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Antimicrobial Activity and Bioactive Compounds of Persian Gulf Sea Cucumber (*Holothuria leucospilota*)

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Bioactive compounds of the body wall of the sea cucumber (*Holothuria leucospilota*) collected from the Persian Gulf were extracted using methanol and analyze separation was determined by GC-MS. Extracts were evaluated for their antibacterial and antifungal activities against Gram positive and Gram negative bacteria and also *Candida* species by using disk diffusion and broth microdilution susceptibility tests. Efficacy of extract was evaluated against sessile yeast and bacterial cells by the time-dependent killing assay. GC-MS analysis clearly showed the presence of twenty important constituents. The major determined bioactive compound in methanolic extract was fatty acids. The preliminary test showed significant antimicrobial activity for all isolates tested (with zone of inhibition ranging from 7.1 ± 0.56 to 14.1 ± 0.91 mm). The inhibition zones for *Candida* species tested were ranged from 7.4 ± 0.75 to 14.7 ± 0.46 mm. The MICs value were ranged from 5 ± 0.02 to 80 ± 0.75 mg/ml for bacteria tested, while *Candida* species were more susceptible to extract and MICs were ranged from 0.75 ± 0.01 to 25 ± 0.90 mg/ml. All isolates tested were showed significant reduction of cells started from 2-6 h incubation. It is also indicated that *Candida albicans* was more susceptible in terms of decreasing the number of cells after 24 h incubation time in compare to other species.

Key words: Antimicrobial activity; bioactive compounds; *Holothuria leucospilota*.

The continuous use of antibiotics has resulted in multidrug-resistant microbial strains which cause urgent need to research and discover new antimicrobial compounds with diverse novel mechanisms of action[1]. Sea cucumber, a marine invertebrate of the phylum Echinoderm, represents a novel class of dietary antioxidants[2] with a variety of biomedical activities[3]. Sea cucumber use has been proposed in functional foods, known as "Gamat" in Indonesia are used in traditional

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medicine to cure several health problems including, asthma, hypertension, rheumatism, burns, internal and external wounds and cancer[4,5]. Some sea cucumber species have also been used in modern Chinese medicine to treat arteriosclerosis, skin diseases, hypertension, inflammation and cancer[6,7]. Biomedical properties of sea cucumbers can be linked to the presence of a wide array of bioactive constituents including essential fatty acids, sulfated polysaccharides, polyphenol, triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan, sterols (glycosides and sulfates), cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and terpenoids[8-10].

A number of published reports have revealed that some of sea cucumber species contain several potentially antimicrobial compounds. Haug *et al*.[11] examined and found high antibacterial activity against Gram positive bacteria in the fractions of eggs of *Cucumaria frondosa*. Jawahar *et al*.[12] studied antimicrobial effect of *Holothuria atra* and *Holothurias cabra* , they found that *Escherichia coli*, *Aeromonas hydrophila*, *Enterococcus* species, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi*, and *Vibrio harveyi*, and fish-generated *Aspergillus* species were sensitive and only *Bacillus* species, was resistant. Another study by Mariana *et al*.[13] found that methanol extract of *Stichopus badionotus* inhibited the growth of methicillin-resistant *S. aureus*. Also AL-Haj *et al*.[14] found in methanolic extracts of *Gracilaria changii* inhibited growth of *S. aureus* and *S. pyogenes*. Despite of several worldwide studies that have revealed the efficacy of some sea cucumber species as potential sources of antibacterial compounds, there is lack in information about levels of these activities in most Iranian sea cucumber species. Therefore, this study was propose to investigate the bioactive compound and antimicrobial activities of extracts from Persian Gulf sea cucumbers as part of a research hopes to find new potential antimicrobial compounds in Persian Gulf sea cucumbers.

MATERIALS AND METHODS

Sample collection and extraction

Samples of the sea cucumber species, *Holothuria leucospilota* were collected from Persian Gulf coastal areas in Iran. The samples were dissected to remove internal organs, and packed immediately with ice prior sending to the lab and kept at -80 °C until extracted. Extraction procedures using organic solvent was carried out according to the modified method of Yuan[15]. Ground freeze-dried Persian Gulf sea cucumber samples (10 g) were extracted with 100 ml of methanol at room temperature for 72 h. The samples were then filtered with Whatman filter paper no. 1 and the solvent was removed under vacuum at 40 °C using a rotary evaporator giving a viscous mass.

