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Antibacterial Potential of some Seaweeds Species to Combat Biofilm-producing Multi-drug Resistant *Staphylococcus aureus* of Nile Tilapia

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THERE is an increasing worldwide demand for seafood due to the awareness of fish as an important protein source for a growing population. However, the diseases caused by multi-drug resistant (MDR) bacterial infections and the low survival rate of the fish represent significant challenges to fish farmers. The virulence and pathogenicity of bacteria are often enhanced when growing as a biofilm. Therefore, a variety of new antimicrobial drugs has attracted wide attention in treating fish pathogen bacteria. Herein, the efficiency of macroalgal extracts as an antibacterial agent against MDR *Staphylococcus aureus* bacteria isolated from Nile tilapia (*Oreochromis niloticus*) was studied. Out of 200 bacterial isolates, 50 strains were identified as *S. aureus*. Of these strains, 37 were MDR and showed a potential role in the production of virulence factors, including staphylokinase (70.2%), lecithinase (81%), protease (56.7%) and lipase (59.4%). Significant production of biofilm virulence factor by MDR *S. aureus* strains was also observed from the quantitative and qualitative analysis. Four algal species namely *Jania rubens*, *Ulva lactuca*, *Sargassum vulgare,* and *Sargassum fusiforme* were tested for their antibacterial activity against MDR *S. aureus* strains. Of those, *S. vulgare* diethyl ether extract showed the highest antibacterial. In addition, GC-MS analysis revealed 20 identified components in *S. vulgare* diethyl ether extract, in which Longifolene was dominant (16.5%). This study thus established the possibility of developing an antibacterial agent to combat developing MDR *S. aureus* and biofilm-related infections in Nile tilapia.

Keywords: Biofilm, Multi-drug resistance, Nile tilapia, Seaweeds, *Staphylococcus aureus*.

Introduction

There is an increasing worldwide demand for seafood due to the awareness of fish as an important protein source for a growing population (Hamed et al., 2018). Wild fisheries are currently in a state of decline because of overfishing, climate change, pollution and marine habitat destruction among other factors (Hamed et al., 2018; Eladel et al., 2019). Previous studies agreed that the response of fish to stress conditions depends on the stressor (temperature, crowding, hypoxia, the presence of heavy metals, etc.) and on the characteristics of the fish themselves (e.g. fish species, age or sex). Such stressful conditions could enhance the spread of pathogenic bacteria and cause serious outbreaks of disease (Annabi et al., 2013; Allam et al., 2019). High fish concentrations and lack of sanitary barriers facilitates the spread of pathogens and hence producing high mortality levels (Quesada et al., 2013). In order to avoid such high economic losses in fish, several veterinary drugs are applied in aquaculture as a trial to prevent or treat disease outbreaks (Rico et al., 2013). However, Seyfried et al. (2010) concluded that the extensive use of veterinary drugs is becoming restricted due to their high-risk factors and rapid development of multi-drug resistance bacteria (MDR) (Tolba et al., 2019). Also, the wide use of synthetic antimicrobials as trichlorfon or praziquantel has led to resistance

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development (Umeda et al., 2006). Besides, the potential harm of veterinary drug treatments on both human health and environments (Ali et al., 2016). The majority of pathogens associated with the fish health, being opportunistic and, therefore, natural sources should be applied as an alternative solution against microbial fish infections (Iguchi et al., 2003).

Staphylococcus aureus has been regarded as one of the most critical pathogens isolated from the community and healthcare settings in Egypt and all over the world. The significance of *S. aureus* is attributed to the rapid emergence of antibiotic resistance among most of its isolates, as well as an array of cell-surface and secreted virulence factors that contribute to its invasive potential and pathogenicity (El-baz et al., 2017). Its pathogenicity is mainly related to a combination of genetic characteristics mediating virulence, invasive capacity, immune evasion, and antibiotics resistance (Ahmed et al., 2019). It is considered as the third most important cause of foodborne illnesses reported worldwide and one of the most common agents in food poisoning outbreaks (Arfatahery et al., 2015). It is capable of acquiring antibiotic resistance determinants and therefore *S. aureus* isolates often exhibit resistance to multiple classes of antimicrobial agents and therefore they are often regarded as multidrug-resistant bacteria (MDR) (Papadopoulos et al., 2018). It has evolved four general resistance mechanisms, including trapping of the drug, modification of the drug target, enzymatic inactivation of the drug and the activation of transmembrane efflux pumps to fend off an attack from antimicrobials (Pantosti et al., 2007). Besides antibiotic-resistant genes, in recent years, biofilm formation is believed to play an important role in staphylococcal resistance (Mootz et al., 2015). Bacteria enclosed in a self-produced extracellular polysaccharide matrix exhibited a high level of antibiotics tolerance and resistance to host defense. These characteristics facilitate the adherence and colonization of *S. aureus*, which often leads to persistent infections (Feng et al., 2017).

Biofilm is the most important factor that participates in pathogenesis by increasing resistance of the constituent microbes to antibiotics leading to a protected environment against the host`s defenses (El-baz et al., 2016; Ali et al., 2019). Nearly all strains of *S. aureus* secrete several extracellular enzymes whose function is thought to be the disruption of host tissues and/or inactivation of host antimicrobial mechanisms. These exoenzymes include lipases, lecithinase, nuclease proteases, hyaluronidase and staphylokinase (Costa et al., 2013). The marine ecosystem provides an important source of chemical compounds, which have many therapeutic applications such as antibacterial, antiviral and anticancer activities because of its biodiversity (Abdel-Aziz et al., 2019; El-Fallal et al., 2019; Saeed et al., 2019). The ability of seaweeds to produce biologically active secondary metabolites has been widely documented and there are several reports regarding the broad range of biological activity of compounds derived from macroalgae (Kosanić et al., 2015; El-Shouny et al., 2018; Abdel-Hamid & Galal, 2019; Khairy & El-Sheekh, 2019).

