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Characterization of ß-lactamase genes in clinical isolates and determination of the inhibitory effects of Algerian seaweeds extracts on recombinant ß-lactamases GES-22 and OXA-1

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In the name of Allah, the Beneficent, the Merciful

Dedication

I am dedicating this thesis, First and foremost, to my loving parents
my father, Ahmed,
May Allah grant him Jannah Firdaws.
and My mother ***MAIZA Hafsa***
for their love, endless support and encouragement

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ملخص

هدفت هذه الدراسة الى تحديد نوع الجينات المشفرة لبناء الانزيمات المسماة بيتالاكتماز و كذا شيوعها في عزلات بكتيرية سالبة الغرام جمعت من خارج و داخل مستشفيات جزائرية. هدفت أيضا الى التحقق من قدرة مستخلصات ميثانولية لسبعة طحالب نامية على الساحل الجزائري [*Ulva intestinalis, Codium tomentosum, Bryopsis pulmosa, Sargassum vulgare, Dictyota dichtoma, Halopteris scoparia, Cystoseira compressa*] على تثبيط نوعين من بيتالاكتماز و هما GES-22 و OXA-1. بينت النتائج أن العزلات تحوي على الأقل جينا واحدا مشفرا لبناء بيتالاكتماز (*bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{CTX-M1-M2}$, *bla*$_{GES}$, *bla*$_{PER-2}$, *bla*$_{NDM}$, *bla bla*$_{OXA-M\ (23,24,51,58)}$) و لا تحوي أي من الجينات (*bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$) لكنها بالمقابل تحوي الأجزاء المحفوظة بالعليبات الجينية للانتجرونات قسم I و II منفصلة او مجتمعة. تختلف الطحالب السبعة من حيث محتواها من عديدات الفينول الذي تتغير قيمته من 0.65 ± 0.93 و 0.91 ± 3.22 mgGAEs/g DW .تفوق هذه القيمة عند الطحالب الخضراء بمرتين تلك للطحالب البنية. في حين، كان محتوى هذه الأخيرة من فيتامين E أعلى من الطحالب الخضراء. بينت الدراسة التحليلية وجود مركبات مشتركة بين المستخلصات المثانولية للطحالب السبعة و هي حمض الفا-لينولينيك (C18: 3 ω3) ، حمض اللينولييك (C18: 2 ω 6) ، حمض الأولييك (C18: 1 ω9) ، وحمض الأراكيدونيك (C20: 4) ω6). علاوة على هذا، تحوي *U.intestinalis* و *D. dichtoma* على بيكالين ($C_{15}H_{10}O_5$) وكركمين ($C_{21}H_{20}O_6$). يحوي *B.pulmosa* على بايكالين ودايدزين ($C_{15}H_{10}O_4$)؛ يحوي *C. tomentosum* على كيرسيتين ($C_{15}H_{10}O_7$) و يحوي *C.compressa* على (S) نارينجين ($C_{15}H_{12}O_5$). تراوحت قيم IC_{50} المحددة على منحى العلاقة Logit/Log بين 13.01 ± 0.46 (*D. dichtoma*) µg/mL إلى 41.24 ± 0.23 (*C. compressa*) µg/mL و من 13.22 ± 0.96 (*B.pulmosa*) µg/mL إلى 62.39 ± 1.96 (*C. compressa*) µg/mL مع GES-22 و OXA-1 ، على التوالي. أظهر المستخلص المثانولي للطحلب *D. dichtoma* تأثيرًا مثبطًا على GES-22 أكبر من مثبطات β-lactam الكلاسيكية المسماة كلافولانات، سولباكتام و تازوباكتام (IC50= 68.38±0.17µg/mL, 52.68±0.64µg/mL, 29.94±0.01) على التوالي . لم يثبط سولباكتام و كلافولانات الانزيم OXA-1، خلافا للتازوباكتام (IC$_{50}$ =243.03± 4.53 µg/mL). أظهر المستخلص الميثانولي لـ *B. pulmosa* تأثيرًا مثبطًا على OXA-1. (IC$_{50}$ =13.22 ± 0.9 µg/mL) من نتائج نفس الاختبارات تصنف كل المستخلصات المثانولية كمثبطات غير تنافسية لـ OXA-1 و GES-22 ، باستثناء المستخلصين لـ *B.pulmosa* و *C. compressa* اللذان يثبطان GES-22 بطريقة تنافسية ولا تنافسية ،على الترتيب. بينت دراسات المحاكاة إلى أن قوة التثبيط ربما ترجع إلى تكوين روابط هيدروجينية وكارهة للماء بين المركبات التي تم الكشف عنها في المستخلصات المثانولية ومركبات اخرى طبيعية من جهة والأحماض الأمينية للموقع النشط في كل من Serine وmetallo-β-Lactamases من جهة اخرى. أظهرت جميع المركبات التي تم الكشف عنها في المستخلصات المثانولية للطحالب libdock score وCDOCKER interaction energy أكبر من مثبطات β-lactam الكلاسيكية مع 15 ß-lactamases. يمكن اقتراح hesperidin وdihydromyrecitin و curcumin كمثبطات جيدة على أساس libdock score وCDOCKER interaction energy.

الكلمات المفاتيح: بيتالاكتماز، محاكاة، *Ulva intestinalis, Codium intestinalis, Bryopsis pulmosa, Sargassum vulgare, Dictyota dichtoma, Halopteris scoparia, Cystoseira compressa*

Abstract

The main aim of this study was to characterize the type and the prevalence of β-lactamases coding genes in community and nosocomial Algerian Gram negative bacterial isolates, as well as to investigate the inhibitory effects of methanolic extracts of seven seaweeds Of Algerian coast [*Ulva intestinalis, Codium tomentosum, Bryopsis pulmosa, Sargassum vulgare, Dictyota dichtoma, Halopteris scoparia,* and *Cystoseira compressa*] against GES-22 and OXA-1 β-lactamases variants. The results showed that isolates harboring at least, one β-lactamase coding gene (*bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{CTX-M1-M2}$, *bla*$_{GES}$, *bla*$_{PER-2}$, *bla*$_{NDM}$, *bla bla*$_{OXA-M\ (23,24,51,58)}$); neither *bla*$_{KPC}$ nor *bla*$_{VIM}$, *bla*$_{IMP}$ gene was detected. The conserved regions of class-I and II integron gene cassettes were determined alone and together in these isolates. The TPC varied among the seven seaweeds species, ranging between 0.93 ± 0.65 and 3.22 ± 0.91 mg GAEs/g DW. TPC in the MEs of green seaweed species were found to be nearly two times greater than that in brown seaweeds. However, vitamin E contents of brown seaweed MEs were higher than those of green seaweeds. Furthermore, qualitative analysis showed the existance of common compounds in the MEs including α-linolenic acid (C18:3 ω3), linoleic acid (C18:2 ω6), oleic acid (C18:1 ω9), and arachidonic acid (C20:4 ω6). Moreover, *U. intestinalis* and *D. dichtoma* contain baicalein ($C_{15}H_{10}O_5$) and curcumin ($C_{21}H_{20}O_6$). *B. pulmosa* contains baicalein and daidzein ($C_{15}H_{10}O_4$); *C. tomentosum* contains quercetin ($C_{15}H_{10}O_7$) and *C. compressa* contains (S) naringenin ($C_{15}H_{12}O_5$). Values of IC_{50} of MEs determined by linear computerized regression analysis after logit/log transformation, are ranged from 13.01 ± 0.46 µg/mL (*D. dichtoma*) to 41.24 ± 0.23 µg/mL (*C. compressa*) and from 13.22 ± 0.96 µg/mL (*B. pulmosa*) to 62.39 ± 1.96 µg/mL (*C. compressa*) with GES-22 and OXA-1, respectively. ME of *D.dichtoma* exhibited significant inhibitory effect on GES-22 more than classical β-lactam inhibitors, clavulanate, sulbactam and tazobactam (IC_{50} = 68.38 ± 0.17 µg/mL, 52.68 ± 0.64 µg/mL, and 29.94 ± 0.01 µg/mL, respectively). OXA-1 was not inhibited by sulbactam and clavulanate and was moderately inhibited by tazobactam (IC_{50} =243.03± 4.53 µg/mL). However, ME of *B. pulmosa* exhibited significant inhibitory effect on OXA-1 (IC_{50} =13.22 ± 0.96 µg/mL). Data from the same tests categorizing all MEs as mixed inhibitors of OXA-1 and GES-22, except *B. pulmosa* and *C. compressa* wich inhibit GES-22 with competitive and non-competitive manner, respectively. Docking studies indicated that the potency of inhibition is probably due to the formation of hydrogen bonds and hydrophobic interactions between ligands (compounds identified in MEs of seaweeds and other natural compounds) and active amino-acids site of both Serine and metallo-β-Lactamases. All compounds identified in the MEs of seaweeds have shown libdock score and CDOCKER interaction energy higher than the classical inhibitors clavulanate and sulbactam towards 15 ß-lactamases. Hesperidin, dihydromyrecitin and curcumin can be suggested as good ligands on the basis of libdock score and CDOCKER interaction energy.

Key words: β-lactamases, GES-22, OXA-1, docking, *Ulva intestinalis, Codium tomentosum, Bryopsis pulmosa, Sargassum vulgare, Dictyota dichtoma, Halopteris scoparia,* and *Cystoseira compressa*.

Résumé

Le but principal de cette étude était de déterminer le type et la prévalence des gènes codants pour les β-lactamases dans des isolats bactériens Gram négatif algériens communautaires et nosocomiaux, ainsi que de rechercher les effets inhibiteurs d'extraits méthanoliques (EMs)de sept algues de la cote algérienne [*Ulva intestinalis*, *Codium tomentosum*, *Bryopsis pulmosa*, *Sargassum vulgare*, *Dictyota dichtoma*, *Halopteris scoparia* et *Cystoseira compressa*] sur deux variants de β-lactamases, GES-22 et OXA-1. Les résultats ont montré que les isolats contenant au moins un gène codant pour la β-lactamase (*bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{CTX-M1-M2}$, *bla*$_{GES}$, *bla*$_{PER-2}$, *bla*$_{NDM}$, *bla bla*$_{OXA-M\ (23,24,51,58)}$); ne portent ni le gène *bla*$_{KPC}$ ni le gène *bla*$_{IMP}$ ni le gène *bla*$_{VIM}$. Les régions conservées des cassettes de gènes des intégrons de classe I et II ont été déterminées seules et ensemble dans ces isolats. Le contenu en polyphénols totaux des sept espèces d'algues variait entre 0,93 ± 0,65 et 3,22 ± 0,91 mg GAEs/g poids sec. Ceux des EMs des algues vertes étaient presque deux fois plus élevés que ceux des algues brunes. Cependant, les teneurs en vitamine E de EMs des algues brunes étaient plus élevées que celles de MEs des algues vertes. En outre, une analyse qualitative a montré l'existence de composés communs aux MEs des sept algues, incluant l'acide α-linolénique (C18: 3 3), l'acide linoléique (C18: 2 ω6), l'acide oléique (C18: 1 ω9) et l'acide arachidonique (C20: 4). ω6). De plus, *U. intestinalis* et *D. dichtoma* contiennent de la baicaleine ($C_{15}H_{10}O_5$) et de la curcumine ($C_{21}H_{20}O_6$). *C. tomentosum* contient de la quercetine ($C_{15}H_{10}O_7$) et *C. compressa* contient de la (S) naringenine ($C_{15}H_{12}O_5$). Les valeurs de IC_{50} des ME déterminées par analyse de régression linéaire informatisée après transformation logit / log, variaient de 13,01 ± 0,46 µg/mL (*D. dichtoma*) à 41,24 ± 0,23 µg/mL (*C. compressa*) et de 13,22 ± 0,96 µg/mL. (*B.* pulmosa) à 62,39 ± 1,96 µg/mL (*C. compressa*) avec GES-22 et OXA-1, respectivement. L'EM de *Dichtoma* a montré un effet inhibiteur plus significatif sur GES-22 que les inhibiteurs β-lactames classiques, le clavulanate, le sulbactam et le tazobactam (IC_{50} = 68,38 ± 0,17 µg/mL, 52,68 ± 0,64 µg/mL et 29,94 ± 0,01 µg/mL, respectivement). Le sulbactam et l'acide clavulanique n'ont pas inhibé l'OXA-1, mais le tazobactam l'a inhibé modérément (IC_{50} = 243,03 ± 4,53 µg/mL). Cependant, l'EM de *B. pulmosa* a montré un effet inhibiteur significatif sur OXA-1 (IC_{50} = 13,22 ± 0,96 µg/mL). Des données des memes tests classaient tous les EMs comme inhibiteurs non compétitifs d'OXA-1 et de GES-22, à l'exception de l'EM de *B. pulmosa* et de *C. compressa*, qui inhibent GES-22 de manière compétitive et non compétitive, respectivement. Des études d'amarrage ont montré que la puissance de l'inhibition est probablement due à des liaisons hydrogène et aux interactions hydrophobes entre les ligands (composés identifiés dans les EMs d'algues et d'autres composés naturels) et les acides aminés du site actif des sérine et des métallo-β-lactamases. Tous les composés identifiés dans les EMs d'algues ont montré un score de libdock et une énergie d'interaction CDOCKER supérieurs à ceux du clavulanate et du sulbactam pour les 15 β-lactamases. L'hespéridine, la dihydromyrécitine et la curcumine peuvent être suggérées comme de bons ligands sur la base de leur score de libdock et de l'énergie d'interaction CDOCKER.

Mots clés: β-lactamases, GES-22, OXA-1, docking, *Ulva intestinalis, Codium tomentosum, Bryopsis pulmosa, Sargassum vulgare, Dictyota dichtoma, Halopteris scoparia, Cystoseira compressa*

LIST OF ABBREVIATIONS

***ABTS* :** 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid);

AMPc : Chromosomal located cephalosporinase

ATCC : Amiricain Type Collection culture

C3G : Third-generation cephalosporins

CA-Ab : Community acquired *A. baumanni*

CA-SFM : Comité français de l'antibiogramme de la société française de microbiologie

CDOCKER : CHARMM-based DOCKER

CHARMM : Chemistry at HARvard Macromolecular Mechanics)

CLSI : Clinical and Laboratory Standards Institute

CTX : Cefotaximase

DDST : Double Disc Synergy test

DPPH : 2,2'-diphenyl-1-picrylhydrazyl

DS : Discovery Studio

EDTA : Ethylene diamine tetraacetic acid

ESβLs : Extended Spectrum β-lactamases

GES : Guyana extended spectrum β-lactamases.

HAIs : Hospital acquired infections

HPLC-ESI-MS : High Performance Liquid Chromatography Time of Flight- Mass Spectrometry

HPLC-TOF-MS : High Performance Liquid Chromatography electrospray ionisation tandem-Mass Spectrometry

IC_{50} : half maximal inhibitory concentration

ICUs: intensive care units

IMP : Imipenemase and

IPTG: Isopropyl-β-D-thiogalactopyranoside

K_M : *Michaelis constant*

KPC : *Klebsiella pneumonia* Carbapenemases

MβLs : metallo-β-lactamases

MEs : Methanolic extracts

MHT : Modified Hodge Test

MDR : Multidrug-resistant

MDR-GNB : Multi-drug resistant Gram negative bacteria

MROs : Multidrug-resistant organisms

NDM : New Delhi MβL

NFGNB : *Non-fermenting gram-negative bacilli*

OprD : Outer membrane *porin* D

OXA : oxacillinase

PBA : Phenylboronic acid

PCDDT : Phenotypic Confirmatory Disc Diffusion Test

PDB : Protein Data Bank

PER : *Pseudomonas* Extended Resistance

RMSD : Root Mean Square Deviation

S : Substrate

SβLs : Serine β-lactamases

SHV : Sulfhydryl variable

SD : Standard deviation

SDS-PAGE : Sigle anglophone de sodium dodecyl sulfate polyacrylamide gel electrophoresis

TEM : Temoneira

VEB : Vietnam Extended-spectrum β-Lactamase.

VIM : Verona integron-encoded

Vmax : maximum velocity

WHO : World Health Organization

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DISCUSSION

Introduction

β-lactams are the most widely used antibiotic family in the world. This wide use is due to their broad spectrum of action, and their low toxicity (Handal and Olsen, 2000). This family comprises a large number of molecules, all characterized by the presence of a β-lactam ring. The introduction of third-generation cephalosporins (C3G) into clinical practice in the early 1980s to control infections with penicillinase-producing organisms was followed in 1983 by the description of the first Extended Spectrum β-lactamases (ESβLs) capable of hydrolyzing the extended-spectrum cephalosporins in Germany in strains of *Klebsiella ozaenae* (Knothe *et al*., 1983). Carbapenems, the last antibiotics of the β-lactam class, are bactericidal antibiotics often used as a last treatment option for severe infections caused by ESβLs-producing isolates (Wadekar and Swarooparani, 2017). However, extensive use of these molecules has facilitated the emergence of carbapenem resistant bacteria and hence the production of carbapenemases.

Gram-negative bacteria have become increasingly resistant to ß-lactams. Production of β-lactamases especially ESβLs and carbapenemases, is the main mechanism of resistance to these molecules by hydrolysing the amide linkage of the β-lactam ring (Philippon and Arlet, 2006). To inhibit the hydrolyzing activity of β-lactamases, β-lactam antibiotics are used with inhibitors (tazobactam, clavulanate, and sulbactam). These β-lactamases inhibitors are co-administered with β-lactam antibiotics and they are effective against some class A β-lactamases whereas ineffective against class B and most of the class C and D β-lactamases. As a result of the presence of a β-lactam ring in these inhibitors and their extensive use in combination with β-lactam antibiotics, the β-lactamase in bacteria mutate continually developing their activity even against newly developed β-lactam (Drawz and Bonomo, 2010). Hence, new, non-toxic and potential β-lactamases inhibitors is an urgent need.

It is well known that, over the past decades, seaweeds have been attracting attention in the search for bioactive compounds to develop new drugs and healthy foods among which polyphenols, lipids, polysaccahrides, sterols, terpenes. Furthermore, various seaweed extracts were reported to have high antioxidant, anti-cancer activity and to influence anti-inflammatory responses. It is in this context that the present study, whose main objective is to characterize the type of β-lactamases produced by Gram-negative bacilli strains collected from community and hospitals in Algeria and to investigate the inhibitory effect of seven Algerian seaweeds [*Ulva intestinalis, Codium tomentosum, Bryopsis pulmosa, Sargassum vulgare, Dictyota dichtoma, Halopteris scoparia,* and *Cystoseira compressa*] against GES-22 and OXA-1 β-lactamases variants, produced recombinantly. In order to develop this aspect, we have adopted the following methodology

- Antibiotic susceptibility pattern of isolates to β-lactam antibiotics and other antibiotic classes.
- Phenotypic detection of ESβLs and carbapenemases using several tests;
  - Double Disc Synergy test (DDST)
  - Phenotypic Confirmatory Disc Diffusion Test (PCDDT)
  - Modified Hodge Test (MHT)
  - inhibition tests
- Characterization of the β-lactamase genes (*bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{CTX-M1-M2}$, *bla*$_{PER-2}$, *bla*$_{VEB,}$ *bla*$_{GES}$, *bla*$_{KPC}$, *bla*$_{OXA-M\ (23,24,51,58)}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$) and the integrons class I and II by PCR technique among one hundred and eight clinical isolates collected.
- Investigation of the inhibitory effects (*in vitro*) against GES-22 and OXA-1 β-lactamases variants, produced recombinantly in methanolic extracts of the seven studied seaweeds.
- Identification of the compounds of methanolic extracts by HPLC-ESI-MS and HPLC-ESI-MS-TOF.

  Finally, the prediction (*in silico*) of the structure of a molecular complex (β-lactamases-compounds identified in MEs and other molecules) by molecular docking using Discovery Studio (DS) 2016 software (Accelrys Software Inc., San Diego).

Review

I. Nosocomial infections

I.1. Definition

Nosocomial infection term lies the problem of the relationship between the hospital and the infection acquired by the patients who stay there. Indeed, the word "nosoconium" means "hospital", it comes from Greek meaning: nosos=disease and komien= to care for. Nosocomial infections -also known as hospital acquired infections (HAIs) are defined by the World Health Organization (WHO) as:

"*… infections acquired during a hospital stay that were neither present nor in incubation at admission. Infections occurring more than 48 hours after admission are usually considered nosocomial*." (WHO, 2002). Infections in newborn babies that occurred during or after delivery are considered nosocomial (Orrett *et al*., 1998). Nosocomial infections are considered to be the most common complication affecting hospitalized patients. The WHO estimates that developing countries have as much as a 20 times higher risk of nosocomial infections than developed countries (WHO, 2007).

I.2. Causative Agents

Nosocomial infections are usually caused by bacteria in ninety percent of cases. Moreover, viruses, protozoa and fungi are rarely involved (Bereket *et al*., 2012). A range of Gram-negative organisms are responsible for HAIs, the *Enterobacteriaceae* family being the most commonly identified group overall. Gram bacilli negative represent 60% of which *E.coli* is predominantly involved. Gram-positive cocci represent 30% (Thiolet *et al*., 2013).

The increased concern about infections led to increased use of antibiotics. Widespread use of these molecules is often cited as a cause of the emergence of multidrug-resistant organisms (MROs) as nosocomial pathogens. The multi-drug resistant Gram negative bacteria (MDR-GNB) are by far the most important and costly today, as the vast majority of nosocomial infections are caused by this type of bacteria (Livermore, 2012). Microorganisms usually implicated in these infections include among others *Escherichia coli*, *Klebsiella species*, *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Staphylococcus aureus* which are rapidly gaining resistance because of the broad spectrum antibiotics used in an attempt to control them. MROs are bacteria that have become resistant to numerous antimicrobial agents, which have inadvertently led to increased morbidity and mortality from HAIs (NHMRC, 2010). Globally, methicillin-resistant Staphylococcus aureus and MDR GNB are described as

problematic MROs (WHO, 2015). A large proportion of HAIs are caused by MROs; however, specific numbers have not been identified or provided (WHO, 2015). Unfortunately, MROs including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and extended-spectrum β-lactamases (ESβLs) or carbapenemase-producing Enterobacteriaceae, are increasingly being reported worldwide (Peleg and Hooper, 2010).

II. Gram-negative bacilli

Gram-negative bacilli, frequently isolated from bacteriology laboratories, occupy an important place in human pathology. Generally, they are divided in two groups: enterobacteria which can grow in the presence of bile salts and use lactose as an energy source on MacConkey's agar and non-fermenting Gram-negative bacilli that cannot use lactose (so-called "non-lactose fermenters") include, most prominently *Pseudomonas* and *Acinetobacter* species as well as less common organisms *Stenotrophomonas, Burkholderia*, and *Achromobacter* species (Liassin, 2000; Mehrad *et al*., 2015). In this literature review, we will focus on enterobacteria, *Pseudomonas sp* and *Acinetobacter ssp*, because they are the germs targeted in this study.

II.1. *Enterobacteriaceae*

II.1.1. History and Taxonomy

Enterobacteria are a large group of bacteria with a strong similarity. The creation of this group was proposed by Rahn, (1937) as *Enterobacteriaceae*, in which he collected the bacterial genera such as *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella* in the unique genus *Enterobacter*.

Enterobacteria are Eubacteria. Phylogenetic studies place them in the phylum Proteobacteria, Class Gammaproteobacteria, Order Enterobacteriales and to the family *Enterobacteriaceae*. This family comprise a large group of genetically and biochemically related bacteria (Brenner *et al*., 2005). The family name is still maintained, however, the classification of bacteria in the family has evolved a lot. Until the early 1960s bacterial classification was largely based on phenotypic characteristics and culture-based observations. Currently, *Enterobacteriaceae* are classified on the basis of the genetic relationships between members and their similarity to other closely related bacteria using 16S rRNA gene sequences analysis (Joly and Reynaud, 2002).

II.1.2. Biological characteristic and Habitat

The name *Enterobacteriaceae*, was given because the germs of this family are mainly pathogens of the humans and animals digestive tract and others are normal colonizers of this digestive tract (*Escherichia coli*, *Enterobacter spp.*, *Klebsiella spp.*). Enterobacteriaceae are a major component of the normal human intestinal microbiota but relatively uncommon at other body sites (Balows *et al*., 1992). However, some species are insect- or plant-associated (Janda and Abbott, 1998). Although, they are also present in the environment. They are found in water, soil and some foods. They are able to disseminate easily by hand-carried transmission or via the contamination of the water and the food.

II.1.3. *Enterobacteriaceae* Infections

Most of the bacteria belonging to the *Enterobacteriaceae* family are harmless and not causing disease symptoms. A large group of bacteria from this family are opportunistic microorganisms, posing a threat to the elderly, weakened, sick or in a state of immunological suppression. The vast majority of bacteria present in the digestive tract is harmless to humans, however, some species of the *Enterobacteriaceae* family are considered to be pathogenic organisms responsible for various types of infection, including intestinal diseases (Jarząb *et al*., 2011). Nosocomial infections are mainly urinary tract infections, wounds surgical procedures, pulmonary infections, septicemia, and other localizations. More bacteria already mentioned in community infections with a multi-resistance profile mention are of *Enterobacter sp.*, *Serratia sp*. However, community acquired infections are mainly urinary tract infections caused by *E. coli*, pulmonary infections caused by *K. pneumoniae* and food poisoning caused by *Salmonellae*.

II.1.4. Species belonging to the family *Enterobacteriaceae*

Enterobacteriaceae family is defined by a set of general common bacteriological characteristics. These are Gram-negative bacilli, non-sporulating, optional aero or anaerobes, motile or immotile, growing rapidly on media ordinary, not having oxidase, reduce nitrates to nitrites, ferment D-glucose with or without gas production (Eyquem *et al*., 2000; *al*., Avril2000). Possess a common antigen called Kunin antigen or ECA (enterobacterial common antigen) (Delarras, 2014). The majority of *Enterobacteriaceae* targeted in this study are: *E.coli* and *K. pneumoniae*. For that, it seems necessary to briefly recall some general characteristics of these bacteria.

II.1.4.1. *Escherichia coli*

E. coli was first isolated in 1885 by Theodore Escherich, a German pediatrician, in stool of infants, which he called *Bacterium coli commune* (Kaper *et al*., 2004). The name *E. coli* is given in 1919 by Castellani and Chaombers (Grimont, 1987). Since then, *E. coli* has become the best-known bacterium that has been the most studied by fundamentalists for physiology and genetics search (Avril *et al*., 2000). It is the dominant species in the commensal flora, particularly in the digestive tract of the human. It colonizes from the first hours of birth (Bonacorsi *et al*., 2001; Janda and Abbott 1998). However, following the acquisition and combination of virulence factors, this bacterial species can also behave as an intestinal or extraintestinal localized pathogen (Levine, 1987; Pohl, 1993). Moreover, *E. coli* is very responsive in the environment: water, soil, and in food (Baraduc *et al*., 2001).

E. coli has particular biochemical characteristics that allowing to differentiate it from other species among which, the production of indole from tryptophan, lack of use of citrate as a carbon source and lack of production acetoin (Joly and Reynaud, 2002), in addition to the common bacteriological characteristics of *Enterobacteriaceae,* non-sporulating, generally motile, aero-anaerobic facultative, with respiratory and fermental metabolism, negative oxidase, catalase positive and nitrate reductase positive (Bidet and Bingen, 2011). *E. coli* is naturally sensitive to antibiotics. However, face to the intensive use of these molecules and under their conjugate effects and resistance gene transfer, it may become, resistant (Adjidé *et al*., 2006).

II.1.4.2. *Klebsiella pneumoniae*

Carl Friedlander (1882) first described *Klebsiella pneumoniae*, as an encapsulated bacillus under the name of Friedlander's pneumobacillus isolated from the lungs of patient who had died from pneumonia. The genus *Klebsiella* described for the first time by Trevisan (1885) and he was named it to honor Klebs Edwin, a German microbiologist (Trevisan, 1887).

Klebsiella pneumoniae, is a very widespread germ in environment, it is frequently isolated from plants, soil and water. In humans, it exists as part of the normal microbiota of the nasopharynx and gastrointestinal tract. This species colonizes on the mucosal surfaces of mammals such as humans, horses, or swine (Cruz-Córdova *et al*., 2014). The type species *K. pneumoniae* are facultative anaerobic bacilli, non-spore-forming, capsulated, non-motile, oxidase negative, positive nitrate reductase, positive urease, positive Voges-Proskauer reaction,

which ferments mannitol and glucose with gas production, , (Janda and Abbott, 2006; Cruz-Córdova *et al*., 2014).

K. pneumoniae is considered one of the most important opportunistic pathogens associated with nosocomial and community-acquired infections (Podschun and Ullmann, 1998). It is among the most pathogen with high morbidity and mortality rates worldwide. This species is the chief cause of various HAIs involving upper and lower respiratory tract and urinary tract infections. It is also responsible for gastrointestinal infections and liver abscess in community. (Podschun and Ullmann, 1998; Tsai *et al*., 2008)

II.2. *Non-fermenting gram-negative bacilli* (NFGNB)

II.2.1. Genus *Acinetobacter*

II.2.1.1. History and Taxonomy

The history of the genus *Acinetobacter* began in 1911 with the discovery of a microorganism called Micrococcus calcoaceticus by the Dutch microbiologist Beijerinck from soil sampling (Baumann *et al*., 1968). Schaub and Hauber rediscover this bacterium in 1948, from samples of the human urinary system (Schaub and Hauber, 1948). Brisou and Prévost (1954) propose the designation of the genus *Acinetobacter* (from the Greek akinetos: "non motile" to group a heterogeneous collection of immobile, Gram-negative and positive or negative oxidase reaction. In 1968, Baumann *et al*., restricted the genre *Acinetobacter* to only negative oxidase strains and have recognized a unique species that they have proposed to name *Acinetobacter calcoaceticus*. Three years later, this proposal will be endorsed by the "*Subcommittee on Moraxella and Allied Bacteria*". In 1974, the genus *Acinetobacter* has been listed in the edition of Bergey's Manual of Systematic, with the description of a single species, *Acinetobacter calcoaceticus* as typical strain for genus (*A. calcoaceticus* ATCC 23055). In 1986, Bouvet and Grimont by DNA / DNA hybridization techniques, distinguished 12 genomic species, some are clearly named as *A.baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junii and A. lwoffii* (Bouvet and Grimont, 1987).

The species' names have endured substantial taxonomic changes over the years due to the advanced understanding of molecular methods of the genetic make-up of this group of microorganisms. Recent classifications which seem to have gained wide acceptance among bacterial taxonomists have categorized the genus *Acinetobacter* in the domain of Bacteria, phylum Proteobacteria, class of Gammaproteobacteria, order of Pseudomonodal and is

classified in the family *Moraxellaceae*. The species *A. baumannii*, *A. haemolyticus* and *A. calcoaceticus* are of clinical significance (Almasaudi, 2018).

II.2.1.2. Biological characteristic and Habitat

Bacteria of the genus *Acinetobacter* are Gram-negative bacilli or coccobacilli, non-sporulating, sometimes capsulated, strict aerobic, oxidase-negative, catalase positive, non-motile and non-fermenting (Jans *et al*., 2004; Peleg *et al*., 2008). These are, often grouped in pairs or in chains of variable length. In the exponential phase, their diameter typically ranges from 0.9 to 1.6 μm and their length from 1.5 to 2.5 μm (Doughari *et al*., 2011; Jung and Park, 2015).

Members of the genus *Acinetobacter* are considered ubiquitous organisms (Peleg *et al*., 2008). Whose main habitat is soil, sewage, as well as food sources that are stored in chilled conditions (Tomaras *et al*., 2003). In fact, not all species of the genus *Acinetobacter* have their natural habitat in the environment (Peleg *et al*., 2008). In humans, *Acinetobacter spp.* are part of the flora of healthy skin, mucous membranes and the pharynx, and human respiratory secretions (Almasaudi, 2018). In hospitalized patients ‘”are often found in the skin, oropharynx, and digestive tract (Jung and Park, 2015).

II.2.1.3. *Acinetobacter baumannii* Infections

A. baumannii is the most frequently species involved in nosocomial and community-acquired infections, followed by *A. pittii* and *A. nosocomialis* within the genus *Acinetobacter*, (Peleg *et al.*, 2008). *A. baumannii* has become in the recent years a clinically relevant pathogen, involved in a wide range of infections. Infections due to *A. baumannii* are most often nosocomial, rarely community-based (Bergogne-berezin and towner, 1996). *A. baumannii* is able to survive for several days in the hospital environment on abiotic surfaces (Tomaras *et al*., 2003). This persistence can be explained no only by its great adaptability but also by its antimicrobial resistance (Gaddy and Actis, 2009). The main severe nosocomial infections due to *A.baumannii* are pneumopathies. *A. baumannii* nosocomial pneumonia occurs in intensive care units (ICUs) with a frequency of 3–5% and with death rates of 30–75% (Doughari *et al*., 2011). However, in patients who require prolonged mechanical ventilation, this latest is a major risk factor of pneumonia. In addition, this micro-organism can cause infections of the skin and soft tissue, including the wounds (Johnson *et al*., 2007). Bacteremia and secondary meningitis are also among the severe nosocomial infections caused by *A. baumannii* (Peleg *et al*., 2008).

The incidence of *A.baumannii* varies from one site to another. However, this microorganism is the second most common etiologic agent among all the Gram-negative bacteria (Almasaudi, 2018).

Community acquired *A. baumanni* (CA-Ab) infections are rare but serious cause of community-acquired pneumonia, predominantly occur in countries with tropical or sub-tropical climates. They are more likely to occur in the humid months of the year. Moreover, CA-Ab infections affect individuals with risk factors, which include excess alcohol consumption, diabetes mellitus, smoking and chronic lung disease (Dexter *et al*., 2015).

II.2.2. Genus *Pseudomonas*

II.2.2.1. History and Taxonomy

The family *Pseudomonadaceae* belongs to a large group of bacteria that in the past has been conventionally referred to as "non-fermenting" (Hansen, 1991). The classification into genera and species within this family has long been based on simple phenotypic orientation. The simplification of this classification was carried out by Stanier who studied mainly the assimilation of carbonaceous substances, and by Palleroni who classified *Pseudomonas* species into 5 genomic groups (Martin, 2007).

The genus *Pseudomonas* was described by Walter Emil Friedrich August Migula, a Botanist devoted to plant taxonomy (Palleroni, 2003). This genus is classified in the family *Pseudomonadaceae*, the order of *Pseudomonadales*, the class of *Gammaprotobacteria*, division of *Proteobacteria*, branching of *Prokaryotes* and reign of *Bacteria* (Garrity *et al*., 2010)

II.2.2.2. Biological characteristic and Habitat

Bacteria of the genus *Pseudomonas* correspond to bacilli Gram-negative non sporulating, strict aerobes, usually motile by a polar ciliature, do not ferment carbohydrates, do not fix nitrogen and are not photosynthetic, chemotropic, with oxidative metabolism. They are oxidase positive or negative, catalase positive. The strains are easily grown on the usual culture media, nutrient agar (Yumoto *et al*., 2001). This genus includes fluorescent species producing pigments specific. The two most common and characteristic pigments are pyocyanin and pyoverdine which are soluble in culture media. The two pigments are produced by *Pseudomonas aeruginosa*, but this latest can be lost the pigments by mutation. *P. fluorescens*, *P. putida*, *P.syringae*, and *P. cichorii* produce only pyoverdine (Matewish and Lam, 2004). However, some strains don’t produce these pigments such as *P. alcaligenes* and *P. stutzeri*

(Martin, 2007). The word '*aeruginosa*' comes from the Latin word for verdigris or copper rust. This describes the blue-green bacterial pigment seen in cultures of *P. aeruginosa*. This species was first obtained in 1882 in pure culture by Gessard from wounds that had produced blue-green discoloration (Forkner, 1960).

Pseudomonas is ubiquitous in nature. It has been found in many surface waters and soils and infects a number of common plants. It is frequent in warm waters in which there has been human or animal activity. They are considered a commensal flora in humans or animals. Some play a pathogenic role including *Pseudomonas syringae* in plants and *Pseudomonas aeruginosa* in humans and animals. In hospitals, *Pseudomonas* are found in the environment patients (sinks, faucets and sanitary equipment). This ubiquity is associated with their metabolic versatility and their ability to adapt to the most hostile conditions (Meghdas *et al*., 2004).

II.2.2.3. *Pseudomonas aeruginosa* Infections

The genus *Pseudomonas* is one of the most complex of Gram-negative bacteria. Their mechanism of action varies according to the species and the host. Some species are pathogenic for humans, animals (Nishimori *et al*., 2000) and plants (Akkermans *et al*., 1996, Munsch and Alatossava, 2002) while others are useful. *P. aeruginosa* is an opportunistic pathogen for humans. It is an important cause of nosocomial infections and community acquired infections in immunocompromised patients and patients with structural lung disease (John *et al*., 2017).

P. aeruginosa is the primary cause of ventilated, associated pneumonia in the intensive care unit and, lead to a broad spectrum of disease such as urinary, burn, respiratory infections, and septicemia (Fazeli *et al*., 2012). Hospital-acquired infections caused by this organism are often associated with high morbidity and mortality because these microorganisms are virulent and have a limited susceptibility to antimicrobials (Dwivedi *et al*., 2009).

III. Resistance of Gram-negative bacilli to β-lactams

ß-lactams form a broad class of antibiotics that includes derivatives of penicillin, cephalosporins, monobactams, carbapenems and ß-lactamase inhibitors. These antibiotics all contain a common element in their molecular structure known as ß-lactam ring which confers the antibiotic activity. For over 60 years, β-lactams have been the first line of antibiotic treatments for many community- and hospital-acquired infections, including those caused by multidrug-resistant pathogens. Despite the enormous efforts to develop new β-lactam

derivatives to overcome growing bacterial resistance, so far no single molecule escapes from hydrolysis by several of the thousands of β-lactamases described (Juan *et al*., 2017). Gram-negative bacteria have become increasingly resistant to ß-lactams, rendering infection by these strains very challenging to treat. For this, antibiotic development programs have placed greater emphasis on the identification of natural antibiotics other than β-lactams, such as aminoglycosides and tetracyclines, followed by optimization of (fluoro) quinolone molecules as a way of circumventing β-lactam resistance in Gram-negative bacteria (Bush, 2016). Gram-negative bacteria are either naturally resistant to β-lactams, or they have acquired resistance.

III.1. Types

III.1.1. Inherent (natural) resistance

Antibiotic resistance is the ability of a bacteria to resist the effects of an antibiotic. Natural or intrinsic resistance is caused by the structural characteristics of bacteria and it is not associated with the use of antibiotics. It is common to all bacteria of the same species. This resistance is due to the presence of common chromosomal genes to all the bacteria of the same species and transmitted to the offspring. Intrinsic resistance determines the Wild-type phenotype of bacterial species against antibiotics (Mayer *et al*., 2000).

The intrinsic resistance in Gram-negative bacilli is manifested by chromosomal cephalosporinases which express themselves either constitutively or inducible (Cavallo *et al*., 2004) or due to an outer membrane that establishes a permeability barrier against the antibiotic. For example, Gram-negative bacteria are intrinsically resistant to penicillin G by virtue of their double membrane structure which prevents the antibiotic from accessing the cell wall target. In addition to this, the expression of oxacillinase in *A. baumannii.*

III.1.2. Acquired resistance

Bacteria developed several mechanisms in order to acquire resistance to antibiotics previously sensitive. All require either the modification of existing genetic material (mutation) or the acquisition of new genetic material from another source. Acquired resistance occurs from (i) acquisition of exogenous genes by plasmids (conjugation or transformation), transposons (conjugation), integrons and bacteriophages (transduction), (ii) mutation of cellular genes, and (iii) a combination of these mechanisms (Giedraitienė *et al*., 2011). To counter the adverse effects of ß-lactam antibiotics, bacteria have evolved in diverse ways: i) mutations leading to loss or under-expression of porins that disallow entry of ß-lactams, ii) production of new

penicillin binding proteins that have low affinity to ß-lactams, iii) expulsion of ß-lactams from periplasmic space mediated by efflux pumps and iv) production of enzymes that hydrolyze ß-lactam rings. The main mechanism of resistance acquired to β-lactams in Gram bacilli negative is the production of β-lactamases (cavallo *et al*., 2004).

III.2. β-lactamases

Before the use of ß-lactams in medicine, certain bacteria produced already ß-lactamases. These enzymes may have played a minor role in metabolism of the cell wall, or to protect the bacteria against ß-lactams produced by fungi in the environment. Whatever their function, it is the increased and uncontrolled human use of these antibiotics that has favored the emergence of bacteria carriers of ß-lactamases (Livermore, 1998). Dissemination of resistant strains and the emergence of new mechanisms for resistance, particularly production of new β-lactamases, pose serious problems for the medical world. ß-lactamases degrading/modifying the ß-lactam ring by cleaving the amine linkage of ß-lactams (Yamaguchi *et al*., 2005; Zhang and Hao, 2011). These flexible enzymes have been detected in both Gram-positive and Gram-negative bacteria, but these enzymes are especially important in Gram-negative bacteria as they are the most common cause of β-lactam resistance in this group of bacteria (Bush *et al*. 1995; Livermore, 1995).

III.2.1. Classification

There are two classifications of β-lactamases: structural classification of Ambler and functional classification of Bush-Jacoby-Medeiros. The most used in current medical practice is currently that of Ambler. This classification is based on the amino acid sequence homology of β-lactamases. It divides these inactivating enzymes into four groups (A to D) according to the primary structure of the enzyme. The enzymes of classes A, C and D are called active serine (serine type), which require an active site serine residue to catalyse the ring opening of the ß-lactams and are mostly penicillininases or cephalosporinases; while class B groups are metallo-β-lactamases (MβLs) (metallo-enzymes type), which require one or two zinc ions in their active site for their activity (Philippon *et al*., 2016). Depending on the hydrolyzed substrate, ß-lactamases are therefore calling penicillinases, cephalosporinases, extended-spectrum ß-lactamases or carbapenemases. The highly problematic strains carry ESβL and carbapenemase genes.

III.2.1.1. Extended-spectrum ß-lactamases (ESβLs)

III.2.1.1.1. History and Definition

The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983. It was discovered in Germany in strains of *Klebsiella ozaenae* (Knothe *et al*., 1983). The term ESβL was first proposed in 1988 to distinguish plasmid β-lactamases giving resistance to broad-spectrum cephalosporins, hence the name "E-spectrum β-lactamases". In the late of 1980s, this new term was assigned to TEM and SHV. Once they are commonly seen in *E.coli* and *Klebsiella spp*., but now they were documented worldwide and reported t in other members of the *Enterobacteriaceae* and in NFGNB such as *A. baumannii* and *P. aeruginosa* (Jacoby and Munoz-Price, 2005).

ESβLs are plasmid-mediated β-lactamase produced by certain Gram-negative bacteria that confer significant resistance to penicillin, narrow- and extended-spectrum (third and fourth generation) cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) and monobactams (aztreonam) except carbapenems and cephamycins; β-Lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam are generally inhibit ESβL-producing strains. These bacteria are frequently co- or multi-resistant, exhibiting resistance to other antimicrobial classes such as fluoroquinolones, aminoglycosides, and trimethoprim-sulphamethoxazole (Heuvelinka *et al*., 2019). This resistance was caused by point mutations in the amino sequence of the classical plasmid mediated TEM-1, TEM-2, OXA-1 and SHV-1 β-lactamases. With the exception of OXA-type enzymes (which are class D enzymes), the ESβLs are of molecular class A (Paterson and Bonomo *et al*., 2005).

III.2.1.1.2. Types

a. Ambler class-A ESβLs

- **TEM-type**

TEM refers to the name Temoneira. This designation was made because TEM-1 was first reported named *Tem*oneira in 1965 from an *E.coli* isolate from a patient in Athens, Greece (hence the designation TEM). This enzyme has substrate and inhibition profiles similar to those of SHV-1. It is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid. The first derivative of TEM-1 was TEM-2; it had a single amino acid substitution and has the same hydrolytic profile as TEM-1 (Barthelemy *et al*., 1985) but differs by a difference in isoelectric point. TEM-1, TEM-2 are not ESβLs.

The TEM-type ESβLs are derivatives of TEM-1 and TEM-2. TEM-3 was the first TEM variant reported with increased activity against extended spectrum cephalosporins; arose from point mutations in the TEM-2 gene which resulted in two amino acid changes: glutamic acid to lysine at position 104 and glycine to serine at position 238 (Sougakoff *et al*., 1988a). TEM-3 was originally named CTX-1 because of its enhanced activity against cefotaxime. This plasmid-mediated β-lactamase was detected in France in *Klebsiella pneumoniae*. In retrospect, TEM-3 may not have been the first TEM-type ESβL (Soughakoff *et al*., 1988b). In 1982, in Liverpool (England), a *Klebsiella oxytoca* strain came from a neonatal unit which had been stricken by an outbreak of *K. oxytoca* producing TEM-1 (Du Bois *et al*., 1995) harboring a plasmid carrying a gene encoding ceftazidime resistance. The β-lactamase responsible of this resistance was what is now called TEM-12 (Paterson and Bonomo *et al*., 2005).

- **SHV-type**

SHV refers to Sulfhydryl variable. This designation was made because it was thought that the inhibition of SHV activity by *p*-chloromercuribenzoate was substrate-related, and was variable according to the substrate used for the assay (Sykes and Bush, 1982). The first ESβL described, SHV-2, was identified in an isolate of *Klebsiella ozaenae* in Germany in 1983. Analysis of the SHV-2 gene showed that it was a result of a point mutation in the SHV-1 gene which resulted in an amino acid change from glycine to serine at position 238. The SHV-type ESβLs may be more frequently found in clinical isolates than any other type of ESβLs (Jacoby *et al*., 1997).

- **CTX-M-type**

CTX reflects the potent hydrolytic activity of these β-lactamases against cefotaxime. In 1986, a non-TEM and a non-SHV ESβL cephalosporinase was discovered in a cefotaxime-resistant *E. coli* (Matsumoto *et al*., 1988). In 1989, cefotaxime-resistant strains were found in Germany, France and Argentina (Bauernfeind *et al*., 1990; Bauernfeind *et al*., 1992). The CTX-Ms showed a much higher degree of activity to cefotaxime than to ceftazidime (Walther-Rasmussen and Hoiby 2004). The origin of the CTX-M enzymes is different from that of TEM and SHV ESβLs. While SHV-ESβLs and TEM-ESβLs were generated by amino acid substitutions of their parent enzymes, CTX-M ESβLs were acquired by the horizontal gene transfer from other bacteria using genetic apparatuses such as conjugative plasmid or transposon. CTX-M type ESβLs are subdivided into five groups on the basis of amino acid

sequence similarity: CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group, and CTX-M-25 group (Shaikh *et al*., 2015).

- **PER-type**

PER refers to *Pseudomonas* Extended Resistance. The PER-1 enzyme, initially discovered in 1993 in *P. aeruginosa* in Turkey. It was common in *P. aeruginosa* and *Acinetobacter spp*. but was also detected in *S. enterica*, *serovar typhimurium*, *Providencia spp*., *Proteus mirabilis* and *Alcaligenes faecalis*. This enzyme is weakly associated to other ESβLs and confers resistance to Penicillins, cefotaxime, ceftazidime, and aztreonam but variably to carbapenems and cephamycins. Its activity is well inhibited by clavulanate, sulbacteram and tazobactam. These enzymes share low sequence homology (25–27%) with TEM, SHV or CTX-M enzymes (Bauernfeind et al., 1996). PER-2 was found in *S. enterica* strains in Argentina in 1996, and subsequently in other Gram-negative bacteria. PER-2 enzyme share 86% of amino acid homology with PER-1 (Naas *et al*., 2008).

- **VEB-type**

VEB refers to Vietnam Extended-spectrum β-Lactamase. The enzyme VEB-1 (38% identity with PER-1) was first described in 1996 in an isolated *E. coli* strain from a Vietnamese patient and then in *P. aeruginosa* in Thailand (Naas *et al*., 2000). Since then, VEB-1 was detected in *P. aeruginosa* in Kuwait, China, India and Bangladesh and in *A. baumannii* in France, Belgium and Argentina and *P. stuartii* in Algeria, *Enterobacter cloacae* and *Achromobacter xylosoxiin* in France, and *E. coli* in Canada. Several national epidemics have been reported: *A. baumannii* VEB-1 in France and Belgium; *P. Mirabilis* VEB-1 in South Korea; and *E. cloacae* in China (Naas *et al*., 2008). The VEB-1 β-lactamase confers a higher level of resistance to ceftazidime than to cefotaxime. The $bla_{\text{VEB-1}}$ was a class 1 integron located n the chromosome or on plasmids (Cae *et al*., 2002) and can therefore be associated with other resistance genes such as qnrA, a plasmid determinant of resistance to quinolones (Naas *et al*., 2008).

- **GES-type**

GES refers to Guyana extended spectrum β-lactamases. GES-1 was reported for the first time in 1998 in a strain of *K. pneumoniae* isolated from a rectal specimen of a hospitalized newborn in the Guyenne hospital and then it reported in Argentina, Brazil, Portugal and the Netherlands. GES-type ESβLs are increasingly isolated from GNB including *P. aeruginosa*, *E.*

coli, and *K. pneumoniae* (Naas *et al*., 2008). GES has a hydrolysis profile similar to class A ESβLs, including activity on Penicillins and broad-spectrum cephalosporins, but not on cephamycins, aztreonam, and carbapenems, and is inhibited by clavulanate and tazobactam (Poirel *et al*., 2000, Poirel *et al*., 2008).

- **GES-22**

In 2014, two strains recovered in 2011 carried GES-22 from Turkey. This enzyme had an extended-spectrum β-lactamase profile (Castanheira *et al*., 2014). bla_{GES-22} gene had one amino acid substitution compared to GES-11 (M169L; 99.7% identity), showing the greatest homology, and two amino acid differences compared to GES-1 (G243A and M169L; 99.3% homology). GES-11 was initially described from an *A. baumannii* isolate recovered during 2008 in a French hospital, and this gene was carried in a class 1 integron that also harbored *aac*(*6'*)-Ib and *dfrA7*. This substitution confers on GES-22 more efficient hydrolysis of the mechanistic inhibitors, clavulanic acid and sulbactam (Saral *et al*., 2016).

b. Ambler class-D ESβLs

- **OXA-type**

The denomination OXA was due to the capacity of this group to hydrolyze isoxazolylpenicillin oxacillin faster than the classical penicillins and the fact that they are not inhibited by clavulanic acid and EDTA. However, today this definition is not valid because since enzymes have been recently described that inactivate Penicillins M (oxacillin, cloxacillin) weakly, but all OXA β-lactamases are active against amino and carboxipenicillins (Opazo *et al*., 2012).

