immigrants to the Mediterranean (Bebbington, 1975). In the Red Sea, five species of *Aplysia* were recorded, of which *A. oculifera* Adams and Reeve is the most common (Eales, 1979).

Some sea hare species spout ink when disturbed or attacked, and they swim away using their broad wing-like flaps or parapodia. Their ink is extracted from their algal diet rather than being synthesized (Zsilavecz, 2007). However, sea hares have two main secretory glands; purple gland giving off a red or purple fluid, or, in some species, a white ink and opaline gland giving off a white opaque secretion. The animal chemically defends to avoid being eaten by predators through detecting chemicals released, called alarm signals, and known from many species (Wyatt, 2003).

Antimicrobial activities of *A. fasciata* crude extract

The antibacterial and antifungal activity was investigated by using the standard techniques. Generally, the activities via well-cut diffusion method were calculated in terms of inhibition zone diameter (mm), and then expressed as absolute unit (AU). The activities via pouring technique were expressed in terms of suppression percentage (%). In the present investigation, a pronounced antimicrobial activity has been observed against some bacterial and fungal strains. The crude extract of *A. fasciata* showed a considerable activity against many of bacterial and fungal strains. However, the highest

antibacterial activity was detected against *P. aeruginosa* (AU = 3.4), followed by *E. coli* (AU = 2.9), then by *B. subtlis* (AU = 2.7). The other bacterial pathogens were not affected at all (Table 2).

Table 2. The antibacterial activity of *A. fasciata* crude extract against the bacterial reference strains via well-cut diffusion technique.

Bacterial pathogens	Gram stain category	Antibacterial activity (AU*)
B. subtlis	G +ve	2.7
B. cerues		NI
S. aureus		NI
S. epidermids		NI
E. faecalis		NI
P. aeruginosa	G –ve	3.4
K. pneumoniae		NI
E. coli		2.9

*AU refers to the antibacterial activity which was calculated according to the equation mentioned in methodology section. NI means inhibition was not happened.

Table 3. The antifungal activity of *A. fasciata* crude extract on the fungal reference strains via pouring method.

Fungal pathogens	Antifungal activity (Suppression %*)
P. crustosum	~50
P. notatum	NS
A. terreus	NS
A. niger	~10
A. flavus	NS
F. solani	~20
F. oxysporum	NS

*Suppression % refers to the antifungal activity which was calculated according to the equation mentioned in methodology section. NS means suppression not taken place.

Likewise, the maximum fungal suppression, via pouring method, was observed against *P. crustosum* (50%). AUs against both *F. solani* and *A. niger* were 20 and 10%, respectively, while there was no activity recorded against the others. This result is shown in Table 3 and Fig. 3. Additionally, the antifungal activity via well-cut diffusion technique conducted that the highest AU (6.8) was recorded against *A. flavus* followed by AU=4.8 against *F. solani*, then 1.8 against *P. crustosum* (Table 4). However, the crude extract had no activity against any other tested fungi. Furthermore, the antifungal activity against reference yeast strains (Table 4) ranged between 3.1 and 6.8. The highest activity was recorded against *C. tropicalis* (6.8), followed by AU (4.8) against *R. mucilaginosa*. The AUs of the antifungal activities were as 3.1 and 3.2 against *C. glabrata* and *C. krusei*, respectively. However, the crude extract had no activity against *C. albicans.*

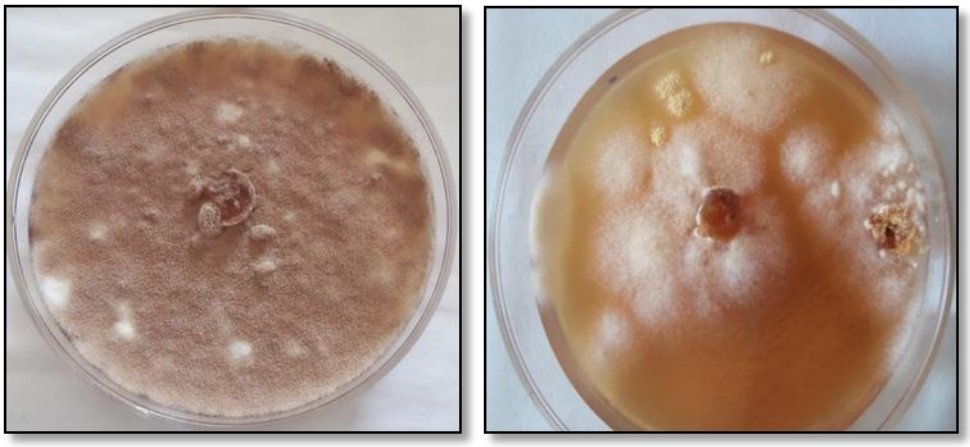

Fig. 3. Antifungal activity of *A. fasciata* crude extract via pouring method against *P. crustosum;* control (Left) and treated (Right).

