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Preliminary bioassay on antibacterial effects of *Tripneustes gratilla* extracts from the Red Sea, Egypt

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ABSTRACT

Tripneustes gratilla are entirely depended on powerful innate immune systems in order to stay healthy. An attempt is conducted to evaluate the antibacterial effects of extracts obtained from different tissues (i.e. gonad, gut, and spine) of the sea urchin *T.gratilla* from the Red Sea against some selected bacterial pathogens. Potentially bioactive metabolites extracted from different organs, using methanol and chloroform, were examined against seven pathogenic bacterial species; *Staphylococcus aureus* 25923, *Enterococcus faecalis 29212*, *Pseudomonas aeruginosa* 9027, *Bacillus subtilis 6633*, *Escherichia coli* 8739, *Vibrio fluvialis* and *Vibrio damsela*. The methanolic gut extract showed the highest antibacterial activity against the examined pathogenic bacteria. The minimum inhibitory concentration of the methanolic gut extract was determined at different concentrations (200- 1000 µg / ml) using the broth dilution method. FTIR and GC-MS were determined. The study indicated that the sea urchin *T. gratilla* gut extract seems to be a promising source of antibacterial compounds.

INTRODUCTION

The growing number of antibiotic resistant bacteria is becoming a serious problem that affects most of the countries worldwide. Resistance to multiple antibiotics has developed among many common pathogens, such as *staphylococci*, *pneumococci*, and *Pseudomonas* organisms **(Olofsson and Cars, 2007)**. It poses an increase that drew the researchers to accomplish studies on new antimicrobial agents from different novel sources, mostly are natural sources. Thus, the methods that was used to develop drugs and therapeutic agents are greatly enhanced by newly developed formulations of pharmaceutical products from novel natural resources, catering the needs of alternative treatments that are more effective. Nevertheless, infectious diseases tend to be a limiting factor for public health all over the world. Consequently, a lot of researches have been done to find an effective tool for preventing or curing the disease **(Marimuthu *et al.*, 2015)**.

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For a long period of time, marine environments have been a worthwhile fountain for natural products in support of maintaining the human health, especially in the last decade, with the more attention for natural based remedies. On the other hand, the microbial communities in seawater and sediments are highly abundant that its concentration may reach 10^6 and 10^9 per milliliter, respectively **(Austin, 1988)**. In reference, marine organisms are exposed in far tougher condition compared with the terrestrial ones. They are continuously exposed to high concentrations of bacteria, fungi and viruses, all of which might be pathogenic. This declares their dependence on successful antimicrobial pathways to protect them against microbial infections. This high diversity provides an excellent opportunity to discover new bioactive compounds **(Abubakar *et al*., 2012)**. Consequently, the marine environment considered a rich source of new compounds for the development of new antibacterial medicines. Like many other marine invertebrates, sea urchins have been regarded as a bioactive source of biomedical compounds. The shells are known to contain various polyhydroxylated naphtoquinone pigments, spinochromes **(Anderson *et al*., 1969)** as well as their analogous compound, echinochrome A, of which bactericidal effect was reported by **Service and Wardlaw (1984)**. Polyhydroxylated napthoquinone pigment commonly was isolated from sea urchin spines as well **(Pena-Cabrera *et al*., 2002)**. The sea urchin gonads polyhydoxylated naphthoquinone, echinochrome A, showed potential antioxidant activity **(Lebedev *et al*., 2001**). The antibacterial activity of sea urchin is generally characterized using various extracts in organic solvents, for example acetone, methanol – toluene, ether and chloroform. Several studies demonstrated the presence of antimicrobial factors in several tissues of sea urchin **(Kazemi *et al*., 2016)**. The methanol and chloroform extracts of gut, gonad, spines and mouth parts from sea urchin *Tripneustes gratilla* manifest antimicrobial properties against an array of pathogenic bacteria **(Abubakar *et al*., 2012 and Ambag *et al*., 2016)**.

The present work has been focused on studying the potentiality of the extracts of *Tripneustes gratilla* organs (spines, gonads and gut) as antibacterial agents in addition to characterizing the most promising compounds.

MATERIALS AND METHODS

1. *Experimental animals and collected samples*

Tripnestus gratilla samples were collected from Hurgada, Red Sea. Samples were collected by snorkeling at depth ranged between 1.0 and 2.5 m. Specimens diameters ranged between 60 and 110 mm were collected and transported to the laboratory immediately. Samples were washed by filtered sea water and processed for further studies.