Most OXA-type β-lactamases do not hydrolyze the third generation cephalosporins to a significant degree and are not regarded as true ESBLs. However, OXA-10 one of many derivates of the classical plasmid-mediated OXA-1 β-lactamase, weakly hydrolyzes cefotaxime, ceftriaxone and aztreonam, giving reduced susceptibility to these antibiotics. Other OXA-type ESβLs include OXA-11, OXA-14, OXA-17 (Paterson and Bonomo, 2005). These enzymes are most commonly found in *P. aeruginosa*, but they have also been reported from other Gram-negative bacteria (Paterson and Bonomo *et al*., 2005).

- **OXA-1**

OXA-1 β-lactamase, like most OXAs, hydrolyzes amino and ureidopenicillins (piperacillin) significantly and hydrolyzes narrow-spectrum cephalosporins weakly. Morever, *it* hydrolyzes broad-spectrum cephalosporins, conferring reduced susceptibility to cefepime and

cefpirome (Sugumar *et al*., 2014). OXA-1 is exhibiting resistance to the clinically available β-lactamase inhibitors (tazobactam, sulbactam, and clavulanate) (Che *et al*., 2014). The $bla_{\text{OXA-1}}$ gene has been found in plasmid and integron locations in a large variety of Gram-negative organisms. This gene has frequently been found to be associated with genes encoding the worldwide-spread CTX-M-15 ESβL. This association of $bla_{\text{OXA-1}}$ with $bla_{\text{CTX-M}}$ genes makes isolates resistant to β-lactam-β-lactamase inhibitor combinations (Sugumar *et al*., 2014). The location of the $bla_{\text{OXA-1}}$ gene was in a Tn*21*-derived transposon, inserted between the *aad* gene encoding aminoglycoside resistance and its promoter (Evans and Amyes, 2014).

III.2.1.2. Carbapenemases

III.2.1.2.1. History and Definition

Carbapenems are bactericidal antibiotics often used as a last treatment option for severe infections caused by isolates producing by ESβLs and AmpC producers (Wadekar and Swarooparani, 2017). However, extensive use of these molecules has facilitated the emergence of carbapenem resistant bacteria and hence the production of carbapenemases. From the first report of these enzymes in the 1980s, the last decade is marked with the rapid spread of carbapenemases-producing organisms, becoming a public health concern worldwide. Carbapenemases are produced mostly by *Enterobacteriaceae* and NFGNB such as *P. aeruginosa* and *A. baumannii* (Bedenić and Sardelić, 2018).

III.2.1.2.2. Types

a. Ambler class-A carbapenemases

- **GES-type**

The family of GES β-Lactamase have a high activity towards carbapenems, when this activity is low, these enzymes are considered as ESβLs (Walther-Rasmussen and Hiby, 2007). Unlike most of ESβLs, GES-1 does not hydrolyze aztreonam and, above all, GES-2 hydrolyzes carbapenems by being less susceptible to β-Lactamase inhibitors. By a single mutation in the omega loop (G170N), GES-2 is the first example of ESβLs with an expansion of the activity spectrum to carbapenems.

- **KPC-type**

KPC refers to *Klebsiella pneumonia* Carbapenemases. The first KPC-1-producing strain was isolated in 1996 in South Carolina. It was a strain of *K. pneumoniae* resistant to all β-

lactams. This first description was quickly followed by the publication of another variant KPC-2. Then seven other variants have been reported (KPC-3 to KPC-9), distinguished by at least two amino acid substitutions (Cuzon *et al*., 2010). Phenotypically, KPC-1, KPC-2 and KPC-3 confer a high level of resistance to C3G. The KPC genes are plasmid and readily transmitted by conjugation (phillipon and Arlet, 2006), these latter frequently carry other resistance genes including aminoglycosides and/or fluoroquinolones, sometimes even other β-lactamases CTX-M-15. KPC-type enzymes are capable of hydrolyzing almost all β-lactamines: Penicillins, cephalosporins, carbapenems, and monobactam. Only the activity of cephamycins and ceftazidime is poorly altered. Among the third-generation cephalosporins, cefotaxime is the most hydrolyzed antibiotic, and among the carbapenems, KPC possesses the highest affinity for meropenem, they are weakly inhibited by clavulanic acid and tazobactame (Cuzon *et al*., 2010).

b. Ambler class-B carbapenemases

The class B β-lactamases represent the metallo-β-lactamases and were found 40 years ago. They are clinically the most relevant carbapenemases. These enzymes are able to degrade all classes of β-lactams except monobactams. Additionally, they have an efficient activity against carbapenems (Bedenić and Sardelić, 2018). Those MβLs are usually located within different integron structures, and these integrons are associated with mobile plasmids or transposons facilitating the transfer of resistance genes between bacteria. The metallo6β-lactamases are resistant to therapeutic β-lactamase inhibitors. The metallo-β-lactamases are zinc-dependant and so called EDTA-inhibited enzymes and are mostly found in *Pseudomonas sp.* and *Serratia sp.* (Bebrone, 2007). The metallo-β-lactamases are predominantly chromosomally encoded but can also be found on plasmids. The most frequently clinically relevant MβLs are Verona integron-encoded (VIM), imipenemase (IMP), and New Delhi MβL (NDM).

c. **Ambler class-D carbapenemases**

- **OXA-type**

Class D enzymes, also referred to as OXA-type. The first β-lactamase of this type is OXA-23 (ARI-1), initially identified in *A. baumannii* was obtained from a strain isolated in 1985 in a Scottish hospital, named ARI-1 (Acinetobacter Resistant to Imipenem) (Paton *et al*., 1993). They can be subdivided into five subgroups, namely the OXA-23, -24/40, -48 and -58 carbapenemases, which are mainly plasmid encoded, and the OXA-51 carbapenemase, which

is chromosomally encoded and intrinsic in *A.baumannii*. Class D enzymes are not inhibited by clavulanic acid or EDTA (Bebrone, 2007).

III.2.2. Active site and mechanism of action

III.2.2.1. Serine β-lactamases

According to Ambler, serine β-lactamases (SβLs) are grouped into three class A, C and D (Ambler 1980). The comparison of the primary structures of serine β-lactamases shows the existence of functional amino acids and/or conserved in the composition of elements forming the active site of these enzymes. Element 1 is an amino acid tetrad. However, element 2 and 3 are a triad amino acid (**Table. 1**) (Lamotte-Brasseur *et al*., 1995). These protected regions are known to be involved in the formation of hydrogen bonds and other bonds which are involved in stabilizing the substrate-enzyme interaction during enzyme reactions. Confused, they participate directly or indirectly in the hydrolysis of beta-lactamine (Charlier *et al*., 1998).

Serine β-lactamases catalyze the cleavage of the amide linkage of the betalactam ring through a serine residue of their active site in a three-step reaction. In a first step, the enzyme and beta-lactam interact to form the non-covalent Michaelis-Menten complex. In a second step called the acylation step, following a necleophilic attack of the carbonyl of the β-lactam ring by the hydroxyl group of the amino acid serine, a covalent acyl-enzyme complex is formed. In a third step called deacylation step, the intervention of a water molecule previously activated or deprotonated by an amino acid which may be a lysine, or glutamate, or a tyrosine, causes the dissociation of the acyl-enzyme complex and formation acidic compounds such as penicilloic acid or cephalosporoic acid which are inactive (Chen and Herzberg, 2000, Minasov *et al*., 2000).

Table 1. Comparison of the three conserved regions in group A, C and D active-site serine β-lactamases according to the Ambler classification.

Ambler class	Conserved elements		
	Element 1	**Element 2**	**Element 3**
Groupe A	Ser^{70} -X-X-Lys (S-X-X-K)	Ser^{130}-Asp-Asn/Ser (S-D-N) (S-D-S)	Lys/Arg^{234}-Thr/Ser-Gly (K-T-G, K-S-G, R-S-G, R-T-G)
Groupe C	Ser^{64} -X-X-Lys (S-X-X-K)	Tyr^{150}-Ala-Asn (Y-A-N)	Lys^{314} -Thr-Gly (K-T-G)
Groupe D	Ser^{70} -X-X-Lys (S-T-F-K)	Tyr^{144}-Gly-Asn (Y-G-N)	Lys^{214} -Thr-Gly (K-T-G)

III.2.2.2. Metallo-β-lactamases

Ambler class B groups are metallo-β-lactamases, which require one or two zinc ions in their active site for their activity (Philippon *et al*., 2016). This group of enzymes is subdivided into three subclasses B1, B2 and B3 according to the percentage of homology of primary structures (Gonzalez *et al*., 2007). It ranges from approximately 25% to 40% in the same subclass and from 10% to 20% between two subclasses (Garau *et al*., 2005). The alignment comparison of the primary structures reveals the existence of four residues strictly conserved among all the MBLs (His^{118}, Asp^{120}, His^{196} and His^{263}) (Rossolini and Docquier, 2007).

Several catalytic mechanisms have been proposed for mono and binuclear active-site MβLs. Abriata *et al*. (2008) proposed a mecanisme of hydrolysis of a class B1 MβL (BCII), an MβL with an active site that can be mono or bi-nuclear. In the case of the mono-nuclear active site, the main stages of the catalytic mechanism are: (i) the formation of the Michaëlis complex: the OH of the water deprotonated by the asparagine residue at the position 120, established a bond with the Zn ion and Attacks the carbonyl carbon of the β-lactam ring. This leads to the formation of a negatively charged tetrahedral intermediate complex. The four bonds established by the zinc ion serve as a stabilizer for this intermediate. (ii) Catalysis step: Asp120 residue donates a proton to the nitrogen of the β-lactam ring resulting in the cleavage of the C-N bond of this ring and its opening. (iii) Release of the product: the C-N bond cleavage of the β-lactam ring ionizes the carbonyl carbon of the same ring allowing it to establish a double bond with oxygen charged with the intermediate complex formed following the first step. This results in the release of the product and the active site of the enzyme (De Seny *et al*., 2002).

In the case of the bi-nuclear active site, the main stages of the catalytic mechanism are always the same: (i) the formation of the Michaelis complex: the hydroxyl bridging ion Zn1-Zn2 is responsible for the nucleophilic attack, which results to a negatively charged intermediary. (ii) Catalysis: The zinc-bound apical water molecule is optimally positioned to protonate the nitrogen resulting in the cleavage of the betalactam ring C-N bond and its opening. (iii) Release of the product: the C-N bond cleavage of the betalactam ring ionizes the carbonyl carbon of the same cycle allowing it to establish a double bond with oxygen charged with the intermediate complex formed following the first step. The newly formed hydroxide ion of Zn2 gives way to a molecule of water to bind to Zn1 followed by dissociation of the product from the active site of the enzyme (Gonzalez *et al*., 2007; Abriata *et al*., 2008).

IV. Molecular Docking

The research and the development of a drug molecule "drug discovery" is often an extremely long, tedious and very expensive process at high risk of failure. For each new drug, the time elapsing between the first step, during which a relevant biological target in a given disease process is identified, until a drug is placed on the market is estimated at an average of 12 to 14 years with a minimum overall cost of 800 million of dollars (Morgan *et al*., 2011). An alternative approach, such as virtual screening or rational drug design, are now routinely used to guide drug discovery. The use of these *in silico* techniques could improve the efficiency and save the cost of drug discovery (Bai *et al*., 2014).

Virtual screening approaches based on the structure of the target, more specifically the molecular docking, predict possible modes of interaction between a ligand and the therapeutic target and provide a way of studying interactions at the molecular level and are, therefore, an indication of the biological activity of new molecules taking into account only structural criteria (Cheng *et al*., 2007). Molecular docking is considerably easier to implement, cheaper and faster than using the experimental methods. Docking software is therefore very useful tools in biology, pharmacy and medicine, because most of the active pharmaceutical ingredient are small molecules (ligand) that interact with a biological macromolecule of therapeutic interest, usually protein (target), in order to influence the mechanism in which this protein is involved. It consists in predicting and reproducing protein-ligand interactions.

IV.1. Docking methods and scoring functions

Protein-ligand docking involves two complementary steps. The first is to look for complex conformation prediction of the ligand able to establish ideal interactions with the receiver (docking algorithm) The second is the evaluation of these conformations by mathematical functions called score functions, it is based on a quick calculation of their interaction energy with this receiver (scoring function). Docking can be interpreted qualitatively by observing the ligand in the cavity protein and quantitatively by processing the data from score functions.

IV.1.1. Docking methods

Protein-docking involves a large amount of calculation. For this, different algorithms have been developed to predict the protein-ligand interactions. Classically, docking quality is judged by measuring the RMSD, called redocking test (Root Mean Square Deviation) on the

heavy atoms between the pose obtained in docking, and the pose of the original molecule. Based on the treatment of algorithms of ligand flexibility, the searching algorithms of the ligand flexibility can be divided into three basic categories: systematic conformational search, stochastic (or random) search and simulation (or deterministic) search (Huang and Zou, 2010).

IV.1.1.1 Systematic search

Systematic search protein-ligand docking algorithms allow ligands to rotate in all directions from 0 to 360 °, using an incremental step selected, which often will lead to high cost on future evaluation time. The advantage of this method is that it can evaluate all the possible interactions between protein and ligand. As a result, this quickly causes a very significant increase in number of conformations created that it calls combinatorial explosion (Sousa *et al*., 2006). One of the methods to deal with this problem is to define an active site region and let the ligand just rotate within this site, which can greatly reduce the amount of calculation. Another way is to divide the ligand into rigid and flexible fragments. Docking these fragments separately into the active site and then link them together to rebuild the ligand (**Figure. 1**). At first the ligand is cut into rigid and flexible parts. Between the points where rotations are possible, one or more rigid "anchors" are defined, then a first rigid part is put in interaction with the receiver then the flexible parts are added of successively with an exploration of the torsion angles. The most important is the choice of basic fragments to be placed first in the site because it is very difficult for the algorithms to compensate for a bad initial position. This type of algorithm is used in several docking programs such as Dock (Rarey *et al*., 1996) and FlexX (Apfel *et al*., 2001).

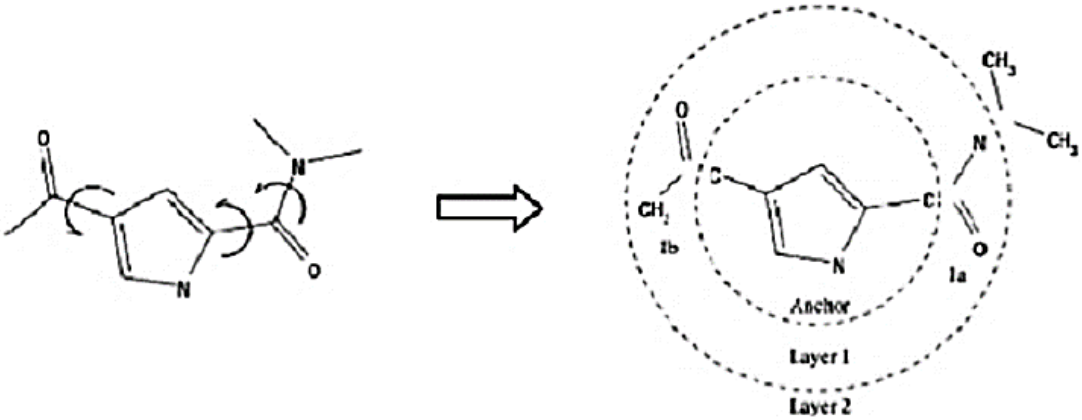


Figure 1. Demonstration of an incremental reconstruction whose molecule is fragmented and then docked and rebuilt layer by layer in the active site (Ewing *et al*., 2001).

IV.1.1.2. Stochastic search

The stochastic approach consists of making random changes in the structure or the position of ligand, which are accepted or rejected on the basis of a probability function "fitness" (Kitchen *et al*., 2004). One of the weakness points of this method is the uncertainty of convergence. To avoid this, it is necessary to multiply the calculations, independently of each other. There are four types of stochastic algorithms: Monte Carlo (MC) methods, evolutionary algorithms (EA), Tabu search methods, and swarm optimization (SO) methods (Huang and Zou, 2010).

IV.1.1.3. Simulation search

In the simulation search, the initial state of the ligand determines the movements to be performed to generate the next state. This state must be equal to or less than the initial state. The problem of this search is that some choice of initial state will lead to local minima instead of the real near-native structure. Another issue is that it normally requires high computational cost to get the potential protein-ligand complex structure. CHARMM (Chemistry at HARvard Macromolecular Mechanics) is a highly versatile and widely used program for molecular simulation and modeling. It uses energy minimization techniques to optimize the conformations, performs a normal mode or molecular dynamics simulation, and analyzes the simulation results to determine structural, equilibrium, and dynamic properties (Brooks *et al*., 2009).

IV.1.2. Scoring functions

Docking results usually consist of different positions of the ligand in the site interaction, corresponding to several favorable conformations that the ligand can adopt. Scoring methods make it possible to evaluate the binding energy of the complex formed and to give a score to the poses obtained during the docking step (Gohlke and Klebe, 2002). The score is a numerical datum useful for quantifying the degree to which a ligand is complex to a receiver. This is an approximation of the free energy resulting from transition from the free form of the protein and the ligand to the combination as a complex. The thermodynamic principle is as follows:

$$\Delta G = \Delta G_{complex} - \Delta G_{ligand} - \Delta G_{protein}$$

Scoring functions can be divided into the following three categories: force-field-based, empirical, and knowledge-based scoring functions.

IV.1.2.1. Force-field-based

Force field scoring functions are a mathematical function that expresses the energy of a system by the sum of various terms derived from molecular mechanics. Its use as a score function in the calculating of the free energy of a system is the result of the work of Prof. Irwin Kuntz at the University of California at San Francisco USA (Kuntz *et al.*,1982), which was followed by others (*Totrov and Abagyan, 1997; Rester, 2006)*. The force-field-based scoring function can evaluate the potential energy of a system, as the sum of different particles (ligand and protein) in the system. The score is calculated by summing the protein-ligand interaction (intermolecular interactions) energy and the energy internal ligand (Ferrara *et al*., 2004).

IV.1.2.2. Empirical

Empirical scoring functions calculate the free energy of binding for protein-ligand complexes was developed by Hans-Joachim Bohm (Bohm, 1994). These scoring functions describing the ionic interactions, hydrophobic interactions, bridges or hydrogen bonds and interactions generated by the change of entropy. These functions sum up these different terms by weighting them with terms describing the different types of molecular interactions (Perez *et al*.,1998).

IV.1.2.3. Knowledge-based

Knowledge-based scoring functions are built from rules based on structural analysis of three-dimensional experimental models of protein-ligands complexes to statistically analyze preferences of interactions between different type of atoms or chemical groups. These methods employ the assumption that highly observed types of interactions are energetically favorable (Muegge *et al*., 2006). Not like empirical methods, knowledge-based methods do not need any additional analysis on the training dataset, which reduces the amount of calculation. But it is limited by the size of the database used.

IV.2. Docking Algorithms used

LibDock and CDOCKER, two common docking algorithms in Discovery Studio software utilized to evaluate the applicability for the docking studies.

IV.2.1. LibDock

The LibDock algorithm is an interface to the Discovery studio docking program that was developed by Diller and Merz (2001). This algorithm is one of the few commercially

available docking programs (Holloway *et al*., 2007) using protein binding site features referred to as HotSpots to guide docking. It has four functional aspects: first, conformation generation of the ligands, creating an image to the binding site (HotSpots identification), matching this image and the ligand, and a final optimization step is performed before the poses are scored (Rao *et al*., 2007). HotSpots consist of two types: apolar and polar. An apolar HotSpot is preferred by an apolar atom (for example a carbon atom). A polar Hotspot is preferred by a polar ligand atom (for example a hydrogen bond donor or acceptor).

The LibDock is a flexible docking module (Alam and Khan, 2018). It uses the systematic conformational search algorithm to dock ligands freely to the receptor and rank the compounds via the default scoring function LigScore (Krammer *et al*., 2005). Higher LibDock score means a high chance of ligand-protein binding (Alam and Khan, 2018).

IV.2.2. C-DOCKER

CDOCKER (CHARMM-based DOCKER) algorithm is a molecular dynamics (MD) simulated-annealing-based algorithm. It is an implementation of a CHARMM based docking tool. CDOCKER provides better accuracy than other methods. However, it is still among the more compute-intensive methods (Gagnon *et al*., 2016). CDOCKER energy (Kcal/mol) and CDOCKER energy interaction (Kcal/mol) were the scoring function of the CDOCKER algorithm. The CDOCKER energy indicated the energy of the ligand-protein complexes, and the CDOCKER energy interaction indicated the energy of the ligand (Duan *et al*., 2014).

V. Studied seaweeds

Algae are photosynthetic organisms with a great diversity of forms and sizes. They can exist from unicellular microscopic organisms (microalgae) to multicellular of great size (macroalgae or seaweeds). Some algae are organisms that live in complex habitats submitted to extreme environmental conditions (salinity, temperature, nutrients, UV-Vis irradiation...). To survive in these adverse conditions, they must adapt rapidly by producing a great variety of secondary (biologically active) metabolites, which cannot be found in other organisms. So, Algae can be an interesting natural source of novel compounds with biological activity (Munir *et al*., 2013). In the present study, Seven Algerian seaweeds (*Ulva intestinalis, Codium tomentosum, Dictyota dichotoma, Halopteris scoparia, Sargassum vulgare, Cystoseira*

compressa and *Bryopsis corticulans*) were chosen according to different criteria as a potential and promising source of β-lactamases inhibitors.

V.1. Green seaweeds

V.1.1. *Ulva intestinalis* Linnaeus, 1953

V.1.1.1. Description and Taxonomy

Ulva intestinalis is a green macroalgae known as intestinal herb with length of 10-15cm and width of 1-8 mm, characterized by smooth tubular shape similar to the intestine usually unbranched (**Figure. 2**). This seaweed is floating in aquatic habitats and appears as green to dark green when becoming aggregated near the shore. Is consists of semi-circular cells of 10-22µm diameter, with vegetative cells containing laminated chloroplast (Ruangchuay et *al*. 2012). This alga grows through the year, which is widely distributed, especially in the Asian countries and grows in salt water habitats, brackish water and fresh water (Bellinger and Sigee, 2015). This species belongs to the kingdom of Plantae, phylum of Chlorophyta, class of Ulvophyceae, order of Ulvales, and to the Family of *Ulvaceae* (Guiry et *al*., 2018).

Figure 2. Photograph of *Ulva intestinalis*

V.1.1.2. Biological activities

Previous studies demonstrated that *U. intestinalis* has many biological activities. Jiao *et al*. (2009) reported that polysaccharides compounds isolated from this species tested in *vitro* and in *vivo* against tumor cell type Sarcoma has been effective against the tumor. Polysaccharides compounds have been also proved their effectiveness as anti-tumor and immunomodulating in *vivo* by using laboratory rats (Jiao et *al*., 2010). Another study of Paul and Kundu, (2013) examined the activity of the methanolic extract of *U. intestinalis* against

HeLa cancer cells in *vivo*, showed that this extract was effective against cancer cells. Al-Jaber et *al*. (2015) evaluated the cytotoxic activity of two extracts on HeLa cells in *vitro*. They reported that both extracts are effectiveness on cancer cells with the superiority of methanol extract. Wang et *al*. (2014) reported that sulfated polysaccharides compounds isolated from this species was active against hepatoma HepG2. In addition, acetone extract of *U. intestinalis* revealed a highly effective on Human colon carcinoma LS174 cells, Human lung carcinoma A549 cells, malignant melanoma Fem-x and Chronic myelogeneous leukemia K562 (Kosanic et *al*. 2014).

V.1.2. *Codium tomentosum* Stackhouse, 1797

V.1.2.1. Description and Taxonomy

Codium tomentosum is a green macroalgae, that is organized in branches that form spongy, dark green, coenocytic thalli of intertwined filaments (**Figure. 3**), being commonly found on exposed rocks and/or deep rock pools on the lower seashore. It is native to the North East of the Atlantic Ocean but has also been found around the coasts of Africa and in various other parts of the world (Guiry et *al*., 2018; Regoa et *al*., 2014). Phylogenetic studies place this species in the kingdom of Plantae, phylum of Chlorophyta, class of Ulvophyceae, order of Bryopsidales, and the Family of *Codiaceae* (Muller, 2004; Guiry et *al*., 2018).

Figure 3. Photograph of *Codium tomentosum*

V.1.2.2. Biological activities

Codium species are known to be an important source of bioactive compounds with many properties, such as antioxidant, antigenotoxic, antitumor and hypoglycemic activities (Valentao

et *al*., 2010, Lordan et *al*., 2011). In fact, these seaweeds species and their extracts have demonstrated strong antioxidant activity (Yuan and Walsh, 2006). Powerful antioxidant molecules found in macroalgae like proteins, phenolic compounds, such as flavonoids and coumarins, tocopherols, nitrogen containing compounds including alkaloids, chlorophyll derivatives, amino acids and amines, as well as other compounds like carotenoids, ascorbic acid, glutathione and uric acid have been elucidated in this species (Celikler et *al*., 2009). However, anti-tumourigenic (El-Masry et *al*., 1995) and hypoglycemic activities (Lamela et *al*., 1989) have been attributed for *Codium tomentosum*. In addition, Augusto *et al*., (2016) investigated the application of edible seaweeds *Codium tomentosum* extracts as post-harvest treatment in minimally processed Fuji apple fruit.

V.1.3. *Bryopsis pulmosa* (Hudson) C.Agardh, 1823

V.1.3.1. Description and Taxonomy

Bryopsis pulmosa is a green macroalgae consisting of tubular multinucleate (siphonous) axes. Its thallus has a cylindrique ramified axis, which can measure between 3 and 15 cm in height. Lateral ramules are arranged in almost regular rows on both sides of the axis. The diameter of the axis and ramules decreasing in size towards the apex gives them the appearance of a feather-like barbs (**Figure. 4**) (the term plumosa comes from Latin and means" that looks like a feather" (Cabioc'h *et al*., 1992). This species belonging to the kingdom of Plantae, phylum of Chlorophyta, class of Ulvophyceae, order of Bryopsidales, and the Family of *Bryopsidaceae* (Guiry et *al*., 2018).

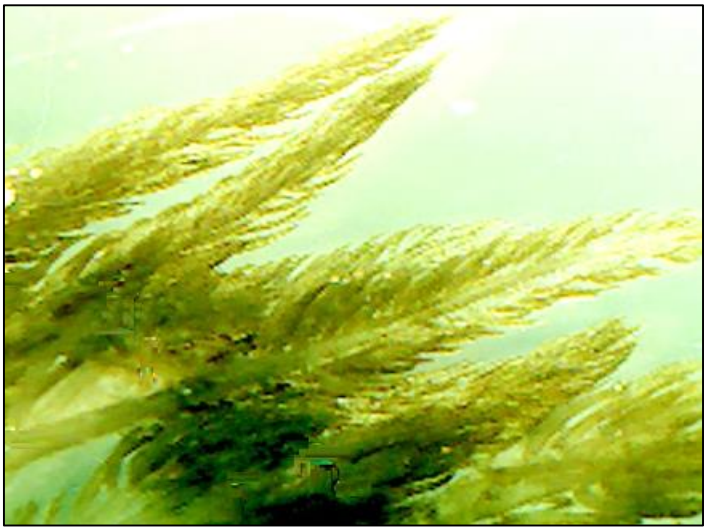


Figure 4: Photograph of *Bryopsis plumosa*

V.1.3.2. Biological activities

A number of algal lectins were considered as potential microbicide candidates to prevent sexual transmission of HIV through topical applications (Huskens and Schols, 2012). Algal lectins are used in biomedical research for antiviral, antinociceptive, anti-inflammatory, anti-tumor activities, etc. and in pharmaceutics for the fabrication of cost-effective protein expression systems and nutraceutics. *B. plumosa* is a good source of lectins. A D-mannose specific lectin have been purified from *B. plumose. The agglutinating activity of horse and sheep erythrocyte was inhibited by this lectin* (Han *et al*., 2011). Lectins have interesting chemical properties in relation to immunological and cancer applications. Human A-type specific agglutinating activity has been reported from *B. plumosa* (Jung *et al*., 2010). On other hand, Biju *et al.* (2014) reported that *B. plumosa* had antifeedant properties against larvae of moth *Hyblaea puera* and was able to reduce the protein and fat content of the treated larvae.

V.2. Brown seaweeds

V.2.1. *Sargassum vulgare* C. Agardh, 1820

V.2.1.1. Description and Taxonomy

Sargassum vulgare is is characterized by a short stem carrying primary branches (20 to 70 cm) and secondary branches (5 to 10 cm). The elongate "leaves", crossed by a median vein, denticulate and have at their base vesicles, yellowish, spherical and hollow constituting floats (**Figure. 5**). Irregular rhizoidal branches helps in attachment of alga to the substratum (Vishnupriya Sowjanya and Raja Sekhar, 2017). This species belonging to the kingdom of Chomista, phylum of Ochrophyta, class of Phaeophyceae, order of Fucales, and the Family of *Sargassaceae* (Guiry et *al*., 2018).

Figure 5. Photograph of *Sargassum vulgare*

V.2.1.2. Biological activities

Previous studies had demonstrated that alginates from *Sargassum sp*. showed a considerable activity against various murine tumors. The alginate and the biomass of *Sargassum spp.* have been reported to be suitable substrates for heavy-metal adsorption, and the adsorption parameters for Cd, Au, Cu, Fe, Ni, Pb, and Zn have been investigated (Torres et *al*., 2007). Davis et *al*. (2004) showed that the amount of guluronic acid and of uronic acid diads and triads are the critical parameters in the use of alginate from Sargassum *spp*. for metal biosorption strategies.An extract of *Sargassum vulgare* at 5% concentration has shown 78.8% syncytium inhibitory activity toward the human T-cell lymphotropic virus (HTLV-1), and it may be useful in preventing infection (Romanos et *al*., 2002). Another potential application of this seaweed is as biosorbent for heavy metals (Cd and Cu).

V.2.2. *Dictyota dichotoma* (Hudson) J.V.Lamouroux, 1809

V.2.2.1. Description and Taxonomy

Dictyota dichotoma is a cosmopolitan brown seaweed. It is also one of the most abundant seaweeds in tropical marine habitats (Siamopoulou et *al*. 2004). It is reported to occur in abundant amounts in southern coastal areas (Sohrabipour et *al*. 2004). *D. dichotoma* is a brown algae witch has a flat yellowish brown thallus (10-14 cm high and 2-8 mm wide), with a short flattened base and dichotomically divided thongs (= longitudinally and regularly divided into 2 parts) (**Figure. 6**), without rib with rounded or bilobed end. The seaweed attaches to the substrate by rhizoids which end in adhesive discs (Vishnupriya Sowjanya and Raja Sekhar, 2017). This species belonging to the kingdom of Chomista, phylum of Ochrophyta, class of Phaeophyceae, order of Fucales, and the Family of *Dictyotaceae* (Guiry et *al*., 2018).

Figure 6. Photograph of *Dictyota dichotoma*

V.2.2.2. Biological activities

Brown seaweeds produce different polysaccharides, namely alginates, laminarans and fucoidans. Rabanal et *al*. (2014) reported that the galactofucan fractions of *D. dichotoma* exhibited a moderate antiviral activity (against HSV-1 and CVB3). However, the remaining fractions were inactive against CVB3, but some of them, rich in xylose, mannose and uronic acid were also active against HSV-1. *D. dichotoma* showed an anti-cancer activity on MCF-7 cells with an IC_{50} of 17.2 ng. Flow cytometry analyses demonstrated that the methanolic extract of this species was strongly induced apoptosis and moderate viability inhibition on MCF10A cells with an IC_{50} of 49.3 ng mL-1 (Celenk et *al*., 2016).

V.2.3. *Halopteris scoparia* (Linnaeus) Sauvageau, 1904

V.2.3.1. Description and Taxonomy

After being subjected to sun-drying, *Halopteris scoparia* is currently made into commercial products consumed primarily in Portugal (Campos et *al*., 2019). It is characterised by shrub-like thallus (**Figure. 7**) with a brown colouration and about 20 cm high. It binds itself to the substrate with a fibrous and sponge-like basal disk. Its ramification is dense, irregularly alternate and distichous. Due to morphological features and environmental versatility *H. scoparia* is considered an excellent alga to host epifitic communities of associated macrofauna (Guerra-García *et al*., 2009). This species belonging to the kingdom of Chomista, phylum of Ochrophyta, class of Phaeophyceae, order of Sphacelareales, and the Family of *Stypocaulaceae* (Guiry et *al*., 2018).

Figure 7. Photograph of *Halopteris scoparia*

V.2.3.2. Biological activities

Various studies on the biological activities of *H. scoparia* have been reported. Significant antioxidant activity of *H. scoparia* was detected with *n*-hexane extract in DPPH and methanol extract in $ABTS^{+}$. Güner *et al*. (2019) reported that each *n*-hexane, chloroform and methanol extracts of *H. scoparia* L. Sauvageau cause a significant reduction in cell viability, especially in HeLa cells. When the apoptotic gene expressions were examined after treatment of extracts, the expression of many pro-apoptotic genes in both caspase-independent and caspase-dependent intrinsic and extrinsic pathways increased. These findings suggest that, considering that it had not led to irritation and toxicity in *vivo*, edible *H. scoparia* is a natural antioxidant and its apoptotic/cytotoxic activities can potentially be used against human cancers (Güner et *al.*, 2019). Kartal et *al*. (2009) have also prouved antioxidant and anticholinesterase activity of *H. scoparia* ethanol extract. Another study by Campos et *al*. (2019) reported that *H. scoparia* ethanolic extract demonstrated cyclooxyge-nase-2 (COX-2) inhibitory capacities between 40% and 79%, indicating anti-inflammatory activity.

V.2.4. *Cystoseira compressa* (Esper) Gerloff & Nizamuddin, 1975

V.2.4.1. Description and Taxonomy

Cystoseira compressa is a big brown algae (**Figure. 8**). Its height varies from a few cm in a beaten environment to more than 50 cm and up to 1 m in calm environment. It is fixed on the rock by a small discoid base from which there are several short, erect, cylindrical or flattened axes measuring 2 to 3 cm. These axes, which are practically non-existent in young people, carry long primary branches flattened at the base and branched in a plane. These branches then ramify to the third or fourth order. This species belonging to the kingdom of Chomista, phylum of Ochrophyta, class of Phaeophyceae, order of Fucales, and the Family of *Sargassaceae* (Guiry et *al*., 2018).

V.2.4.2. Biological activities

Several biological activities of the *Cystoseira* genus have been reported. The genus *Cystoseira* contains a wide variety of secondary metabolites, namely lipids, terpenoids, steroids, carbohydrates, phlorotannins, phenolic compounds, pigments and vitamins. About sixty interesting biological properties in *Cystoseira compressa* have been attributed to these compounds, which often displayed antioxidant, anti-inflammatory, cytotoxicity, anticancer cholinesterase inhibition, anti-diabetic, and anti-herpetic activities (Mhadhebi et *al*., 2012, 2014). Antibacterial, antifungal and anti-parasitic activities as antimalarial and antileishmanial

were also reported, but less often (Bruno De Sousa et *al*., 2017). *Cystoseira compressa* species is known to contain fucoidans and other compounds with bioactive properties, such as Antiinflammatory, antioxidant, and gastroprotective (Hadj Ammar et *al*., 2015, 2016).

Figure 8. Photograph of *Cystoseira compressa*

Material and Methods

MATERIAL AND METHODS

I. Biological material

I.1 Bacterial isolates

Our study took place during a period of 16 months from January 2015 to April 2016, it concerns both out and in-patients in different hospitals in Algeria. The isolates were obtained from the cultures of various pathological products (urine, pus, stool, blood culture, cerebrospinal fluid (CSF), breast and nipple discharge, catheters). Samples received at the laboratory are accompanied by an information sheet that includes: Full name, age, sex, hospitalization service and type of samples.

I.2 Seaweeds

The samples were collected in May 2015 by scuba diving, from the southern Mediterranean Sea (36 ° 35'N-2 ° 29'E) in the region located on the coast of Bou Ismaïl Bay, Kouali, Algeria **(Figure. 9).** The collected samples were kept in seawater at 0 ° C, after which the seaweeds were successively rinsed with water and distilled water to remove epiphytes and necrosis. The seaweeds identified as *Ulva intestinalis*, *Codium tomentosum*, *Bryopsis pulmosa*, *Sargassum vulgare*, *Dictyota dichtoma*, *Halopteris scoparia*, and *Cystoseira compressa* at the National Higher School of Marine Sciences and Coastal Management. Then they were dried in the shade and ground by a blender to a fine powder.

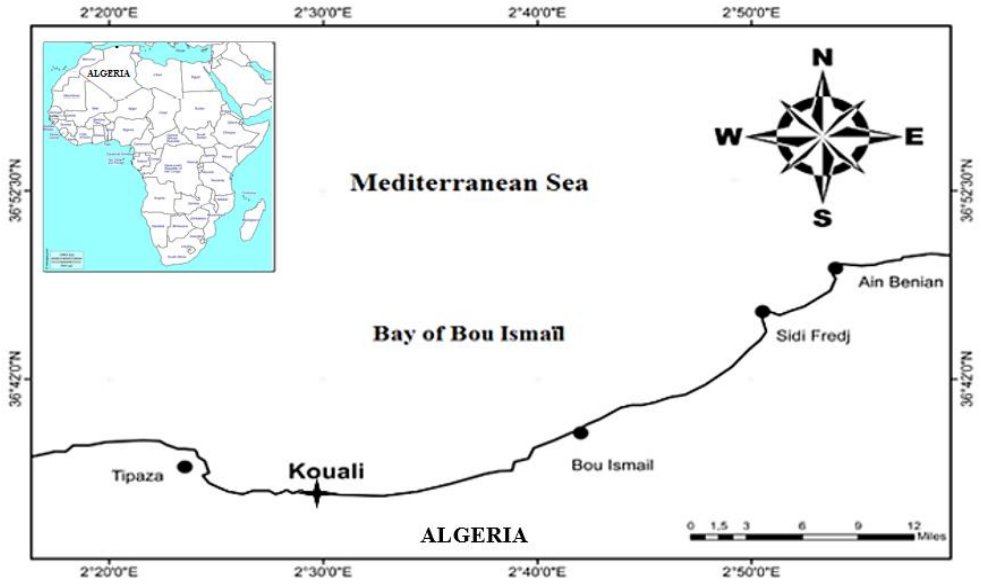


Figure 9. Geographical map centered on the sampling site (Kouali, Algeria)

II. Methods

II.1. Antibiotics susceptibility of MDR and detection of β-lactamases producing strains

II.1.1. Antibiotics susceptibility

The isolates were identified using API 20E/NE system (Bio-Mérieux, France), then the susceptibility test of 108 non-repetitive Gram negative isolates (*E.coli* (36), *K. pneumoniae* (21), *P. aeruginosa* (20), *A. baumannii* (20) and 11 other enterobacterial species) to β-lactams and other families of antibiotics was carried out by the standard Mueller Hinton agar diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2014).

Well-isolated bacterial colonies in the growth phase were transferred to tubes containing physiological saline until a turbidity close to that of Mc Farland 0.5 (corresponding to about 10^8 bacteria / ml) (Densimat, BioMérieux®). Subsequently, whole surfaces of Mueller Hinton agar previously cast into Petri plates were inoculated with this microbial suspension by the swabbing technique (Kirby-Bauer method). The antibiotic discs to be tested are deposited, and the plates are incubated for 16-18 hours at 35 °C.

To validate the results of the antibiogram, the control strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 are tested in parallel.

The interpretation in sensitive (S) intermediate (I) or resistant (R) is performed according to criteria defined by CLSI 2014 recommendations, after measuring the different diameters of the inhibition zones obtained around the discs of antibiotics. Strains with reduced diameter at third cephalosporins generation were retained. **Tables 2, 3 and 4** show lists of antibiotics tested.

Table 2. Abbreviation and Potency of antibiotic discs used with *Enterobacteriaceae*

Class	Antibiotic	Abbreviation	Potency (µg/disc)
β-lactams	Ampicillin	AMP	10 µg
	Amoxicillin / clavulanic acid	AMC	30 µg
	Ticarcillin	TIC	75 µg
	Piperacillin	PIP	100 µg
	Mecillinam	MEL	10 µg
	Cefazolin	CZ	30 µg
	Cephalothin	CF	30 µg
	Cefoxitin	FOX	30 µg
	Cefotaxime	CTX	30 µg
	Ceftazidime	CAZ	30 µg
	Cefepime	FEP	30 µg
	Imipenem	IPM	10 µg
	Ertapenem	ETP	10 µg
	Aztreonam	ATM	30 µg
Aminoglycosides	Amikacin	AN	30 µg
	Gentamicin	GM	15 µg
	Netilmicin	NET	30 µg
	Tobramycin	TM	10 µg
Quinolones	Nalidixic acid	NA	30 µg
	Ciprofloxacin	CIP	5 µg
	Levofloxacin	LVX	5 µg
Phenicol	Chloramphenicol	C	30 µg
Sulfamides trimethoprim	Trimethoprim/ Sulfamethoxazole	SXT	1,25/ 23,75 µg
Polypeptides	Colistin	CT	50µg
Tetracyclines	Tetracycline	TE	30 µg
Fosfomycins	Fosfomycin	FOS	50 µg
Furans	Nitrofurantoin	FT	300 µg

Table 3. Abbreviation and Potency of antibiotic discs used with *P. aeruginosa*

Class	Antibiotic	Abbreviation	Potency (µg/disc)
β-lactams	Ticarcillin	TIC	75 µg
	Piperacillin	PIP	100 µg
	Ticarcillin / Clavulanic acid	TCC	75/10 µg
	Ceftazidime	CAZ	30 µg
	Cefepime	FEP	30 µg
	Cefpirome	CPO	30 µg
	Imipenem	IPM	10 µg
	Aztreonam	ATM	30 µg
Aminoglycosides	Amikacin	AN	30 µg
	Gentamicin	GM	15 µg
	Tobramycin	TM	10 µg
	Netilmicin	NET	30 µg
Fluoroquinolones	Ciprofloxacin	CIP	5 µg
	Levofloxacin	LVX	5 µg
Polypeptide	Colistin	CS	10 µg
Ansamycin	Rifampicin	RD	30 µg
Fosfomycin	Fosfomycin	FOS	50 µg

Table 4. Abbreviation and Potency of antibiotic discs used with *A. baumannii*

Class	Antibiotic	Abbreviation	Potency (µg/disc)
β-lactams	Ticarcillin	TIC	75 µg
	Piperacillin	PIP	100 µg
	Ticarcillin / clavulanic acid	TIM	75+10 µg
	Ceftazidime	CAZ	30 µg
	Cefepime	FEP	30 µg
	Cefpirome	CPO	30 µg
	Imipenem	IPM	10 µg
	Aztreonam	ATM	30 µg
Aminoglycosides	Gentamicin	GM	15 µg
	Amikacin	AN	30 µg
	Tobramycin	TM	10 µg
	Netilmicin	NET	30 µg
Fluoroquinolones	Ciprofloxacin	CIP	5 µg
	Levofloxacin	LVX	5 µg
Tetracyclin	Doxycycline	DO	5 µg
Sulfamids	Trimethoprim / Sulfamethoxazole	SXT	1,25/ 23,75 µg
Polymyxin	Colistin	CS	10 µg
Ansamycin	Rifampcin	RD	30 µg

II.1.2. Detection of β-lactamases

II.1.2.1. Phenotypic detection

II.1.2.1.1. Extended Spectrum Beta-Lactamas

a. Double disc synergy test

The production of ESβLs was detected by the test of synergy (Jarlier *et al*., 1988). It consists to apply an AMC disc (20 µg amoxicilline+10 µg clavulanate) to a medium previously inoculated with Enterobacteriaceae at 30mm (Center a center) of a disc: ceftazidime (CAZ 30µg), cefotaxime (CTX 30µg), cefepime (FEP 30µg) and aztreonam (ATM 30µg). Plates were then incubated at 35±2 °C for 24 h. The presence of ESβLs is inferred when the inhibition zone around the AMC disc and the ceftazidime, cefotaxime, cefepime or aztreonam discs was enhanced on the side of the clavulanic-containing disc, resulting in a characteristically shaped zone referred to as champagne-cork, keyhole, ellipsis or phantom image.

b. Phenotypic confirmatory disc diffusion test

ESβLs derived from Ambler class A enzymes are inhibited by ß-lactamase inhibitors used in therapy (clavulanic acid, sulbactam and tazobactam). The association of ESβLs production with other resistance mechanisms such as hyperproduction of cephalosporinase make their detection more difficult. The purpose of the double-disc test is to confirm the presence or absence of ESβLs in strains that do not exhibit a synergistic image, with a decrease in the diameter of third-generation cephalosporins.

All bacterial species were subjected to the Phenotypic Confirmatory Test (PCDDT). In this test, four discs one of cefotaxime (CTX-30 µg) and other of ceftazidime (CAZ-30 µg), and each of these in combination with clavulanic acid, were placed on the surface of the lawn of bacteria.

After incubation at 35±2 °C for 16-18 h, a minimum difference of 5 mm between the zone of inhibition of a cephalosporin disc and in combination with clavulanic acid, referred to an as positive ESβLs as per Clinical and Laboratory Standards Institute guidelines (CLSI, 2014).

II.1.2.1.2. Carbapenemases

a. Modified Hodge Test

This test demonstrates the production of carbapenemase enzyme hydrolyzing imipenem Ambler Carbapenemase class A (KPC) and class B in strains resistant to carbapenem using a sensitive strain.

A bacterial suspension of *E.coli* ATCC 25922, was prepared at 0.5 MF and used to inoculate the agar plates. After 3 to 5 minutes, an ertapenem disc (10 µg) was placed in the center of the plate. The tested and two reference strains (*K. pneumoniae* ATCC BAA-1705: carbapenemase positive and *K. pneumoniae* ATCC BAA-1706: carbapenemase negative) were streaked in a straight line from the edge of ertapenem disc to the edge of the plate.

After overnight incubation at 35±2 °C, a clover leaf-like indentation of *E. coli* ATCC 25922 indicates the production of a carbapenemase that hydrolyzes imipenem by the strain tested (Lee *et al*., 2010).

b. Inhibition tests

These tests aim to classify the carbapenemases detected in imipenem-resistant strains, into Ambler class A or class B. Ambler class A carbapenemases can be detected by a synergy between boronic acid and carbapenems (one or more). This test was performed using an imipenem disc (10 µg) and a blank sterile disc soaked with 20 µl of phenylboronic acid solution (APB 400 µg). The test is positive when the difference between the inhibition zone of PBA is greater than or equal to 5mm in comparison with imipenem after incubation at 35±2 °C for 24 hours (Spyros *et al*., 2010).

The Metallo-beta-lactamases are carbapenemases that require one or two zinc ions for their catalytic function, and consequently are able to be inhibited by EDTA (divalent cation chelator). This chelation property is used in synergy tests to differentiate the metallo-β-lactamases (MβLs) from the other β-lactamases hydrolyzing the carbapenems. Practically, two discs spaced 25mm each containing 10µg of imipenem were applied to surfaces of MH inoculated with microbial suspension and a quantity of 730 µg of EDTA (0.5M, pH 8) (Junsei Chemical, Tokyo, Japan) was subsequently added to one of them. The test is positive when the inhibition zone around imipenem plus EDTA disc is greater than or equal to 7mm in comparaison with imipenem disc alone, after incubation at 35±2 °C for 24 hours (Yong *et al*., 2002).

II.1.2.2. Molecular detection

This study involved the molecular characterization of *bla*$_{\beta\text{-lactamase}}$ gene by PCR in 108 non-repetitive isolates phenotypically identified as ESβLs and/or carbapenemase producers: *E.coli* (36), *K. pneumoniae* (21), *P. aeruginosa* (20), *A. baumannii* (20) and 11 other enterobacterial species.

a. DNA extraction

Genomic DNAs of Gram (-) bacteria were obtained from overnight culture of bacteria grown in Luria-Bertani Broth with shaking incubator at 37 ˚C. A volume of 1.5 µl of bacterial suspension was centrifuged at 13.000 rpm for five minutes. Supernatant was removed, and pellet was suspended in 500 µl distilled water and subsequently boiled in a water bath for 10 minutes. Then, it was centrifuged at 13.000 rpm for 5 minutes and debris was removed. Supernatant was transferred into a new tube from which 5 µl was used as the DNA template for PCR reaction (Ausubel *et al*., 1995).

b. Amplification of the β-lactamase genes by PCR

PCR was used to amplify the ESβLs and carbapenemase genes and integrons using the primes listed in **Table 5**. PCR reactions were performed in 25 µL volume containing 2.5 µl of 10X PCR buffer (100 mM Tris-HCl [pH 8.8], 500 mM KCl, Triton X-100 to 1%), (Promega), 1.5 µl of $MgCl_2$ (25 mM, Promega), 1.25 µl of 4 dNTPs (4 mM stock, Promega), 1 µl of the primers positive and 1 µl of the negative primers, 0.2 µl (5U / µL) of the Hot Start Taq DNA polymerase (Promega), 5 µl of DNA and 12.55 µl of ultrapure water (Qiagen). The amplification reactions were performed as mentioned in **Table 6** (T100, Bio Rad). The amplification products were stored at 4 °C until use.

