Biological Synthesis of Zinc Oxide Nanoparticles Using *Bacillus Cereus* and Its Antibacterial Activity

K. Chitra[1], *Dr. R. Janet Rani*[2], ***Dr. M.M. Abdul Kader Mohideen***[3] **and** *S. Peer Mohamed*[4]

Abstract

Today the development of reliable, eco-friendly processes for the synthesis of nanomaterials is an important aspect of nanotechnology.In this work we have developed Zinc Oxide (ZnO) nanoparticles using Bacillus cereusculture.ZnO is the metal oxide nanoparticles, ZnO nanoparticle is non-toxic. The Preliminary confirmation for the ZnO nanoparticles synthesis is the formation of White precipitate in the reaction mixture. The wurtzite crystalline structure of the ZnO nanoparticles is identified by X-ray diffractometer (XRD).The Scanning Electron microscope (SEM) showed the spherical shaped ZnO nanoparticles synthesized using Bacillus cereus culture.The antibacterial effect of ZnO nanoparticles synthesized using Bacillus cereus was examined against Escherichia coli (E.coli). Disc diffusion method is used to examine the antibacterial activity of ZnO nanoparticles.The minimum inhibitory concentration is 50 µl of ZnO nanoparticles against E.coli. The synthesized ZnO nanoparticles have the ability to kill the bacteria. Thus ZnO could be used in various clinical applications.

Keywords: *Zinc oxide nanoparticles, Bacillus cereus, XRD, Antibacterial activity*

Introduction

Nanotechnology has gained attraction in recent years,because of the controlled synthesis of the materials (less than 100 nm in size). Nanomaterial is similar to naturally occurring proteins and biomolecules in

1 Assistant Professor, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.
2 Assistant Professor and Head, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.
3 Assistant Professor, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.
4 Assistant Professor, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.

the cell due to its ultra- small size (McNeil, 2005; Manokari et al., 2016). There are various chemical and physical methods used for the synthesis of nanoparticles. There are several demerits in the synthesis of nanoparticles using physical and chemical methods such as high energy requirements in physical methods, the use of organic solvents, toxic reducing agents and capping agents in chemical method and both the methods are costly and produce toxic byproducts. Thus, there is a need to develop a clean, biocompatible, nonhazardous, inexpensive, energy efficient eco-friendly method (Chokriwal et al., 2014; Gade et al., 2011; Xie et al., 2011; Musarrat et al., 2011).

An alternative method to synthesis nanoparticles by biological origin has been developed in recent years. The unicellular and multicellular organisms such as actinomycetes, bacteria, fungus, plants, viruses and yeast are involved in the synthesis of nanoparticles. The synthesis of nanoparticles using biological origin provides a clean, non-toxic and environment-friendly method to fabricate nanoparticles (Shah *et al.*, 2015). Recently, microorganisms such as bacteria and fungi are considered interesting nanofactories because microorganisms such as bacteria and fungi play an important role in remediation of toxic metals through reduction of the metal ions (Bhima et al., 2010).

Due to low cost, metal oxide nanoparticles such as Zinc Oxide and titanium oxide are more preferable than silver nanoparticles. Owing to its unique photo-catalytic, electrical, electronic, optical, dermatological and antibacterial properties, zinc oxide has a wide range of applications. Zinc oxide is a semiconductor with a direct wide band gap and large excitation Binding energy, piezoelectric, biosafe, are the three key characters that make the possibility of using Zinc oxide in many fields (Yadav et al., 2006; Kathirvelu et al., 2009).

In the present work, we aim at the synthesis of Zinc oxide nanoparticles using *Bacillus sp*. The synthesized Zinc Oxide nanoparticles were characterized using X-ray diffractometer for analysing the nature of the nanoparticles and Scanning electron Microscope for analysing the morphology of the nanoparticles. The bactericidal activity of Zinc oxide nanoparticles was carried out using disc diffusion method.

Materials and Methods

Isolation and Identification of Bacteria

The soil sample was collected from agricultural land using spatula. Isolation of bacteria was carried out using Serial dilution technique. The morphological and physiological characterization of the isolate was

Scanned with CamScanner

performed according to the methods described in Bergey's Manual of determinative bacteriology. The isolated bacteria was identified using gram's staining and various biochemical reactions. The obtained results were compared with Bergy's Manual of determinative bacteriology.

Preparation of Zinc oxide Nanoparticles using *Bacillus cereus*

The 24 hours *Bacillus cereus* culture was inoculated in Nutrient broth and incubated for 24 hours in orbital shaker at room temperature. After 24 hours of incubation the culture was centrifuged and the supernatant was collected in conical flask. The extracellular aqueous media was used for the synthesis of Zinc oxide nanoparticles extra cellulary. 1mM of Zinc oxide was added to the aqueous media, and then the flask was incubated at room temperature in orbital shaker for 24 hours. After 24 hrs the reaction mixture was centrifuged for 15 min at 5000 rpm, and the obtained pelletwas dried at 60 °C for 6 hrs. The dried pellet powder was used for the characterization studies.

Characterization of biosynthesized Zinc oxide nanoparticles

The air dried zinc oxide nanoparticle sample was used for characterization technique. Powder X-Ray diffractometer used to analyze the nature of the nanoparticles. Scanning electron Microscope is used to identify the morphology of the synthesized zinc oxide nanoparticles.

Antibacterial activity

The antibacterial activity of the ZnO nanoparticles was examined against *Escherichia coli*.Disc diffusion method was used for examine the antibacterial activity of synthesized ZnO nanoparticles. The disc was prepared using whatman No.1 filter paper. Different concentrations of ZnO nanoparticles (10 µl to 50 µl) were used to examine the antibacterial activity of ZnO nanoparticles. The different concentrations of colloidal ZnO nanoparticles were poured on disc separately. The 24 hrs of old *E.coli*culture was swabbed on Muller Hinton agar plate and the discs were placed on the culture swabbed plate. The nanoparticles free disc was used as a control. The plates were incubated at room temperature and the susceptibility of the tested organisms was observed by Zone of inhibition (Parthasarathy et *al.,* 2017).

Results and Discussion

Identification of Bacterial culture

The isolated microorganisms were examined using various staining, cultural and biochemical test for identification. On growth media the isolated organism is abundant and white colony, highly motile. Gram's

staining gives a result, as gram positive, rod shape organism. The obtained characteristics described that the isolated organism is to be *Bacillus cereus.*

Extracellular synthesis of Zinc oxide Nanoparticles

The visual observation of colour formation is the preliminary confirmation for the synthesis of Zinc oxide nanoparticles. Figure 1 (A) shows the extracellular culture is yellow color before the addition of zinc oxide, (B) shows after the addition of zinc oxide, the extracellular culture color was changed to white and (C) Formation of white precipitate after 24 hours of reaction. Jayaseelan et al (2012) synthesized zinc oxide using *Aeromonashydrophila*, and obtained the similar changes during the synthesis of zinc oxide nanoparticles.

X-ray diffractometer

Figure 2, shows the XRD pattern of the synthesizedZnO nanoparticles. The peaks diffractions from various planes correspond to hexagonal close packed structure of ZnO. The peaks at 2θ = 31.73º, 33.02 º, 36.19 º, 47.73 º, 56.57 º, 59.05 º, 67.85 º are allotted to (100), (002), (101), (102), (110), (103), (112).The crystalline size of the particles was determined using Debye-Scherrer equation. The size of the synthesized ZnO was calculated using three main diffraction peaks by scherrer formula and the average crystalline size of the zinc Oxide nanoparticle was 110 nm (Moghaddamet *al.,* 2017).

Scanning Electron Microscope

The SEM image (Figure 3) shows the spherical shaped agglomerated ZnO nanoparticles synthesized extra cellularly using *Bacillus cereus* culture. The SEM image was taken at x10, 000 magnification. The shape of the biosynthesized ZnO nanoparticle is spherical. Jayaseelanet *al* (2012) synthesized zinc oxide using *Aeromonashydrophila*. They obtained spherical zno nanoparticles.

Antibacterial activity of ZnO nanoparticles

Disc diffusion method was used to provide evidence for the antibacterial activity of biosynthesized ZnO nanoparticles against *E.coli*.The inhibitory effect of various concentrations of ZnO nanoparticles (10µl-50µl) was examined by the zone of inhibition. Figure 4 shows the antibacterial effect of ZnO nanoparticles against *E.coli*on Muller Hinton agar medium. The minimum inhibitory concentration of growth was obtained at 50 µl. Figure 4shows the antibacterial effect of ZnO nanoparticles synthesized using *Bacillus cereus* against *E.coli* on Muller Hinton agar medium. The mechanism is not recognized for the antibacterial activity of nanoparticle

Scanned with CamScanner

but some researchers explained that nanoparticles may bind on the surface of the bacterial cell membrane and leads to cell death (Parthasarathyet al., 2015)

Table 1: Cultural and Biochemical Characteristics Analysis of the Isolate

S.No.	Biochemical Tests	*Bacillus cereus*
1	Gram staining	Gram positive, Rod
2	Agar slant	Abundant, white growth
3	Motility	Positive
4	Spore staining	Positive
5	Indole	Negative
6	Methyl red	Negative
7	VogesProskauer	Positive
8	Citrate Utilization	Positive
9	Starch hydrolysis	Positive
10	Oxidase	Negative
11	Catalase	Positive

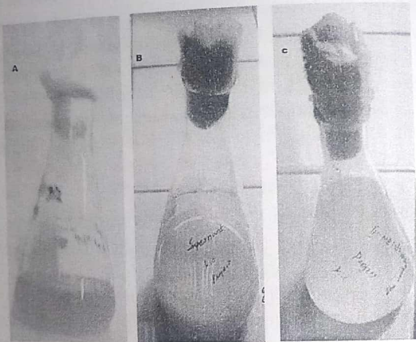


Figure 1 (A) shows the extracellular culture is yellow color before the addition of zinc oxide, (B) shows after the addition of zinc oxide, the extracellular culture colour changed to white and (C) formation of white precipitate after 24 hours of reaction

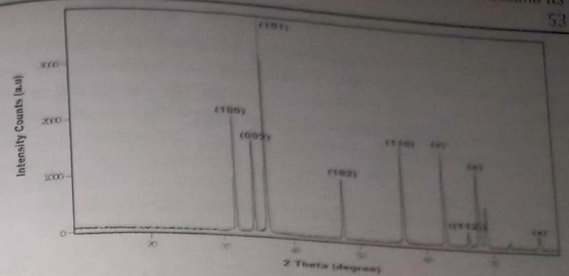


Figure 2: XRD pattern of the biosynthesized ZnO nanoparticles

Figure 3: Scanning Electron Microscope image of synthesized ZnO nanoparticles

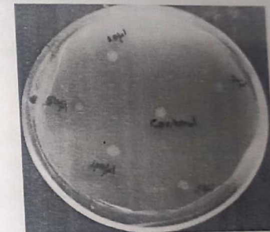


Figure 4 shows the antibacterial effect of ZnO nanoparticles synthesized using *Bacillus cereus* against *E.coli* on Muller Hinton agar medium

Scanned with CamScanner

Conclusion

The microbial mediated synthesis of nanomaterials has recently been recognized as a promising source for mining nanomaterials. The newly identified *Bacillus cereus* efficiently synthesized ZnO nanoparticles was confirmed by X-ray diffractometer and Scanning electron microscope. The X-ray diffractometer result confirmed that the synthesized Zinc oxide nanoparticles were crystalline in nature. The scanning electron microscopy showed the spherical shape of theZnO nanoparticles. The synthesized ZnO nanoparticles showed efficient antibacterial activity against *E.coli*.

References:

[1]. Bhima BV, Nath N, Sinha P. Characterization and antibacterial analysis of silver nanoparticles synthesized by the marine fungi Hypoceralixii MV1 isolated from mangrove sediment soil. Journal of Pharmac Research 2011; 4: 477-479.

[2]. Chokriwal A, Sharma MM, Sigh A. Biological synthesis of Nanoparticles using Bacteria and their Applications. American Journal of Pharmtech Research 2014; 4: 39-61

[3]. Gade A, Ingle A, Whiteley C, Rai M. Mycogenic Metal Nanoparticles: Progress and Applications. BiotechnolLett 2011; 32: 593–600.

[4]. Kathirvelu S, Souza LD, Dhurai B. UV protection finishing of textiles using ZnO nanoparticles. Indian Journal of Fibre & Textile Research 2009;34: 267-273.

[5]. Manokari M, Shekhawat MS. Production of Zinc oxide nanoparticles using extracts of passifloraedulis Sims f.flavicarpa Deg. World Scientific News 2016; 47: 267-278.

[6]. McNeil SE. Nanotechnology for biologist. Journal of Leukocyte Biology 2005; 78: 585-594.

[7]. Moghaddam AB, Moniri M, Azizi S, Rahim RA, Ariff AB, Saad WZ, Namvar F, Navaderi M, Mohamad R. Biosynthesis of ZnO nanoparticles by a new PichiaKudriavzevii Yeast strain and evalution of their antimicrobial and antioxidant activities. Molecules 2017; 872:1-18.

[8]. Musarrat J, Dwivedi S, Singh BR, Saquib Q, Khedhairy AA. Microbially synthesized nanoparticles: Scope and applications. Springer-Verlag Berlin Heidelberg, Germany 2011; DOI 10.1007/978-1-4419-7931-5

[9]. Parthasarathy G, Saroja M, Venkatachalam M, Evanjelene VK. Biological Synthesis of Zinc oxide nanoparticles from leaf extract of Curcuma neilgherrensis Wight. International Journal of Materials Science 2017; 12:73-86.

[10]. Parthasarathy G, Saroja M, Venkatachalam M. Biosynthesized nano-formulation of zinc oxide-aloe vera and to study their characterization and antibacterial activities against multiple pathogens. International Journal of Pharmaceutical Sciences and Research 2017; DOI: 10.13040/IJPSR.0975-8232.8 (2).900-07

[11]. Shah M, Fawcett D, Sharma S, Tripathy SK, Poinern GEJ. Green synthesis of Metallic Nanoparticles via Biological Entities. Materials 2015; 8: 7278-7308.