GC-MS analysis

Analyte separation was determined by GC–MSS using an Agilent HP-6990 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent HP-5973 mass selective detector in the electron impact mode (ionization energy: 70 eV) operating in external configuration with triple axis detector in the electron ionization (EI) mode using an autosampler. Separations were carried out in a HP-5MS capillary column (30m×0.25mm i.d., 0.25-µm film thickness) of fused silica gel with 5% methoxypolysiloxane. Initial oven temperature was set at 70 °C, held for 3.5 min; ramped at 25 °C min^{-1} up to 180 °C, held for 3 min; ramped at 10 °C min^{-1} up to 300 °C and held for 5 min. Helium (purity 99.999) was employed as carrier gas with a constant flow of 1.0 ml min^{-1}. Qualitative analysis was obtained in the m/z SCAN range of 45 to 800 amu. PTV injections were carried out in four steps: injection, solvent evaporation, analyte transfer and cleaning. In the injection step, the split valve was open at 20 ml min^{-1} and 50 µl samples were introduced into a Siltek deactivated liner with frit (Restek) at 70 °C. During the evaporation step, the temperature was raised to 85 °C at a rate of 30 °C min^{-1} for 30 s to eliminate the solvent, which was vented through the split valve at a flow of 36 ml min^{-1}. In the transfer step, the split valve was closed and the temperature increased to 300 °C at a rate of 75 °C min^{-1} in splitless mode for 3min. The injector was kept at 300 °C with a purge flow of 50 ml min^{-1} for cleaning purposes until the end of the run. The mass spectrometer was operated in EI mode at 70 eV. A parent ion was chosen for each compound by taking the m/z and relative abundance of parent ions as high as possible in order to increase sensitivity. Identification of components was based

on retention indices (RI) relative to computer matching with the Wiley 275.L and Wiley 7n.L libraries. Good quality secondary spectra for every compound were obtained selecting a non-resonant waveform.

Evaluation of antimicrobial activity of Persian Gulf sea cucumber extract

Preparation of isolates

Five standard isolates of Gram positive and Gram negative bacteria including *Lactobacillus helveticus* LBK-16H, *L. shirota* UPM, *Befidio bacterium longum* ATCC 15707, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 8739 and also twelve clinical and ATCC isolates of different *Candida* species from microbiological laboratories of University of Malaya including *Candida albicans* 2696*, C. albicans* ATCC 14053, *C. rugosa* 3114*, C. rugosa* ATCC 10571, *C. krusei* 2639*, C. krusei* ATCC 6258, *C. parapsilosis* 2603*, C. parapsilosis* ATCC 22019, *C. glabrata* 2744, *C. glabrata* ATCC 2001, *C. tropicalis* 4355 and *C. tropicalis* ATCC 750 were obtained and cultured on Nutrient Agar (NA) and Sabouraud Dextrose Agar (SDA) for bacteria and yeast, respectively. The cultured plates were incubated at 37 °C for 48-72 h. Anaerobic bacteria were incubated in a candle jar.

Disc diffusion agar test

Antimicrobial disk susceptibility test was performed to obtain the preliminary screening of antimicrobial activity of Persian Gulf sea cucumber extract. According to Clinical and Laboratory Standards Institute (CLSI) protocol (document M02-A11) with slight modifications all isolates were cultured on NA and SDA for bacteria and yeast, respectively and then passaged twice to ensure viability and purity. Subsequently the stock suspensions of 1×10^6 to 5×10^6 cells/ml of yeast and 1×10^8 to 5×10^8 cells/ml of bacteria were made and then poured and plated on SDA (for yeast) and Mueller Hinton Agar (MHA) for bacteria. For the next step 0, 10, 20 and 50 µl of the sterile Persian Gulf sea cucumber extract were impregnated on separate paper disks and put on the surface of cultured media. Fluconazole and tetracycline were also used as the control positive for yeast and bacteria, respectively. Eventually the cultured plates were incubated at 37 °C for 48 hand then the inhibition zone was measured[16].