Table 4. The effect of *A. fasciata* crude extract on different fungal reference strains (molds and yeasts) via well-cut diffusion technique.

Fungal pathogens	Antifungal activity (AU*)
Molds:	
P. crustosum	1.8
P. notatum	NI
A. terreus	NI
A. niger	NI
A. flavus	6.8
F. solani	4.8
F. oxysporum	NI
Yeasts:	
C. albicans	NI
C. tropicalis	6.8
C. krusei	3.2
C. glabrata	3.1
R. mucilaginosa	4.8

*AU refers to the antifungal activity which was calculated as mentioned in before. NI means inhibition was not occurred.

Numerous researchers were interested in many secondary metabolites, to be applied as; anti-cancer, anti-tumor, and anti-viral agents, which are very useful in the pharmacological industry. However, a series of antitumor peptide/macrolides isolated from *Dolabella auricularia* (Kamiya *et al.*, 2006) and antitumor from *A. fasciata* (Mona *et al.*, 2016). Although *Aplysia* are rich sources of secondary metabolites, these substances have been mainly studied in the context of bioprospecting for bioactive molecules (Pereira *et al.*, 2016). Early, Yamazaki (1993) extracted novel antimicrobial from several sea hares; *A. kurodai*, *A. juliana*, and *D. auricularia*. They have designated them as aplysianins, julianins and dolabellanins, respectively. In addition, he noticed that the factors were active for Gram-positive and Gram-negative bacteria and some fungi, and their action was not cytocidal but cytostatic. Another series of bioactive peptide/macrolides, known as aplyronines, have been isolated from Japanese sea hares and showed an antitumor activity (Kamiya *et al.*, 2006). Interestingly, the active principles in *Aplysia* species and *D.auricularia* were shown to be L-amino acid oxidase

(LAAO). Possible antibacterial activity and cytotoxic activity mechanisms of these proteins were detected (Kamiya *et al*., 2006).

Several bioactive substances are found in the ink alone and some in opaline alone, and others are generated only when ink and opaline are co-secreted and mixed in the mantle cavity (Kicklighter *et al*., 2005; Derby *et al*., 2007). A huge number of molecules have been discovered from *Aplysia* species possess secondary metabolites (Abe Kawsar *et al*., 2010). However, there are not more information available about the bioactive substances extracted from *Aplysia* species.

Successfully, LAAOs in the albumen gland and egg mass of sea hares have antimicrobial activity (Jimbo *et al*., 2003; Cummins *et al*., 2004). Regarding to this, Barbieri *et al*. (1997) stated that because sea hares lay eggs in strings on the substrate, using antimicrobials to prevent fouling is an obvious advantage and one that is accomplished by many invertebrates through diverse molecules. In addition, Iijima *et al.* (2003) isolated a novel antimicrobial peptide from the sea hare *D. auricularia*. They and other workers (Kamiya *et al.*, 2006; Yamada *et al*., 2009) reported that *Aplysia* species had specific biological active substances, including antibacterial factors, toxins, and chemical defense substances. For example, Orthologs of Escapin (oxidizes Larginine) exist in all other sea hares examined, including *A. dactylomela* (Derby, 2007). Some of these have had bacteriostatic and bactericidal effects (Kamio *et al.*, 2009). Yang *et al.* (2005) have isolated an antibacterial protein (Escapin) from the defensive secretions of the sea hare *A. californica*. This protein has been characterized as bacteriostatic and bacteriocidal agents. Recently, Derby *et al.* (2018) investigated the antimicrobial activity of Escapin, an L-amino acid oxidase from the ink of sea hares.