Table 5: Resistance genes with sequences of primers

Genes	primers	Sequence (5'→3')	Size of products (pb)	Ref
***bla*$_{TEM}$**	a216 (+) a217 (-)	F: AGTATTCAACATTTYCGTGT R: TAATCAGTGAGGCACCTATCTC	860	Cicek *et al*., 2013
***bla*$_{SHV}$**	os-5 (+) os- 6 (-)	F: ATGCGTTATATTCGCCTGTG R: TTAGCGTTGCCAGTGCTC	843	Cicek *et al*., 2013
***bla*$_{CTX-M1}$**	CtxM1 (+) CtxM1 (-)	F: GCGTGATACCACTTCACCTC R: TGAAGTAAGTGACCAGAATC	260	Cicek *et al*., 2013
***bla*$_{CTX-M2}$**	CtxM2(+) CtxM2 (-)	F: TGATACCACCACGCCGCTC R: TATTGCATCAGAAACCGTGGG	341	Cicek *et al*., 2013
***bla*$_{GES}$**	Ges (+) Ges(-)	F: ATGCGCTTCATTCACGCAC R: CTATTTGTCCGTGCTCAGGA	863	Moubareck *et al*., 2009
***bla*$_{PER-2}$**	Per (+) Per (-)	F: ATGAATGTCATCACAAAATG R: TCAATCCGGACTCACT	927	Celenza *et al*., 2006
***bla*$_{VEB}$**	Veb (+) Veb (+)	F: ATTTCCCGATGCAAAGCGT R: TTATTCCGGAAGTCCCTGT	542	Moubareck *et al*., 2009
***bla*$_{KPC}$**	Kpc (+) Kpc (-)	F: ATGTCACTGTATCGCCGTCT R: TTTTCAGAGCCTTACTGCCC	893	Poirel *et al*., 2004
***bla*$_{VIM}$**	Vim (+) Vim (-)	F: ATTGGTCTATTTGACCGCGTC R: TGCTACTCAACGACTGAGCG	780	Jeon *et al*., 2005
***bla*$_{NDM}$**	Ndm (+) Ndm (-)	F: GAGATTGCCGAGCGACTTG R: CGAATGTCTGGCAGCACACTT	497	Cicek *et al*, 2014
***bla*$_{IMP}$**	Imp (+) Imp (-)	F: CATGGTTTGGTGGTTCTTGT R: ATAATTTGGCGGACTTTGGC	488	Jeon *et al*., 2005
***bla*$_{OXA-48}$**	Oxa-48 (+) Oxa-48 (-)	F: TTGGTGGCATCGATTATCGG R: GAGCACTTCTTTTGTGATGGC	733	Poirel *et al*., 2004
***bla*$_{OXA-23}$**	Oxa-23 (+) Oxa-23 (-)	F: GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCCAT	501	Woodford *et al*., 2006
***bla*$_{OXA-24}$**	Oxa-24 (+) Oxa-24 (-)	F: GGTTAGTTGGCCCCCTTAAA R: AGTTGAGCGAAAAGGGGATT	246	Woodford *et al*., 2006
***bla*$_{OXA-51}$**	Oxa-51 (+) Oxa-51 (-)	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353	Woodford *et al*., 2006
***bla*$_{OXA-58}$**	Oxa-58 (+) Oxa-58 (-)	F: AAGTAT TGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	599	Woodford *et al*., 2006
Class-I.Int	5'-CS 3'-CS	F: GGCATCCAAGCAGCAAG R: AAGCAGACTTACCTGA	Variable	Levesque *et al*., 1995
Class-II.Int	hep-51 hep-74	F: GATGCCATCGCAAGTACGAG R:CGGGATCCCGGACGGCATGCACGATTTGTA	Variable	White *et al*., 2001

Pb : Pair bases ; Class-I.Int: Class I Integron ; Class-II.Int: Class II Integron.

Table 6: Specific PCR programs for the different ESβL genes

Genes	Initial denaturation 94 °C	Denaturation 94 °C	Hybridation 1 min. (°C variable)	Elongation 72 °C (Variable)	Finale elongation 72 °C	Storage 4 °C
	1 cycle	34 cycles			1 cycle	
TEM	5 minutes	1 minutes	56 °C	1 minutes	7 minutes	∞
SHV	5 minutes	1 minutes	55 °C	1 minutes	7 minutes	∞
CTX-M1	5 minutes	1 minutes	55 °C	40 seconds	7 minutes	∞
CTX-M2	5 minutes	1 minutes	55 °C	40 seconds	7 minutes	∞
GES	5 minutes	1 minutes	56 °C	50 seconds	7 minutes	∞
PER	5 minutes	1 minutes	46 °C	1 minutes	7 minutes	∞
VEB	5 minutes	1 minutes	48 °C	1 minutes	7 minutes	∞
KPC	5 minutes	1 minutes	55 °C	1 minutes	7 minutes	∞
VIM	5 minutes	1 minutes	58 °C	50 seconds	7 minutes	∞
NDM	5 minutes	1 minutes	57 °C	40 seconds	7 minutes	∞
IMP	5 minutes	1 minutes	54 °C	50 seconds	7 minutes	∞
OXA-48	5 minutes	1 minutes	55 °C	50 seconds	7 minutes	∞
OXA-23	5 minutes	1 minutes	52 °C	30 seconds	7 minutes	∞
OXA-24	5 minutes	1 minutes	52 °C	30 seconds	7 minutes	∞
OXA-51	5 minutes	1 minutes	52 °C	30 seconds	7 minutes	∞
OXA-58	5 minutes	1 minutes	52 °C	30 seconds	7 minutes	∞
Class-I. Int	5 minutes	1 minutes	55 °C	5 minutes	10 minutes	∞
Class-II. Int	5 minutes	1 minutes	53 °C	5 minutes	7 minutes	∞

c. Electrophoresis and detection

The PCR products were run in 1% of agarose gel with ethidium bromide solution (Promega). The products were first mixed with equal volume of 1X bromophenol blue solution and then loaded into the wells of the gel. The gel was run for one hour at 110 V using 1X TAE buffer (Tris acetate 40 mm, EDTA 1 mm, pH 8.0, Invitrogen). The gel was visualized by transillumination with UV light (λ = 320 nm).

II.1.2.3. Sensitivity and Specificity

The performance of the phenotypic tests for the detection of ESßLs among Gram-negative bacilli producing various ß-lactamases (ESßLs and/or carbapenemases) was evaluated using PCR as the gold standard (Ilstrup, 1990). For each test, the sensitivity was calculated based on the ratio (**a*100)/(a+c)**, where **a** represented the number of strains that were correctly

identified as ß-lactamases producers (true positives) by the tested assay and **c** represented the number of true ß-lactamases producers incorrectly identified as non-producing strains (false negatives), while the specificity was calculated based on the ratio **d*100/(b+d)**, where **d** represented the true number of strains not producing MBLs correctly identified (true negatives) by the tested assay and **b** represented the number of strains that were incorrectly identified as MBL producers (true positives).

II.2. Expression and purification of recombinant GES-22 and OXA-1 proteins

The β-lactamase GES-22 and OXA-1 were produced recombinantly according to Saral *et al*., 2016 and Çalık, 2016, respectively. Briefly pET28a-GES-22 and pET28a-OXA-1 expression vectors were transformed into *E. coli* BL21 (DE3) for overexpression seperately. Then a single colony harboring the pET28a-GES-22 and pET28a-OXA-1 vectors were selected and cultured at 37 ° C in 500 mL of LB medium containing 30 µg / ml of kanamycin. The cultures were induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.6 OD_{600} and kept at 28 ° C for 5 h. The cells were collected by centrifugation and suspended in lysis buffer (50 mM Tris-H_2SO_4 (pH 7.4) with 20 mg / ml lysozyme) before sonication. After sonication, the lysate was centrifuged at 9000 rpm for 30 minutes at 4 ° C. The supernatant was transferred into a new tube and loaded onto a nickel resin equilibrated with 50 mM Tris-H_2SO_4, 500 mM NaCl, 2 mM DTT, pH 7.4. The column was firstly washed with 50 mM Tris-H_2SO_4, 500 mM NaCl, 2 mM DTT, 25 mM imidazole, pH 7.4 and then with 50mM Tris-H_2SO_4, 500 mM NaCl, 2 mM DTT, 100 mM Imidazole, pH 7 4. The recombinant proteins were eluted with 50 mM Tris-H_2SO_4, 500 mM NaCl, 2 mM DTT, 300 mM imidazole pH 7.4. Fractions containing OXA-1 or GES-22 were pooled and dialyzed against 50 mM Tris-H_2SO_4, pH 7.4 overnight. Protein purity was controlled by SDS-PAGE. Protein samples were stored at -20 °C.

II.3. Methanolic extracts of seaweeds

II.3.1. Preparation

The dried seaweeds samples (10 g) were extracted with 100 ml of methanol at 20 °C in an ultrasonic bath (Elma Clean Box, Elma). The liquid extract was centrifuged at 4000 rpm for 10 min at 4 °C. Then, the supernatants were pooled and concentrated using a rotary evaporator (EZ-2 Evaporator, GeneVac) at 37 °C. The resulting extract was lyophilized and stored under dark conditions in the freezer at -20 °C until analysis. The yields of the extracted material were expressed as a percentage (w/w) of dried seaweed powder.

II.3.2. Phytochemical analysis

a. Total phenolic content

The total phenol content of the methanolic extracts was determined spectrophotometrically at 765 nm, according to the Folin-Ciocalteu colorimetric method (Singleton *et al*., 1999). A volume of 200 µl of methanolic extract solution was added to 1 ml of Folin-Ciolcalteu reagent previously diluted with 10 ml of deionized water (10%). After 4 min, 800 ml of saturated sodium carbonate (75 g / L) was added. After 2 hours of incubation at room temperature, the absorbance at 765 nm was measured. Quantification of the total phenol compounds was based on a standard gallic acid curve and the results were expressed in milligrams of gallic acid equivalent (mg GAE / g extract).

b. Vitamin E content

Vitamin E content was determined using a method described by Prieto *et al.,* (1999). The samples were homogenized with hexane and shaken for 1 h at 4 ˚C in the dark. After centrifugation at 6000 g for 10 min, the supernatant was transferred to new tubes. A 0.1 mL hexanic extract of seaweeds was mixed with 1 mL phosphomolybdenum reagent solution and incubated for 90 min at 37 ˚C with vigorous shaking. The absorbance was measured at 695 nm. Vitamin E content was expressed as milligrams of α-tocopherol equivalent per gram extract (mg α-tocopherol/ g extract).

II.3.3. HPLC analysis

a. Sample preparation

The standard solutions of chrysin, emodin, kaempferol, quercetin, esculin, genistin, resveratrol, daidzein, formononetin, aloe-emodin, hesperetin, myricetin, baicalin, 7-hydroxy-isoflavone, genistein, (S) naringenin, rhein, curcumin, rutin, (R) pinocembrin, baicalein, puerarin, myricetrin, hesperidin, α-linolenic acid, linoleic acid, oleic acid, abietic acid, arachidonic acid and isosteviol were all prepared in methanol at concentrations of 200 ng/mL, and then diluted to 100 ng/mL with purified water for further LC-MS studies. Each dried extract was dissolved in LC-MS grade methanol, then diluted to 500 µg/mL (HPLC-ESI-MS preliminary experiment), 1 mg/mL (HPLC-ESI-MS) and 5 mg/mL (HPLC-ESI-MS-TOF)

concentrations with purified water for further studies. The final solutions contained half methanol and half water.

b. HPLC-ESI-MS

The analyzes were carried out using Agilent technologies 1200 series high performance liquid chromatography equipped with G1379B degasser, G1311A quaternary pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector (Agilent Technologies, USA). All the compounds were separated on a ZORBAX SB-C_{18} column with a length of 2.1 × 100 mm, and an internal diameter of 3.5 μm, with temperature at 35 ºC. The mobile phase works at a flow rate of 0.3 mL/min using acetonitrile (A)–water (B) contain 0.05% formic acid (v/v). A gradient elution was applied by setting solvent (A) at 10% in 0 min and then gradually increasing it to 90% in the next 90 min, followed by re-equilibration at 10% (A) until 95 min. The injection volume was 10 μL.

For the identification of compounds, an Agilent 6110 quadrupole mass spectrometer with electrospray ionization source (ESI) was operated in negative ion mode. The conditions of the ESI source were set with a drying gas flow of 10.0 L/min, a drying gas temperature of 350 °C, a nebulizer pressure of 35.0 psig and a capillary voltage of 3000 V. The determination was performed using both negative full scan mode and the negative selected ion monitoring (SIM) mode (m/z = 253.2; 269.2; 285.2; 301.2; 339.2; 431.3; 227.2; 253.2; 267.2; 296.2; 301.2; 317.2; 445.3; 237.2; 269.2; 271.2; 283.2; 267.3; 609.5; 255.2; 269.2; 415.3; 463.3; 609.5; 277.4; 279.4; 281.4; 301.4) for chrysin, emodin, kaempferol, quercetin, esculin, genistin, resveratrol, daidzein, formononetin, aloe-emodin, hesperitin, myricetin, baicalin, 7-hydroxy-isoflavone, genistein, (s) naringenin, rhein, curcumin, rutin, (r) pinocembrin, baicalein, puerarin, myricetrin, hesperidin, α-linolenic acid, linoleic acid and oleic acid, respectively, for comparison standard compounds.

c. HPLC-ESI-MS-TOF

The HPLC ESI-MS-TOF analysis was performed with a Waters Acquity Ultra Performance LC system (Waters, USA). Chromatographic separation was carried out at 35°C on a ZORBAX SB-C_{18} column (2.1 mm×100 mm, 3.5 μm). The mobile phase works at a flow rate of 0.3 mL/min using acetonitrile (A)–water (B) contains 0.05% formic acid (v/v). A gradient elution was applied by setting solvent (A) at 10% in 0 min and then gradually increasing it to 90% in the next 60 min, followed by re-equilibration at 10% (A) until 65 min. The injection volume was 10μL.

For the identification of compounds, a Waters Xevo G2 QTof MS (Waters MS Technologies, Manchester, UK) with electrospray ionization source (ESI) was operated in both positive and negative ion modes.

Data analysis

Computer software Agilent chemstation (version B.04.02.) and MassLynx (version 4.1) were used for data acquisition and qualitative analysis of the HPLC-ESI-MS and HPLC-ESI-MS-TOF, respectively.

II.4. GES-22 and OXA-1 β-lactamases inhibition assays

II.4.1. Kinetic study

The aim of this study is to determine the two classical kinetic parameters which are the specific activity (Vmax) and Michaelis-Menten constant (K_M) of GES-22 and OXA-1. For this, we proceed as well as: a reaction mixture was prepared in 96 well microplates, consisting of sufficient quantity for volume of 200 µL sodium phosphate buffer 100 mM (pH: 7.4), 7µL (0.3 µM) GES-22 or OXA-1 was added at the wells. The nitrocefin used as substrate at different finals concentrations (10-120µM) was added to initiate the reaction mixture. The microplate was immediately read at 482 nm using a UV-visible microplate reader for 5 minutes at 25 °C. All tests were performed in triplicate and the specific activity (Vmax) and the K_M were determined from a Lineweaver-Burk graph where the reciprocal of the substrate concentration (1/[S]) on the x-axis is plotted against the initial velocity V_0 on the y-axis.

II.4.2. Determination of IC_{50} parameters

The inhibitory effect on the hydrolysate activity of GES-22 and OXA-1 by crude methanolic extracts of seaweeds was determined by a spectrophotometric test using nitrocefin as a chromogen substrate at a wavelength of 482 nm (ε_{482} = 15000 M^{-1} cm^{-1}). In this test, the classical inhibitors of β-lactamase (tazobactam, clavulanate, and sulbactam) were used as a positive control. The reaction mixture was prepared in 96 well microplates, consisting of 158 µL sodium phosphate buffer 100 mM (pH: 7.4), 5 µL of each extract or control at different concentrations (500-1.25 µg/mL), subsequently 7µL (0.3 µM) GES-22 or OXA-1 was added at the wells and incubated for 10 minutes at 25 °C. After incubation, the nitrocefin (30 µL) was added to initiate the reaction mixture. The microplate was immediately read at 482 nm using a

UV-visible microplate reader. All tests were performed in triplicate and the percentage inhibition was calculated using the following equation:

% Inhibition = ([ABS (Control)-ABS (sample)])/ (ABS (Control)) x 100.

Where ABS (Control) is the absorbance of the activity of GES-22 or OXA-1 without inhibitors, and ABS (sample) is the activity of GES-22 or OXA-1 in the presence of inhibitors.

Percentage inhibitions values were used to determine IC_{50} by linear computerized regression analysis after logit/log transformation. Experiments were performed in triplicate and results are presented as mean values ± Standard deviation (SD). the significance of the difference between IC_{50} of MEs versus the controls were assessed using one-way analysis of variance (ANOVA) followed by Dunnett post-test; in GraphPad Prism version 6.0.

II.4.3. Determination of inhibition type

To examine the type of inhibition of seaweeds ME, the activity of GES-22 or OXA-1 was monitored as before with increasing concentrations of nitrocefin 10–120 μM, in the absence or presence of ME (the concentration of ME was kept constant at finale concentrations near the IC_{50} values). Vmax and the K_M values were determined from a Lineweaver-Burk graph where the reciprocal of the substrate concentration (1/[S]) on the x-axis is plotted against the reciprocal of initial velocity ($1/V_i$) on the y-axis.

II.5. Molecular docking

II.5.1. Evaluation test of the program used

In this study the Libdock and CDOCKER flexible docking protocols in Discovery Studio (DS) 2016 software (Accelrys Software Inc., San Diego) have been used to investigate the binding mode of ligands in the crystal structure of β-lactamases (Xu *et al*., 2015).

The performance of the docking approach of Libdock and CDOCKER were evaluated by the root mean square deviation (RMSD) corresponds to the mean of the deviation of each of the ligand atoms of the software-designed model compared to those of the original molecule. The allowed ratio is a maximum difference of 2 angstroms beyond which the prediction is considered inadequate.

II.5.2. Process of molecular docking

The docking procedure by Discovery Studio 2016 is broken down into 5 steps:

a. Preparation of the protein

The crystal structures of β-lactamases were obtained from the Protein Data Bank in PDB form via the protein bank (http: //www.pdb.org). Totally, 15 protein structures were used for docking. The water molecules in protein were removed and the protein was prepared by adding hydrogen and correcting the incomplete residues using Clean Protein tool of DS, then the protein was refined with CHARMM.

b. Preparation of the ligand

The ligands used in this work are downloaded as SDF from the PubChem databank (http://pubchem.ncbi.nlm.nih.gov). Next, the two-dimensional (2D) chemical structure of 40 compounds obtained from Pubchem (**Appendix A**), should be prepared and was followed by hydrogen addition, conversion into 3D structures, pH-based ionization and charge neutralization. The ligands were set to be flexible, and the enzyme was prepared as a rigid body.

c. Detection of the binding site

The sites present in the protein are identified using the command: define and edit binding site (**Figure. 10**).

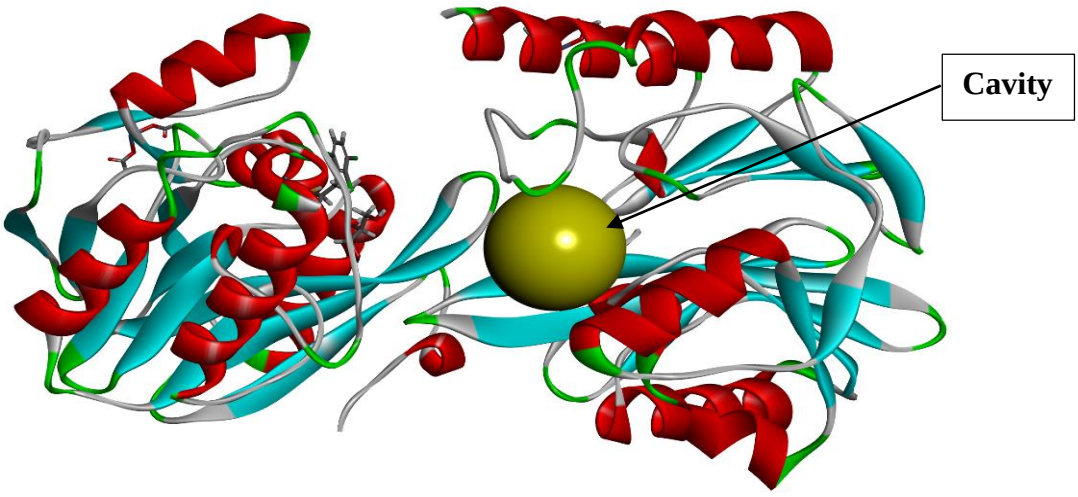


Figure 10. Binding sites (cavities) detected in the receptor

d. Docking

LibDock is a high-throughput algorithm for docking ligands into an active receptor site. Ligand conformations are aligned to polar and apolar receptor interaction sites (hotspots) and the best scoring poses are reported. Ligand conformations can be pre-calculated or generated

on the fly using Catalyst. Since some of the output poses may have hydrogen atoms in close proximity to the receptor, a CHARMM minimization step can be enabled to optimize the docked poses further (increases CPU time). CDOCKER uses molecular dynamics (MD) with CHARMM force field scheme to dock ligands into a binding site of targeted protein. The binding site of β-lactamases was defined with the original ligand in the PDB structure.

e. Results analysis

The best ligand deposited in the active site of the enzyme was chosen according to the best Libdock score or CDOCKER energy / CDOCKER energy interaction.

Results

RESULTS

I. Distribution of MDR isolates

From January 2015 to April 2016, 108 MDR isolates were isolated and identified out of 173 strains of gram-negative bacilli isolated from various clinical samples of which 68.52% (74/108) were from hospitalized patients and 31.48% (34/108) are from outpatients and then their distribution was performed according to different criteria.

I.1. Distribution according to species

Among all strains, *Enterobacteriaceae* strains are more frequent (68/108). This group is constituted of 33.33 % (36/108) *Escherichia coli*, 19.44 % (21/108) *Klebsiella pneumoniae*, and 10.18 % (11/108) other enterobacterial species (2 *Citrobacter freundii*, 2 *Proteus vulgaris*, 2 *Proteus mirabilis*, 1 *Klebsiella oxytoca*, 1 *Enterobacter cloacae*, 1 *Morganella morganii*, 1 *Providencia stuartii*,1 *Serratia marcescens*). Strains different from *Enterobacteriaceae* are *Pseudomonas aeruginosa* 18.51 % (20) and *Acinetobacter baumannii* 18.51 % (20).

I.2. Distribution according to biological sample

Among the 108 strains collected, the majority (43.52%) is isolated from urine, the rest from blood culture (17.59%), pus (14.81%), stool (12.03%) and catheters (09.26%) (**Table. 7**).

Table 7. Distribution of MDR isolates according to the biological sample

Type of specimen	Number of MDR
Urines	47
Blood culture	19
Pus	16
Stool	13
Catheters	10
breast and nipple discharge	02
CSF	01
Total	108

I.3. Distribution according to age

The age of patients varies from 1 days (newborn) to 93 years. the highest rate of MDR isolates is noted with children age under 02 years (31.48%) and patients over 60 years (27.77 %), followed by the groups which the age ranging from 2 to 18 years with rate of 19.44%. In conclusion, children and elderly patients are the most affected by the phenomenon of resistance (**Table. 8**).

Table 8. Distribution of MDR isolates according to the age, both from out and in-patients

Age (years)	Number of MDR
< 2	34
2-18	21
18-35	14
35-60	09
> 60	30
Total	108

I.4 Distribution according to sex

According to the table below (**Table. 9**), the rate of MDR isolates in male patients 67 (62.03%) is higher than in female 41 (37.96%).

Table 9. Distribution of MDR isolates according to the sex, both from out and in-patients

Sex	Number of MDR
Male	67
Female	41
Total	108

I.5 Distribution according to service (in-patients)

After analysis, certain hospital services appeared to be more affected by the resistance to antibiotics problem, notably the intensive care unit and the pediatric and the women's medicine services. Related incidence percentage are 36.94%, 25.68%, 17.57%, respectively (**Table. 10**). The rate in the other services was distributed as follows: Surgery (08.11%), pneumology (06.75%), gynecology (05.40%).

Table 10. Distribution of MDR isolates according to services

In-patients		
Service	**Number of MDR**	**Species**
Intensive care unit	n=27	*P. aeruginosa* (n=08)
		A. baumannii (n=08)
		E. coli (n=06)
		K. pneumonaie (n=04)
		E. cloaceae (n=1)
Pediatric	n= 19	*E. coli* (n=08)
		P. aeruginosa (n=04)
		K. pneumonaie (n=05)
		A. baumannii (n=01)
		K. oxytoca (n=01)
Woman's medicine	n= 13	*E. coli* (n=04)
		K. pneumonaie (n=04)
		P. aeruginosa (n=03)
		A. baumannii (n=02)
Surgery	n= 6	*E. coli* (n=05)
		A. baumannii (n=01)
Pneumology	n= 5	*A. baumannii* (n=03)
		E. coli (n=02)
Gynecology	n= 4	*E. coli* (n=02)
		Proteus (n=02)
Total	74	

II. Antibiotics susceptibility of MDR isolates

II.1. *Enterobacteriaceae*

All *Enterobacteriaceae* isolates (68/108) were tested against 27 antibiotic molecules **(Table. 11)** belonging to different class including 14 ß-lactams, 4 aminoglycosides, 3 quinolones, and 6 others**.** This test was performed using the disc diffusion method and the results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014).

II.1.1. *Escherichia coli*

The results of the susceptibility study of *E. coli* show that all strains (100%) are resistant to β-lactams: ampicillin, ticarcillin, piperacillin, cefazolin, cephalothin, cefotaxime, ceftazidime and monobactam. This percentage decrease to 66.66 % when it comes to the association of amoxicillin and clavulanic acid. A medium resistance to mecillinam (55.55%), a decrease resistance toward the cefoxitin (13.88%) and no resistance was observed with respect to imipenem and only one strain was resistant to ertapenem.

In the case of aminoglycosides, the percentage of resistance strains increased gradually when netilmicin, gentamycin, tobramycin and amikacin are tested. Related values are 05.55 %, 38.88% 66.66%, 66.66%, respectively. A same observation is noted for quinolones, 52.77%, 55.55 and 69.44% of strains are respectively resistant to nalidixic acid, levofloxacin and ciprofloxacin.

Nitrofurantoin, colistin and fosfomycin are the most active antibiotics, with a sensitivity rate of 100% for the two last antibiotics and 91.67% for nitrofurantoin.

II.1.2. *Klebsiella pneumoniae*

K. pneumoniae strains have very high levels of resistance to β-lactams: 100% toward ampicillin, ticarcillin, piperacillin, cefazolin, cephalothin, cefotaxime, ceftazidime, cefepime and aztreonam; 71.43 % toward the association amoxicillin and clavulanic acid; 47.62% to mecillinam; and only two strains were resistant to imipenem and ertapenem.

For aminoglycosides and quinolones medium to high resistance rate were also observed: 52.39% for amikacin, 71.43% for gentamycin, 80.96 and 85.72% for netilmicin and tobramycin, respectively. 71.43%, 90.47% and 61.91% for nalidixic acid, levofloxacin and ciprofloxacin, respectively.

Colistin and fosfomycin are the most active antibiotics, on all strains with a 100% sensitivity rate.

II.1.3. other enterobacterial species

The results of the susceptibility test of 11 strains: 2 *Citrobacter freundii*, 2 *Proteus vulgaris*, 2 *Proteus mirabilis*, 1 *Klebsiella oxytoca*, 1*Enterobacter cloacae*, 1 *Morganella morganii*, 1 *Providencia stuartii* and 1 *Serratia marcescens* showed that all strains have very high levels of resistance toward β-lactams excepting carbapenems: 100% toward ampicillin, ticarcillin, piperacillin, cefazolin, cephalothin, cefotaxime, ceftazidime, cefepime and aztreonam. Only the two strains *Proteus mirabilis* were sensitive to the association amoxicillin and clavulanic acid. Amikacin is the most active antibiotic on all strains with a 100% sensitivity rate. Regarding other antibiotics, all strains presented a resistance with varying degrees

II.2. *Acinetobacter baumannii*

Antibiograms of the twenty *A. baumannii* strains isolated and identified (**Table. 12**) show that only 10 strains were resistant to imipenem. The rest was susceptible to all β-lactams including imipenem.

For aminoglycosides, the highest rate was observed for amikacin (65%) and gentamicin (60%), following by netilmicin (55%) and tobramycin (50%).

Regarding quinolones and sulfamide-trimethoprim association, resistance levels ranged from 80% for levofloxacin to 95% for trimethoprim+ sulfamethoxazole. In contrast to colistin which remains the most active antibiotic on *A. baumannii* strains with 80% sensitivity, doxycycline and rifampicin have a moderate antibacterial activity against this species.

Table 11. Antibiotic susceptibility of *Enterobacteriaceae* (n=68)

Class	ATB	*E.coli* (n=36) (%)	*K.pneumoniae* (n=21) (%)	Other enterobacterial species (n=11)								Total (%)
				K.o n=1	Pm n=2	Pv n=2	Cf n=2	M.m n=1	S.m n=1	E.c n=1	P.s n=1	
β-lactams	AMP	100	100	R	R	R	R	R	R	R	R	100
	AMC	66.66	71.43	I	S	I	R	R	R	R	R	70.58
	TIC	100	100	R	R	R	R	R	R	R	R	100
	PIP	100	100	R	R	R	R	R	R	R	R	100
	MEL	55.55	47.62	S	R	R	I	R	R	S	S	55.88
	CZ	100	100	R	R	R	R	R	R	R	R	100
	CF	100	100	R	R	R	R	R	R	R	R	100
	FOX	13.88	00	S	S	R	R	R	S	R	R	17.64
	CTX	100	100	R	R	R	R	R	R	R	R	100
	CAZ	100	100	R	R	R	R	R	R	R	R	100
	FEP	36.11	100	R	R	R	R	R	R	R	R	66.17
	IPM	02.77	09.53	R	R	S	S	S	S	S	S	07.35
	ETP	02.77	09.53	S	S	S	S	S	S	S	S	04.41
	ATM	100	100	R	R	R/I	R	R	R	R	R	100
Amino-glycosides	AN	05.55	52.39	S	S	S	S	S	S	S	S	19.11
	GM	66.66	71.43	S	S	R	R	S	R	R	R	67.64
	NET	38.88	80.96	S	S	R	S	R	R	S	S	51.47
	TM	66.66	85.72	R	S	R	R	S	R	R	S	72.05
Quinolones	NA	52.77	71.43	R	S/R	S	R	R	S	I	R	60.29
	CIP	69.44	90.47	R	S/R	S	R	S	S	I	R	73.52
	LVX	55.55	61.91	R	S/R	I	R	S	S	S	R	58.82
Phenicols	C	77.77	38.10	S	S/R	R	I	R	R	S	I	64.70
Sulfamides trimethoprim	SXT	58.33	95.24	R	S/R	R	R	R	S	R	R	73.52
Polymyxines	CT	00	00	S	R	R	S	R	R	S	R	10.29
Tetracyclines	TE	75	80.96	R	R	R	R	R	R	R	R	80.88
Fosfomycin	FOS	00	00	S	S/R	S/R	S	R	S	S	S	02.94
Furans	FT	8.33	52.39	R	R	R	R	R	R	R	R	36.76

R: resistant, S: sensitive, I: intermediate

K.o: *Klebsiella oxytoca***; P.m:** *Proteus mirabilis***; P.v:** *Proteus vulgaris***; C.f:** *Citrobacter freundii***; M.m:** *Morganella morganii* **; S.m:** *Serratia marcescens***; E.c:** *Enterobacter cloacae***; P.s:** *Providencia stuartii.*

Table 12: Antibiotic susceptibility of *A.baumannii* (n=20)

Class	Antibiotics	Resistance rate (%)
β-lactams	Ticarcillin (TIC)	100%
	Piperacillin (PIP)	100%
	Ticarcillin/Clavulanic acid (TCC)	100%
	Ceftazidime (CAZ)	100%
	Cefepime (FEP)	100%
	Cefpirome (CPO)	100%
	Imipenem (IPM)	50%
	Aztreonam (ATM)	100%
Aminoglycoside	Amikacin (AN)	65%
	Gentamicin (GM)	60%
	Netilmicin (NET)	55%
	Tobramycin (TM)	50%
Quinolone	Ciprofloxacin (CIP)	85%
	Levofloxacin (LVX)	80%
Polymyxine	Colistin (CT)	20%
Tetracyclines	Doxycycline (DOX)	50%
Rifampycin	Rifampicin	45%
Sulfamide trimethoprim	Trimethoprim+ Sulfamethoxazole (SXT)	95%

II.3. *Pseudomonas aeruginosa*

The antibiotic susceptibility test (**Table. 13**) revealed that all strains of *P. aeruginosa* are resistant to rifampicin but sensitive to colistin. A very remarkable resistance towards the ß-lactams is noted. Levels of resistance to such antibiotics are 95% for ticarcillin, 90% for ceftriaxone, 80% for ticarcillin/clavulanic acid, 75% for imipenem, 75% for piperacillin, 70% for ceftazidime and cefepime, 80% for cefoperazone and aztreonam. Whereas intermediate resistance was recorded towards aminoglycosides with a level of resistance ranging from 40% to 65%. Colistin, is the most active antibiotics on the growth of *P. aeruginosa* strains with 95% sensitivity.

Table 13. Antibiotic susceptibility of *P. aeruginosa* (n=20)

Class	Antibiotics	Resistance rate (%)
β-lactams	Ticarcillin (TIC)	95%
	Piperacillin (PIP)	75%
	Ticarcillin/Clavulanic acid (TCC)	80%
	Ceftazidime (CAZ)	70%
	Cefepime (FEP)	70%
	Ceftriaxone (CPO)	90%
	Cefoperazone (CFP)	80%
	Imipenem (IPM)	75%
	Aztreonam (ATM)	80%
Aminoglycosides	Amikacin (AN)	40%
	Gentamicin (GM)	40%
	Netilmicin (NET)	50%
	Tobramycin (TM)	40%
Quinolones	Ciprofloxacin (CIP)	65%
	Levofloxacin (LVX)	45%
Polymyxine	Colistin (CT)	05%
Rifampycin	Rifampicin	100%
Phosphonic	Fosfomycin	55%

III. Detection of β-lactamases

III.1. Phenotypic detection

III.1.1. Extended Spectrum Beta-Lactamases

a. Double disc synergy test (DDST)

ESβLs are preliminary detected in *Enterobacteriaceae* by the search for a synergy between the clavulanic acid and cephalosporins or monobactam. The inhibition zone around the AMC disc was enhanced, resulting in a characteristically shaped zone referred to as champagne-cork, keyhole, ellipsis or phantom image. These synergistic image was obtained with 55 strains out of 68; of which 32/36 *E. coli*; 17/21 *K. pneumoniae*; and 06/11 other Enterobacterial species. The figure below (**Figure. 11**) shows an example of DDST results.

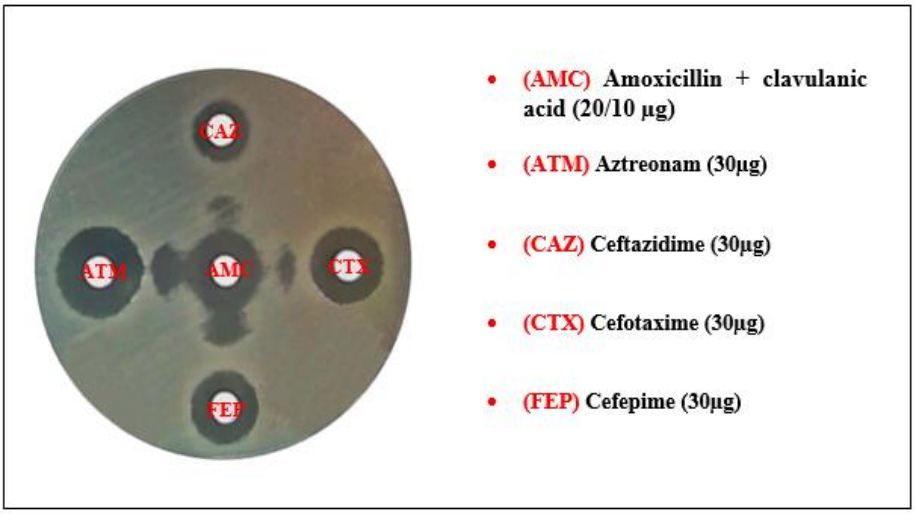


Figure 11. Positive test of synergy obtained with an *E.coli* strain

b. Phenotypic confirmatory disc diffusion test (PCDDT)

The phenotypic confirmatory disc diffusion test is performed to detect the production of an ESβLs that can be masked by another mechanism of resistance in strains that do not show a synergistic image after preliminary tests (DDST). The PCDDT was positive (**Figure. 12**) with 77 isolates out of 108; of which are 62 *Enterobacteriaceae* (34/36 *E. coli*; 20/21 *K. pneumoniae*; and 08 other species); 01/20 is *P. aeruginosa* and 14/20 are *A. baumannii*. The following figure shows a positive PCDDT.

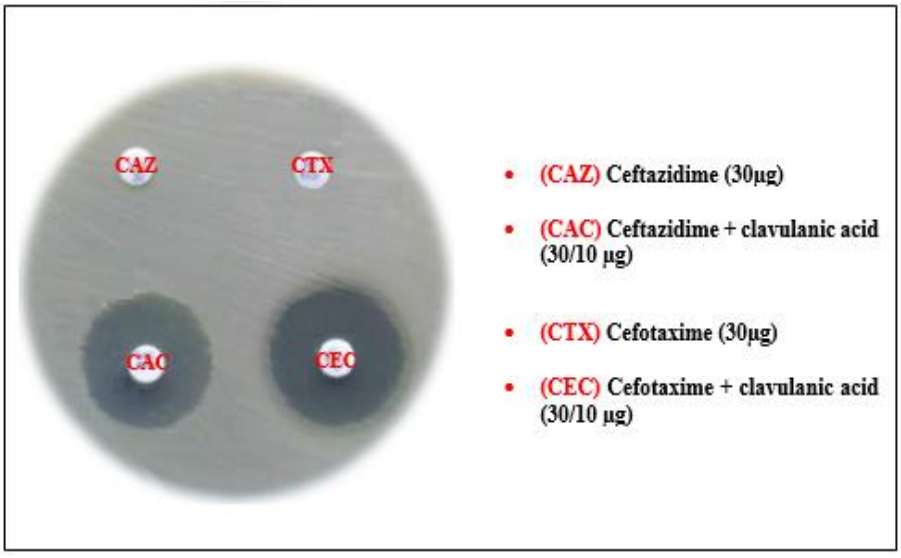


Figure 12. Positive combined disc diffusion test obtained with a *K. pneumoniae* strain

III.1.2. Carbapenemases

a. Modified Hodge Test (MHT)

The clover leaf-like indentation of *E.coli* ATCC 25922 was visible with 01 *P.aeruginosa* and 28 *Enterobacteriaceae* (19 *E.coli*, 07 *K.pneumoniae*, 01 *K.oxytoca* and 01 *P.mirabilis*). however, this clover leaf-like indentation is slightly visible with 07 *A.baumannii* IMP-R isolates (**Figure. 13**).

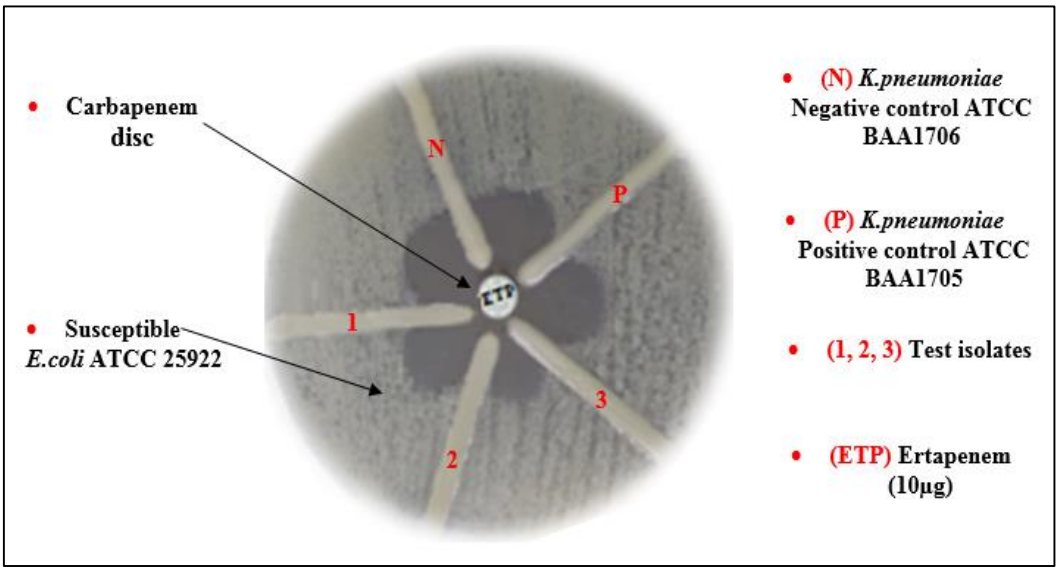


Figure 13. Positive modified Hodge test

b. Inhibition tests

- **Ambler class A carbapenamases**

The test of detection of the Ambler class A carbapenamases was negative with all strains.

- **IMP-EDTA Combined Disc Test**

The synergistic effect of IMP with EDTA was obtained with 04 *A. baumannii*, 02 *K.pneumoniae* and 08 *P.aeruginosa* (**Figure. 14)** other Enterobacterial species resistant to imipenem were negative with IMP-EDTA Combined Disc Test.

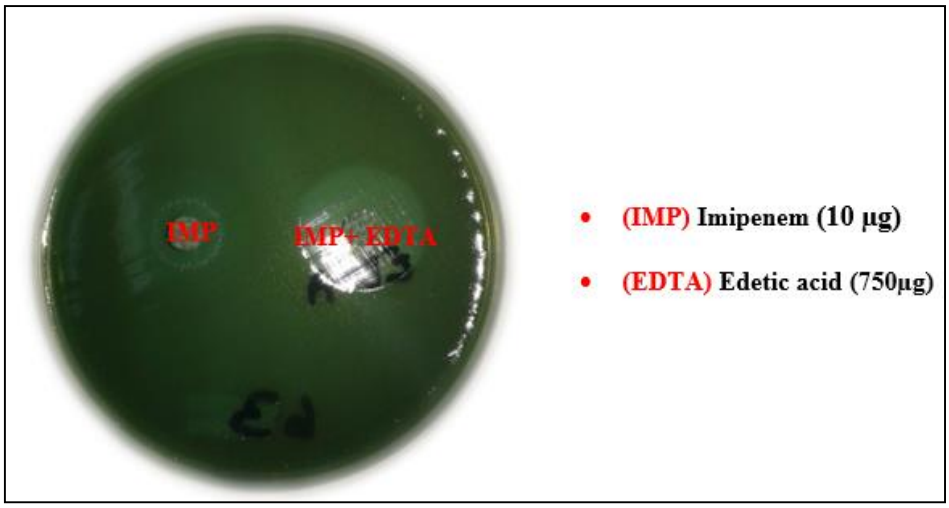


Figure 14. Positive IMP-EDTA Combined Disc test obtained with *P. aeruginosa* strain

III.2. Molecular detection

III.2.1. β-lactamase genes in strains

III.2.1.1. *Enterobacteriaceae*

a. *Escherichia coli*

As seen in the **Table. 14**, several β-lactamase coding genes such as bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{CTX-M} and bla_{OXA-48}, were detected in *E. coli*. At least, one β-lactamase coding gene was amplified in the strains. $bla_{CTX-M1/M2}$ was detected in all strains except two strains 32 and 60.

b. *K.pneumonaie*

As seen in the **Table. 15**, β-lactamase coding genes of ESβLs (bla_{TEM}, bla_{SHV}, $bla_{CTX-M1/M2}$) and carbapenemases (bla_{OXA-48}, and bla_{NDM}) were detected in *K. pneumonaie.* At least, one β-lactamase coding gene was amplified in the strains.

c. other enterobacterial species

As seen in the **Table. 16**, β-lactamase ESβLs type coding genes of bla_{TEM}, bla_{SHV}, bla_{PER} and bla_{CTX-M} were detected in some of the other Enterobacterial species. In addition, bla_{OXA-48} carbapenemase coding gene is detected in two strains *K.oxytoca* and *P.mirabilis.* No β-lactamase coding gene was amplified in *P.vulgaris, M.morganii* and *E.cloacae* strain. bla_{TEM} was mostly found in the other Enterobacterial species.

Table 14. β-lactamase coding genes in *E. coli* strains (n=36)

Strain N°	ESßLs				Carbapenemases	Integrons	
	TEM	SHV	PER	CTX-$_{M1/M2}$	OXA-$_{48}$	IC1	IC2
07	+	+		+	+	+	+
08	+	+		+		+	+
09	+	+		+		+	
10	+	+		+		+	
12	+			+		+	+
13	+	+		+			
15	+	+		+	+		
22				+			
25	+			+		+	+
27	+			+		+	
28	+			+		+	
32	+						
38	+	+		+		+	
40	+			+		+	
44	+			+		+	+
47	+			+		+	+
49	+			+		+	
53	+		+	+			
54	+		+	+			+
56	+	+		+		+	
58	+			+			+
60	+	+					
61	+			+			
65	+	+		+			
66				+			
67				+			
70		+		+		+	
71				+		+	
72				+		+	
73	+	+		+		+	
77				+			
81				+			
83	+			+		+	
85	+	+		+		+	
87	+	+		+		+	+
92	+			+		+	

+: presence of genes. The empty boxes indicated the absence of genes.
IC1: Integron class I; IC2: Integrons class II

Table 15. β-lactamase coding genes in *K. pneumonaie* strains (n=21)

Strains N°	ESβLs			Carbapenemases		Integrons	
	TEM	SHV	CTX-$_{M1/M2}$	OXA-$_{48}$	NDM	IC1	IC2
11		+	+			+	
14	+	+	+	+			
23	+	+	+		+	+	
24	+	+	+				
35	+	+	+				
36	+	+	+			+	
42	+		+				
43	+	+	+		+		
48	+	+	+			+	
55	+					+	
57	+	+	+				
64	+	+	+				
68	+	+	+			+	
69	+	+	+				
74	+	+	+				
76		+	+				
79	+		+				
80	+	+	+				
82			+			+	
84	+	+	+			+	+
86	+	+					

+: presence of genes, the empty boxes indicated the absence of genes.

Table 16. β-lactamase coding genes were investigated in the other Enterobacterial species (n=11)

Strain N°	Name of bacteria	ESβLs				Carbapenemases
		TEM	SHV	PER	CTX-$_{M1/M2}$	OXA-$_{48}$
06	*K.oxytoca*	+	+		+	+
46	*C.freundii*	+				
50	*C.freundii*	+			+	
39	*P.mirabilis*	+				
51	*P.mirabilis*	+		+	+	+
63	*P.vulgaris*	+		+		
75	*P.vulgaris*					
30	*M.morganii*					
37	*S.marcescens*	+				
29	*E.cloacae*					
19	*P.stuartii*	+	+			

+: presence of genes, the empty boxes indicated the absence of genes.

III.2.1.2. *Pseudomonas aeruginosa*

β-lactamase coding genes were investigated in all *P.aeruginosa* strains. As seen in the **Table. 17**, several β-lactamase coding genes such as *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{GES}$, *bla*$_{PER}$ and *bla*$_{CTX-M1/M2}$ were detected in *P.aeruginosa* strains. No β-lactamase coding gene was detected in 13 strains (21, 78, 89, 90, 100, 103, 121, 122, 124, 127, 128, 129 and 130).

Table 17. β-lactamase coding genes in *P. aeruginosa* strains (n=20)

Strain N°	**ESβLs**				**Carbapenemases**	**Integrons**
	TEM	SHV	PER	CTX-$_{M1/M2}$	GES	IC1
3	+	+				+
4	+	+			+	+
5	+	+	+	+		+
21						+
78						+
89						+
90						+
91	+			+		+
100						+
103						+
121						+
122						+
123	+					+
124						+
125	+					+
126	+					+
127						+
128						+
129						+
130						+

+: presence of genes, the empty boxes indicated the absence of genes.

III.2.1.3. *Acinetobacter baumannii*

As seen in the **Table. 18**, β-lactamase coding genes of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{PER}$, *bla*$_{OXA-M(23,24,51,58)}$ and *bla*$_{CTX-M1/M2}$ were detected in *A. baumannii*. At least, one β-lactamase coding gene was amplified in the strains, except strains N° 96, 101, 102 and 107.

Table 18. β-lactamase coding genes in *A. baumannii* strains (n=20)

Strain	ESβLs				Carbapenemases			Integrons	
N°	TEM	SHV	PER	CTX-$_{M1/M2}$	NDM	OXA-$_{48}$	OXA-$_{M\ (23,24,51,58)}$	IC1	IC2
20			+				+		
33			+				+		
41	+	+					+		
45					+		+		
52	+		+	+				+	+
59	+						+		
62	+			+			+		
93	+					+			
94		+				+			
95						+			+
96									
97	+			+				+	
98	+	+		+					
99				+				+	
101									
102									
104	+						+		
105	+								
106	+	+		+					
107									

+: presence of genes, the empty boxes indicated the absence of genes.

In summary, molecular determination of β-lactamase coding genes in 108 isolates phenotypically identified as ESβLs and/or carbapenemases producers revealed a prevalence of 65.74 % (71 strains) carriers of a gene *bla*$_{TEM}$, this included 28 (77.77%) of *E.coli*, 18 (85.71%) of *K. pneumonaie*, 10 (50%) of *A. baumannii*, 7 (35%) of *P. aeruginosa* and 8 of other Enterobacterial species. The *bla*$_{SHV}$ gene was detected in 40 strains (37.03%) (17 in *K.pneumonaie* (80.95%), 14 in *E. coli* (38.88%), 04 in *A. baumannii* (20%), 03 in *P. aeruginosa* (15%) and 01 in *K. oxytoca* and 01 *P. stuartii*). The *bla*$_{PER}$ gene was detected in 8 strains (7.4%) (03 in *A. baumannii* (15%), 02 in *E. coli* (5.55%), 01 in *P. aeruginosa* (05%), 01 in *P. mirabilis* (50%), 01 *P. vulgaris* (50%).

bla$_{CTX-M1/M2}$ gene was found in 34 *E.coli* (94.44%), 19 *K.pneumonaie* (90.47%), 06 *A.baumannii* (30%), 02 *P. aeruginosa* (10%), 01 *K. oxytoca*, 01 *C. freundii* and 01 *P. mirabilis.*

Eight strains were detected positive for *bla*$_{\text{OXA-48,}}$ (03) in *A. baumannii* (15%), 02 in *E.coli* (5.55%), 01 in *K.pneumonaie* (4.76%), 01 *K.oxytoca* and 01 *P.mirabilis* and seven (35%) *A.baumannii* strains harboured *bla*$_{\text{OXA-M(23,24,51,58)}}$ gene. *bla*$_{\text{NDM}}$ gene was infrequent and found in two strains of *K.pneumonaie* (9.52%) and in a single strain of *A.baumannii* (5%). Likewise, only one isolate was detected positive for *bla*$_{\text{GES}}$ in *P.aeruginosa* (5%). none of *bla*$_{\text{KPC,}}$ *bla*$_{\text{IMP}}$, and *bla*$_{\text{VIM}}$ genes was found in our isolates. The figure below (**Figure. 15**) shows a Polymerase chain reaction (PCR) of ß-lactamases found in these Algerian MDR isolates.

