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A study on the effect of two algal extracts on modulating the antibiotic activity to resist Methicillin-resistant *Staphylococcus aureus* strains.

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قسم علم النبات

دراسة تأثير مستخلصات طحلبين على تعديل نشاطية المضادات الحيوية لمقاومة عثرات من ال *Staphylococus aureus* المقاومة للميثيسلين.

موسى علي خليفة الكصك

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University of Tripoli (2022)

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections. MRSA infections are very difficult to cure because MRSA strains are resistance against almost all clinically available antibiotics. In this study two brown algae extracts, *Petalonia fascia* (*P. fascia*) and *Dictyota dichotoma* (*D. dichotoma*) collected from Tajura western coast of Libya, were evaluated individually and in combination with Amoxicillin for antibacterial activity against five strains of MRSA (S119,S120,S122,S130,S283), using hole-plate diffusion method, and by absorption measurements using spectrophotometer. The MIC and MBC of algae extracts were determined, as well as the probable mode of their anti-bacterial effect, was evaluated using bacterial physiological indicators, including intracellular potassium ion efflux and nucleotide leakage. Also hemolytic activity of algae extracts was assessed as an indicator of their relative cytotoxicity on human red blood cells . The following extract-antibiotic combinations were used (1:1), (1:2), (2:1). Results showed that methanolic extracts of *P. fascia* and *D. dichotoma* have a significant inhibitory action against all tested MRSA strains, with a marked advantage of *P.fascia* against all tested strains. Also the two algae extracts showed spectrum of bactericidal effect with a ratio MBC/MIC ≤ 4 obtained on all tested MRSA strains. Compared Amoxicillin alone, the bacterial growth of all MRSA strains was completely inhibited as absorption of each sample decreased dramatically indicating reduced bacterial growth, due to the effect of algal extract enhancing the antibacterial activity of Amoxicillin. Also the combination between tested algae extracts and Amoxicillin showed significantly induced a distinct release of nucleotide and potassium ions out of cell membrane, compared with Amoxicillin as alone, which did not showed significant results. which indicates that is a disruption in the cell membrane permeability and leakage of intracellular materials, or a rupture of the cell membrane and cell wall and exit of all the contents of cell to the outside environment. Moreover the results also showed that algae extracts possess a considerable low hemolytic activity and considered at the safe level. Therefore, our results revealed the importance of *D. dichotoma* and *P. fascia* crude extracts when associated with Amoxicillin to control MRSA strains.

Key words: MRSA, combination, cytotoxicity, algae extracts, mode of action.

دراسة تأثير مستخلصات طحلبين على تعديل نشاطية المضادات الحيوية لمقاومة عثرات من ال *Staphylococus aureus* المقاومة للميثيسلين.

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المستخلص

تعتبرالمكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) ،أحد الأنواع الرئيسية المسببة لعدوى المستشفيات، يصعب علاج عدوى هذه المكورات لأن أغلب عثراتها تقاوم تقريبًا جميع المضادات الحيوية المتاحة سريريًا. في هذه الدراسة ، تم تقييم فعالية مستخلصين لإثنين من الطحالب البنية كمضادات للبكتيريا ، وهما *Petalonia fascia* و *Dictyota dichotoma*، ضد خمس سلالات من المكورات العنقودية المقاومة للميثيسيلين(S119,S120,S121,S130,S283) ، باستخدام طريقة الحفر على الأطباق (hole-plate diffusion method)،وأيضا تقييم فعالية الدمج للمستخلصين مع المضاد الحيوي أموكسيسيلين (Amoxicillin)، كمضادات للبكتيريا وذالك عبر قياس العكارة باستخدام مقياس المطياف الضوئي. تم تحديد أقل تركيز مثبط (MIC) و أقل تركيز قاتل (MBC) لمستخلصي كلا الطحلبين ، وكذلك تم تقييم آلية العمل للمستخلصين كمضادات للبكتيريا ضد العثرات المختبرة ، باستخدام بعض المؤشرات الفسيولوجية البكتيرية ، بما في ذلك تدفق أيونات البوتاسيوم خارج الخلايا البكتيرية وتسرب النيوكليوتيدات إلى الخارج أيضا، كما تم تقييم النشاط الانحلالي لمستخلصي كلا الطحلبين كمؤشر لسميتها الخلوية على خلايا الدم الحمراء للإنسان. تم استخدام المدمج للمستخلصين والمضادات الحيوية،[بنسب (1:1) و (1:2) و(2:1)]. أظهرت النتائج أن مستخلصي كلا الطحلبين لها تأثير مثبط ملحوظ ضد جميع عثرات MRSA المختبرة ، مع أفضلية ملحوظة لطحلب ال *P.fascia* ضد جميع العثرات المختبرة. كما أظهر مستخلصي الطحلبين تأثيرا قاتلا بنسبة MBC / MIC≤ 4 ضد جميع عثرات MRSA المختبرة. أظهرت نتائج الدمج بين مستخلصي الطحالب المختبرة والأموكسيسيلين، بالمقارنة مع الأموكسيسيلين منفردا ، أن النمو البكتيري لجميع عثرات ال MRSA إنتهى تمامًا بسبب تأثير مستخلص الطحالب الذي عزز النشاط المضاد للبكتيريا للأموكسيسيلين. أيضا أظهرت نتائج الدمج بين مستخلصي الطحالب المختبرة والأموكسيسيلين زيادة في تدفق أيونات البوتاسيوم والنوكليوتيدات خارج غشاء الخلية البكتيرية، مقارنة مع الأموكسيسيلين بمفرده. وهو ما يشير إلى أنه قد حدث خلل في تنظيم نفاذية غشاء الخلية وتسرب هذه المواد بكميات كبيرة إلى خارج الخلية البكتيرية ، أو تمزق غشاء الخلية والجدار الخلوي، وخروج جميع محتويات الخلية للخارج. علاوة على ذلك ، أظهرت النتائج أيضًا أن مستخلصي كلا الطحلبين تمتلك نشاطًا انحلاليًا منخفضًا ضد كريات الدم الحمراء ،بشكل ملحوظ وتعتبر ضمن المستوى الآمن للإستخدام. في المجمل العام تشير كل النتائج السابقة إلى أهمية الدمج بين المستخلصات الإيثانولية للطحلب *P.fascia* و *D. dichotoma* مع الأموكسيسلين في السيطرة على سلالات المكورات العنقودية الذهبية المقاومة للميثيسيلين.

الكلمات الدالة: المكورات العنقودية الذهبية المقاومة للميثيسيلين ، الدمج، السمية، المستخلصين الطحلبيين، آلية العمل.

Dedication

I dedicate this work to the pure soul of my father, who was my role model to endure all odds, and also to my dear mother, who gave me all the support throughout the research period, this dedication is also extended work to all my wonderful family members.

Acknowledgement

I convey my sincere gratitude to Allah for sustaining me throughout the study period, my supervisors: Prof. Salah M. Azwai (Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli), and Prof. Rabia O. Alghazeer (Department of Chemistry, Faculty of Sciences, University of Tripoli), for their moral support, precious advice and patience throughout this study and I would like to express my deep pride for them, that I was one of their students. I thank Dr. Fatiem Gammoudi (Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli), for her help in training to deal with bacteria in Lab. I thank Ms Sana Alguhmassey and Mr. Moammar A.Sidati, (researchers in the Biochemistry Laboratory, Department of Chemistry, University of Tripoli), for helping me run the experiments and providing support in the lab, and I would like to express my sincere gratitude to them and wish them success in their scientific career. I thank Mr. Obeyda Dia Addein , to help me carry out statistical analysis. I thank Ms. Mahasn Kubbat who helped me conducting potassium leak experiment, in the Tajoura Water Quality Analysis Center. I would like to thank Tajoura Marine Biology Research Center, for all the support it provided me to conduct this study, including the collection and authentication of the tested algal samples.

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List of Abbreviations

Abbreviations	Expression or meaning
AR	Antimicrobial Resistance
Cfu	colony-forming unit
DIZ	Diameter of inhibition zone
ESBL	Extended Spectrum β-lactamase
FabI	enoyl-acyl carrier protein reductase
HRBCs	Human Red Blood Cells
LMPs	Low molecular weight of phlorotannins
MBC	Minimum bactericidal concentrations
MIC	Minimum inhibitory concentrations
MDR	Multidrug-resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
SD	Standard deviation
SPSS	Statistical Program for Social Sciences
VISA	Vancomycin Intermediate *Staphylococcus aureus*

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1. Introduction

Antibiotics are probably one of the most successful forms of chemotherapy in the history of medicine. The introduction of antibiotics not only helped in the treatment of infections but also have a major role in decreasing mortality and morbidity (Aminov, 2009; Jumaa and Karaman, 2015).

The term antibiotic was coined from the word antibiosis, which literally means against life. In the past, antibiotics were considered organic compounds produced by one microorganism, which is toxic to other microorganisms. As a result of this notion, an antibiotic was originally, broadly defined as a substance, produced by one microorganism or of biological origin, which at low concentrations can inhibit the growth of or are lethal to other microorganisms (Etebu and Arikekpar, 2016). However, this definition has been modified in modern times to be natural or synthetic chemicals products able to affect the survival of microorganisms through inhibiting their growth or killing them (Davies and Davies, 2010). Generally, antibacterials can be classified on the basis of type of action: bacteriostatic and bactericidal, Antibacterials, which destroy bacteria by targeting the cell wall or cell membrane of the bacteria, are termed bactericidal and those that slow or inhibit the growth of bacteria are referred to as bacteriostatic (Ullah and Ali, 2017).

The discovery of penicillin by Alexander Fleming and the subsequent therapeutic use of antibiotics were major turning points in modern medicine. Once life threatening infectious diseases like pneumonia, syphilis, or sepsis caused by wound infections became easily curable with little to no side effects. Sparked the success of penicillin to discovery of numerous new antibiotics. Within a few decades most of the antibiotic classes known today were discovered and commercialized, a time often referred to as the golden era of antibiotics.

Nowadays, antibiotics are one of the pillars of modern medicine and used to treat numerous bacterial infections, they have become one of the most important medical interventions needed for the development of complex medical approaches such as cutting edge surgical procedures, solid organ transplantation and management of patients with cancer, and neonatal care events (Munita and Ari , 2016 and Knopp, 2018).

The struggle of humanity against infectious diseases is well known, the discovery of antibiotics led to optimism that infections could be controlled and prevented. However, infections are still the leading cause of death in developing world. Both aerobic and anaerobic bacteria have been implicated in wound infections, which commonly occur under hospital environment and result in significant morbidity, prolonged hospitalization, and huge economic burden. Especially when the efficiency of antibiotics is compromised by the growing number of antibiotic-resistant pathogens. It appears that the emergence of antimicrobial resistance (AR) is inevitable to almost every new drug, and it is recognized as a major problem in the treatment of microbial infections in both hospitals and community. Inappropriate and irrational use , addition to quality of drugs , poor infection control in health care settings, and poor management of medical waste, all of these conditions are responsible for emergence of resistant microbial populations (Lin *et al.*, 2013; Trojan *et al.*, 2016; Gupta and Birdi, 2017 and Kapoor *et al.*, 2017).

Antibiotic resistance has turned into a severe global health crisis and yet people are not completely aware of the threat it poses at the individual level as well as the community level. However, it is a widely recognized and growing problem that causes over 700,000 deaths each year worldwide. The using antibiotics was in ever-increasing amounts for the last half century, and it is estimated that the AR plague may have claimed 500 million lives worldwide (Rather *et al.*, 2017; Massey and Ross, 2017 ; Strachan and Davies, 2017).

Moreover, according to a recent report, antibiotic resistance is estimated to cause around 300 million premature deaths by 2050, with a loss of up to $100 trillion to the global economy. The Multidrug-resistant bacteria (MDR) is considered a threat to public health, not only by increasing the number of mortality , but also those who have weak immunity become an easy target for these bacteria, also causing high medical cost and prolonging the morbidity period, for that the World Health Organization (WHO) has named antibiotic resistance as one of the three most important public health threats of the 21st century (Prestinaci *et al*., 2017and Munita and Arias, 2016).

From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic attack, bacteria can acquire resistance via horizontal gene transfer, by mobile genetic elements and bacteriophages and mutations in gene often associated with the mechanism of action of the compound. Alteration of target sites, active efflux of drugs and enzymatic degradations are the strategies employed by the

pathogenic bacteria to develop intrinsic resistance to antibiotics (Munita and Arias, 2016; Frieri *et al.*, 2016 and Gupta *et al.*, 2017).

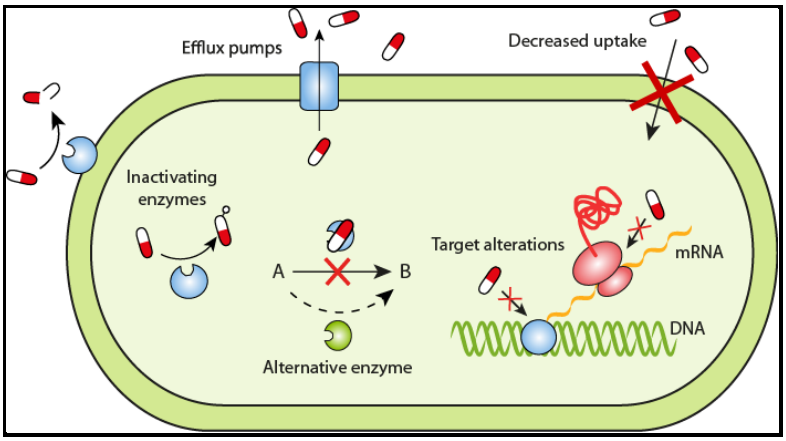

Figure1: Mechanisms which bacteria become resistance to the antibiotic (ReActgroup.org)

Antimicrobial resistance is ancient and it is the expected result of the interaction of many organisms with their environment (Munita and Arias, 2016).

1.1 *Staphylococcus aureus*

Staphylococcus genus is widely spread in nature being part of the indigenous microbiota of skin and mucosa of animal and birds. *S. aureus, S. epidermidis, S. saprophyticus a*nd *S. haemolyticus* are the most important species as community and nosocomial human infection causing agents (Coutinho *et al*., 2009).

Staphylococcus aureus is a major pathogen associated with serious community and hospital acquired diseases, it is a Gram-positive, nonmotile, small, round-shaped bacteria, typically found growing in grape-like clusters. This bacterium is facultative anaerobic, mesophilic, catalase-positive and oxidase negative. It grows within a temperature range from 7 °C to 48 °C and in a pH range from 4.2 to 9.3. Its distinctive feature is halotolerance equivalent to minimal water activity (*aw*) values ranging from 0.83 to 0.85 (Valik *et al.*, 2008; Altalib *et al.*, 2014 and Mcbirney *et al.*, 2016).

It has ability to produce several factors that protect against host derived innate immune factors, such as complement and antibodies. It is one of the leading causes of human infections of the skin, soft tissues, bones and joints, abscesses, and normal heart valves. *S. aureus* flourishes in the hospital setting and is associated with bloodstream and surgical wound infections and has become important nosocomial organism. Penicillinase producing *Staphylococcus aureus* strains were isolated shortly after penicillin G became available. Methicillin Resistant *S. aureus'* (MRSA) first

recognized in the 60s is a multi-resistant strain that has been documented worldwide showing risen resistance to different classes of antimicrobials (Matsuo *et al*., 2011; Abouzeed *et al.*, 2013 and Altalib *et al.*, 2014).