The present study aimed to evaluate the antimicrobial activity of macroalgal extracts collected from Alexandria, Egypt against multidrug-resistance (MDR) bacteria isolated from Nile tilapia fish. In addition, the effect of algal extract application on fish growth performance was conducted.

Materials and Methods

Samples collection

This study was conducted on 250 Nile tilapia fish (*Oreochromis niloticus*) species collected from Kafr Elzayat city EL-Gharbia Governorate, Egypt, during the period from November 2015 to January 2017. They were transferred immediately alive or recently dead to Bacteriology Laboratory at the Faculty of Veterinary Medicine, Zagazig University, Egypt. Clinical examination was done on a live fish and any abnormal swimming fish behavior, fish activity, and body coloration were observed. Fishes were subjected to clinical and postmortem examinations for the presence of any lesions involving the skin, fins, gills, internal organs, and abdominal cavity. In total, five specimens (skin ulcers, gills, liver, kidney, and spleen) of each fish species were obtained for bacteriological examination. Fish with abnormal characters (n=107) were selected for further studies.

Isolation and identification

Bacterial swabs were isolated from the diseased organs of Nile tilapia fish which included gills, skin ulcers, spleen, liver, and kidney. Samples were streaked on prepared plates of Mannitol Salt Agar (Oxoid, UK) and incubated using incubator

at 37°C for 18–24h. After incubation, isolated colonies suspected to be *S. aureus* were allowed to grow on nutrient agar plates (HiMedia India) and then identified microscopically, biochemically, and serologically (Carnicer-Pont et al., 2006; Abdel et al., 2010). All the suspected strains of

S. aureus were identified using preliminary tests such as Gram staining, catalase and coagulase as previously described (Akobi et al., 2012).

Antibiotic susceptibility test

Antibiotic susceptibility was determined by the agar diffusion method (Bauer et al., 1966). A single pure colony of each isolate was inoculated into a test tube containing 2ml nutrient broth (Oxoid, UK) and incubated overnight. The young physiological overnight culture of each isolate was diluted with sterile distilled water until the turbidity matched 0.5 McFarland (approximately 10^8 CFU/ml). A sterile swab stick was dipped into the adjusted suspension and streaked over the surface of an already prepared Mueller-Hinton Agar (Oxoid, UK) plates. The antibiotic discs: Tetracycline (30mg), Minocycline (30mg), Imipenem (10mg), Meropenem (10mg), Amoxicillin/clavulanic acid (30mg), Ampicillin/sulbactam (30mg), Amikacin (30mg), Gentamicin (10mg), Ciprofloxacin (5mg), Levofloxacin (5mg), Azithromycin (15mg), Clarithromycin (15mg), Chloramphenicol (30mg), Sulfadiazine (100mg), Sulphamethoxazole (50mg), Cefoperazone (75mg), Cefotaxime (50mg), Ampicillin (30mg), Oxacillin (30mg) (Abtek®) were applied on to the inoculated plates maintaining a distance of 30mm edge to edge. The plates were incubated for 18–24h at 37°C. The diameter of the inhibition zones was measured using a ruler and interpreted according to the criteria recommended by the (CLSI) (Wayne, 2015).

Phenotypic detection of virulence factors Detection of lipase enzyme

Isolates were screened for lipolytic activity by inoculation of the isolate on nutrient agar plates containing lipoidal emulsion 30ml L^{-1} (1ml tween 80 and 100ml olive oil per 500ml water) (Atlas, 2010). The plates were incubated at 37°C for 48h then flooded with a saturated solution of copper sulfate solution for 15min, greenish-blue color around growth indicates positive production of lipase enzyme.

Detection of protease production

Proteolytic activity of the tested isolates was assessed according to the method of Sánchez-Porro et al., with some modification (Sánchez-Porro et al., 2003). A pure colony was inoculated on nutrient agar plate supplemented with 1% skimmed milk and 1% casein. The plate was incubated at 37°C for 48h. The protease activity was indicated by the appearance of a clear zone around growth that was clarified after keeping the plates overnight in the refrigerator.

Detection of lecithinase production

Lecithinase activity of the tested isolates was detected on egg yolk agar plates (Sharaf et al., 2014). The plates were prepared by the addition of 10% of egg yolk suspension to prewarmed (45°C) tryptic soy agar (TSA) supplemented with 1% NaCl. A positive result was defined by the appearance of white precipitate around the colonies after incubation at 37°C for 24-72h.

Screening of staphylokinase activity

Human plasma was separated and heated at 56°C in a water bath for 20min then mixed with melted nutrient agar in ratio 3:1 nutrient agar to plasma (Mohana Srinivasan et al., 2013). A volume of 20µl of the concentrated enzyme was applied into wells cut in the heated plasma agar plates, incubated at 37°C for 24h and checked for a halo zone around wells.

Detection of hemagglutination ability

All MDR isolates were checked for their ability to agglutinate human erythrocytes by their fimbriae by slide method as described by Vagarali et al. (Vagarali et al., 2008). Clumping of erythrocytes within five mints of stirring was considered a positive result of hemagglutination.