[12]. Xie J, Lee JY, Wang DIC, Ting YP. Identification of Active Biomolecules in the High Yield Synthesis of Single Crystalline Gold Nanoplates in Algal Solutions. Small 2011; 3: 672- 682.

[13]. Yadav A, Prasad V, Kathe AA, Raj S., Yadav D, undaramoorthy C, Vigneshwaran N. Functional finishing in cotton fabrics using zinc oxide nanoparticles. Bulltein Material Science 2006; 29: 641-645.

Scanned with CamScanner

BIOMEDICAL POTENTIAL OF MEDICINAL PLANTS EXTRACTS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

M.Manohar[a*]., P.Pandiselvi[a], Dr.R.Jannet Rani[a], K,Chitra[a] and Feroz Khan[a]
PG and Research Department of Microbiology,
Sadakathullah Appa College (Autonomous) Tirunelveli – 627006
***Corresponding Author:** gansun1@gmail.com

Abstract - The major problem existing in the field of medicine is drug resistance. The organisms were developing resistance to almost all the existing drugs. Among such organisms MRSA pose a major threat. The prevalence of MRSA infection is also increasing steadily. So we are in need of alternative therapy to cure such infections. One such remedy could be the use of extracts from medicinal plants. In the present study we used the extracts of some herbal plants such as Punica granatum, Solanum trilobatum, Ocimum tenuiflorum, Ripes uva-crispa, Psidium guajava, Cajanus cajan, Euphorbia hirta, Phyllanthus niruri, Phyllanthus amarus against MRSA. Among these plant methanol extract of P. granatum (inhibition zone 20mm) showed remarkable results against MRSA. We also checked the synergistic effect of some antibiotics with Punica granatum. They showed synergistic effect with Amoxicillin Cephalothin and Cefepime.

Keywords: MRSA, Solanum trilobatum, Psidium guajava. Antibacterial activity

INTRODUCTION

All over the ages, humans have relied on nature for their basic needs, such as food, shelter, clothing, transportation, fertilizers, flavors , medicine etc, (Critchley, A.T. (1983).). For thousands of years, plants formed the basis of traditional medicine systems that have been existing and continue with new remedies to mankind. Eventhough some of the therapeutic properties of the plants have been proven. It is based on the empirical findings of thousands of years (Heinrich *et al*., 2004). Various types of plants used in herbalism and many of these plants have medicinal properties. 3.3 billion Peoples in the developing countries utilize the medicinal plants which are the "backbone" of traditional medicine (Davidson-Hunt I *et al*., 2000).

Fruits and vegetables are most popular due to their dietetic value worldwide and rich sources of beneficial vitamins and fibers, anti-oxidants, minerals. The standard consumption of fresh fruits and vegetables may diminish the risk of cardiovascular diseases, stroke and certain cancers. Usually fruits are processed into beverage, squash juice and syrups. However by-products can be used as functional food ingredients such as phytochemicals, pharmaceuticals, essential oils, seed oil, pectin, food products, and dietary fibers (Azad *et al*., 2014). Therefore, fruits by-products not only superior source of bioactive compounds but also could be used as several value-added products (Noor *et al*., 2014).

In conventional chemistry and pharmacology plants might provide a useful source for the production of new effective medicines and this may be used to replace existing drugs . In general traditional medicine is turned out to be very useful in the discovery of natural products such as pharmaceutical drugs (Patwardhan *et al*., 2004). The shrub *Prosopsis juliflora* (Sw.) DC. (*P. juliflora*) commonly identified as mesquite has been used as a traditional medicine on various continents for curing catarrh, inflammation, colds, sore throat, excrescences, flu, measles, diarrhea, dysentery and hoarseness, for the healing of wounds (Hartwell *et al*., 1971).

METHODOLOGY

Isolation of *Staphylococcus aureus*

For the isolation of *Staphylococcus aureus*, pus samples were collected from wounds. The specimens were collected from Bose Clinical Lab, Madurai. Fluid Thioglycollate medium was used as an enriched medium for *Staphylococcus aureus*. The specimen was inoculated on Mannitol salt agar, Mac Conkey agar and Blood agar plates. The plates were incubated aerobically at 37^0C overnight. The test organisms further confirmed by their physiological and biochemical characters.

Detection of MRSA

Disk Susceptibility Test (Acar and Goldstein, 1991). The Kirby-Bauer disk diffusion test was developed for this study.

The sensitivity pattern of MRSA strains to β-lactam antiboiotics

Disk susceptibility test (Acar and Goldstein., 1991).

The Kirby –Bauer disk diffusion test was developed for these studies as described above with the antibiotic disks methicillin, amoxyclav, amoxicillin, cefdin, cephalothin, cefepime, cefaclor, vancomycin and teicoplanin. The sensitivity pattern of the test organism was recorded.

Determination of the degree of methicillin resistance

The Kirby-Bauer disk diffusion test was developed for this study. MRSA strains were individually tested for their degree of methicillin resistance with methicillin 5 µg and 10 µg.

Collection of herbal plants

The following fresh plant materials and sea weeds were collected from Vilathikulam, Thoothukudi district. Plants such as *Punica granatum, Prosopis juliflora, Ocimum tenuiflorum,* and sea weeds such as *Sargassum muticum* and *Jania . rubens.*

Preparation of plant extract

Water extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of sterile distilled water. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no:1 filter paper.

Methanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of methanol. They were placed in shaker for 24 hours. The methanol extract was filtered by using Whatmann No:1 filter paper.

Ethanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of ethanol. They were placed in shaker for 24 hours. The ethanol extract was filtered by using Whatmann no:1 filter paper.

Petroleum ether

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of petroleum ether. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no: 1 filter paper.

Determination of antibacterial activities of plant extract against MRSA strain

Loading sterile disks with plant extract

Antibacterial activity was measured using disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI). Briefly petriplates containing approximately 25-30 ml of Mueller-Hinton agar medium were inoculated using a cotton swab with a 4-6 fold culture of the bacteria. (Acar and Goldstein, 1991).

Disk susceptibility test

The Kirby- Bauer disk diffusion test was developed for these studies as described above with the disks of plant extract. The sensitivity pattern of the test organism was recorded.

Determination of synergetic effect of plant extract with some antibiotics against MRSA

The Kirby- Bauer disk diffusion test was developed for these studies as described above. To access possible interactions between the extract of plant materials and β-lactam antibiotics, disk containing the extract of plant materials and β-lactam antibiotics were placed on a plate that had been inoculated with MRSA strain. The distance between the disks was equal to the sum of the radii of their zones of inhibition when examined alone.

Checkerboard method to determine the antimicrobial combinations against MRSA

The checkerboard (or chessboard) method is the technique used most frequently to assess antimicrobial combinations in vitro, presumably because its rationale is easy to understand, the mathematics necessary to calculate and interpret the results are simple, it can readily be performed in the clinical laboratory using microdilution systems that are obtain able commercially, and it has been the technique most frequently used in studies that have suggested an advantage of synergistic therapy in the treatment of neutropenic patients with Gram-negative septicemia. The term "checkerboard" refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested in concentration equal to, above, and below their minimal inhibitory concentrations for the organisms being tested.

RESULTS

Isolation and characterization of *staphylococcus aureus*

Eight numbers of MRSA strains were isolated from pus. Various media (Table 3) were used to identify the pathogenic strain. Mannitol salt agar was used as a differential and selective medium for recovering strain from the specimen. *Staphylococcus aureus* produced yellow colour colonies in Mannitol Salt agar.

Table 1.Cultural characterization of *Staphylococcus aureus*

S. No	Medium	Colony Morphology	Interpretation
1	MacConkey Agar	Pink colour colonies	Lactose fermentation positive
2	Blood Agar	Yellow to cream colonies or white colonies with clear zone	β-haemolytic colonies
3	Mannitol Salt Agar	Yellow colonies	Mannitol fermentation positive

Table.2. Degree of Methicillin Resistance of MRSA strains (Fig. 20)

S. No	Methicillin Disk Content	Zone diameter of inhibition (mm)	Reaction of the Organism
1	Methicillin (5 μg)	-	Resistant
2	Methicillin (10 μg)	-	Resistant
3	Methicillin (30 μg)	-	Resistant

Determination of antibacterial activities of plant extract against MRSA strain

The antimicrobial activity of various extracts of *Punica granatum* and *Prosopis juliflora* were tabulated (Table 7 -9). (Fig. 5-12).

Table.3. Antimicrobial activity of various extracts of *Punica granatum*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	10	15	15	_
MRSA 1	Partial	10	15	_
MRSA 2	Partial	10	20	_
MRSA 3	Partial	15	20	_
MRSA 4	10	15	21	_
MRSA 6	10	15	20	_
MRSA 7	Partial	10	15	_
MRSA 8	10	15	20	_

Table 4.Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Table 5. Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Fig. 1. ***P. granatum*** **(pomegranate)**

Fig.2. ***P. juliflora*** **(karuvelam)**

Fig. 3 ***S. muticum***

Fig. 4. ***J.rubens***

Plate 1. Synergistic effect between ***P. granatum*** **and methicillin 10 µg**

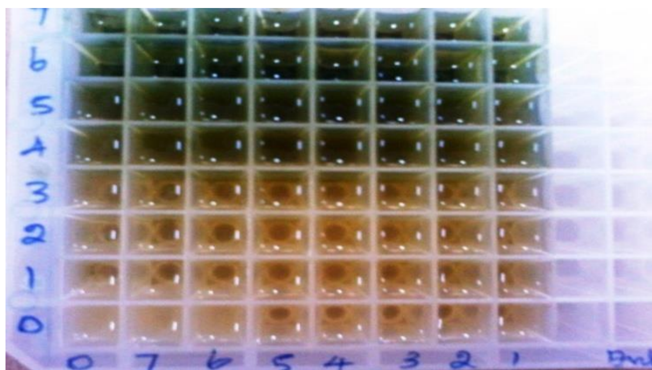
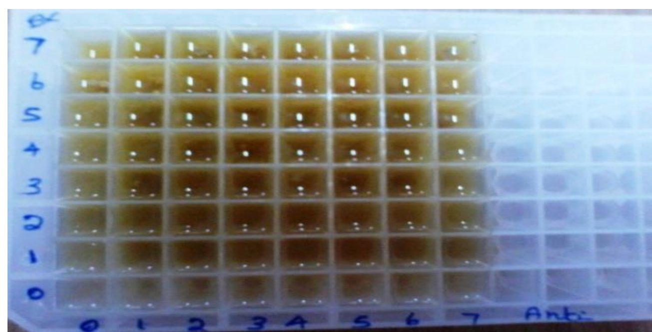
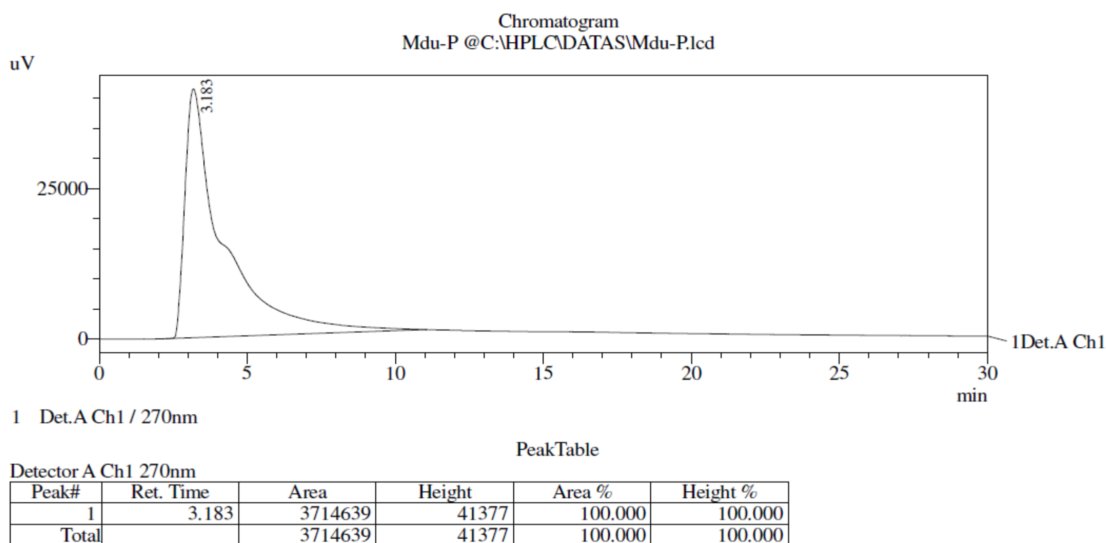


Plate 2. Synergistic of ***P.juliflora*** **with Amoxyclav**





PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.183	3714639	41377	100.000	100.000
Total		3714639	41377	100.000	100.000

Fig. 6. HPLC analysis of *P. granatum*

DISCUSSION

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years, the World Health Organization advocated that countries should interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origin (WHO. 1978).

The increasing occurrence, particularly in hospitals, of *S. aureus* resistant not only to methicillin but to a wide range of antimicrobial agents, including all kinds of β-lactams, has made therapy more difficult (Unci Ney *et al.,*2006).. Although strategies have been proposed in an attempt to control the spread, the search for new ways to treat MRSA infections stimulates the investigation of natural compounds as an alternative treatment of these infections.In the present study, the analysis of the growth inhibition activity by the disk diffusion method showed

that 10 medicinal plants (*P. juliflora*, *P. granatum*, *S.trilobatum*, *O.tenuiflorum*, *R.uva-crispa*, *P.guajava*, *C. cajan*, *E. hirta*,*P.niruri* and *P. amarus*),were active against hospital strains of MRSA under test conditions with crude extract concentrations as high as 5g/20ml.(Williams, et al.,2007)

In the present study, 2 plant extracts (*P. juliflora*, *P. granatum*) were effective against MRSA, MRSA1, MRSA2, MRSA 3, MRSA 4, MRSA 6, MRSA 7, MRSA 8 strains. We were using water, ethanol, methanol, petroleum ether extracts for all the 8 strains. Methanol extract inhibit the growth of 8strains of MRSA. The synergistic method were also performed for the methonal extracts with 10 antibiotics of methicillin (5µg), methicillin (10µg), amoxyclav (30 µg 10 µg), amoxicillin (30 µg 10µg), cefdin (5µg), cephalothin (30µg), cefepime (30µg), cefaclor (30µg), vancomycin (30 µg) and teicoplanin (30µg) in MRSA strain.