2. *Preparation of extracts*

Tissue samples of spines, gonads and guts of *T.gratilla* (4gm each) were grinded down and homogenized with two solvents; 10 volumes (v/w) of 70% (v/v) methanol and chloroform and agitated for 24 h in a shaker (90 rev/min.) at 10°C. The crude extracts were then centrifuged (12,000g) at 4°C for 5 minutes. The supernatants were collected, filtered through Whatman No. 1 sterile filter paper and collected in a Beckman sterilized tube and stored at -20°C. The sterile filtrates were used for antibacterial assay through the agar disc diffusion process **(Abubakar *et al*., 2012 and Marimuthu *et al*., 2015)**.

3. Antibacterial assay

The antibacterial activity of natural products was assessed against seven pathogenic bacterial species; namely, *Staphylococcus aureus* 25923, *Enterococcus faecalis 29212*, *Pseudomonas aeruginosa* 9027, *Bacillus subtilis 6633*, *Escherichia coli* 8739, *Vibrio fluvialis* and *Vibrio damsela* provided by the stuff members of National Institute of Oceanography and Fisheries-Alexandria Branch. Agar diffusion well-variant was used to test the efficiency of *T. gratilla* extracts. The pathogenic indicator bacteria were inoculated in nutrient agar media (50 ml) in each plate. Upon solidification, wells were punched out using 0.5 cm cork borer. The organic extracts were prepared by transferring 50 mg of the extract to 1mL of dimethyl sulfoxide (DMSO) (Merck, Germany). The extract was then transferred to the well and incubated at 37°C for 24 h, under aerobic conditions. After incubation, the detection of clear inhibition zone around wells is an indication of antibacterial activities of the different organs of sea urchin extracts **(Shushizadeh *et al*., 2019).**

4. Minimum inhibitory concentration (MIC)

The broth dilution method was used; a stock solution of 1mg/ml of the extract was prepared in the nutrient broth medium and was serially diluted to obtain concentrations of 200, 400, 600, 800 and 1000 µg/ml. 0.5 ml of each dilution was transferred to a test tube containing 3.0 ml of nutrient broth, then inoculated with 0.5 ml of one of the pathogenic indicator bacterial culture. A test tube set containing broth alone was used as control. Both test and control tubes were incubated for 24h at 37°C. The tube containing the least extract concentration showing no visible sign of growth was determined as the minimum inhibitory concentration after the incubation period **(Maheswaran *et al*., 2015)**.

5. Fourier-transform infrared spectroscopy analysis (FTIR)

Bio-transformed products found in the gut were frozen, then diluted with potassium bromide (ratio 1:100). The sample FTIR spectrum was tracked on a diffuse reflectance mode (DRS-800) mounted FTIR instrument. All measurements were performed in the range from 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1}. The spectral data recorded were compared with the reference chart to classify the functional groups identified in the tested sample **(Bashari *et al*., 2019)**. The test was performed at the Regional Center for Mycology and Biotechnology, Al-Azhar University; Cairo, Egypt.

6. Gas-liquid chromatography mass spectra (GLC)

A high-performance 5890 gas-liquid chromatography (GLC) (Hewlett Packard) coupled with 5989B series mass spectrometer (MS) (Shimadzu (EI) Japan) was used to identify chemical constituents of crude extract from sea urchin gut. The percentage of each compound was calculated as the ratio of the peak area to the chromatographic total area. The initial oven temperature was scheduled for keeping at 90°C for 1 minute and then rising at 8°C /min. to 300°C for 30 minutes. Helium was used at a flow rate of 1.5 ml /min. as a carrier gas. The volume of injection for each sample was 1 µl in split less mode where the temperature of the injector was 290°C. The mass distribution was run at 70 eV and the mass range was between 60 and 600 amu (**Hassan and Shobier, 2018**).