Biofilm formation

Detection of biofilm formation as a mechanism of resistant

The qualitative assay for biofilm formation was performed according to the method described by Christensen et al. (Christensen et al., 1985), with certain modifications. Glass tubes (13×100mm) filled with 2.6ml of tryptic soy broth (TSB) containing 0%, 0.25%, 1% or 2.5% glucose, were inoculated with a loopful of a pure culture of a strain from TSA plates containing 0%, 0.25%, 1% or 2.5% glucose, respectively. A total of 0.6ml of inoculated broth was removed from each tube for the microtiter-plate test. Tubes containing only TSB were included in the test as negative controls. After overnight incubation at 37°C in air, the The

content of each tube was carefully removed with a pipette, and 2ml of a 0.25% safranin solution for Gram staining (bioMerieux) was immediately added. After 1 min, the tubes were emptied with a pipette and placed upside down without a wash step in between. Following overnight standing at room temperature, the results of the test were read.

The test was considered positive when there was an adherent layer of stained material on the inner surface of the tube. The adherence was estimated as absent (0), weak (+), moderate (++), or strong (+++). The presence of stained material at the liquid-air interface was not considered to be indicative of biofilm formation. The test was repeated three times for each strain.

Quantitative assay of the biofilm

Three wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid (Spektar, Cacak, Yugoslavia) were filled with 200µl of bacterial suspension each. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24h at 37°C. Then, the content of each well was aspirated, and each well was washed three times with 250µl of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200µl of 99%´ methanol (Destilacija, Teslic, Yugoslavia) per well, and after 15min plates were emptied and left to dry. Then, plates were stained for 5min with 0.2ml of 2% Hucker crystal violet used for Gram staining (bioMerieux) per well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air-dried, the dye bound to the adherent cells was resolubilized with 160µl of the adherent 33% (v /v) glacial acetic acid (Zorka Pharma, Sabac, Yugoslavia) per well. The optical density (OD) of each well was measured at 570nm by using an automated ICN Flow Titertek Multiscan Plus reader. The reading was performed two times: (i) before the addition of glacial acetic acid, as in standard microtiter-plate test and (ii) after glacial acetic acid was added.

For the purposes of comparative analysis of test results, we introduced a classification of adherence capabilities of tested strains into four categories. Commonly used by Christensen et al. (Christensen et al., 1985), classification of the results obtained by the microtiter-plate test has only three categories. All strains were classified into the following categories: non-adherent (0), weakly (+), moderately (++), or strongly (+++) adherent, based upon the optical density (OD) of bacterial films. We defined the cut-off OD for the microtiter-plate test as three standard deviations above the mean OD of the negative control. Strains were classified as follows:

OD ≤ OD	Non-adherent
ODC < OD ≤ 2 x OD	Weakly adherent.
2xODC < OD≤ 4xOD	Moderately adherent
4xODC < OD	Strongly adherent

Macroalgae collection and extract preparation

Macroalgae were collected from Abu-Qir, Alexandria coast by a quadrate technique using a 50×50cm steel quadrate (Russell, 1977). Three quadrate samples were taken at the collection site. All algal populations within the quadrate were collected carefully and washed with seawater to remove epiphytes and other marine organisms. The collected macroalgae were transported to the laboratory in sterile polythene bags, identified according to Taylor (Taylor, 1960) and then dried in an oven at 40°C until constant dry weight. The biomass was determined for each species as gram dry weight per square meter (g m^{-2}). Organic solvents (diethyl ether, ethanol, methanol, and chloroform) were used for extraction. Each powdered sample (5g) was soaked overnight in 25ml of the desired solvent. The resultant crude extracts were filtered and then concentrated in a rotary evaporator at 40°C. The crude extracts were weighed and were suspended in the dimethyl sulfoxide (DMSO) to a final concentration of 200mg ml^{-1} and stored in a refrigerator at 4°C.

Antimicrobial testing assay

The tested bacterial isolates were grown overnight on nutrient agar. The inoculum turbidity was adjusted equivalent to 0.5 McFarland (approximately 10^{8} CFU/ml). MDR isolates were selected and the most prevalent drug-resistant isolate was tested against the collected algal extracts. The inocula were spread uniformly over the agar plates surface by a glass rod. To test the antibacterial activity, the macroalgal extract was prepared. In regular wells (0.7mm) diameter, 50µl of tested algal extracts were added. Wells with DMSO were considered as a negative control. In order to determine the possible inhibitory activity

of the dilutant tested extracts, all agar plates were incubated for 24h at 37°C. Inhibitory zone (mm) was measured as an indicator of antibacterial activity.

Gas chromatography-mass spectrometry (GC-MS)

The promising algal extract was identified using gas chromatography-mass spectrum (GC-MS) analysis after concentrating in a desiccator. GC-MS model Agilent 8790B /5977B MSD was used in the analysis that employs Agilent 19091s-433UI column (325°C) and the components were separated using Helium (He) as a carrier gas at a constant flow of 0.8ml min^{-1} at ionization energy (70eV). The sample extract (1µl) was injected into the instrument. The injector temperature was set at 250°C and throughout the analysis, temperature flow was set at 100°C with Hold time 1min and Run time 1min and the speed of increasing by three intervals (Ramp1 25°C min^{-1}) with Hold time 1min and Run time 4.8min, (Ramp2 2°C min^{-1}) with Hold time 1min and Run time 35.8min and (Ramp3 25°C min^{-1}) with Hold time 2min and Run time 39.8min. Identification of the compounds was done by using the National Institute of Standard and Technology (NIST) library spectra databases, where the spectrum of the unknown component was compared with that of known components stored in this library. The name, molecular weight, compound nature and a molecular formula of the test sample were identified based on the NIST library. In addition, the biological activities of the identified compounds were identified by a literature survey.