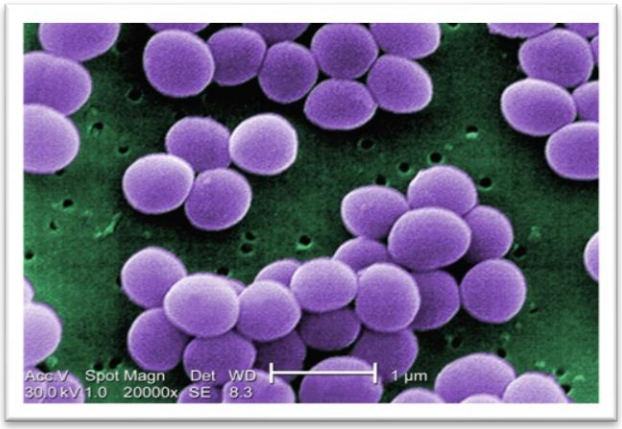

Figure2: Shows a strain of *Staphylococcus aureus* bacteria using scanning electron micrograph, SDC (2001)

Methicillin was the first but had the disadvantage of being acid labile. It was superseded by the acid stable, penicillin and Amoxicillin. Acquired infections in community or hospital due to Vancomycin Intermediate *Staphylococcus aureus* (VISA), Methicillin Resistant *S. aureus* (MRSA) or ESBL (extended spectrum β-lactamase) were observed. These strains showed resistance to a wide range of antibiotics thus limiting the treatment options to very few agents such as vancomycin and teicoplanin (Altalib *et al.*, 2014; Foster, 2017 and Gupta *et al.*, 2017).

Penicillins including Amoxicillin, together with the Cephalosporins, are the major β-lactam antibiotics. But most types of bacteria, including *Staphylococcus aureus*, have begun to show resistance to most of the β-lactam family, this resistance to β-lactam family may occur due to a) Preventing the drug from reaching its target: diminished permeability of the bacterial cell to the antibiotic; b) Altering the target: alteration(s) of the penicillin-binding proteins; c) Inactivating the antibiotic: bacterial production of inactivating enzymes, referred to as β-lactamases. Therefor compounds able to inhibit those mechanisms, or able to potentiate the antimicrobial activity of old antibiotics by the bacteria are needed.

The global epidemic of bacterial resistance to existing antibiotics has prompted the search for naturally occurring candidate drugs and food preservative agents from terrestrial and marine source. Nature is an amazing source of chemical diversity and

naturally derived compounds have unique pharmacological properties (Soares *et al*., 2012 ;Shannon and Abu-Ghannam, 2016 and Martins *et al*., 2018).

Marine algae represent a great source of biomolecules with a wide spectrum of effects useful in different biotechnological fields such as in food and textiles industries, biochemistry, pharmacology, in human and veterinary medicine. Many investigations revealed that macro algae have potential use in pharmacology researches as antibacterial anticoagulant, antiviral, antioxidant and anticancer activities. Their inhibitory effect is related to the presence of bioactive compounds as secondary metabolites including polyphenols, carotenoids, saponins, flavonoids, alkaloids, tannins and cardiac glycosides (Rizzo *et al*., 2017; Saleh and Mariri., 2017).

These secondary metabolites from algae are not required for normal growth or reproduction adapt themselves to the environmental conditions and to protect against predation, herbivory and to compete for space, osmotic stress, high levels of UV light, oxygen, and salinity(Mashhadinejad et al., 2017).

In addition, an average of one million bacterial cells are present in each milliliter of seawater, are proposed to be the main factors controlling their production (Shannon *et al*., 2016).

Recent studies suggest, the natural product combinations have become a research priority of considerable interest. The synergy of naturally occurring substances with antibiotics can be an effective strategy for enhancing or restoring antibiotics that are currently ineffective for diseases caused by multi-resistant bacteria, including MRSA (Vaou *et al*., 2021). Mechanism of modulating action of extracts on bacterial antibiotic susceptibility probably related to their antioxidant activity. Ability of natural bioactive extracts to act synergistically with antibiotics is considered a new approach that helps in solving the problem of bacterial resistance. In addition, using synergistic substances, it is possible to reduce the toxicity of the antibiotic by reducing the working dose of the drug. However, not much is understood about the combination of algal antibacterial extracts and antibiotics and their effect on bacterial cells (Samoilova *et al*., 2014 ; Alghazeer *et al.*, 2016 and Manisha *et al.*,2017).

One of the features of algae extracts is changes in permeability and integrity of the cell membrane and cell wall, which can facilitate the penetration of antibiotics into the cytoplasm of bacteria. After that, the mechanism of destruction of the pathogen will be carried out by the action of the antibiotic on the vital functions of the microbe,

replication, transcription, or translation of DNA, depending on the type of antibiotic (Besednova *et al*., 2020)

The aim of this study was to investigate the *in vitro* change in the resistance of antibiotic activity by two algae extracts, against multi-resistant strains of Methicillin-resistant *Staphylococcus aureus.*

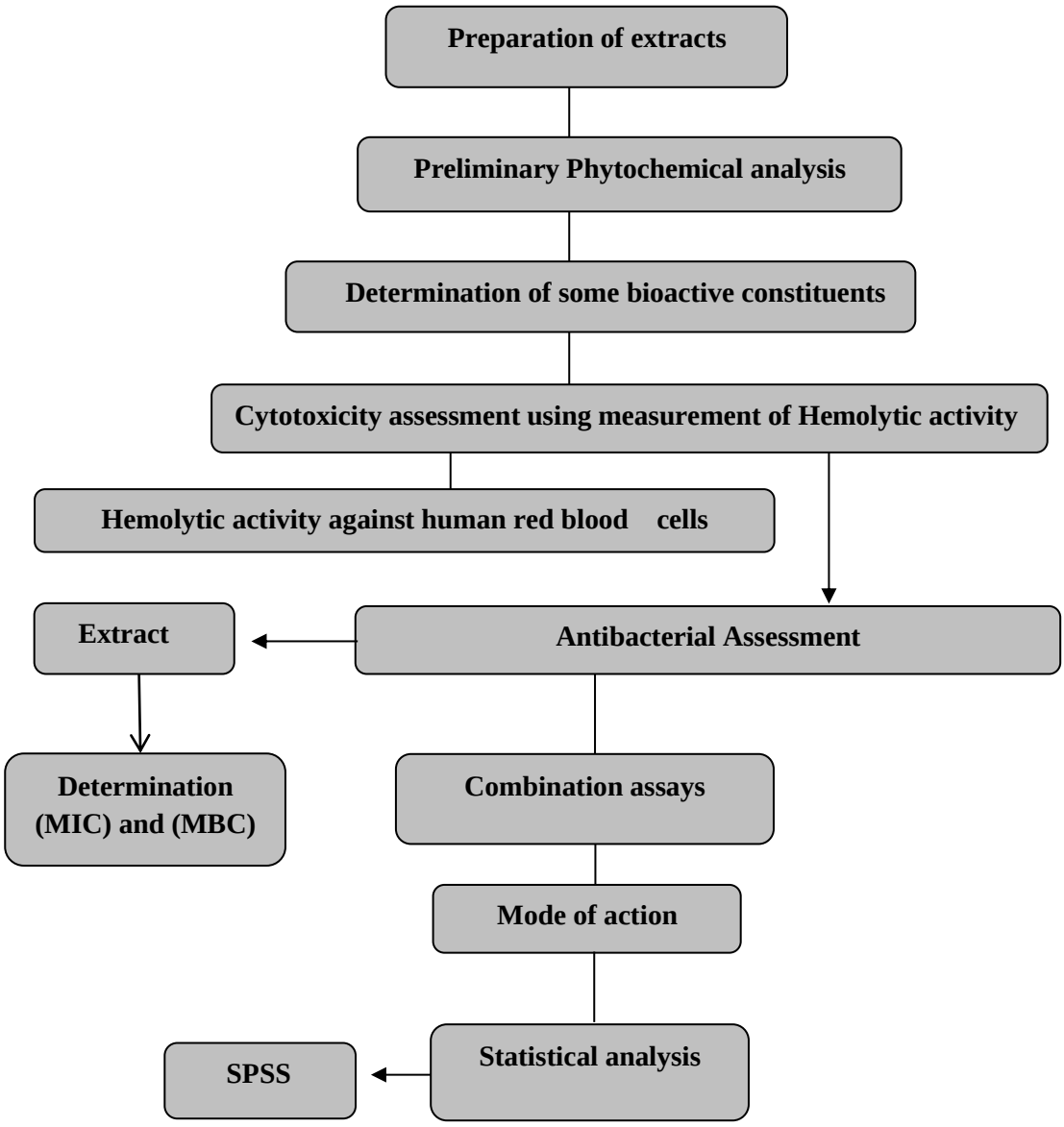

Figure 3: Shows the outline of study

2.Literature review

2.1. Antibacterial activity of algae extracts

The antimicrobial activity of methanol, dichloromethane and hexane extracts, as well as the essential oils of brown algae (Phaeophyta) *Colpomenia sinuosa*, *Dictyota dichotoma*, *Dictyota dichotoma* var. *implexa*, *Petalonia fascia* and *Scytosiphon lomentaria*, from the Aegean Sea, were evaluated by Demirel *et al*., (2009). Antimicrobial activities of the algae extracts were assessed against Gram positive including *S. aureus* and Gram negative bacteria and one yeast strain by the disk diffusion method. The results showed that the methanolic extracts of *Dictyota dichotoma* inhibited *S. aureus*. According to the results, the dichloromethane extracts generally showed more potent antimicrobial activity than the methanol and hexane extracts.

A study by Ibtissam *et al,*. (2009).The antibacterial activity of methanolic extracts from 32 macroalgae, 13 species of *Chlorophyta*, and 19 species of Phaeophyta from the Atlantic and Mediterranean coast of Morocco were evaluated for the production of antibacterial compounds against five strains from Gram positive and Gram negative bacteria, including *S. aureus* ATCC 25923 their results indicate that these species of seaweed collected from the Atlantic and Mediterranean coast of Morocco presented a significant capacity of antibacterial activities, which makes them interesting for screening for natural products.

A study by Oumaskour *et al,*. (2012), in this study six organic extracts prepared with different solvents (methanol, acetone, hexane, chloroform and dichloromethane-methanol), and aqueous extract of 27 species of marine algae belonging to the Chlorophyta and Phaeophyta were collected from the coast of Sidi Bouzid (Maroco), their antibacterial activities against pathogenic microorganism, eight Gram-positive bacteria including *S. aureus* spp. *aureus* and two Gram-negative bacteria were investigated **.** The best activity was observed in methanolic extract followed by acetonic extract and that prepared with methanol–dichloromethane. Algae belonging to Phaeophyta were the most active in comparison with Chlorophyta. The Gram-positive bacteria presented a sensibility superior to the Gram-negative and *S. aureus* ssp. *aureus* was the more sensitive.

Taskin *et al*., (2012), studied eight spices of marine algae, three spices of Rhod - ophyceae, two species of phaeophyceae including *Dictyota dichotoma*, and three species of Chlorophyceae, were collected from Cyprus coast (Eastren Mediterranean Sea) and were examined as antibacterial agents, against some common food-borne pathogens species of bacteria including (*S. aureus*) through the methanolic solvent. *S. aureus* was the most sensitive bacterium, since it was inhibited by most of the methanolic extracts, generally six species of algae including *D. dichotoma* were shown inhibitor activity against food-related pathogens.

Alghazeer *et al*., (2013), studied the antibacterial activity of methanolic, and hot water extracts, from nineteen marine algal species (6 Chlorophyta, 8 Phaeophyta, 5 Rhodophyta), were collected from the western coast of Libya. Their flavonoids and alkaloid extracts were tested against Gram positive bacteria *(S. aureus, Bacillus subtilis*, *Bacillus sp*, and *S. epidermidis*) and Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Klebsiella sp* and *Pseudomonas aeroginosa*). The alkaloid extracts from green algae inhibited all the tested bacteria, but the maximum effect was exhibited by brown and red algae species, as well as flavonoids extracts from seven algae species.

Some of green, red and brown algae species, were collected from Red sea coastal waters of Jeddah, Saudi Arabia by Al-Saif *et al*., (2013), and were evaluated for their potential for bioactivity. Extracts of this species were prepared using ethanol, chloroform, petroleum ether and water, and assayed for antibacterial activity against four strains from Gram positive and Gram negative bacteria, including *S. aureus* ATCC 29213. It was found that chloroform was most effective followed by ethanol, petroleum ether and water for the preparation of algal extract with significant antibacterial activities, respectively. Results also indicated that the extracts of some red alga species were more efficient against the tested bacterial strains followed by some green alga, and brown algae.

Alshalmani *et al*., (2014) carried out a study where four species from macroalgae, including *Cystoseira compressa*, *Entero morpha intestinals*, *Corallina* and *Ulva lactuca*, were collected from Benghazi coast. Four different solvents namely
water, ethanol, methanol, and methylated spirit were used for extraction, the results showed zones of inhibition ranged between 8 to 16 mm in aqueous extract and up to 16 mm in methanol extract. The maximum activity (16 mm) was recorded from methanol extract of *Ulva lactuca* against *S. aureus* and minimum activity (8mm) recorded by *Cystoseira compressa* against *S. aureus*.

A study by Kolsi *et al*., (2015), was conducted to evaluated the antimicrobial and antifungal activity of hexane, ethyl acetate and methanol extracts, from thirteen species of marine algae, five *pheophytea* including (*Dictyota dichotoma*), five *cholo- rophytea* and three *Magnoliophytea*, were collected at Tunisian coastline (Chebba and Sfax), were tested against eight human pathogenic bacteria, which included *S. aureus*, and two human pathogenic yeast, and a fungi (*Aspergillus niger)*. The results of thirteen marine species extracts have shown significant antimicrobial activity and the maximum inhibitory activities were observed in the brown algae, it was more active on bacteria than the fungi.

The antimicrobial for the marine algae *Enteromorpha sp. Cystoseria indica, Sargassum swartzii, Gracilaria corticata, Caulerpa taxifolia and Caulerpa racemosa,* from Kodinar coast, Gujarat India was evaluated by Karthikeyan *et al*., (2015). In the presence of methanol, ethanol, chloroform and diethyl ether solvents were used for marine algae extraction, the antibacterial activity against Gram positive included *S. aureus*, and Gram negative bacteria were exhibit. The maximum antibacterial activity was observed in the ethanol extract of all the seaweeds except *C. racemosa*.

Another study by Alghazeer *et al*., (2016), examined the flavonoids extracts from *Padina pavonica and Cystoseira compressa*, which were collected from Tajura coast east of Tripoli and evaluated its antibacterial activity against 17 isolates from pathogenic bacteria including (5 isolates), *S. aureus sub sp.aureus* strains isolated from meat, meat products, milk and dairy products. The results showed that all of tested isolates were multi drug resistant with high Multiple Antibiotic Resistances (MAR) index. The algal flavonoids extracted from *C. compressa* exhibited stronger antibacterial activities against the seventeen tested isolates, as compared with the flavonoids extracted from *P. pavanica* that showed positive effect against nine isolates with low inhibition zone. Flavonoids extracted from *C. compressa* also displayed the best spectrum ofbactericidal effect with a ratio MBC/MIC$\leq$4 obtained on all susceptible tested bacterial strains.