RESULTS

1. Antibacterial activity

The methanol and chloroform extracts of the three selected tissues were compared for its antibacterial activity (Fig. 1 and table 1). All *T. gratilla* extracts with a concentration of 1000 µg/ ml exhibited antibacterial activity in vitro. The methanol gut extracts mostly showed the highest antibacterial activity against all the tested pathogenic bacteria. The methanol spine extracts showed slightly higher effect than gut only in case of *S. aureus* 25923. The methanol gonad extracts represented similar effect like gut extracts in case of *Escherichia coli* 8739. Methanol spine, gonads and gut extracts showed similar effect against *Pseudomonas aeruginosa* 9027. In general, little or no activity was observed in both the spines and gonads chloroform extracts. In case of the methanol gut extracts, maximum zone of inhibition (20.0 mm) was observed against *Enterococcus faecalis* 29212 followed by *Bacillus subtilis* 6633 and *Vibrio damsel* (18.0 mm). In comparison, the lowest zone of inhibition was determined against *P. aeruginosa* 9027 (10.0mm), see Fig.(1) and table (1).

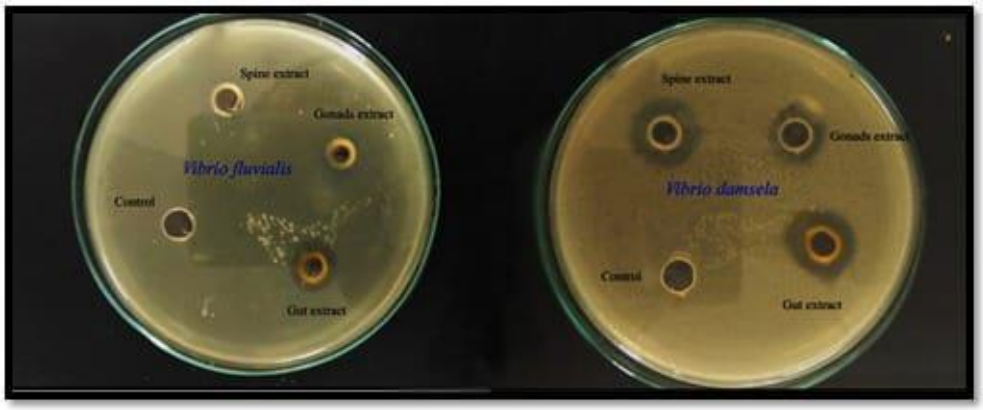

Fig. 1. Antibacterial activity of the different sea urchin *T. gratilla* parts using methanolic extracts.

Table 1. Antibacterial activities of methanol and chloroform extracts, from organs of the sea urchin *T. gratilla* against selected pathogenic bacteria.

Indicator bacteria	Antibacterial activity (mm) of the sea urchin *T. gratilla* methanol extracts			Antibacterial activity (mm) of the sea urchin *T. gratilla* chloroform extracts		
	Spine	**Gonads**	**Gut**	**Spine**	**Gonads**	**Gut**
P. aeruginosa 9027	10.0	10.0	10.0	08.0	06.0	08.0
E. coli 8739	10.0	15.0	15.0	00.0	08.0	10.0
E . faecalis 29212	00.0	10.0	20.0	00.0	10.0	08.0
S. aureus 25923	12.0	00.0	10.0	00.0	00.0	06.0
B. subtilis 6633	00.0	10.0	18.0	00.0	00.0	00.0
V. fluvialis	00.0	00.0	10.0	00.0	00.0	06.0
V. damsela	15.0	10.0	18.0	06.0	06.0	00.0

2. *Minimum inhibitory concentration (MIC)*

Table (2) shows the minimum inhibitory concentration of methanol gut extract of the sea urchin *T. gratilla*. The results revealed that *Enterococcus faecalis* 29212 was arrested at a low concentration of 400 µg/ ml, while *Bacillus subtilis* 6633 was arrested at a concentration of 600 µg/ ml. Registered minimum inhibitory concentration at 800 µg/ml was recorded for *Escherichia coli* 8739 and *Vibrio damsela*. *Staphylococcus aureus* 25923 was reported at the concentration of 1000 µg/ ml with *Vibrio fluvialis*.

Table 2. Minimum inhibitory concentration of methanolic gut extracts of *T. gratilla* against examined pathogenic bacteria.