Susceptibility of commercial antibiotics

On comparison level, the activity of several commercial antibiotics (mm) was examined and then compared to the results of *A. fasciata* crude extract activity (mm) (Table 5). Basically, Gram positive showed obvious susceptibility towards most of the tested antibiotics. In fact, *B. subtlis* was sensitive towards Cephalexin, Rifampicin**,** and Piperacillin**,** while it was resistant towards both Metronidazole and Amikacin. Also, *B. cerues*, was sensitive towards Cephalexin, Rifampicin**,** and Amikacin**,** while it was resistant towards both Piperacillin and Metronidazole. As well as, *S. aureus* was sensitive toward both Cephalexin and Metronidazole, while it was resistant towards Rifampicin, Piperacillin, andAmikacin. On contrary, *S. epidermids*, *E. faecalis*and *K. pneumonia* exhibited clear resistance towards all tested antibiotics. Also, *P. aeruginosa* behaved like them except for Cephalexin and Metronidazole, where they showed intermediate sensitivity. In addition, *E. coli* was only susceptible against Cephalexin**.** However, data confirmed that the Gram negative bacteria were more resistant than Gram positive ones and the inhibition of *A. fasciata* crude extract was lower than all commercial antibiotics. Our study confirmed that the Gram negative bacteria were more resistant than Gram positive ones. This may be due to Gram negative bacteria have a largely impermeable thick cell wall (Exner *et al*., 2017). Also, the inhibition of *A. fasciata* crude extract was lower than all commercial antibiotics may due to the bioactive substance in such crude is exposed to the dilution effect. So, it did not show high clearance zone around the tested microorganism.

GC-MS/MS analysis of *A. fasciata* crude extract

In particular, the results of GC-MS/MS of ethanolic crude extract of *A. fasciata* proved the presence of several bioactive constituents, with 18 major compounds (Fig. 3 & Table 6). The chemical profiles of them are mainly: 1,3,2-Dioxathiolane, 2-oxide (12.97%), S-Methyl methane thiosulphonate (64.29%), 4,25-Secoobscurinervan-4-one, O-acetyl-22-ethyl-15,16-dimethoxy-, (22à) (17.85%), Carbonotrithioic acid, dimethyl ester (13.73%), 2-Amino-3-(4-hydroxyphenyl)-propanoic acid (27.18%), Cyclohexasiloxane, dodecamethyl (6.05%), 1,2-Dithiolane (96.70%), Benzenesulfonic acid, 4-amino-3-nitro (8.51%), unidentified compound (3.24%), Cycloheptasiloxane, tetradecamethyl (6.52%), 2-Cyclopropene-1-carboxylic acid, 2-(1,1-dimethyl-5-oxohexyl)-, methyl ester (90.25%), 4-Piperidineacetic acid, 1-acetyl-5-ethyl-2-[3-(2-hydroxyethyl)-1H-indol-2-yl]-à-methyl-, methyl ester (12.78%), Ethyl iso-allocholate (6.51%), 2,7-Diphenyl-1,6-dioxopyridazino[4,5:2',3']pyrrolo[4',5'-d]pyridazine (9.44%), Cyclooctasiloxane, hexadecamethyl (9.14%), Glycocholic acid (78.58%), (5á)Pregnane-3,20á-diol, 14à,18à-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate (0.24%), and Spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R) (0.22%).

Table 5. Effect of different commercial antibiotics on bacterial reference strains in comparing to crude extract from *A. fasciata*.

Reference strain	Inhibition zone (mm)* of *A. fasciata* crude extract	Inhibition zone (mm)/Antibiotic (disc/µg)				
		Cephalexin (CL, 30 µg)	Rifampicin (RF, 30 µg)	Piperacillin (TZP, 10 µg)	Metronidazole (MTZ, 20 µg)	Amikacin (AMK, 30 µg)
B. subtlis	10	23	21	13	7	0
B. cerues	0	25	14	6	0	22
S. aureus	0	30	9	9	29	8
S. epidermids	0	10	0	0	9	0
E. faecalis	0	6	6	6	6	0
K. pneumoniae	0	0	0	7	0	9
P. aeruginosa	11	0	0	0	12	0
E. coli	10	23	0	0	0	0

*These values taken were representative as the highest average from Table 2. Susceptible/sensitive is considered when clearance inhibition zone detected around well or disc. 0; no activity (Resistant), ~10 mm; moderate activity, ~ 15 mm; high activity, and ~20 mm very high activity.