Statistical analysis

Results are presented as the mean ± standard deviation (SD) from three replicates. The statistical analysis was carried out using PC-ORD (ver.5). Data obtained were analyzed statistically to determine the degree of significance using one-way analysis of variance (ANOVA) at probability level $P \leq 0.05$.

Results

Clinical examination showed 107 out of 250 tilapia fish were unhealthy and showed loss of equilibrium, mild lesions, hemorrhaged or inflamed fins, bulging eyes and scale loss with red spots and deep ulcers. 200 bacterial isolates were isolated from the gills, spleen, liver, kidney, and skin ulcers of the unhealthy tilapia fish. *S. aureus* (n= 50) was the most predominant bacterial species isolated from diseased fish. According to a source of isolates of *S. aureus* the skin ulcers isolates had the highest drug-resistant percentage (74%) of the total 50 isolates (Fig. 1), gills isolates had the highest virulence factors abundant percentage (69%) (Fig. 2, Table 1), spleen isolates had the highest mean of optical density for biofilm formation (Fig. 3, Table 2), but with the insignificant statistical difference in comparing with other isolates from a different sources (P= 0.9).

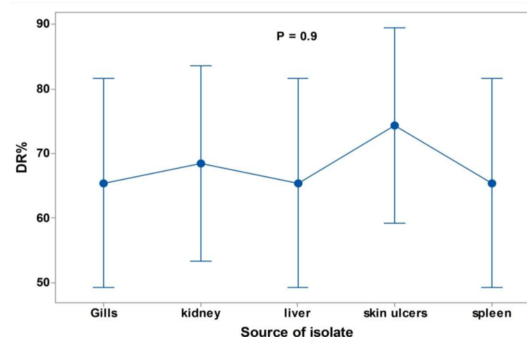

Fig. 1. Drug resistance percentage (DR %) in isolates of *S. aureus* depending on its source of isolation.

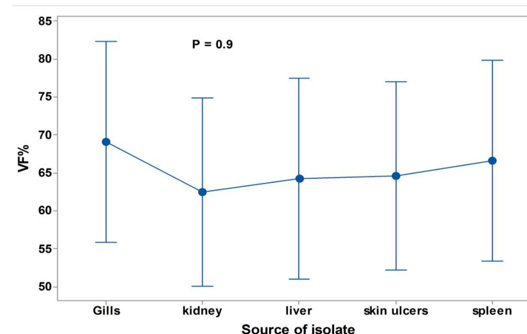

Fig. 2. Virulence factors percentage (VF %) in isolates of *S. aureus* depending on its source of isolation.

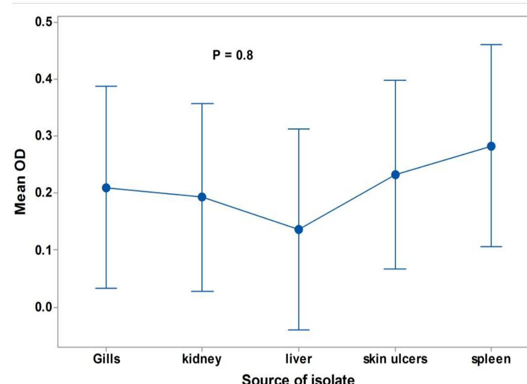

Fig. 3. The optical density (OD) of biofilm of different isolates among *S. aureus*.

TABLE 1. Drug resistance and virulence factors among different sources of *S. aureus* (n= 37).

Source of isolate	DR (%)		VF (%)	
	Mean	SD	Mean	SD
Gills (n= 7)	65.41	20.58	69.05	15
Kidney (n= 8)	68.42	20.86	62.5	17.25
Liver (n= 7)	65.41	20.35	64.29	17.82
Skin ulcers (n= 8)	74.34	22.23	64.58	16.52
Spleen (n= 7)	65.41	20.58	66.67	19.25
P$	0.9		0.9	

- DR: Drug resistance, VF: Virulence factors, SD: Stander deviation.
- $One way ANOVA, P considered significant if < 0.05.

TABLE 2. The incidence of biofilm-producing isolates among the Nile tilapia.

Isolate source	Optical density (OD_{570})	
	Mean	SD
Gills	0.21	0.24
Kidney	0.19	0.21
Liver	0.14	0.19
Skin	0.23	0.25
Spleen	0.28	0.25
P$	0.8	

- The mean optical density at 570nm and the standard deviation (SD) of the mean indicate Biofilm formation. The isolates were classified as weak biofilm producer (0.1< OD≤ 0.2), moderate biofilm producer (0.2< OD≤ 0.4) and strong biofilm producer (0.4< OD).
- $One way ANOVA, P considered significant if < 0.05.

The frequency of antimicrobial resistance in *S. aureus* isolates to different tested antibiotics ranged between 24% for clarithromycin and 100% for oxacillin and ampicillin (Table 3). In addition, most of the isolates showed a high frequency of MDR, as shown in the cluster dendrogram (Fig. 4), which included 37 isolates of *S. aureus* (SA) and 19 of the examined antibiotics. The dendrogram results revealed three isolates (SA10, SA24, and SA39) of the total 37 isolates to have drug resistance activity toward less than 50% toward of the total examined antibiotics (n= 19), on the other hand, 6 isolates (SA6, SA17, SA19, SA27, SA38, and SA43) of 37 showed drug resistance activity 100% of the total examined antibiotics. The remaining isolates had drug resistance activity towards less than 100% and more than 50% of the total examined antibiotics.