Broth microdilution test for determination of relative MICs

Broth microdilution test was performed using 96 microplates according to CLSI protocols for bacteria and yeast with slight modifications and MICs value were obtained. The endpoint was defined as the lowest concentration of Persian Gulf sea cucumber extract that caused 50% and 90% growth inhibition compared to control-growth[17].

Study of inhibition of sessile cells by Persian Gulf sea cucumber extract

Five ml of Persian Gulf sea cucumber extract equalled 1 × MIC concentration was added to the same volume of bacteria and yeast cell suspensions with 10^8 cell/ml and 10^6 cell/ml, respectively and incubated at 37 °C for 48 h. At time intervals (0, 2, 6, 12, 24, 36 and 48 h), 100 µl of the former mixture was plated on the surface of MHA (for bacteria) and SDA (for yeast) and then incubated at 37 °C for 24-48 h for determination of viable colony counts[18].

RESULTS AND DISCUSSION

Chemical composition analysis of Persian Gulf sea cucumber

The spectrum of the unknown components was compared with the spectrum of known components stored in the GC-MS library. The name, molecular weight and structure of the components of the test materials were ascertained. GC chromatogram analysis of the methanolic extract of Persian Gulf sea cucumber showed several peaks which indicating the presence of twenty important constituents (Figure 1). On comparison of the mass spectra of the constituents with the GC-MS library, the twenty compounds were characterized and identified (Table 1). GC-MS analysis exhibited that the most of determined compound in methanolic extract are fatty acids.

Fatty acids of sea cucumber are well known as vital components for tissue repair and conglutination of wound[19]. Various long chain fatty acids were obtained from methanolic extract of Persian Gulf sea cucumber which is essential for growth of body and construction of cell membrane. The results are shown in Table 1. The fatty acids have been identified as a main element responsible for treatment and protective effect on cancer and cardiovascular, and asthma[20].

For examples; Eicosapentaenoic acid (entry 6, 12.95%) is ω-3 fatty acids which have antimicrobial effects and beneficial effects on vascular, immune system and resolve inflammatory processes. Methyl arachisate (entry 8, 2.66%) has been indicated as a useful fatty acid in cell membrane. (Z)-9-Octadecenoic acid methyl ester (entry 7, 4.7%) is a ω-6 fatty acid which is essential

Table 1. GC-MS analysis of Persian Gulf sea cucumber

No.	RT (min)	Name of compound	Molecular formula	MW	Peak area%
1	9.865	Methyl myristate	$C_{15}H_{30}O_2$	242	1.04
2	11.740	Methyl,(7E)-7-hexadecenoate	$C_{17}H_{32}O_2$	268	4.01
3	11.925	Methyl tridecanoate	$C_{14}H_{28}O_2$	228	5.49
4	13.640	Methyl oleate	$C_{19}H_{36}O_2$	296	11.02
5	13.843	Methyl isostearate	$C_{19}H_{38}O_2$	298	2.73
6	15.092	cis-5,8,11,14,17-Eicosapentaenoic acid, methyl ester	$C_{21}H_{32}O_2$	316	12.95
7	15.345	(Z)-9-Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	296	4.70
8	15.580	Methyl arachisate	$C_{21}H_{42}O_2$	326	2.66
9	16.183	cis-9-Octyldecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	2.02
10	17.021	17-Octadecynoic acid	$C_{18}H_{32}O_2$	280	2.42
11	17.784	Methyl palmitoleate	$C_{17}H_{32}O_2$	268	3.57
12	18.533	Methyl nervonate	$C_{25}H_{48}O_2$	380	1.53
13	19.134	(22E)-Ergosta-5,22-dien-3-yl acetate	$C_{30}H_{48}O_2$	440	4.382
14	19.709	1-Heptatriacontanol	$C_{37}H_{76}O$	536	9.77
15	20.077	Cholesteryl formate	$C_{28}H_{46}O_2$	414	16.00
16	20.405	Crinosterol	$C_{28}H_{46}O$	398	2.05
17	20.866	Stigmasterol acetate	$C_{31}H_{50}O_2$	454	2.05
18	21.154	(22E)-Stigmasta-5,22-dien-3-yl acetate	$C_{31}H_{50}O_2$	454	3.32
19	21.622	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	4.47
20	22.329	3-(Methoxymethoxy) cholest-4-ene	$C_{29}H_{50}O_2$	430	2.96