Cystoseira barbata, were collected from Red Sea coastal water (Safaga, Egypt), and were studied for its bioactivities potential by Abdel-Raouf *et al*., (2017). The algal extract proved a potent activity against bacterial and fungal strains ranged between medium and high suppression action. It showed that Gram positive bacteria *Bacillus subtilis* and *S. aureus* were more sensitive than Gram negative bacteria *Serratia*

marcescens, Pseudomonas aeuroginosa. The results indicated scope for utilizing this alga as a source of antibacterial and antifungal substances.

A study by Moubayed *et al*., (2017), the antibacterial activity of selected brown and green marine algae which collected from Saudi Arabia Red Sea and Arabian Gulf were demonstrated. The methanolic and acetone extracts were tested against Gram positive, including methicillin resistant *S. aureus* (MRSA) and Gram negative bacteria and *Candida albicans*. Results showed both brown algae extracts species *Sargassum latifolium B* and *Sargassum platycarpum* more active against Gram positive than Gram negative, while *Cladophora* methanolic extract showed an obvious effect on (MRSA). Furthermore, in this study the Spectrometer analysis together with the high performance liquid chromatography provided a detailed description of the possible functional constituents and the major chemical components present in marine macroalgae particularly in brown seaweeds to be mainly of phenolic nature to which the potent antimicrobial activity has been attributed.

Ghalem *et al*., (2018), studied diethyl ether and chloroform extracts from the *Ulva lactuca, Dictyota dichotoma and Corallina elongate,* which were collected along the Coast of Mostaganem-Stidia in northwest of Algeria. The extracts were screened for their antibacterial activity against two bacterial pathogens (*Escherichia coli* and *S. aureus*). The antibacterial activities were assessed by standard protocol of Disc Diffusion Method. Chloroform extracts being most potent towards the tested bacterial strains than diethyl ether, which showed no antibacterial activity to both *E. coli and S. aureus.*

A study by Kim *et al*., (2018), the ethanolic extract of edible brown algae *Ishige okamurae*, were used as antibacterial agent against Cutaneous bacterial pathogens including *S. aureus*, and its synergistic antibacterial effect against *Pseudomonas aeruginosa.* The ethanolic extract of *Ishige okamurae* exhibits potent antibacterial activity against cutaneous bacterial pathogens. Among the ethanol soluble fractions, the n-hexane (Hexane)-soluble fraction exhibited the strongest antibacterial activity against the pathogens with MIC values ranging 64 to 512 μg/mL and with minimum bactericidal concentration values ranging from 256 to 2048 μg/mL. Furthermore, the combination with Hexane fraction and antibiotics (ceftazidime, ciprofloxacin, and meropenem) exhibited synergistic effect.

2.2. Combination effect

Fucoidan is a sulfated polysaccharide that is primarily extracted from brown seaweeds, was tested against clinic isolates methicillin-resistant *S. aureus* (MRSA) 1 - 20 either alone or with antibiotics, using broth dilution method checkerboard and time kill assay, (Mi Choi *et al*., 2015). Minimum inhibitory concentrations (MICs)/Minimum bactericidal concentrations (MBCs), were reduced to one half-eighth as a result of the combination of the fucoidan with antibiotics. The results showed increase of the rate of killing in units of CFU/mL to a greater degree begins 2 - 6 hours of treatment with 1/2 MIC of fucoidan with 1/2 MIC, compared was observed with alone.

These results suggest that fucoidan could be employed as a natural antibacterial agent against multi-drug bacteria.

The methanol extract of *H. pedunculatum* was combined with eight first-line antibiotics and they were investigated as antibacterial agents by (Olayinka *et al*., 2009). Against five bacterial strains responsible for wound infections including *S. aureus* strains . Results showed in all, 60% of the interactions were synergistic, but on the other side, all combination regimes on *S. aureus* ATCC 6538 yielded no synergistic effect . Finally the study propose that extracts of *Helichrysum pedunculatum* could be of relevance in combination therapy, and as a source of resistance modifying principles, that could be useful as treatment options for wound infections.

Nshimiyumukiza *et al*., (2015), they studied the synergistic Antibacterial Activity of *Ecklonia cava*, when combination with streptomycin against *Listeria monocytogenes*. Results showed the MIC values of streptomycin in combination with the ethyl acetate (EtOAc) fraction were markedly reduced up to 64-fold, suggesting that the antibacterial activity of the antibiotic was restored when combined with the EtOAc fraction.

Another study from University of Fort Hare, South Africa, by Olajuyigbe and Afolayan (2013), the combination between ethanolic extract of *Ziziphus mucronata* and seven first-line antimicrobial agents to which most of them have become resistant, were evaluated against seven types of bacteria strains. Although, the agar diffusion assay, results suggested that the interactions between the ethanolic extract of *Z. mucronata* and the antibiotics were both synergistic and additive in nature, the fractional inhibitory concentration indices (FICI) showed that the interactions were synergistic (54.17%), additive (27.78%), indifferent (16.67%), and antagonistic (1.39%). which indicates that the synergistic effects implied that the antibacterial

combinations would be more effective and useful in the treatment of multicausal and multidrug-resistant bacteria than a single monotherapy of either antibacterial agent.

The *in vitro* interaction between different extracts of *Thymbra spicata* and certain antimicrobial drugs of different mechanisms, including ampicillin, cefotaxime, amikacin and ciprofloxacin, against multidrug-resistant strains of *S. aureus* and *Klebsiella pneumoniae* were evaluated by Haroun and Al-Kayali (2016), from Syria. The results showed synergistic, additive and indifference interaction between plant crude extracts and used antibiotics depending on the strain. The best synergistic capacity appeared with cefotaxime against *S. aureus* strains, where the activity of cefotaxime was increased from 8 to 128 fold. These results suggesting a possible utilization of this herb in combination therapy against emerging multidrug-resistance *S. aureus* and *K. pneumoniae*.

A study by Jouda *et al*., (2015), from Gaza, Palestine. The synergistic effect to four medicinal plants extracts, namely *Artemisia herba-alba, Lantana camara, Allium sativum* and *Eucalyptus camaldulensis*, with eighteen commercial antibiotic, against *Escherichia coli, S. aureus and Pseudomonas aeruginosa*, were assessed using disk diffusion method. The results showed that ethanolic extracts used against *E. coli, S. aureus* and *P. aeruginosa* were showed antimicrobial and synergistic effect with most antibiotics better than methanolic and aqueous extracts. These results revealed the importance of plant extracts when being associated with antibiotic in control of Pathogenic bacteria.

A study by Kim *et al*., (2016), they studied the synergistic antimicrobial effect of *Sargassum serratifolium* extract ,with commercial antibiotics against Human Skin Pathogens. Results showed the hexane fraction exhibited a synergistic antimicrobial activity with commercial antibiotics used in the treatment of acne vulgaris.

Nafis *et al*., (2021), studied the chemical profile, antimicrobial properties, and synergistic effect with known antibiotics of essential oil extracted from the marine red macroalgae *Centroceras clavulatum* against some of pathogenic species of bacteria including *Staphylococcus aureus* . As the synergistic effects of its application combined with the antibiotics ciprofloxacin and fluconazole, by the checkerboard method. A significant synergic action was observed when the oil was applied in combination with ciprofloxacin and fluconazole, with fractional inhibitory concentration index values ranging from 0.31 to 0.50. Synergy was found in 80% of the combinations and a 2 to 16-fold reduction of antibiotics MIC was observed.

2.3. Mode of action

The crud extract of algae are containing many bioactive compounds, such as phenols, terpenes, indoles, fatty acids and volatile halogenated hydrocarbons, etc. So with many of this compounds, the mechanism of action cannot be determined in one direction, that what showed a study by El Shafay *et al*., (2015), in this study the antibacterial activity of diethyl ether, methanol, ethanol and chloroform extracts of some algae including *Sargassum vulgare, Sargassum fusiforme,* were evaluated against ten multidrug resistant clinical isolates of Gram positive and Gram negative bacteria including *S. aureus* and *Klebsiella pneumonia.* Transmission electron microscopy was applied for determining the morphological changes in *S. aureus* and treated with 100µl diethyl ether extract of *S. fusiforme* and 50 µl ethanol extract of *S. vulgare*, respectively. Perforation of cell wall, leakage of cytoplasmic contents, severe distortion of outer cell shape, inner chromatin mild scattered cytoplasmic vacuolation, rupture of cell wall, and decreased cell size for both bacterial isolates were observed. However, some studies were focused on some of this bioactive compounds and how it worked as antibacterial.

Holanda *et al*., (2005) were isolated a lectin from the red alga *Solieria filiformis* and was evaluated for its effect on the growth of 8 Gram-negative and 3 Gram-positive including *S. aureus*. The lectin (500 µg/mL) stimulated the growth of the ram positive species *Bacillus cereus* and inhibited the growth of 6 from 8 species of the Gram negative bacteria at 1000 µg/mL. But the lectin (10-1000 µg/mL) had no effect on the growth of 2 gram-positive bacteria including *S. aureus* and 2 Gram negative bacteria (*Escherichia coli* and *Salmonella typhimurium*). The study suggested the interaction of *S. filiformis* lectin with the cell surface receptors of Gram-negative bacteria promotes alterations in the flow of nutrients, which would explain the bacteriostatic effect.

Low molecular weight of phlorotannins (LMPs) from *Sargassum thunbergii* against *Vibrio parahaemolyticus* were investigated by Wei *et al*., (2016) to understand their mode of action. Effects of LMPs on the growth curve and the content changes of some substances in culture media, and those on morphological and ultra-microstructure were also observed by scanning electron microscope and transmission electron microscopy(TEM). The results showed that LMPs inhibited the growth at logarithmic phase and damaged the cell membrane and cell wall of tested bacteria, which led to the leakage of cytoplasm and the deconstruction of membrane permeability.

A study by Zheng *et al*., (2005), in this study unsaturated fatty acids derivatives showed inhibitory effect against some of resistance pathogenic bacteria, and by using acetate incorporation assay, the mechanism for this antimicrobial activity was exhibited the linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis.

Alghazeer *et al*. (2021), studied antibacterial activities and mode of action of algal alkaloid-rich extracts against isolates of multidrug resistant *S. aureus* and enterohaemorrhagic *Escherichia coli* (EHEC) O157. The probable mode of action on this isolates was evaluated by two bacterial physiological indicators including, potassium ion efflux and nucleotide leakage. The results showed both of alkaloids extracts presented antibacterial activity against all tested isolates and they significantly induced a distinct release of nucleotide and potassium ion out of the cell membrane, indicating that they cause a change in the fluidity or permeability or both of the cell membrane.

3.Materials and Methods

3.1 Materials

3.1.1. Sampling area, Collection and Identification of the algae Material.

After determining of some bioactive compounds, tow algae species named *Petalonia fascia* (*P. fascia*) and *Dictyota dichotoma* (*D. dichotoma*), were selected from many of algae samples, that were collected from coast Tajura western coast of Libya between August and Mares 2018-2019, (SA 01, N 32°53'45.47 E 13°21'3.16; SA 02, N 32°53'51.95 E 13°21'4.25; SA 03, N 32°53'54.19 E 13°20'54.10; SA 04, N 32°53'46.23 E 13°20'50.90) (Figure 2). The algal samples were identified and classified, at Marine Biology Research Center (MBRC), Tajura - east of Tripoli Libya. The routine procedures, which included, removing the epiphytes and necrotic parts, followed by rinsing with sterile distilled water, were carried out on the collected samples . The samples were dried in the shade at ambient temperature for seven days and immediately subjected to extraction.

Figure 4: Localization of the collection site of algae

3.1.2.Bacterial strains

The staphylococcal isolates used in this study, were originally isolated from meat and meat products and molecularly identified as *S. aureus* at the Department of food hygiene, Faculty of Veterinary Medicine, University of Tripoli, Libya. When tested for antibiotics susceptibility, their antibiogram revealed that, they were MDR and with the

exception of *S. aureus* 120 (S2), the remaining; *S. aureus* 119 (S1), *S. aureus* 121 (S3), *S. aureus* 130 (S4) and *S. aureus* 283 (S5) were all MRSA (Naas *et al*., 2019).

3.1.3. McFarland standard

McFarland standard was used to standardize the approximate number of bacteria in liquid suspension with that of the McFarland standard, the McFarland standard was prepared as described in Kirby Bauer disk diffusion susceptibility test protocol (Hudzicki 2009).

3.1.4.Antibiotics

Amoxicillin (500mg) was obtained from Bristol Laboratories Ltd/UK. While Amoxicillin/Clavulanic Acid (Augmentin, 1g) was obtained from Dar Al Dawa /Jordan. Antibiotics were dissolved in Dimethyl sulfoxide (DMSO) 0.01%.

3.1.5.Chemicals

1. Solvents (Methanol, Ethanol, Dimethyl sulfoxide, potassium persulfate, and Phosphate buffer saline)
2. Reagents (Dragendorff, Ninhydrin solution, Ammonia solution)
3. Media (Nutrient agar, Mueller Hinton agar and Nutrient broth).

3.1.6. Equipments

Table1: Equipments used in the study

Equipment	Company
Incubator with inner Orbital shaking mechanism (15-50ºC)	GFL ,Germany
Spectrophotometer (UV-VIS 6305)	PerkinElmer, LAMBDA 25, USA
Laminar flow cabinet-biosafety 5-130 EHRET	EHRET, Germany
Autoclave, Sterilization	GMBH,KSG, Germany
Water distilling apparatus(12L/h)	GFL, Germany
Ordinary centrifuge (6000 revolution/min) Hettic EBA20	EBA20, Germany
Rotary evaporator (Heidolph300 LabroRota)	Germany
Hot plate (0-450 ºC) with magnetic stirrer (0-1200/min)	IKA labor technik, USA
Flame Photometer	BMW Technologies, UK

3.2. Methods:

3.2.1. Extraction and phytochemical screening

Powdered samples of *P.fascia* and *D.dichotoma* were extracted with methanol (99%) as the amount of solvent used was twice the mass of the specimens. After extraction (3 times) for 72 hours at room temperature (25-30ºC) with shaking, the crude

extract was filtered by Whatman filter paper No.1, and the solvent was con- centrated by rotary evaporator (Stuart RE300) at 40ºC under reduced pressure, the resultant residue kept at -20ºC until use (Musbah *et al*., 2019).

3.2.2. Phytochemical screening

The qualitative detection of alkaloids, flavonoids, saponins, tannin, proteins, anthraquinones, steroids, quinones, carbohydrates, coumarins, terpenoids and lipids was performed according to(Kumar *et al*., 2013; Banu and Cathrine., and Deyab *et al*.,2016). The color intensity or the precipitate formation was used as analytical responses to these tests.

3.2.2.1.Test for alkaloids: about 0.3 g of each algal extract was mixed with 5 ml of 1% HCl, warmed and filtered, then 2 ml of the filtrate were treated with (Dragendorff's) reagent, after that the alkaloids were observed by presence or absence of the turbidity or precipitate formation.

3.2.2.2. Test for Flavonoids: 5 ml of the dilute ammonia solution was added to the portion of the aqueous filtrate of each algal extract followed by the addition of concentrated H_2SO_4. A yellow colouration observed in each extract indicated the presence of flavonoids.