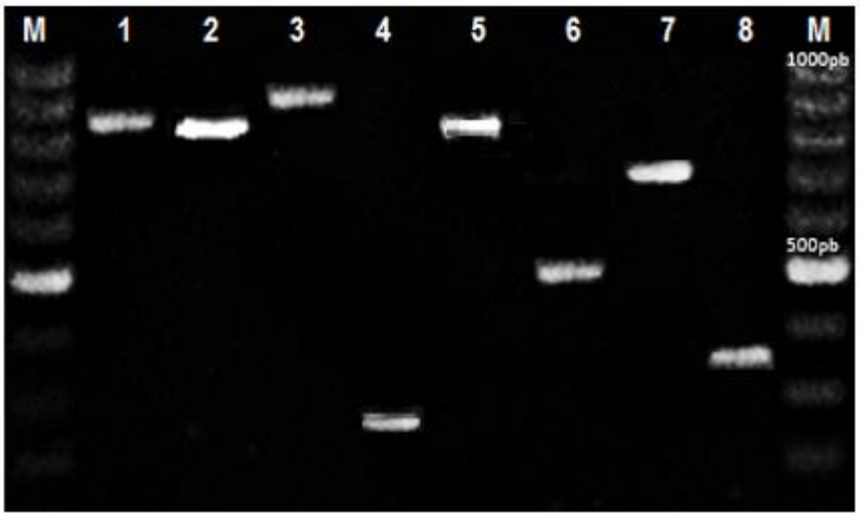


Figure 15. Polymerase chain reaction (PCR); Lane M Molecular size marker (100 bp); lane 1, *bla*$_{\text{TEM}}$; **lane 2,** *bla*$_{\text{SHV}}$; **lane 3,** *bla*$_{\text{PER}}$; **lane 4,** *bla*$_{\text{CTX-M1}}$; **lane 5,** *bla*$_{\text{GES}}$; **lane 6,** *bla*$_{\text{NDM}}$; **lane 7,** *bla*$_{\text{OXA-48}}$; **lane 8,** *bla*$_{\text{OXA-51}}$.

III. 2.2. Class-I and Class-II integrons

We amplified different size of class-I and class-II integron gene cassettes by PCR. All isolates were tested for the presence of the conserved regions of class I and class II integrases. Class-I integronss were determined in twenty *P. aeruginosa* strains (n=20) (strain N°: 3, 4, 5, 21, 78, 89, 90, 91, 100, 103, P1, P8, P9, P12, P13, P14, P15, P17, P19 and P20). Whereas no Class-II integrons were determined in this species.

For *A.baumannii* strains, class-I and II integrons together determined in one strain (n=52), class-I integron gen cassette were determined in two strains (n= 97 and 99), class-I integrons gen cassette were determined in one strains (n=95).

In *E. coli*, class-I integrons alone were determined in 19 strains (9, 10, 12, 25, 27, 28, 38, 40, 44, 47, 49, 56, 70, 71, 72, 73, 83, 85 and 92), class-II integrons alone were determined in two strains (strain no: 54 and 58) and class-I and II integrons together were determined in seven strains together (strain no: 7, 8, 12, 25, 44, 47 and 87).

In *K. pneumonaie* strains, class-II integrons were determined in one sample with class-I integrons gen cassette (n=84) and class-I integrons gen cassette were determined in seven strains (strain no: 11, 23, 36, 48, 55 68 and 82).

In other species, class-I and II integrons gene cassettes were determined alone and together as seen in the **Table. 19**.

Table 19. Integrons class I and II in the other Enterobacterial species (n=11)

N°	Strain N°	Name of bacteria	Integrons	
			IC1	IC2
	06	*K.oxytoca*	+	+
	46	*C.freundii*	+	
2.	50	*C.freundii*	+	
3.	39	*P.mirabilis*		+
4.	51	*P.mirabilis*	+	+
5.	63	*P.vulgaris*		
6.	75	*P.vulgaris*		
7.	30	*M.morganii*		+
8.	37	*S.marcescens*		
9.	29	*E.cloacae*		+
10.	19	*P.stuartii*		

+: presence of genes, the empty boxes indicated the absence of genes.

III.3. Performance of Phenotypic detection tests

The performance characteristics of phenotypic detection of ESβLs and carbapenemases tests are shown in **Table 20**. The sensitivities of ESβLs detection tests ranged from 14.28 to 100%. However, the specificities of DDST and PCDDT with all species are 100%. PCDDT

was poorly sensitive with *P.aeruginosa* and highly sensitive with *A. baumannii* and *Enterobacteriaceae.*

The sensitivities of carbapenemases detection tests ranged from 70 to 100 % and specificities from 46.66 to 100%. The ability of the CDT to detect MBLs in *Enterobacteriaceae* is very satisfactory, sensitivity and specificity were 100%.

Table 20. Sensitivities and specificities of phenotypic methods for detection of ESBLs and carbapenemases production with molecular identification (PCR) as the reference method for *Enterobacteriaceae*, *A.baumannii* and *P.aeruginosa* isolates (n=108)

	SENSITIVITY (%)			SPECIFICITY (%)		
	Enterobacteriaceae	***A.baumannii***	***P.aeruginosa***	***Enterobacteriaceae***	***A.baumannii***	***P.aeruginosa***
ESBLs						
DDST	84.61	-	-	100	-	-
PCDDT	95.4	100	14.28	100	100	100
CARBAPENEMASES						
MHT	71.43	70	100	74.07	100	100
CDT	100	100	-	100	66.66	46.66

IV. Expression and purification of GES-22 and OXA-1

E. coli BL 21 (DE3) bacteria transformed with vectors carrying the genes coding for β-lactamases were cultured for GES-22 and OXA-1. The purified recombinant proteins (GES-22 and OXA-1) were analyzed on acrylamide gel and then stained with Coomassie blue. For each protein, a majority band is observed close to the theoretical mass expected. GES-22 and OXA-1 proteins were obtained recombinantly at high purity (more than 95%) (**Figure. 16**). The molecular weight of GES-22 and OXA-1 are 31,19 and 30,88 kDa respectively.

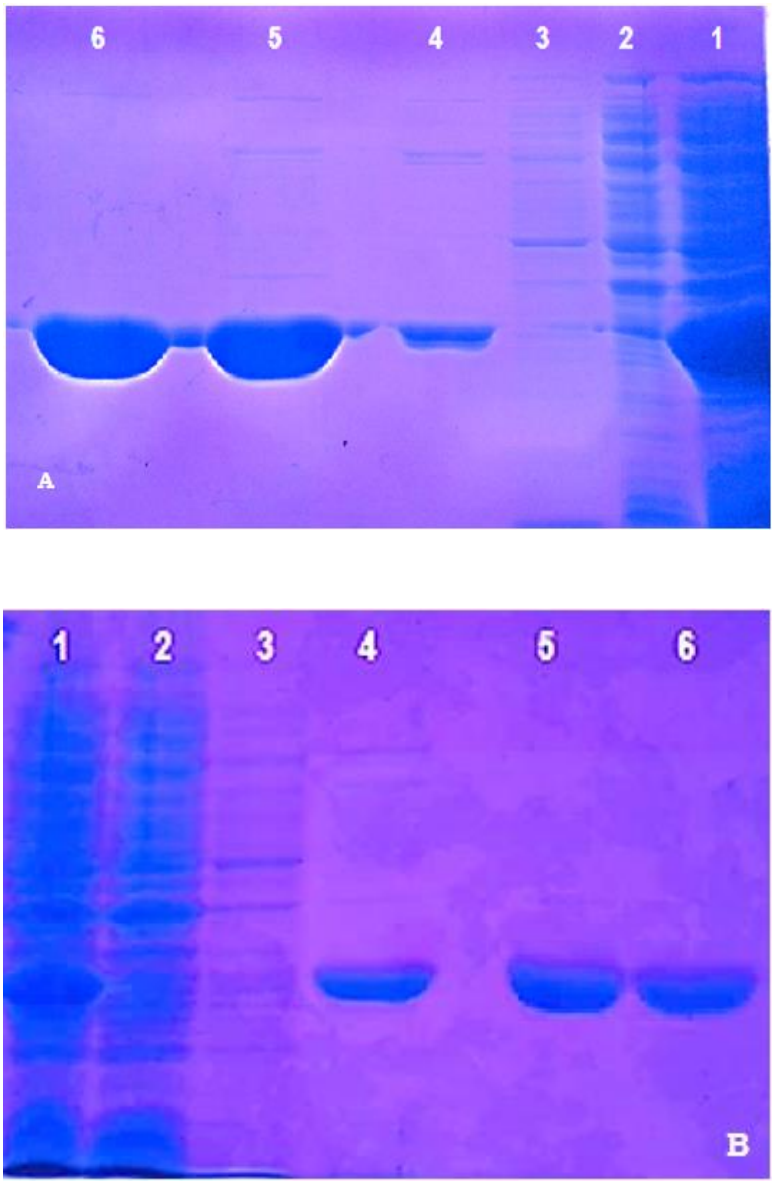


Figure 16. SDS-PAGE gel analysis and purification of (A) GES-22 and (B) OXA-1 expression in *Escherichia coli*. GES-22 and OXA-1 have a molecular weight of around 31,19 and 30,88 kDa, respectively. The band darkens denoting protein transcription and growth due to IPTG induction. Lane 1: supernatant, lane 2: flow, lane 3: 25 µM, lane 4: 100 µM, lane 5: 300 µM, lane 6: 300 µM

V. Phytochemical analysis

The total bioactive compound contents of ME of seaweeds are summarized in **Table 21**. Determined bioactive compounds are total phenolic content, total protein and vitamins E.

V.1. Total phenolic content (TPC)

Extraction yields are presented in the **Table 21**. Compared to other seaweeds, the higher yield (20.61 %) is obtained with *C. tomentosum.*

The amount of total phenolic content of ME of seaweeds are determined as mg GAE/g dry weight extract. The TPC varied among the seven seaweeds, ranging between 2.12±0.14 and 3.22 ± 0.91 mg GAE/g in ME of green seaweeds and 0.93 ± 0.65 and 1.42±0.51 in ME of brown seaweeds. The highest level of polyphenols was recorded in *B. pulmosa* (Chlorophyceae) followed by *C. tomentosum* (2.66±1.33mg GAE/g DW), *U. intestinalis* (2.12±0.14 mg GAE/g DW), *D. dichtoma* (1.89±0.22 mg GAE/g DW), *C. compressa* (1.42±0.51 mg GAE/g DW), *S.vulgare* (1.11±0.77 mg GAE/g DW), and finally *H. scoparia* (phaeophyceae).

V.2. Vitamin E content

The quantitative analysis of vitamin E occurring in ME of seaweeds is ranging between 0.73±0.13 (*U. intestinalis*) mg α-tocopherol/g and 0.83±0.08 (*C. tomentosum*) mg α-tocopherol/g in green seaweeds and 1.07±0.16 (*H.scoparia*) and 1.33±0.58 (*C. compressa*) mg α-tocopherol/g dry weight extract in brown seaweeds. The highest level of total vitamin E was recorded in *C. compressa* (phaeophyceae).

V.3. HPLC analysis of ME of seaweeds

In order to identify the constituents of the ME of seaweeds, the high-performance liquid chromatography with time-of-flight mass spectrometry (HPLC-TOF-MS) was used in positive and negative ion modes. ME of seaweeds were not purified and directly used for simultaneous determination of common compounds by HPLC-ESI-MS, and HPLC-ESI-MS-TOF without using any matrices. Furthermore, the MS data including t_R, values of m/z was got from HPLC-TOF-MS analysis. The total ion chromatograms (TICs) of ME in electrospray ionization (ESI) negative and positive modes are listed in the **Appendix C.**

Table 21. Total phenolic content in methanolic extracts of studied seaweeds

Seaweeds species	Extraction yeild[a] (%)	TPC (mg GAE/g)[b]	Vitamin E (mg α-tocopherol/g)[b]
Green seaweeds			
U.intestinalis	10.55	2.12±0.14	0.73±0.13
C.tomentosum	20.61	2.66±1.33	0.83±0.09
B.pulmosa	17.26	3.22±0.91	0.67±0.08
Brown seaweeds			
S.vulgare	13.33	1.11±0.77	1.29±0.32
D.dichtoma	14.22	1.89±0.22	1.14±0.01
H.scoparia	15.15	0.93±0.65	1.07±0.16
C.compressa	15.40	1.42±0.51	1.33±0.58

a: values expressed as % of dry seaweeds weight. b: Values are expressed as mean ± SD of three parallel measurements; SD: standard deviation; GAE: Gallic Acid equivalent.

The identification of common compounds in ME of seaweeds was performed based on their mass spectrometry and retention times by comparison with those of different reference substances. The spectroscopic characteristics and the retention times of these compounds are listed in **Table 30**. Compounds 1, 2, 3 and 4 (t_R = 48.414; 52.193; 56.448; 48.888 and 51.391 min, $[M-H]^-$ ion presented at m/z 277.216; 279.232; 281.247 and 301.216) corresponded to the molecular formula of $C_{18}H_{30}O_2$; $C_{18}H_{32}O_2$; $C_{18}H_{34}O_2$ and $C_{20}H_{32}O_2$. They were identified as α-linolenic acid, linoleic acid; oleic acid; arachidonic acid, respectively, in the ME of the seven seaweeds (**Appendix C**). Compound 5 (t_R = 21.686 min, $[M-H]^-$ ion presented at m/z 269.24), corresponded to the molecular formula of $C_{15}H_{10}O_5$, was identified as baicalein in the ME of *U. intestinalis* , *D. dichtoma* and *B. pulmosa***.** Compound 6 (t_R = 30.505 min, $C_{21}H_{20}O_6$, $[M-H]^-$ ion presented at m/z 367.4) is identified in *U. intestinalis* and *D. dichtoma* as Curcumin (**Appendix C**). Compound 7 (t_R = 17.594 min, $C_{15}H_{10}O_7$, $[M-H]^-$ ion presented at m/z 301.2), 8 (t_R = 16.252 min, $C_{15}H_{10}O_4$, $[M-H]^-$ ion presented at m/z 253.2) and 9 (t_R = 19.94 min, $C_{15}H_{12}O_5$, $[M-H]^-$ ion presented at m/z 271.2) are identified only in *C.tomentosum*, *B.pulmosa*, *C.compressa* as quercetin, daidzein and (S) naringenin, respectively (**Appendix C**).

Table 22. Compounds identified in methanolic extracts from ME of seaweeds by HPLC-ESI-MS and HPLC-ESI-MS-TOF.

Compound	Formula	M.W	$[M-H]^-$	Retention time (min)	Identification	Found in
1	$C_{18}H_{30}O_2$	278.434	277.216	48.414	α-Linolenic acid	All ME
2	$C_{18}H_{32}O_2$	280.452	279.232	52.193	Linoleic acid	
3	$C_{18}H_{34}O_2$	282.468	281.247	56.448	Oleic acid	
4	$C_{20}H_{32}O_2$	304.474	303.232	51.391	Arachidonic acid	
5	$C_{15}H_{10}O_5$	270.24	269.24	21.686	Baicalein	UI, DD, BP
6	$C_{21}H_{20}O_6$	368.38	367.4	30.505	Curcumin	UI and DD
7	$C_{15}H_{10}O_7$	302.24	301.2	17.594	Quercetin	CT
8	$C_{15}H_{10}O_4$	254.24	253.2	16.252	Daidzein	BP
9	$C_{15}H_{12}O_5$	272.25	271.2	19.94	(S) Naringenin	CC

UI: *Ulva intestinalis*, **DD:** *Dictyota dichtoma*, **BP:** *Bryopsis pulmosa*, **CT:** *Codium tomentesum*, **CC:** *Cystoseira compressa*.

VI. GES-22 and OXA-1 β-lactamases inhibition assays

VI.1. Determination of kinetic parameters (K_M and V_{max}) of GES-22 and OXA-1

Firstly, we have verified if our β-lactamases enzymes are actives or not on nitrocefin. For this, hydrolysis of a fixed sample of nitrocefin (60µM) was started by addition of 7µL of OXA-1 or GES-22 and evolution of absorbance was recorded at 480nm for 5min. As it shown in **Figure 17**, nitrocefin absorbance enhances with time indicating that enzymes are actives.

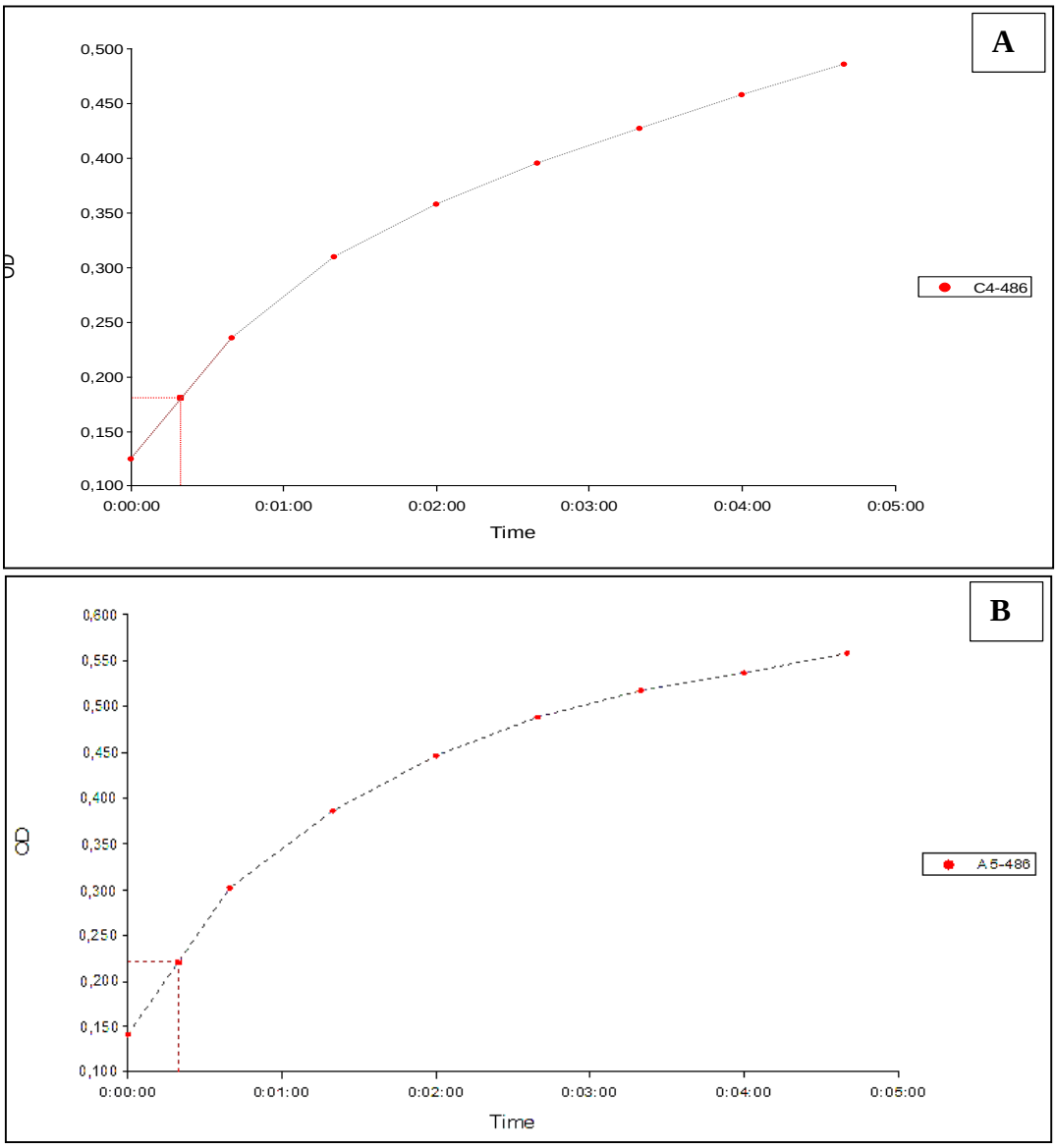


Figure 17. Evolution of the absorbance of 60 µM nitrocefin in the presence of 07 µl of a solution of OXA-1 (A) and GES-22 (B) proteins recombinants

After this test, a Lineweaverburk plot is obtained using the reciprocal of increasing final concentrations of nitrocefin (1/[S]) on the x-axis and the reciprocal of the initial velocity (V_i)

of reaction started by GES-22 or OXA-1 recombinant proteins on the y-axis **(Figure. 18)**. The values of the Michaelis-Menten constant (K_M) determined from this representation are 255.11 µM and 223.78 µM, respectively. Those of maximum velocity are 1.71 x 10^{+2} and 1.63 x 10^{+2} µmol/min/mg protein, respectively.

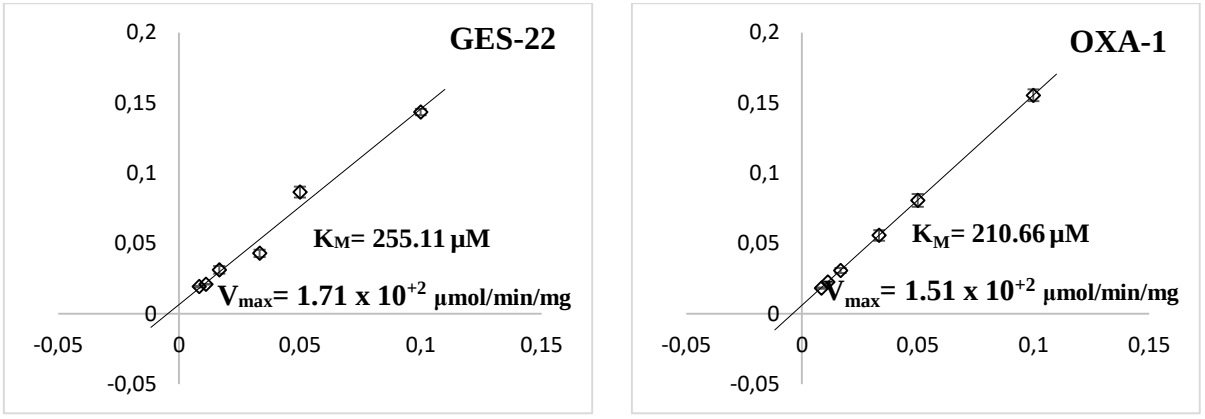


Figure 18. Lineweaver-burk curve and values of K_M (µM) and Vm (µmol/min/mg) obtained with GES-22 and OXA-1 and nitrocefin as substrate

VI.2. Determination of IC_{50} parameter

VI.2.1. Case of clavulanic acid, tazobactam and sulbactam

The measurement of the activity of β-lactamases GES-22 and OXA-1 on a fixed concentration of nitrocefin (60 µM) in absence and presence of an increasing concentrations (1.25 to 300 µM) of clavulanic acid, tazobactam or sulbactam, shows that these latter inhibit the two enzymes in a dose-dependent manner. Values of IC_{50} determined by linear computerized regression analysis after logit/log transformation are respectively 68.38 ± 1.17, 29.94 ± 1.12 and 52.68± 2.64 with GES-22. Class D β-lactamases OXA-1 was not inhibited by sulbactam and clavulanate and was moderately inhibited by tazobactam **(Table. 23).**

Table 23. Values of IC_{50} of clavulanic acid, tazobactam or sulbactam obtained with GES-22 and OXA1

β-lactamases	**IC_{50} (µM)**		
	Tazobactam	Sulbactam	Clavulanate
GES-22	29.94 ± 1.12	52.68± 2.64	68.38 ± 1.17
OXA-1	243.03± 4.53	n.d	n.d

n.d: non determined

VI.2.2. Case of methanolic extract of seaweeds

As obtained with clavulanic acid, tazobactam or sulbactam, the measurement of the activity of β-lactamases GES-22 and OXA-1 started by a fixed concentration of nitrocefin (60 µM) in absence and presence of an increasing concentrations (1.25 to 500 µg/mL) of methanolic extract of *Ulva intestinalis*, *Codium tomentosum*, *Dictyota dichtoma*, *Halopteris scoparia*, *Sargassum vulgare*, *Cystoseira compressa* or *Bryopsis pulmosa*, shows that all these extracts inhibit the two enzymes in a dose-dependent manner. Values of IC_{50} determined by linear computerized regression analysis after logit/log transformation are ranged from 13.01 ± 0.46 (*D. dichtoma*) to 41.24 ± 0.23 (*C. compressa*) and from 13.22 ± 0.96 (*B. pulmosa*) to 62.39 ± 1.96 (*C. compressa*), respevtively with GES-22 and OXA-1 **(Table. 24)**.

Table 24. IC_{50} of methanolic extract of seaweeds

GES-22		OXA-**1**	
Seaweeds	**IC_{50} (µg/ml)**	**Seaweeds**	**IC_{50} (µg/ml)**
D. dichtoma	13.01 ± 0.46	*B. pulmosa*	13.22 ± 0.96
C. tomentosum	16.54 ± 0.48	*U. intestinalis*	14.36 ± 0.70
U. intestinalis	16.87 ± 0.10	*D. dichtoma*	19.19 ± 0.88
B. pulmosa	22.12 ± 0.22	*C. tomentosum*	28.21 ± 1.21
H. scoparia	25.72 ± 0.87	*S. vulgare*	31.65 ± 1.11
S. vulgare	35.32 ± 0.91	*H. scoparia*	51.69 ± 1.01
C. compressa	41.24 ± 0.23	*C. compressa*	62.39 ± 1.96

VI.3. Determination of inhibition type

The inhibition type of GES-22 and OXA-1 activity by each ME of seaweeds was evaluated using Lineweavere Burk plots in absence and presence of a fixed concentration of inhibitor **(Figures. 19 and 20)**. The different apparent V_{max} and K_M values and the inhibition type are shown in the **Table. 25**.

The apparent V_{max} and K_M in the presence of ME of *U. intestinalis*, *C. tomentosum*, *D.dichtoma*, *H. scoparia* and *S. vulgare* against GES-22 was less than the control. These data,

categorizing them as mixed inhibitors. The apparent V_{max} in presence of ME of *C.compressa* and the apparent K_M in presence of ME of *B. corticulans* are not changed categorizing them as competitive and non-competitive inhibitors, respectively. Among the inhibitors, the affinity for GES-22 was found highest for the ME of *S.vulgare.*

The apparent V_{max} and K_M in the presence of ME of *U. intestinalis*, *C. tomentosum*, and *H. scoparia* against OXA-1 were less than the control categorizing them as mixed inhibitors. The data indicated that the ME of *B.pulmosa* has the higher affinity for OXA-1.

Table 25. Apparent kinetic parameters (K_M and V_{max}) of GES-22 and OXA-1 and type of their inhibition by ME of seaweeds

Samples	GES-22			OXA-1		
	Specific activity[b]	**K_M (µM)**	**Inhibition type**	**Specific activity[b]**	**Km (µM)**	**Inhibition type**
Control[a]	1.71 x 10^{+2}	255.11	-	1.51 x 10^{+2}	210.66	-
U. intestinalis	1.11 x 10^{+2}	190.37	M	1.03 x 10^{+2}	177.02	M
C.tomentosum	1.12 x 10^{+2}	185.48	M	1.12 x 10^{+2}	185.48	M
D. dichtoma	0.98 x 10^{+2}	167.26	M	0.34 x 10^{+2}	47.78	M
H. scoparia	1.54 x 10^{+2}	204.32	M	1.19 x 10^{+2}	204.32	M
B. pulmosa	1.26 x 10^{+2}	254.99	NC	0.52x 10^{+2}	77.46	M
S. vulgare	0.39 x 10^{+2}	60.31	M	0.97x 10^{+2}	174.34	M
C. compressa	1.72x 10^{+2}	423.47	C	1.12x 10^{+2}	194.06	M

[a] reaction mixture without ME,[b]µmol of Nitrocefin hydrolysed per min and per mg of protein (µmol. $min^{-1}.Mg^{-1}$)
M: Mixed inhibitor, NC: non-competitive inhibitor, C: competitive inhibitor

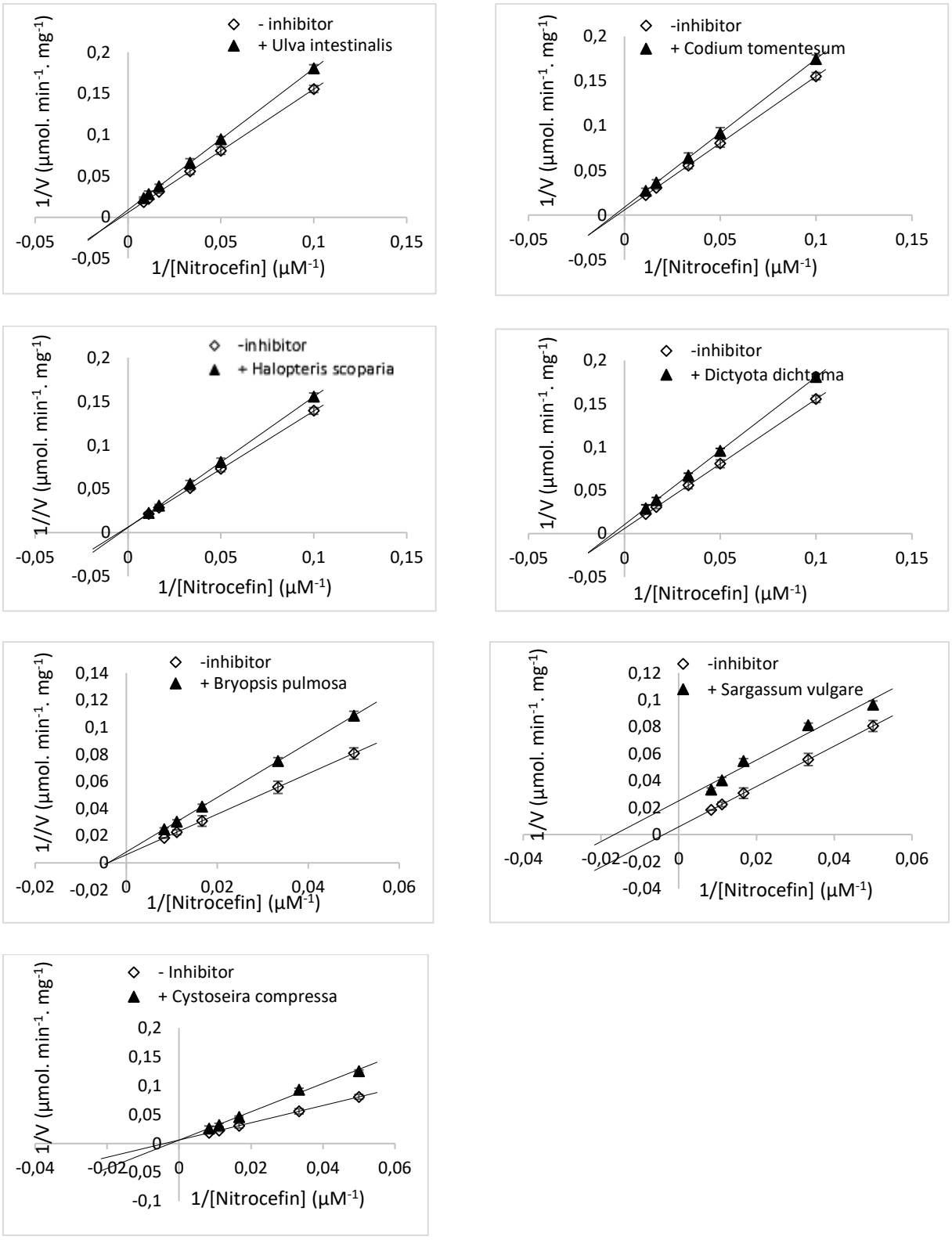
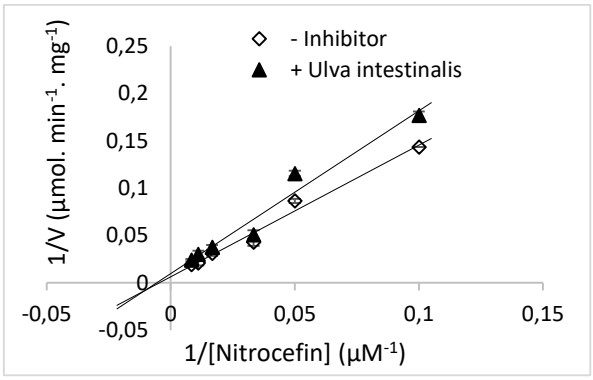
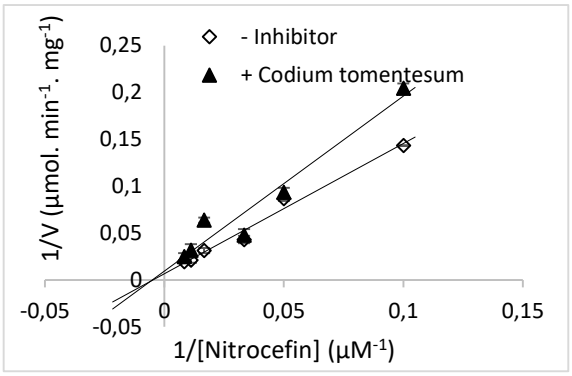
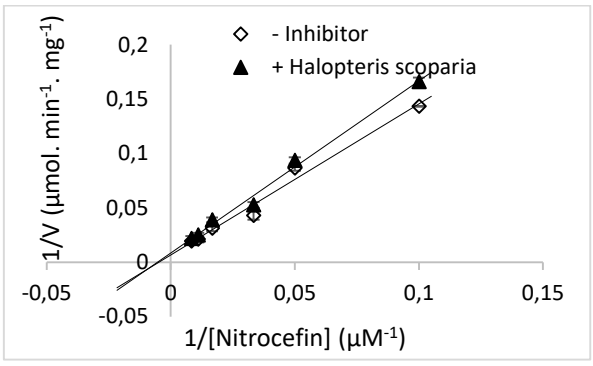
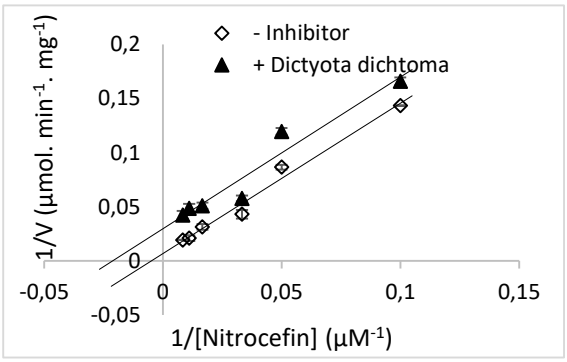


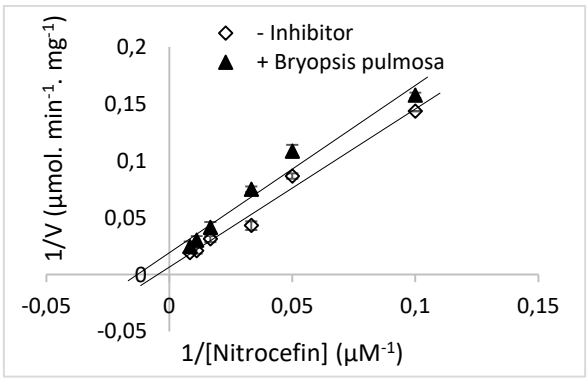
Figure 19. Lineweaver burk plots obtained when activity of GES-22 is started with increasing concentration of nitrocefin in presence (▲) and absence (◇) of each ME of seaweeds

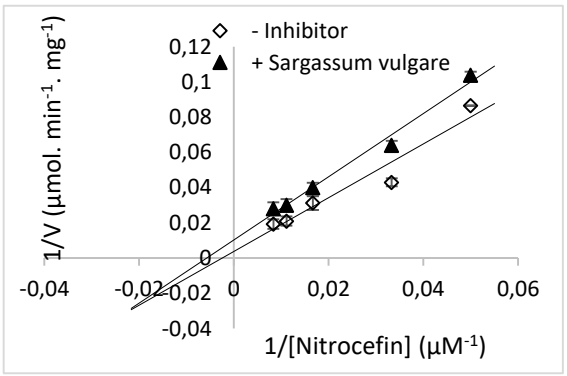












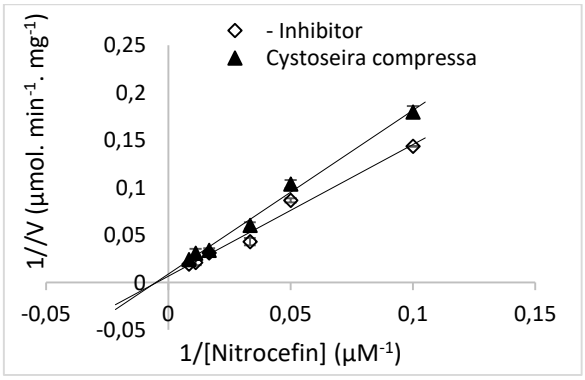


Figure 20. Lineweaver burk plots obtained when activity of OXA-1 is started with increasing concentration of nitrocefin in presence (▲) and absence (◇) of each ME of seaweeds.

VII. Molecular docking

VII.1. Evaluation test of the program used

Several methods are used to evaluate the performance of the different programs of docking for each application. The ability of an algorithm to find the correct location of the ligand in relation to its receptor is usually determined by means of the deviation root mean-square-deviation (RMSD) of the model designed by the software against the crystal structure. To check if the protocols that we have developed is correct, we have found it useful to first evaluate the performance of the docking approach of Libdock and CDOCKER by the root mean square deviation (RMSD) (**Table. 26**).

The accepted value is a maximum difference of 2 angstroms beyond which the prediction is considered as inadequate. The RMSD test was performed on 23 β-lactamases complexes available in the PDB. It is more precisely, complexes formed between the β-lactamases enzyme and different ligands. The PDB codes of the complexes and their ligands as well as the values of their RMSDs obtained are mentioned in **Table 26**. Among the several complexes tested proposed by the PDB, we chose the betalactamases mentioned in the following table, because the value of the mean square deviation or RMSD of the model designed by the software towards the structure of the crystal does not exceed 2A°.

Based on the obtained results we can conclude that the Libdock and CDOCKER docking approach selected of the discovery studio used is sufficiently performance.

VII.2. Docking study

In subsequent step, we have studied each complex of 15 β-lactamases formed with 40 ligands and 3 β-lactamase inhibitors. The results of DS simulation represented by Libdock score and -CDOCKER energy/-CDOCKER inreraction energy obtained are reported in the **tables 27 and 28**. These tables represented respectively, the score obtained with 6 ligands, of which the first one is the ligand with high score compared to all ligands and the five others are the compounds founded in the ME of our seaweeds.

Table 26. RMSD of different β-lactamases complexes studied

	PDB ID	TYPE	Method	RMSD (A°)
1.	4LEN	CTX-M-9	Libdock	1.2329
2.	4GOG	GES-1	Libdock	1.2343
3.	1TDG	SHV-1	Libdock	1.7476
4.	3P98	TEM-72	Libdock	1.435
5.	1NXY	TEM-1	Libdock	1.1013
6.	4ZBE	KPC-2	Libdock	1.3194
7.	4S2O	OXA-10	Libdock	1.5731
8.	4JF4	OXA-23	Libdock	1.7428
9.	4WM9	OXA-24	CDOCKER	1.4192
10.	4WMC	OXA-48	Libdock	1.2982
11.	5L2F	OXA-51	Libdock	1.836
12.	4Y0U	OXA-58	Libdock	0.9611
13.	4PVT	VIM-2	Libdock	1.3644
14.	1DD6	IMP-1	Libdock	1.5316
15.	3Q6X	NDM-1	CDOCKER	1.2444

RMSD: Root-mean-square-deviations, PDB ID: Protein Data Bank IDentifier

Table 27. Libdock score ENERGY of the best 5 compounds and standards binding to β-lactamases

PDB ID	N	PUBCHEM CID	NAME	LIBDOCK SCORE
4LEN (CTX-M9)		161557	Dihydromyricetin	115.839
	1-	969516	Curcumin	104.659
	2-	439246	Naringenin	96.5941
	3-	5281605	Baicalein	92.0442
	4-	5280934	α-Linolenic acid	90.9981
	5-	444899	Arachidonic acid	89.9683
	STANDARDS			
	1-	123630	Tazobactam	97.0757
	2-	5280980	clavulanic acid	73.4964
	3-	130313	Sulbactam	70.1717
PDB ID		**PUBCHEM CID**	**NAME**	**LIBDOCK SCORE**
4GOG GES-1)		3594	Hesperidin	164.583
	1-	969516	Curcumin	123.159
	2-	444899	Arachidonic acid	116.093
	3-	5280450	Linoleic acid	109.004
	4-	439246	Naringenin	107.989
	5-	5280934	α-Linolenic acid	107.486
	STANDARDS			
	1-	123630	Tazobactam	97.7716
	2-	5280980	clavulanic acid	88.2229
	3-	130313	Sulbactam	77.9537
PDB ID		**PUBCHEM CID**	**NAME**	**LIBDOCK SCORE**
1TDG SHV-1)		3594	Hesperidin	166.465
	1-	969516	Curcumin	124.686
	2-	444899	Arachidonic acid	111.65
	3-	445639	Oleic acid	103.236
	4-	5280450	Linoleic acid	102.56
	5-	5280934	α-Linolenic acid	101.882
	STANDARDS			
	1-	123630	Tazobactam	90.5248
	2-	5280980	clavulanic acid	70.3992
	3-	130313	Sulbactam	66.2064
PDB ID		**PUBCHEM CID**	**NAME**	**LIBDOCK SCORE**
3P98 (TEM-72)		425990	Paeoniflorin	120.553
	1-	969516	Curcumin	95.3096
	2-	439246	Naringenin	94.4492
	3-	444899	Arachidonic acid	88.2373
	4-	445639	Oleic acid	81.9631
	5-	5280934	α-Linolenic acid	81.7332
	STANDARDS			
	1-	123630	Tazobactam	94.705
	2-	5280980	clavulanic acid	79.302
	3-	130313	Sulbactam	70.4396

PDB ID		PUBCHEM CID	NAME	LIBDOCK SCORE
1NXY (TEM-1)		64982	Baicalin	150.43
	1-	969516	Curcumin	123.079
	2-	445639	Oleic acid	105.135
	3-	5280934	α-Linolenic acid	103.263
	4-	444899	Arachidonic acid	102.421
	5-	439246	Naringenin	100.958
	STANDARDS			
	1-	123630	Tazobactam	103.565
	2-	5280980	clavulanic acid	79.1524
	3-	130313	Sulbactam	70.1912
PDB ID		PUBCHEM CID	NAME	LIBDOCK SCORE
4ZBE (KPC-2)		5281673	Myricitrin	139.773
	1-	969516	Curcumin	116.16
	2-	439246	Naringenin	102.626
	3-	444899	Arachidonic acid	100.423
	4-	5280934	α-Linolenic acid	96.9752
	5-	5280450	Linoleic acid	93.9008
	STANDARDS			
	1-	123630	Tazobactam	96.3408
	2-	5280980	clavulanic acid	78.9632
	3-	130313	Sulbactam	67.312
PDB ID		PUBCHEM CID	NAME	LIBDOCK SCORE
4S2O (OXA-10)		5281417	Esculin	93.9122
	1-	439246	Naringenin	86.0493
	2-	445639	Oleic acid	84.9145
	3-	444899	Arachidonic acid	83.3292
	4-	5281605	Baicalein	82.4423
	5-	969516	Curcumin	82.2015
	STANDARDS			
	1-	123630	Tazobactam	86.9125
	2-	5280980	clavulanic acid	66.7339
	3-	130313	Sulbactam	67.9105
PDB ID		PUBCHEM CID	NAME	LIBDOCK SCORE
4JF4 (OXA-23)		3594	Hesperidin	139.531
	1-	969516	Curcumin	106.697
	2-	444899	Arachidonic acid	103.598
	3-	445639	Oleic acid	97.6381
	4-	5280450	Linoleic acid	97.3625
	5-	5280934	α-Linolenic acid	96.0991
	STANDARDS			
	1-	123630	Tazobactam	91.2057
	2-	5280980	clavulanic acid	73.5823
	3-	130313	Sulbactam	76.0117
PDB ID		PUBCHEM CID	NAME	LIBDOCK SCORE
		161557	Dihydromyricetin	99.6997

4WMC (OXA-48)	1-	969516	Curcumin	92.0739
	2-	439246	Naringenin	85.431
	3-	444899	Arachidonic acid	85.0914
	4-	5280934	α-Linolenic acid	84.0353
	5-	445639	Oleic acid	81.8462
	STANDARDS			
	1-	123630	Tazobactam	86.038
	2-	5280980	clavulanic acid	70.9416
	3-	130313	Sulbactam	68.7698
PDB ID		**PUBCHEM CID**	**NAME**	**LIBDOCK SCORE**
5L2F OXA-51)		3594	Hesperidin	159.947
	1-	969516	Curcumin	119.953
	2-	444899	Arachidonic acid	112.597
	3-	439246	Naringenin	103.577
	4-	445639	Oleic acid	102.726
	5-	5280450	Linoleic acid	101.478
	STANDARDS			
	1-	123630	Tazobactam	100.193
	2-	5280980	clavulanic acid	81.2853
	3-	130313	Sulbactam	78.9262
PDB ID		**PUBCHEM CID**	**NAME**	**LIBDOCK SCORE**
4Y0U (OXA-58)		161557	Dihydromyricetin	105.662
	1-	5280343	Quercetin	99.8038
	2-	439246	Naringenin	96.7315
	3-	444899	Arachidonic acid	96.5965
	4-	5280934	α-Linolenic acid	96.3819
	5-	5280450	Linoleic acid	92.4908
	STANDARDS			
	1-	123630	Tazobactam	92.2091
	2-	5280980	clavulanic acid	74.9686
	3-	130313	Sulbactam	72.6283
PDB ID		**PUBCHEM CID**	**NAME**	**LIBDOCK SCORE**
4PVT (VIM-2)		425990	Paeoniflorin	130.398
	1-	444899	Arachidonic acid	111.425
	2-	5280934	α-Linolenic acid	110.427
	3-	969516	Curcumin	107.85
	4-	439246	Naringenin	105.442
	5-	5280450	Linoleic acid	104.145
	STANDARDS			
	1-	123630	Tazobactam	111.573
	2-	5280980	clavulanic acid	82.3481
	3-	130313	Sulbactam	83.385
PDB ID		**PUBCHEM CID**	**NAME**	**LIBDOCK SCORE**
		5280805	Rutin	199.392
	1-	969516	Curcumin	164.791
	2-	444899	Arachidonic acid	128.087

1DD6 (IMP-1)	3-	5280343	Quercetin	125.657
	4-	439246	Naringenin	123.632
	5-	5280450	Linoleic acid	120.649
	STANDARDS			
	1-	123630	Tazobactam	112.471
	2-	5280980	clavulanic acid	78.6306
	3-	130313	Sulbactam	79.1643

Table 28. –CDOCKER energy/–CDOCKER inreraction energy of the best 5 compounds

PDB ID		**Pubchem CID**	**NAME**	**-CDOCKER energy (kcal/mol)**	**- CDOCKER interaction energy (kcal/mol)**
3Q6X (NDM-1)		10168	Rhein	127.529	119.212
	1-	5280343	Quercetin	98.0867	104.277
	2-	5280450	Linoleic acid	66.2534	94.4663
	3-	5280934	α-Linolenic acid	50.1126	93.7072
	4-	445639	Oleic acid	81.0329	93.5745
	5-	444899	Arachidonic acid	31.6	88.7078
	STANDARDS				
	1-	123630	Tazobactam	61.1765	86.1801
	2-	5280980	clavulanic acid	33.7385	79.4313
	3-	130313	Sulbactam	64.4129	83.4184
4WM9 (OXA-24)		5280805	Rutin	19.5352	69.8565
	1-	5280343	Quercetin	61.7539	63.0256
	2-	445639	Oleic acid	29.3655	43.3471
	3-	444899	Arachidonic acid	17.5803	40.388
	4-	5281605	Baicalein	40.4853	39.7871
	5-	439246	Naringenin	21.1582	39.4533
	STANDARDS				
	1-	123630	Tazobactam	9.14269	32.5336
	2-	5280980	clavulanic acid	18.7857	28.4225
	3-	130313	Sulbactam	11.5383	27.8947

VII.3. Molecular interaction studies

A total of 15 β-lactamases as target proteins of which 12 are SβLs and 3 are MβLs were tested for interaction with the higher score compounds. Confused, all these compounds establish many different covalent and non-covalent bonds with active aminoacids site of both SβLs and MβLs targets. Covalent bonds are frequently carbon and Pi-hydrogen bonds; non-covalent bonds are conventional hydrogen bonds **(Table. 29)**.