Table 2. Antimicrobial activity of Persian Gulf sea cucumber extract using disk diffusion agar test

Isolates	Zone of inhibition (mm) ± SD				
	Sea cucumber extract (µl)			Standard drug (10 µg)	
	10	25	50	Tetracycline	Fluconazole
L. helveticus LBK-16H	-	-	7.3 ± 0.91	16.9 ± 0.56	-
L. shirota UPM	-	-	7.7 ± 0.37	19.2 ± 0.79	-
B. longum ATCC 15707	-	7.1 ± 0.56	9.4 ± 0.39	14.4 ± 0.87	-
S. aureus ATCC 25923	8.3 ± 0.61	10.4 ± 0.87	13.2 ± 0.78	16.6 ± 0.96	-
E. coli ATCC 8739	9.4 ± 0.67	10.6 ± 0.77	14.1 ± 0.91	17.5 ± 0.65	-
*C. albicans*ATCC 14053	8.7 ± 0.77	12.5 ± 0.54	14.7 ± 0.46	-	21.2 ± 0.82
C. albicans 2696	7.9 ± 0.57	12.2 ± 0.74	14.3 ± 0.59	-	20.4 ± 0.89
C. tropicalis ATCC 750	7.6 ± 0.65	9.8 ± 0.45	13.8 ± 0.64	-	21.3 ± 0.59
C. tropicalis 4355	8.2 ± 0.75	10.1 ± 0.84	13.5 ± 0.77	-	20.8 ± 0.52
C. parapsilosis ATCC 22019	7.8 ± 0.38	9.6 ± 0.74	12.8 ± 0.64	-	19.5 ± 0.75
C. parapsilosis 2603	7.4 ± 0.75	9.7 ± 0.86	13.1 ± 0.93	-	18.6 ± 0.61
C. glabrata ATCC 2001	8.1 ± 0.55	8.9 ± 0.48	12.1 ± 0.54	-	18.9 ± 0.48
C. glabrata 2744	7.9 ± 0.61	8.7 ± 0.65	12.5 ± 0.84	-	18.5 ± 0.85
C. rugosa ATCC 10571	7.7 ± 0.74	8.4 ± 0.28	11.9 ± 0.52	-	18.1 ± 0.46
C. rugosa 3114	7.8 ± 0.66	9.2 ± 0.76	12.3 ± 0.47	-	18.4 ± 0.71
C. krusei ATCC6258	8.3 ± 0.46	9.3 ± 0.49	13.2 ± 0.58	-	19.6 ± 0.61
C. krusei 2639	8.6 ± 0.72	9.7 ± 0.75	13.4 ± 0.66	-	19.8 ± 0.78

for synthesizing prostaglandins[21] muscle growth[22,23], brain and very effective in reducing alzheimer's disease symptoms[24,25].

Antimicrobial activities of Persian Gulf sea cucumber extract

The preliminary test showed significant antibacterial activity of Persian Gulf sea cucumber extract for all isolates tested with zone of inhibition ranging from 7.1 ± 0.56 to 14.1 ± 0.91 mm. Among from bacterial tested *Lactobacillus* species was resistant to Persian Gulf sea cucumber extract with 10 and 25 µl volumes, while the others were susceptible in all volume tested (Table 2).

The antibacterial activity of sea cucumber extract was reported by some investigators [3,26,27]. It is demonstrated that a peptide originated from *Cucumaria frondosa* could be able as the main agent of the echinoderm to show antimicrobial activities.

On the other hand, sea cucumber extract was able to show antifungal activity[28]. Indeed it is indicated that a triterpene glycosides called holotoxins from *Stichopus japonicas* showed fungicidal activity against *C. albicans*[29]. Moreover, in the present study all *Candida* species tested were susceptible to Persian Gulf sea cucumber extract in all volumes tested (Table 2). Interestingly the inhibition zones of Persian Gulf sea cucumber extract for *Candida* species tested were ranged from 7.4 ± 0.75 to 14.7 ± 0.46 mm.

All isolates were susceptible to standard drugs tested and resistance phenomena were not obtained. Table 3 shows the relative MICs value of Persian Gulf sea cucumber extract against microorganisms tested. The MICs value were ranged from 5 ± 0.02 to 80 ± 0.75 mg/ml for bacteria tested, while *Candida* species were more susceptible to Persian Gulf sea cucumber extract and MICs were ranged from 0.75 ± 0.01 to 25 ± 0.90 mg/ml.