In the present study, detection of various virulence factors revealed that among the 37 tested isolates of *S. aureus*, 22 (59.4%) isolates had shown biofilm production with different intensities as seen by naked eyes, 30 (81%) isolates were lipase producers, proteolytic activity was detected in 21 (56.7%) isolates, 22 (59.4%) isolates produced lecithinase enzyme. The plasmolytic effect of staphylokinase enzyme was detected in 26 (70.2%), 24 (64.8%) isolates had the ability to agglutinate human RBCs (Table 4), the cluster dendrogram includes 37 isolates of *S. aureus* and 6 examined virulence factors and it showed that; only one isolate (SA21); its virulence factors abundance was less than 50%, while the remaining 36 had virulence factors abundance more than 50%, two of them (SA17 and SA33) showed abundance of the whole 6 examined virulence factors (Fig. 5). Biofilm formation strength was determined by OD and included from mild and moderate to the strong formation (Table 5).

Four species of seaweeds were identified as *Padina pavonica, Sargassum fusiforme, Sargassum vulgare,* and *Ulva lactuca*. The antibacterial activities of the collected four macroalgal species extracted by four different organic solvents (ethanol, methanol, diethyl ether, chloroform) were measured against MDR *S. aureus*. Macroalgal diethyl ether extracts of *S. vulgare* showed the highest antibacterial activity at 200µl with inhibition zone diameters of 29.2mm. The mean inhibition zone (mm) was affected by the different organic solvent extractions from different algal species against *S. aureus* isolates with MDR characters. Figure 6 shows the significant inhibitory effect of all organic extract from *U. lactuca* species in comparison with other algal species using different concentrations of the organic extracts it also reveals the insignificant inhibitory effect of each organic extract compared to one another. The results in Fig. 6 also showed a significant directly proportionate increase in the inhibitory effect of all organic extract (regardless of the producing algal species) with the increase in their concentration.

TABLE 3. The incidence of drug resistance among 37 isolates of *S. aureus.*

Antibiotics	No. of resistant isolates	Percentage (%)
Tetracycline (TE)	24	64.86
Minocycline (MI)	19	51.35
Imipenem (IPM)	33	89.19
Meropenem (MEM)	27	72.97
Amoxicillin/clavulanic acid (AMC)	24	64.86
Ampicillin/sulbactam (SAM)	24	64.86
Amikacin (AK)	22	59.46
Gentamicin (CN)	24	64.86
Ciprofloxacin (CIP)	13	35.14
Levofloxacin (LEV)	17	45.95
Azithromycin (AZM)	34	91.89
Clarithromycin (CLR)	9	24.32
Chloramphenicol (C)	34	91.89
Sulfadiazine (SD)	26	70.27
Sulphamethoxazole (SMZ)	17	45.95
Cefoperazone (CEP)	31	83.78
Cefotaxime (CTX)	26	70.27
Ampicillin (AMP)	37	100
Oxacillin (OXA)	37	100
Total	37	100

GC analyzed the diethyl ether extract of *S. vulgare*-MS. Twenty compounds were identified, of which longifolene (16.5%) (Fig. 7), 9,12-Octadecadienoyl chloride (15.9%), hexadecanoic acid methyl ester (14.2%) and Octadecanoic acid methyl ester (11.2) were recognized as major components (Table 6).

Discussion

Studies of *S .aureus* related to food generally focus on fish, dairy, poultry, livestock and handling contact (Normanno et al., 2005, 2007; Simon & Sanjeev, 2007; De Boer et al., 2009; Yan et al., 2012; Carfora et al., 2015; Li et al., 2015; El Shafay et al., 2016). Different processing units storage temperature, retailing environment, and product types may cause a diverse contamination level of *S. aureus*, which has rarely been considered in previous studies.

S. aureus is capable of acquiring antibiotic resistance determinants and therefore *S. aureus* isolates often exhibit resistance to multiple classes of antimicrobial agents (Rybak & LaPlante, 2005). In this respect, we focus on detection of antibiotic resistance among *S. aureus* isolated from Nile tilapia, 74% of total isolates have the ability to resist multiple classes of antibiotics (Tetracyclines, Carbapenems, β-lactamase inhibitor, Aminoglycosides, Quinolones, Macrolides, Chloramphenicol, Sulfonamide, Cephalosporin, penicillins), The MAR (multi-antibiotic resistance) index analysis revealed that the most isolates had a very high MAR index value ranged from 47% to 100% which indicated that the MAR values are an indication of the extent of microbial exposure to antibiotics used within the community (Olayinka et al., 2009). On observing the multidrug resistance (MDR), it was found that all the tested bacterial isolates showed high-frequency multiple drug resistance (MDR) (9–19 agents). Tetracyclines are a type of antibiotics that possess the naphthacene ring system and inhibit protein synthesis in a bacteria cell by interfering with the 30S ribosome function, the main mechanism of resistance in bacteria towards it is through efflux pump by membrane transporters, macrolides action is the inhibition of protein synthesis in bacteria, mechanism of resistance through methylation of ribosome active site by Altered target, β-lactams possess a beta-lactam ring in their structure and the mode of action is by inhibition of cell wall synthesis, mechanism of resistance through the secretion of Penicillinase enzyme (Madigan et al., 2012), Quinolone antibiotics inhibit DNA synthesis by targeting two essential type II topoisomerases, DNA gyrase and topoisomerase IV (Topo IV), mechanism of resistance related to chromosomal mutations in genes encoding the protein targets, or mutations causing reduced drug accumulation, either by a decreased uptake or by an increased efflux and plasmid-located genes associated with quinolone resistance (Fàbrega et al., 2009). The widespread use of antibiotics has been identified as the main factor responsible for the increased incidence of antibiotic resistance (Khalil et al., 2015). Janda (2002) reported that many bacterial strains are known to harbor mobile elements that encode antibiotic resistance and can be transferred among themselves or to other bacterial species, to evolve multiple antibiotic resistances.