References:

1. Critchley, A.T. (1983). *Sargassum muticum*: a taxonomic history including world-wide and western pacific distributions. *Journal of the Marine Biological Association of the United Kingdom* 63: 617–625.
2. Davidson-Hunt. I. (2000). Ecological ethno botany: stumbling toward new practices and paradigms. MASA J 16:1–13, 2000.
3. Azad, A.K.M., M. A. Ali, M.S. Akter, M.J. Rahman and M. Maruf Ahmed. (2014). Isolation and Characterization of Pectin Extracted from Lemon Pomace during Ripening. *Journal of Food and Nutrition Sciences* Vol. 2, No. 2, pp. 30-35.
4. Noor, F., M.J. Rahman, M.S. Mahomud, M.S. Akter, M.A.I. Md. Aminul Islam Talukder and M. Ahmed. (2014). Physicochemical Properties of Flour and Extraction of Starch from Jackfruit Seed. *International Journal of Nutrition and Food Sciences* Vol. 3, No. 4, pp. 347-354.
5. Patwardhan, B., A. D. Vaidya and M. Chorghade. (2004). Ayurveda and natural products drug discovery. *Curr Sci* 86: 789-799.
6. Hartwell, J.L. (1971). Plants used against cancer. A survey. *Lloydia* 34: 386-425.
7. Acar, J.F. and F. W. Goldstein, (1991). Disk Susceptibility Test. In: Lorian V. (Ed). Antibiotics in Laboratory Medicine. Williams and Wilkins, London.
8. WHO. (2002). Traditional medicine. Report, EB111/9, World Health Organization, Geneva.
9. Williams, S. L. and J. E. Smith. (2007). A Global Review of the Distribution, Taxonomy, and Impacts of Introduced Seaweeds. *The Annual Review of Ecology, Evolution and Systematics*, 38, 327-59.
10. Unci Ney, J.Y., W.Y. Shieh, Jeng and W.L. Huang. (2006). Antibiotic activity of lectins from marine algae against marine Vibrio. *Journal of International Microbiology and Biotechnology* 30: 433-439.

Biological Synthesis of Zinc Oxide Nanoparticles Using *Bacillus Cereus* and Its Antibacterial Activity

K. Chitra[1], Dr. R. Janet Rani[2], Dr. M.M. Abdul Kader Mohideen[3] and S. Peer Mohamed[4]

Abstract

Today the development of reliable, eco-friendly processes for the synthesis of nanomaterials is an important aspect of nanotechnology.In this work we have developed Zinc Oxide (ZnO) nanoparticles using Bacillus cereusculture.ZnO is the metal oxide nanoparticles, ZnO nanoparticle is non-toxic. The Preliminary confirmation for the ZnO nanoparticles synthesis is the formation of White precipitate in the reaction mixture. The wurtzite crystalline structure of the ZnO nanoparticles is identified by X-ray diffractometer (XRD).The Scanning Electron microscope (SEM) showed the spherical shaped ZnO nanoparticles synthesized using Bacillus cereus culture.The antibacterial effect of ZnO nanoparticles synthesized using Bacillus cereus was examined against Escherichia coli (E.coli). Disc diffusion method is used to examine the antibacterial activity of ZnO nanoparticles.The minimum inhibitory concentration is 50 µl of ZnO nanoparticles against E.coli. The synthesized ZnO nanoparticles have the ability to kill the bacteria. Thus ZnO could be used in various clinical applications.

Keywords: *Zinc oxide nanoparticles, Bacillus cereus, XRD, Antibacterial activity*

Introduction

Nanotechnology has gained attraction in recent years,because of the controlled synthesis of the materials (less than 100 nm in size). Nanomaterial is similar to naturally occurring proteins and biomolecules in

---

1 Assistant Professor, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.
2 Assistant Professor and Head, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.
3 Assistant Professor, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.
4 Assistant Professor, Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli, Tamil Nadu, India.

the cell due to its ultra- small size (McNeil, 2005; Manokari et al., 2016). There are various chemical and physical methods used for the synthesis of nanoparticles. There are several demerits in the synthesis of nanoparticles using physical and chemical methods such as high energy requirements in physical methods, the use of organic solvents, toxic reducing agents and capping agents in chemical method and both the methods are costly and produce toxic byproducts. Thus, there is a need to develop a clean, biocompatible, nonhazardous, inexpensive, energy efficient eco-friendly method (Chokriwal et al., 2014; Gade et al., 2011; Xie et al., 2011; Musarrat et al., 2011).

An alternative method to synthesis nanoparticles by biological origin has been developed in recent years. The unicellular and multicellular organisms such as actinomycetes, bacteria, fungus, plants, viruses and yeast are involved in the synthesis of nanoparticles. The synthesis of nanoparticles using biological origin provides a clean, non-toxic and environment-friendly method to fabricate nanoparticles (Shah *et al*., 2015). Recently, microorganisms such as bacteria and fungi are considered interesting nanofactories because microorganisms such as bacteria and fungi play an important role in remediation of toxic metals through reduction of the metal ions (Bhima et al., 2010).

Due to low cost, metal oxide nanoparticles such as Zinc Oxide and titanium oxide are more preferable than silver nanoparticles. Owing to its unique photo-catalytic, electrical, electronic, optical, dermatological and antibacterial properties, zinc oxide has a wide range of applications. Zinc oxide is a semiconductor with a direct wide band gap and large excitation. Binding energy, piezoelectric, biosafe, are the three key characters that make the possibility of using Zinc oxide in many fields (Yadav et al., 2006; Kathirvelu et al., 2009).

In the present work, we aim at the synthesis of Zinc oxide nanoparticles using *Bacillus sp*. The synthesized Zinc Oxide nanoparticles were characterized using X-ray diffractometer for analysing the nature of the nanoparticles and Scanning electron Microscope for analysing the morphology of the nanoparticles. The bactericidal activity of Zinc oxide nanoparticles was carried out using disc diffusion method.

Materials and Methods

Isolation and Identification of Bacteria

The soil sample was collected from agricultural land using spatula. Isolation of bacteria was carried out using Serial dilution technique. The morphological and physiological characterization of the isolate was

performed according to the methods described in Bergey's Manual of determinative bacteriology. The isolated bacteria was identified using gram's staining and various biochemical reactions. The obtained results were compared with Bergy's Manual of determinative bacteriology.

Preparation of Zinc oxide Nanoparticles using *Bacillus cereus*

The 24 hours *Bacillus cereus* culture was inoculated in Nutrient broth and incubated for 24 hours in orbital shaker at room temperature. After 24 hours of incubation the culture was centrifuged and the supernatant was collected in conical flask. The extracellular aqueous media was used for the synthesis of Zinc oxide nanoparticles extra cellulary. 1mM of Zinc oxide was added to the aqueous media, and then the flask was incubated at room temperature in orbital shaker for 24 hours. After 24 hrs the reaction mixture was centrifuged for 15 min at 5000 rpm, and the obtained pelletwas dried at 60 °C for 6 hrs. The dried pellet powder was used for the characterization studies.

Characterization of biosynthesized Zinc oxide nanoparticles

The air dried zinc oxide nanoparticle sample was used for characterization technique. Powder X-Ray diffractometer used to analyze the nature of the nanoparticles. Scanning electron Microscope is used to identify the morphology of the synthesized zinc oxide nanoparticles.

Antibacterial activity

The antibacterial activity of the ZnO nanoparticles was examined against *Escherichia coli.*Disc diffusion method was used for examine the antibacterial activity of synthesized ZnO nanoparticles. The disc was prepared using whatman No.1 filter paper. Different concentrations of ZnO nanoparticles (10 µl to 50 µl) were used to examine the antibacterial activity of ZnO nanoparticles. The different concentrations of colloidal ZnO nanoparticles were poured on disc separately. The 24 hrs of old *E.coli*culture was swabbed on Muller Hinton agar plate and the discs were placed on the culture swabbed plate. The nanoparticles free disc was used as a control. The plates were incubated at room temperature and the susceptibility of the tested organisms was observed by Zone of inhibition (Parthasarathy et *al.,* 2017).

Results and Discussion

Identification of Bacterial culture

The isolated microorganisms were examined using various staining, cultural and biochemical test for identification. On growth media the isolated organism is abundant and white colony, highly motile. Gram's

staining gives a result, as gram positive, rod shape organism. The obtained characteristics described that the isolated organism is to be *Bacillus cereus.*

Extracellular synthesis of Zinc oxide Nanoparticles

The visual observation of colour formation is the preliminary confirmation for the synthesis of Zinc oxide nanoparticles. Figure 1 (A) shows the extracellular culture is yellow color before the addition of zinc oxide, (B) shows after the addition of zinc oxide, the extracellular culture color was changed to white and (C) Formation of white precipitate after 24 hours of reaction. Jayaseelan et al (2012) synthesized zinc oxide using *Aeromonashydrophila*, and obtained the similar changes during the synthesis of zinc oxide nanoparticles.

X-ray diffractometer

Figure 2, shows the XRD pattern of the synthesizedZnO nanoparticles. The peaks diffractions from various planes correspond to hexagonal close packed structure of ZnO. The peaks at 2θ = 31.73º, 33.02 º, 36.19 º, 47.73 º, 56.57 º, 59.05 º, 67.85 º are allotted to (100), (002), (101), (102), (110), (103), (112).The crystalline size of the particles was determined using Debye-Scherrer equation. The size of the synthesized ZnO was calculated using three main diffraction peaks by scherrer formula and the average crystalline size of the zinc Oxide nanoparticle was 110 nm (Moghaddamet *al.,* 2017).

Scanning Electron Microscope

The SEM image (Figure 3) shows the spherical shaped agglomerated ZnO nanoparticles synthesized extra cellularly using *Bacillus cereus* culture. The SEM image was taken at x10, 000 magnification. The shape of the biosynthesized ZnO nanoparticle is spherical. Jayaseelanet *al* (2012) synthesized zinc oxide using *Aeromonashydrophila*. They obtained spherical zno nanoparticles.

Antibacterial activity of ZnO nanoparticles

Disc diffusion method was used to provide evidence for the antibacterial activity of biosynthesized ZnO nanoparticles against *E.coli.*The inhibitory effect of various concentrations of ZnO nanoparticles (10µl-50µl) was examined by the zone of inhibition. Figure 4 shows the antibacterial effect of ZnO nanoparticles against *E.coli*on Muller Hinton agar medium. The minimum inhibitory concentration of growth was obtained at 50 µl. Figure 4shows the antibacterial effect of ZnO nanoparticles synthesized using *Bacillus cereus* against *E.coli* on Muller Hinton agar medium. The mechanism is not recognized for the antibacterial activity of nanoparticle

Scanned with CamScanner

but some researchers explained that nanoparticles may bind on the surface of the bacterial cell membrane and leads to cell death (Parthasarathyet al., 2015)

Table 1: Cultural and Biochemical Characteristics Analysis of the Isolate

S.No.	Biochemical Tests	*Bacillus cereus*
1	Gram staining	Gram positive, Rod
2	Agar slant	Abundant, white growth
3	Motility	Positive
4	Spore staining	Positive
5	Indole	Negative
6	Methyl red	Negative
7	VogesProskauer	Positive
8	Citrate Utilization	Positive
9	Starch hydrolysis	Positive
10	Oxidase	Negative
11	Catalase	Positive

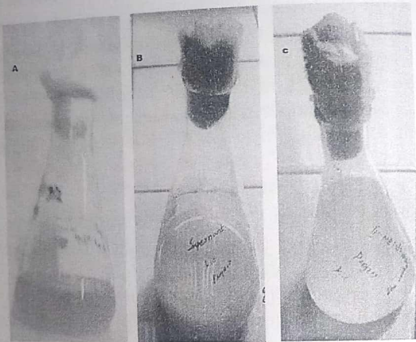


Figure 1 (A) shows the extracellular culture is yellow color before the addition of zinc oxide, (B) shows after the addition of zinc oxide, the extracellular culture colour changed to white and (C) formation of white precipitate after 24 hours of reaction

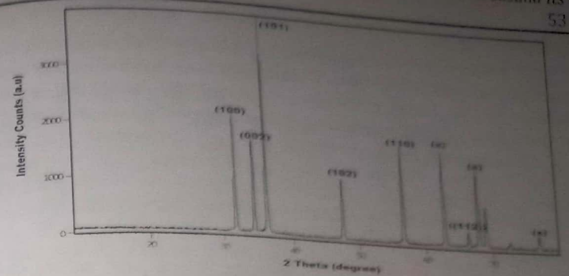


Figure 2: XRD pattern of the biosynthesized ZnO nanoparticles

Figure 3: Scanning Electron Microscope image of synthesized ZnO nanoparticles

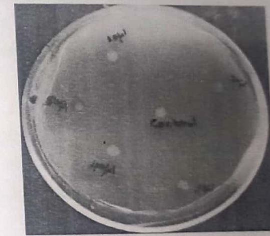


Figure 4 shows the antibacterial effect of ZnO nanoparticles synthesized using *Bacillus cereus* against *E.coli* on Muller Hinton agar medium

Scanned with CamScanner

Conclusion

The microbial mediated synthesis of nanomaterials has recently been recognized as a promising source for mining nanomaterials. The newly identified *Bacillus cereus* efficiently synthesized ZnO nanoparticles was confirmed by X-ray diffractometer and Scanning electron microscope. The X-ray diffractometer result confirmed that the synthesized Zinc oxide nanoparticles were crystalline in nature. The scanning electron microscopy showed the spherical shape of theZnO nanoparticles. The synthesized ZnO nanoparticles showed efficient antibacterial activity against *E.coli*.