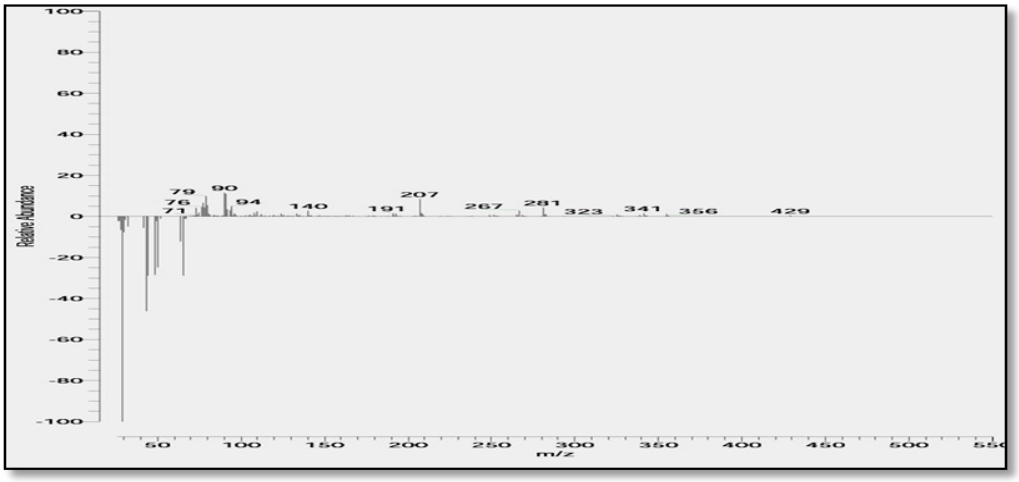

Fig. 3. GC-MS/MS chromatogram of *A. fasciata* crude extract showing the retention time of the identified compounds.

Table 6. Chemical constituents detected in of *A. fasciata* ethanolic crude extract by GC-MS/MS.

Peak No.	Compound name	RT (min)	Molecular formula	MW (m/z)	Hit	SI	RSI	Prob. (%)
1	1,3,2-Dioxathiolane, 2-oxide	5.71	$C_2H_4O_3S$	108	1	537	879	12.97
2	S-Methyl methanethiosulphonate	6.01	$C_2H_6O_2S_2$	126	1	615	803	64.29
3	4,25-Secoobscurinervan-4-one, O-acetyl-22-ethyl-15,16-dimethoxy-, (22à)	6.31	$C_{27}H_{36}N_2O_6$	484	1	507	512	17.85
4	Carbonotrithioic acid, dimethyl ester	6.71	$C_3H_6S_3$	138	2	537	553	13.73
5	2-Amino-3-(4-hydroxyphenyl)-propanoic acid (DL-Tyrosine)	6.89	$C_9H_{11}NO_3$	181	1	592	787	27.18
6	Cyclohexasiloxane, dodecamethyl	7.11	$C_{12}H_{36}O_6Si_6$	444	1	570	701	6.05
7	1,2-Dithiolane	7.22	$C_3H_6S_2$	106	1	834	857	96.70
8	Benzenesulfonic acid, 4-amino-3-nitro	7.67	$C_6H_6N_2O_5S$	218	1	535	831	8.51
9	Unidentified compound	8.01	$C_{26}H_{36}O_8$	476	4	505	511	3.24
10	Cycloheptasiloxane, tetradecamethyl	9.13	$C_{14}H_{42}O_7Si_7$	518	3	547	563	6.52
11	2-Cyclopropene-1-carboxylic acid, 2-(1,1-dimethyl-5-oxohexyl)-, methyl ester	9.51	$C_{13}H_{20}O_3$	224	1	827	874	90.25
12	4-Piperidineacetic acid, 1-acetyl-5-ethyl-2-[3-(2-hydroxyethyl)-1H-indol-2-yl]-à-methyl-, methyl ester	9.72	$C_{23}H_{32}N_2O_4$	400	1	554	615	12.78
13	Ethyl iso-allocholate (Steroid)	9.83	$C_{26}H_{44}O_5$	436	5	534	553	6.51
14	2,7-Diphenyl-1,6-dioxopyridazino[4,5:2',3']pyrrolo[4',5'-d]pyridazine	10.61	$C_{20}H_{13}N_5O_2$	355	4	579	593	9.44
15	Cyclooctasiloxane, hexadecamethyl	11.07	$C_{16}H_{48}O_8Si_8$	592	4	559	603	9.14
16	Glycocholic acid	12.30	$C_{26}H_{43}NO_6$	465	1	765	849	78.58
17	(5á)Pregnane-3,20á-diol, 14à,18à-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate	12.80	$C_{28}H_{43}NO_6$	489	10	560	569	0.24
18	Spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)	14.36	$C_{27}H_{40}O_4$	428	9	521	604	0.22

Generally, the bioactive compounds detected in the crude extract of our *A. fasciata* were organic acids and their derivatives, besides much other of organic alcohols, steroids and terpenoids. However, the antimicrobial activities of the most of these constituents have been identified and established (Ibrahim, 2012; Moustafa *et al*., 2013; Hussein *et al*., 2016; Ibrahim *et al*., 2018).