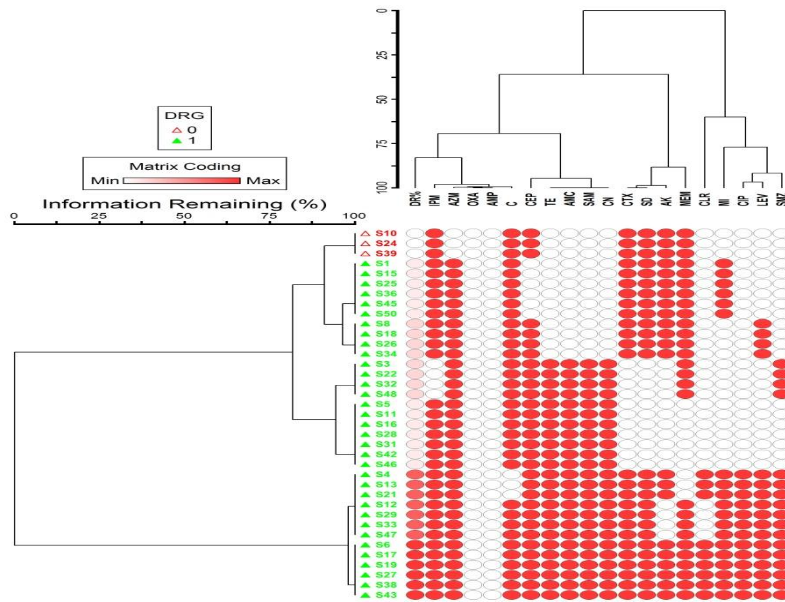

Fig. 4. Clustering analysis of 37 *S. aureus* isolates.

TABLE 4. Incidence of virulence factors among *S. aureus* (n= 37).

Virulence factors	No. of isolates	Percentage (%)
Lipase enzyme	22	59.46
Protease production	21	56.76
Staphylokinase activity	26	70.27
Hemagglutination ability	24	64.86
Lecithenase production	30	81.08
Biofilm formation	22	59.46
Total	37	100

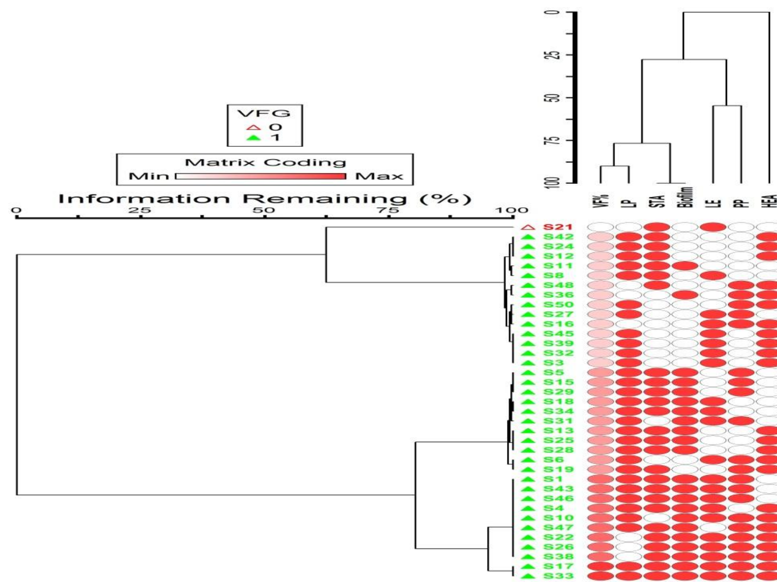

Fig. 5. Clustering analysis of *S. aureus*-producing virulence factors.

TABLE 5. The efficiency of biofilm production among biofilm-producing *S. aureus* (n= 22).

Strength	No. of isolates	Percentage (%)
Mild	11	29.73
Moderate	4	10.81
Strong	7	18.92

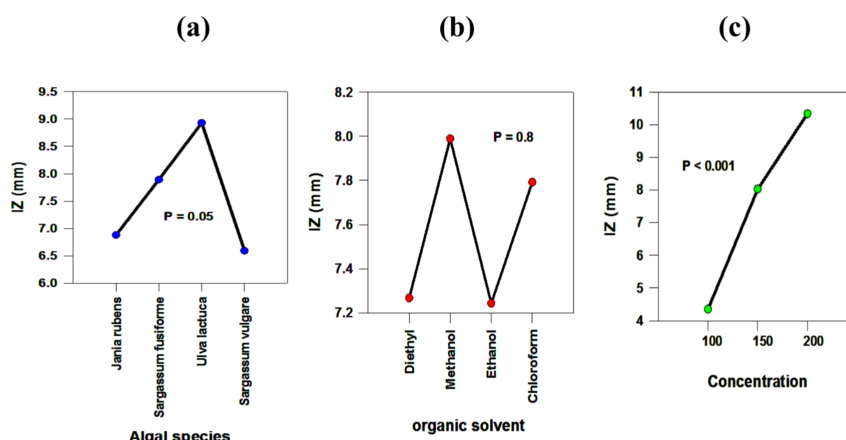

Fig. 6. Mean inhibition zones (mm) of different algal extracts against MDR *S. aureus* isolates.