In the case of SβLs, which are six among class A and six among class D, ligands with high score interact with SβLs targets by covalent and conventional hydrogen bonds established with different aminoacids of conserved elements of active site. These aminoacids are frequently SER70, SER130, ASN132, THR234, LYS205, 234, GLY235 when SβLs are of class A **(Figures 21 to 26)** and SER67 and 70, 79, 80, 83, 126, 216, 218, 219, 257 and LYS124, 125 and 216 when when SβLs are of class D **(Figures. 27 to 32)**

In case of MβLs which are VIM-2, IMP-1 and NDM-1, paenoflorin establishes two conventional hydrogen bonds with HIS118, ARG228 and three carbon hydrogen bonds with ASP120, GLY232, HIS263 of VIM-2 active site. In addition, this ligand establishes a Pi-alkyl interaction with HIS196 of the active site of the same MβLs **(Figure. 33)**. Rutin establishes five conventional hydrogen bonds with PRO32, SER71 and 80, TYR163, GLY164 and five covalent bonds with TRP78, LYS161, GLY166, HIS197, SER198 and other non-covalent interactions with HIS79 and Zn of IMP-1 **(Figure. 34)**. Rhein forms two conventional hydrogen bonds with HIS189 and 250 and other non-covalent interactions with two Zn and LYS211 of NDM-1 **(Figure. 35)**

Table 29. Amino acid residues of β-lactamases targets showing more frequent interactions in the company of the high score ligands predicted using Discovery studio

Target	Ligand	β-lactamases amino acid residues showing different interactions	
		Covalent bond (Carbon hydrogen bond + Pi-donor hydrogen bond)	**Conventional hydrogen bond**
SβLs class A			
CTX-M-9	**Dihydromyrecitin**	GLY236	SER70, ASN132, SER237
GES-1	**Hesperidin**	GLU98, PRO162, GLY231, CYS233	SER64, ASN127, GLU161
SHV-1	**Hesperidin**	GLY283	ILEU221 and 279, MRD 800
TEM-72	**Paeoniflorin**	ASN132 and 170, GLY236	SER70 and 130, ALA237
TEM-1	**Baicalin**	SER130	ASN132 and 170, VAL216, LYS234
KPC-2	**Myrecitin**	PRO106, GLY235	SER69 and 129, ASN131, GLU165, THR234 and 236
SβLs class D			
OXA-10	**Esculin**	GLY207	SER67, PHE208
OXA-23	**Hesperidin**	SER 79 and 126	LYS124 and 216, THR217, TRP219, ASP222
OXA-24	**Rutin**	SER257 and 259	SER219
OXA-48	**Dihydromyrecitin**	SER70	TYR211, ARG250
OXA-51	**Hesperidin**	SER80, GLY219	LYS125, SER218, TRP222, ARG260, ACT 302
OXA-58	**Dihydromyrecitin**	ALA82, SER83 and 130	LEU170, TRP223, ALA226, ARG263
MβLs			
VIM-2	**Paeoniflorin**	ASP120, GLY232, HIS263	HIS118, ARG228
IMP-1	**Rutin**	TRP78, LYS161, GLY166, HIS197, SER198	PRO32, SER71 and 80, TYR163, GLY164
NDM-1	**Rhein**	HIS189 and 250	_

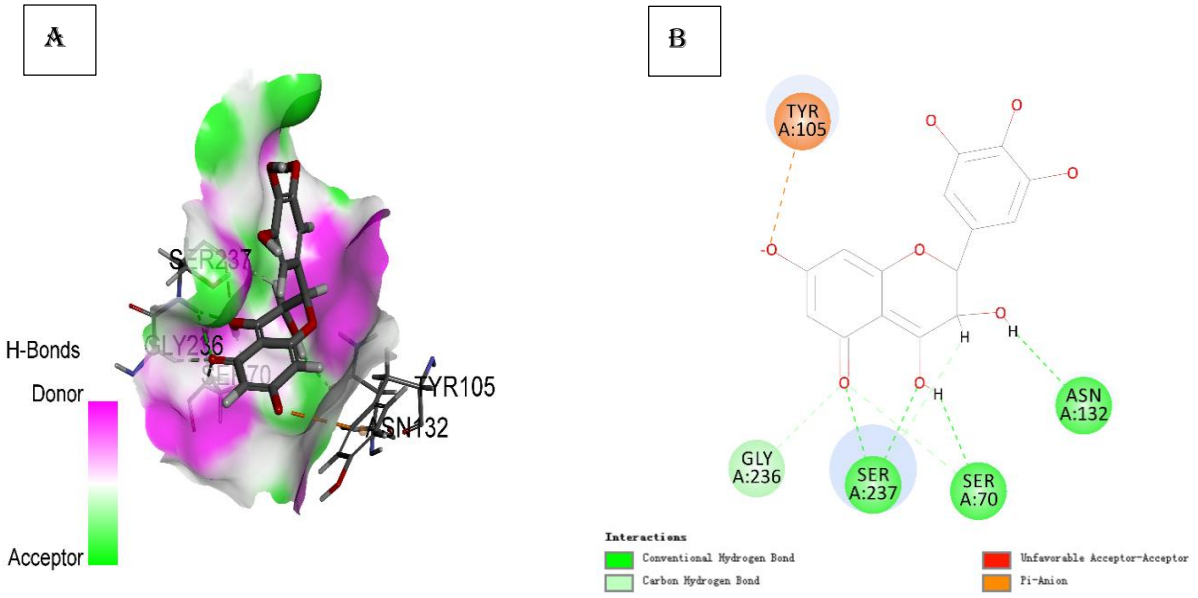


Figure 21. Interaction of Dihydromyrecitin and CTX-M-9. A: 2D interaction diagram. B: Fisher projection

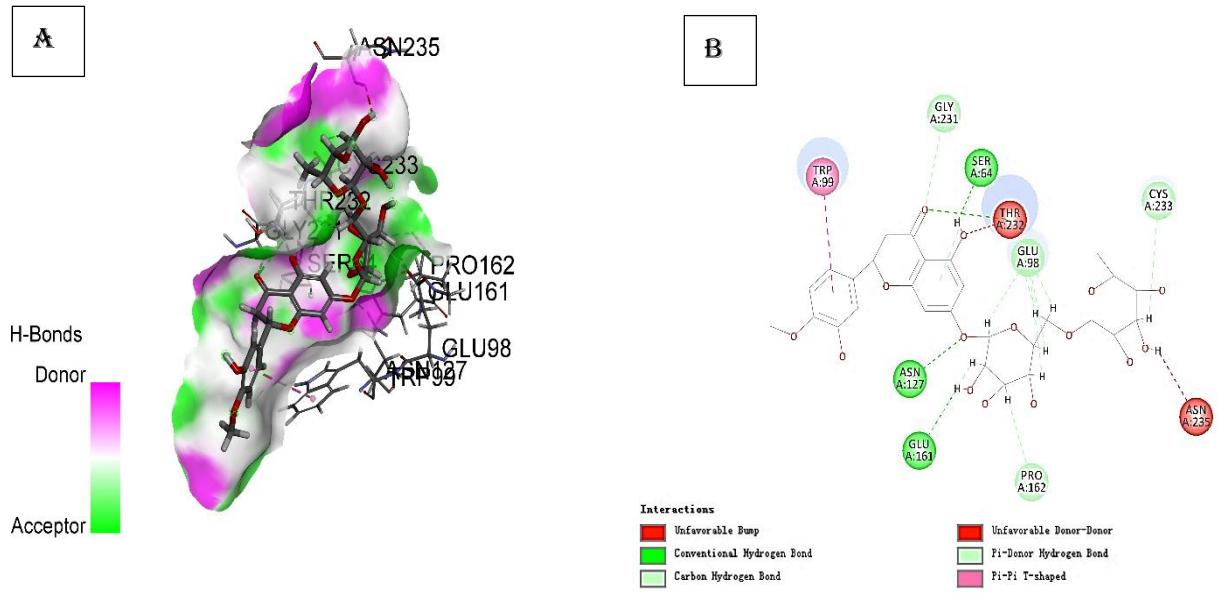


Figure 22. Interaction of Hesperidin and GES-1. A: 2D interaction diagram. B: Fisher projection

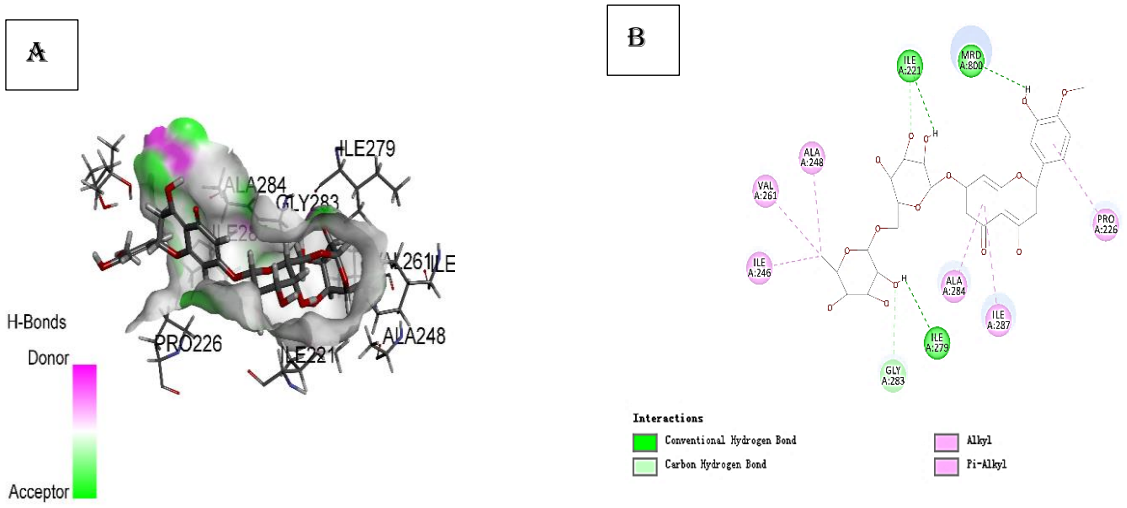


Figure 23. Interaction of Hesperidin and SHV-1. A: 2D interaction diagram. B: Fisher projection

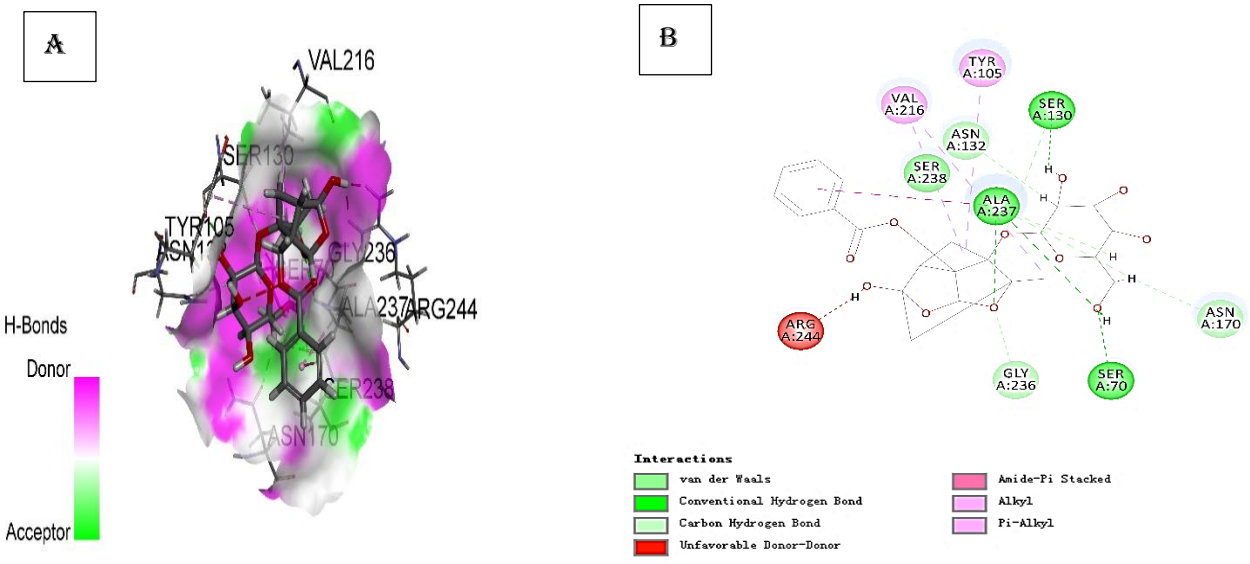


Figure 24. Interaction of Paeoniflorin and TEM-72. A: 2D interaction diagram. B: Fisher projection

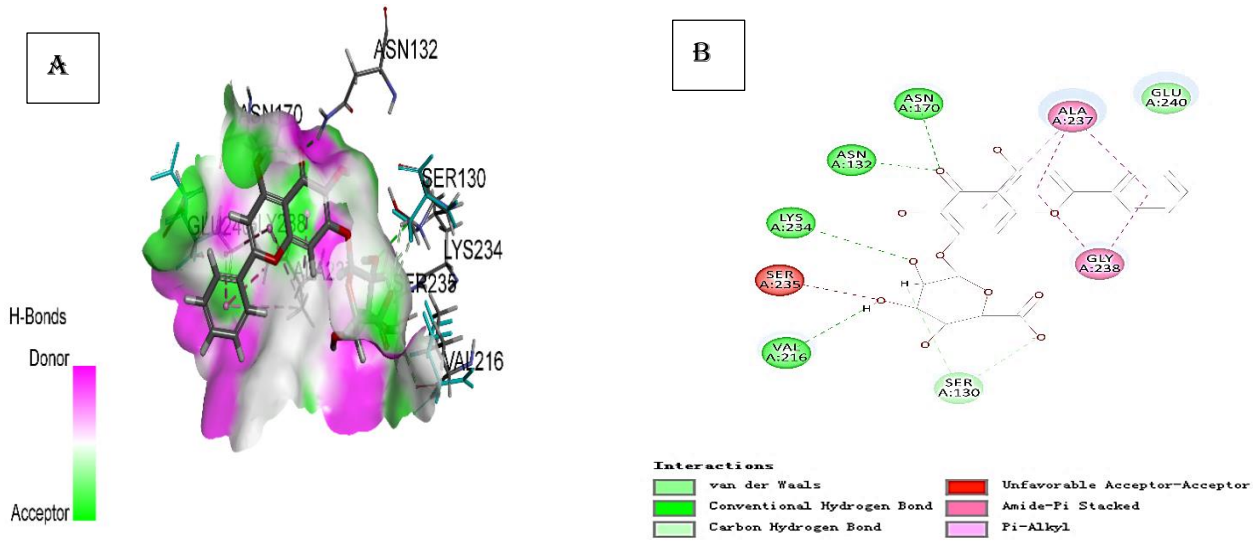


Figure 25. Interaction of Baicalin and TEM-1. A: 2D interaction diagram. B: Fisher projection

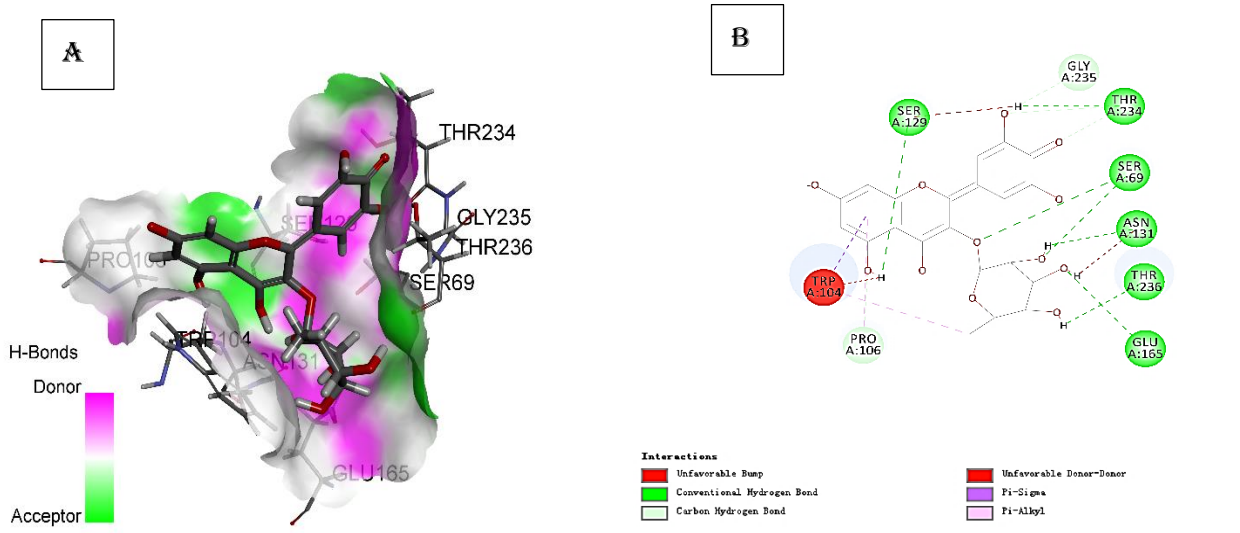


Figure 26. Interaction of Myrecitin and KPC-2. A: 2D interaction diagram. B: Fisher projection

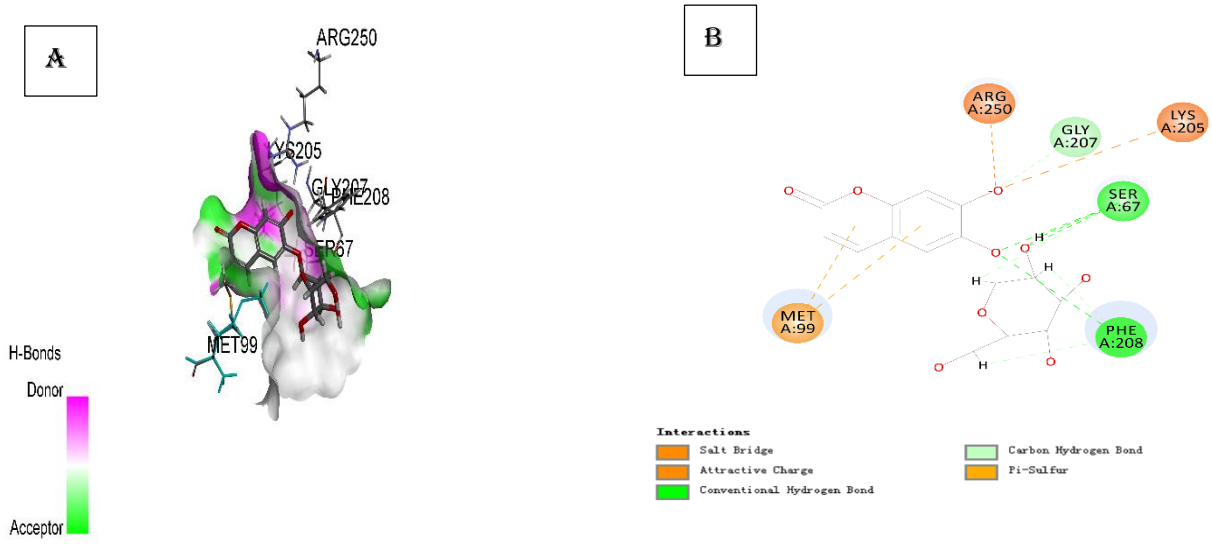


Figure 27. Interaction of Esculin and OXA-10. A: 2D interaction diagram. B: Fisher projection

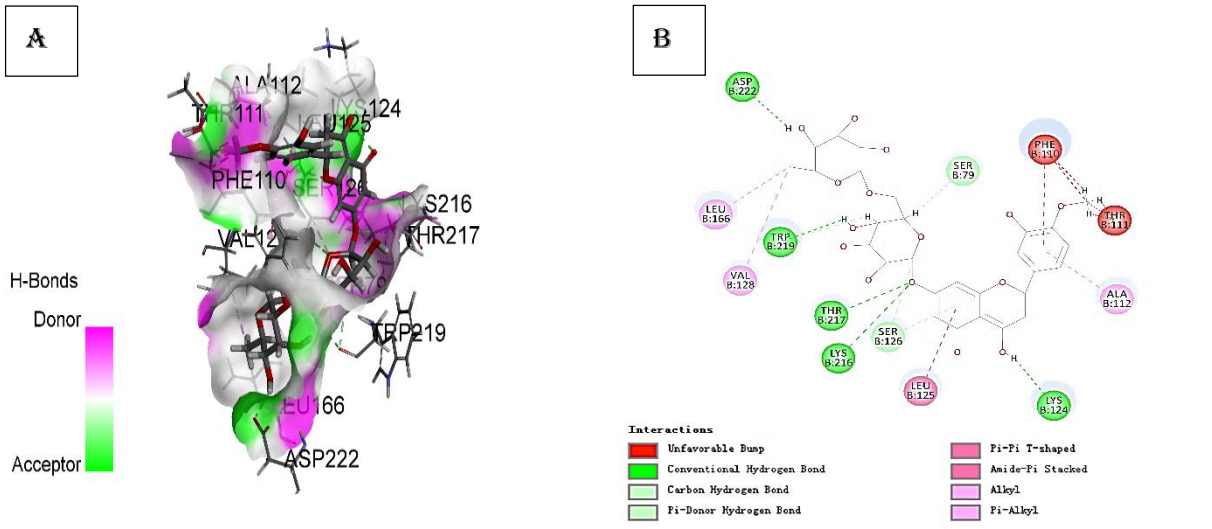


Figure 28. Interaction of Hesperidin and OXA-23. A: 2D interaction diagram. B: Fisher projection

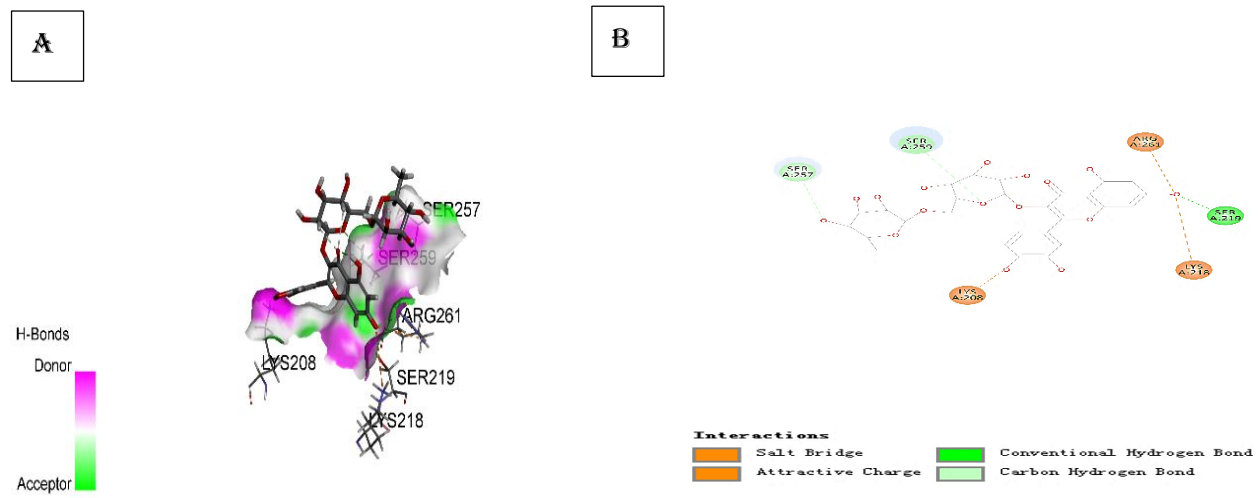


Figure 29. Interaction of Rutin and OXA-24. A: 2D interaction diagram. B: Fisher projection

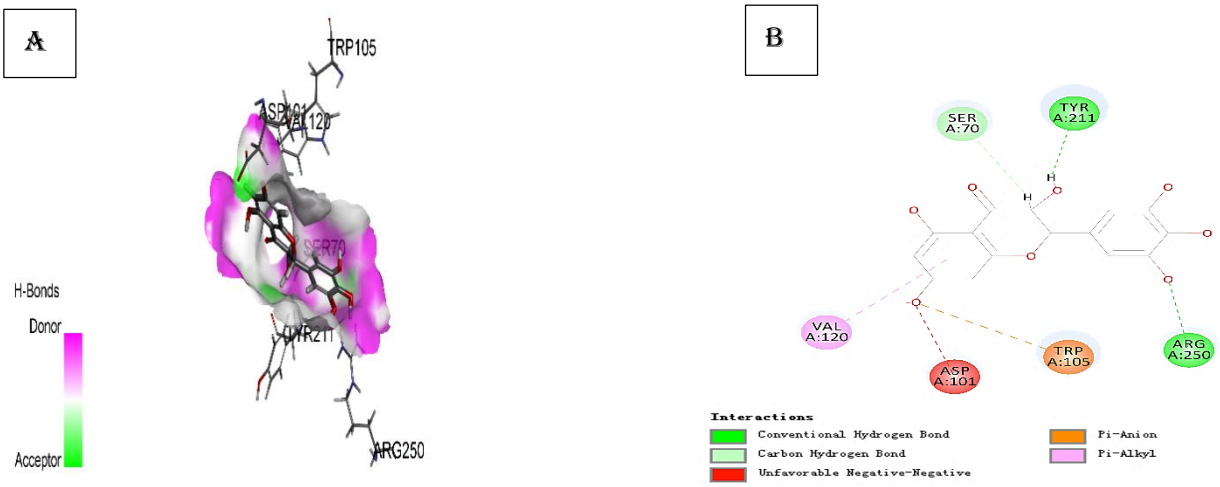


Figure 30. Interaction of Dihydromyrecitin and OXA-48. A: 2D interaction diagram. B: Fisher projection

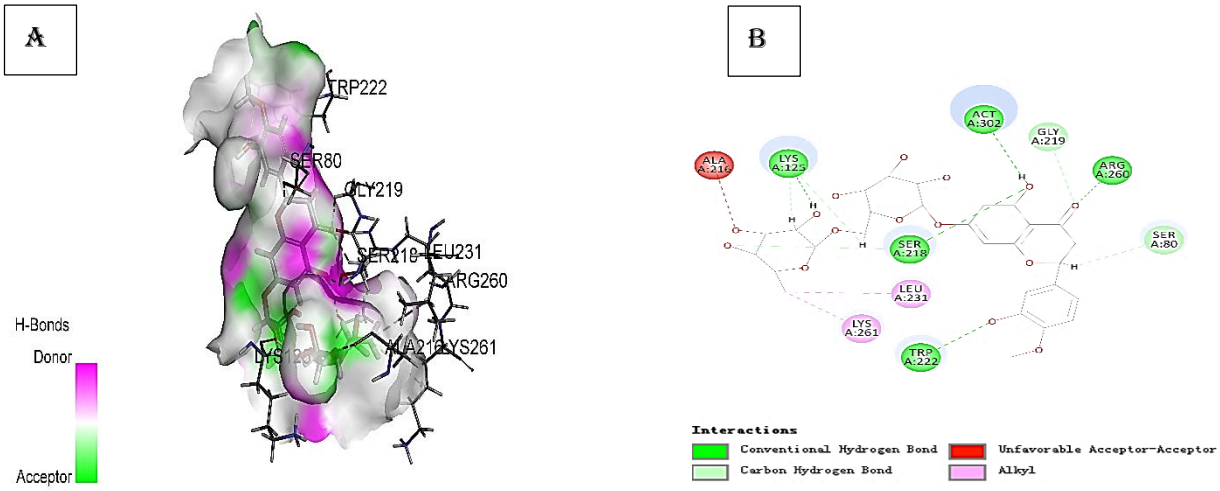


Figure 31. Interaction of Hesperidin and OXA-51. A: 2D interaction diagram. B: Fisher projection

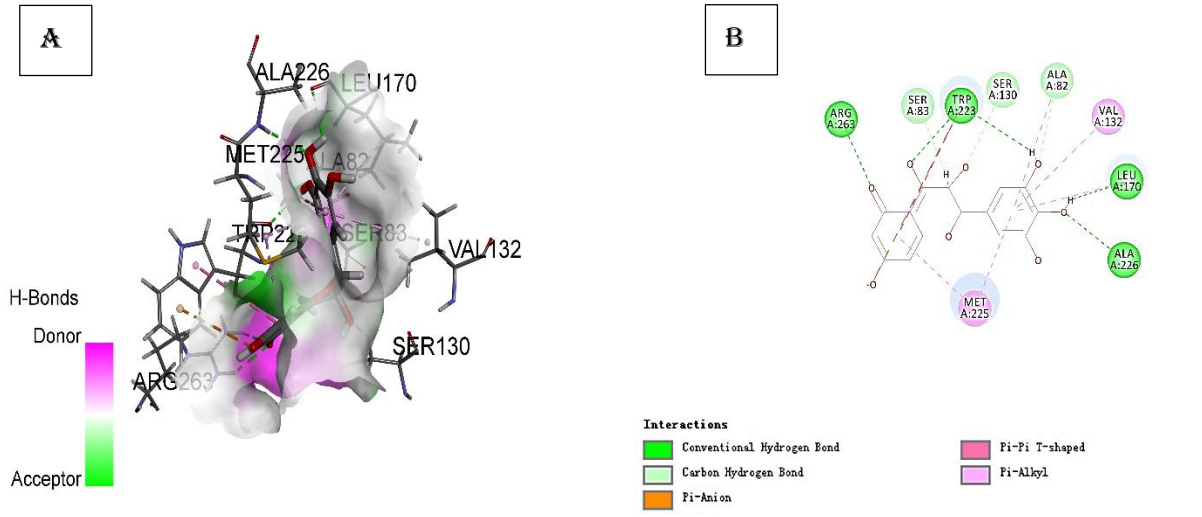


Figure 32. Interaction of Dihydromyrecitin and OXA-58. A: 2D interaction diagram. B: Fisher projection

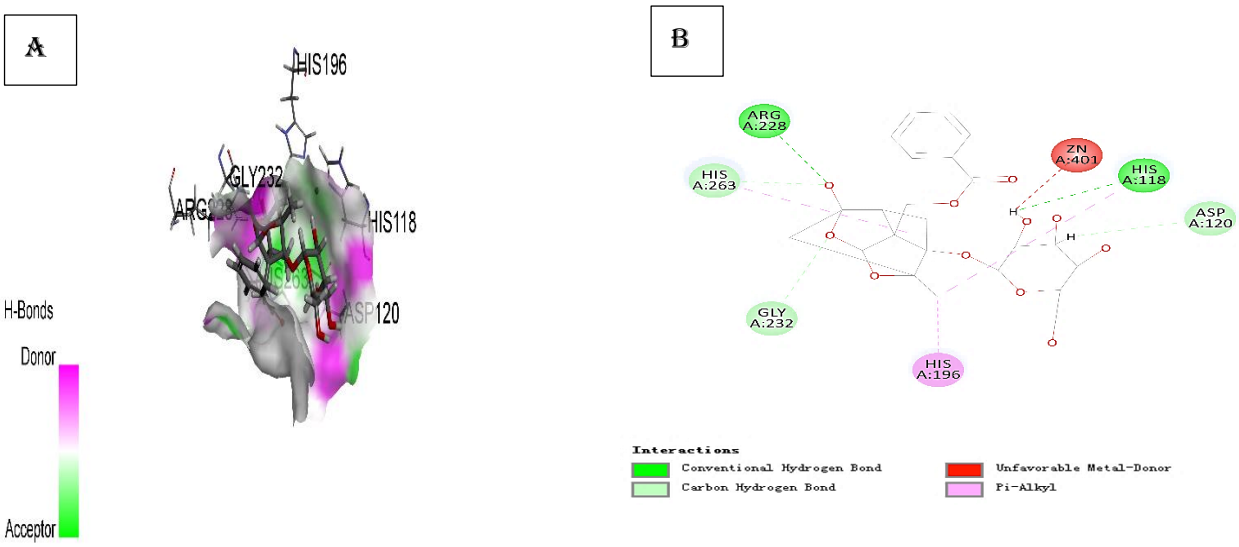


Figure 33. Interaction of Paeoniflorin and VIM-2. A: 2D interaction diagram. B: Fisher projection

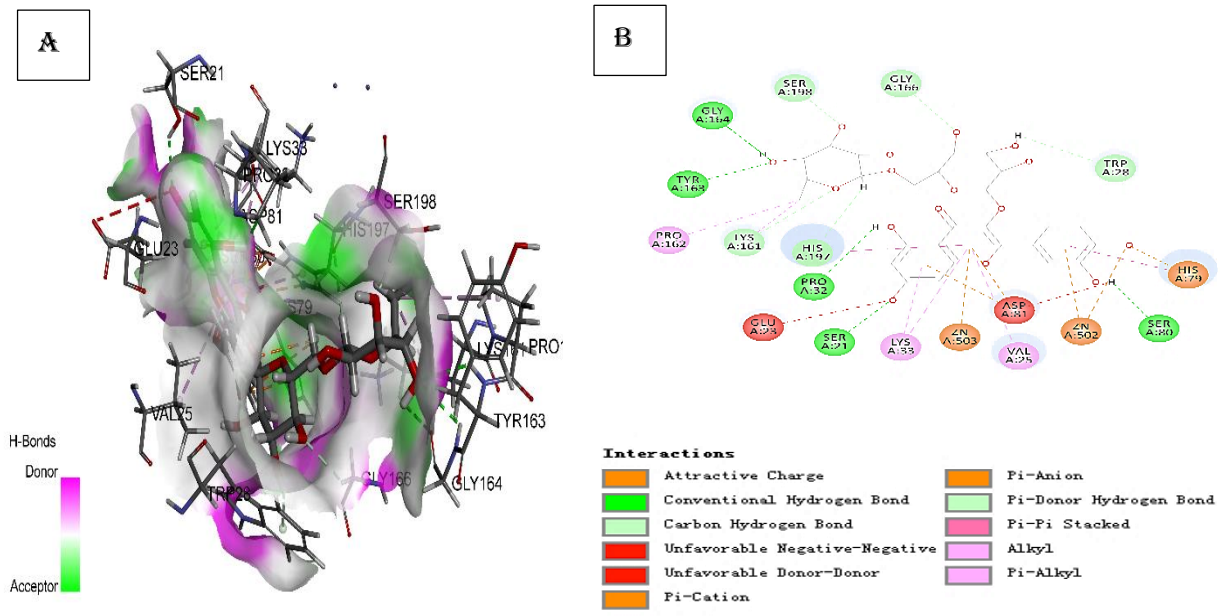


Figure 34. Interaction of Rutin and IMP-1. A: 2D interaction diagram. B: Fisher projection

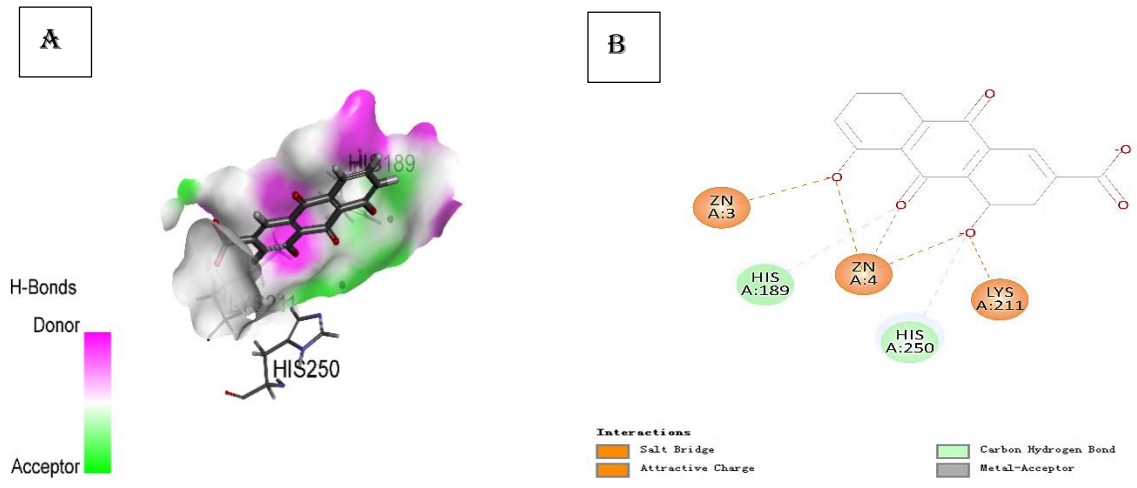


Figure 35. Interaction of Rhein and NDM-1. A: 2D interaction diagram. B: Fisher projection

Discussion

DISCUSSION

I. Distribution of MDR isolates

There is no consensual definition of a Multidrug-resistant bacteria. According to the recommendations of the antibiogram committee of the French Microbiology Society, resistant strains (R + I) with at least one antibiotic from three different antibiotic families (β-lactams, quinolones, aminoglycosides, sulfonamides (cotrimoxazole)) were defined as multi-resistant (CA-SFM, 1999). Some researchers require resistance to three classes of antibiotics, by different mechanisms (Carlet, 2006). Boutoille (2012) has defined MDR, any bacteria not susceptible to at least one antimicrobial agent among at least 3 categories to be tested for the germ. However, Hawkey *et al*., 2018 have defined MDR bacteria, bacteria that remain susceptible to only one or two antibiotics. As a result, these terms are used arbitrarily creating great confusion among researchers, health care professionals and the public (Falagas and Karageorgopoulos, 2008). For the purpose of this study "MDR" will be used to denote isolates resistant to at least three classes of antimicrobial agents. These multi-resistance leading to therapeutic impasses are mainly found in Gram-negative bacilli, including *Enterobacteria*, *P.aeruginosa* and *A. baumannii* (Nordmann, 2014). Studies conducted in many developing countries, have indicated high antibiotic resistance among GNB to commonly used antibiotics (Le Doare *et al*., 2015, Kumburu *et al*, 2017). These bacteria are emerging internationally, and it poses a major problem in different hospitals in Algeria (Drissi *et al*., 2010, Berrazeg *et al*., 2013, Mesli *et al*., 2013). In our study, the rate of MDR-GNB is 62.42%. Our results are inferior to those reported by Agyepong *et al*. (2018) in a teaching hospital in Ghana where Multidrug resistance was observed in 89.5% of the bacterial isolates and superior to those reported by Siwakoti *et al*. (2018) who found that MDR-GNB infections was not uncommon in intensive care units and it accounted for 47 MDR-GNB cases per 100 ICU admission. The majority of MDR strains, in this study, are from nosocomial source 68.52%, compared with 31.48% from community sources. The appearance of multidrug resistance in the community would be a serious health problem. This epidemic phenomenon is particularly worrying because of the rapidity of its diffusion and its geographical extent. Faced with this threat, it is therefore urgent to adopt the rules of "good use" and "less use" of antibiotics.

The most common cause of bacterial resistance to β-lactam antibiotics is the production of β-lactamases (Sasirekha *et al*., 2010). In the current study, Seventy MDR-GNB were defined phenotypically and molecularly as ESßLs producers of which 58/68 are *Enterobacteriaceae* (34

E. coli, 18 *K. pneumoniae* and 06 other Enterobacterial species), 06/20 are *A.baumannii* and 06/20 are *P.aeruginosa*. Two *A.baumannii* strains were defined as NDM carbapenemase producers and sixteen were defined as ESßLs and carbapenemases coproducers of which 07/68 are *Enterobacteriaceae* (02 *E. coli*, 03 *K. pneumoniae*, 01 *K. oxytoca* and 01 *P. mirabilis*), 08/20 are *A. baumannii* and 01/20 is *P.aeruginosa* strains. In totally, 65/68 (95.6%), 16/20 (80%) and 07/20 (35%) of *Enterobacteriaceae*, *A. baumannii* and *P. aeruginosa* strains harboring at least one β-lactamases coding gene, respectively. These results are closely related to that reported in Algeria by Baba Ahmed-Kazi Tani *et al*., 2013 where 100% (71/71) of the MDR Enterobacterial isolates (17 *E. coli*, 50 *K. pneumoniae* and 4 *E. cloacae*) were defined as ESßL producers. Moreover, Pokhrel *et al*., 2006 reported that all ESßL-producing strains were found only in MDR cases. The genes that code for production of ESßL are often linked to other resistance genes (Thomson, 2001) causing extended spectrum of drug resistance (Ahmed and Salam, 2002).

I.1. Distribution according to species

In the present study, *Enterobacteriaceae* was the most predominant family with a prevalence of 62.96% followed by 18.51% *A. baumannii* and 18.51% *P. aeruginosa*. The predominant species among *Enterobacteriaceae* were *E.coli* (52.94%) followed by *K.pneumoniae* (30.88%) This finding is comparable to the results obtained by many studies (Sader *et al*., 2003; Andrade *et al*., 2006; Mansouri and Abbasi, 2010). However, *P. mirabilis* that is reported in European countries and the USA (Chanal *et al*., 2000, Karlowskyet *al*., 2003) to be the second most common *Enterobacteriaceae* isolate, in our study occupied the penultimate place with virtually identical isolation rates 2.94% (2/68) with *P. vulgaris* and *C.freundii*. *K. oxytoca, E. cloacae*, *M. morganii*, *P. stuartii* and *S. marcescens* occupied the last place with isolation rates 1.47% (1/68).

I.2. Distribution according to biological sample

The distribution of the specimen types showed that the highest proportion of MDR-GNB isolates were from urine specimens followed by blood culture with rate of 43.52% and 17.59%, respectively. Urinary tract infection is a common pathology in daily practice, it ranks first in all infections acquired in the hospital or in community. These results are consistent with those reported in other studies (Belmonte et *al.*, 2010; Mayoral et *al.*, 2010, Agyepong *et al*., 2018).

I.3. Distribution according to age

The most colonized patients by MDR-GNB are infant and advancing age-groups. Our result was relatively similar with those reported by Donetti and Barbe and their collaborators. They reported that age greater than 60 years was a significant risk factor for MDR bacteria (Donetti et *al*., 1998, Barbe *et al*., 2010). Children under 02 years, are commonly associated with risk factors due to their lack of fully developed immunity, malnutrition as well as inadequate hygiene. Whilst, the reduced immunity, co-morbid diseases such as diabetes mellitus, chronic heart diseases and the neurogenic bladder are the main causes of colonized of elderly patients by MDR-GNB (Agyepong *et al*., 2018).

I.4. Distribution according to sex

The different MDRs studied were more frequent in mal than in female with a sex ratio M/F=1.63. The data from the literature are insufficient, which makes it impossible to determine with certainty that sex is a risk factor for infections related to MDR-GNB. No other study to our knowledge has shown that elderly men patients are more vulnerable that women and even women were more immunocompetent. However, Bréaud and Guys (2005) demonstrated the beneficial effect of circumcision on upper urinary tract infections in children during the first years of life. This study reported a one-tenth decrease in resistance rates of circumcised boys compared to uncircumcised boys. Thus, it is known in our customs that children, sex male, are circumcised generally from the age of two.

I.5. Distribution according to service (in-patients)

A higher proportion of MDR-GNB were recovered from in-patients 74 (68.52%) compared to the out-patients 34 (31.48%). The use of invasive procedures (catheterization and mechanical ventilation) promotes the appearance of higher infections rates by MDR strains among in-patient (Chang *et al*., 2011). Anupurba *et al*. (2006) reported that, the duration of the hospital stay was directly proportional to a higher prevalence of the infection. However, Fortes Déguénonvo *et al*. (2015). found that venous catheterization, antibiotic therapy before MDRs isolation, immunodepression and bladder catheterization were the main risk factors for the nosocomial infections by MDRs with the respectively rates of 94%, 76%, 51.2%, 50%. Our study showed that the prevalence of MDR bacteria in intensive care unit was 36.94%. This rate was similar to that reported in a study conducted by Saidani *et al*., 2006 in Tunisia where, the rate is 38.4%. Agyepong *et al,* (2018) reported that the highest number of MDR isolates was collected from the medical intensive care unit (ICU) followed by Child Health, thus these

results agrees with ours. Other studies in Tunisia and Morocco have reported prevalence less important in adults from 17 to 20% in intensive care unit (Kallel *et al*., 2005, Arslan *et al*., 2010).

II. Antibiotic resistance patterns

The antibiogram was developed in the 1950s for the reason of the appearance and diffusion of bacterial resistance. It is a routine test but nevertheless complex to ensure a reliable result. Its purpose is to predict the outcome of antibiotic treatments (Billy, 2003). Levels of bacterial resistance vary from country to country and from year to other. Indeed, our results show a considerable resistance rate to the majority of antibiotics tested.

II. 1. *Enterobacteriaceae*

Resistance to β-lactams in *Enterobacteriaceae* is currently dominated by ESßLs, problem among community and hospital isolates (Vodovar *et al*., 2012). The main characteristics of ESßL-mediated resistance in *Enterobacteriaceae* are the resistance to amino- and carboxy-penicillins, as well as to second-generation and one or several third- and fourth-generation cephalosporins or aztreonam (Drieux *et al*., 2008). *Enterobacteriaceae* resistant to third-generation cephalosporins (C3G) is the main cause of nosocomial infections. These bacteria become more and more resistant to antibiotics and start to cross the boundaries of the hospital to emerge in the community. In the present study, 85.3% of MDR *Enterobacteriaceae* were defined as ESßLs producers. *Enterobacteriaceae* strains are resistant to all ß-lactams with high rates except carbapenems and cefoxitin. We reported also a high degree of resistance to multiple classes of antibiotics. Our findings are similar to that of Sharma, Khalifa and Chakraborty who reported that ESβLs producing *E.coli* organisms were frequently resistant to non β-lactam antibiotics such as aminoglycosides, fluoroquinolones and cotrimoxazole (Sharma *et al*., 2007; Khalifa *et al*., 2009; Chakraborty *et al*., 2013). The genes encoding for ESßLs enzymes are located on large plasmids that can harbor resistance genes to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole (Kanj and Kanafani, 2011). Decreased susceptibility to the third-generation cephalosporins, ceftazidim (100 %) and cefotaxime (100%), may suggest high expression or production of ESßLs. In this study, All *E.coli* strains harboring at least one $bla_{\text{EßSL}}$ gene. Two strains harboring both $bla_{\text{EßSL}}$ and $bla_{\text{carbapenemase}}$ genes (OXA-48). However, all *K.pneumoniae* strains harboring at least two $bla_{\text{EßSL}}$ genes except two strains harboring one gene of ESßLs, In addition, one *K.pneumoniae* strains co-expressed $bla_{\text{EßSL}}$ and $bla_{\text{carbapenemase}}$ gene (NDM). NDM gene is carried on plasmids

which also carry a number of other genes conferring resistance to aminoglycosides, macrolides and sulphamethoxazole, thus making strains harboring this gene multidrug resistant (Franklin *et al*., 2006). All other Enterobacterial species harboring $bla_{\text{EßSL}}$ gene except *K.oxytoca* and *P.mirabilis* co-expressed $bla_{\text{EßSL}}$ and $bla_{\text{carbapenemase}}$ gene (OXA-48) *P.vulgaris*, *M.morgannii* and *E.cloaceae* harboring neither $bla_{\text{EßSL}}$ nor $bla_{\text{carbapenemase}}$ genes. In *Enterobacteriaceae* strains, $bla_{\text{CTX-M}}$ was the most prevalent ESßLs gene, followed by bla_{TEM} then bla_{SHV}. These results are similar to those reported in previous studies (Giske *et al*., 2008, Hussain *et al*., 2011).

II.2. *Acinetobacter baumannii*

A.baumannii has become an important human opportunistic pathogen due to the increase in the number of infections caused by this microorganism, and the emergence multi-resistant strains (MDR). Most often, it is responsible for nosocomial infections that are difficult to control and treat, particularly in intensive care units (Mc Connell *et al*., 2013). In our study 75% of *A.baumannii* responsible for nosocomial infections of which 53.33 % are from patients in the intensive care units. Many other studies have reported the prevalence of *A.baumannii* infections in intensive care units (Elouennass *et al*., 2003, Al-Agamy *et al*., 2014). The results in terms of antibiotic resistance found in this study were alarming. The resistance levels were very high for ß-lactams and reached 100% for all antibiotics except imipenem (45%). These results are closely related to those obtained by Al-Agamy *et al*. (2014) and Alkasaby and El Sayed Zaki (2017) whereas, they reported a high level of resistance to imipenem. Indeed *A.baumannii* has naturally mechanisms of resistance to ß-lactams including hyperproduction of chromosomal cephalosporinase to which will be added its ability to easily acquire resistance by involving several mechanisms of enzymatic resistance, efflux and impermeability. Decreased susceptibility to cephalosporins, as cefotaxime, ceftazidime, and cefepime, (0%) may suggest high expression or production of ESßLs (Zhanel *et al*., 2013). Carbapenem resistance in the genus *Acinetobacter* is mainly related to the production of oxacillinases (ß-lactamases class D) with carbapenemase activity or metallo-β-lactamases (ß-lactamases class B). In the present study, 40% of *A.baumannii* isolates were producing both ESBLs and carbapenemases, 30% producing only ESßLs and 10% producing only carbapenemmases and only one isolate coproducing MBLs and carbapenemases (5%). However, Safari *et al*. (2015) showed that most of *A.baumannii* isolates were producing MßLs (99%), but not ESßLs (7%). Ciprofloxacin resistance rate (85%) was similar to studies conducted in Egypt which showed the same resistance rate (Al-Agamy *et al*., 2014). As for colistin, which is part of the family Polymyxins, and which is often the only therapeutic alternative for carbapenem-resistant *A.baumannii*

strains. Our strains have kept a sensitivity of (80%). Alkasaby and El Sayed Zaki (2017) confirm this result but with slightly higher sensitivity rates (97%).

II.3. *Pseudomonas aeruginosa*

P. aeruginos is one of the most important pathogens causing nosocomial infections; it is naturally resistant to many antimicrobial agents. It has a distinctive capacity to become resistant to many available antimicrobial agents via multiple mechanisms. In our study, the rate of isolation of *P.aeruginosa* was higher in in-patients (75%) as compared to that in the out-patients (25%) of which 53.33 % are from patients in the intensive care units. Similarly, a study conducted by Anupurba *et al*., 2006 reported that the isolation of *P. aeruginosa* was more common in in-patients (73.42%). The antibiogram of *P.aeruginosa* isolates showed an increased resistance against ß-lactams, the cephalosporin third generation, ceftazidime, is used for the treatment of infections caused by *P. aeruginosa*. Ceftazidime resistance is mainly mediated by production of ESßLs besides other mechanisms. In the present study, we observed a high resistance level to various antibiotics other than ß-lactams such us aminoglycosides, quinolones. A decreased susceptibility of *P. aeruginosa* to the commonly used antibiotics has already been reported by previous researchers (Jaykumar and Appalraju, 2007; Aggarwal *et al*., 2008; Peshattiwar and Peerapur, 2011). The resistance to ceftazidime antibiotic is increasing at an alarming rate due to its indiscriminate use as a broad-spectrum agent for empirical therapy. In our study 70% of isolates were resistant to ceftazidime. Our results are similar to those reported by Dwivedi *et al.*, 2009 who reported that resistance rate to the ceftazidime is 63%. In contrast, Ibukun et *al.*, 2007 reported a low resistance for ceftazidime (20.6%). The prevalence of ESBL-producing *P. aeruginosa* is 35%. This rate is slightly higher than the one reported by Aggarwal *et al.* (2008) (20.27) and even lower than the rate that has been reported by Haider *et al*. (2014) (59.45%). These results suggest that the ESBLs are now increasing in *P. aeruginosa*, which were usually widespread among members of *Enterobacteriaceae.* High frequency of ESBLs in *P. aeruginosa* is due to horizontal spread of genes (Vinita *et al*., 2018). In this study, 20% producing at least 2 ESßL-type. This varied ESßL production could be due to inappropriate use of antibiotics or due to environmental influence (Vinita *et al*., 2018).

III. Phenotypic and molecular detection of ß-lactamases

Early detection of β-lactamases producing strains is crucial for optimal treatment of critically in patients (Pitout *et al*., 2005). However, detection of these enzymes is a great challenge in the clinical microbiology laboratory (Steward *et al*., 2000). The basic phenotypic

strategy to detect ESβLs producing strains is to use a cephalosporin indicator to screen for likely producers, then to seek the synergy between cephalosporin and clavulanic acid, which confirms ESβLs production (Tenover *et al*., 2002; NCCLS, 2002). Whereas, strategy to detect carbapenemases producing strains is the use of the cloverleaf technique (modified Hodge test) for the detection of diffusible carbapenemases based upon inactivation of carbapenem, or the use of the carbapenemases inhibitors. These are the boronic acid for detection of Ambler class A carbapenemases, the metal-chelating agent EDTA and dipicolinic acid to detect Ambler class B carbapenemases, and cloxacillin against Ambler class C carbapenemases AmpC (Giske *et al*., 2011; Seah *et al*., 2011). However, PCR was considered the gold standard method for detection of β-lactamases (Ghonaim *et al*., 2018). For us, several methods of β-lactamases detection were employed to detect and characterize β-lactamases (ESβLs and Carbapenemases) in Algerian bacterial strains and to compare both phenotypic and molecular detection methods. ESβLs producing isolates were characterized phenotypically for ESβLs production, using double disc synergy test as initial screening test, phenotypic confirmatory disc diffusion test (PCDDT) as a confirmation test for strains that do not show a synergistic image after initial screening test (DDST). This to detect the production of an ESβLs that can be masked by another mechanism of resistance. Carbapenemase phenotypic confirmation is investigated based on the detection of diffusible carbapenemases (evaluated in the Modified Hodge Test (MHT)) and on the inhibition of the activity of carbapenemases, by the synergy between inhibitors of Ambler class B (MβLs), EDTA, or Ambler class A (KPC), boronic acid, and carbapenems. However, the molecular characterization is based on PCR-based technique. This later was used to amplify the ESβLs and carbapenemase genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M1\text{-}M2}$, *bla*$_{PER\text{-}2}$, *bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{OXA48}$, *bla*$_{OXA\text{-}M}$ [23,24,51,58]) and the conserved regions of the class I and II integrons.