References:

[1]. Bhima BV, Nath N, Sinha P. Characterization and antibacterial analysis of silver nanoparticles synthesized by the marine fungi Hypoceralixii MV1 isolated from mangrove sediment soil. Journal of Pharmac Research 2011; 4: 477-479.

[2]. Chokriwal A, Sharma MM, Sigh A. Biological synthesis of Nanoparticles using Bacteria and their Applications. American Journal of Pharmtech Research 2014; 4: 39-61

[3]. Gade A, Ingle A, Whiteley C, Rai M. Mycogenic Metal Nanoparticles: Progress and Applications. BiotechnolLett 2011; 32: 593–600.

[4]. Kathirvelu S, Souza LD, Dhurai B. UV protection finishing of textiles using ZnO nanoparticles. Indian Journal of Fibre & Textile Research 2009;34: 267-273.

[5]. Manokari M, Shekhawat MS. Production of Zinc oxide nanoparticles using extracts of passifloraedulis Sims f.flavicarpa Deg. World Scientific News 2016; 47: 267-278.

[6]. McNeil SE. Nanotechnology for biologist. Journal of Leukocyte Biology 2005; 78: 585-594.

[7]. Moghaddam AB, Moniri M, Azizi S, Rahim RA, Ariff AB, Saad WZ, Namvar F, Navaderi M, Mohamad R. Biosynthesis of ZnO nanoparticles by a new PichiaKudriavzevii Yeast strain and evalution of their antimicrobial and antioxidant activities. Molecules 2017; 872:1-18.

[8]. Musarrat J, Dwivedi S, Singh BR, Saquib Q, Khedhairy AA. Microbially synthesized nanoparticles: Scope and applications. Springer-Verlag Berlin Heidelberg, Germany 2011; DOI 10.1007/978-1-4419-7931-5

[9]. Parthasarathy G, Saroja M, Venkatachalam M, Evanjelene VK. Biological Synthesis of Zinc oxide nanoparticles from leaf extract of Curcuma neilgherrensis Wight. International Journal of Materials Science 2017; 12:73-86.

[10]. Parthasarathy G, Saroja M, Venkatachalam M. Biosynthesized nano-formulation of zinc oxide-aloe vera and to study their characterization and antibacterial activities against multiple pathogens. International Journal of Pharmaceutical Sciences and Research 2017; DOI: 10.13040/IJPSR.0975-8232.8 (2).900-07

[11]. Shah M, Fawcett D, Sharma S, Tripathy SK, Poinern GEJ. Green synthesis of Metallic Nanoparticles via Biological Entities. Materials 2015; 8: 7278-7308.

[12]. Xie J, Lee JY, Wang DIC, Ting YP. Identification of Active Biomolecules in the High Yield Synthesis of Single Crystalline Gold Nanoplates in Algal Solutions. Small 2011; 3: 672- 682.

[13]. Yadav A, Prasad V, Kathe AA, Raj S., Yadav D, undaramoorthy C, Vigneshwaran N. Functional finishing in cotton fabrics using zinc oxide nanoparticles. Bulltein Material Science 2006; 29: 641-645.

Scanned with CamScanner

BIOMEDICAL POTENTIAL OF MEDICINAL PLANTS EXTRACTS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

M.Manohar[a*]., P.Pandiselvi[a], Dr.R.Jannet Rani[a], K,Chitra[a] and Feroz Khan[a]
PG and Research Department of Microbiology,
Sadakathullah Appa College (Autonomous) Tirunelveli – 627006
***Corresponding Author:** gansun1@gmail.com

Abstract - The major problem existing in the field of medicine is drug resistance. The organisms were developing resistance to almost all the existing drugs. Among such organisms MRSA pose a major threat. The prevalence of MRSA infection is also increasing steadily. So we are in need of alternative therapy to cure such infections. One such remedy could be the use of extracts from medicinal plants. In the present study we used the extracts of some herbal plants such as Punica granatum, Solanum trilobatum, Ocimum tenuiflorum, Ripes uva-crispa, Psidium guajava, Cajanus cajan, Euphorbia hirta, Phyllanthus niruri, Phyllanthus amarus against MRSA. Among these plant methanol extract of P. granatum (inhibition zone 20mm) showed remarkable results against MRSA. We also checked the synergistic effect of some antibiotics with Punica granatum. They showed synergistic effect with Amoxicillin Cephalothin and Cefepime.

Keywords: MRSA, Solanum trilobatum, Psidium guajava. Antibacterial activity

INTRODUCTION

All over the ages, humans have relied on nature for their basic needs, such as food, shelter, clothing, transportation, fertilizers, flavors , medicine etc, (Critchley, A.T. (1983).). For thousands of years, plants formed the basis of traditional medicine systems that have been existing and continue with new remedies to mankind. Eventhough some of the therapeutic properties of the plants have been proven. It is based on the empirical findings of thousands of years (Heinrich *et al*., 2004). Various types of plants used in herbalism and many of these plants have medicinal properties. 3.3 billion Peoples in the developing countries utilize the medicinal plants which are the "backbone" of traditional medicine (Davidson-Hunt I *et al*., 2000).

Fruits and vegetables are most popular due to their dietetic value worldwide and rich sources of beneficial vitamins and fibers, anti-oxidants, minerals. The standard consumption of fresh fruits and vegetables may diminish the risk of cardiovascular diseases, stroke and certain cancers. Usually fruits are processed into beverage, squash juice and syrups. However by-products can be used as functional food ingredients such as phytochemicals, pharmaceuticals, essential oils, seed oil, pectin, food products, and dietary fibers (Azad *et al*., 2014). Therefore, fruits by-products not only superior source of bioactive compounds but also could be used as several value-added products (Noor *et al*., 2014).

In conventional chemistry and pharmacology plants might provide a useful source for the production of new effective medicines and this may be used to replace existing drugs . In general traditional medicine is turned out to be very useful in the discovery of natural products such as pharmaceutical drugs (Patwardhan *et al*., 2004). The shrub *Prosopsis juliflora* (Sw.) DC. (*P. juliflora*) commonly identified as mesquite has been used as a traditional medicine on various continents for curing catarrh, inflammation, colds, sore throat, excrescences, flu, measles, diarrhea, dysentery and hoarseness, for the healing of wounds (Hartwell *et al*., 1971).

METHODOLOGY

Isolation of *Staphylococcus aureus*

For the isolation of *Staphylococcus aureus*, pus samples were collected from wounds. The specimens were collected from Bose Clinical Lab, Madurai. Fluid Thioglycollate medium was used as an enriched medium for *Staphylococcus aureus*. The specimen was inoculated on Mannitol salt agar, Mac Conkey agar and Blood agar plates. The plates were incubated aerobically at 37^{0}C overnight. The test organisms further confirmed by their physiological and biochemical characters.

Detection of MRSA

Disk Susceptibility Test (Acar and Goldstein, 1991). The Kirby-Bauer disk diffusion test was developed for this study.

The sensitivity pattern of MRSA strains to β-lactam antiboiotics

Disk susceptibility test (Acar and Goldstein., 1991).

The Kirby –Bauer disk diffusion test was developed for these studies as described above with the antibiotic disks methicillin, amoxyclav, amoxicillin, cefdin, cephalothin, cefepime, cefaclor, vancomycin and teicoplanin. The sensitivity pattern of the test organism was recorded.

Determination of the degree of methicillin resistance

The Kirby-Bauer disk diffusion test was developed for this study. MRSA strains were individually tested for their degree of methicillin resistance with methicillin 5 µg and 10 µg.

Collection of herbal plants

The following fresh plant materials and sea weeds were collected from Vilathikulam, Thoothukudi district. Plants such as *Punica granatum, Prosopis juliflora, Ocimum tenuiflorum,* and sea weeds such as *Sargassum muticum* and *Jania . rubens.*

Preparation of plant extract

Water extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of sterile distilled water. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no:1 filter paper.

Methanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of methanol. They were placed in shaker for 24 hours. The methanol extract was filtered by using Whatmann No:1 filter paper.

Ethanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of ethanol. They were placed in shaker for 24 hours. The ethanol extract was filtered by using Whatmann no:1 filter paper.

Petroleum ether

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of petroleum ether. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no: 1 filter paper.

Determination of antibacterial activities of plant extract against MRSA strain

Loading sterile disks with plant extract

Antibacterial activity was measured using disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI). Briefly petriplates containing approximately 25-30 ml of Mueller-Hinton agar medium were inoculated using a cotton swab with a 4-6 fold culture of the bacteria. (Acar and Goldstein, 1991).

Disk susceptibility test

The Kirby- Bauer disk diffusion test was developed for these studies as described above with the disks of plant extract. The sensitivity pattern of the test organism was recorded.

Determination of synergetic effect of plant extract with some antibiotics against MRSA

The Kirby- Bauer disk diffusion test was developed for these studies as described above. To access possible interactions between the extract of plant materials and β-lactam antibiotics, disk containing the extract of plant materials and β-lactam antibiotics were placed on a plate that had been inoculated with MRSA strain. The distance between the disks was equal to the sum of the radii of their zones of inhibition when examined alone.

Checkerboard method to determine the antimicrobial combinations against MRSA

The checkerboard (or chessboard) method is the technique used most frequently to assess antimicrobial combinations in vitro, presumably because its rationale is easy to understand, the mathematics necessary to calculate and interpret the results are simple, it can readily be performed in the clinical laboratory using microdilution systems that are obtain able commercially, and it has been the technique most frequently used in studies that have suggested an advantage of synergistic therapy in the treatment of neutropenic patients with Gram-negative septicemia. The term "checkerboard" refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested in concentration equal to, above, and below their minimal inhibitory concentrations for the organisms being tested.

RESULTS

Isolation and characterization of *staphylococcus aureus*

Eight numbers of MRSA strains were isolated from pus. Various media (Table 3) were used to identify the pathogenic strain. Mannitol salt agar was used as a differential and selective medium for recovering strain from the specimen. *Staphylococcus aureus* produced yellow colour colonies in Mannitol Salt agar.

Table 1.Cultural characterization of *Staphylococcus aureus*

S. No	Medium	Colony Morphology	Interpretation
1	MacConkey Agar	Pink colour colonies	Lactose fermentation positive
2	Blood Agar	Yellow to cream colonies or white colonies with clear zone	β-haemolytic colonies
3	Mannitol Salt Agar	Yellow colonies	Mannitol fermentation positive

Table.2. Degree of Methicillin Resistance of MRSA strains (Fig. 20)

S. No	Methicillin Disk Content	Zone diameter of inhibition (mm)	Reaction of the Organism
1	Methicillin (5 µg)	-	Resistant
2	Methicillin (10 µg)	-	Resistant
3	Methicillin (30 µg)	-	Resistant

Determination of antibacterial activities of plant extract against MRSA strain

The antimicrobial activity of various extracts of *Punica granatum* and *Prosopis juliflora* were tabulated (Table 7 -9). (Fig. 5-12).

Table.3. Antimicrobial activity of various extracts of *Punica granatum*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	10	15	15	_
MRSA 1	Partial	10	15	_
MRSA 2	Partial	10	20	_
MRSA 3	Partial	15	20	_
MRSA 4	10	15	21	_
MRSA 6	10	15	20	_
MRSA 7	Partial	10	15	_
MRSA 8	10	15	20	_

Table 4.Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Table 5. Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Fig. 1. ***P. granatum*** **(pomegranate)**

Fig.2. ***P. juliflora*** **(karuvelam)**

Fig. 3 ***S. muticum***

Fig. 4. ***J.rubens***

Plate 1. Synergistic effect between ***P. granatum*** **and methicillin 10 µg**

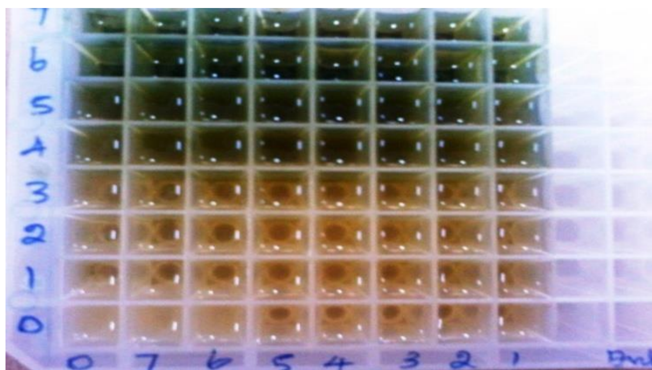
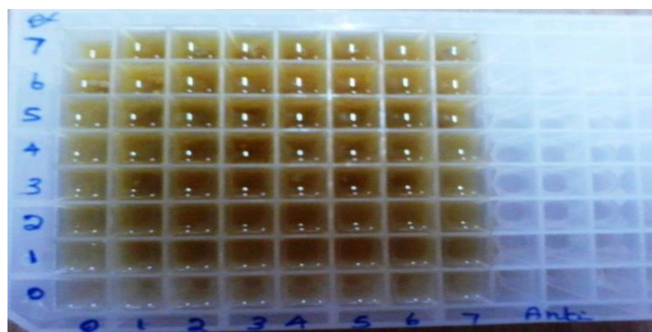
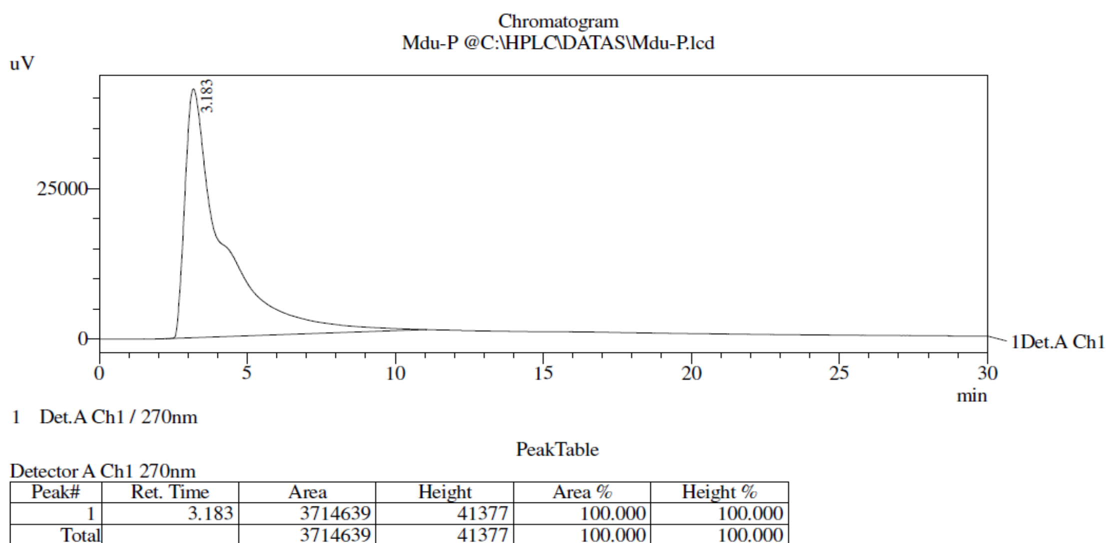


Plate 2. Synergistic of ***P.juliflora*** **with Amoxyclav**





Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.183	3714639	41377	100.000	100.000
Total		3714639	41377	100.000	100.000

Fig. 6. HPLC analysis of *P. granatum*

DISCUSSION

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years, the World Health Organization advocated that countries should interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origin (WHO. 1978).