3.2.2.3. Test for saponins: about 2.0g of the powdered algal material were boiled in 20ml distilled water in a test tube in boiling water bath and filtered, then 10 ml of the filtrate were mixed with 5 ml of distilled water and were shaken vigorously to the formation of stable persistent froth, then the frothing was mixed with 3 drops of olive oil and shaken vigorously for the formation of emulsion, which indicates the presence of saponins.

3.2.2.4. Test for Tannins: about 0.5 g of each dried powdered samples was boiled in 10 ml distilled water and then filtered. Few drops of 1% aqueous Iron chloride (FeCl) solution were added to the filtrate. The Formation of brownish green, purple, blue or black color indicated the presence of tannins.

3.2.2.5. Test for Proteins: about 0.2g of each dried powdered tested sample was boiled in 2 ml of 0.2% Ninhydrin solution. Appearance of violet color indicate the presence of proteins.

3.2.2.6.Anthraquinones: few drops of 10% ammonia solution were added to 1 ml of algae extracts, appearance of pink colored precipitate indicates the presence of anthra-quinones.

3.2.2.7.Test for Steroids: To 1 ml of algal extracts equal volume of chloroform was added and a few drops of sulphuric acid was added appearance of brown ring indicates the presence of steroids.

3.2.2.8.Test for Quinones: To 1 ml of crude algal extract, 1 ml of concentrated sulphuric acid was added. Formation of red color indicates presence of quinones.

3.2.2.9.Test for Carbohydrates: The presence of carbohydrates was confirmed when 2 ml of extract were treated with 1 ml of Molisch's reagent and few drops of sulphuric acid which resulted in the formation of purple or reddish color after very gentle shaking.

3.2.2.10.Test for Coumarins : 1 mL of 10 % NaOH was added to 1 mL of algal extract. Formation of yellow color indicates the presence of coumarins.

3.2.2.11.Test for Terpenoids : about 2 mL of chloroform along with concentrated Sulphuric acid were added to 0.5 ml of the algal extract. Formation of reddish brown color at the interface indicates the presence of Terpenoids.

3.2.2.11.Test for lipids: A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oils.

3.2.3.Quantitative analysis of phytochemical substances in algal extracts

The quantitative estimation of polyphenols, flavonoids, tannins, terpenoids, alkaloids, phlorotannins, steroids and coumarins, were performed according to (Deyab *et al*., 2016; Hashmi *et al*., 2021)**.**

3.2.3.1.Total of polyphenols content : One mL of 10 % Folin-Ciocalteu reagent was added to 20 µL of algal extract or standard. The reagents were mixed well and incubated for 5 min before adding 700 µL of 10 % Na_2CO_3. The solutions were further incubated for 2 h before reading the absorbance at 765 nm. Gallic acid in the range of 20–200 mg/L was used to construct a calibration curve. Estimation of the total phenols was carried out in triplicate. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram of dry weight.

3.2.3.2.Total of flavonoids content : 0.5 ml of 2% aluminium chloride in methanol was mixed with the same volume of algal extract. After 1 hour incubation at room temperature, the absorbance of the mixtures was measured at 415 nm. Rutin in the range of 20–200 mg/L was used to construct a calibration curve. Estimation of the total flavonoids was carried out in triplicate.

3.2.3.3.Total of tannins content : Briefly, 50 µl of algal extract were mixed with 1.5 ml of 40% vanillin (prepared with methanol), and then 750 µl of HCl were added. The solution was shaken vigorously and left to stand at room temperature for 20 min in

darkness. The absorbance of the mixtures was measured at 500 nm. Catechin in the range of 20–200 mg/L was used to calibration curve**.** Total tannin content was expressed as mg tannic acid equivalents (TE) per gram of dry weigh**.**

3.2.3.4.Total of terpenoids content: Powder form of 10 g of each extract was soaked in alcohol for a day. Later on it was filtered and petroleum ether was use for purpose of extraction. The extracted material was calculated and considered as terpenoids.

3.2.3.5.Total of alkaloids content : The algae extracts (1 mg) was dissolved in methanol, 1 ml of 2 N HCl was added and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3, and 4 ml chloroform by vigorous shaking and collected in a 10 ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (20, 40, 60, 80 and 100 μg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm. The total alkaloid content was expressed as mg of AE/g of extract.

3.2.3.6.Total phlorotannins content: One milliliter algae extracts (100 mg/mL) was mixed with 5 mL of Folin-Ciocalteu reagent (10% in distilled water). After 5 min, 4 mL of sodium carbonate (7.5% in distilled water) was added. The samples were incubated for 2 h at room temperature in the dark. The absorbance was measured at 725 nm. Total Phlorotannin content of the extracts was expressed as milligrams of phloroglucinol equivalent per gram of extract (mg PgE /g).

3.2.3.7.Total steroids content: 1ml of methanolic extract of steroid solution was transferred into 10 ml volumetric flasks. Sulfuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyano ferrate solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70±20 °C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm ,against the reagent blank.

3.2.3.8.Total coumarins content :500 μl of algae extracts, 2 ml distilled water and 500 μl of lead acetate (5%, w/v) solution were added in a test tube. After shaking thoroughly, 7 ml of distilled water was added and mixing well, 2 ml of this solution was taken in another test tube and 8 ml of 0.1 M (v/v) hydrochloric acid solution was added. The solution was kept for 30 minutes at room temperature and absorbance was recorded at 320 nm. The total coumarin content was expressed as mg of coumarin equivalents per gm of sample extract (mg CE/g).

3.2.4. Anti-Hemolytic Activity

The Anti-hemolytic activity of algal extract was evaluated against human red blood cells (RBCs) from healthy, non-smoking volunteers (Kalaivani *et al.,* 2010). RBCs were centrifuged at 3000 rpm for 10 min, washed, and re-suspended in saline. Algal extracts at concentrations of 62.5, 125, 250, 500, µg/mL were mixed with 1mL RBCs to obtain a 2 mL 4% erythrocyte, and were incubated at 37°C for 1 h. Cultures were centrifuged, and the absorbance of the supernatants (released hemoglobin) was measured at 414 nm. Zero and 100% hemolysis was determined in normal saline and 0.1% Triton X-100, respectively, using an ultraviolet–visible spectrophotometer (PerkinElmer, LAMBDA 25, USA). The negative control was Aspirin and the extract's hemolytic effect was calculated using the following formula, and the result was expressed as percentage hemolysis.

$$\% \text{ Hemolysis} = \times 100 \quad \frac{\text{Abs414 nm of sample solution} - \text{Abs414 nm of saline}}{\text{Abs414 nm of 0.1\% Triton X-100} - \text{Abs414 nm of saline}}$$

where Abs control is the absorbance without algal material and Abs sample is the absorbance of algal extract or standard**.**

3.2.5. Antibacterial assay

The antibacterial activity of two algae crud extracts (*D. dichotoma* and *P. fascia*) against the five tested MRSA strains, (S119, S120, S121, S130, S283), was conducted *in vitro,* by using "hole-plate diffusion method" (Irshad*etal*,, 2012) Extract at concentration of 250 mg/ml was loaded onto wells in triplicate and incubated at 37C° for 18 h. Diameters of inhibition zones (DIZ) were measured in mm and the results were recorded as the mean of triplicate experiments. Methanol was used as negative control, Amoxicillin (AML) (40µg) and amoxicillin/clavulanic acid (AMC) (Augmentin,120µg) were used as reference.

3.2.5.1. Determination of Minimum Inhibitory Concentration (MIC) and Minimum bactericidal concentration (MBC)

The MIC was conducted by using well agar diffusion method (Natrah.,*et al.*, 2015). Different concentrations of algae extracts ranging from 200 to 30 mg/ml, diluted in methanol and were inoculated with tested MRSA strains(1.5×10^6 CFU/ml) and incubated at 37C° for 18 h. After the incubation the lowest concentration that

completely inhibits the growth of tested bacterium within 24 h was considered as MIC. While the MBC of algal tested extract were measured by two fold micro-dilution agar method, (Alghazeer *et al.*, 2017). Bacterial strains were cultured in Mueller–Hinton broth (MHB) to reach approximately (1.5×10^6 CFU/ml). Serial dilution of the extract was prepared using Mueller-Hinton (M-H) broth and used as diluents to achieve a decreasing concentration ranging from 200 to 30 mg/mL and was inoculated with equal volume of bacterial suspension. All tubes were incubated at 37^0C for 18 h. The lowest concentration without any visible growth was taken as the MBC. Each test was repeated three times in a separate time.

3.2.6. Combination effect assays

3.2.6.1. Antibiotics and extract preparation

The stock solutions of the test materials were prepared by dissolving the methanol extract of P. fascia in absolute methanol to a final concentration of 125 mg/ml into Dimethyl sulfoxide (DMSO) 0.01% while the antibiotics AML and AMC were dissolved in Dimethyl sulfoxide (DMSO) 0.01% to a final concentration of 40 ug/ml and 120ug/ ml, respectively which was used for the assay. The working solutions of the tested extract and the antibiotic were prepared by calculating the MIC for each one of the stock solutions. The potentiation action of the extract- antibiotic combinations used for the treatment of staphylococcal isolates was investigated. The following extract-antibiotic combinations were used: C1; extract: antibiotic (1:1), C2: extract: antibiotic (1:2), C3: extract :antibiotic (2:1).

3.2.6.2. Potentiation of antibiotic activity

To evaluate the capability of the *P. fascia* and *D.dichotoma* extracts to potentiate the effects of antibiotic against the MRSA strains, two methods were used; Antimicrobial Susceptibility Test, and Time-kill growth rate (%).

3.2.6.2.1. Antimicrobial Susceptibility

Combination effect of the tested algae extracts (*D. dichotoma* and *P. fascia*) with antibiotic (Amoxicillin) against MIRSA strains, were determined by depending absorption measurements using spectrophotometer (Mabhiza *et al.*, 2015). After culturing bacteria on Mueller-Hinton broth, the tested algae extracts was added as alone to each tube containing bacterial suspension of each tested strain to reach the effective

concentration of extract(250mg/ml). Also, a tested antibiotic was added in the same way to reach the effective concentration of each antibiotic 40µg/ml to Amoxicillin. In addition to that, the combination between algal extracts with Amoxicillin was conducted in a ratio of (1:1/1:2/2:1) for each treatment. Then the tested samples were incubated for 18h at 37 ℃ with shaking at 115 rpm. Bacterial strains without treatment was determined as a positive control. Cell growth was monitored by measurement of the optical density at 600 nm.

3.2.6.2.2. Time-kill growth assay

Bacterial culture for viable count assay was prepared in MHB medium. Freshly prepared bacterial suspension (6×10^8 CFU/mL) was added to each sample to reach MIC and incubated at 37 °C under agitation condition, each bacterial suspension was inoculated with MHB only as negative control. Samples for viable cell counts were carried out at 0, 40, 80, 120 and 160 min time intervals. After incubation, the culture was 10-fold diluted and spread on Mueller–Hinton agar (MHA). Colony counting was performed after incubation. Each assay was performed in triplicate, (Wang *et al.*, 2019).

3.2.7. Mechanism of action:

3.2.7.1. Measurement of Nucleotide Leakage

To evaluate the effect of samples on the damaged cell membrane by measuring the leakages of intracellular components such as nucleotides. Nucleotide leakage was assessed according to the method by (Silva ja *et al*., 2014). The bacterial suspension at the logarithmic growth phase (6×10^8 CFU/mL) was incubated with extract (1MIC) alone and Phosphate Buffer Saline (PBS) pH 7.9 as a negative control, as well as antibiotic and mixtures samples was incubated at 37 °C at different time durations. Cells were then centrifuged, and the obtained supernatants were diluted. The absorbance of supernatants was determined at 260 nm using an ultraviolet–visible spectrophotometer (PerkinElmer, LAMBDA 25, USA).

3.2.7.2.Potassium efflux

The amount of potassium ions released from bacterial was measured using the method as described by (Lou *et al.*, 2011). The MRSA tested strains were refreshed for 18h at 37 ℃ , then strains were washed and resuspended in (PBS) pH 7.9. Isolated strains (6×10^8 CFU/mL) were cultured with extract (2MIC) alone and (PBS) pH 7.9 as a negative control solvent (negative control), antibiotic and mixture samples at 37 °C,

were incubated at different time durations. The potassium ion concentration of the supernatant was measured using a flame photometer (BMW Technologies, UK).

3.2.8.Statistical Analysis

Data were expressed as mean ± standard deviation (SD) of triplicate determination. All statistical analyses were performed using SPSS v.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). Statistical difference between extract activities and effect of combination were determined using one-way analysis of variance(ANOVA) . Difference were considered statistically significant at $P < 0.05$.

4. Results

4.1. Taxonomic of tested algae:

4.1.1. *Petalonia fascia* (O.F.Müller) Kuntze (1898)

Empire: Eukaryota; Kingdom: Chromista; Phylum: Ochrophyta; Class: Phaeophyceae ;Subclass: Fucophycidae; Order: Ectocarpales; Familly: Scytosiphonaceae; Genus: Petalonia; *Petalonia fascia*.

Figur5: *Petalonia fascia* (O.F.Müller) Kuntze

Description: Plants with olive or yellow-brown fronds to 300mm long and 15-20 mm wide. Narrows to short stalk and attached by small disc. Generally growing on stones and shells in shallow pools in sheltered places, particularly in harbours.

4.1.2. *Dictyota dichotoma* (Hudson) J.V.Lamouroux 1809

Empire: Eukaryota; Kingdom: Chromista; Phylum: Ochrophyta; Class: Phaeophyceae; Subclass: Dictyotophycidae; Order: Dictyotales; Familly: Dictyotacae; Genus: *Dictyota*; *Dictyota dichotoma.*

Figure6: *Dictyota dichotoma* (Hudson) J.V.Lamouroux

Description: Thallus flat with fairly regular dichotomous branches with parallel sides to 30 cm long, the tips usually bifid. Outer layer of small cells enclosing a single layer of large cells no more than one cell thick even near the base. Branches 3 to 12 mm wide, membranous without a midrib.

4.2. Phytochemical analysis

Results of phytochemical analysis are showed in Table 2. In the current study, the methanol crude extract of *D.dichotoma* and *P.fascia* showed different results to most important phytoconstituents in both tested algae, The results indicated presence of flavonoids and steroids in higher rates in both algae (+++), while proteins and anthraquinones were absent in both tested algae (-).In contrast, the results indicated that saponins were present at good ratios in *P.fascia* (++), while in *D.dichotoma* was absent.

Table 2: Phytochemical screening results of *Dictyota dichotoma* and *Petalonia fascia*

Phytochemical	Methanolic extracts	
	D. dichotoma	***P. fascia***
Alkaloids	++	++
Anthraquinones	-	-
Carbohydrates	+++	++
Coumarins	+++	++
Flavonoids	+++	+++

Lipids	++	+
Proteins & amino acids	-	-
Quinones	-	-
Saponins	-	++
Steroids	+++	+++
Tannins	++	++
Terpenoids	+++	-

(+++) = Copiously present, (++) = Moderately present, (+)= Slightly present (-)= Absent.