Basically, Kiyoyuki and Hideo (1997) described the chemistry of the bioactive compounds isolated from the sea hares belonging mainly to two genera: *Aplysia* and *Dolabella*. These compounds are classified as; i) polyketides, ii) terpenes, iii) peptides and depsipeptides. Special emphasis is placed on the chemistry of cytotoxic compounds such as aplyronines and dolastatins that were obtained. Actually, many of bioactive substances from sea hares species are terpenoids especially sesquiterpenoids and diterpenoids, as well as, organic acids and their esters (Benkendorff, 2010; Bornancin *et al*., 2017). For instance, Datta *et al.* (2019) found that the proteinogenic amino acid tyrosine acted as potent antimicrobial agents against *Shigella flexneri 2a* and methicillin-resistant *S. aureus*. Also, Ethyl iso-allocholate (steroid) has antimicrobial activity (Muthulakshmi *et al*., 2012). As well as, Schmidt *et al.* (2001) and Sannasiddappa *et al.* (2017) mentioned that cationic cholic acid derivatives, such as Glycocholic acid, displayed potent and broad-spectrum activity against multidrug-resistant Gram-negative and -positive bacteria. They confirmed that specific examples of these compounds were effective permeabilizers of the outer membranes of many strains of multidrug-resistant Gram-negative bacteria and sensitized these to hydrophobic antibiotics. Moreover, Kim *et al.* (2006) conducted that the acyclic thiosulfinates (1, 2-

Dithiolane) possess antimicrobial, antiparasitic, antitumor and cysteine protease inhibitory activity while the natural 1, 2-dithiolane-1-oxides are growth inhibitors. Also, Benkeblia *et al*. (2007) confirmed the effectiveness of the natural biologically active S-Methyl methane thiosulphonate in developing potent antifungal agents.

CONCLUSION

The current study has observed that the investigations carried out on the sea hares, specifically on *Ablyzia fasciata*, are little and still need much more efforts especially in recent manner. Although, according to the results obtained during our study, *A. fasciata* habited in the Egyptian Mediterranean Sea can be useful in the industry of pharmaceutical products, since it possess significant bioactive capacities and its crude extract showed good spectrum of antimicrobial activity against the tested microbial species. Further studies have to be carried out to separate and then elucidate the structure of the most effective bioactive substance. Finally, the unidentified compounded (with $C_6H_6N_2O_5S$ and molecular weight equals 476), detected by the precise tool; GC-MS/MS, may be promising if it takes a chance in future study.

REFERENCES

Abe Kawsar, S.M.; Ozeki, Y.; Mamun, S.M.A. and Rahman, Md.S. (2010). Growth inhibitory effects on microorganisms by a D-galactose-binding lectin purified from the sea hare (*Aplysiakurodai*) eggs: An *in vitro* study. Nat. Sci., USA 8(2): 82-89.

Amer, M.S. and Ibrahim, H.A.H. (2019). Chitosan from marine-derived *Penicillumspinulosum* MH2 cell wall with special emphasis on its antimicrobial and antifouling properties. Egy. J. Aqua. Res., 45: 359-365.

Atlas, R.M. (1997). Handbook of media for environmental microbiology (p. 265, 412). **Boca Raton**., FL: CR Press.

Barash, A. and Danin, Z. (1971). Opisthobranchia (mollusca) from the Mediterranean waters of Israel. Int. J. Zool., 20: 151-200.

Barash, A. and Danin, Z. (1982). Mediterranean mollusca of Israel and Sinai: composition and distribution. Israel Acad Sci. Human., 405 pp.

Barbieri, L.; Valbonesi, P.; Bonora, E.; Gorini, P.; Bolognesi, A. and Stirpe, F. (1997). Polynucleotide: adenosine glycosidase activity of ribosome-inactivating proteins: effect on DNA, RNA and poly(A). Nucleic Acids Res., 25: 518-522.

Bebbincton, A. (1975). On a collection of Apfysia species from Naples with a note on the distribution of *Aplysia parvula* (Gastropoda, Opisthobranchia) in the Mediterranean. Pubbl. Staz. Zool. Napoli, 39: 121-128.

Bebbington, A. and Hughes, G.M. (1973). Locomotion in *Aplysia* (Gastropoda, Opisthobranchia). *Proceedings of the Malacological Society, London, 40*: 399-405.