Efficacy of Persian Gulf sea cucumber extract was evaluated against sessile yeast and bacterial cells by the time-dependent killing assay (Figure 2, 3). All isolates tested were showed significant reduction of cells after treatment with Persian Gulf sea cucumber extract started from 2-6 h incubation ($p < 0.001$). It is also indicated that *C. albicans* treated with Persian Gulf sea cucumber extract was more susceptible in terms of decreasing the number of cells after 24 h incubation time in compare to other species ($p \leq 0.05$).

Indeed the data in this study provides a better understanding of the antimicrobial activity of Persian Gulf sea cucumber. Preliminary tests such as disc diffusion agar assay, broth microdilution assay and also time kill study conducted in this study as documented earlier have shown that all clinical and standard isolates tested were sensitive to Persian Gulf sea cucumber. Little is known about the molecular mechanisms of action of sea

Table 3. Relative minimum inhibitory concentrations (MICs) of Persian Gulf sea cucumber extract against microorganisms tested

Isolates	MIC_{50} (mg/ml)	MIC_{90} (mg/ml)
L. helveticus LBK-16H	25 ± 0.32	40 ± 0.61
L. shirota UPM	20 ± 0.54	80 ± 0.75
B. longum ATCC 15707	10 ± 0.18	20 ± 0.43
S. aureus ATCC 25923	5 ± 0.02	25 ± 0.21
E. coli ATCC 8739	7.5 ± 0.03	12.5 ± 0.16
C. albicans ATCC 14053	0.75 ± 0.01	10 ± 0.04
C. albicans 2696	1.25 ± 0.03	10 ± 0.02
C. tropicalis ATCC 750	10 ± 0.07	25 ± 0.29
C. tropicalis 4355	7.5 ± 0.09	10 ± 0.06
C. parapsilosis ATCC 22019	5 ± 0.03	12.5 ± 0.09
C. parapsilosis 2603	5 ± 0.59	10 ± 0.15
C. glabrata ATCC 2001	12.5 ± 0.21	25 ± 0.35
C. glabrata 2744	10 ± 0.18	25 ± 0.26
C. rugosa ATCC 10571	2.5 ± 0.12	10 ± 0.21
C. rugosa 3114	5 ± 0.43	10 ± 0.76
C. krusei ATCC6258	12.5 ± 0.95	25 ± 0.90
C. krusei 2639	10 ± 0.56	20 ± 0.69

cucumber in terms of antimicrobial activity. A physicochemical basis for the antimicrobial action of fatty acids on protoplasts and whole cells was discussed by some researcher. Fatty acids of chain length > C10 induced lysis of protoplasts[30]. Long chain fatty acids are well-known to be inhibitory on Gram positive bacteria even at low concentrations[31]. Although, antimicrobial activity