Indicator bacteria	Concentrations of the methanolic gut extracts				
	200 µg/ml	400 µg/ml	600 µg/ml	800 µg/ml	1000 µg/ml
***P. aeruginosa* 9027**	+++	+++	++	+	-
***E. coli* 8739**	+++	+++	+	*	-
E. faecalis 29212	+	*	-	-	-
***S. aureus* 25923**	+++	+++	++	+	*
B. subtilis 6633	++	+	*	-	-
V. flvialis	+++	+++	++	+	*
***V. damsel*a**	+++	++	+	*	-

3. *Fourier-transform infrared spectroscopy analysis (FTIR)*

Fourier-transform infrared spectroscopy was used to identify the chemical bond functional groups present in the methanolic extract of the gut. The various functional groups in methanolic solvent extracts of the *T. gratilla*'s gut were analyzed from graph represents in Fig. (2). The gut extract represented absorption at 3417.86 cm^{-1}showing O-H stretch alcohol group. The 2924.09 cm^{-1}, 1465.90 cm^{-1} and 2854.65 cm^{-1}show the presence of C-H stretch alkane group. The 2075.41 cm^{-1} represent the presence of N=C=S stretch isothiocyanate group (strong). The range between 1635.64 and 918.12 cm^{-1} show the presence of aromatic C=C. The peaks at 1411.89 and 1388.75 cm^{-1} represent alcoholic group O-H bend alcohol's group. The peak of 1334.74 cm^{-1} represents the occurrence of C-N stretch aromatic amine group. The peaks of 1211.30 and 1111.00 cm^{-1} show C-O stretching. Finally, the peak of 1041.56 cm^{-1} show group of CO-O-CO stretching (strong, broad).

4. *Gas-liquid chromatography mass spectra analysis (GC-MS)*

The GC-MS chromatogram of crude methanol extracts from the gut showed different bioactive compounds. A total of 16 peaks for different bioactive compounds with specific retention time intervals with identical peak area were identified. The principal constituents and the relative percentage of the defined compounds represented in Fig. (3) and table (3). The corresponded component to the peaks were determined as follows: Cyclohexene, 1-methyl-4-(1-methylethenyl)-(S)-, 4,8-dimethyl-1,3,7-nonatriene, Isooctyl chloride, cyclohexane,1,2,3-trimethyl-, (1à,2á,3à)-, 6,9,12-octadecatrienoic acid,

methyl ester, 7-epi-cis-sesquisabinene hydrate, 5-Bromo-2-methyl-2-pentene, 6,11-Dimethyl-2,6,10-dodecatrien-1ol,15-Methyltricyclo|6.5.2[13,14].0[7.15]|pentadeca -1,3,57,9,11,13-heptene, Phosphoric acid, tributyl ester, Methyl tetradecanoate, 2H-Pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl),[3S[3à,6à(R*)]]-, 9-hexadecenoic acid, methyl ester, (z), 9,12-Octadecadienoic acid (Z, Z)-, methyl ester, 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)- and cis-11-Eicosenoic acid, methyl ester.

Table 3. Gas-liquid chromatography mass spectrum analysis of methanol extract of bioactive compounds from *T.gratilla* gut

No	R T Value (In Min.)	Compound	Molecular Formula	Molecular Weight	Peak area (%)
1	6.91	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-	$C_{10}H_{16}$	136	1.65
2	9.33	4,8-dimethyl-1,3,7-nonatriene	$C_{11}H_{18}$	150	4.79
3	11.08	Isooctyl chloride	$C_8H_{17}Cl$	148	5.91
4	13.83	cyclohexane,1,2,3-trimethyl-, (1à,2á,3à)-	C_9H_{18}	126	10.82
5	15.47	6,9,12-octadecatrienoic acid, methyl ester	$C1_9H_{32}O_2$	292	0.32
6	15.58	7-epi-cis-sesquisabinene hydrate	$C_{15}H_{26}O$	222	0.23
7	15.90	5-Bromo-2-methyl-2-pentene	$C_6H_{11}Br$	162	1.91
8	17.46	6,11-Dimethyl-2,6,10-dodecatrien-1-ol	$C_{14}H_{24}O$	208	1.23
9	19.76	15-Methyltricyclo|6.5.2[13,14].0[7.15] |pentadeca-1,3,57,9,11,13-heptene	$C_{16}H_{14}$	206	0.87
10	23.06	Phosphoric acid, tributyl ester	$C_{12}H_{27}O_4P$	266	7.56
11	24.67	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.86
12	25.07	2H-Pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl)-, [3S-[3à,6à(R*)]]-	$C_{15}H_{26}O_2$	238	0.57
13	28.42	9-hexadecenoic acid, methyl ester, (z)	$C_{17}H_{32}O_2$	268	0.65
14	32.08	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	$C_{19}H_{34}O_2$	294	3.74
15	35.01	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	$C_{21}H_{34}O_2$	318	0.43
16	35.77	cis-11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	324	1.57