In this part of our study, recall that 108 MDR GNB were isolated and identified between January 2015 to April 2016, from different hospital in Algeria, of which 62.96% are Enterobacteriaceae strains (68/108). This group is constituted of 33.33 % (36/108) *Escherichia coli*, 19.44 % (21/108) *Klebsiella pneumoniae*, and 10.18 % (11/108) other Enterobacterial species (2 *Citrobacter freundii*, 2 *Proteus vulgaris*, 2 *Proteus mirabilis*, 1 *Klebsiella oxytoca*, 1 *Enterobacter cloacae*, 1 *Morganella morganii*, 1 *Providencia stuartii*,1 *Serratia marcescens*). Strains different from Enterobacteriaceae, represented 37.04 %, are *Pseudomonas aeruginosa* 18.52 % (20), *Acinetobacter baumannii* 18.52 % (20).

III.1. Extended Spectrum β-Lactamases

Nosocomial infections caused by ESßL-producing Gram-negative bacteria often complicate therapy, limit the therapeutic options and cause treatment failures (Rossolini *et al*., 2007, Zahar *et al*.,2009). Therefore, phenotypic detection of ESßLs is important for limiting the spread of resistance mechanisms as well as epidemiological purposes. Some ESßLs such as TEM-3 and SHV-2 confer high levels of resistance to cephalosporins, in contrast, some others, such as TEM-7 and TEM-12, confer low levels of resistance, which makes their detection more difficult with the antibiotic susceptibility tests (d'Azevedo *et al*.,2004). Moreover, the coexistence of ESBLs with derepressed chromosomal cephalosporinases, plasmid-mediated AmpCs, and carbapenemases may lead to difficulties in identifying the production of each enzyme (Poulou *et al*.,2014). Thus, confirmatory tests have therefore been recommended for the correct detection of ESßLs activity. This is crucial for appropriate individualized antibiotic treatment, infection control purposes, and epidemiological evaluations (Finch *et al*., 2012).

III.1.1. *Enterobacteriaceae*

Enterobacteriaceae-producing ESßLs have emerged gradually during the last decades and their prevalence reach alarming rates. The prevalence and the distribution of ESBLs genotypes are different from one year to another and even vary greatly in different geographical areas (Poulou *et al*.,2014). In the last years, CTX-M ß-lactamases have become widespread in both *Escherichia coli* and *Klebsiella spp.* in nosocomial and community settings (Brahimi *et al*., 2018). In Algeria, The CTX-M-3 and CTX-M-15 were identified in both community and nosocomial isolates of various *Enterobacteriaceae*. Touati *et al*., 2006 describes for the first time the emergence of CTX-M-3 and CTX-M-15 ß-lactamases among clinical isolates of *Enterobacteriaceae*. Iabadene *et al*. (2008) described SHV-12 in clinical *E.cloacae* isolates. In 2019, CTX-2 and SHV-12 were described by Boujamaa *et al*. (2019) for the first time in MDR *P.mirabilis* isolates. Similarly, our study reports a widespread diffusion of CTX-M-1/M-2 followed by TEM and SHV. The presence of the $bla_{\text{CTX-M-1/2}}$ gene in 94.44% of *E.coli,* 90.5% of *K.pneumoniae* and 72.8% of other Enterobacterial species reflects the wide spread of this enzyme in both community and nosocomial Algerian *Enterobacteriaceae* isolates. This agrees with previous studies, thus confirming the predominance of CTX-M type in *Enterobacteriaceae* in Algeria (Ramdani-Bouguessa *et al*., 2006, Baba Ahmed-Kazi Tani *et al*., 2013, Ayad *et al*., 2016). Coexpression of TEM, CTX-M-1/M-2, and SHV genes was reported in 13/36 *E.coli,* 14/21 *K.pneumoniae,* 1/1 *K.oxytoca* strains. Souna *et al*. (2014) reported the coexistence of genes TEM, CTX-M-1, and SHV in 2 strains of *E. cloacae.*

Moreover, we reported the expression of *bla*$_{PER-2}$ in 02 *E.coli,* 01 *P.mirabilis* and 01 *P.vulgaris* strains. The DDST is the most widely used test due to its simplicity and ease of interpretation (Ho *et al*., 2000). By using this test, 55 of 68 *Enterobacteriaceae* isolates included in this study, were confirmed as ESßLs producers, indicating a 100% specificity and 84.61 % sensitivity. Our results corroborated with those observed by other authors, in which, Singh *et al*., 2014 reported that sensitivity and specificity reached 83.61% and 100% by using the double disc synergy test at 30 mm, however, sensitivity achieved 88.52% when the distance was kept at 20 mm apart. It is sometimes necessary to adjust the disc spacing. It is worth to note that reducing the distance between the clavulanate and the third-generation cephalosporin disc significantly improves the sensitivity test (Thomson and Sanders, 1992; Tzelepi *et al*., 2000). Ho *et al*. (2000) reported the sensitivity of DDST to be 83.8% at an interdisc width of 30 mm. However, the sensitivity can be increased to 97.9% by decreasing the interdisc width to 20 mm. Thus, a second test, phenotypic confirmatory combined disc test, has therefore been applied for the detection of ESßLs. As previously reported, Linscott and Brown, showed that the ability of the combined disc method to detect ESßLs is very satisfactory, and sensitivity can reach 100% when testing both cefotaxime and cefepime against group 1 and 2 *Enterobacteriaceae* (Linscott and Brown *et al*., 2005). Singh and collaborators demonstrated that this method achieved the highest sensitivity (93.44%) among all other phenotypic tests applied. Whereas, sensitivity remained the same even with the combination of cefotaxime and cefepime (Singh *et al*., 2014). However, Garrec *et al*. (2011) showed that the sensitivity after testing the two latter drugs was not different from that of cefotaxime alone. In this study, PCDDT was positive with 62/68 *Enterobacteriaceae* strains with 95.4% sensitivity and 100% specificity. This test was able to pick up 07 more isolate as ESßLs producers, of which 3 were harboring the *bla*$_{OXA-48}$ gene that DDST failed to identify. Poulou *et al*. (2014) reported that the standard CLSI ESßL confirmatory test was unable to detect the vast majority of ESßL producers when MßLs, or both KPCs and MßLs, were coproduced. Also, it was negative in several isolates coproducing ESßLs with KPCs, plasmid mediated AmpCs, or derepressed chromosomal cephalosporinases. However, it is worth noting that in this study, PCDDT sufficiently detected ESBLs among OXA-48-possessing isolates, since OXA-48 derivatives only weakly hydrolyze cephalosporins (Poirel *et al*., 2004, Poirel *et al*., 2012). Although coexpression of the MßLs with ESßLs, can mask the presence of latter ß-lactamases, so that, in terms of phenotypic screening, the prevalence of ESßLs may be underestimated (Poulou *et al*., 2014). We suggest that this is the case of our false negative *Enterobacteriaceae*, in which 02/03 of these strains harboring the *bla*$_{NDM}$ gene.

III.1.2. *Acinetobacter baumannii*

Production of ESβLs is one of the most important resistance mechanisms of *A. baumannii* strains. A high prevalence of ESßL-producing *A. baumannii* strains has been documented by various studies around the world (Hamzeh *et al*., 2012; Shareek *et al*., 2012). The results of this study indicated that 70% of *A. baumannii* are ESßL-producing strains. The prevalence of bla_{TEM}, $bla_{CTXM1/M2}$, bla_{SHV} and bla_{PER-2} is 71.43%, 42.86%, 28.57% and 21.43%, respectively. Chaudhary and Payasi., 2012reported that the prevalence of ESßL-producing *A. baumannii* is 83.6%, among that, the prevalence of TEM-type ESßLs (bla_{TEM-1}, bla_{TEM-2}, and bla_{TEM-50}) ranged from 82% to 87%, followed by SHV-type ESßLs (bla_{SHV-1} and bla_{SHV-10}) 67 to 78% then CTX-M type ESßLs (bla_{CTXM-9} and $bla_{CTXM-15}$) 60 to 67% in all ESßL-producing isolates. In contrast to our study, this study, showed a high prevalence of the bla_{SHV} in comparaison of bla_{CTXM} gene. However, bla_{TEM} gene remained in the first position in terms of prevalence rate in both studies. A research conducted by AL-Thahab (2013) showed that 83.8% of *A. baumannii* isolates were positive to the ESßL test of which 8.3% harbored bla_{TEM} and 25% bla_{SHV} genes. Another research conducted by Al-Agamy *et al*., 2014 found that the most prevalent Ambler class A ß-lactamase-encoding gene was bla_{TEM}, which was identified in 87.5% isolates, however, the next most prevalent gene was bla_{PER}, which was identified in 55% of the isolates. PER has been documented in *Acinetobacter spp.* isolates from France (Poirel *et al*., 1999), Turkey (Vahaboglu *et al*., 2001), South Korea (Yong *et al*., 2003), Americas (Pasteran *et al*., 2006), Belgium (Naas *et al*., 2006), India (Litake *et al*., 2009), Iran (Farajnia *et al*., 2013), Egypt (Al-Agamy *et al*., 2014) and Nepal (Tada *et al*., 2017), but never in Algerian *A.baumannii* strains. Thus, to our knowledge this is the first report of PER-2-producing *A.baumannii* strains in Algeria. Detection by PCDDT was highly sensitive and indicated the presence of 14/14 ESßL producing strains. Sensibility and sensitivity of PCDDT is 100%. Litake *et al*. (2015) showed that out of 18 ESßL producers none of the isolates showed a clear-cut positive double disc synergy test. Only two isolates showed little indication of positive DDST, and the findings of DDST were in doubts, using augmentin disc in the center and ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30µg), cefepime (30 µg), aztreonam (30 µg) around it. However, all isolates showed positive PCDDT. They found that the results of PCDDT were convincing compared to DDST.

III.1.3. *Pseudomonas aeruginosa*

Resistance of *P.aeruginosa* to many antibiotics is due to the production of different classes of extended spectrum β-lactamases (ESßLs). Our research showed that *P. aeruginosa*

isolates harboured *bla*$_{TEM}$, *bla*$_{CTXM1/M2}$, *bla*$_{SHV}$ and *bla*$_{PER-2}$ genes. TEM-type enzymes were most prevalent in ESBL-producing *P. aeruginosa* (100%). The prevalence of *bla*$_{SHV,}$ *bla*$_{CTXM1/M2}$, and *bla*$_{PER-2}$ genes is 42.85, 28.57 and 14.28 %, respectively. Moreover, this study reported the presence of *bla*$_{GES}$ gene in one strain harboring *bla*$_{TEM}$ and *bla*$_{SHV}$. Lin *et al*., 2012 reported that TEM-type enzymes were most prevalent (92.3%). Whereas they not detected CTX-M and PER ß-lactamases enzyme. Detection of these enzymes by phenotypic methods showed that only one *P.aeruginosa* was positive for the PCDDT. The sensitivity of these test was 14.28% and its specificity was 100%. There is no standardized method for the detection of ESßL-producing *P. aeruginosa*. PCDDST based on the inhibitory effect of clavulanic acid on the activities of ESßLs by restoring the activity of extended-spectrum cephalosporins, is inadequate for the detection of ESßLs in *P.aeruginosa*. These results correlate with the findings of other researchers. Jiang *et al*. have compared various screening methods for detecting ESßLs in *P. aeruginosa* with well characterized ß-lactamases, they reported that only 29.4% were positive for a PDDST. However, the sensitivity was increased to 82.4% in DDST that contained 250 mg/mL cloxacillin (Jiang *et al*., 2006). Thus, the inhibition of AmpC enzyme activity with the addition of cloxacillin could enhance the abilities of DDST to detect ESßLs in *P. aeruginosa* (Lin *et al*. 2012).

III.2. Carbapenemases

Carbapenemases are β-lactamases having a hydrolytic activity against carbapenems. These enzymes belong to three classes according to the Ambler classification: the class A (KPC, and GES), class B corresponds to metallo-β-lactamases (VIM, IMP and NDM) and class D essentially corresponds to oxacillinase enzymes (OXA-type). In this study, we characterized these enzymes phenotypically by MHT and inhibition test and moleculary by the amplification the *bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{OXA48}$ and *bla*$_{OXA-M\ (23,24,51,58)}$ genes.

III.2.1. *Enterobacteriaceae*

Carbapenemase-producing *Enterobacteriaceae* were almost nonexistent up to the 1990s. Today, carbapenemases belonging to Ambler classes A, B, and D have been found worldwide among the *Enterobacteriaceae*. VIMs, IMPs, KPCs, NDMs and OXA_{48}-like enzymes are the most carbapenemases described in *Enterobacteriaceae* (Poirel *et al*., 2007; Doi and Paterson, 2015; Matsumura *et al*., 2018). Compared to non-fermentative pathogens, carbapenem-resistant *Enterobacteriaceae* have been rarely reported in Algeria (Bourafa *et al*., 2018). The *bla*$_{OXA-48}$ gene was first identified in a *K. pneumoniae* isolate from Turkey (Poirel

et al., 2004). The first description of OXA-$_{48}$-producing *E. coli* in Algeria was reported by Agabou *et al*. (2014) with the prevalence of 1.1%. They also reported in 2016 the detection of an OXA-$_{48}$-producing *E. coli* strain isolated from a patient in the Constantine region (Agabou *et al*., 2016). Recently, Bourafa *et al*. (2018) describes for the first time the emergence of OXA-$_{48}$-producing *E.coli* and *K.pneumoniae* in the community in Algeria. These reports coincided with our study; thus, this is the second report of OXA-$_{48}$-producing *E. coli* and *K.pneumoniae* from outpatients with 2 cases, strain of *K. pneumoniae* N° 02 and strain of *E. coli* N° 07. In addition, we described *bla*$_{\text{OXA-48}}$ in *E. coli* and *K. oxytoca* of in-patients and for the first time in Algeria, OXA-$_{48}$-producing *P. mirabilis*. *bla*$_{\text{VIM19}}$ was the first carbapenemase belonging to the Ambler class B described in *Enterobacteriaceae* in Algeria (Robin *et al*., 2010). Sassi *et al*. (2014) reported the detection of the *bla*$_{\text{NDM-5}}$ gene in *E.coli*. Following that the *bla*$_{\text{KPC-3}}$ gene was reported in *K. pneumoniae* (Bakour *et al*., 2015). To the best of our knowledge, no reports are available on NDM-producing *K. pneumoniae* isolates from Algeria thus, this is the first report on the presence of *bla*$_{\text{NDM}}$ in 02 *K. pneumoniae* strains (strains N°23 and 43). This finding may reflect the current spread of carbapenemases in out and in-patients *Enterobacteriaceae* strains throughout Algeria. Among 68 *Enterobacteriaceae* strains of which 36 are *E.coli*, 21 are *K.pneumoniae*, and 11 are other Enterobacterial species (2 *Citrobacter freundii*, 2 *Proteus vulgaris*, 2 *Proteus mirabilis*, 1 *Klebsiella oxytoca*, 1 *Enterobacter cloacae*, 1 *Morganella morganii*, 1 *Providencia stuartii*,1 *Serratia marcescens*) MHT revealed 28 positive results. However, only 05 are true positives (02 OXA-$_{48}$ producing *E.coli*, *01 P.mirabilis* an 01 *K.oxytoca* and 01 *K. pneumoniae* strains). False-negative results were obtained for 02 IMP-R NDM-producing *K. pneumoniae*. Our results indicated that MHT is sensitive (71.43%) for detecting class A and D carbapenemase activity, whereas this technique was limited by the occurrence of false detection of carbapenemase class B-NDM production (sensibility 74.07%). These results are in accordance with those from previous studies (Seah *et al*., 2011, Girlich *et al*., 2012). Seah *et al*., 2011 found that the false-positive results display in CTX-M-producing strains with impermeability, and thus, we suggest that this is the case of our false positive *Enterobacteriaceae*, in which all these strains harboring the *bla*$_{\text{CTXM1-M2}}$ gene. Moreover, Pasteran and collaborators have raised the problem of difficulties in the interpretation of the MHT especially with MßLs in *Enterobacteriaceae* for the weak carbapenemase producers (Pasteran *et al*., 2009). Among 68 *Enterobacteriaceae*, 05 (12.4%) were screened by IMP-EDTA-CDT for the MßLs detection based on their reduced susceptibility to carbapenems (imipenem and/or ertapenem). This test was positive with the 02 IMP-R NDM-producing *K. pneumoniae*. IMP-EDTA-CDT was highly sensitive (100%). El-Ghazzawy *et al*. (2015)

concluded that EDTA-IMP-CDT could be a sensitive, easy to perform, and interpret phenotypic rapid method. Many other previous studies also found that the combined disc test was highly sensitive (Galani *et al*., 2008, Picao *et al*., 2008, Bora *et al*., 2014) for the detection of MßLs in *Enterobacteriaceae* and this is in a good agreement with our findings.

III.2.2. *Acinetobacter baumannii*

Resistance to carbapenems, molecules considered as treatment of choice of *A. baumannii* infections and is particularly problematic since it drastically decreases the therapeutic possibilities. *A. baumannii* has a gene on its chromosome that naturally encodes an oxacillinase (or class-D β-lactamase), the main representative of which is OXA-$_{51}$, with many variants described. Carbapenem resistance in *A. baumannii* is often achieved by acquired oxacillinases with carbapenemase properties (CHDLs) such as OXA-$_{23}$, OXA-$_{24/40}$, OXA-$_{58}$ or OXA-$_{143}$ (Poirel *et al*., 2006, Peleg *et al*., 2008); metallo-b-lactamases (MBLs) such as IMP, VIM, SIM or NDM, or carbapenemases belonging to Ambler group A as KPC or certain GES variants. In our study, the presence of these carbapenemases genes was searched by PCR in 10 Algerian IMP-R *A.baumannii* strains (50%). The results showed that all IMP-R *A.baumannii* strains were positive for at least one carbapenemases gene (*bla*$_{NDM}$ and *bla*$_{OXA-48}$, *bla*$_{OXA-M(23,24,51,58)}$), suggesting that these carbapenemases significantly contributed to the imipenem resistance in our *A.baumannii* strains. According to Zhou *et al*. (2007) imipenem is hydrolysed by carbapenemases of the class B or D that may be present in *Acinetobacter ssp* strains. The first NDM-1 in Algeria have been identified in *A. baumannii* strains isolated in Algerian patients Transferred to France (Boulanger *et al*., 2012) and Belgium (Bogaerts *et al*., 2012). Mesli *et al*. (2013) also reported the presence of NDM-1 in *A. baumannii* strains in western Algeria. Resistance to carbapenems in *A. baumannii* is often associated with the production of oxacillinases. In Algeria, OXA-$_{58}$ was detected for the first time in 2010 in Tlemcen (Drissi *et al*., 2010). OXA-$_{23}$, OXA-$_{24}$, OXA-$_{58}$ and OXA-$_{72}$ was reported in many other studies (Bakour *et al*., 2012, Touati *et al*., 2012). OXA-$_{23}$ is the most widespread and seems to be endemic in Algeria (Khorsi *et al*., 2015). However, OXA-$_{48}$ and its variants are widespread in *Klebsiella pneumoniae* and other *Enterobacteriaceae*. OXA-$_{48}$-like-producing *A. baumannii* was detected for the first time in 2013 by Gonçalves *et al*, in the fecal flora of nursing home residents in Northern Portugal (Gonçalves *et al*., 2013). OXA-$_{48}$ in multidrug-resistant *A. baumannii* strains is detected for the second time by Mathlouthi *et al.* (2015) in Libya. These reports coincided with our study; thus, to our knowledge, this is the first report of OXA-$_{48}$-producing MDR *A.*

baumannii in Algeria and the third description of an OXA-$_{48}$-like carbapenemases outside of the *Enterobacteriaceae* in the world.

Detection of carbapenemases-producing *A. baumannii* by Modified Hodge Test indicated the presence of the clover leaf-like indentation image with 7 strains *A. baumannii* IMP-R out of 10 strains indicating that they produce carbapenemase. These clover images are not very visible. The detection of carbapenemase by the polymerase chain reaction indicated the presence of *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-23-24-51-58}}$ and/or *bla*-$_{\text{NDM}}$ genes in all *A.baumannii* IMP-R strains (10/10). The MHT gave negative results with the *A. baumannii* strain that detected as NDM-producing strain (strain N° 45) by PCR and 02 OXA-type producers (strain N° 41 and 62). This test was specific (100%) but poorly sensitive (70%) for the detection of carbapenemase activity. Our results are closely related to the results of Bonnin *et al*. (2012) that investigated seven NDM-positive *Acinetobacter baumannii* isolates of worldwide origin to evaluate the best technique for their identification. They found that the MHT gave negative results for all tested NDM-producing *A. baumannii* strains. However, VIM-, IMP- and some OXA-type producers gave weak synergistic images. According to them, MHT was poorly sensitive and specific for detecting the carbapenemase activity of any carbapenemase producers. IMP-EDTA CDT revealed the presence of the synergistic images in the presence of EDTA in 04 *A.baumannii* IMP-R, the strain N° 45 which is detected as MβLs producer by PCR harboring *bla*-$_{\text{NDM}}$ gene and strains N° 33,59 and 62 which are detected as carbapenemases producer by PCR harboring *bla*$_{\text{OXA-23-24-51-58}}$ genes. The increase of inhibition zone diameter in the presence of EDTA is 14 mm with strain N° 45 and 8,10 and 9 mm with strains N°33,59 and 62 respectively. In this study, some MβLs negative strains harboring OXA-$_{\text{M(23-24-51-58)}}$ gave false-positive results suggesting that IMP-EDTA CDT was highly sensitive (100%) and poorly specific (66.66%) with *A.baumannii* strains. The false-positive results that is not associated with true MBL production may be due to the action of EDTA on membrane permeability, which increasing the susceptibility to imipenem (Denny *et al*., 2003), or to the affection of OXA enzymes by the EDTA inhibitory effect, which would also lead to false interpretations of MBL synergy tests (Danel *et al*., 2001). The results of this test conform the studies of Bonnin *et al*. (2012) who reported that several MBL-negative strains producing OXA-$_{23}$ or OXA-$_{40}$ gave false-positive results and that the intrinsic effect of EDTA on *A. baumannii* might interfere in the specificity of this test.

III.2.3. *Pseudomonas aeruginosa*

Imipenem-resistant *P.aeruginosa* is a significant and current concern, because of the limited therapeutic options for this pathogen strains (Franco *et al*., 2010). The carbapenem resistance in *P.aeruginosa* has been due mostly to the impermeability because of oprD porin loss and the production of carbapenemases that have been extensively described in *P. aeruginosa* during the last years (Touati *et al*., 2013). These carbapenemases are whose activity is partly inhibited by clavulanic acid, such as the plasmid β-lactamase GES-2 which is an ESßLs with carbapenemase properties that was identified in epidemic strains of South Africa, and MβLs including those of the IMP, VIM, SPM, GIM, SIM, AIM-1, FIM-1, and NDM families (Cornaglia *et al*., 2011, Hammami *et al*., 2011, Ocampo-Sosa *et al*., 2012, Pollini *et al*., 2013). In Algeria, the first carbapenemases identified in *P.aeruginosa* was VIM-2 by Touati *et al*. (2013) in Annaba and Sefraoui *et al*. (2014) in Oran, followed by VIM-4 that reported by Mellouk *et al*. (2017) in Annaba and Skikda. Our results reported the first report of GES-producing *P. aeruginosa* strain in Algeria.

In our study, out of 20 strains of *P. aeruginosa*, 15 isolates (75%) were resistant to imipenem. Modified Hodge Test indicated the presence of carbapenemases in 01 strain out of the fifteenths. *bla*$_{GES}$ carbapenemase coding gene was detected in this strain (strain N° 04) by PCR. For the detection of Ambler class B β-Lactamases, IMP-EDTA CDT was done for all imipenem resistant isolates and the results revealed that 08 out of 15 *P. aeruginosa* isolates was positive (MβLs producer). According to the results of PCR, none of imipenem resistant and sensitive strains were carrying genes for MβLs production. These results indicating that phenotypic methods may lead to false positive results. Marra *et al*., 2006 found a 69.6% false MβLs detection rate when they used EDTA as the inhibitor agent. These results suggest that the likely phenotypes of the resistance of *P.aeruginosa* strains to imipenem are the hyperproduction of natural cephalosporinase associated with impermeability alterations or loss of porin oprD and not the production of MβLs. The alteration of the oprD gene was the main mechanism for imipenem resistance in *P. aeruginosa* clinical strains, as previously described in Algeria (Sefraoui *et al*., 2014, Mellouk *et al*., 2017, Bourafa *et al*., 2018) and in other countries (Al Bayssari *et al*., 2014, Grall *et al*., 2011). Marra *et al*. (2006) found that 81.1% of the strains were resistant to imipenem without MβLs production and demonstrate that other resistance mechanisms are involved, such as permeability mutations via the loss of porins or the up-regulation of efflux systems.

Integron class I and II

Integrons, especially class I and II, have contributed to the dissemination of antimicrobial resistance genes mainly in many gram-negative bacteria (Sung and Oh, 2014). In the present study, screening of class-I and class-II integron gene cassettes by PCR showed that 34 (50%) and 15 (22.05%) Enterobacterial isolates were positive for class I and class II integrons, respectively. Ten (6.8%) were positive for both *intI*1 and *intI*2 among which 07 *E. coli*, 01 *K. pneumonaie*, 01 *K. oxytoca* and 01 *P.mirabilis*. Class-I integronss were determined in all *P. aeruginosa* strains. Whereas no Class-II integrons were determined in this species. For *A. baumannii* strains, class-I and II integrons together determined in one strain, class-I integron gen cassette were determined in two strains and class-I integrons gen cassette were determined in one strains.

The number and rate of antibiotic resistance gene carried on integrons have been increasing by misuse of antibiotics and horizontal gen transfer. Integron-mediated resistance gene transport in clinical and community isolates should be screened at specific intervals, and measures should be taken about the use of antibiotics inactivating by resistance genes in the integron gene cassettes.

IV. Phytochemical analysis

Our study reveals and confirms the dissemination and the high prevalence of ß-Lactmases producing isolates (ESßLs and/or carbapenemases) especially among Gram negative bacteria in both community and hospitals in Algeria. This is distressing because it constitutes a serious health problem. Hence, new and potential β-lactamases inhibitors is an urgent need. It is well known that, over the past decades, seaweed has been attracting attention in the search for bioactive compounds to develop new drugs and healthy foods. Seven seaweeds were chosen to evaluate their Inhibitory effect against two purified recombinant ß-Lactmases, GES-22 A-class and OXA-1 D-class. The investigation of these seaweeds effect involves different stages, the main one being the preparation of extracts which are complex mixtures, the identification of the bioactive molecules in these extracts by phytochemical analysis and finally the evaluation of their inhibitory effect.

Our samples were collected from the southern Mediterranean Sea (36 ° 35'N-2 ° 29'E) in the region located on the coast of Bou Ismaïl Bay, Kouali, Algeria, during spring time. This site was chosen because of its richness in algal populations and it is free of any discharge. Kouali is a reference area which is free of any human impact. These included *Ulva*

intestinalis, Codium tomentosum and *Bryopsis pulmosa* belonging to Chlorophyceae and *Sargassum vulgare, Dictyota dichtoma, Halopteris scoparia* and *Cystoseira compressa* belonging to the class of Phaeophyceae.

Extraction with organic and inorganic solvents is an essential step in the preparation of seaweed extracts. However, the choice of extraction solvents influences the yields of secondary metabolites of the extracts. Yan *et al*. (1998) reported that methanolic extracts of various seaweed samples had a high antioxidant activity and hence methanol was considered as the most effective solvent for seaweed extract preparation. In addition, Athukorala *et al*. (2003) found that methanolic extract showed the highest antioxidant activity in all assays performed and it contained a high amount of phenolic compounds in comparison to hot water, ethanol, acetone, ethyl acetate, chloroform, ether, hexane and carbon tetrachloride extracts. In the present study, extraction is prepared by methanol, as this solvent is able to increase the permeability of cell walls and facilitate the extraction of a large number of polar compounds as well as compounds of medium and low polarity (Seidel, 2005).

The total polyphenol contents vary considerably between the methanolic extracts of the seven of seaweeds extract tested. The Folin-Ciocalteu reagent assay gives an evaluation of all the phenolic compounds of an extract, it is not specific to the polyphenols, but many compounds can react with the reagent to give a high apparent phenolic level (Tawaha *et al*., 2007). The TPC in the green seaweed species (*B. pulmosa, C. tomentosum, U. intestinalis*) were found to be nearly two times greater than that in brown seaweeds. Yildiz *et al*. (2014) reported that green seaweeds (*U. rigida* and *C. tomentosum*) revealed higher levels of polyphenols than red seaweeds. It is difficult to compare TPC obtained in this study with TPC of other studies described above, because of variations in extraction solvent and TPC assay conditions, in addition to the lack of common reference sample in these previous reports.

Seaweeds are an important unconventional source of vitamins (liposoluble and hydrosoluble). The profile of vitamin in seaweeds can vary according to algal species, growth stage, environmental parameters and season (Kumar *et al*., 2008). Vitamin E is the major lipid-soluble antioxidant responsible for protecting the polyunsaturated fatty acids in membranes against lipid peroxidation, free radicals and singlet oxygen species (Machlin and Bendich, 1987). In general, brown seaweeds contained higher levels of tocopherols than green and red seaweeds. Brown algae contain alpha, beta and gamma tocopherol while the green and red algae only contain the alpha tocopherol (Lobo and Rani, 2013). In nature, α-tocopherol is the

most common form of vitamin E. It is also the most biologically active form. In this study vitamin E contents of brown seaweed extracts (*S.vulgare, D.dichtoma, H.scoparia* and *C.compressa*) were higher than those of green seaweeds (*U. intestinalis, C. tomentosum* and *B. pulmosa*). However, Yildiz *et al*. (2014) reported that the vitamin E contents of the green seaweed *U. rigida* and *C. tomentosum* were remarkable higher than those of two red seaweeds.

The preliminary phytochemical screening by HPLC analysis indicates the presence of various compounds in the methanolic extract of green and brown seaweeds (**Appendix B**). Based on their mass spectrometry and retention times by comparison with those of different reference substances commercially available, this study identified four common compounds in the MEs of seaweeds. These compounds are α-linolenic acid (C18:3 ω3), linoleic acid (C18:2 ω6), oleic acid (C18:1 ω9), and the eicosanoid precursor ‘‘arachidonic acid’’ (C20:4 ω6). Moreover, *U. intestinalis* and *D. dichtoma* contain baicalein ($C_{15}H_{10}O_5$) and curcumin ($C_{21}H_{20}O_6$). *B. pulmosa* contains baicalein and daidzein ($C_{15}H_{10}O_4$), *C. tomentosum* contains quercetin ($C_{15}H_{10}O_7$) and *C. compressa* contains (S) naringenin ($C_{15}H_{12}O_5$).

Mono and polyunsaturated fatty acids have been frequently found in seaweeds. Previous reports have found that oleic acid was the most monounsaturated fatty acids abundant in seaweeds (Khotimchenko *et al*., 2002; Tabarsa *et al*., 2012; Silva *et al*., 2013). Tabarsa *et al*. (2012) reported that all the brown seaweeds tested contained the essential fatty acids linolenic acid (C18:3 ω3), linoleic acid (C18:2 ω6) and arachidonic acid (C20:4 ω6), which have also been reported in previous studies in seaweeds (Dawczynski *et al*., 2007). However, various phenolic compounds including flavones such as lutein, flavonoles such as quercetin and myricetin, iso-flavones such as genistin and daidzein, and flavanones such as hesperidin were found in methanolic extract of red, brown, and green seaweeds (Yoshie *et al*., 2000; Santoso *et al.*, 2004; Yangthong *et al.*, 2009).

V. GES-22 and OXA-1 β-lactamases inhibition assays

β-Lactamase production is the most important mechanism by which Gram-negative pathogens become resistant to β-lactam antibiotics. To inhibit the hydrolyzing activity of β-lactamases, β-lactam antibiotics are used with inhibitors (tazobactam, clavulanate, and sulbactam). These β-lactamases inhibitors are co-administered with β-lactam antibiotics and they are effective against some class A β-lactamases whereas ineffective against class B and most of the class C and D β-lactamases. As a result of the presence of a β-lactam ring in these inhibitors and their extensive use in combination with β-lactam antibiotics, the β-lactamase in

bacteria mutate continually developing their activity even against newly developed β-lactam (Drawz and Bonomo, 2010). The present study reported that these enzymes are widely disseminated in gram negative bacteria from hospital and community in Algeria. Hence, new, non-toxic and potential β-lactamases inhibitors is an urgent need. We purified and expressed both GES-22 and OXA-1 of A- and D-class β-lactamases, respectively, to investigate the inhibitory effect within seven seaweed methanolic extracts. The purity of the recombinant GES-22 and OXA-1 β-lactamases was shown to be more than 95% by SDS-PAGE. These two β-lactamases were chosen because OXA-1 is a highly active class D penicillinase and exhibiting resistance to the clinically available β-lactamase inhibitors (tazobactam, sulbactam and clavulanate). However, GES-22 was firstly identified in a clinical *A. baumannii* isolate and it encodes a new variant of A-class β-lactamase (Cicek *et al*., 2014). It differs from GES-11 by a single substitution M169L (99.7% identity). This substitution at the omega loop confers on GES-22 more efficient hydrolysis of the classical inhibitors, clavulanic acid and sulbactam (Saral *et al*., 2016). To the best to our knowledge, this is the first report of the inhibition of GES-22 activity. Whereas Bethel *et al*. (2008) proposed that penems inactivate OXA-1 efficiently by forming an unusual acyl-enzyme complex. Likewise, Che *et al*. (2014) reported that the penems are good inhibitors of OXA-1 β-lactamase.

In previous studies, seaweeds are act as antioxidant (Chew *et al*., 2008; Valentão *et al*., 2010; López *et al*., 2011; Chernane *et al*., 2014), antimicrobial (Shanmughapriya *et al*., 2008; Ibrahim and Lim, 2015), anti-inflammation (Kazłowska *et al*., 2010; Lin *et al*., 2016), antiviral (Rabanal *et al*., 2014), anticancer (Moussavou *et al*., 2014; Çelenk *et al*., 2016), antidiabetic (Sharifuddin *et al*., 2015; Lin *et al*., 2017) and as Efflux pump inhibitors (Lu *et al*., 2019). Despite these activities, their potential as β-lactamases inhibitors has not yet been evaluated. Thus, this is the first report of β-lactamases inhibition by seaweeds.

The nitrocefin assay shows that nitrocefin hydrolysis by GES-22 and OXA-1 can be inhibited by the methanolic extract of seaweeds in a dose dependent manner with different IC_{50} values. The seven ME exhibited stronger inhibition effect against OXA-1 in comparison to the β-lactamase inhibitors (tazobactam, sulbactam and clavulanate) that are not effective against class D β-lactamases OXA-1. The MEs of *D.dichtoma*, *C.tomentosum, U.intestinalis*, *B.pulmosa* and *H.scoparia* exhibited, in order, higher inhibition effect against GES-22 in comparison to the β-lactamase inhibitor tazobactam. Whereas, all ME exhibited hight inhibition activity against GES-22 in comparison to the β-lactamase inhibitors sulbactam and clavulanate.

β-lactamase kinetic assay demonstrated that, in the presence of all MEs, both the apparent Km and apparent Vmax of OXA-1 activity decreased with increase in inhibitors concentration as compared with the control. The type of inhibitors is categorized from these data as mixed. Likewise, *U.intestinalis, C.tomentosum, D.dichtoma,* and *H.scoparia* exhibited mixed inhibition against GES-22 activity, whilst *B. pulmosa* and *C.compressa* exhibited non-competitive and competitive inhibition, respectively. The only ME that inhibits GES-22 activity with a competitive manner is the ME of *Cystoseira compressa* seaweed. Foti *et al.* (1994) and Ruberto *et al*. (2001) reported that the mediterranean marine algae of genus *Cystoseria* possessed antioxidant activity comparable to that of α-tocopherol.

The results of the present study suggest that there is no correlation between total phenolic content and inhibitory effect. The TPC of the ME of *D. dichotoma* were lower than that of *U. intestinalis* and *C. tomentosum*, however its inhibitory effect against GES-22 was better than theirs, and better than the inhibitory effect of *C. tomentosum* against OXA-1. These data suggest that methanolic extracts are mixtures of biological active compounds included some non-phenolic compounds, which can contribute to the inhibitory activity. Furthermore, inhibitory effect of seaweed extracts on β-lactamases vary based upon the chemical content of the extract.

It is thought that the inhibitory activity of our MEs of seaweeds could be due to the individual effect of their compounds identified (α-linolenic acid, linoleic acid, oleic acid, arachidonic acid, baicalein, curcumin, daidzein, quercetin and (S) naringenin or to their synergistic effect; as it may also be due to the effect of other molecules that have not yet identified. The responsible compounds related to the inhibitory activity of seaweed extracts are not yet cleared. It was only seen that these extracts were able to inhibit the hydrolyze activity of GES-22 and OXA-1. Further studies are required in order to identify the inhibitory compound(s) to turn them a good purpose in the fight against β-lactam antibiotics resistance.

VI. Molecular docking

Molecular docking studies were carried out in order to identify a potent inhibitor to block the ß-lactamase activity and to evaluate the binding affinity and binding energy of the compounds identified in methanolic extracts of seaweeds towards ß-lactamase. We selected 40 natural compounds from various secondary metabolites classes among which the compounds identified in methanolic extracts of seaweeds. These compounds were subsequently docked in the active site of 15 ß-lactamases (12 SβLs and 3 MβLs). The absence of the 3D-structure for

GES-22 in PDB and the high RMSD of OXA-1 prompted us to use other β-lactamases among which ESβLs and carbapenemases found in gram negative bacteria from hospital and community in Algeria. The well-known classical inhibitors (clavulanate, sulbactam and tazobactam) were chosen as reference compound.

Docking results have shown that ligands establish different covalent and non-covalent bonds with active amino-acids site of both SβLs and MβLs targets. In the case of SβLs, which are six among class A and six among class D, ligands with high score interact with SβLs targets by covalent and conventional hydrogen bonds established with different aminoacids of conserved elements of active site. These aminoacids are frequently SER70, SER130, ASN132, THR234, LYS205, 234, GLY235 when SβLs are of class A and SER67 and 70, 79, 80, 83, 126, 216, 218, 219, 257 and LYS124, 125 and 216 when when SβLs are of class D**.** In case of MβLs which are VIM-2, IMP-1 and NDM-1, paenoflorin establishes two conventional hydrogen bonds with HIS118, ARG228 and three carbon hydrogen bonds with ASP120, GLY232, HIS263 of VIM-2 active site. In addition, this ligand establishes a Pi-alkyl interaction with HIS196 of the active site of the same MβLs. Rutin establishes five conventional hydrogen bonds with PRO32, SER71 and 80, TYR163, GLY164 and five covalent bonds with TRP78, LYS161, GLY166, HIS197, SER198 and other non-covalent interactions with HIS79 and Zn of IMP-1. Rhein forms two conventional hydrogen bonds with HIS189 and 250 and other non-covalent interactions with two Zn and LYS211 of NDM-1. These covalent bonds that established with key amino-acids of both SβLs and MβLs targets suggest that these compounds can irreversibly inactivate β-Lactamases. Considering the low toxicity of these compounds, they may be used as potential lead compounds for the development of ß-lactamases inhibitors.

Binding energies in the protein ligand interactions explain how the ligand fits or bind to the ß-lactamases. All compounds identified in the MEs of seaweeds have shown libdock score and CDOCKER interaction energy higher than the classical inhibitors clavulanate and sulbactam towards the 15 ß-lactamases. Curcumin has the best libdock score among compounds identified in the MEs with 10/15 ß-lactamases among which 9 SβLs and 1 MβL. However, hesperidin and dihydromyrecitin have the best libdock score among the 40 compounds, these ligands bind to 4/15 and 3/15 SβLs, respectively. On the basis of libdock score and CDOCKER interaction energy hesperidin, dihydromyrecitin and curcumin can be suggested as good ligands.

Hesperidin is a flavone glycoside of citrus fruit, abundantly found in sweet orange and lemon (Garg *et al*., 2001). Various studies on the inhibition effects of this compounds have

reported. Sakata *et al*. (2003) indicated that hesperidin inhibits cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) proteins, which might be related to the anti-inflammatory and anti-tumorigenic efficacies. Hesperidin inhibits the naturally induced CYP1A1 associated 7-ethoxyresorufin-O-deethylase (EROD) activity and glutathione S-transferase (GST) activities of fish *in vitro* (Arinç *et al*., 2015). It comes out as a multi-potent phytochemical agent in Alzheimer's disease therapeutics exhibiting strong proteases ß-secretase-1 binding ability (BACE1), high amyloid-ß peptide (Aß) aggregation inhibition, from a phytochemical library of 200 phytochemicals through the multi-target screening to identify multi-potent (Chakraborty *et al*., 2016). Hesperidin show noncompetitive inhibition against BACE1 and cholinesterases *in vitro* and *in silico* (Lee *et al*., 2018). Furthermore, hesperidin is the most active potential NDM-1 inhibitor, it acts directly on key residues near the NDM-1 active site using docking (Shi *et al*., 2019).

Dihydromyricetin (DHM), also known as ampelopsin belonging to flavonoid family, is isolated from *Ampelopsis grossedentata* (Li *et al*., 2017). The inhibition effects of DHM have been reported by various studies. Tang *et al*. (2016) suggested that DHM was a potent natural inhibitor of NF-κB. DHM inhibited the aggregation of calcium oxalate crystals and decreased the level of Calcium/creatinine in the urine (Wang *et al*., 2016). Wu *et al*. (2017) reported that DHM inhibited cardiac apoptosis and restored autophagy, which might be related to the increased AMPK phosphorylation and downstream target UNC-51 like kinase (ULK1) expression. To the best of our knowledge there is no information about the inhibition of ß-lactamases by DHM.

Curcumin is a component of turmeric, a yellow spice widely used as a food flavoring and coloring agent. Inhibition of enzymes by curcumin has been the subject of many published papers. Zhu *et al*. (2014) found that from a library of about 10,000 compounds from 300 different Chinese medicine caffeic acid, curcumin, salvianolic acid E, ferulic acid and p-coumaric acid have high binding score with the three clinical inhibitor resistant TEM β-lactamase mutants M69I, S130G and R244S according to the results of molecular docking. Moreover, curcumin was found to optimally fit within the binding pocket of inhibitor of glycogen synthase kinase-3b (GSK-3b) via several attractive interactions with key amino acid using simulated docking experiments with *in vitro* and *in vivo* validations (Bustanji *et al*., 2009). Inhibition of the cytochromes P450 (CYP) isoenzymes by curcumin has been demonstrated in cells cultured *in vitro* (Firozi *et al*., 1996). Furthermore, curcumin potently inhibits a multitude of key oncogenic signaling factors among which c-Jun/AP-1 (Huang *et al*., 1991), ornithine

decarboxylase (Lu *et al*., 1993), NF-kB (Singh and Aggarwal, 1995), cycloxygenase-2 (Plummer *et al*., 1999), histone acetyltransferase activity (Balasubramanyam *et al*., 2004; Morimoto *et al*., 2008).

Conclusion & Outlook

CONCLUSION AND OUTLOOK

In conclusion, our study reveals and confirms the dissemination of ESβLs and carbapenemase-producing Gram-negative bacilli in community and Algerian hospitals. Thus, this constitutes a serious problem because carbapenems are in many cases considered as the last line of therapy. These strains harboring at least, one β-lactamase coding gene (*bla*$_{TEM}$, *bla*$_{SHV,}$ *bla*$_{CTX-M1-M2}$, *bla*$_{GES}$, *bla*$_{PER-2}$, *bla*$_{NDM}$, *bla bla*$_{OXA-M\ (23,24,51,58)}$), neither *bla*$_{KPC}$ nor *bla*$_{VIM}$, *bla*$_{IMP}$ gene was detected. The conserved regions of class-I and II integron gene cassettes were determined alone and together in these isolates. To control and detect the spread of these β-lactamases-producing strains, both optimized phenotypic and molecular analysis are recommended in the routine diagnostic of clinical laboratories because the emergence of strains harboring several β-lactamases simultaneously can raise a diagnostic and therapeutic problem.

Phytochemical analysis showed that the total phenolic contents in the MEs of green seaweed species were found to be nearly two times greater than that in brown seaweeds. However, vitamin E contents of brown seaweed MEs were higher than those of green seaweeds. Qualitative analysis showed the existance of common compounds in the MEs including α-linolenic acid (C18:3 ω3), linoleic acid (C18:2 ω6), oleic acid (C18:1 ω9), and arachidonic acid (C20:4 ω6). Moreover, *U. intestinalis* and *D. dichtoma* contain baicalein ($C_{15}H_{10}O_5$) and curcumin ($C_{21}H_{20}O_6$). *B. pulmosa* contains baicalein and daidzein ($C_{15}H_{10}O_4$), *C. tomentosum* contains quercetin ($C_{15}H_{10}O_7$) and *C. compressa* contains (S) naringenin ($C_{15}H_{12}O_5$).

Nitrocefin hydrolysis activity of GES-22 and OXA-1 can be inhibited by the MEs of seaweeds in a dose dependent manner with different IC_{50} values. All ME exhibited stronger inhibition effect against OXA-1 in comparison to the β-lactamase inhibitors (tazobactam, sulbactam and clavulanate) that are not effective against class D β-lactamases OXA-1. The MEs of *D. dichtoma, C. tomentosum, U. intestinalis, B. pulmosa* and *H. scoparia* exhibited, in order, higher inhibition effect against GES-22 in comparison to the β-lactamase inhibitor tazobactam. Whereas, all MEs exhibited hight inhibition activity against GES-22 in comparison to the β-lactamase inhibitors sulbactam and clavulanate. Docking studies indicated that the potency of inhibition is probably due to the formation of hydrogen bonds and hydrophobic interactions between ligands (compounds identified in MEs of seaweeds and other natural compounds) and active amino-acids site of both SβLs and MβLs. All compounds identified in the MEs of seaweeds have shown libdock score and CDOCKER interaction energy higher than the classical inhibitors clavulanate and sulbactam towards 15 ß-lactamases.

The results obtained during this study remain preliminary and deserve to be exploited and supplemented by an epidemiological study to accurately evaluate the distribution of these β-lactamases that could emerge even more in the future. In the development of the present study, the isolation, the purification and the characterization of the responsible molecule for the inhibition of β-lactamases activity detected in these extracts remain interested. It could be nice if we can do the analysis of the composition in different seasons, layers of Algerian sea to discover the best season and the best zone that can give more yield and may be more substances. It would be also interesting to carry out a more in-depth study to studying the mechanisms of action *in vivo* and to broaden the study to other natural molecules.