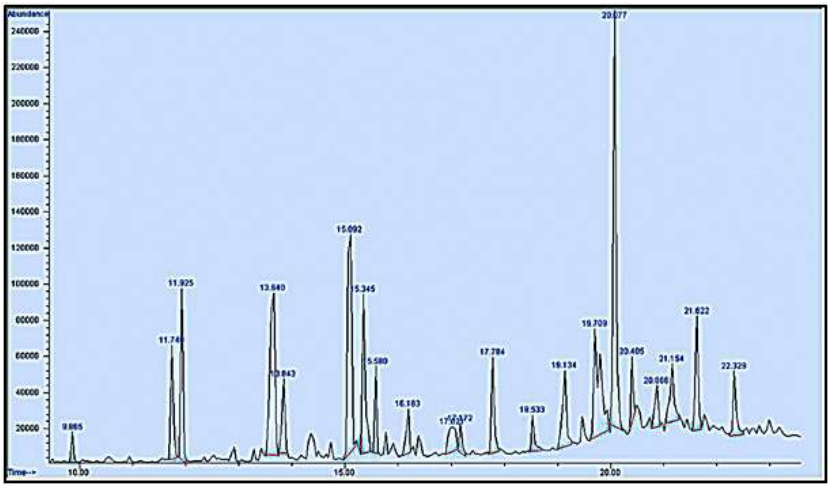

Fig. 1. GC spectrum of Persian Gulf sea cucumber

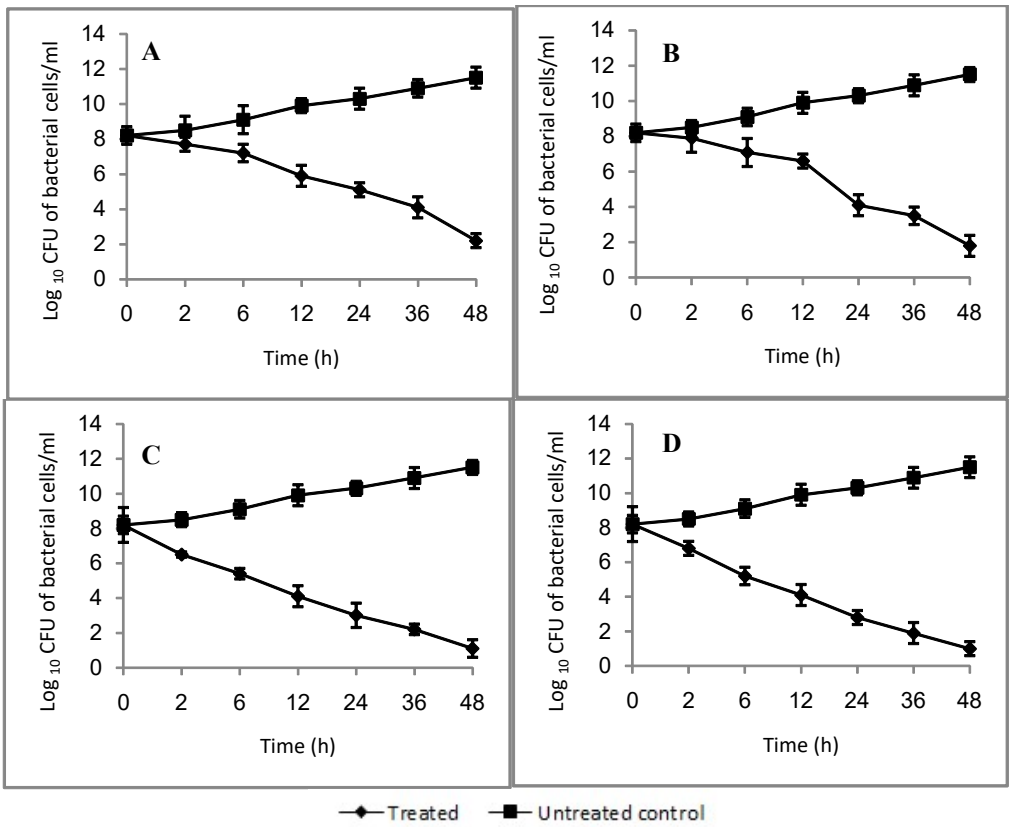

Fig. 2. Time dependent killing of sessile bacterial cells treated by *Holothuria leucospilota* extract. (A) *Lactobacillus helveticus* LBK-16H, (B) *Befidiobacterium longum* ATCC 15707, (C) *Staphylococcus aureus* ATCC 25923 and (D) *Escherichia coli* ATCC 8739

has been more attributed to long-chain unsaturated fatty acids (C16- C20) such as palmitoleic, oleic and linolenic acids, other long-chain saturated fatty acids, are known to have the same effect although to a lesser extent[32]. Even though it was not possible to conclude that the large amounts of fatty acids, observed in this study, were the responsible elements for the antimicrobial activity found, it cannot be forgotten that these compounds do play an important role in this activity and more work may be done in order to clarify this function by working with pure compounds and assessing their