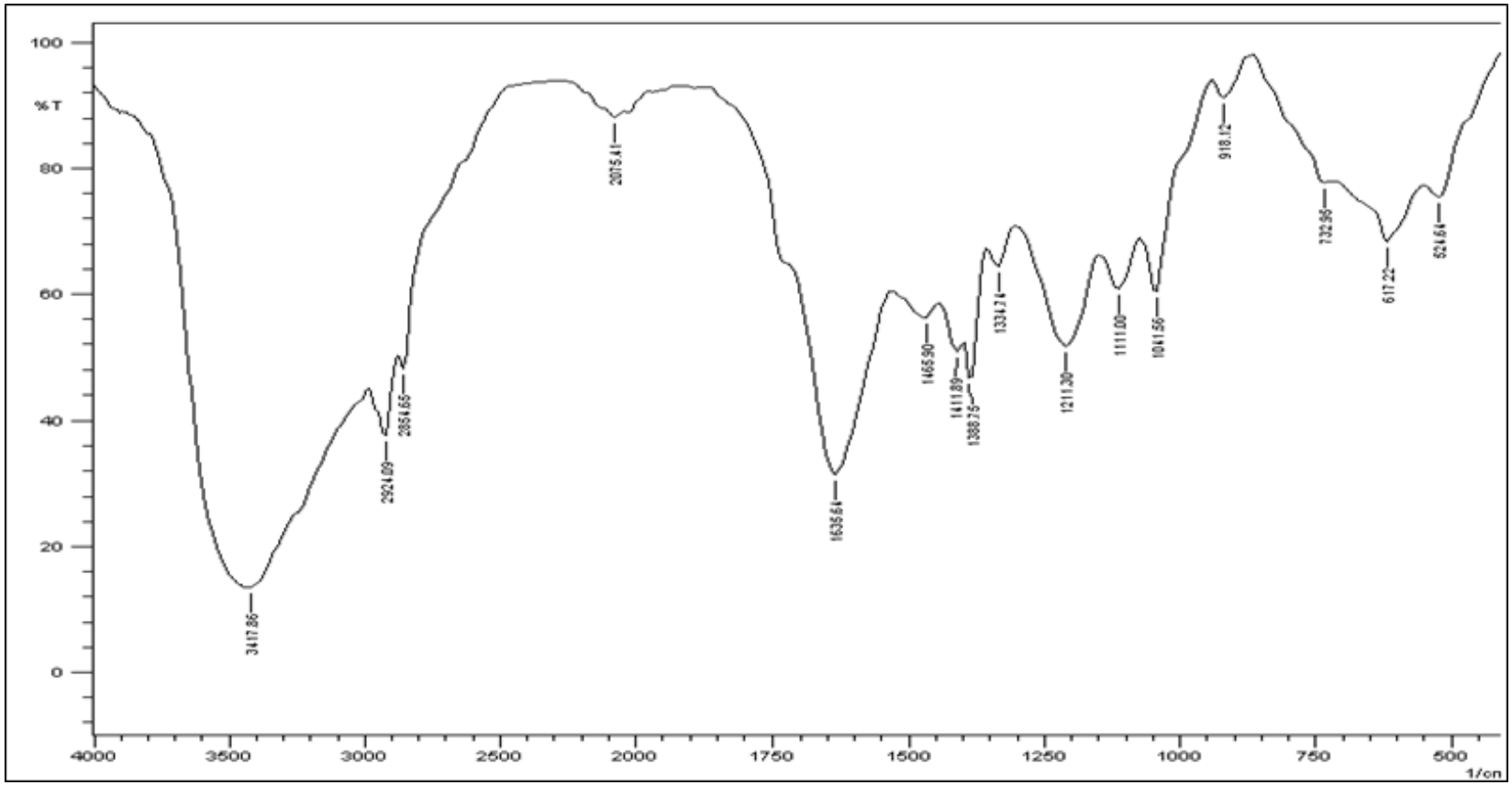

Fig. 2. FTIR spectrum of the methanolic gut extract produced from *T. gratilla*.