4.2.1. Quantitative phytochemical screening

Total phenol, tannin, phlorotannin, flavonoids, alkaloids, steroids, and coumarins content of crude methanol extract of *P. fascia* and *D. dichotoma* are presented in table 3. From The results, phenols were the highest content (1367.44±17.51 **mg GAE/g dw**) for *P. fascia* and(1176.89±25.89 **mg GAE/g dw**) for *D. dichotoma* followed by phlorotannin, (357.60±38.44 **mg PhgE/g dw**), and (292.46 ±40.24 **mg PhgE/g dw**) respectively , whereas alkaloids (1.55±0.08 **mg AE/g dw**), and (2.02 ±0.05**mg AE/g dw**) respectively, were the lowest in content for extracts.

Table3: Mean total of some selected phytochemicals content of *Dictyota dichotoma and Petalonia fascia*

phytochemicals		methanolic extracts	
		D. dichotoma	***P. fascia***
Polyphenols	**mg GAE/g dw**	**1176.89±25.89**	**1367.44±17.51**
Tannins	**mg TAE/g dw**	**7.31±0.54**	**14.42±0.60**
Phlorotannins	**mg PhgE/g dw**	**292.46 ±40.24**	**357.60±38.44**
Flavonoids	**mg RE/g dw**	**233.86±4.64**	**113.22±0.69**
Alkaloids	**mg AE/g dw**	**2.02±0.05**	**1.55±0.08**
Steroids	**mg SE/g dw**	**88.40±10.37**	**89.06±7.87**
Coumarin	**mg CE/g dw**	**14.74±0.20**	**10.87±0.13**

mgGAE/gdw= milligrams of Gallic acid equivalent per gram dry weight; Phg= Phloroglucinol, R = Rutin, A = Atropine, E = Estrone, C: Coumarin; Data are presented as mean value ± standard deviation (SD) of triplicate readings (n = 3).

4.3. Anti-hemolytic activity

Extracts were tested for toxic effects on human RBCs (Table 4). No toxicity effect was found for the extract to the concentrations tested for antimicrobial activity. Although there was an increase in the hemolytic activity as the concentration increased as well as the hemolysis activity of extract was higher than the negative control, the results were still considered at the safe level.

Table 4: Anti-hemolytic activity of *Dictyota dichotoma* and *Petalonia fascia* methanol extracts against human erythrocytes (HRBCs)

Concentration of extract (µg/ml)	Hemolysis activity (%)		
	D. dichotoma	*P. fascia*	Aspirin
62.5	1.67 ± 0.04	1.74 ± 0.05	0.57 ± 0.03
125	2.38 ± 0.07	2.68 ± 0.08	0.88 ± 0.02
250	2.94 ± 0.02	4.73 ± 0.25	1.86 ± 0.01
500	5.09 ± 0.02	5.84 ± 0.01	2.87 ± 0.10

Data are presented as mean value ± standard deviation (SD) of triplicate readings (n = 3).

4.4. Antibacterial activity

In the primary antibacterial screening of both *D.dichotoma* and *P.fascia* methanol extracts were active against all assayed strains, with a slight preference for *P.fascia* extract, with diameter of inhibition zone ranged from 16-21mm, compared to *D.dichotoma* extract, which exhibited an inhibition zone ranged from 13-15mm (Table 5). The zone of inhibition to the extracts against assayed strains, was lower compared to positive control except *S. aureus* 130 wherein the inhibition zone diameter was greater than positive control (Augmentin). The maximum inhibition zone of 21 mm was observed from *P.fascia* extract against *S. aureus* 283.

Table 5: Diameters of inhibition zones (mm) in primary screening of *Dictyota dichotoma and Petalonia fascia* extract against five isolates of *S. aureus* by hole-plate diffusion method.

Isolates	*D.dichotoma*	*P. fascia*	*Augmentin*	*Amoxicillin*
S119	15.0 ± 1.0	18 ± 1.00	19	7
S120	15.5± 1.00	18.5 ± 1.0	22	8
S121	17.0±1.00	16.0±0.00	21	7
S130	16.0 ± 1.00	19 ± 0.00	14	-

S283	**13.0±0.00**	**21.0±0.00**	**32**	**20**

DIZ diameter of inhibition zone. Data are presented as the mean± SD, *n*=3; diameter of hole =6mm

Table 6 and Table 7 shows the values of minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) of *P. fascia* and *D.dihotoma* extracts tested on five strains of *S. aureus*, the extract showed the same MIC and MBC (50 mg/ml and 100 mg/ml) respectively, except their values with *P. fascia* against S120, which showed a higher MIC and MBC of 62.5 and 187.5 mg/ml respectively. Algal extracts also displayed the best spectrum of bactericidal effect with a ratio MBC/MIC <4 obtained on five tested bacterial strains (Table 6&7).

Table 6: Minimum inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and MBC/ MIC ratio of *Petalonia fascia* extract against tested bacterial strains

Isolates	MIC*	MBC*	MBC/MIC
S119	50	100	2
S120	62.5	187.5	3
S121	50	100	2
S130	50	100	2
S283	50	100	2

*: mg/ml

Table 7: Minimal inhibitory concentration (MIC), minimal bactericidal concentrion (MBC) and MBC/ MIC ratio of *Dictyota dihotoma* extract against tested bacterial strains.

Isolates	MIC*	MBC*	MBC/MIC
S119	50	100	2
S120	50	100	2
S121	50	100	2
S130	50	100	2
S283	50	100	2

*: mg/ml

4.5. Antibacterial activity of the three different combinations between tested algae extracts and Amoxicillin

Assays strains were sensitive to Augmentin and resistant to Amoxicillin, so amoxicillin was used to study the ability of extract to modulate its activity. To achieve that the algal extract was mixed with amoxicillin in the three different combination

between *P.fascia* and amoxicillin ratios of 1:1 (C1), 1:2 (C2) and 2:1 (C3) as well as between *D.dichotoma* and amoxicillin ratios of 1:1 (C4), 1:2 (C5) and 2:1 (C6) that were incubated with assayed strains for 18 hr. At the end of the incu- bation period, bacterial growth was completely inhibited as absorption of each sample decreased dramatically indicating reduced bacterial growth, due to the effect of algal extract enhancing the antibacterial activity of Amoxicillin (Figure 7,8).

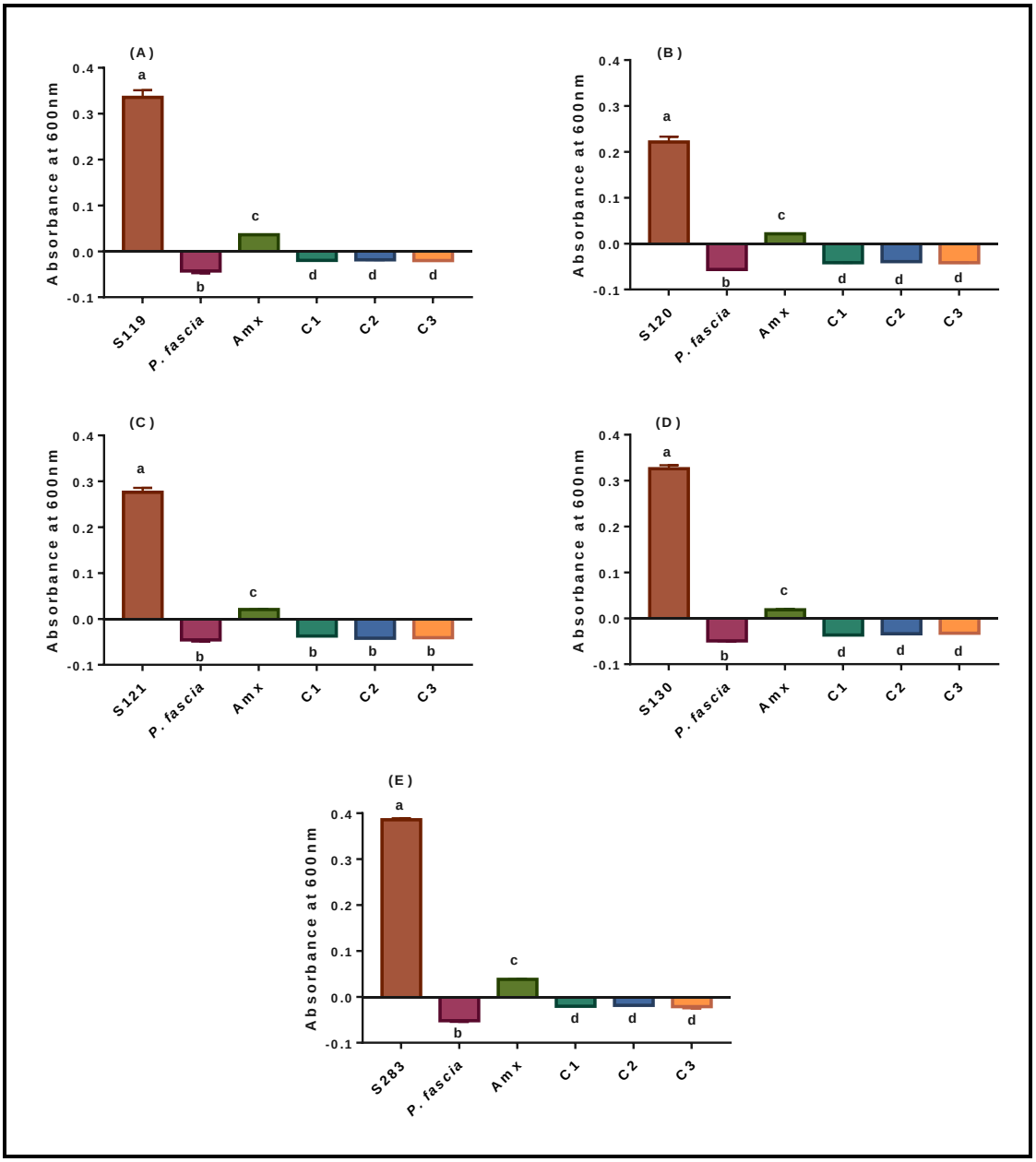

Figure7: Antibiotic modifying activity of the *P. fascia* extract in combination with amoxicillin beta-lactam antibiotic. Amx:(Amoxicillin), P: *P. fascia*, C1: (P: Amx; 1:1), C2: (P: Amx; 1:2), C3: (P: Amx; 2:1); Each value is represented as mean ±SD (n=3) , Values followed by different letters were significantly different according to tukey multiple comparison test post hoc test at ,($P < 0.05$).

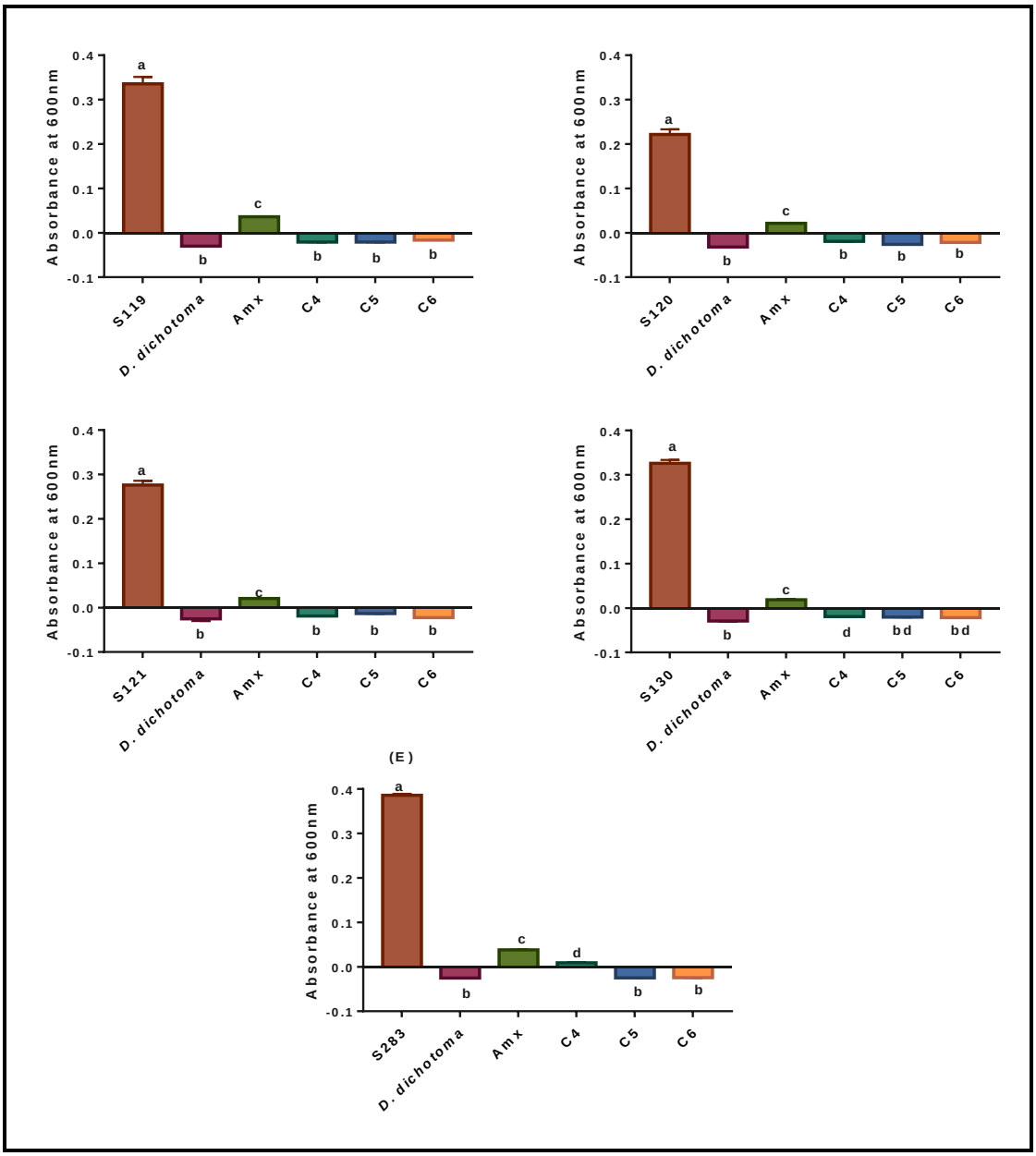

Figure8: Antibiotic modifying activity of the *Dictyota dichotoma* extract in combination with amoxicillin beta-lactam antibiotic. Amx:(Amoxicillin), D: *D. dichotoma*, C4: (D: Amx; 1:1), C5: (D: Amx; 1:2), C6: (D: Amx; 2:1); Each value is represented as mean ±SD (n=3) , Values followed by different letters were significantly different according to tukey multiple comparison test post hoc test at ($P < 0.05$) .

4.6. Time-kill assay

In order to confirm the modulation of antibiotic activity when being combined with tested algae extracts, the C1, C2, C3, C4, C5 and C6 combinations were subjected to time-kill assay (Table 8,9). Results of viable bacteria population (%) for *S. aureus* strains treated with algal extract alone, amoxicillin alone, and combination extract-amoxicillin in different ratios within 160 min are tabulated in tables 7&8. Generally, algal extract alone and its combinations, (C1, C2, and C3) for *P.fascia* with amoxicillin as well as its combinations, (C4, C5 and C6) for *D.dichotoma* with amoxicillin, showed a stronger inhibitory activity on the five tested MRSA strains, and the effect was in a time-dependent manner. A positive effect on the antibacterial activity of amoxicillin was observed when it has combined with algal extract at different ratios indicating the ability of the extract to modulate the antibiotic activity. Compared to amoxicillin, C1 and C3, significantly enhanced the amoxicillin activity as the percent of viable bacterial population reached 0% after 80 min incubation time against three of the tested strains (S120, S121 and S130), while C2 showed a slower decrease in the population of viable bacteria wherein the rate of viable population reach between 4-1% at 160 min of incubation time. On the other hand C4 and C6 significantly enhanced the amoxicillin activity as the percent of viable bacteria population reach 0% after 80 min incubation time against all assayed strains, compared C5 was viable bacteria population reach 0% after 120 min.