The increasing occurrence, particularly in hospitals, of *S. aureus* resistant not only to methicillin but to a wide range of antimicrobial agents, including all kinds of β-lactams, has made therapy more difficult (Unci Ney *et al.,*2006).. Although strategies have been proposed in an attempt to control the spread, the search for new ways to treat MRSA infections stimulates the investigation of natural compounds as an alternative treatment of these infections.In the present study, the analysis of the growth inhibition activity by the disk diffusion method showed

that 10 medicinal plants (*P. juliflora*, *P. granatum*, *S.trilobatum*, *O.tenuiflorum*, *R.uva-crispa*, *P.guajava*, *C. cajan*, *E. hirta*,*P.niruri* and *P. amarus*),were active against hospital strains of MRSA under test conditions with crude extract concentrations as high as 5g/20ml.(Williams, et al.,2007)

In the present study, 2 plant extracts (*P. juliflora*, *P. granatum*) were effective against MRSA, MRSA1, MRSA2, MRSA 3, MRSA 4, MRSA 6, MRSA 7, MRSA 8 strains. We were using water, ethanol, methanol, petroleum ether extracts for all the 8 strains. Methanol extract inhibit the growth of 8strains of MRSA. The synergistic method were also performed for the methonal extracts with 10 antibiotics of methicillin (5µg), methicillin (10µg), amoxyclav (30 µg 10 µg), amoxicillin (30 µg 10µg), cefdin (5µg), cephalothin (30µg), cefepime (30µg), cefaclor (30µg), vancomycin (30 µg) and teicoplanin (30µg) in MRSA strain.

References:

1. Critchley, A.T. (1983). *Sargassum muticum*: a taxonomic history including world-wide and western pacific distributions. *Journal of the Marine Biological Association of the United Kingdom* 63: 617–625.
2. Davidson-Hunt. I. (2000). Ecological ethno botany: stumbling toward new practices and paradigms. MASA J 16:1–13, 2000.
3. Azad, A.K.M., M. A. Ali, M.S. Akter, M.J. Rahman and M. Maruf Ahmed. (2014). Isolation and Characterization of Pectin Extracted from Lemon Pomace during Ripening. *Journal of Food and Nutrition Sciences* Vol. 2, No. 2, pp. 30-35.
4. Noor, F., M.J. Rahman, M.S. Mahomud, M.S. Akter, M.A.I. Md. Aminul Islam Talukder and M. Ahmed. (2014). Physicochemical Properties of Flour and Extraction of Starch from Jackfruit Seed. *International Journal of Nutrition and Food Sciences* Vol. 3, No. 4, pp. 347-354.
5. Patwardhan, B., A. D. Vaidya and M. Chorghade. (2004). Ayurveda and natural products drug discovery. *Curr Sci* 86: 789-799.
6. Hartwell, J.L. (1971). Plants used against cancer. A survey. *Lloydia* 34: 386-425.
7. Acar, J.F. and F. W. Goldstein, (1991). Disk Susceptibility Test. In: Lorian V. (Ed). Antibiotics in Laboratory Medicine. Williams and Wilkins, London.
8. WHO. (2002). Traditional medicine. Report, EB111/9, World Health Organization, Geneva.
9. Williams, S. L. and J. E. Smith. (2007). A Global Review of the Distribution, Taxonomy, and Impacts of Introduced Seaweeds. *The Annual Review of Ecology, Evolution and Systematics*, 38, 327-59.
10. Unci Ney, J.Y., W.Y. Shieh, Jeng and W.L. Huang. (2006). Antibiotic activity of lectins from marine algae against marine Vibrio. *Journal of International Microbiology and Biotechnology* 30: 433-439.

ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/341622119

EFFICACY OF ALLIUM CEPA AND ALLIUM SATIVUM AGAINST DERMATOPHYTES

Article · May 2020

CITATION
1

READS
506

4 authors, including:

S Muthu Raj
K L University

9 PUBLICATIONS **2** CITATIONS

SEE PROFILE

All content following this page was uploaded by S Muthu Raj on 25 May 2020.

The user has requested enhancement of the downloaded file.

EFFICACY OF ALLIUM CEPA AND ALLIUM SATIVUM AGAINST DERMATOPHYTES

M.Mohammed Latheef[a] , S.Muthu Raj[a*] , MAbdul Salam[a] , Ragavi[a] , and K.Chitra[a]
PG and Research Department of Microbiology Sadakathullah Appa College(Autonomous)
Tirunelveli-627011
***Corresponding Author:muthurajmicrobiology@gmail.com**

***Abstract:** Medicinal plants such as Allium cepa L. (Onion) and Allium sativum (Garlic) have become the focus of intense study regarding to gather conservation and potential pharmacological effects. Allium cepa L. (Onion) which belongs to the family Alliaceae is also known as 'garden onion' or 'bulb' onion (Yin & Cheng, 1998). Allium sativum (Garlic) has been used as herbal medicine in world wide and it help prevent various diseases and disorders. Allicin is the main biologically active component present in freshly crushed garlic cloves it is an antioxidant compounds found to have been health-protecting factor. Primary sources of antioxidants Allicin compounds are naturally present in whole grains, fruits and vegetables (Lawson et al., 1991). In the present study, two important medicinal plants namely Allium cepa (Onion) and Allium sativum (Garlic), assessed the phytochemical components and antifungal activity against skin pathogens with different concentration and combination.*

***Keywords:** Allium cepa, Allium sativum, Allicin, Alliaceae*

I. Introduction:

Allium cepa L. (Onion):

About 80% of individuals developed countries used traditional medicine, which has compound derived being medicinal plants. Medicinal plants such as *Allium cepa* L. (Onion) and *Allium sativum* (Garlic) have become the focus of intense study regarding to gather conservation and potential pharmacological effects. *Allium cepa* L. (Onion) which belongs to the family *Alliaceae* is also known as 'garden onion' or 'bulb' onion (Yin & Cheng, 1998). It is an oldest cultivated vegetables in history. The bulbs from in onion family have being utilized as a food source for millennia. The bulb grows underground and is used for energy storage. The leaves are bluish green and hollow, the bulbs are large, fleshly and firm. Three main varieties of onion are available red, white and purple skinned. Onions are easily propagated, transported and stored (Nivas et al., 2006).

Allium sativum (Garlic):

Allium sativum (Garlic) has been used as herbal medicine in world wide and it help prevent various diseases and disorders. Allicin is the main biologically active component present in freshly crushed garlic cloves it is an antioxidant compounds found to have been health-protecting factor.

Primary sources of antioxidants Allicin compounds are naturally present in whole grains, fruits and vegetables (Lawson et al ., 1991). All of the background research involving allicin has been done by either garlic powder from different manufactures or processing the garlic cloves through the many different chemicals in order to obtain allicin. It is produced by the interaction of non-protein amino acid allin with used enzyme alliinase (Ponnulakshmi *et al*., 2013).

Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Garlic has traditional dietary and medicinal applications as anti-infective agent.

In vitro evidence of the antimicrobial activity of fresh and freeze-dried garlic extract against many bacteria , fungi, virus (Rahman M.M et al., 2012).

II. Materials and Method:

In the present study, two important medicinal plants namely *Allium cepa* (Onion) and *Allium sativum*(Garlic), assessed the phytochemical components and antifungal activity against skin pathogens with different concentration and combination.

The following objectives were carried out in this study:

- The organic solvents viz., Petroleum ether, Chloroform, Methanol, Acetone, Ethyl acetate, water was used for the extraction of plant material.
- The extracts were screened for the presence of phytochemical compounds and also test antifungal activity against *Aspergillus* spp, *Mucor* spp *Altertaria* spp, and *Culvularia* sp

The selected medicinal plants used in different concentration with different combinations to analyze the antibiogram activity.

Collection plant materials:

The plant materials, red onion and white garlic were selected for the investigation of antimicrobial activities. Fresh Onion and Garlic were procured from the local market in Tirunelveli Dt, Tamil Nadu, India.

III. Extraction Plant Materials:

i) Aqueous extract:

Fresh onions and garlic were peeled of the outer layer and 1 kg of onion and garlic washed thoroughly a distilled water and the bulb has cut the pieces and was made into a crude paste. The paste was soaked in 1litre of sterile distilled water for 24 hours at 4ºC and it was then filtered thrice using a sterile muslin cloth. The filtrate was poured into a beaker and concentrated on a water bath at 100°c to obtain semi-solid residues and they aqueous extract was weighted and this was immediately subjected to preliminary phyto-chemical and antifungal analysis using standard method (Ponnulakshmi *et al*., 2013).

ii) **Ethanolic extract :**

After cleaning 1kg of onion and garlic as described earlier they were made into a paste was soaked in 500ml of ethanol for 15 days at room temperature than it were filtered using sterile muslin cloth and the filtrate was poured into a beaker and concentrated on a water bath at 70-80ºC to obtain semi-solid residue. The weight of the yield was noted and this was subjected in preliminary phyto-chemical and antifungal analysis using standard method.(Mazhood Khan *et al.,* 2012)

iii) **Chloroform extract**:

After making a paste of 1 kg onion and garlic of different varieties as described earlier, they were separately soaked in 300 ml of chloroform for a week at room temperature the filtered using sterile muslin cloth and filtrate was concentrated in a beaker at 60-62°C to obtain semi-solid residue. This have weighted and subjected to preliminary phyto-chemical and antifungal analysis.(Prakash s. et al., 2016)

iv) **Petroleum ether extract:**

Following the earlier procedure, onion and garlic bulb of different varieties was prepared and soaked in 200ml of petroleum ether for 15 days at room temperature. It was filtered and the filtrate we concentrated at 40-60ºc. The extract were weighted and subjected to preliminary phyto-chemical and antifungal analysis standard method. (Gulsen Gonagul *et al* 2010)

Test Pathogens:

Antifungal activities of aqueous, ethanol, chloroform, petroleum ether extracts of various *A.cepa* and *A.sativum* varieties small onion and garlic were studied. Ketoconazole was used as standard drug. The microorganisms, maintained on Potato Dextrose agar. (Four species of fungi, *Aspergillus* spp, *Mucor* spp *Altertaria spp,* and *Culvularia* sp were used in study.

Processing of Clinical Sample:

The skin scraping is collected from wound infected patients. The sample was emulsed in saline water.

Isolation:

The skin scrapping was further transfer to Nutrient agar plate for isolation of bacteria. The skin scrapping was inoculated with Potato Dextrose agar for the isolation of Fungi.

Selective Medium:

The isolated organisms of *Staphylococcus* sp, were streaked on the selective medium of Mannitol Salt agar. The isolated organisms of *Bacillus* sp, were streaked on the selective medium of Nutrient agar. The isolated organisms of *Pseudomonas* sp, were streaked on the selective medium of King's medium.