Benkeblia N. and Lanzotti V. (2007) Allium thiosulfinates: Chemistry, biological properties and their potential utilization in food preservation. Food-Global Science Books, 1(2): 193-201.

Benkendorff, K. (2010). Molluscan biological and chemical diversity: secondary metabolites and medicinal resources produced by marine molluscs. Biology Review of the Cambridge philosophical Society, 85(4): 757-775.

Bornancin, L.; Bonnard I.; Mills, S.C. and Banaigs, B. (2017). Chemical mediation as a structuring element in marine gastropod predator-prey interactions. Nat. Prod. Rep., 34(6): 644-676.

Cosgrove, S.E. (2006). The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. Clin. Infect. Dis**.**, 15; 42 Suppl 2: S82-S89.

Cummins, R.A.; Davern, M.T.; Eckersley, R.; Lo, S.K.; Okerstrom, E. and Woerner, J. (2004). Australian Unity Wellbeing Index, Survey 10, Report 10.0. The wellbeing of Australians. Health and Body Weight. *Australian* Center on Quality of Life, Melbourne, Australia,

Datta, L.P.; Dutta, D.; Chakraborty, A. and Das, T.K. (2019). Tyrosine based cationic acrylates as potent antimicrobial agents against shigellosis. Biomat. Sci., 7(6): 2611-2622.

Derby, C.; Gilbert, E.S and Tai, P.C. (2018). Molecules and mechanisms underlying the antimicrobial activity of escapin, an l-amino acid oxidase from the ink of sea hares. Biol. Bull., 235(1): 52-61.

Derby, C.D. (2007). Escape by inking and secreting: marine molluscs avoid predators through a rich array of chemicals and mechanisms. Biol. Bull., 213: 274-289.

Derby, C.D.; Kicklighter, C.E.; Johnson, P.M. and Zhang, X. (2007). Chemical composition of inks of diverse marine molluscs suggests convergent chemical defenses. J. Chem. Ecol., 33: 1105-1113.

Diáz-Granados, C.A.; Cardo, D.M. and McGowan-Jr, J.E. (2008). Antimicrobial resistance: international control strategies with a focus on limited resource settings. Int. J. Antimicrob. Agents, 32(1): 1-9.

Eales, N.B. (1979). The Aplysiidae from the Red Sea. Argamon Israel J. Malacol., 7: 1 9 pp.

Exner, M.; Bhattacharya, S.; Christiansen, B.; Gebel, J.; Goroncy-Bermes, P.; Hartemann, P.; Heeg, P.; et al. (2017). Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria? GMS hygiene and infection cont., 12: Doc05.

Freeman, M.; Howes, R.; Wasserman, J.; Casci, T. and Smith, R. (1997). EGF receptor signaling strategies in the Drosophila eye. *A. Dros. Res. Conf., 38: 5C.*

Guinea, J.; Peláez, T.; Alcalá, L. and Bouza, E. (2005). Evaluation of Czapeck agar and Sabouraud dextrose agar for the culture of airborne *Aspergillus conidia*. Diag. Microbiol. Infec Dis., 53 (4): 333-334.

Hussein, H.M.; Hameed, I.H. and Ibraheem, O.R. (2016). Antimicrobial activity and spectral chemical analysis of methanolic leaves extract of *Adiantum capillus-veneris* using GC-MS and FTIR spectroscopy. Int. J. Pharmacog. Phytochem. Res., 8(3): 369-385.

Ibrahim, H.A.H. (2012). Antibacterial carotenoids of three *Holithoria* species in Hurghada, Egypt. Egyp. J. Aquatic Res., 38: 185-194.

Ibrahim, H.A.H.; Ahmed, H.O.; Abd El Razek, F.A. and Elmasry, E. (2018). Proteolysis and heat-sensitive antibacterial agents from several levantine sponge species**.** Int. J. Adv. Res., 6(2): 14-27.

Ibrahim, H.A.H.; Elshaer, M.M.; Elatriby, D.E. and Ahmed, H.O. (2020). Antimicrobial activity of the sea star (*Astropecten spinulosus*) collected from the Egyptian Mediterranean Sea, Alexandria. Egy. J. Aqua. Biol. Fish., 24(2): 507-523.

Iijima, R.; Kisugi, J. and Yamazaki, M. (2003). A novel antimicrobial peptide from the sea hare *Dolabella auricularia*. Develop. Comp. Immunol., 27(4): 305-311.