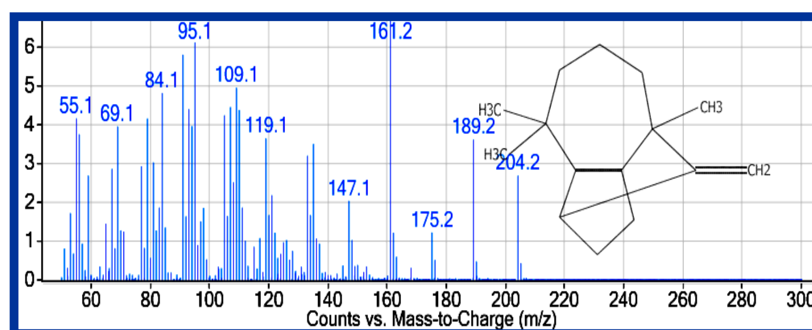

Fig. 7. The chemical structure of longifolene obtained from the GC- MS analysis of *S. vulgare* diethyl ether extract.

The pathogenicity of *S. aureus* is related to a number of virulence factors including biofilm, that allows it to adhere to the surface of the host cell, avoid the immune system causing a harmful effect to host (Bien et al., 2011). Extracellular enzymes like lipase and lecithinase enzymes have been associated with *S. aureus* associated with infections. In this study, about 81% of isolates presented a lipolytic activity, a variety of bacterial lipases are able to hydrolyze triglycerols for nutrient acquisition (El-baz et al., 2016). It has also been postulated that lipase enhances adhesion to the host surface. Undoubtedly, lipase plays a supreme role in the severity of infection (El-baz et al., 2016). The toxic effect of lecithinase relies on the fact that the enzyme usually acts on cell membranes, either perforating them, resulting in cell lysis or by breaking down the phospholipids (Sharaf et al., 2014). Shetty et al. (2009) reported that lecithinase causes lysis of red blood cells, myocytes, fibroblasts platelets, and leukocytes. Another virulence determinant in *S. aureus* is bacterial proteases among the tested isolates, 56.7% of isolates showed proteolytic activity. Previous studies had shown that staphylococcal proteases could cleave and degrade a number of important host proteins causing the destruction of tissues (Karlsson & Arvidson, 2002). Coagulases are proteins that nonenzymatically activate prothrombin, which, in turn, converts fibrinogen to fibrin, leading to blood clotting. The time of the clot generation varies for humans and animals plasmas. This is due to differences in the activity of coagulase-prothrombin complexes,

as well as to the existence of host-specific variants of von Willebrand factor-binding protein (vWpb) encoded on host-specific mobile genetic elements. Coagulases are staphylococcal key virulence factors contributing to the development of pseudo-capsule that promote abscess formation and infection persistence as well as staphylococcal bacteremia and endocarditis (Bonar et al., 2018). All *S. aureus* isolates were tested for their ability to agglutinate human erythrocytes. There is only limited knowledge of the role of staphylokinase in staphylococcal infection. Staphylokinase might facilitate *S. aureus* to bind host plasminogen through bacterial cell surface receptors and thereby to promote invasion of host tissues (Kuusela & Saksela, 1990).

In our study, 59.4% of the total isolates showed biofilm formation. (18.9 %) of our isolates presented strong adherence, Besides the production of the formation of highly organized multicellular complexes by bacteria, known as biofilms, is increasingly being recognized as an important virulence factor in staphylococci (Melchior et al., 2006; Sonbol et al., 2015).

Biofilm formation occurs in two sequential steps by initial attachment of the bacteria to a solid surface and through the proliferation and accumulation of cells in multilayers and enclosing of the bacterial community in a polymeric matrix this matrix is essentially constituted by exopolysaccharides, but it can also contain surface proteins responsible for the bacteria attachment to surfaces (Oliveira et al., 2006), also contributing to the evasion of the immunological defenses and to the difficulty of pathogen eradication, often resulting in persistent infections (Melchior et al., 2006; El-Shouny et al., 2019).

In mature biofilms, the release of planktonic cells occurs from the outer layer, which might be responsible for infection re-occurrence (Melchior et al., 2006).

TABLE 6. The GC-MS analysis of the diethyl ether extract of *S. vulgare* diethyl ether extract.

No.	Compounds	RT	Percentage (%)
1	3-Cyclohexene-1-methanol, 5-hydroxy-.alpha.,.alpha.,4-trimethyl-, (1S-trans)-	5.352	5.1833
2	Carveol	7.525	0.4995
3	Fenchol	10.595	3.3742
4	3-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-	11.964	0.5122
5	Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-, (1.alpha.,3.beta.,4. beta.,5.alpha.)-	14.123	5.5486
6	2-Caren-4-ol	17.282	0.3402
7	Isoborneol	17.638	0.7607
8	Cyclohexanol, 2-methyl-5-(1-methylethenyl)-, (1.alpha. 2. alpha., 5.beta.)-	19.023	0.7668
9	Cyclopentaneacetic acid	20.466	1.399
10	Terpinen-4-ol	21.987	2.5312
11	Thymol	22.537	0.9244
12	Longifolene	24.236	16.571
13	Isopropyl myristate	25.266	4.6147
14	Hexadecanoic acid methyl ester	28.25	14.2233
15	Oleic acid methyl ester	29.19	2.2784
16	9,12-Octadecadienoyl chloride	31.915	15.941
17	Nerolidol isobutyrate	33.405	1.7853
18	Octadecanoic acid methyl ester	34.668	11.2218
19	Octacosane	38.102	8.1343
20	Heneicosane	39.375	1.3888

RT: Retention time.

Pradhan et al. (2012) concluded that various algal extracts, are a good source of new bioactive compounds from a pharmaceutical point of view, showing positive antibacterial activity against different strains of fish and shellfish pathogens. The variation in antibacterial activity may be attributed to the type of solvents, method of extraction and algae collection season. Zheng et al. (2005) referred to the promising role of unsaturated fatty acids and their derivatives as antibacterial agents against *S. aureus* through their inhibitory effect on FabI (enoyl-ACP reductase enzyme), an essential component of bacterial fatty acid.