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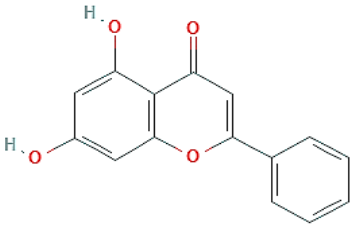
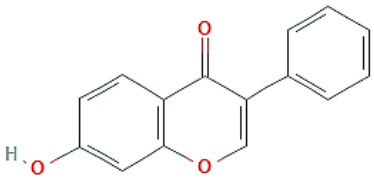
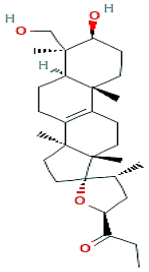
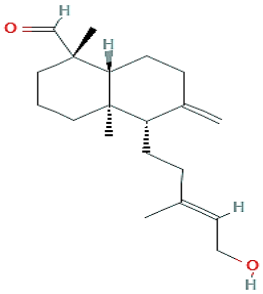
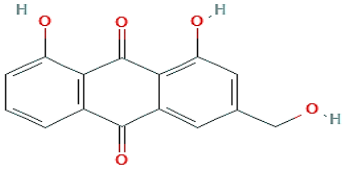
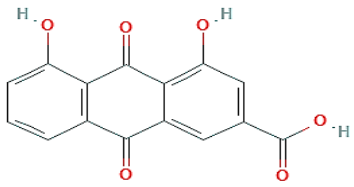
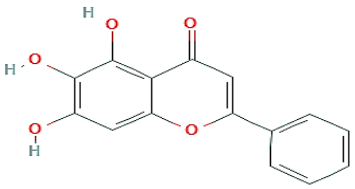
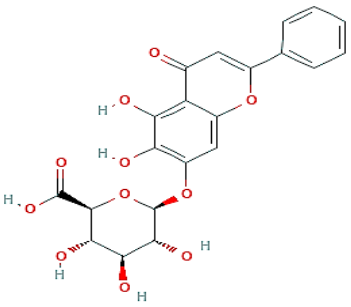
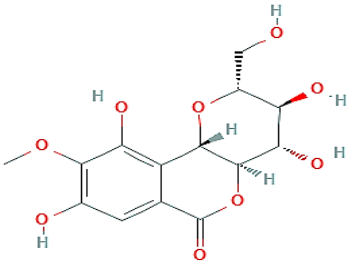
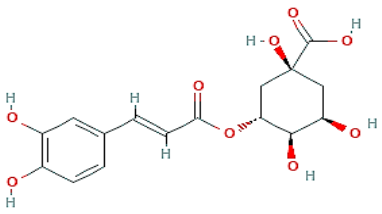
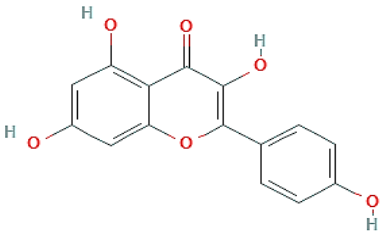
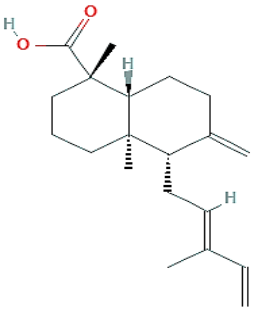
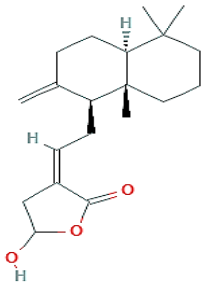
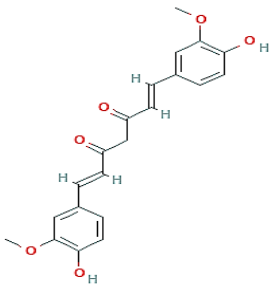
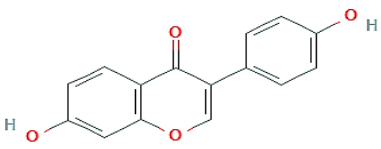
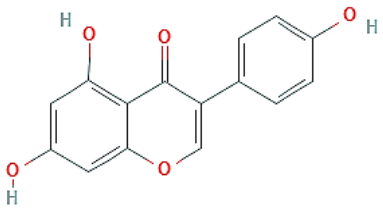
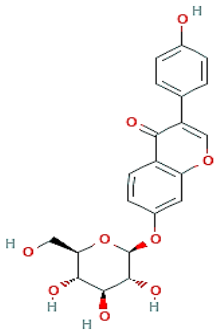
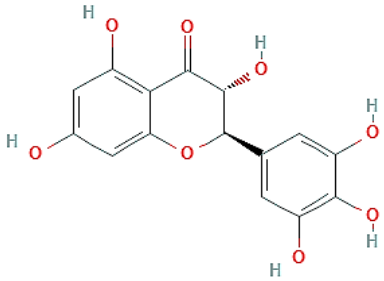
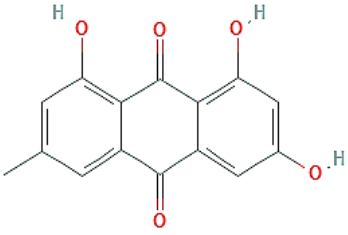
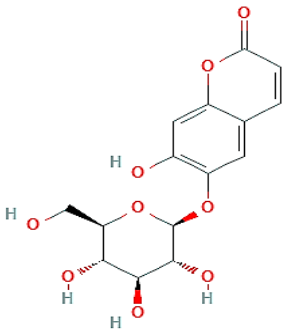
Appendices

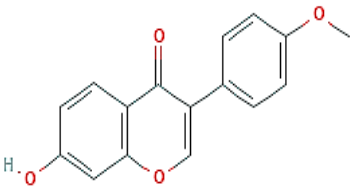
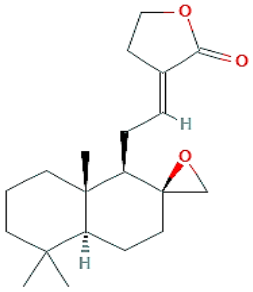
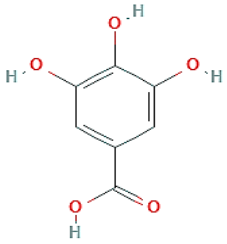
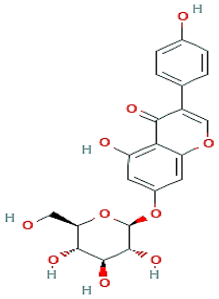
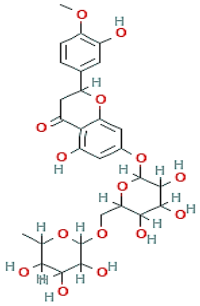
Appendix A. Chemical structures and pubchem CID of the compounds used

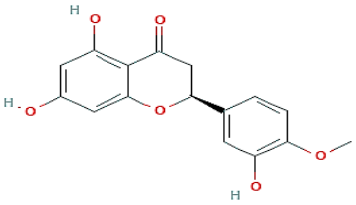
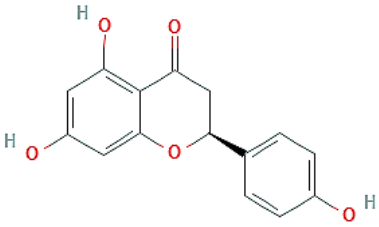
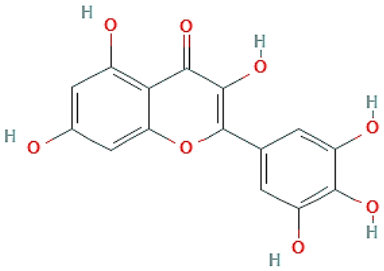
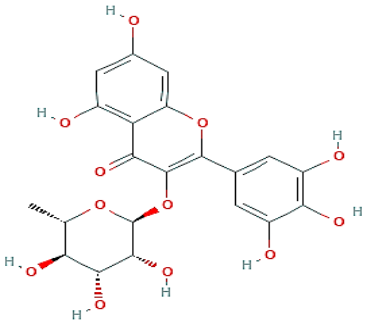
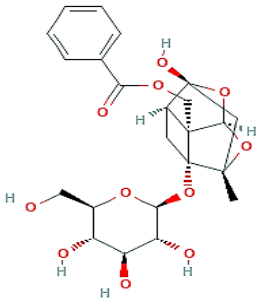
N°	Molecules	Pubchem CID	Structures	Class
1	Chrysin ($C_{15}H_{10}O_4$)	5281607		Diterpenoids
2	7-hydroxyisoflavone ($C_{15}H_{10}O_3$)	5376891		Isoflavones
3	15-deoxoeucosterol ($C_{29}H_{46}O_4$)	21632988		Isoflavanones
4	Agatholal ($C_{20}H_{32}O_2$)	15559800		Diterpenoids
5	Aloe-emodin ($C_{15}H_{10}O_5$)	10207		Anthraquinones

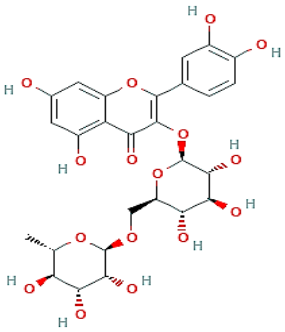
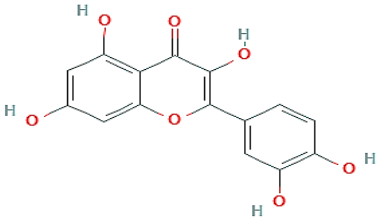
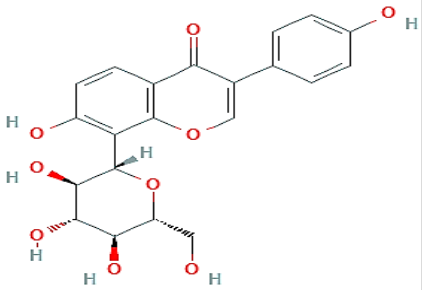
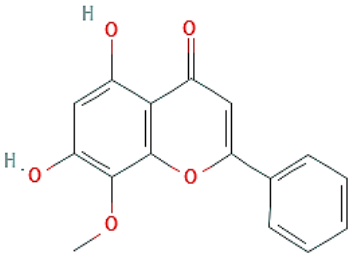
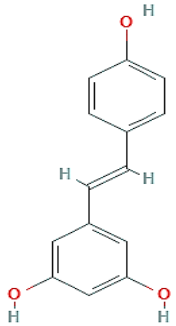
6	Rhein ($C_{15}H_8O_6$)	10168		Anthraquinones
7	Baicalein ($C_{15}H_{10}O_5$)	5281605		Flavone
8	Baicalin ($C_{21}H_{18}O_{11}$)	64982		Flavone
9	Bergenin ($C_{14}H_{16}O_9$)	66065		Isocoumarin
10	Chlorogenic acid (CGA) ($C_{16}H_{18}O_9$)	1794427		Polyphenols

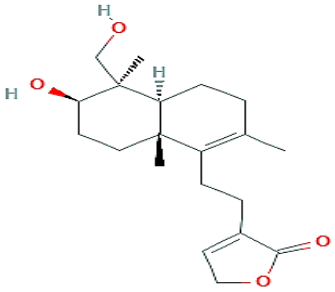
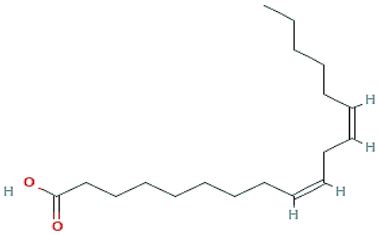
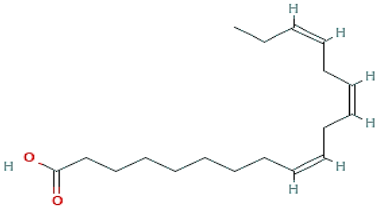
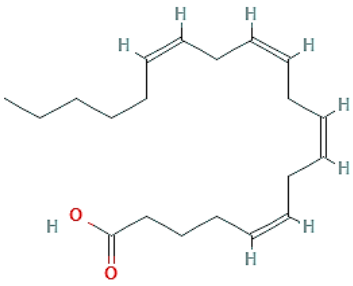
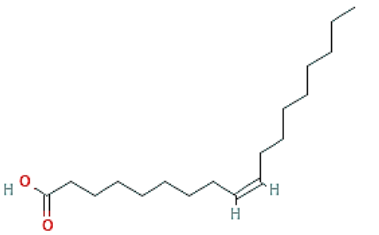
11	Kaempferol ($C_{15}H_{10}O_6$)	5280863		Flavanols
12	Communic acid ($C_{20}H_{30}O_2$)	637125		Diterpenoids
13	Coronarin D ($C_{20}H_{30}O_3$)	52947373		Diterpenoids
14	Curcumin ($C_{21}H_{20}O_6$)	969516		Polyphenols
15	Daidzein ($C_{15}H_{10}O_4$)	5281708		Isoflavones

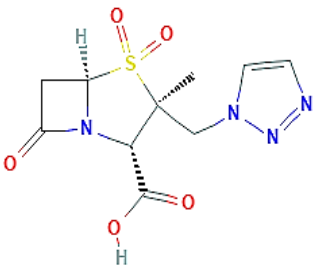
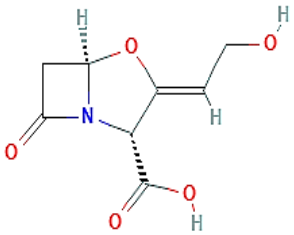
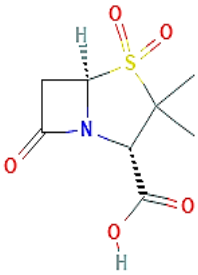
16	Genistein ($C_{15}H_{10}O_5$)	5280961		Isoflavone
17	Daidzin ($C_{21}H_{20}O_9$)	107971		Isoflavones
18	Dihydromyricetin Ampelopsin ($C_{15}H_{12}O_8$)	161557		Flavanonols
19	Emodin ($C_{15}H_{10}O_5$)	3220		Anthraquinones
20	Esculin ($C_{15}H_{16}O_9$)	5281417		Coumarin glucoside

21	Formononetin ($C_{16}H_{12}O_4$)	5280378		Isoflavones
22	Galanolactone ($C_{20}H_{30}O_3$)	11141699		Diterpenoids
23	Gallic acid ($C_7H_6O_5$)	370		phenolic acids
24	Genistin ($C_{21}H_{20}O_{10}$)	5281377		Isoflavones
25	Hesperidin ($C_{28}H_{34}O_{15}$)	3594		Flavanones

26	Hesperetin ($C_{16}H_{14}O_6$)	72281		Flavanones
27	Naringenin ($C_{15}H_{12}O_5$)	439246		Flavanones
28	Myricetin ($C_{15}H_{10}O_8$)	5281672		Flavonoids
29	Myricitrin ($C_{21}H_{20}O_{12}$)	5281673		Glycosyloxyflavone
30	Paeoniflorin ($C_{23}H_{28}O_{11}$)	442534		Monoterpene glycoside

31	Rutin ($C_{27}H_{30}O_{16}$)	5280805		Flavonol glycoside
32	Quercetin ($C_{15}H_{10}O_7$)	5280343		Flavonoids
33	Puerarin ($C_1H_0O_1$)	5281807		Isoflavones
34	Wogonin ($C_{16}H_{12}O_5$)	5281703		Dihydroxyflavone
35	Resveratrol ($C_{14}H_{12}O_3$)	445154		Polyphenols

36	Deoxyandrographolide ($C_{20}H_{30}O_4$)	21679042		Labdane diterpenoid
37	Linoleic acid ($C_{18}H_{32}O_2$)	5280450		Polyunsaturated omega-6 fatty acid
38	α-Linolenic acid ($C_{18}H_{30}O_2$)	5280934		Polyunsaturated omega-3 fatty acid
39	Arachidonic acid ($C_{20}H_{32}O_2$)	444899		Polyunsaturated omega-6 fatty acid
40	Oleic acid ($C_{18}H_{34}O_2$)	445639		Unsaturated omega-9 fatty acid

N°	Molecules	Pubchem CID	Structures	Inhibitors
01	Tazobactam ($C_{10}H_{12}N_4O_5S$)	123630		ß-lactamase inhibitor
02	Clavulanic acid ($C_8H_9NO_5$)	5280980		ß-lactamase inhibitor
03	SULBACTAM ($C_8H_{11}NO_5S$)	130313		ß-lactamase inhibitor

Appendix B. Total Ion Chromatogram (TIC) Chromatograms

1.1. *Ulva intestinalis*

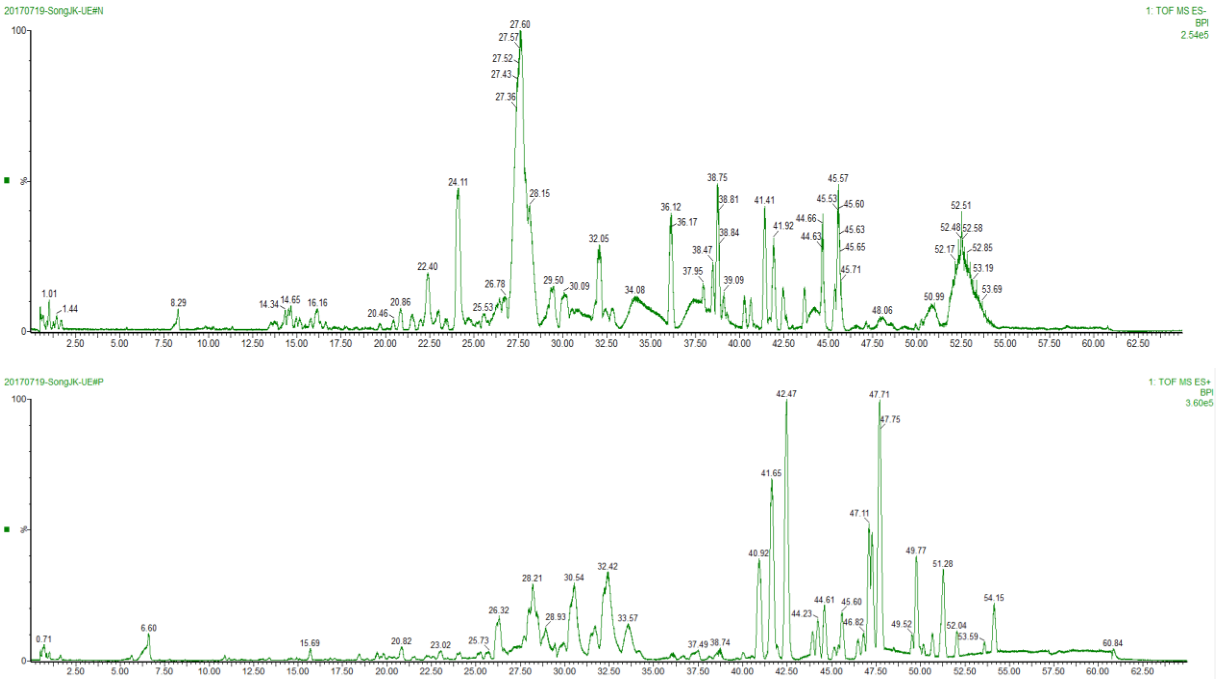


Figure 01: TIC Chromatogram of *Ulva intestinalis* by HPLC-ESI-MS-TOF

1.2. *Dictyota dichtoma*

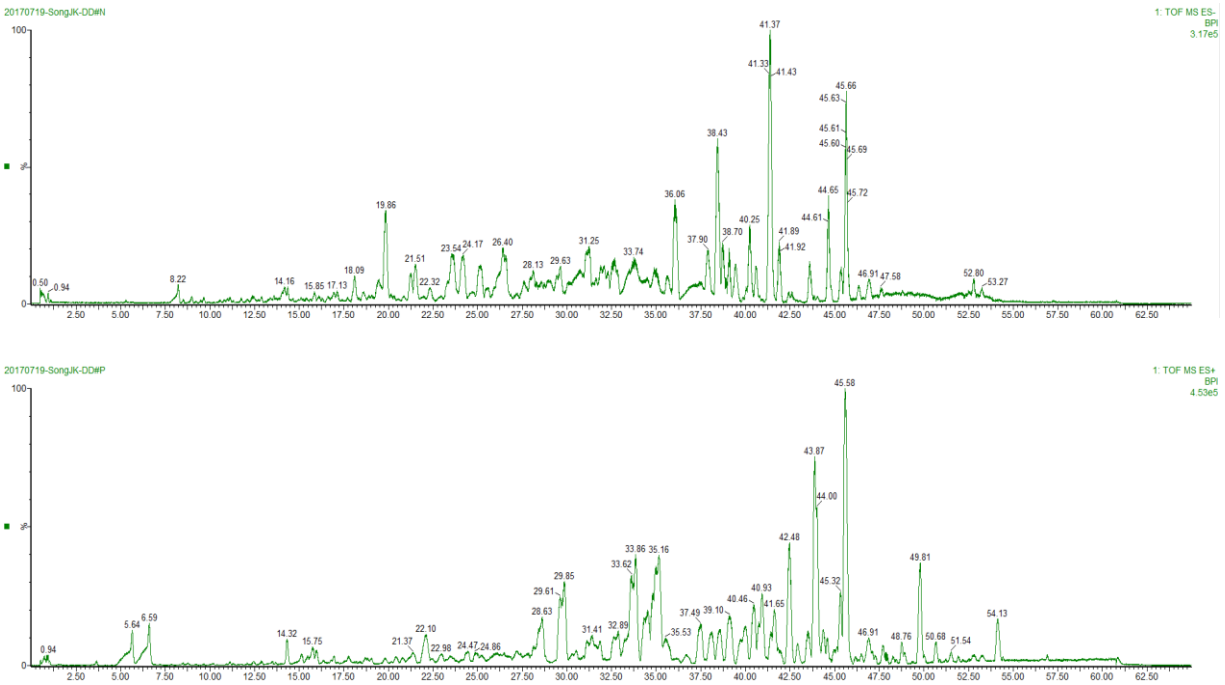


Figure 02: TIC Chromatogram of *Dictyota dichtoma* by HPLC-ESI-MS-TOF

1.3. *Halopteris scoparia*

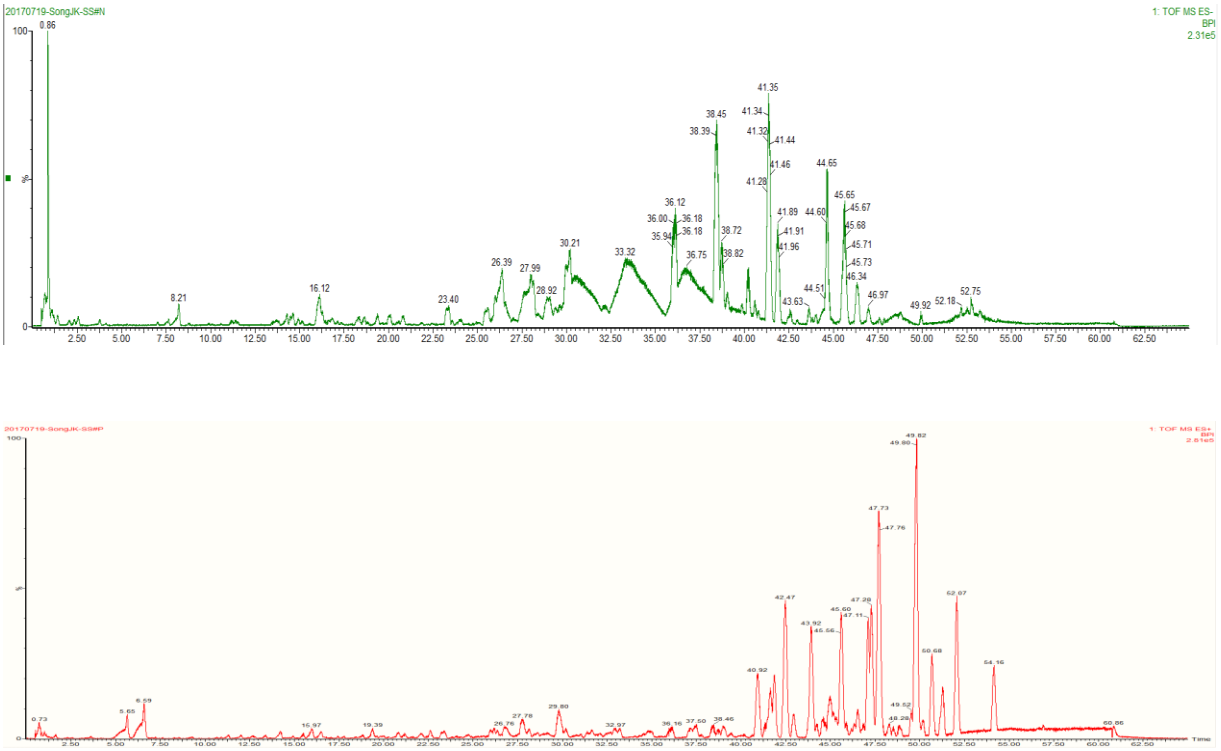


Figure 03: TIC Chromatogram of *Halopteris scoparia* by HPLC-ESI-MS-TOF

1.4. *Sargassum vulgare*

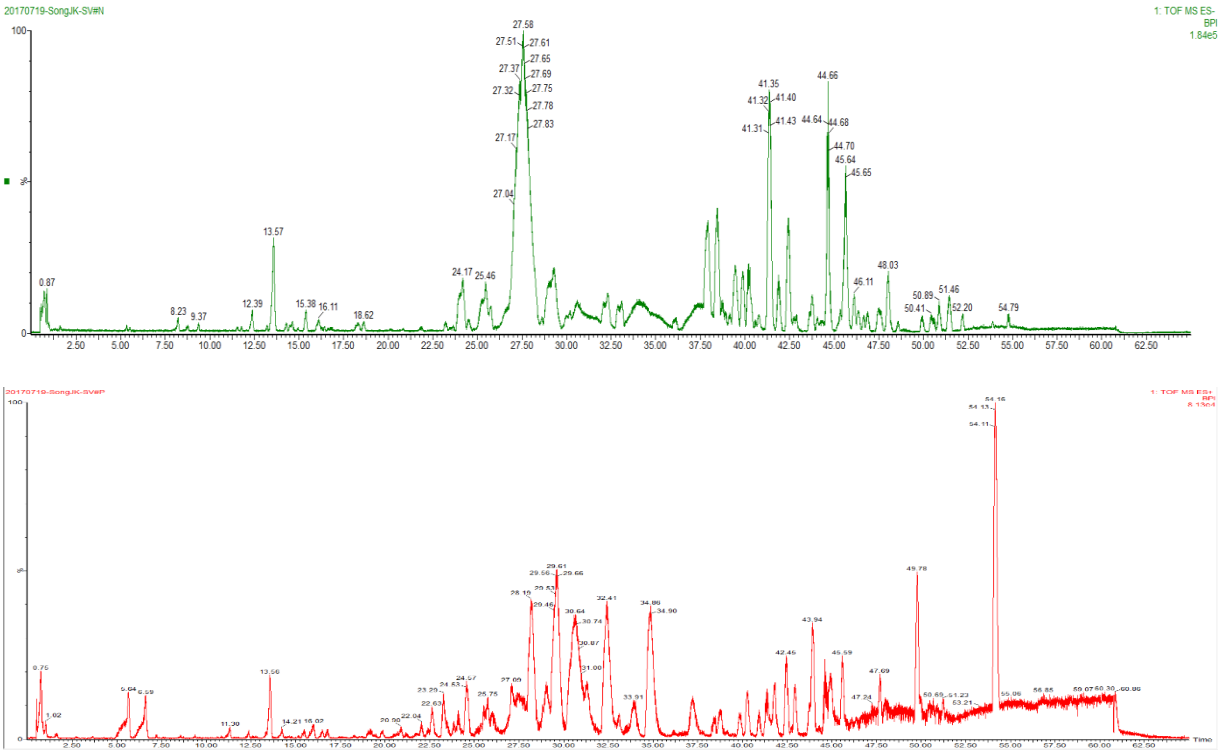


Figure 04: TIC Chromatogram of *Sargassum vulgare* by HPLC-ESI-MS-TOF

1.5. *Codium tomentosum*

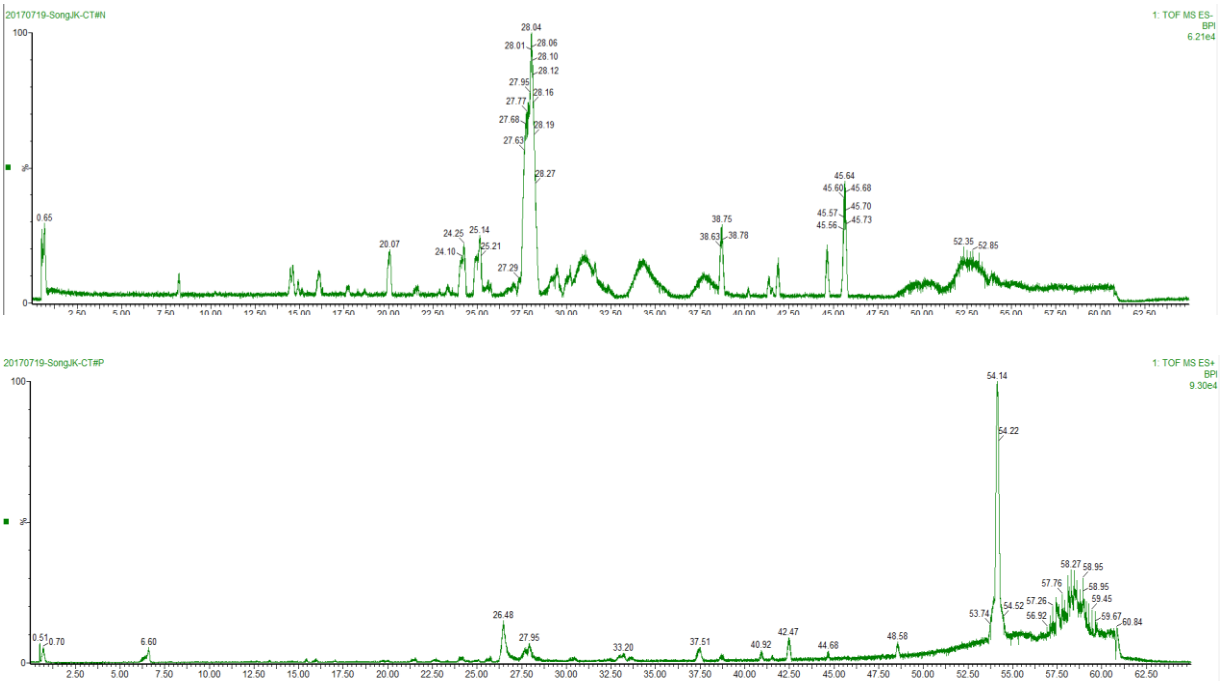


Figure 05: TIC Chromatogram of *Codium tomentosum* by HPLC-ESI-MS-TOF

1.6. *Cystoseira compressa*

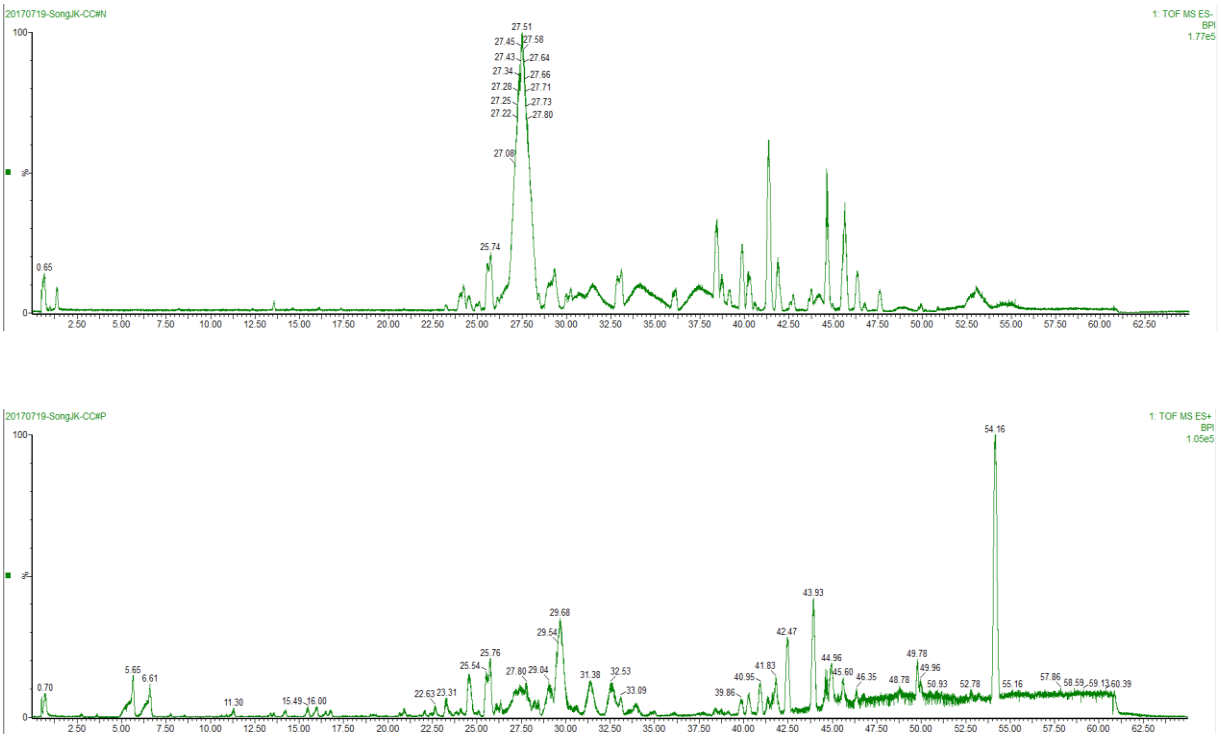


Figure 06: TIC Chromatogram of *Cystoseira compressa* by HPLC-ESI-MS-TOF

1.7. *Bryopsis pulmosa*

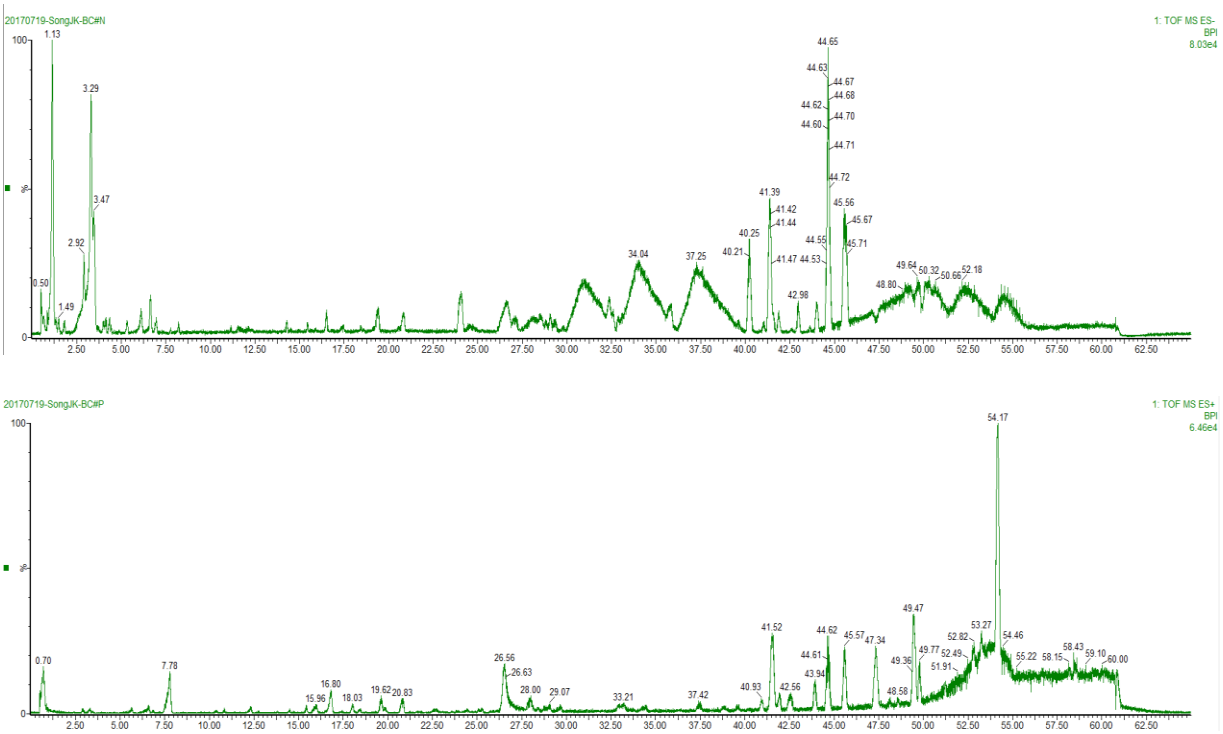


Figure 07: TIC Chromatogram of *Bryopsis pulmosa* by HPLC-ESI-MS-TOF

Appendix C. Qualitative analysis chromatograms using HPLC

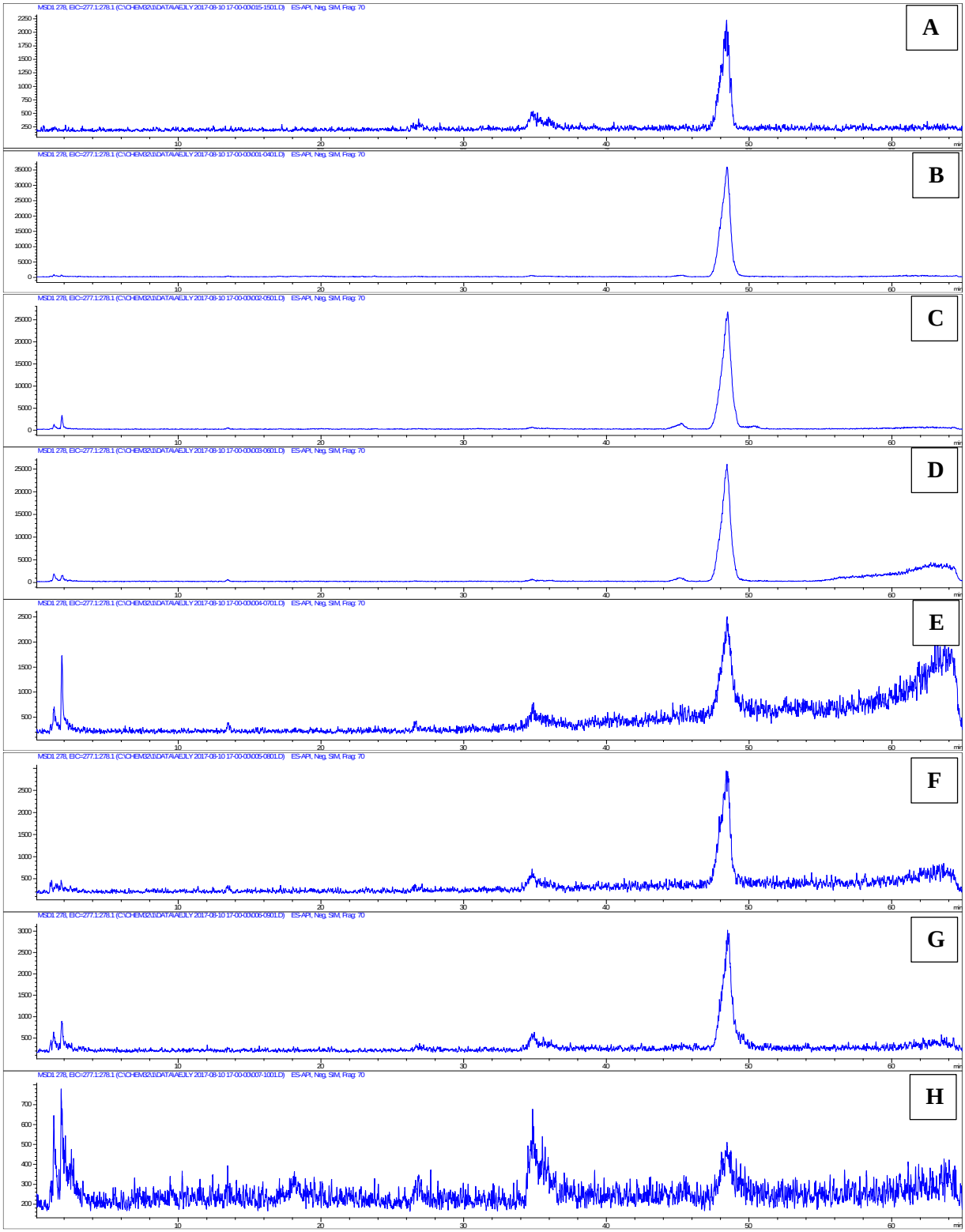


Figure 08. Qualitative analysis chromatograms using HPLC of a solution of α-linolenic acid as standard $[M-H]^-$: 277.4 **(A)**, *U.intestinalis* **(B)**, *D.dichtoma* **(C)**, *H.scoparia* **(D)**, *S.vulgare* **(E)**, *C.tomentosum* **(F)**, *C.compressa* **(G)**, *B.pulmosa* **(H)**

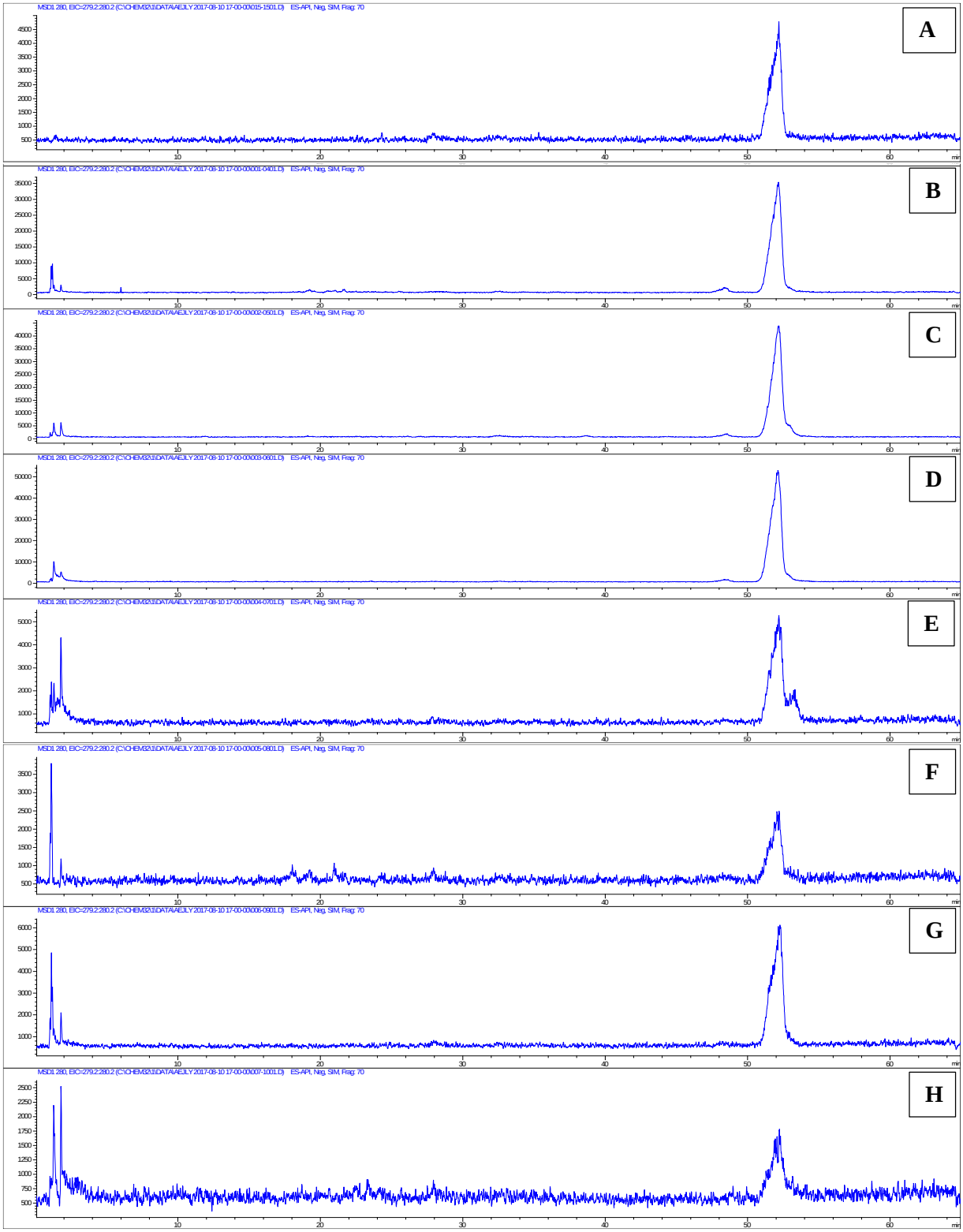


Figure 09. Qualitative analysis chromatograms using HPLC of a solution of linoleic acid as standard $[M-H]^-$: 279.5 **(A)**, *U.intestinalis* **(B)**, *D.dichtoma* **(C)**, *H.scoparia* **(D)**, *S.vulgare* **(E)**, *C.tomentosum* **(F)**, *C.compressa* **(G)**, *B.pulmosa* **(H)**

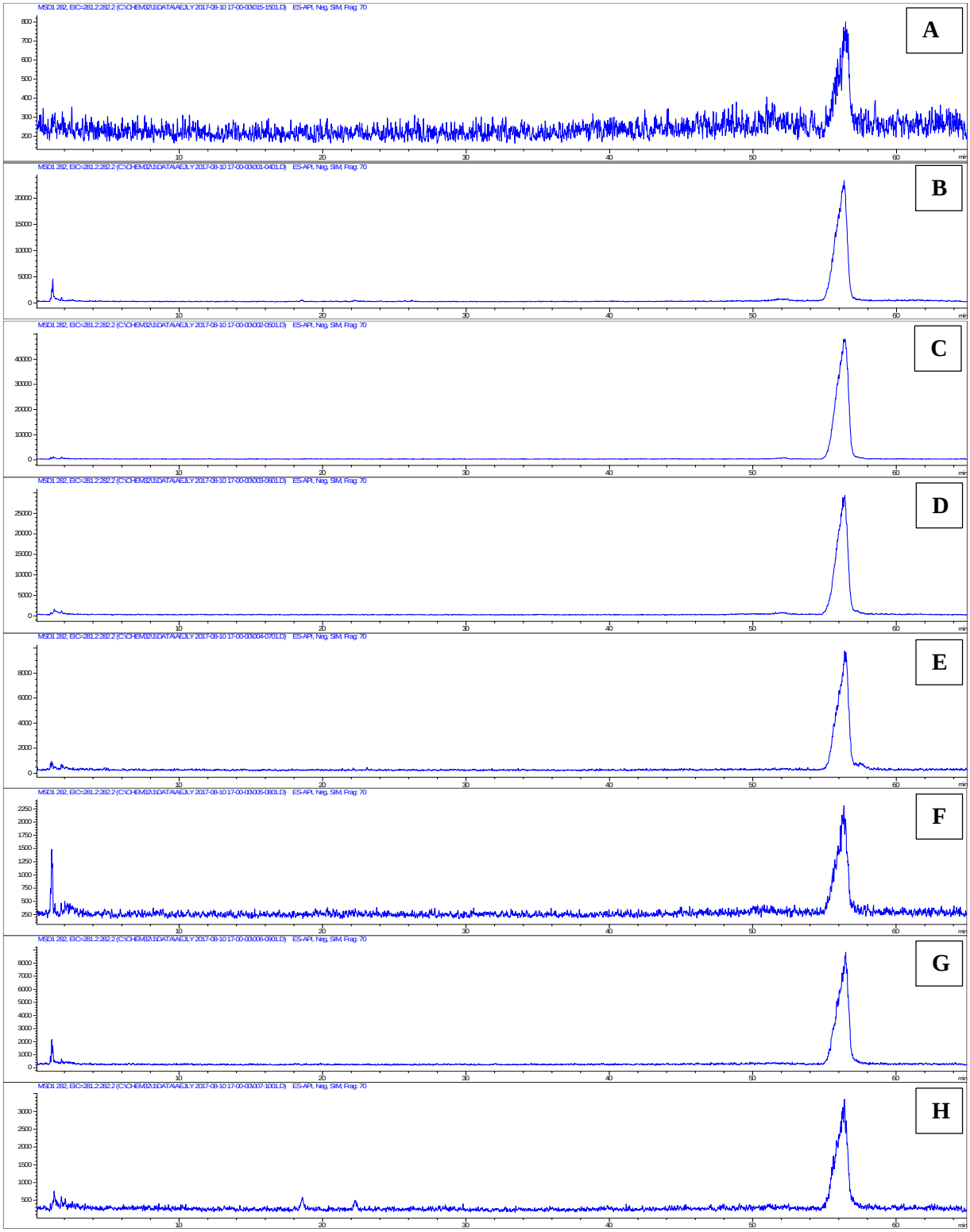


Figure 10. Qualitative analysis chromatograms using HPLC of a solution of oleic acid as standard [M-H]⁻: 281.5 **(A)**, *U.intestinalis* **(B)**, *D.dichtoma* **(C)**, *H.scoparia* **(D)**, *S.vulgare* **(E)**, *C.tomentosum* **(F)**, *C.compressa* **(G)**, *B.pulmosa* **(H)**

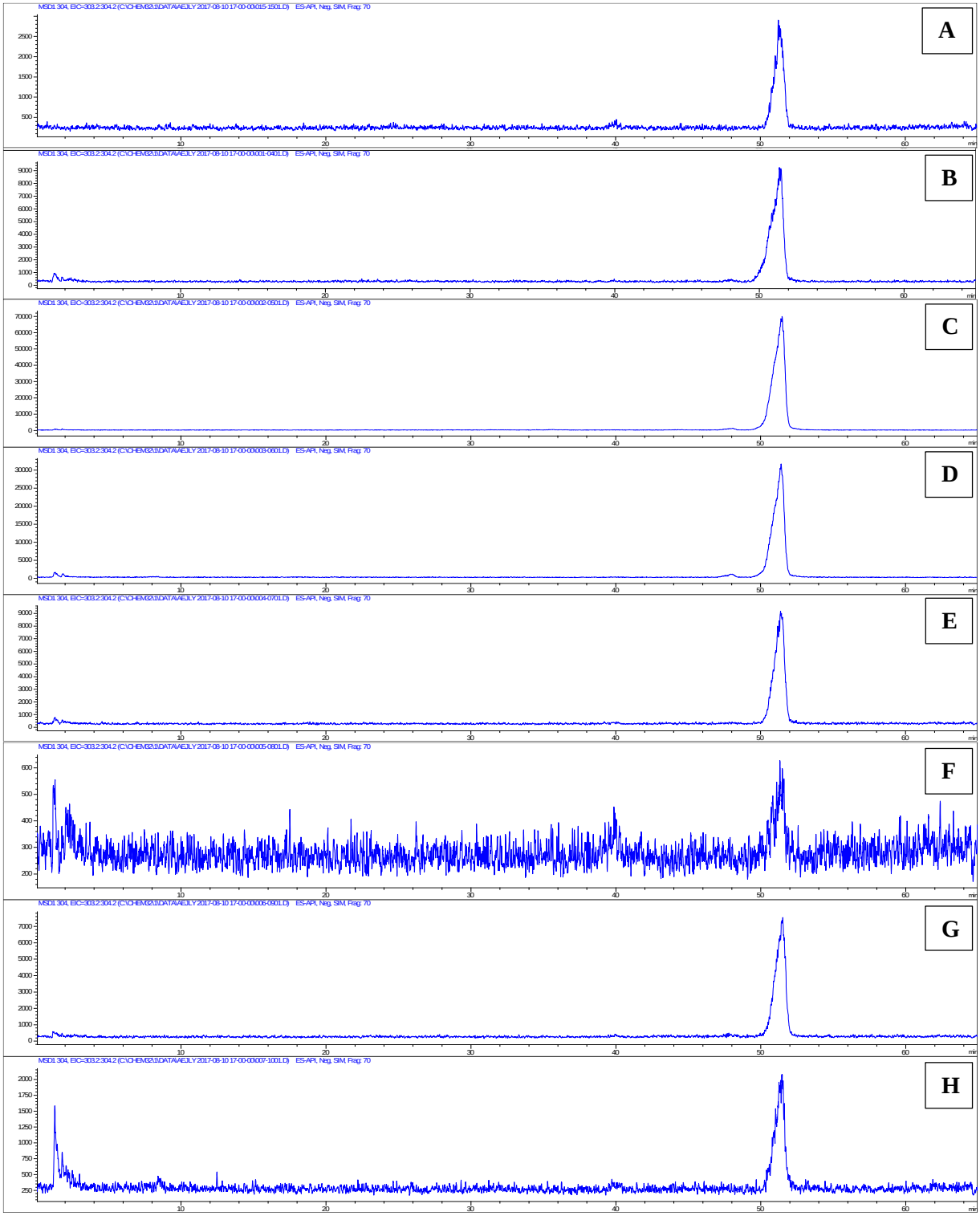


Figure 11. Qualitative analysis chromatograms using HPLC of a solution of arachidonic acid as standard [M-H]$^-$: 277.4 **(A)**, *U.intestinalis* **(B)**, *D.dichtoma* **(C)**, *H.scoparia* **(D)**, *S.vulgare* **(E)**, *C.tomentosum* **(F)**, *C.compressa* **(G)**, *B.pulmosa* **(H)**