Table 8: Time-kill growth rate (%) of combination of *Petalonia fascia* crude extract with amoxicillin and amoxicillin alone against MRSA strains.

MRSA strains	Time (min)				
	0	40	80	120	160
S119					
P. fascia	17.24±2.00	000.0±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Amx	88.50±2.00	65.63±3.21	74.00±4.00	74.23±3.78	80.13±4.00
C1	48.00±1.00	17.6±1.00	11.15±0.58	0.00±0.00	0.00±0.00
C2	20.94±2.52	12.14±2.00	14.87±2.52	2.57±0.58	3.1±1.15
C3	27.58±2.00	4.8±1.15	4.59±1.00	5.28±1.53	1.1±1.00
S120					
P. fascia	8.00±2.52	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Amx	66.34±3.00	59.3±2.52	65.38±2.00	65.4±2.00	68.28±2.00
C1	33.65±2.00	8.65±2.00	1.00±1.00	0.00±0.00	0.00±0.00

C2	41.02±1.53	4.80±1.00	3.84±1.00	5.12±0.58	4.80±1.00
C3	50.00±1.53	13.77±1.53	7.69±1.00	0.00±0.00	0.00±0.00
S121					
P. fascia	15.44±1.53	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Amx	64.88±4.00	88.84±2.00	90.33±2.08	88.37±2.52	68.52±2.52
C1	44.79±2.52	14.67±2.08	3.47±2.00	0.00±0.00	0.00±0.00
C2	47.1±3.51	8.1±2.00	7.78±1.53	4.99±2.08	2.31±1.00
C3	30.89±2.52	10.42±2.00	3.85±1.53	0.00±0.00	0.00±0.00
S130					
P. fascia	6.97±2.52	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Amx	67.00±2.00	65.67±3.06	58.67±1.53	70.67±1.53	0.00±0.00
C1	52.6±1.53	9.63±1.53	6.97±1.53	0.00±0.00	0.00±0.00
C2	51.88±2.00	5.18±2.08	7.18±0.58	7.33±1.53	3.33±1.00
C3	50.00±2.00	9.63±2.08	5.18±1.53	0.00±0.00	0.00±0.00
S283					
P. fascia	33.33±2.52	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Amx	63.51±3.00	61.15±1.53	64.91±2.52	62.9±2.00	57.22±3.51
C1	43.56±2.53	24.66±2.08	5.77±1.53	6.29±1.00	2.33±2.00
C2	47.67±1.52	14.07±2.00	8.1±1.53	5.97±1.15	3.3±1.00
C3	38.32±1.53	6.67±2.08	0.00±0.00	0.00±0.00	0.00±0.00

Amx:(Amoxicillin), P: *P. fascia*, C1: (P: Amx; 1:1), C2: (P: Amx; 1:2), C3: (P: Amx; 2:1); Each value is represented as mean ±SD (n=3).

Table 9: Time-kill growth rate (%) of combination of *Dictyota dichotoma* crude extract with amoxicillin and amoxicillin alone against MRSA strains.

MRSA strains	Time (min)				
	Zero	40	80	120	160
S119					
D. dichotoma	28.8±1.00	5.5±1.53	0.00±0.00	0.00±0.00	0.00±0.00
Amx	77.02±2.00	65.7±3.21	87.5±4.00	75.1±3.78	80.24±4.00
C4	43.68±1.00	4.1±1.00	0.00±0.00	0.00±0.00	0.00±0.00
C5	36.8±3.00	6.2±1.00	11.7±2.00	0.00±0.00	0.00±0.00
C6	35.7±3.61	12.3±2.00	0.00±0.00	0.00±0.00	0.00±0.00
S120					
D. dichotoma	37.5±2.00	5.6±1.00	0.00±0.00	0.00±0.00	0.00±0.00
Amx	66.35±3.00	57.72±2.52	61.27±2.00	71.6±2.00	68.27±2.00

C4	31.74±2.00	5.0±1.53	0.00±0.00	0.00±0.00	0.00±0.00
C5	**37.5±1.00**	**7.41±2.00**	**1.9±2.00**	**0.00±0.00**	**0.00±0.00**
C6	**27.00±1.00**	**12.32±1.53**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
S121					
D. dichotoma	**23.17±1.53**	**2.33±1.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
Amx	**92.41±4.00**	**88.42±2.00**	**91.05±2.08**	**74.55±2.52**	**64.5±2.52**
C4	**52.66±3.06**	**5.82±2.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
C5	**51.39±1.00**	**7.68±1.53**	**3.5±3.05**	**0.00±0.00**	**0.00±0.00**
C6	**55.00±2.00**	**22.1±1.53**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
S130					
D. dichotoma	**24.00±4.58**	**3.4±2.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
Amx	**67.00±2.00**	**65.67±3.06**	**58.67±1.53**	**70.67±1.53**	**63.6±0.00**
C4	**50.32±1.15**	**6.67±2.00**	**6.46±2.00**	**0.00±0.00**	**0.00±0.00**
C5	**28.75±1.53**	**7.78±4.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
C6	**35.42±2.00**	**3.4±1.53**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
S283					
D. dichotoma	**36.4±2.00**	**7.5±2.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
Amx	**63.51±3.00**	**61.15±1.53**	**64.91±2.52**	**62.9±2.00**	**57.22±3.51**
C4	**36.4±2.52**	**5.41±1.53**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
C5	**42.40±2.52**	**11.12±0.00**	**5.9±1.53**	**0.00±0.00**	**0.00±0.00**
C6	**36.39±1.53**	**6.75±1.53**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**

Amx:(Amoxicillin), D: *D. dichotoma*, C4: (D: Amx; 1:1), C5: (D: Amx; 1:2), C6: (D: Amx; 2:1); Each value is represented as mean ±SD (n=3).

4.7.Mode of action

4.7.1. Potassium efflux

Figure(9,10) demonstrated the potassium ion (K^+) concentration when MRSA strains were treated with different concentrations of tested algae extracts with amoxicillin from 3 h to 12 h. The osmotic effect of these extracts as alone appeared in a strong and effective way against all the tested strains since the beginning of the three hours after treatment time. *P.fascia* extract had a marked advantage in terms of effect on all tested strains. The results indicated that after treating bacterial strains with tested

algae extracts ,*P.fascia* combined with Amoxicillin (C1, C2 andC3) and *D.dichotoma* combined with amoxicillin (C4,C5and C6), the release of K^+ ions increased significantly compared amoxicillin alone and was extract concentration dependent , while no noticeable changes in the level of K^+ in the control sample.

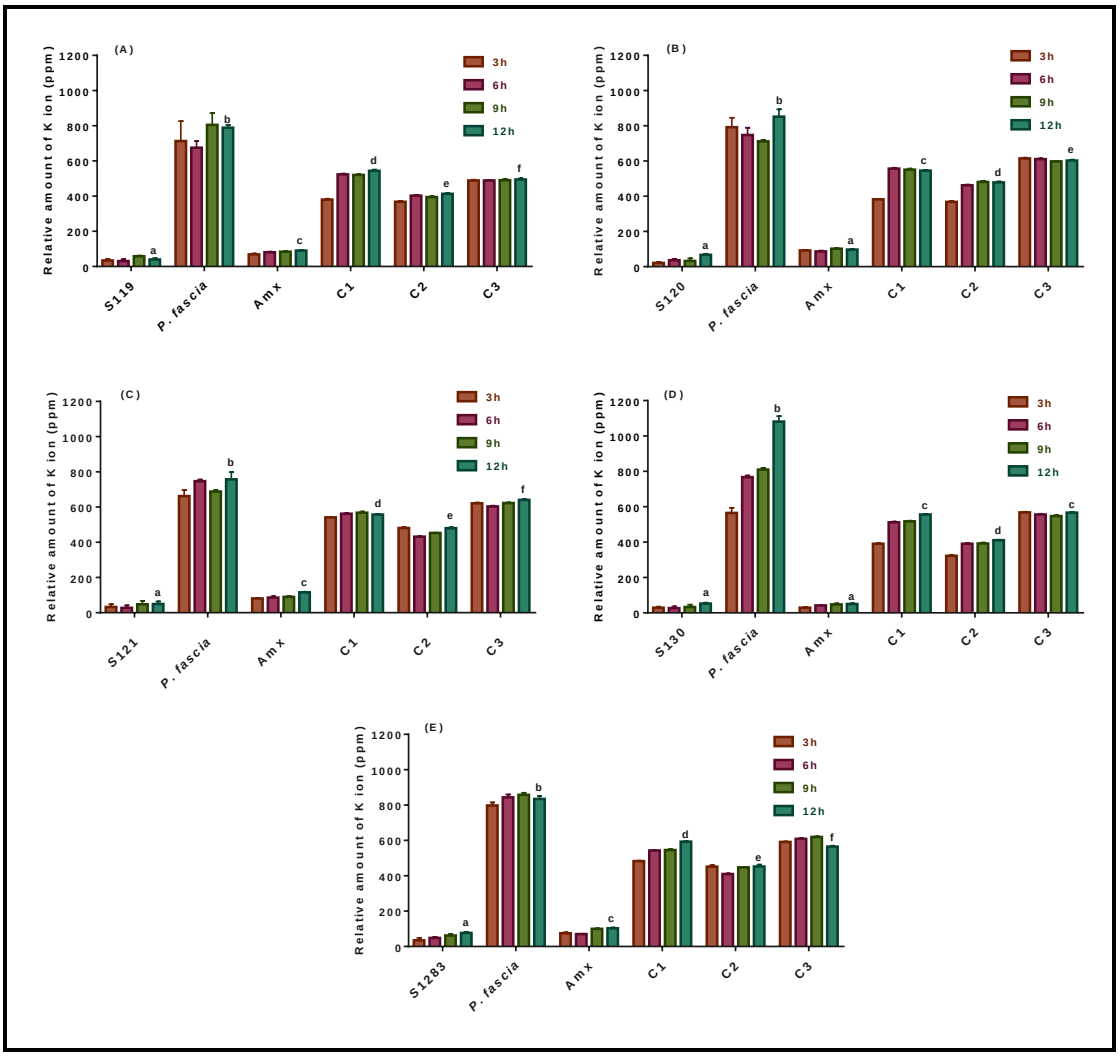

Figure 9: Effect of minimum inhibitory concentration (MIC) of ***P. fascia*** extract and its combination with amoxicillin on the amount of potassium ions (K^+) released from ***S. aureus*** strains. **A**: *S. aureus* 119; **B**: *S. aureus* 120; **C**: *S. aureus* 121; **D**: *S. aureus* 130 and **E**:*S. aureus* 283. Amx:(Amoxicillin), P: *P. fascia*, **C1**: (P: Amx; 1:1), **C2**: (P: Amx; 1:2), **C3**: (P: Amx; 2:1); Each value is represented as mean ±SD (n=3).

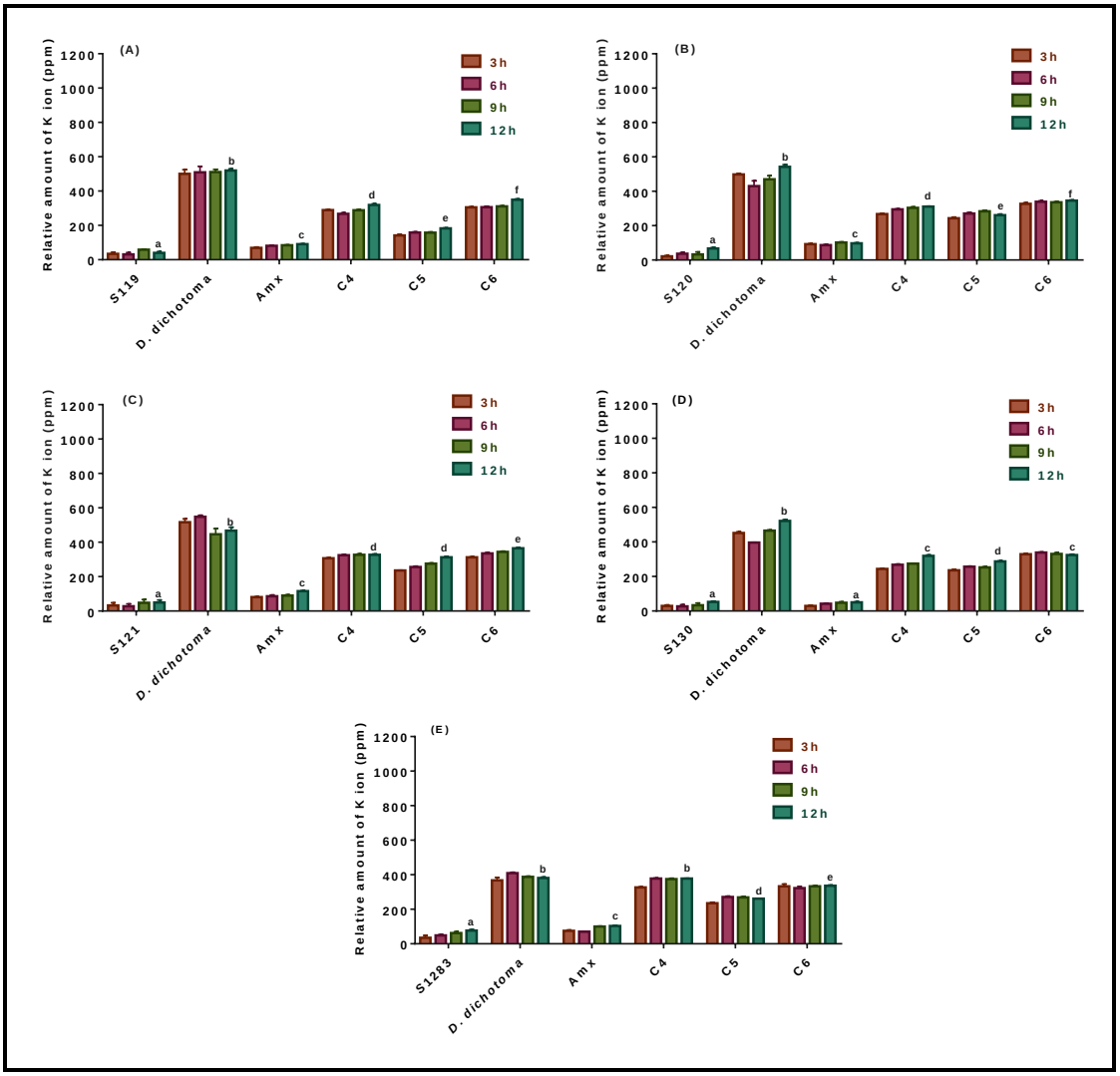

Figure10: Effect of minimum inhibitory concentration (MIC) of ***D. dichotoma*** extract and its combination with amoxicillin on the amount of potassium ions (K^+) released from ***S. aureus*** strains. **A**: *S. aureus* 119; **B**: *S. aureus* 120; **C**: *S. aureus* 121; **D**: *S. aureus* 130 and **E**:*S. aureus* 283. Amx:(Amoxicillin), D: *D. dichotoma*, **C4**: (D: Amx; 1:1), **C5**: (D: Amx; 1:2), **C6**: (D: Amx; 2:1); Each value is represented as mean ±SD (n=3).