In the present study, twenty components were identified and the major components were longifolene, 9, 12-Octadecadienoyl chloride, hexadecanoic acid methyl ester, and octadecadienoic acid methyl ester. 9,12-octadecadienoyl chloride is reported as an anti-inflammatory, cancer preventive, anti-acne, anti-eczema, antihistaminic (Sawant & Mane, 2017), Hexadecanoic acid, is known to have potential antimicrobial activity it is also has shown antioxidant and anticancer activities (Suliman & Nour, 2017), also reported to be active at micro-molar concentrations, against multidrug-resistance *S. aureus* (Desbois et al., 2008). Octadecadienoic was known to have potential antifungal and antibacterial activities (Chandrasekaran et al., 2008).

According to previous reports, the antimicrobial activity of seaweeds results from the presence of steroids, phytochemicals, tannins, terpenoids, phenolic compounds and fatty acids in their extracts (Abdel-Raouf et al., 2017). Algal extracts could have an inhibiting or activating effect on microbial growth depending on their composition and concentration (Reguant et al., 2000). Free fatty acids from marine micro- and macro-algae were reported as an antibacterial against several test organisms, especially the gram-positive bacteria *S. aureus*, *S. epidermidis* and *B. subtilis* (Desbois et al., 2008).

Ali et al. (2016) concluded that the destructive effects of *S. vulgare* ethanolic extract against *A. hydrophila* appeared as rupture of the bacterial cell wall that leads to the outflow of cytoplasmic contents, totally deformation that led to severe cell destruction in accordance with these findings the potential of algal extract against MDR *S. aureus* could be explained on the basis of the presence of hexadecanoic acid methyl ester, 9,12–octadecadienoyl chloride and Octadecanoic acid methyl ester as a major constituent of the algal extract.

The present study suggests that diethyl ether extract of *S. vulgare* could provide protective and nutritive effects to induced the health of Nile tilapia infected with *S. aureus*. Further investigations should be conducted on the algal extract nutritive value for Nile tilapia, furthermore characterization and structure elucidation of the exact active components of *S. vulgare* responsible for the antibacterial activity with their respective possible mechanisms of action.

Conclusion

The solvent extracts of four different seaweeds used in the present study showed significant inhibitory action against MDR *S. aureus*, among the four seaweeds screened for their antibacterial activity, the diethyl ether extract of brown algae

S. vulgare showed the highest inhibitory activity against *S. aureus* isolated from Nile tilapia fish. The antimicrobial activity of seaweeds results from the presence of steroids, phytochemicals, tannins, terpenoids, phenolic compounds and fatty acids in their extracts. This study thus established the possibility of developing an antibacterial agent to combat developing MDR *S. aureus* and biofilm-related infections in Nile tilapia.

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كفاءة بعض أنواع الطحالب ضد البكتيرة العنقوديه استافيلوكوكس أوريس المقاومه للعديد من المضادات الحيويه والمنتجه للبيوفيلم المعزوله من أسماك البلطى النيلى

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تتجه أنظارالناس على مستوى العالم فى زيادة الطلب على المؤكلات البحريه من الأسماك لأنها مصدر غنى بالبروتين لمؤائمة الزيادة السكانية ولكن الأمراض التى تحدث بسبب العدوى بالبكتيريا المقاومه للعديد من المضادات الحيويه يؤدى إلى موت الأسماك داخل المزارع حيث أن البكتيريا الممرضه تكون ما يسمى بالبيوفيلم وعليه فالأدويه المضادة للميكروبات أخذت انتباه واسع لمقاومة البكتيريا الممرضه للأسماك. تم عمل دراسة على كفاءة مستخلصات الطحالب التى تعمل كمضادات للبكتيريا المقاومة للعديد من المضادات الحيوية والمعزولة من أسماك البلطى النيلى. تم أخذ 200 عزله بكتيريه من أسماك البلطى المصابة من بين هذه العزلات تم تعريف 50عزلة كإستافيلوكوكس أوريس من بين هذه العزلات 37 عزله مقاومة للعديد من المضادات الحيوية ومنتجة للعديد من عوامل الضراوة وتشتمل على كلا من إستافيلوكينيز (70.2%) والليثيسينيز (81%) والبروتيز (56.7%) وأخيرا الليبيز (59.4%). وتم تحليل إنتاج البيوفيلم الذى يمكن تقديره كميا ووصفيا. كما تم استخدام أربعة أنواع من الطحالب وهى جنيا روبنز، أولفا لكتيوكا، سراجاسم فولجالرى و سراجاسم فيوزيفورم من حيث القدره على كبح جماح هذه البكتيريا المقاومة للعديد من المضادات الحيوية والمنتجة لعوامل الضراوة من بين هذه المستخلصات كان مستخلص داى إيثيل إيثر لطحلب السراجاسم فولجارى هو أفضل مستخلص له القدرة على كبح جماح هذه البكتيريا وعند عمل تحليل له على جهاز جى سى – إم إس تلاحظ وجود 20 مركب فى هذا المستخلص وكان مركب اللونجوفولين يحتل النسبة الأكبر فى هذا المستخلص (16.5%). حيث أمكن لهذه الدراسه تطوير مواد مضادة لنشاط البكتيريا المقاومة للعديد من المضادات الحيوية والمنتجة للبيوفيلم والمرتبطة بعدوى أسماك البلطى النيلى.