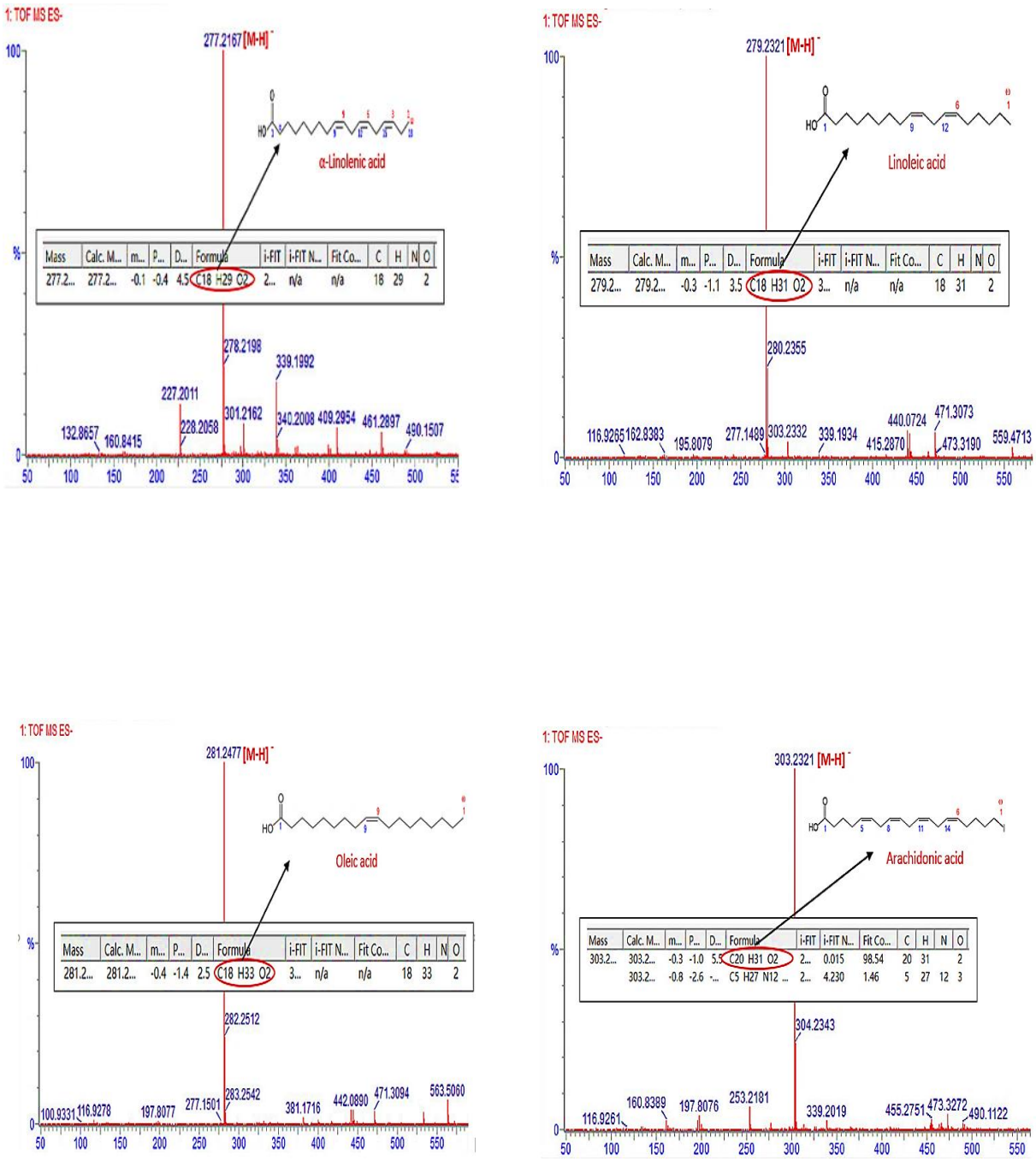


Figure 12. Elemental composition confirmation by accurate mass spectrum of the peak corresponding to the common compounds between all ME of seaweeds and their structural formula

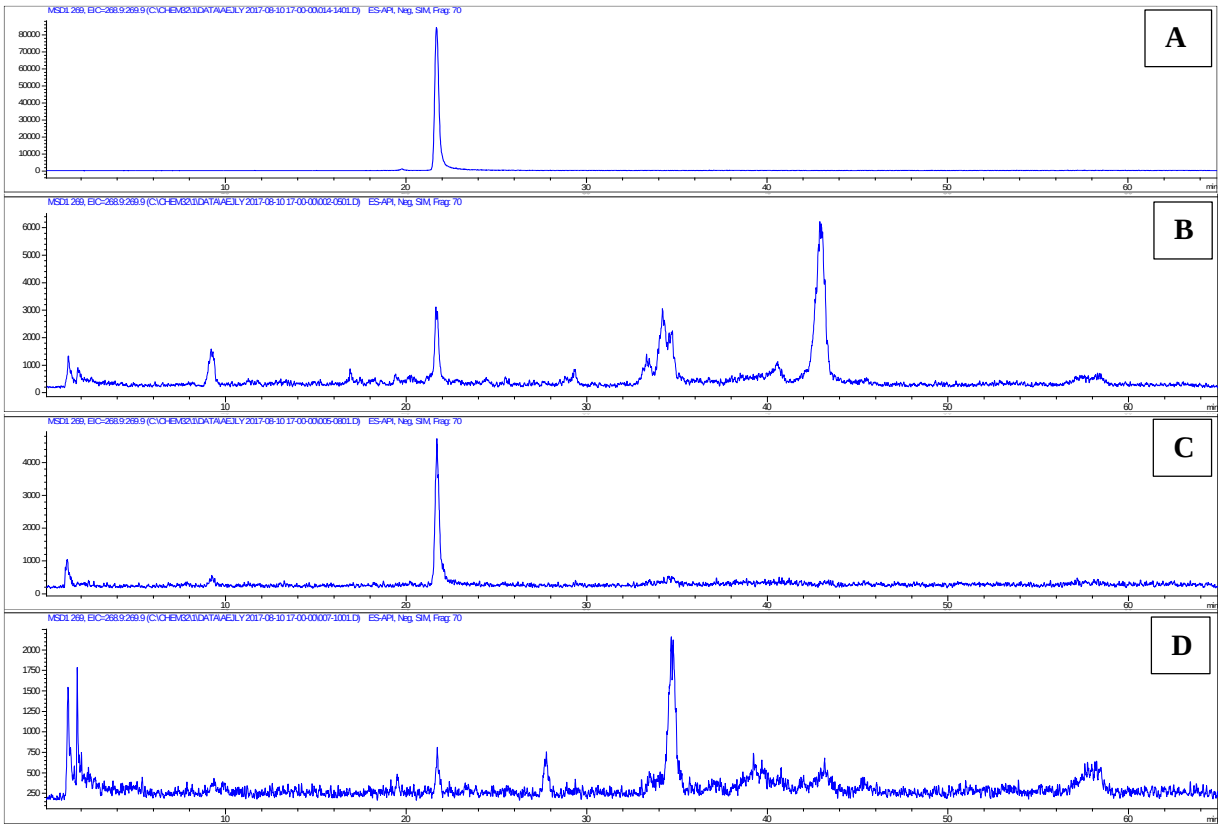


Figure 13. Qualitative analysis chromatograms using HPLC of a solution of baicalein as standard [M-H]$^-$: 269.2 **(A)**, *U.intestinalis* **(B)**, *D.dichtoma* **(C)**, *B.pulmosa* **(D)**

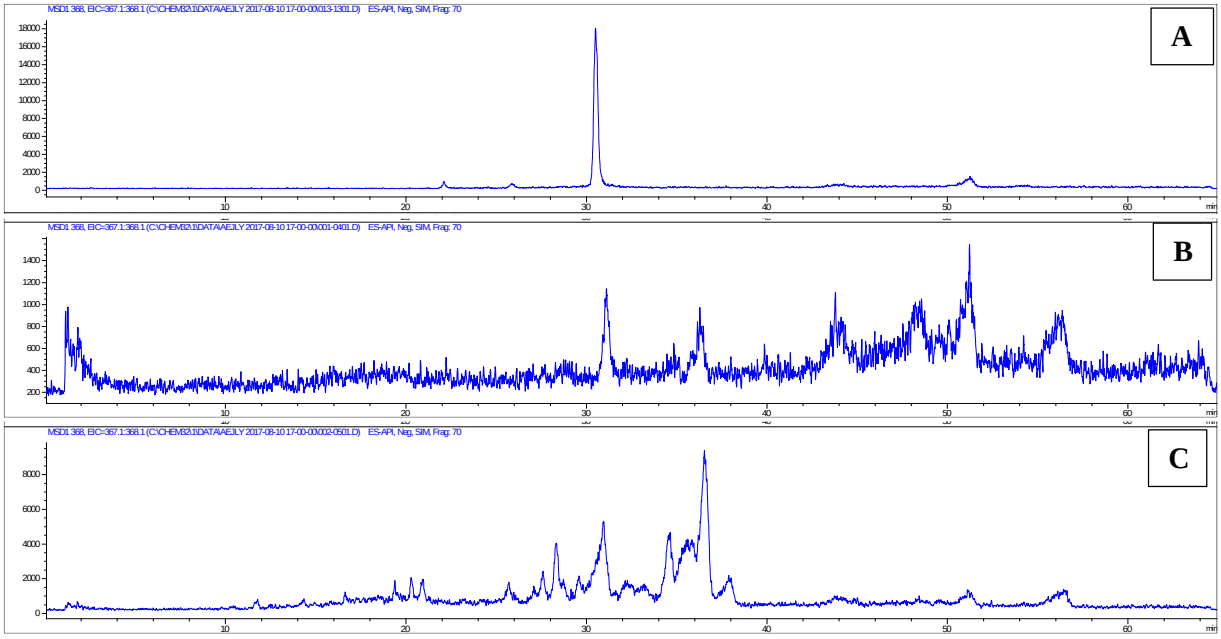


Figure 14. Qualitative analysis chromatograms using HPLC of a solution of curcumin as standard [M-H]$^-$: 367.4 **(A)**, *U.intestinalis* **(B)**, *D.dichtoma* **(C)**

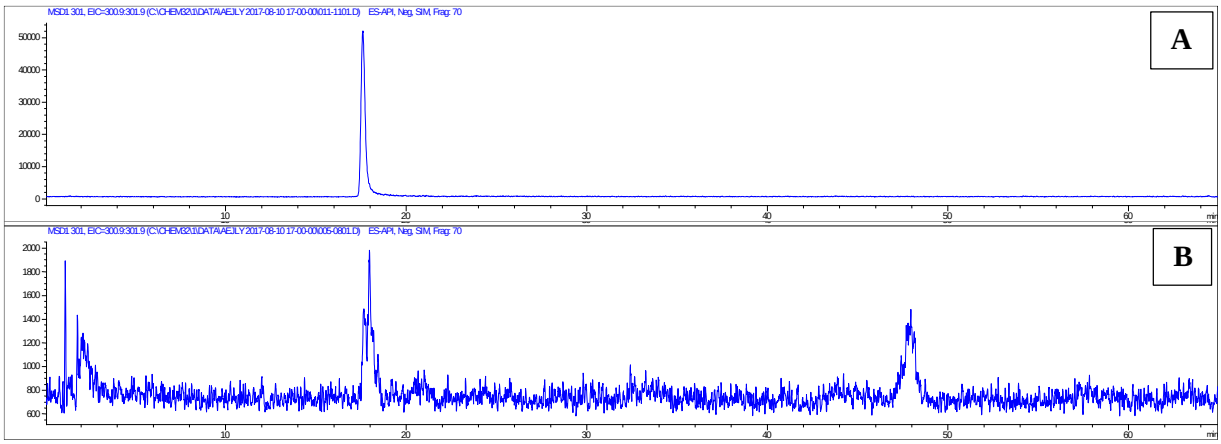


Figure 15. Qualitative analysis chromatograms using HPLC of a solution of quercetin as standard [M-H]⁻: 301.2 **(A)**, *C.tomentosum* **(B)**

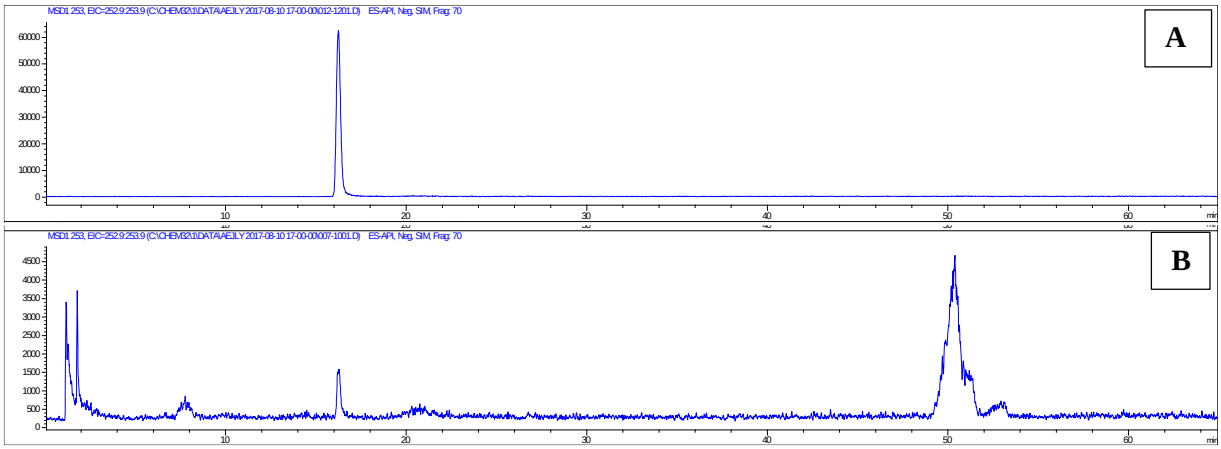


Figure 16. Qualitative analysis chromatograms using HPLC of a solution of daidzein as standard [M-H]⁻: 253.2 **(A)**, *B.pulmosa* **(B)**

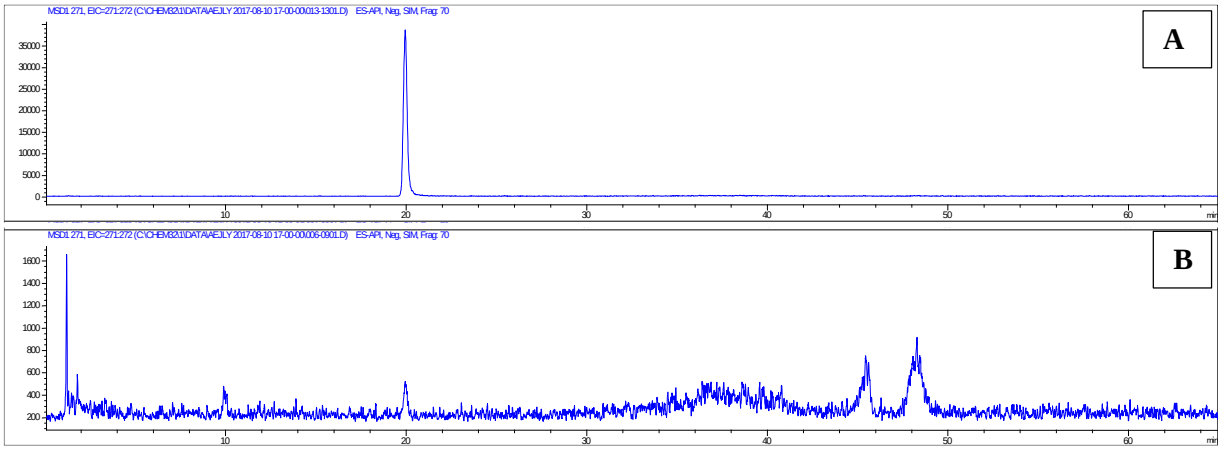


Figure 17. Qualitative analysis chromatograms using HPLC of a solution of (S) naringenin as standard [M-H]⁻: 271.2 **(A)**, *C.compressa* **(B)**

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Investigation of common chemical components and inhibitory effect on GES-type β-lactamase (GES22) in methanolic extracts of Algerian seaweeds

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ABSTRACT

This study aimed to investigate the total phenolic content (TPC), the identification of the common compounds by HPLC-ESI-MS and HPLC-ESI-MS-TOF and the inhibitory effects against class A-type β-lactamase (GES-22 variant, produced recombinantly) in methanolic extracts (MEs) of four Algerian seaweeds [*Ulva intestinalis*, *Codium tomentosum*, *Dictyota dichotoma* and *Halopteris scoparia*]. The TPC varied among the four species, ranging between 0.93 ± 0.65 and 2.66 ± 1.33 mg GAEs/g DW. *C.tomentosum* had higher total phenol content than other seaweeds while, all of them inhibited uncompetitively GES-22 activity in a dose-dependent manner. Nitrocefin was used as chromogenic substrate to evaluate the inhibitory effect on GES-22. The methanolic extract of *D.dichotoma* exhibited significant inhibitory effect on GES-22 (IC_{50} = 13.01 ± 0.046 μg/mL) more than clavulanate, sulbactam and tazobactam (classical β-lactam inhibitors) (IC_{50} = 68.38 ± 0.17 μg/mL, 52.68 ± 0.64 μg/mL, and 29.94 ± 0.01 μg/mL, respectively). IC_{50} of the other ME of *U.intestinalis*, *C.tomentosum,* and *H.scoparia* were 16.87 ± 0.10 μg/mL, 16.54 ± 0.048 μg/mL, and 25.72 ± 0.15 μg/mL, respectively. Except *H. scoparia*, other three seaweed extracts showed almost two times or more inhibition on GES-22. Furthermore, four common compounds in these MEs were identified, α-linolenic acid (C18:3ω3), linoleic acid (C18:2ω6), oleic acid (C18:1ω9), the eicosanoid precursors "arachidonic acid" (C20:4ω6). Baicalein (C15H10O5) was identified in *U.intestinalis* and *D.dichotoma* seaweeds. The fact that all seaweed extracts inhibited the GES-22 better than commercial samples makes these seaweeds candidate for discovering new inhibitors against β-lactamases. Besides that, they contain important components with potential health benefits.

1. Introduction

β-Lactam antibiotics, which include penicillins and cephalosporins, carbapenems, oxacephems and monobactams, constitute over half of the global antibiotic market currently employed in the treatment of bacterial infections [1,2]. However, repeated and prolonged use of β-lactam antibiotics has been associated with the emergence of bacterial antibiotic resistance by production of β-lactamases, which are able to chemically inactivate these molecules by hydrolyzing the endocyclic peptide bond in the β-lactam ring. The frequency of this resistance in microbial pathogens continues to emerge worldwide [3]. Based on the structural similarities and the hydrolytic profiles, β-lactamases are classified into four distinct classes (A, B, C and D) [4], and four groups (1, 2, 3 and 4) [5], respectively. The classical β-lactamases inhibitors (tazobactam, clavulanate, and sulbactam) coadministered with β-lactam antibiotics are effective against class A β-lactamases. They are ineffective against class C and most of the class B and D β-lactamases. Extended spectrum β-lactamases (ESBLs) are a group of enzymes that can hydrolyze third and fourth generation cephalosporins [6], and compromise the efficacy of all β-lactams, except cephamycins and carbapenems. In addition, ESBLs inhibitor exert *in vitro* pressure on ESBLs there by facilitating reverse mutation to less harmful enzymes

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[7]. ESBLs of the GES type have been reported in *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Aeromonas media*, *Aeromonas veronii*, *Klebsiella oxytoca*, *Serratia marcescens* and *Acinetobacter baumannii*. Recently, GES22 (A-class β-lactamase) was characterized from *Acinetobacter baumannii* [8]. It differs from GES-11 by a single substitution M169L (part of the omega loop). By this substitution, the GES-22 variant more efficiently hydrolyses the mechanistic inhibitors such as clavulanic acid and sulbactam [9]. Hence, new and potential β-lactamases inhibitors is an urgent need.

It is well known that, over the past decades, seaweed has been attracting attention in the search for bioactive compounds to develop new drugs and healthy foods. Among which: polyphenols [10], lipids [11], polysaccahrides [12], sterols [13], terpenes [14]. Furthermore, various seaweed extracts were reported to have high antioxidant, anti-cancer activity [15] and to influence anti-inflammatory responses [16]. According to the literature, numerous researchers have attempted to develop new ESBL inhibitors from different source. Very recently, Z Messasma et al., 2018 [17] reported the inhibitory activity of Schiff base ligands on metallo-β-lactamases which are extracted from *Acinetobacter baumannii*. According to this study, Schiff base ligands were revealed to have a good inhibition activity towards MBLs. Additionally, in 2018, R Krishnamoorthy et al. [18], evaluated the antimicrobial effects of a nanoemulsion prepared from *C. viscosa* essential oil on Drug Resistant (DR) ESBL producers and methicillin-resistant *Staphylococcus aureus* (MRSA) and its mode of action. They have, reported that the nanoemulsion formulated from *C. viscosa* essential oil has potential activity against both DR Gram-positive and Gram-negative (MRSA and ESBL-producing) bacterial pathogens and many other studies were evaluated the effect of plant extracts on ESBL producing strains [19,20].

The purpose of this study is to evaluate the inhibitory effect of methanolic extracts of four Algerian seaweeds (*Ulva intestinalis, Codium tomentosum* as Chloprophyceae-green algae, *Dictyota dichotoma* and *Halopteris scoparia* as Phaophycaea-brown algae on the hydrolysis activity of A-class β-lactamase (GES-22) that was identified in *A. baumannii* isolate from Turkey. Additionally, the common compounds in these seaweeds were identified by HPLC-ESI-MS and HPLC-ESI-MS-TOF technics.

2. Materials and methods

2.1. HPLC chemicals and reagents

Chrysin, emodin, kaempferol, quercetin, esculin, genistin, resveratrol, daidzein, formononetin, aloe-emodin, hesperetin, myricetin, baicalin, 7-hydroxy-isoflavone, genistein, (S) naringenin, rhein, curcumin, rutin, (R) pinocembrin, baicalein, puerarin, myricetrin, hesperidin, α-linolenic acid, linoleic acid, oleic acid, abietic acid, arachidonic acid and isosteviol were all purchased from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). Acetonitrile (LC–MS-grade) and methanol (LC–MS-grade) were purchased from J.T. Baker (Seattle, WA, USA). Formic acid (HCOOH, HPLC-grade) was obtained from TEDIA (Fairfield, USA). All the other reagents were of analytical grade. Wahaha purified water (Hangzhou, China) was used throughout the study.

2.2. Algae sampling

The samples were collected in May 2015 by scuba diving, from the Southern of the Mediterranean Sea shores (36°35'N, 2°29'E) in the region located on the coast of the Bay of Bou Ismaïl, KOUALI cove (Fig. 1). This site was selected due to its richness in algal populations and it is also free of any discharges. Collected samples were kept in seawater at 0 °C until they arrived at the laboratory. The algae were successively rinsed with sea water and distilled water to remove Epiphytes and necrotic parts, then they were dried in the shade and ground by a blender to a fine powder.

2.3. Preparation of methanolic extracts

Dried seaweeds samples (10 g) were extracted with 100 mL of methanol for 2 h at 20 °C in an ultrasonic bath, the liquid extract was centrifuged at 4000 rpm for 10 min at 4 °C. Then the supernatants were pooled and concentrated by using a rotary evaporator at 37 °C. The obtained extracts were lyophilized and stored in dark conditions in freezer at −20 °C until the analysis. Yields were expressed as the percentage (w/w) of dry matter (grams) obtained from 100 g of dried algal powder utilized for extraction.

2.4. Estimation of total phenolic content

The total phenolic content of the methanolic extracts were determined spectrophotometrically at 765 nm, according to the Folin-Ciocalteu colorimetric method [21]. A volume of 200 µL of methanolic extract solutions was added to 1 mL of Folin-Ciolcalteu reagent previously diluted with 10 mL of deionized water (10%). After 4 min, 800 µL of saturated sodium carbonate (75 g/L) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Quantification of total phenolic was based on a gallic acid standard curve and the results were expressed as milligram gallic acid equivalent mg GAE/g extract.

2.5. Expression and purification of GES-22

GES-22, a member of A-class β-lactamase, was produced recombinantly in *E. coli* BL21 (DE3) harbored the expression vector of pET28a-GES-22. The bacteria were grown at 37 °C in LB medium containing 60 µg/mL of kanamycin up to OD0.6 at 600 nm. Then, it was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for overnight at 18 °C to maximize the production. The cells were harvested by centrifugation and the pellet was suspended in 50 mM Tris-H2SO4 (pH: 7,4) with 20 mg/mL lysozyme and lysed by sonication. The lysate was centrifuged at 9000 rpm for 40 min at 4 °C. The clarified supernatants were loaded onto a Nickel Resin equilibrated with 50 mM Tris-H2SO4, 500 mM NaCl, 2 mM DTT, pH 7.4. The column was then washed 50 mM Tris-H2SO4, 500 mM NaCl, 2 mM DTT, 25 mM imidazole, pH 7.4 and 50 mM Tris-H2SO4, 500 mM NaCl, 2 mM DTT, 100 mM imidazole, pH 7.4. The purified β-lactamase protein was eluted with 50 mM Tris-H2SO4, 500 mM NaCl, 2 mM DTT, 300 mM imidazole pH 7.4 The GES-22 containing fractions were pooled and dialyzed against 50 mM Tris-H2SO4, pH 7.4 overnight. Protein samples were stored at −20 °C [8].

2.6. β-Lactamase in vitro inhibition assays

Inhibitory effect on the hydrolyzing activity of GES-22 by the crude methanolic extracts of four seaweeds was determined by spectrophotometric test using the nitrocefin as a chromogenic substrate at a wavelength of 482 nm (ε_{482} = 15000 M cm^{-1}). The classical β-lactamase inhibitors (tazobactam, clavulanate, and sulbactam) were used as a positive control. The reaction mixture was prepared in 96-well microplates in 200 µL of final volume and consisted of 158 µL of 100 mM sodium phosphate buffer (pH: 7.4), 5 µL of each extract from different concentrations (500–1.25 µg/mL) and 7 µL (0.3 µM) of GES-22. They were kept 10 min at 25 °C and nitrocefin (30 µL of 60 µM) was added to initiate the reaction. The microplate was immediately read at 482 nm using a UV–visible microplate reader. All assays were carried out in triplicate and the inhibition percentage was calculated using follow the equation: $\%\text{Inhibition} = \frac{[\text{Abs (control)} - \text{Abs (sample)}]}{\text{Abs (control)}} \times 100$. Where Abs (control) is the absorbance of GES-22 activity without inhibitors, and Abs (sample) is GES-22 activity in the presence of inhibitors.

To examine the inhibition type of the seaweed MEs, GES-22 activity was monitored as previously with increasing concentrations of

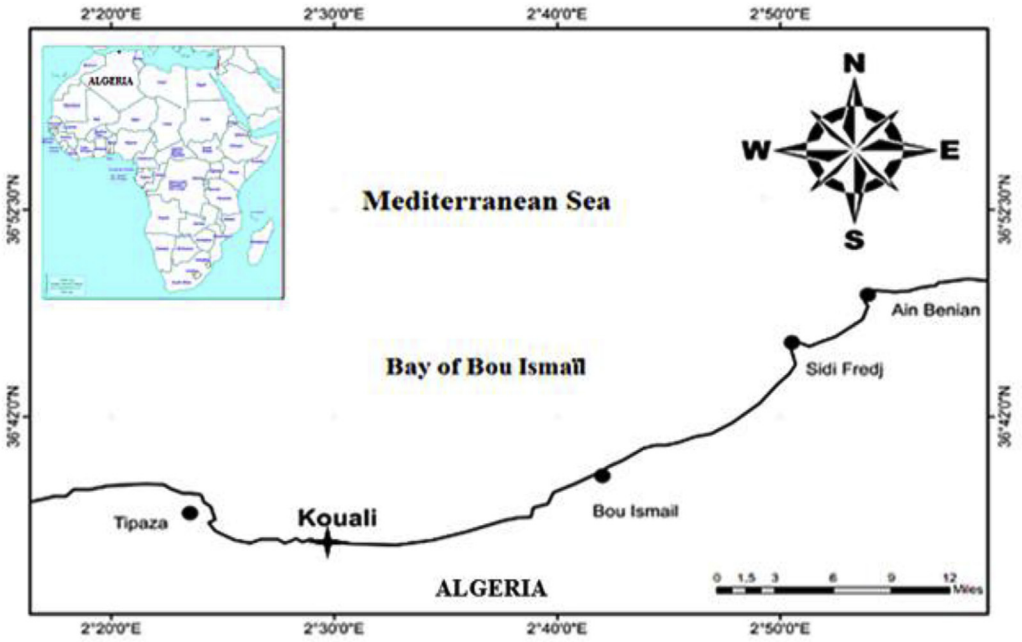

Fig. 1. Geographical map focusing on the sampling site (cove KOUALI, ALGERIA).

nitrocefin from 10 to 150 μM, in the absence or presence of MEs (the concentration of ME was kept constant at 100 μg/mL). The maximum velocity (V_M) and the Michaelis-Menten constant (Km) were determined from a Lineweaver-Burk plot where the reciprocal of the substrate concentration (1/[S]) on the x axis is plotted against the reciprocal of the initial velocity v_0 on the y axis.

2.7. Sample preparation for HPLC analysis

The standard solutions of chrysin, emodin, kaempferol, quercetin, esculin, genistin, resveratrol, daidzein, formononetin, aloe-emodin, hesperetin, myricetin, baicalin, 7-hydroxy-isoflavone, genistein, (S) naringenin, rhein, curcumin, rutin, (R) pinocembrin, baicalein, puerarin, myricetrin, hesperidin, α-linolenic acid, linoleic acid, oleic acid, abietic acid, arachidonic acid and isosteviol were all prepared in methanol at concentrations of 200 ng/mL, and then diluted to 100 ng/mL with purified water for further LC-MS studies. Each dried extract was dissolved in LC-MS grade methanol, then diluted to 500 μg/mL (HPLC-ESI-MS preliminary experiment), 1 mg/mL (HPLC-ESI-MS) and 5 mg/mL (HPLC-ESI-MS-TOF) concentrations with purified water to supply half methanol and half water in final volume.

2.8. HPLC-ESI-MS

The analyzes were carried out using Agilent technologies 1200 series high performance liquid chromatography equipped with G1379B degasser, G1311A quaternary pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector (Agilent Technologies, USA). All the compounds were separated on a ZORBAX SB-C_{18} column with a length of 2.1 × 100 mm, and an internal diameter of 3.5 μm, with temperature at 35 °C. The mobile phase works at a flow rate of 0.3 mL/min using acetonitrile (A)–water (B) contain 0.05% formic acid (v/v). A gradient elution was applied by setting solvent (A) at 10% in 0 min and then gradually increasing it to 90% in the next 90 min, followed by re-equilibration at 10% (A) until 95 min. The injection volume was 10 μL.

For the identification of compounds, an Agilent 6110 quadrupole mass spectrometer with electrospray ionization source (ESI) was operated in negative ion mode. The conditions of the ESI source were set with a drying gas flow of 10.0 L/min, a drying gas temperature of 350 °C, a nebulizer pressure of 35.0 psig and a capillary voltage of 3000 V. The determination was performed using both negative full scan mode and the negative selected ion monitoring (SIM) mode (m/z = 253.2; 269.2; 285.2; 301.2; 339.2; 431.3; 227.2; 253.2; 267.2; 296.2; 301.2; 317.2; 445.3; 237.2; 269.2; 271.2; 283.2; 267.3; 609.5; 255.2; 269.2; 415.3; 463.3; 609.5; 277.4; 279.4; 281.4; 301.4) for chrysin, emodin, kaempferol, quercetin, esculin, genistin, resveratrol, daidzein, formononetin, aloe-emodin, hesperitin, myricetin, baicalin, 7-hydroxy-isoflavone, genistein, (s) naringenin, rhein, curcumin, rutin, (r) pinocembrin, baicalein, puerarin, myricetrin, hesperidin, α-linolenic acid, linoleic acid, cis-9-octadecenoic acid (oleic acid) and abietic acid respectively for comparison standard compounds.

2.9. HPLC-ESI-MS-TOF

Ten μL of samples was used in the HPLC ESI-MS-TOF analysis, that performed by a Waters Acquity Ultra Performance LC system (Waters, USA). Chromatographic separation was carried out at 35 °C on a ZORBAX SB-C_{18} column (2.1 mm × 100 mm, 3.5 μm). The mobile phase works at a flow rate of 0.3 mL/min using acetonitrile (A)–water (B) contains 0.05% formic acid (v/v). A gradient elution was applied by setting solvent (A) at 10% in 0 min and then gradually increasing it to 90% in the next 60 min, followed by re-equilibration at 10% (A) until 65 min.

For the identification of compounds, a Waters Xevo G2 QTof MS (Waters MS Technologies, Manchester, UK) with electrospray ionization source (ESI) was operated in both positive and negative ion modes.

2.9.1. Data analysis

Experiments were performed in triplicate and results are presented as mean values ± Standard Deviation (SD). Values were determined by linear computerized regression analysis after logit/log transformation, the significance of the difference between IC_{50} of MEs versus the controls were assessed using one-way analysis of variance (ANOVA) followed by Dunnett post-test; in GraphPad Prism version 6.0.

Computer software Agilent chemstation (version B.04.02.) and MassLynx (version 4.1) were used for data acquisition and qualitative analysis of the HPLC-ESI-MS and HPLC-ESI-MS-TOF, respectively.

Table 1
Total phenolic content in methanolic extract of studied seaweed.

Samples	Extraction yield[a] (%)	Total phenolic content mg GAEs/g extract
U.intestinalis	10.55	2.12 ± 0.14
C.tomentosum	20.61	2.66 ± 1.33
D.dichotoma	14.22	1.89 ± 0.22
H.scoparia	15.15	0.93 ± 0.65

Values of TPC are expressed as mean ± SD of three parallel measurements; SD: standard deviation; GAE: Gallic Acid equivalents. a: values expressed as % of dry algae weight.

3. Results

3.1. Extraction yield and amounts of total phenolic content

The amount of total phenolic content of seaweeds ME are determined as mg GAEs/g dry weight extract and the data were presented in Table1. The TPC varied among the four species, ranging between 0.93 ± 0.65 and 2.66 ± 1.33 mg GAEs/g DW. The highest level of polyphenols was recorded in *C. tomentosum* (2.66 ± 1.33 mg GAEs/g DW) followed by *U. intestinalis* (2.12 ± 0.14 mg GAEs/g DW), *D. dichotoma* (1.89 ± 0.22 mg GAEs/g DW) and *H. scoparia* (0.93 ± 0.65 mg GAEs/g DW).

3.2. Inhibition of GES-22

The concentrations of the purified GES-22 were determined by measuring absorbance at 280 nm and its purity was shown to be more than 95% by SDS-PAGE. It was found that extracts inhibited the hydrolysis activity of GES-22 in a concentration-dependent manner (Fig. 2).

The IC_{50} values of the MEs of *D.dichotoma*, *C.tomentosum*, *U.intestinalis* and *H. scoparia* were determined as: 13.01 ± 0.046 µg/mL, 16.54 ± 0.048 µg/mL, 16.87 ± 0.10 µg/mL and 25.72 ± 0.15 µg/mL, respectively [Table2].

The MEs of *D. dichotoma*, *C. tomentosum* and *U. intestinalis* exhibited significant inhibitory activity against GES-22 even more than the positive control. Also, ME of *H. scoparia* inhibited GES-22 better than all positive control (tazobactam, clavulanate and sulbactam). The methanolic extracts of *C. tomentosum* and *U. intestinalis* had almost similar inhibitory effect on GES-22.

3.3. The inhibition type

The inhibition type of the MEs of seaweeds against GES-22 activity was evaluated using Lineweavere Burk plots. The K_m, V_{max} values and the inhibition type are shown in the Table3. The apparent V_{max} and K_m in the presence of extracts was less than the control. The data indicated a significant decrease in both apparent parameters of the four seaweeds MEs, categorizing them as uncompetitive type of inhibitors. Among the inhibitors, the affinity for GES-22 was found highest for the ME of *D.dichotoma*.

3.4. HPLC analysis

Seaweed's methanolic extracts were not purified and directly used for simultaneous determination of common compounds by HPLC-ESI-MS, also directly used for HPLC-ESI-MS-TOF without using any matrices. The identification of common compounds in ME of seaweeds was performed based on their mass spectrometry and retention times by comparison with those of different reference substances. The spectroscopic characteristics and the retention times of these compounds are listed in Table 4.

Compounds 1, 2, 3 and 4 (t_R = 48.414; 52.193; 56.448; 48.888 and 51.391 min), $[M-H]^-$ ion presented at *m/z* 277.216; 279.232; 281.247 and 301.216 corresponded to the molecular formula of C18H30O2; C18H32O2; C18H34O2 and C20H32O2. They were identified as α-linolenic acid, linoleic acid; oleic acid; arachidonic acid, respectively, in the ME of the four seaweeds.

Compound 5 (t_R = 21.686 min), $[M-H]^-$ ion presented at *m/z* 269.24, corresponded to the molecular formula of C15H10O5. It was identified as baicalein in the ME of UI (*U.intestinalis*) and DD (*D.dichotoma*).

4. Discussion

The increasing of antibiotic resistance limits the efficacy of antibiotics. Bacteria possess mechanisms to overcome antibiotic pressure and one of them is β-lactamases production. To inhibit the hydrolyzing

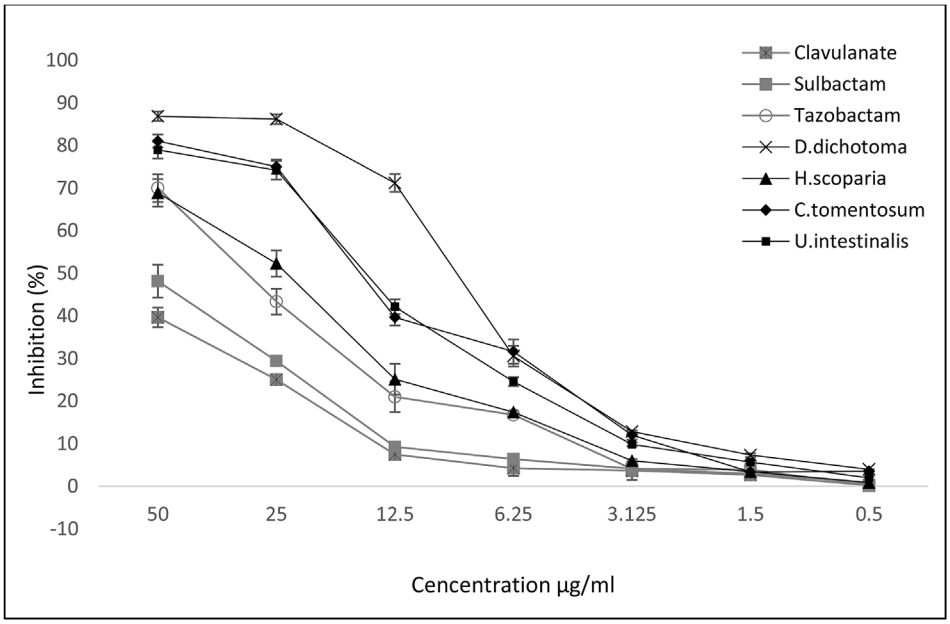

Fig. 2. Evolution of inhibition percentage of GES-22 hydrolysis activity on nitrocefin in presence of different methanolic extracts and the classical inhibitors. Each value was expressed as mean ± SD (n = 3).

Table 2
Inhibitory effects of methanolic extracts on GES-22.

Inhibitors	Methanolic extracts of seaweeds				Positive controls		
	UI	CT	DD	HS	Clavulanate	Sulbactam	Tazobactam
IC_{50} (μg/ml)	16.87 ± 0.10	16.54 ± 0.048	13.01 ± 0.046	25.72 ± 0.15	68.38 ± 0.17	52.68 ± 0.64	29.94 ± 0.01

Values are mean ± standard deviation of the mean of three assays. UI = ***U.intestinalis*; CT** = ***C.tomentosum*; DD** = *D.dichotoma*; HS = *H.scoparia*.

Table 3
Kinetic parameters of GES-22 in the presence and absence of the seaweed's methanolic extracts.

Samples	Specific activity[b]	Km (μM)	Inhibition type
U.intestinalis	$1.11 \times 10^{+2}$	190.37	UC
C.tomentosum	$1.12 \times 10^{+2}$	185.48	UC
D.dichotoma	$0.98 \times 10^{+2}$	167.26	UC
H.scoparia	$1.54 \times 10^{+2}$	204.32	UC
Control[a]	$1.71 \times 10^{+2}$	255.11	–

[a] Reaction mixture without ME.
[b] μmol of Nitrocefin hydrolysed per mn and per mg of protein (μmol. min^{-1}. mg^{-1}), UC: Uncompetitive inhibitor.

activity of β-lactamases, they are used with inhibitores (tazobactam, clavulanate, and sulbactam). The classical β-lactamases inhibitors are coadministered with β-lactam antibiotics and they are effective against class A β-lactamases. They are ineffective against class C and most of the class B and D β-lactamases. As a result of the presence of a β-lactam ring in these inhibitors and their extensive use in combination with a β-lactam antibiotics, the β-lactamase in bacteria mutate continually developing their activity even against newly developed β-lactam [22]. We used recombinant GES-22 variant of A-class β-lactamases to investigate the inhibitory effect in seaweed extracts. bla_{GES-22} allele was firstly identified in a clinical *A. baumannii* isolate and it encodes a new variant of A-class β-lactamase [9]. It differs from GES-11 by a single substitution M169L. This substitution at the omega loop confers on GES-22 more efficient hydrolysis of the classical inhibitors clavulanic acid and sulbactam [8]. It was used to evaluate the inhibitory effect of certain species of seaweed on GES-22 activity. The main finding of the present study was that the four seaweeds, which possess antioxidant [23–25], antimicrobial [26], anticancer [27,28] and antiviral [29] activity, also showed β-lactamase *in vitro* a significant GES-22 inhibitory activity in comparison with the classical inhibitors (Fig. 3) and contain common compounds among themselves.

Significant difference was observed in the phenolic content of four seaweeds tested; the TPC in the three seaweed species (*C. tomentosum*, *U.intestinalis*, *D. dichotoma*) were found to be nearly 2.3 times greater than that in *H. scoparia*. Green algae contained higher amounts of polyphenols than brown algae, these results seem to be in good agreement with those reported before [30]. They reported the amount of TPC of *C. tomentosum* as 2.26 ± 0.08 mg GAEs/g DW. As it can be observed in Table1, our results suggest that there is no correlation between yield and total phenolic content, *U. intestinalis* possessed a lowest extraction yield and almost the highest total phenolic content. The TPC of the ME of *D. dichotoma* were lower than that of *U. intestinalis* and *C. tomentosum*, however its inhibitory effect was better than theirs. These data suggest that methanolic extracts are mixtures of biological active compounds included some non-phenolic compounds, which can contribute to different activities. Furthermore, inhibitory effect of seaweed extracts on β-lactamases vary based upon the chemical content of the extract. β-lactamase kinetic assay demonstrated that, in the presence of MEs, both the apparent K_m and apparent V_{max} decreased with increase in inhibitors concentration as compared with the control. The type of inhibitors is categorized from these data as uncompetitive. The inhibitory activity of seaweed's methanolic extracts can be ranked as follows: *D. dichotoma* > *C. tomentosum* > *U. intestinalis* > *H. scoparia*.

Four common compounds in these MEs were identified by HPLC analysis are α-linolenic acid (C18:3ω3), linoleic acid (C18:2ω6), oleic acid (C18:1ω9), and the eicosanoid precursor "arachidonic acid" (C20:4ω6) (Fig. 4). In addition, another compound identified in *U. intestinalis* and *D. dichotoma* seaweed MEs was baicalein (C15H10O5). Mono and polyunsaturated fatty acids have been frequently found in seaweeds. Previous reports have found that oleic acid was the most monounsaturated fatty acids abundant in seaweeds [31–33]. Tabarsa et al. [32] reported that all the seaweeds tested contained the essential fatty acids linolenic acid (C18:3ω3), linoleic acid (C18:2ω6) and arachidonic acid (C20:4ω6), which have also been reported in previous studies in seaweeds [34]. It is thought that the inhibitory activity of our MEs could be due to the individual effect of their common compounds identified or to their synergistic effect; as it may also be due to the effect of other molecules that have not yet identified. The responsible compounds related to the inhibitory activity of seaweed extracts are not yet cleared. It was only seen that these extracts were able to inhibit the hydrolyze activity of GES-22. Further studies are required in order to identify the inhibitory compound(s) to turn them a good purpose in the fight against antibiotic resistance.

5. Conclusion

To the best of our knowledge, our results are the first published reports on the inhibitory effect of the methanolic extracts of seaweeds on the β-lactamase activity. Also, this study supplied new information about the common compounds in four seaweeds. Their methanolic extracts inhibit uncompetitively *in vitro* the activity of GES-22 and they are more effective than the classical β-lactamase inhibitors, especially the ME of *D.dichotoma*. The mechanism of action of the tested extracts is yet to be tested and further work is in progress to identify the compounds responsible for the inhibition of GES-22 activity from these extracts.

Table 4
Common compounds identified in methanolic extracts from dried seaweeds by HPLC-ESI-MS and HPLC-ESI-MS-TOF.

Compound	Formula	M.W	$[MH]^-$	Retention time (min)	Identification	Found
1	C18H30O2	278.434	277.216	48.414	α-Linolenic acid	All
2	C18H32O2	280.452	279.232	52.193	Linoleic acid	ME
3	C18H34O2	282.468	281.247	56.448	Oleic acid	
4	C20H32O2	304.474	301.216	51.391	Arachidonic acid	
5	C15H10O5	270.24	269.24	21.686	Baicalein	Only UI and DD

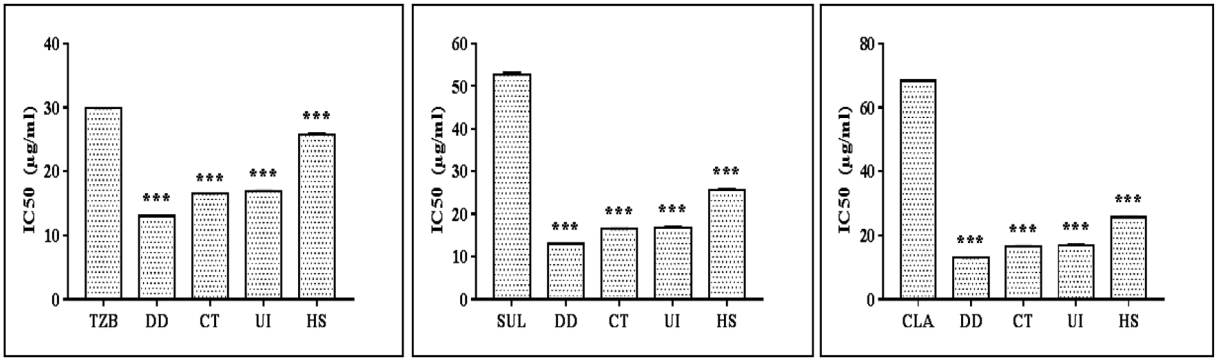

Fig. 3. *In vitro* IC_{50} values of methanolic extracts in comparison with positive controls against GES-22. Results are mean ± SD (n = 3 independent replicates). Statistically significant difference is denoted by $***p < 0.001$, as compared to positive control. TZB = Tazobactam, SUL= Sulbactam, CLA= Clavulanate.

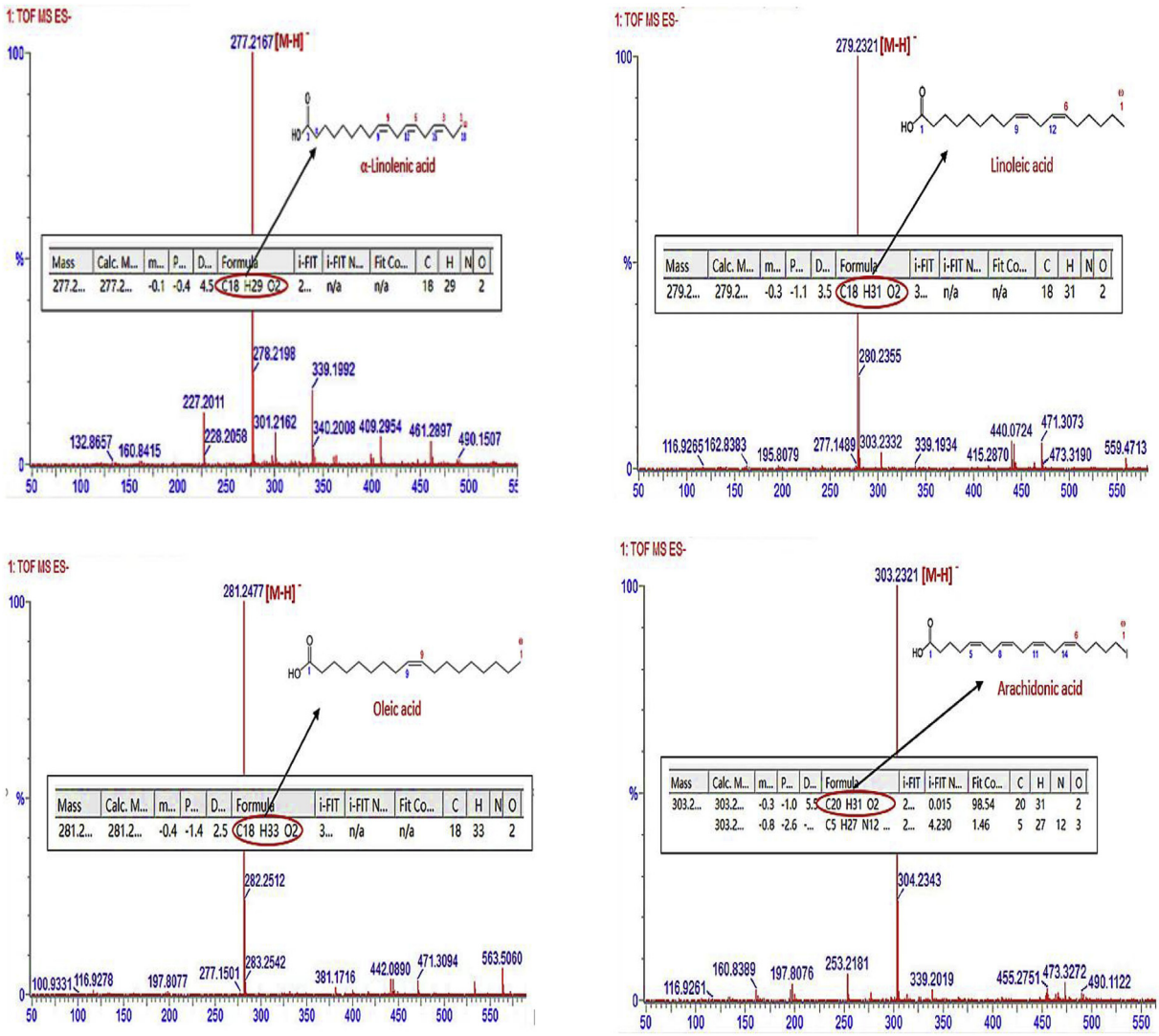

Fig. 4. Elemental composition confirmation by accurate mass spectrum of the peak corresponding to the four common compounds between all ME of seaweeds and their structural formula (Original data).

Conflicts of interest

The authors have no conflicts of interest to declare.

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