IV. Phytochemical Anasysis

a. **Determination of Water content:**

Water content of ***A.cepa*** and *A.sativum* determined using a method of a Thermal drying method was used in a determination in water content of the samples. 10g bulbs of different ***A.cepa*** and *A.sativum* varieties were weighted crucible. This was placed in oven and dried at 105ºC (Hot air oven) for 3 hours. The samples were allowed to cool in a desiccators and then reweighted**.** The percentage of water content has calculated can be expressing he loss the weight on drying as a fraction of the initial weight of sample used and multiple by 100.

b.**Determination of Total ash:**

A known weight of varieties dry bulb which to has and the weight of there by obtained a expressed can be terms of percentage. In a clean crucible, three varieties of *A.cepa* and *A.sativum* dry bulb as taken and weighted. Weighted dish was placed over the tripod stand carefully. The crucible were opened partially and directed to the tip of the flame for gradual heating at 500°c. The onion and garlic samples were heated gently to avoid catching fire. When the smoke subsides the burner was placed the dish. Then the dish was cooled to room temperature and weighted with the contents. Again the sample was heated to effect for any possible and weighted. The process was repeated three consecutive weighing and complete combustion was taken. The total was then determined and recorded. The percentage of an content has calculated using the formula:

$$Ash(\%) = Ma/Ms \times 100$$

Where Má = mass of ash (g) and Mś =Mass of sample used (g)

c.**Determination of pH**:

pH of different *A.cepa* and *A.sativum* varieties were determined per the method of park and the pH values of *A.cepa* were measured. *A.cepa* and *A.sativum* varieties were homogenized with 90 ml of sterile double distilled water, after which, pH values were, measured five times for its concurrency and expressed in average values.

d. **Phytochemical screening**:

The various solvent extracts of powder of leaves of *Leucas asper* were subjected to the phytochemical test for the identification of various active constituents, using the method followed by Malcom and Sofowora ,1969.

i) Test for tannis:

About 2ml of the aqueous extract was mixed with 2ml of distilled water and few drops of $Fe\ Cl_3$ solution were added.

ii) Test for saponins:

3ml of the aqueous extract shaken vigorously with 3ml of distilled water in a test tube and warmed.

ii) Test for phlobatannins:

2ml of aqueous extract was added and 2ml of 1%HCl and the mixture was boiled.

iii) Test for flavonoids:

5ml of aqueous extract was added and 1ml of $H_2\ SO_4$ added. Few minutes wait and observe the result.

iv) Test for Terpenoids:

2ml of aqueous extract was dissolved in 2ml of chloroform and evaporate it. Then 2ml of concentrated $H_2 SO_4$ was added and heated for about 2min.

v) Test for Glycosides:

2ml of aqueous extract was dissolved in 2ml of chloroform and 2ml of acetic acid was added in it. The solution was cooled in ice. Then $H_2 SO_4$ was added carefully. Then observe the result.

vi) Test for Steroids:

2ml of extract was dissolved in 2ml of chloroform and 2ml of concentrated $H_2 SO_4$ was added and observe the result.

vii) Test for Phenols:

1ml of aqueous extract were dissolved in 5ml of alcohol and treaded with few drops of $Fecl_3$ solution.

viii) Test for Protein and Aminoacids:

2ml of plant extract dissolved in 2ml of water. Change the colour indicates the presence of protein.

ix) Test for Alkaloids:

5ml of plant extract was mixed with 1% HCl. The solution obtained was filtered and then 1ml of filtrate was treated with few drops of Mayer's reagent. Formation of turbidity or cream precipitate indicates presence of alkaloids.

x) Test for Carbohydrates:

1ml of aqueous extract was added and few drops of molischs reagent was added, and then few minutes wait. Then few drops of concentrated $H_2 SO_4$ was added and heated for about 2minutes.

xi) Test for Quinins:

1ml of aqueous extract was added and 1ml of $H_2 SO_4$ $_4$ added, few minutes wait and observe the result.

xii) Test for Glycosides:

1ml of aqueous extract was added and 2ml of glacial acetic acid wait for few minutes. Then few drops of 5% ferric chloride was added. Then added 1ml of $H_2 SO_4$ added. Few minutes wait and observe the result.

xiii) Test for coumarin:

1ml of aqueous extract was added and 1ml of 10% of NAOH added, few minutes wait and observe the result.

xiv) Test for Triterpeniods:

1.5ml of aqueous extract was added and 1ml of libeman buchard reagent was added. Then added 1ml of concentrated $H_2 SO_4$ added. And then added few minutes.

xv) Test for anthraquinones:

1ml of aqueous extract was added and few drops of 10% Ammonia solution.

V. RESULTS:

In the present investigation *Allium cepa* and *A.sativum* collected from the Local Markets Tirunelveli Dt., Collected samples processed and analysed their Phytochemical characters. Table 1 show that Flavonoids. Coumanis are positive for the five extracts and Anthrequinones is negative.

Table:1 Phytochemical results for *Allium cepa*

S.No	Chemical Constituents	Petroleum ether	Chloroform	Methanol (H_2O)	Ethyl acetate	Aqueous
1	Carbohydrates	-	+	+	-	-
2	Tannins	-	-	+	_	_
3	Saponins	-	-	_	_	_
4	Flavonoids	+	+	+	+	+
5	Alkaloids	+	+	+	_	+
6	Quinins	-	+	+	+	_
7	Glycosides	-	-	_	_	_
8	Cardiac glycosides	+	+	+	+	+
9	Terpenoids	+	-	+	_	_
10	Phenols	+	-	_	_	_
11	Coumanis	+	+	+	+	+
12	Steroids	-	-	-	+	_
13	Phlobatannins	-	-	-	+	-
14	Triterpenoids	+	+	+	+	-
15	Proteins	+	+	-	+	-
16	Anthrequinones	-	-	-	-	-

Table 2 shows that phytochemical characters of *Allium sativum* from that Coumanis and Cardiac glycosides and Flavonoids shows positive results followed by Triterpenoids, proteins and Anthroquinones showed negative results

Table: 2. Phytochemical results for *Allium sativum*

S.No	Chemical Constituents	Petroleum ether	chloroform	Methanol (H_2O)	Ethyl acetate	Aqueous
1	Carbohydrates	-	+	+	+	+
2	Tannins	-	-	+	_	_
3	Saponins	-	-	_	_	_
4	Flavonoids	+	+	+	+	+
5	Alkaloids	+	+	+	_	+
6	Quinins	-	+	+	+	_
7	Glycosides	-	-	_	_	_
8	Cardiac glycosides	+	+	+	+	+

9	**Terpenoids**	+	-	+	_	_
10	**Phenols**	+	-	_	_	_
11	**Coumanis**	+	+	+	+	+
12	**Steroids**	-	-	-	+	_
13	**Phlobatannins**	-	-	-	+	-
14	**Triterpenoids**	+	+	+	+	-
15	**Proteins**	+	+	-	+	-
16	**Anthrequinones**	-	-	-	-	-

Table: 3 Antifungal activities of *Allium cepa* against *Aspergillus* sp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method (mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 4 Antifungal activities of *Allium cepa* against *Mucor* spp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 5 Antifungal activities of *Allium cepa* against *Altertaria* spp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9

3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 6 Antifungal activities of *Allium cepa* against *Culvularia* sp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 7 Antifungal activities of *Allium sativum* against *Aspergillus* sp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 8 Antifungal activities of *Allium sativum* against *Mucor* spp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 9 Antifungal activities of *Allium sativum* gainst *Altertaria spp*

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Figure 1: Phytochemical Analysis of *A.cep*

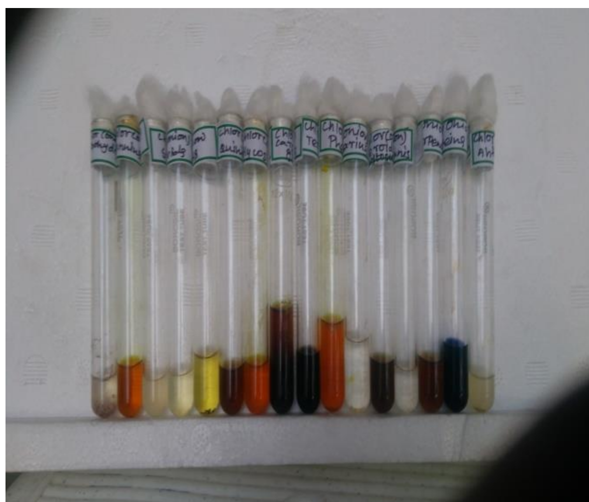
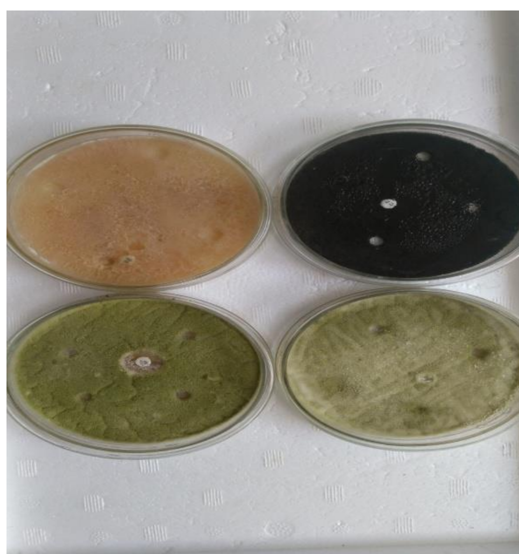


Figure 2: Phytochemical Analysis of *A.sativum*

Figure 3: Fungal pathogens



VI. DISCUSSION:

A.cepa and *A.sativum* extracts has been extensively studied for its antimicrobial activity against a wide range of fungal, and parasitic organisms. However, a limited data is available so far regarding its efficacy against *Aspergillus* spp. Both the extracts exhibited antifungal activity against aspergiius spp. The antifungal activity of purple type of *A.cepa* and *A.sativum* extracts was found to be better as compared to yellow type *of A.cepa* and *A.sativum* extract. Agar well diffusion method showed that EEP both the tested extracts had antifungal activity against *Aspergillus* spp than EEY demonstrated at 12. 5 % and showed no activity at 6.25%. our results are comparable with a study conducted by Nelson at al revealed that ethanolic extract of onion and garlic gave 11 mm zone of inhibition with MIC 0.8 mg /ml against *Mucor* spp and 9 mm of inhibition with MIC 0.8 mg/ml.8.9.In a study conducted by N.Benkebila in Algeria, red/ purple onion and garlic exhibited better antifungal activity as compared to yellow onion and garlic alternoria spp. The zone of inhibition of extracts. In study conducted by Mahash in India, antimicrobial activity of certain plants was evaluated using the disc diffusion method against certain fungi.The onion and garlic bulbs contain numerous organic sulphur compounds including sulfoxide, flavinoides, phenolic acids, sterols including cholesterol, saponins, sugars and a trace of volatile oil compounds may explain the anti - microbial activity of this plants.The phytochemical screening in the present, study, has revealed the presence of triterpenoids,

steroids, glycosides, flavonoids, tannins, carbohydrates and vitamin C in the seeds extract. Further the presence of different phytoconstituents in the four different extracts may be responsible for the therapeutic properties of onion and garlic.The preliminary phyto chemical screening tests may be useful in the detection of the bioactive components and subsequently may lead to the drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacological active chemical compounds.

References:

1. Adetumbi., G.T. Javor, and B.H .S. Lau.(1986). *Allium sativum* (garlic) inhibits lipid synthesis by *Candida albicans''*, *Antimicrob. Agents Chemother*, 30: 499 -501 .

2. Augusti K, (1996). Therapeutic values of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) *Indian J exp Biol*, 34: 634-640.

3. Azu NC, onyeagba RA, Nworie O, kalu J. (2007). Antibacterial Activity of Allium Cepa (onion) and zinger officinale (Ginger) on *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from high vaginal swab. Interent J Trop Med, 3(2) 234-239.

4. C.J. Cavallito, and J.H. Bailey (1994) 'Antibacterial principal of *Allium sativum*'' *J. Am. Chem, Soc*. 66: 1950,

5. EI-meleig, M., Ahme, R., Arafa, N. Ebrahim and EI Kholany., (2010) ''Cytotoxicity of four essential oils on some human and bacterial cells,'' *J. Appl, Sci. in Environ Sanit*., 5, 143 - 159.

6. Griffiths. L., Trueman,T. Crowther,B. Thomas and Smith, B (2002) ''Onions: a global benefit to health'', *Phyto Ther Res*., 16, 603 -615.

7. Hannan, T. Humayun, M. Yasir and Sikandar, S(2010) ''In vitro antibacterial activity of onion (*Allium cepa*) against clinical isolates of *vibro cholera*,'' *J Ayu. Med Coll* , 22 (2) 160 – 163.

8. Helen. K. Krishnakumar,P, Vijayammal. A and Augusti, K. (2000) ''Antioxidant effect of onion oil (*Allium cepa*. Linn) on the damages induced by nicotine in rats as compared to alpha tocopherol'', Toxicol *Lett*., 116, 61 - 68.

9. K.Augusti, (1996) ''Therapeutic values of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.)'', *Indian*. J. Exp. Boil, 34, 634 -640.

10. Lampe.J .(1999) "Health effects of vegetables and fruits: assessing mechanisms of action in human experimental studies,'' *J. Clin. Nutr* 70, 475, 90.

11. Lawson, L.D., Wang, Z. J and Hughes, B. (1991). Identification and HPLC quantization of the sulphides and thiosulpfinates in commercial garlic products. *Planta medica* 57 (4): 363 -70.

12. Lawson, L.D., Wood, S.G and Hughes, B. (1991). HPLC analysis of Allicin and other thiosulfinates in Garlic clove Homogenates. *Planta Medica* 57(3): 263 – 270.

13. Nemeth K, Piskula MK, (2007). Food content, processing, absorption and metabolism of onion flavonoids, Crit . Rev. Food. Sci.Nutr, 47(4): 397 – 409.

14. Parekhm P.J. and Chandam S.V. (2007) " In vitro antimicrobial activity and phytochemical analysis of some Indian medicinal plants'', *Turk J boil*., 31: 53 -58.

15. Rees, S.F. Minney, N. T.plummer, J.H. Slater, and Skyrme. D.A. (1993)'' A quantitative assessment of the antimicrobial activity of garlic'' (*Allium sativum*). *World J. Microbiol. Biotechnol*., 9: 303 – 307.

16. Rose. P Whiteman, M. Moore P. and Zhu Y.(2005). Bioactive cysteine sulfoxide metabolites in the genus *Allium* : the chemistry of potential therapeutic agent, Natural Product Rep, 22, 351 - 368.

17. Wang L, Lee IM, Zhang SM, (2009). Dietary intake of selected flavonoids, flavones, and flavonoid – rich foods and risk of cancer in middle aged and older women, *Am J Clin Nutr*, 89(3): 905 – 912.

18. Weber, D.O. Anderson, J.A. North, B.K. Murry, L. and Hughes, B.G. (1992) ''In vitro viricidal effects of *Allium sativum* (garlic) extract and compounds''. *Planta Med*., 58: 417 – 423.

19. Wilson E A, Dermming - Adams B,, (2007). Antioxidant, anti-inflammatory and antimicrobial properties of garlic and onions, Nutr Food Sci, 37(3): 178 -183.

20. Ying MC, Chang WS, (1998). Antioxidant activity of general *Allium* members. *J. Agri Food Chem*, 46: 4097-4101.

21. Zohri, K. Gwad, and Saber S (1995) ‘’Antibacterial, antidermatophytic and antioxigenic activities of onion (*Allium cepa*) oil’’, *Microbial*. *Res*., 150 , 167 -172.