4.7.2. Nucleotide leakage:

Measuring of UV absorption value at time intervals can reflect the degree of cell membrane damage. Figures (11,12) exhibit an increase in the level of the intracellular constituents, including DNA, released after the incubation of tested algae extracts with tested MRSA strains as alone and as a combination with amoxicillin in three different concentration and amoxicillin with tested MRSA strains for 3, 6, 9 and 12 hr. Tested MRSA strains incubated with tested algae extracts (*P. fascia* or *D.dichotoma*) as alone showed higher nucleotides released than the sample that MRSA strains incubated with amoxicillin as alone. On the other hand the combined effect in three different concentration, showed enhance in constituents nucleotides released compared amoxicillin as alone, while no obvious changes in the OD_{260} values of control.

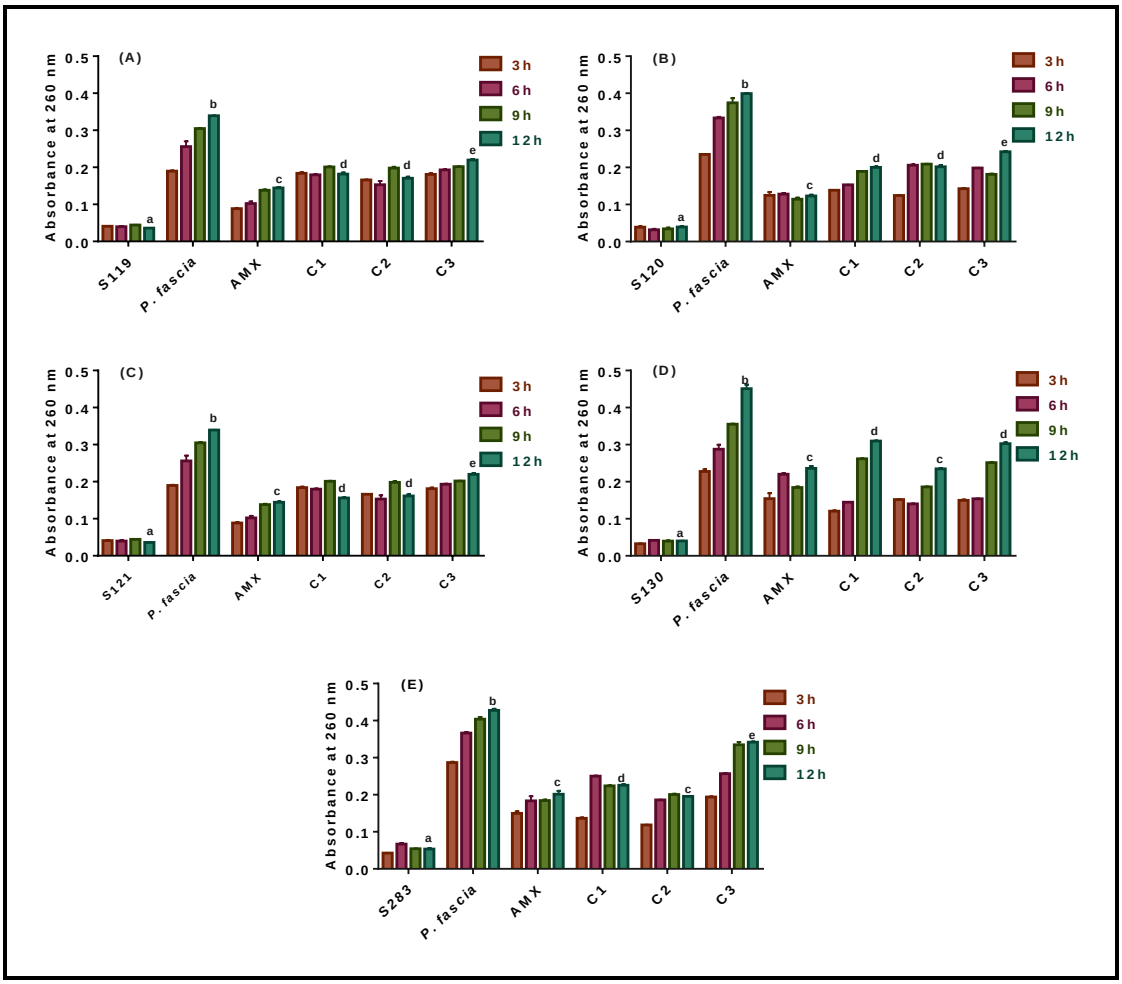

Figure 11: Effect of minimum inhibitory concentration (MIC) of ***P. fascia*** extract and its combination with amoxicillin on the amount of total nucleotide released from ***S aureus*** strains. **A:** *S. aureus* 119; **B:** *S. aureus* 120; **C:** *S. aureus* 121; and **D:** *S. aureus* 130 and **E:***S. aureus* 283. Amx:(Amoxicillin), P: *P. fascia*, C1: (P: Amx; 1:1), C2: (P: Amx; 1:2), C3: (P: Amx; 2:1); Each value is represented as mean ±SD (n=3).

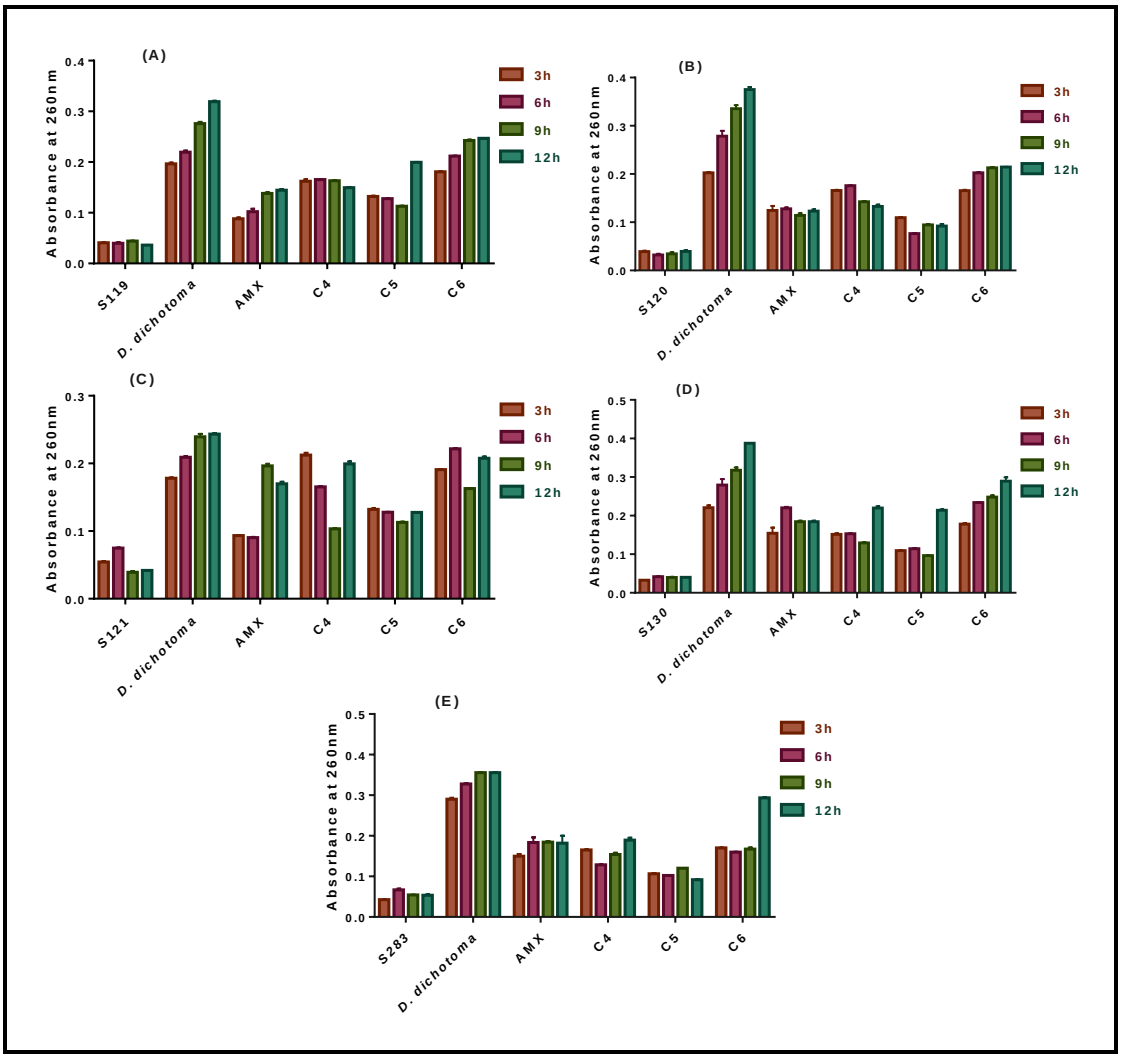

Figure 12: Effect of minimum inhibitory concentration (MIC) of ***Dictyota dichotoma*** extract and its combination with amoxicillin on the amount of total nucleotide released from ***S aureus*** strains. **A:** *S. aureus* 119; **B:** *S. aureus* 120; **C:** *S. aureus* 121; and **D:** *S. aureus* 130 and **E:***S. aureus* 283. Amx:(Amoxicillin), D: *D. dichotoma*, C4: (D: Amx; 1:1), C5: (D: Amx; 1:2), C6: (D: Amx; 2:1); Each value is represented as mean ±SD (n=3).

5. Discussion

The wide use of antibiotics in the treatment of bacterial infections have led to the emergence and spread of resistant strains. Methicillin-resistant *S. aureus* (MRSA) is a major cause of nosocomial infections. MRSA infections are very difficult to cure because MRSA strains are resistant against almost all clinically available antibiotics. As a result, there is an urgent need to develop anti-MRSA agents with novel mechanisms of action. Treatment with antibacterial combinations, using two or more antibacterial agents is one of the most important strategies to overcome multidrug-resistant organism. Potentially harmful side effects associated with use of new chemical entities artificially synthesized and the unsustainably high costs of drug development are slowly shifting the focus to plant derived phytochemicals of medicinal significance (Adwan and Mhanna, 2008; Haroun and Al-Kayali, 2016).

Seaweeds including macroalgae are a rich source of nutrients, but they are also an important source of different kinds of bioactive substances, including sulphated polysaccharides, carotenoid pigments, and phlorotannins, with potential health benefits. Some compounds of algae considered to contain broad spectrum of chemi- cals and biological activities, including antioxidant and free radical scavenging properties, antibacterial, antiviral, anticancer, anti-inflammatory and anti-allergic. Therefore active constituents of various algae extracts could be potential bioactive compounds of interest in the pharmaceutical industry (Alghazeer *et al*., 2017; Corsetto *et al*., 2020).

During the past ten years, several reviews substantiated the effectiveness of combinations of plants with conventional antimicrobials, but using active extracts of algae as a synergic to antibiotics, studies are still very few (Haroun and Al-Kayali, 2016).

In the present study the phytochemical analysis of *D. dichotoma* and *P. fascia* were performed and the results were tabulated according to the presence or absence of the phytochemicals in the algae. Preliminary phytochemical screening of tested algae showed the presence of flavonoids, tannins, steroids, coumarins, carbohydrates and alkaloids with considerable amount in both of tested algae, while quinones, anthraquinones, proteins and amino acids were absent in both of them. The results indicated the presence of terpenoids in higher rates in *D. dichotoma* compared to *P.*

fascia which was absent**.** The presence such of bioactive phytochemicals as reported in this study indicates a wide range of bioactivity applications of the tested algae extracts. These results are somewhat in agreement with previous studies that found of these phytochemicals in brown algae from the same area (Alghazeer *et al*., 2013).

5.1. Anti-hemolytic activity

Limitation of extracts to be used in therapy is their potential to cause damage to mammalian cells (Somaida *et al*.,2020). The hemolytic activity, of crude extract of *D. dichotoma* and *P. fascia* against normal human erythrocytes were examined. Hemolytic activity of crude extract is expressed in % hemolysis methanolic extracts. The results showed weak hemolytic activity was detected for both of *D. dichotoma* and *P. fascia* extracts, even at the highest applied concentrations (500µg/ml), being 5.09 % and 5.84 %, respectively. The hemolytic activity of both tested extracts was higher than the positive control, but still considered at the safe level. These results are broadly consistent with the results of a previous study by Chander *et al*., (2014).

5.2. Antibacterial activity

Antibiotics provide the main basis for the therapy of bacterial infections. However, the high genetic variability of bacteria enables them to rapidly evade the action of antibiotics by developing antibiotic resistance (Alghazeer *et al*., 2017). Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds, nature still remains the richest and the most versatile source for new antibiotics (Habibi *et al.*, 2018). In the present study, the methanolic crude of *D. dichotoma* and *P. fascia* extracts were tested as antibacterial against five MRSA strains using hole-plate diffusion method. Augmentin was used as a positive control. The results of primary screening test exhibited different antibacterial activities and inhibited all tested bacteria, The maximum biological activities were observed by *P. fascia* against S 283 strain, where the inhibition zone diameter was 21mm while in *D. dichotoma* it was 17mm against S121. Generally the diameter of inhibition zones against most tested strains was lower compared to positive control except S130 strain it was greater than positive control. However, these results are more positive compared to a previous study conducted by Alghazeer *et al*., (2013), where the results of treatment with brown algae extracts studied against *Staphylococcus*

aureus showed inhibition zone ranged between 11-14mm. These results seem logical to some extent, given that the algae extract contains a mixture of compounds that are not necessarily all effective. This opinion was confirmed by a study conducted by Alghazeer *et al.*, (2021). Whereas, The results of treatment of alkaloid extracts of two brown algae against isolates of *S. aureus* (128,122,287), indicated that the inhibition zone ranged between 25-41mm. While another study of flavonoid extracts of two brown algae against same isolates of *S. aureus* (128,122,287), indicated a medium inhibition zone that ranged between 11-20mm (Alghazeer *et al*., 2017).

The minimum concentration necessary to kill an organism should be equal to or greater than the MIC for that microbe. A sample is bactericidal when the ratio MBC/MIC ≤ 4 and bacteriostatic when this ratio is >4. It therefore seems to be that antibacterial effects obtained with the *D. dichotoma* and *P. fascia* extracts against, five MRSA strains proved to have best spectrum of bactericidal effect. These results are parallel with a previous results which suggested that some bioactive compounds of algae extracts are capable of bactericidal activity which resulting mostly from the impairment of the cell wall integrity and to cell agglutination as reported by some studies (Alghazeer *et al*., 2021).