Jimbo, M.; Nakanishi, F.; Sakai, R.; Muramoto, K. and Kamiya, H. (2003). Characterization of L-amino acid oxidase and antimicrobial activity of aplysianin A, a sea hare-derived antitumor-antimicrobial protein. Fish**.** Sci**.**, 69, 1240-1246.

Kamio, M.; Ko, K-C.; Zheng, S.; Wang, B.; Collins, S.L.; Gadda, G. et al. (2008). The chemistry of escapin: identification and quantification of the components in the complex mixture generated by an L-amino acid oxidase in the defensive secretion of the Sea Snail *Aplysia californica*. Chem. Euro. J., 15: 1597-1603.

Kamiya, H.; Sakai, R. and Jimbo, M. (2006). Molluscs: bioactive molecules from sea hares. **Cimino, G. and Gavagnin, M.** (Eds.). The chemo-ecological study to biotechnology application. Springer, Berlin-Heidelberg, 215-239.

Khan, Z.A.; Siddiqui, M.F. and Park, S. (2019). Current and emerging methods of antibiotic susceptibility testing. Diagnostics (Basel)., 9(2): 49-55.

Kicklighter, C.; Shabani, S.; Johnson, P.M. and Derby, C. (2005). Sea hares use novel antipredatory chemical defenses. Curr. Biol., 15(6): 549-554.

Kim, S., Kubec, R. and Musah, R.A. (2006). Antibacterial and antifungal activity of sulfur-containing compounds from *Petiveria alliacea*. J. Ethnopharmacol., 104: 188-192.

Kiyoyuki, Y. and Hideo, K. (1997). Bioactive Compounds from the Sea Hares of Two Genera: *Aplysia* and *Dolabella.* Bull. Chem. Soci. Japan, 70(7): 1479-1489.

Lieberman, J.M. (2003). Appropriate antibiotic use and why it is important: the challenges of bacterial resistance. Ped. Inf. Dis. J., 22(12): 1143-1151.

Mander L. and Liu H.-W. (2010). Comprehensive Natural Products II: Chemistry and Biology. 1st Edition. Imprint: Elsevier Science. ISBN: 9780080453811. Pp. 7388

Mona, M.H.; Salem, M.L.; Basiony, M.A. and Ziada, M.A. (2016). *In vitro* antitumor effects of egg extract and purple fluid from marine *Aplysia fasciata*. BFSZU, 38: 194-206.

Morens, D.M.; Folkers, G.K. and Fauci, A.S. (2004). The challenge of emerging and re-emerging infectious diseases. Nat., 430: 242-249.

Moustafa, M.F.M.; Alamri, S.A.; Taha, T.H. and Alrumman, S.A. (2013). *In vitro* antifungal activity of *Argemone ochroleuca* sweet latex against some pathogenic fungi. Afr. J. Biotechnol., 12(10): 1132-1137.

Muller, A.; Duchting, P. and Weiler, E.W. (2002). A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to Arabidopsis thaliana. Planta, 216: 44-56.

Muthulakshmi, A.; Jothibai, M.R. and Mohan, V.R (2012). GC-MS analysis of bioactive components of *Feronia elephantum Correa* (Rutaceae). J. Appl. Pharmace. Sci., 2(2): 69-74.

Nocchi, N.; Soares, A.R.; Souto, M.L. and Pereira, R.C. (2017). Detection of a chemical cue from the host seaweed Laurencia dendroidea by the associated mollusc *Aplysia brasiliana*. PLoS One, 12(11): e0187126.

Palaniveloo, K.; Rizman-Idid, M.; Nagappan, T. and Abdul Razak, S. (2020). Halogenated Metabolites from the Diet of Aplysia dactylomela Rang. Molecules 28, 815: 1-18.

Pereira, R.B.; Andrade, P.B. And Valentão, P. (2016). Chemical diversity and biological properties of secondary metabolites from sea hares of *Aplysia* genus. Mar. Drugs, 14(39): 1-33.

Sannasiddappa, T.H.; Lund, P.A. and Clarke, S.R. (2017). *In Vitro* antibacterial activity of unconjugated and conjugated bile salts on *Staphylococcus aureus.* Front Microbiol., 8: 1581-1589.

Schmidt, E.J.; Boswell, J.S.; Walsh, J.P.; Schellenberg, M.M.; Winter, T.W.; Li, C.; Allman, G.W. et al.. (2001). Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria. J. Antimicrob. Chemother., 47(5):671-674.

Shaaban, M.T.; Ibrahim, H.A.H. and Hanafi, A.A.M. (2020). Antibiotic-resistant bacteria isolated from selected urine and stool human specimens. Biosci. Res., 17(1): 351-365.