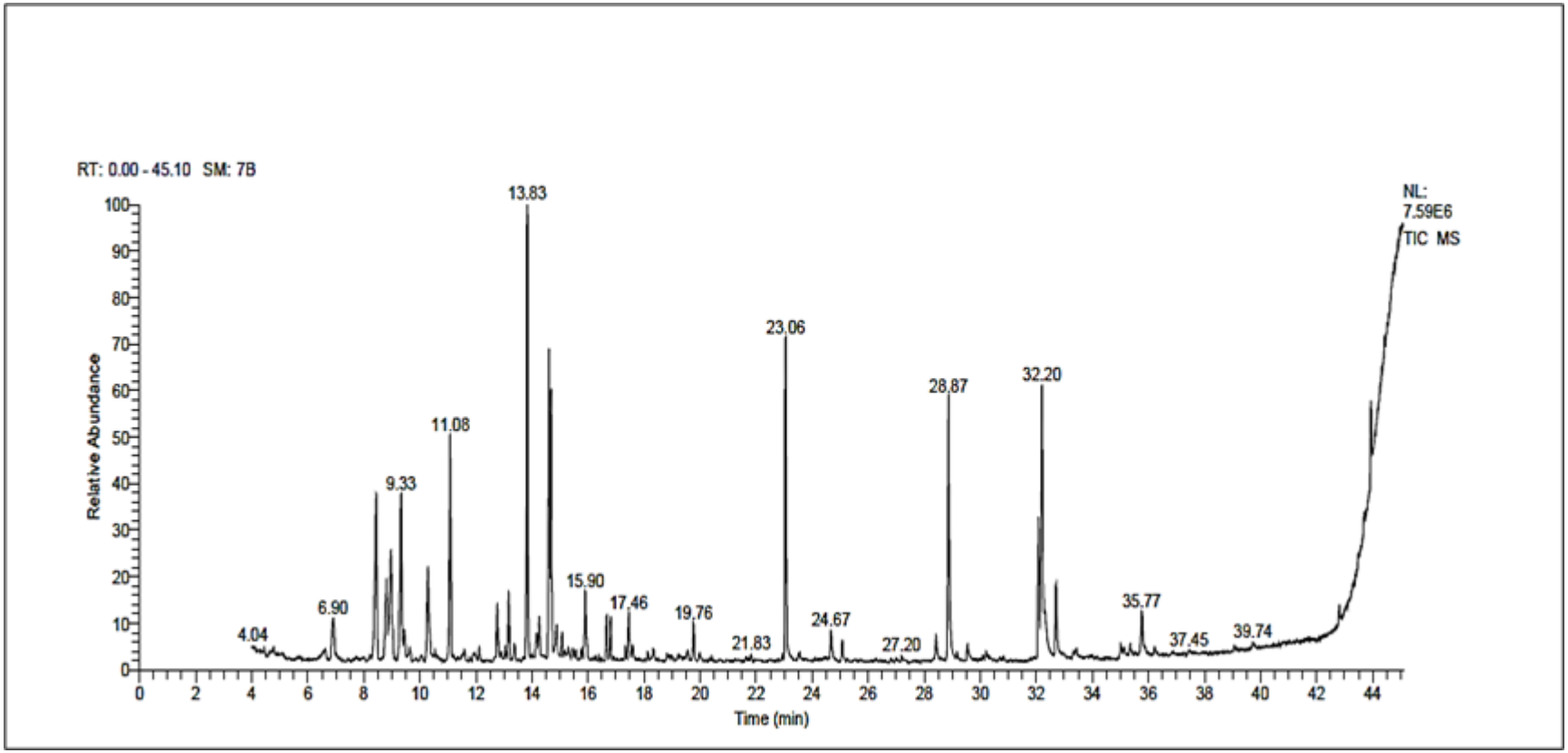

Fig. 3. GC-MC spectrum of the extracted bioactive compounds from *T.gratilla* gut.