5.3. Effect of extracts on antibiotics activity

The ability of natural bioactive extracts to act synergistically with antibiotics is considered a new approach that helps in solving the problem of bacterial resistance (Haroun and Al-Kayali, 2016). Few studies have found that the efficacy of antibacterial agents can be enhanced by combining them with crude extracts (Adwan and Mhanna, 2008). Consequently, the present study was focused on the synergistic activity of algae crude extracts with antibiotics. Assays MRSA strains were sensitive to Augmentin(120µg), but resistant to amoxicillin(40µg), so amoxicillin was used to study the ability of extract to modulate its activity. The tested algae extracts were mixed with amoxicillin in the three different combination between *P.fascia* and amoxicillin ratios of 1:1 (C1), 1:2 (C2) and 2:1 (C3) as well as between *D.dichotoma* and amoxicillin ratios of 1:1 (C4), 1:2 (C5) and 2:1 (C6) that were incubated with assayed strains for 18 hr. Tested MRSA growth was monitored by measurement of the optical density at 600 nm. Compared with amoxicillin alone, the bacterial growth of all MRSA strains were completely inhibited as absorption of each sample decreased dramatically

indicating reduced bacterial growth, due to the enhancing effect of algal extract on the antibacterial activity of amoxicillin. These findings are consistent with those obtained in previous study by Mi Choi *et al.*, (2015), where the study concluded that Fucoidan (primarily extracted from brown algae), exerted synergistic effects when combination with oxacillin or ampicillin and the antimicrobial effect and resistant regulation of Fucoidan against MRSA. In another study by Ha Lee *et al.,* (2014), synergistic effect of phlorotannins isolated from brown algae *Eisenia bicyclis*, was assessed in combination with commercial antibiotics to treat acne-related bacteria. The results of combinations, suggesting that thereby marked or weak synergy effect. Bactericidal activities of combination of tested algae extracts with amoxicillin and amoxicillin alone against tested MRSA strains were also evaluated using Time-kill growth rate (%). Viable counts were conducted at 0, 40, 80, 120, and 160 min after addition the treatments. Generally, the results showed that combinations exhibit a stronger inhibitory activity on five tested MRSA strains and the effect was in a time-dependent manner. This finding showed enhanced the time effect of tested algae extracts with amoxicillin, compared amoxicillin alone which did not show any effect during time duration of treatment. The closest study that indicates the effect of the combination on the time of effectiveness of the treatment is through Mi Choi *et al.,* (2015) within 3 hours of fucoidan treatment with ampicillin or oxacillin resulted in an increased rate of killing as compared to that observed with fucoidan (MIC) alone.

5.4. Mode of action

Each antimicrobial active compound has its own and definite mode of action against specific microorganism targeting different antimicrobial sites. Inhibit cell wall synthesis and block peptidoglycan essential for cell survive and alteration of the cell membrane, cytoplasm leakage and cell destruction, these are some of the mechanisms of action used by the active compounds against bacteria. The irreversible damage of cytoplasmic membrane can be reflected by the leakage of intracellular materials. Measurement of 260 nm absorbing cellular materials usually serves as a marker of cell leakage and can reflect the membrane integrity change compared to unexposed cells. Small ions like potassium ions are also essential for maintaining proper enzyme

activity and can benefit cell membrane functions. However, the transportation of these small ions have to cross a permeability barrier that formed by bacterial plasma membrane (Wang *et al*., 2019; Silva *et al*., 2020 and Alghazeer *et al*., 2021). In this study, the total nucleotide leakage and potassium ions released from tested MRSA strains were determined to study the mechanism of both tested algae extracts as alone and as a combination with amoxicillin in different concentrations. The osmotic effect of these extracts as alone appeared in a strong and effective way against all the tested strains since the beginning of the three hours after treatment time. The results indicated that after treating bacterial strains with tested algae extracts, *P.fascia* combined with amoxicillin (C1, C2 and C3) and *D.dichotoma* combined with amoxicillin (C4,C5 and C6) the release of K^+ ion increased significantly compared amoxicillin as alone and was extract concentration dependent. On the other hand tested MIRSA strains incubated with tested algae extracts (*P. fascia* or *D.dichotoma*) as alone 3-12h, showed higher nucleotides released than the sample that MIRSA strains incubated with amoxicillin as alone. On the other hand the combined effect in three different concentration, showed enhance in constituents nucleotides released compared amoxicillin as alone . These findings probably suggest two possibilities, the first of which is either a disruption in the cell membrane permeability and leakage of intracellular materials, or a rupture of the cell membrane and cell wall and the exit of all the contents of the cell to the outside environment . These results were supported by several previous studies that indicated that several active compounds of natural extracts have a direct effect on the integrity of the plasma membrane and the regulation of cellular permeability (Wang *et al*., 2019; Silva *et al*., 2020). Also a previous study indicated the synergistic and additive interactions between *Thymbra spicata* phytochemicals and antibiotics, including ampicillin which are cell wall inhibitors. Study suggest the exact mechanism for the reduction of β-lactam resistance by the natural antimicrobials are may be due to some structural change in the resistant bacteria, inhibition of the of Penicillinase activity (Haroun and Al-Kayali 2016).

5.5.Conclusion

This study probably suggests the possibility of concurrent use of amoxicillin and tested algae extracts in combination in treating infections caused by MRSA strains. However, it is hard to predict synergistic effects *in vivo* on the basis of the presented *in vitro* evidence alone because it is difficult to estimate the *in vivo* concentration of active ingredients, after treatment with algae extracts. In this study, we explored the antibacterial activity of *D. dichotoma* and *P. fascia* extracts, gave insight into its mode of action on MRSA strains and studied the synergistic effect of both algae with amoxicillin. Based on the results obtained, we concluded that the mechanism of action of both tested brown algae, is disruption in the cell membrane permeability and leakage of intracellular materials. The results also showed that algae extracts possess a relatively low hemolytic activity. Therefore, our results revealed the importance of *D. dichotoma* and *P. fascia* when being associated with amoxicillin to control MRSA strains.

6. Future work

- Extracting some active compounds from the crude extract and evaluating their activity in improving the effectiveness of some ineffective antibiotics.
- Assess the cytotoxicity effect of combine between algae extracts and antibiotic
- Use electron microscopy to assess the morphological changes on the bacterial cell to confirm the hypothesis of the mechanism of action of these extracts.
- Elucidation the *in vivo* antibacterial activity of combine between algae extracts and antibiotic to confirm the synergistic role of the algae extracts.

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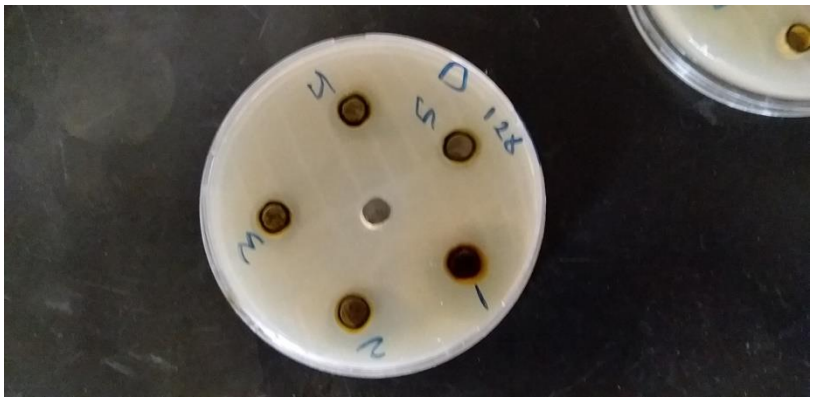
8. Appendices

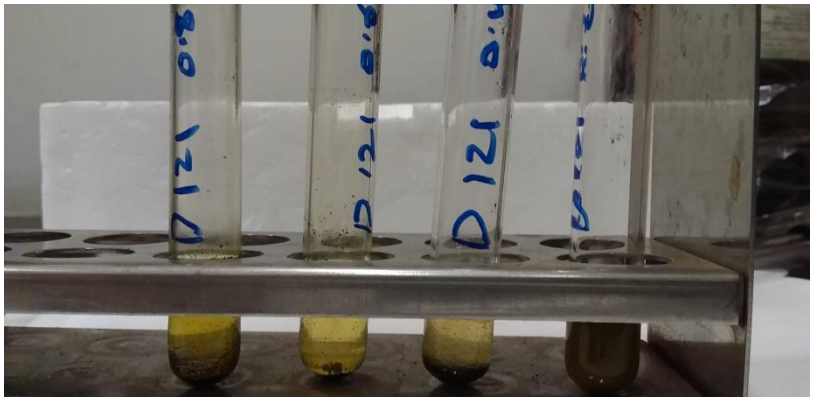
Collection and processing of algal samples

Preparation of the bacterial suspension

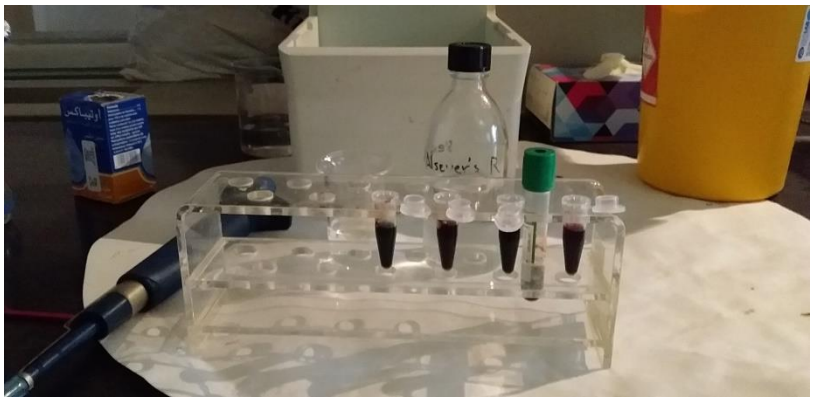


MIC Test

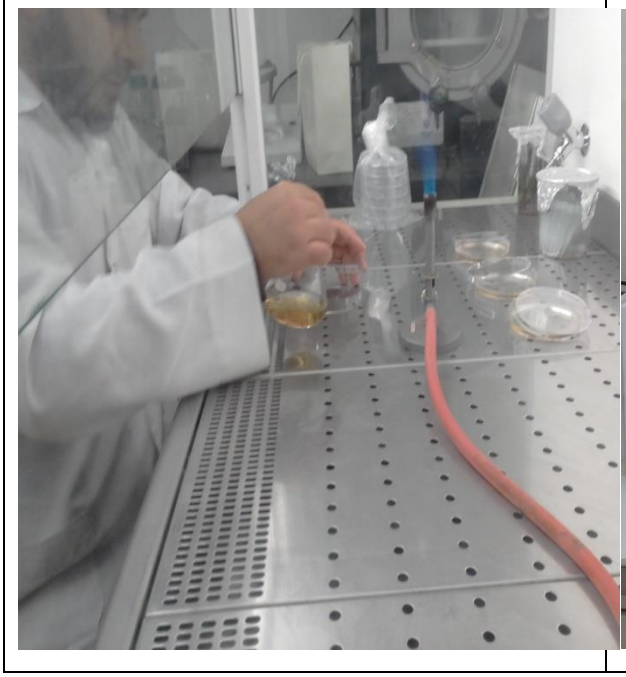
Make holes on medium for extracts injection



MBC Test



Cytotoxicity assay on human red blood cells

Rotary evaporator	Incubator
	
Laminar-flow-cabinet-biosafety	**Flam-photometer**
	

Some of used Equipments

Antibacterial Susceptibility

MRSA Strains	Percentage of synergic effect(Amoxicillin/*P.fascia*)		
	C1	C2	C3
S119	154.9	151.8	155.6
S120	190.8	221.5	201.4
S121	280.6	294.5	297.4
S130	294.0	278.3	272.0
S283	153.8	148.6	155.9

MRSA Strains	Percentage of synergic effect(Amoxicillin/*D.dichotoma*)		
	C4	C5	C6
S119	157.5	158.1	145.5
S120	190.8	221.5	201.4
S121	191.3	164.8	208.7
S130	201.4	208.0	214.1
S283	123.9	164.1	164.6

Extracellular Potassium ion

MRSA Strains	Percentage of synergic effect(Amoxicillin/*P.fascia*) (%)12h		
	C1	C2	C3
S119	501.2	359.7	446.3
S120	457.3	389.8	515.7
S121	383.8	316.1	455.2
S130	997.8	708.6	1019.5
S283	474.4	340.4	446.8

MRSA Strains	Percentage of synergic effect(*D.dichotoma*/ Amoxicillin)(%)12h		
	C4	C5	C6
S119	254.8	102.4	287.8
S120	217.9	182.1	253.9
S121	183.8	169.5	217.1
S130	526.0	469.5	539.1
S283	264.8	153.1	225.5

Nucleotide Leakage

MRSA Strains	Percentage of synergic effect(Amoxicillin/*P.fascia*) 12h		
	C1	C2	C3
S119	8.1	10.2	53.0
S120	62.6	65.2	96.6
S121	9.0	10.2	53.0
S130	31.4	0.11	29.6
S283	19.0	1.6	62.8

MRSA Strains	Percentage of synergic effect(*D.dichotoma*/ Amoxicillin)12h		
	C4	C5	C6
S119	4.2	38.3	70.4
S120	9.0	-24.0	74.1
S121	16.2	-25.0	25.7
S130	22.1	15.1	57.2
S283	1.7	-50.2	56.7

Percentage synergic effect of combination assays

D&AMX	S	*D. dichotoma*	Amx	C4	C5	C6	ANOVA
S119	0.336 ± 0.015^{a}	-0.030 ± 0.0^{b}	0.036 ± 0.001^{c}	-0.021 ± 0.001^{b}	-0.021 ± 0.0^{b}	-0.016 ± 0.0^{b}	< 0.0001
S120	0.221 ± 0.012^{a}	-0.032 ± 0.0^{b}	0.021 ± 0.0^{c}	-0.019 ± 0.0^{b}	-0.026 ± 0.0^{b}	-0.022 ± 0.0^{b}	< 0.0001
S121	0.276 ± 0.01^{a}	-0.025 ± 0.005^{b}	0.021 ± 0.001^{c}	-0.019 ± 0.0^{b}	-0.013 ± 0.0^{b}	-0.022 ± 0.001^{b}	< 0.0001
S130	0.326 ± 0.008^{a}	-0.029 ± 0.001^{b}	0.019 ± 0.001^{c}	-0.019 ± 0.001^{d}	-0.020 ± 0.0^{bd}	-0.021 ± 0.001^{bd}	< 0.0001
S283	0.386 ± 0.003^{a}	-0.025 ± 0.001^{b}	0.038 ± 0.0^{c}	0.009 ± 0.002^{d}	-0.025 ± 0.0^{b}	-0.025 ± 0.001^{b}	< 0.0001

Statistical analysis of the combined effect between *D. dichotoma* and amoxicillin against the tested strains and evaluation of significant differences using Tukey test

P&AMX	S	*P.facsia*	Amx	C1	C2	C3	ANOVA
S119	0.336±0.01	-0.043±0.004	0.036±0.01	-0.020±0.0	-0.019±0.0	-0.020±0.0	< 0.0001
S120	0.221±0.01	-0.056±0.001	0.021±0.00	-0.041±0.00	-0.041±0.0	-0.041±0.00	< 0.0001
S121	0.276±0.010	-0.046±0.004	0.021±0.00	-0.037±0.00	-0.042±0.0	-0.041±0.00	< 0.0001
S130	0.326±0.008	-0.049±0.00	0.019±0.00	-0.036±0.00	-0.034±0.0	-0.032±0.00	< 0.0001
S283	0.386±0.003	-0.052±0.003	0.038±0.00	-0.020±0.000	-0.019±0.0	-0.021±0.004	< 0.0001

Statistical analysis of the combined effect between *P.facsia* and amoxicillin against the tested strains and evaluation of significant differences