Sipahi, O.R. (2008). Economics of antibiotic resistance. Exp. Rev. Anti-Inf. Ther., 6(4): 523-39.

Susswein, A.J.; Gev, S.; Achituv, Y. and Markovich, S. (1984). Behavioral patterns of *Aplysia fasciata* along the Mediterranean coast of Israel. Behav. Neural. Biol., 41: 7-22.

Takamatsu, N.; Shiba, T.; Muramoto, K. and Kamiya, H. (1995). Molecular cloning of the defense factor in the albumen gland of the sea hare *Aplysia kurodai*. FEBS Lett., 377: 373-376.

Wyatt, T.D. (2003). Pheromones and Animal Behaviour. The Press Syndicate of the University of Cambridge, Oxford.

Yamada, K.; Ojika, M.; Kigoshi, H. and Suenaga K. (2009). Aplyronine A, a potent antitumor macrolide of marine origin, and the congeners aplyronines B-H: chemistry and biology. Nat. Prod. Rep., 26: 27-43.

Yamazaki, M. (1993). Antitumor and antimicrobial glycoproteins from sea hares. Compar. Biochem. Physiol. Part C: Compar. Pharmacol., 105(2): 141-146.

Yang, H.; Johnson, P.M.; Kamio, K-C.K.M.; Germann, M.W.; Derby, C.D. and Tai, P.C. (2005). Cloning, characterization, and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*. J. Exp. Biol. 208: 3609-3622.

Zsilavecz, G. (2007). Nudibranchs of the Cape Peninsula and False Bay. ISBN 0-620-38054-3.

Arabic summary

النشاطية المضادة للميكروبات لأرنب البحر (*Aplysia fasciata*) المجموع من البحر الأبيض المتوسط المصري، الاسكندرية

حسن عبد الله حسن إبراهيم[١]، محمد صالح إبراهيم[١]، حمدي عمر أحمد[٢]، ناهد عبد الله حسن[٣]

[١]قسم الميكروبيولوجيا البحرية المعهد القومي لعلوم البحار والمصايد، الاسكّندرية.
[٢]قسم اللافقاريات البحرية المعهد القومي لعلوم البحار والمصايد، الاسكّندرية.
[٣]قسم الكيمياء، مستشفى الباطنة التخصصي، جامعة المنصورة.

تم جمع أنواع من ارنب البحر من البحر الأبيض المتوسط، الإسكندرية، مصر. تم تعريفه بناءً على السمات المورفولوجية والتشريحية العامة على أنه *Aplysia fasciata*. تم فحص الأنشطة المضادة للبكتيريا والفطريات من خلال التقنيات القياسية. أظهرت البيانات التي تم الحصول عليها أنه تم الكشف عن أعلى نشاط مضاد للميكروبات ضد *P. aeruginosa* (AU = 3.4) ، يليه *E. coli* (AU = 2.9)، ثم *B. sublis* (AU =) 2.7. ولم تتأثر الممرضات البكتيرية الأخرى على الإطلاق. بالمثل، لوحظ أقصى تثبيط فطري، عن طريق طريقة الصب، ضد فطر *P. crustosum* (50 ٪). وكانت AUs ضد كل من *F. solani* و *A. niger* 20 و 10٪ على التوالي، بينما لم يتم تسجيل أي نشاط ضد الآخرين. أيضاً، تم إجراء النشاط المضاد للفطريات عن طريق طريقة (well-cut discussion technique)، حيث تم تسجيل أعلى (AU 6.8) ضد *A. flavus*، تلاه AU = 4.8 مقابل *F. solani*، ثم AU = 1.8 ضد *P. crustosum*. علاوة على ذلك، بينما تراوحت قيم التثبيط المضاد للفطريات ضد سلالات الخميرة المرجعية بين3.1 و 6.8. وقد تم تسجيل أعلى مستوى ضد *C. tropicalis* (AU = 4.8) تلاه ضد *R. mucilaginosa*. فيما يخص دراسة فعالية بعض المضادات الحيوية التجارية (ملم)، أكدت البيانات أن البكتيريا السالبة لصبغة جرام كانت أكثر مقاومة من البكتيريا الموجبة لصبغة جرام. على الجانب الآخر، لوحظ أن من خلال نتيجة GC-MS/MS للمستخلص الخام وجود العديد من المكونات النشطة بيولوجيًا، وكان لمعظمها أنشطة مضادة للميكروبات.