DISCUSSION

The resilience of marine biota to survive in a wide range of marine environment and conditions, as well as lack of physical defense or adaptive immunity against pathogens and parasites, turns them as natural sources of the bioactive substances **(Bragadeeswaran *et al*., 2013)**. Like all marine organisms, the echinoid *T. gratilla* are constantly immersed in microorganism's community, thus they entirely dependent on powerful innate immune systems. Several extracts of the sea urchin *T. gratilla* showed in vitro antimicrobial activity **(Abubakar *et al.* 2012 and Ambag *et al*., 2016)**. Thus, the present researches are considered as an attempt to evaluate the antibacterial effects of various extracts in the different tissues of sea urchin *Tripneustes gratilla* from Red Sea against some bacterial pathogens. Identification of bioactive compounds was performed to confirm and correlate the anti-bacterial effects and to highlight its potential in discovering novel compounds. In this study, two solvents were used to extract and screen the *T. gratilla*s spine, gonads and gut for antibacterial activity against array of pathogenic bacteria. The highest activity against all the microorganisms tested was present in case of methanolic extract. This coincides with **Abubakar *et al.* (2012)** as similarly methanol extracts perform inhibition of both gram-positive and gram-negative bacteria. Since the two solvents possess different polarity and regarding the fact that each solvent extract compound of a similar polarity **(Shushizadeh *et al*., 2019)**. Methanol has higher polarity index than chloroform (5.1 and 4.1 respectively). Thus, methanol solvent has more ability to destroy cell wall and causes the components in the cell to disintegrate and dissolve in it **(Lapornik *et al*., 2005)**. This may explain the difference in the antibacterial activity in the test and the higher inhibition performance with methanol.

The maximum zone of inhibition (20.0 mm) for the methanol extract was observed against *Enterococcus faecalis* 29212 (gram + ve bacteria) using gut extract. A similar finding for the high effect of gut extracts were reported by **Ambag *et al.* (2016)** as they estimated that *T. gratilla* manifest antibacterial properties against an array of pathogenic bacteria, wherein the highest antibacterial activity was found in the gut and gonad extracts. Besides, **Abubakar *et al.* (2012)** estimated that the antimicrobial activity of the sea urchin *T. gratilla* are concentrated mainly in the gut and gonad extracts with little or no activity in the spine and mouth extracts.

Several factors could be the cause of the high effect of gut extracts as in general, the gastrointestinal (GI) tract is suitable for finding novel antimicrobials due to the vast array of microbes inhabiting it **(Garcia-Gutierrez *et al*., 2019)**. Besides, gut bacteria showed a role in the host health as in case of the sea urchin *Strongylocentrotus droebachiensis* (**Hakim *et al*., 2019**). Moreover, gut produces several compounds such as., phenolic compounds and other acids that can inhibit the growth of other bacteria. Similarly, they produce different peptides and non-peptidic molecules that showed promising antibacterial properties (**Garcia-Gutierrez *et al*. 2019)**. Understanding the microbial composition of the sea urchin digestive system may elucidate the role of the gut microbiome in the antimicrobial activity.

The lesser effect of gonad extracts in our study may be attributed to the maturity stage of the tested gonad as they were not in the ripe condition on performing the experiment. This suggests further studies on the gonad extracts in relation to its developmental stages. On the other hand, methanol spine extracts of the present study

showed higher effect on the same pathogenic bacteria reported by **Abubakar *et al*. (2012)**. This difference may be attributed to the habitat and the microorganism's community where the sea urchins immersed in.

The minimum inhibitory concentration for bacteria assumed different values in the present study ranging between 400 to 1000 µg/ ml. Disparities between active extracts showed that antimicrobial activity may be mediated by plenty of different compounds. Isolation and purification of active substances in order to determine their chemical character and evaluate their potential as new drugs are needed. Fourier-transform infrared spectroscopy (FTIR) is the chemical method widely used in the most biological extracts for the study of bioactive compounds **(Noh *et al*., 2015)**.Currently, due to its ease of sample preparation, FTIR becomes a well-accepted method, easy, needs little sample size and does not require more economical solvent usage **(Hernández-Martínez *et al*., 2014)**. Numerous studies have shown that, FTIR is used for the study of bioactive compounds in a variety of research specially food technology, pharmaceutical and medicinal **(Gosav and Dinica 2011, Anand and Gokulakrishnan 2012, Sahu and Saxena 2013 and Pervez *et al*., 2015)**.

CONCLUSION

The present study confirms the presence of functional group such as alkanes, isothiocyanate, aromatic amine and alcohol groups which was responsible for the antibacterial effects. The GC-MS of the methanol crude extract from *T. gratilla* determined the principal constituents and the relative percentage of the compounds defined. The identification of bioactive chemical compounds is based on the peak area, retention time, molecular weight and molecular formula. In addition, other earlier studies clearly demonstrated that the above mentioned bioactive compounds possess antimicrobial activity **(Krishnaiah *et al*. 2009, Kumar*et al*., 2010 and Jain *et al*., 2012)**. Therefore, the current study adds evidence to the presence of potent compounds in *T.gratilla* of Red Sea.

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