View publication stats

Journal of Pharmaceutical and Scientific Innovation

www.jpsionline.com (ISSN : 2277 -4572)

Research Article

AN INVESTIGATION ON NANO METAL VITAMIN COMPLEX TO CONTROL OXIDATIVE CELL DAMAGE AND MICROBIAL SPOILAGE IN SEAFOOD

Haris Variyathody [1]* and Manohar M [2]

[1]Manager-Quality, Health, Safety & Hygiene. The Leela Palaces Hotels and Resorts, Mobor, Cavelossim, Salcette, Goa, India

[2]Professor, Department of Microbiology, Vivekanandha College of Arts and Science for women (Autonomous), Elayampalayam, Tiruchengode - TK, Namakkal, Tamilnadu, India

*Corresponding Author Email: harisvt12345@gmail.com

DOI: 10.7897/2277-4572.07497

Received on: 13/06/18 Revised on: 15/07/18 Accepted on: 18/07/18

ABSTRACT

Fish is considered to be one of the most-traded food commodities worldwide with more than half of fish exports by value originating in developing countries. In order to increase its shelf life and maintain its nutritional value, texture and flavour, food preservation becomes necessary. Fish spoilage results from three basic mechanisms: Enzymatic autolysis, oxidation, microbial growth. With advancements in nanotechnology, different metals like silver, copper, zinc etc., have been engineered into a nanometre size and have attracted great research interest for use in different food industries because of their potential antimicrobial property. Vitamins like Pyridoxine, alpha tocopherol acetate, folic acid are commonly used as a dietary supplement and therapeutic agent possessing antioxidant and cell proliferation activity. Therefore, in the present research, copper oxide nanoparticles complexed with vitamin-E (MnV-C) were synthesized, and its potential to control the oxidative damage in the cells of seafood; and the microbial spoilage in seafood is investigated. All the bacterial species were found to be sensitive to the nanometal vitamin complex. The MICs of nanometal vitamin complex ranged from 1.6mg/ml to 6.4mg/ml. Maximum inhibitory zones obtained against *Bacillus cereus and Shigella* sp. were 17mm and 16mm. Thus the metal nanoparticles coupled vitamins (MnV-C) was effective against the oxidative cell damage and pathogenic microbes in seafood. This technology can be used as a preservation technique; can improve the quality fish and fish products.

Keywords: Fish spoilage, Nanometals, Vitamin complex, antimicrobial property, oxidative cell damage

INTRODUCTION

Fisheries and aquaculture remain important sources of food, nutrition, income and livelihoods for hundreds of millions of people around the world. World per capita fish supply reached a new record high of 20 kg in 2014. Moreover, fish continues to be one of the most-traded food commodities worldwide with more than half of fish exports by value originating in developing countries. Recent reports by high-level experts, international organizations, industry and civil society representatives all highlight the tremendous potential of the oceans and inland waters now, and even more so in the future, to contribute significantly to food security and adequate nutrition for a global population expected to reach 9.7 billion by 2050. With the ever growing world population and the need to store and transport the food from one place to another where it is needed, food preservation becomes necessary in order to increase its shelf life and maintain its nutritional value, texture and flavour. Fish is highly perishable commodity and also much in demand One-fourth of the world's food supply[1] and 30% of landed fish[2] are lost through microbial activity alone. Spoilage of food products is due to chemical, enzymatic or microbial activities[3]. During fish spoilage, there is a breakdown of various components and the formation of new compounds. These new compounds are responsible for the changes in odour, flavour and texture of the fish meat. Fish spoilage results from three basic mechanisms: Enzymatic autolysis, oxidation, microbial growth. Low temperature storage and chemical techniques for controlling water activity, enzymatic, oxidative and microbial spoilage are the most common in the industry today. Thus prevention of fish from cell damage and microbial spoilage is an essential part in preservation of seafood[4].

Nanotechnology is an emerging science and with growing use particularly in developing new materials at nanoscale levels[5]. Nanometals have been engineered and have attracted great research interest for use in different food industries. The metal nanoparticles have emerged as a potential alternative to conventional antibiotics because of their potential antimicrobial property. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution. Nanotechnology increasing the shelf life of different kinds of food materials and also help brought down the extent of wastage of food due to microbial infestation[6]. Different nanometals from silver, copper, zinc, etc. can be synthesized which possess anti-microbial, anti-oxidant, anti-biotic and anti-fungal activities etc., Due to a wide presence in nature, implementation of different functions within the majority of living organisms, relatively low cost and environmental safety, copper compounds (Cu) have a high potential for their application as antibacterial agents being capable to replace silver and composites of different precious metals[7]. Cu nanoparticles are hypotoxic and show a high antibacterial effect in relation to the cells of test cultures of gram positives and gram negative organisms. Nanoparticles attached to the microbial surfaces can decrease both cell mobility and nutrient flow between the cell's exterior and interior compartments. It was reported that[8] copper has the potential to disrupt cell function in multiple ways, since several mechanisms

acting simultaneously may reduce the ability of microorganisms to develop resistance against copper. The nonspecific mode of action of nanoparticles against bacteria makes them ideal candidates as antimicrobial agents with less risk of development of bacterial resistance[9]. In addition, no research has discovered any bacteria able to develop immunity to copper as they often do with antibiotics[10].

Vitamins like Pyridoxine, alpha tocopherol acetate, folic acid etc., possess antioxidant and cell proliferation activity. Oxidation occurs as a result of the reaction between atmospheric oxygen and the unsaturated fatty acids. Peroxides are intermediate products in the oxidation process, which in turn break down to odor and flavor-producing compounds. Other studies have shown that supplemental vitamin E improves the stability and flavor of veal fat, frozen poultry, milk and pork[11-14] when added to the feed over a long period of time. Vitamin E and C has ability in preventing oxidative damage of cells and repairing already existing damage[15]. Therefore, the main objective of this research is to synthesis metal nanoparticles complexed with vitamins (MnV-C), and its potential to control the oxidative damage in the cells of seafood; and the microbial spoilage in seafood is investigated.

MATERIALS AND METHODS

Synthesis of copper oxide nanoparticles[16]

Solution I: 6.9g of copper sulphate pentahydarate was dissolved in 100ml of distilled water.

Solution II: 34.6g of sodium potassium taratate and 12g of sodium hydroxide was dissolved in 100ml of distilled water.

About 50ml of solution I and 50ml of solution II was mixed together with vigorous stirring and 5g of glucose (reducing agent) was added and then the mixture was stirred vigorously was 10mins and then keep in boiling water bath at 60°C for 10mins. Then, the obtained mixture is centrifuged and washed with distilled water twice and with ethanol twice and it was air dried and the powdered substance was used for further analysis.

Preparation of Nanometal vitamin complex[17]

A series of Nanometal vitamin complex were synthesized according to the following general procedure: copper oxide nanoparticles were added gradually to magnetically stirred ethanol solution (20ml). Then, to the first reaction mixture, vitamin E oil was added and stirred carefully at about 60-80 °C till the reaction reached equilibrium. Then evaporation of the solvent (by placing the reaction mixture in a fume cupboard) and nanometal vitamin complex is synthesized.

Determination of minimum inhibitory concentration[18] (MIC)

Minimum inhibitory concentration (MIC) of nanometal vitamin complex against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella* sp., and *Shigella* sp. was determined by standard broth macrodilution method. All the test cultures were inoculated in a sterile liquid media (Nutrient broth (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g; Final pH (7.0 ± 0.2) and allowed to attain the growth for 24 to 48hours. To determine the MIC, a set of tubes with 1ml of Nutrient broth was added under sterile conditions. About 150µl of nanometal vitamin complex at different concentrations (25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, 800µg/ml, 1.6mg/ml, 3.2mg/ml, 6.4mg/ml, 12.8mg/ml) was added to the tubes containing broth solution. To this above mixture, 500µl inoculum of each test cultures was added to their respective tubes. All the inoculated tubes were incubated at 37°C ± 0.2°C for 24 to 48hours. Organic solvent, methanol was used as negative control and antibacterial drug gentamicin (100µg/ml) was used as positive control.

Antibacterial activity of Nanometal vitamin complex[19]

The antibacterial activity of Nanometal vitamin complex was evaluated against the five test organisms (*Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella* sp., and *Shigella* sp.) by well diffusion method. Sterile Nutrient Agar (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of each of the bacterial cultures were streaked with the sterile cotton swab three times by turning the plate at 60^0 angle between each streaking. Under sterile conditions, 6mm wells were cut on the agar surface of each Nutrient Agar (NA) plates. About 50µl each of Nanometal vitamin were loaded into the well and the plates were incubated at 37ºC for 24 - 48h. The antibacterial activity was evaluated in terms of zone of inhibition around the wells of each extract in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimetre.

RESULTS AND DISCUSSION

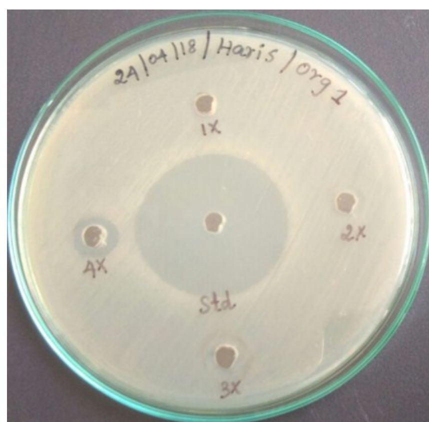
Minimal Inhibitory Concentration of Nanometal vitamin complex

The MICs of Nanometal vitamin complex against five test bacteria, *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella* sp., and *Shigella* sp. were shown in Table 2. The MICs of Nanometal vitamin complex ranged from 1.6mg/ml to 6.4mg/ml. The results were identified to be well coincided with the antibacterial activity presented in Table. All the bacterial species were found to be sensitive to the Nanometal vitamin complex. MIC of 1.6mg/ml was found to be the least concentration for test organisms *Staphylococcus aureus Bacillus cereus*. Similarly *Escherichia coli* and *Salmonella* sp. exhibited 3.2mg/ml as the MIC value. About 6.4mg/ml was found to be a satisfactory MIC value for the test organism *Shigella* sp. The obtained results in the present research were found satisfactory, supportive and also in accordance to the following literature analysis.

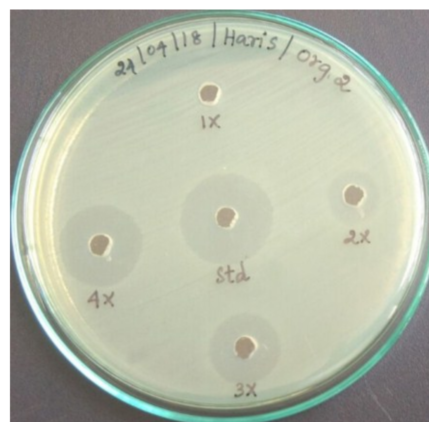
Minimal Inhibitory Concentration of Copper oxide nanoparticles were evaluated by Ruparelia *et al*., 2008[20]. Based on disk diffusion tests, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), the bactericidal effect of silver and copper nanoparticles were compared dispersed in batch cultures. Many bacterial species were found sensitive to nanoparticles. Disk diffusion tests carried out with *E. coli* and *S. aureus* showed silver nanoparticles had better bactericidal effect on bacterial species compared to the copper nanoparticles. On comparing to the other bacterial strains *B. subtilis* was found to be more sensitive to nanoparticles especially copper nanoparticles. In MIC/MBC and disk diffusion test bactericidal effect of copper nanoparticles showed a good negative correlation observed between the inhibitory zones (r2=-0.75). *E. coli* showed strain-specific variation and strain specific-variation for *S. aureus* in MIC/MBC was negligible.

Table: 1 Anti-bacterial activity of Nanometal vitamin complex against test organisms

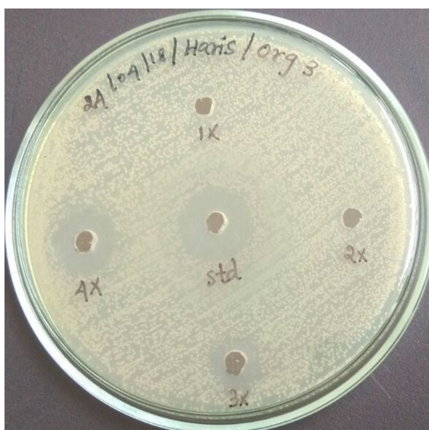
S.No	Test organism	Zone of Inhibition(in mm)				
		1x	2x	3x	4x	Ciprofloxacin
1.	*Staphylococcus aureus*	0	07	10	11	29
2.	*Bacillus cereus*	0	12	14	17	18
3.	*Escherichia coli*	0	0	09	12	15
4.	*Salmonella* sp	8	11	13	14	16
5.	*Shigella* sp	8	13	14	16	17



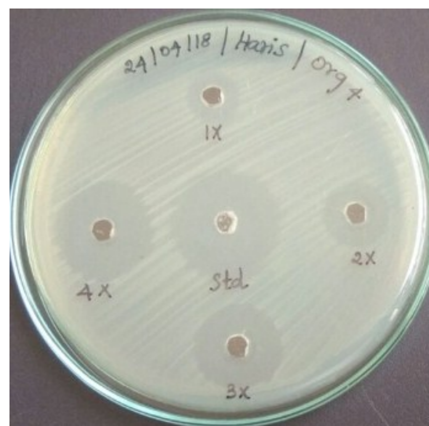
Staphylococcus aureus



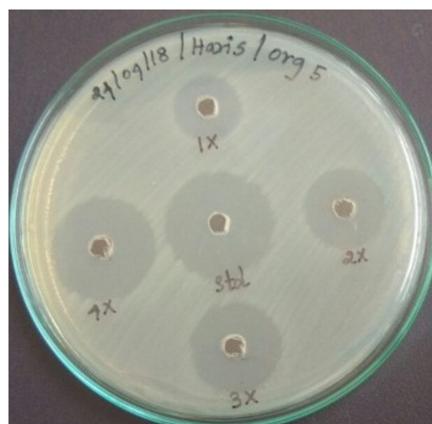
Bacillus cereus



Escherichia coli



Salmonella sp



Shigella sp

Figure 1: Antibacterial activity of Nanometal vitamin complex against different organisms

Silver and copper metal nanoparticles were synthesised by chemical processes. Silver nitrate and copper nitrate was reduced by ascorbic acid in the presence of chitosan[21]. Particle size was increased by reducing the chitosan concentration and increasing the nitrate concentration. Surface zeta potentials for silver and copper nanoparticles produced varied from 27.8 to 33.8 mV. Antibacterial activities of Silver, Copper, Silver and Copper mixtures and bimetallic nanoparticles of Silver or Copper were tested using *Bacillus subtilis* and *Escherichia coli*. The more susceptible organism among *Bacillus subtilis* and *Escherichia coli* was found to be *Bacillus subtilis*. Silver nanoparticles showed higher antibacterial activity than copper and mixtures of nanoparticles. Bimetallic Ag/Cu nanoparticles proved to possess the highest antibacterial activity with with minimum inhibitory concentrations (MIC) of 0.054 and 0.076 mg/L against *B. subtilis* and *E. coli* respectively.