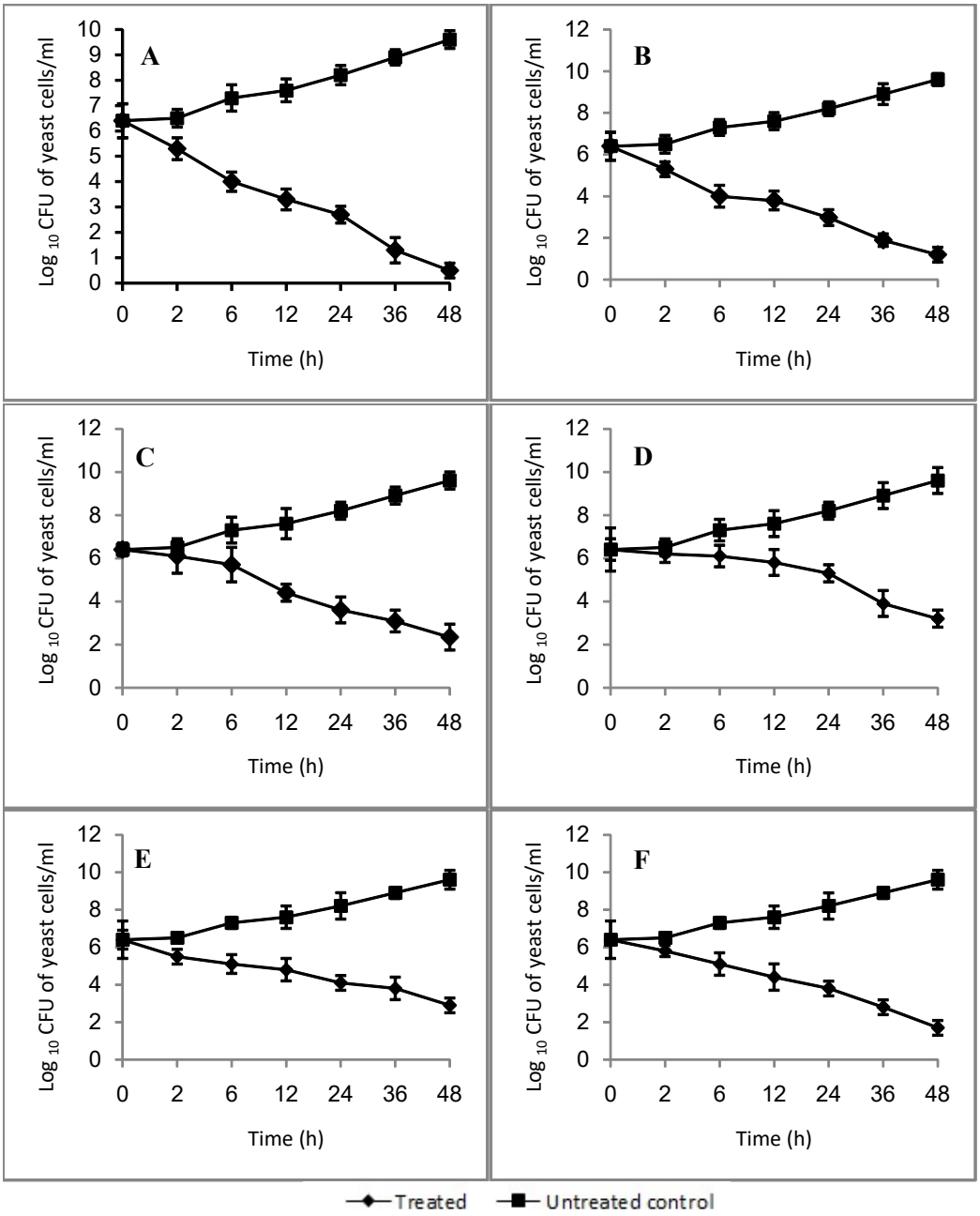

Fig. 3. Time dependent killing of sessile yeast cells treated by *Holothuria leucospilota* extract. (A) *Candida albicans* ATCC 14053, (B) *C. tropicalis* ATCC 750, (C) *C. parapsilosis* ATCC 22019, (D) *C. glabrata* ATCC 2001, (E) *C. rugosa* ATCC 10571 and (F) *C. krusei* ATCC 6258.

effect on test organisms. Mostly, the previous reports have not been able to uncover the molecular evidence in terms of gene expression analysis. On the other hand, there are very little studies that investigated the activity of sea cucumber as an antimicrobial agent in an animal modeling and further investigations will be necessary.

In conclusion, widely available marine sources like sea cucumber are rich sources of fatty acid and have the potential to be an excellent source of pharmaceuticals that target microbes which damage human and animal life. Sea cucumber extracts from Persian Gulf have high concentrations of fatty acid and hence seem to have greater potential in inhibiting the growth of several strains of pathogenic bacteria.

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