Antibacterial activity of Nanometal vitamin complex

The antibacterial activity of Nanometal vitamin complex against five test bacteria, *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella* sp., and *Shigella* sp. were evaluated by agar diffusion method (Figure 1). Four different concentrations of Nanometal vitamin complex were used to determine the antibacterial activity against each organism. Maximum inhibitory zones were obtained against *Bacillus cereus and Shigella* sp at 4x concentration was 17mm and 16mm (Table.1). Thus, antibacterial activity of Nanometal vitamin complex against five test bacterial species showed promising results in the present research. During the analysis, the Nanometal vitamin complex showed good antibacterial activity against *Shigella* sp when compared to other organisms (Table: 1).

The antibacterial activity of copper nanoparticles is due to interaction with the bacterial outer membrane (peptidoglycan), causing the membrane to rupture and killing bacteria[22]. Kim *et al*., 2000[23] emphasized that the bactericidal effects observed in this study might have been influenced by the release of Cu^{2+} ions. Copper ions released by the nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death. Copper ions inside the bacterial cells may bind to deoxyribonucleic acid molecules (DNA) and become involved in cross-linking within and between the nucleic acid strands, resulting in the disorganized helical structure[24].

The antibacterial properties of Cu nanoparitcles (Cu-NPs) were investigated against *Salmonella typhi* by Anamika *et al*., 2016[25]. The Cu-NPs were prepared by the reduction of copper acetate with the help of ethylene glycol (EG), then sample was characterized by XRD for its average particle size identification. The antibacterial activity assessed by well diffusion and disc diffusion method on different concentration of nanoparticles. It was found that these Cu-NPs showed antibacterial activity in form of zone inhibition, wherein, zone of inhibition increased with increase in concentration of Cu-NPs.

Overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms and this lead to search for agents that may have antibacterial effects. Vitamin E emerged as an essential, fat-soluble nutrient in the human body and it is essential, because the body cannot manufacture its own vitamin E, so foods and supplements must provide it. The anti-bacterial analysis of vitamin E against pathogenic bacteria was investigated by Dalia Abd *et al*., 2013[26]. Results showed that gram negative bacteria were shown to be more resistant than gram positive bacteria. The resistance of gram negative bacteria towards antibacterial substances may be related to lipopolysaccharides in their outer membrane.

In a study of the effect of Vitamin E on secondary bacterial infection after influenza infection in young and old mice which was done in 2004 by Gay *et al*., [27] the result was that Vitamin E supplementation abolished the priming effect of influenza infection on *S.aureus* and the researchers concluded that vitamin E may exert its effect by number of mechanisms, including reducing reactive oxygen species (ROS), decreasing proinflammatory cytokines and adhesion molecule expression and production, increasing antioxidant and antimicrobial activity. Most of the animal studies that investigated the effect of Vitamin E on infectious diseases reported a protective effect despite the variations in the dose and duration of the supplementation, infectious organisms involved, and route of administration. Only a limited number of studies have investigated the effect of vitamin E on resistance against infections in humans.

CONCLUSION

In order to increase its shelf life and maintain its nutritional value, texture and flavour food preservation becomes necessary in storage and transport of food products. Nano metals and vitamins having antimicrobial and has potential to control the oxidative damage in the cells of seafood can be used as an alternative for the preservatives in storage of sea foods. The Nanometal vitamin complex against five test bacterial species showed promising results in the present research. The developed Nanometal vitamin complex in the present study revealed the significance of the preservation of stored sea foods preventing oxidative and microbial spoilage. Thus the metal nanoparticles coupled vitamins (MnV-C) was effective against the oxidative cell damage and pathogenic microbes in seafood which can improve the quality fish and fish products and increase their shelf life.

REFERENCES

1. Huis in’t Veld, JHJ. Microbial and biochemical spoilage of foods: An overview. Int. J. Food Microbiol 1996; 33:1-18.
2. Amos B. Analysis of quality deterioration at critical steps/points in fish handling in Uganda and Iceland and suggestions for improvement. United Nations University, Uganda 2007.
3. Baird-Parker TC. The Production of Microbiologically Safe and Stable Foods. In: The Microbiological Safety and Quality of Food, Lund,B.M. and T.C. Baird-Parker (Eds.). Aspen Publishers Inc 2000. Gaithersburg, MD., USA: 3-18.
4. Ghaly AE, Dave D, Budge S, Brooks MS. Fish Spoilage Mechanisms and Preservation Techniques: Review. American J App Sci 2010; 7(7):859-877.
5. Albrecht MA, Evan CW, Raston CL. Green chemistry and the health implications of nanoparticles. Green Chem 2006; 8:417-432.
6. Pradhan N, Singh S, Ojha N, Srivastava A, Barla A, Rai V *et al*. Facets of nanotechnology as seen in food processing, packaging, and preservation industry. BioMed Res. Int 2015:1-17.
7. Veerapandian MS, Sadhasivam S, Choi J, Yun K. Glucosamine functionalized copper nanoparticles: Preparation, characterization and enhancement of anti-bacterial activity by ultraviolet irradiation. Chem Eng J 2012; 209:558-567.

8. Raffi M, Mehrwan S, Bhatti TM, Akhter JI, Hameed A, Yawar W, Hasan MM. Investigations into the antibacterial behavior of copper nanoparticles against Escherichia coli. Annals Microbiol 2010; 60:75-80.
9. Harikumar PS, Anisha Aravind. Antibacterial Activity of Copper Nanoparticles and Copper Nanocomposites against Escherichia Coli Bacteria. Intl J sci 2016;5(2):83-90.
10. Theivasanthi, Alagar. Nano sized copper particles by electrolytic synthesis and characterizations. Intl J Physical Sci 2010; 6(15):3662-3671.
11. King AD Jr, Nagel CW. Growth inhibition of a *Pseudomonas* by carbon dioxide. J Food Sci 1967; 32:575-579.
12. Kraft AA, Ayres JC. Post-mortem changes in stored meats. IV. Effect of packaging materials on keeping quality of self-service meats. Food Technol 1952; 6:8-12.
13. Ledward DA. Metmyoglobin formation in beef stored in carbon dioxide-enriched and oxygen -depleted atmospheres. J. Food Sci 1970; 35:33-37.
14. Leibrandt RL. Atlas of gas analyses by gas chromatography. J Gas Chromatog 1967; 5:518-524.
15. Santoshi R. Ghodake, Adinath N Suryakar , Prabhakar M Kulhalli, Ramchandra K Padalkar, Abdul Kayyum Shaikh. A study of oxidative stress and influence of antioxidant vitamins supplementation in patients with major depression. Current Neurobiology 2012; 3(2):107-111.
16. Kooti M, Matouri L. Fabrication of nanosized cuprous oxide using fehling's solution Transaction F. Nanotechnol 2010; 17(1):73-78.
17. Fazary A, Ju Y, Rajhi A *et al*., Bioactivities of Novel Metal Complexes Involving B Vitamins and Glycine. Open Chem 2016, 14(1):287-298.
18. NCCLS (1997). Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.
19. Meenakshi J, Kalai Gandhi V. Preparation of a Metal and Herbal Nanocomposite (HC_{NC}): Coating onto Wound Dressing Materials and Evaluating its Pharmacological Properties Like Antimicrobial Activity and Biocompatibility. Intl J Pharm 2016; 12(6):633-643.
20. Ruparelia JP, Chatterjee AK, Duttagupta SP, Mukherji S. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomater 2008; 4(3):707-16.
21. Zain NM, Stapley AG, Shama G. Green synthesis of silver and copper nanoparticles using ascorbic acid and chitosan for antimicrobial applications. Carbohydr Polym. 2014;4 Suppl 112:195-202.
22. Subhankari I, Nayak PL. Antimicrobial Activity of Copper Nanoparticles Synthesised by Ginger (*Zingiber officinale*) Extract. World J Nano Sci Technol 2013; 2(1):10-13.
23. Kim J, Cho H, Ryu S, Choi M. Effects of metal ions on the activity of protein tyrosine phosphatase VHR: highly potent and reversible oxidative inactivation by $Cu2^{+}$ ion. Arch Biochem Biophys 2000; 382:72–80.
24. Fatemeh Shaffiey, Sayedeh, Maryam Shapoori, Abbas Bozorgnia, Mohammad Ahmadi. Synthesis and evaluation of bactericidal properties of CuO nanoparticles against *Aeromonas hydrophila*. Nanomed J 2013; 1(3):198-204.
25. Anamika Jaiswal, Gaherwal S, Pavitra Devi Lodhi, Jaiveer Singh, Netram Kaurav, Shrivastava MMP. Synthesis and antibacterial properties of copper nanoparticles for *Salmonella typhi.* AIP Conference Proceedings 2016.
26. Dalia Abd, Al Kader, Fitua Alsaedi, Bahir Aziz M, Abdul Razzaq, Mshimesh Muhanad, Taqwa Jehad. Antibacterial Effects of Vitamin E: in Vito Study . J Biotech Res Center 2013; 7(2):17-23.
27. Gay R, Han SN, Marko M, Belisle S, Bronson R, Meydani SN. The effect of vitamin E on secondary bacterial infection after influenza infection in young and old mice. Ann N Y Acad Sci 2004;1031:418-21.

How to cite this article:
Haris Variyathody and Manohar M. An investigation on nano metal vitamin complex to control oxidative cell damage and microbial spoilage in seafood. J Pharm Sci Innov. 2018;7(4): 139-143.
http://dx.doi.org/10.7897/2277-4572.07497

Source of support: Nil, Conflict of interest: None Declared

Disclaimer: JPSI is solely owned by Moksha Publishing House - A non-profit publishing house, dedicated to publish quality research, while every effort has been taken to verify the accuracy of the content published in our Journal. JPSI cannot accept any responsibility or liability for the site content and articles published. The views expressed in articles by our contributing authors are not necessarily those of JPSI editor or editorial board members.

Silver Nanoparticle Conjugated Marine Invertebrate Antimicrobial Peptides (AgNPs - Amps) against Gram-Negative *ESKAPE* Pathogens

M. Manohar[1] & Muhammad Musthafa[2*] & K. Gopianand[2]
[1]Sadakkathullah Appa College, Tirunelveli, Tamil Nadu, India.
[2]Vivekanandha Arts & Science College for Women, Namakkal, Tamil Nadu, India.

Received: December 09, 2018 Accepted: January 21, 2019

ABSTRACT: *Due to a couple of reasons like increasing bacterial resistance to the commercial antibiotics, re-emerging bacterial diseases, identification of new infectious agents, challenges in the anti-tumor therapy etc., the scientific world is searching for new antibiotics with significant action modes. A group of bacteria, notorious for 'escaping' the actions of antibiotic and forming resistance to them are identified and described as 'the ESKAPE pathogens'.They are Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp.They pose a new threat in pathogenesis, transmission and drug and antibiotic resistance through newly acquired mechanism. The antimicrobial peptides (AMPs) are such molecules of a new promise with multiple mechanisms of action as antibiotics, antifungals, anti-biofilm agents, anti-fouling agents, anti-tumor agents etc. and are the key agents of the only innate immune system of marine invertebrates, which are exposed to a large number of pathogens. On the other hand, the metal nanoparticles which have proved their activity against multidrug resistant bacteria can be conjugated with AMPs to improve the potentiality of action against the MDR pathogens. The present review analyses the chances of conjugating silver nanoparticle (AgNPs) with the antimicrobial peptides (AMPs) to get the anti-MDR bacterial activity multiplied.*

Key Words: *Multidrug resistance; ESKAPE pathogens; marine invertebrates; antimicrobial peptides; silver nanoparticle*

1. Introduction

Over the past two decades the global emergence and spread of antimicrobial resistant strains of commonly encountered pathogens has been observed [1] and their alarming increase has become a serious global health issue threatening the achievements of modern medicine [2]. The World Health Organization warns that the 21st century may be seeing the beginning of a pre-antibiotic era, posing the biggest threat to health, food security and development [3]. Fatalities from antibiotic-resistant infections are predicted to rise into the millions by 2050. [4]. This trend of antimicrobial resistance (AMR) is more serious among bacterial pathogens [5] and a survey of hospital acquired infections (HAIs) in the United States in 2011 reported a total of about 722,000 cases, with 75,000 deaths associated with nosocomial infections [6]. A study conducted in 2002 had already estimated that, taking all types of bacterial infections into the account approximately 1.7 million patients suffered from HAIs, which contributed to the deaths of 99,000 patients per year [7].

The antibiotic resistance can affect anyone of any age, in any country and it leads to longer hospital stays, higher medical costs and increased mortality [3].Infections in hospital-born babies were estimated to account for up to 56% of all neonatal deaths in some under-resourced countries. *Klebsiella pneumoniae, Escherichia coli, Pseudomonas* spp., *Acinetobacter* spp. and *Staphylococcus aureus* were the most frequent causative pathogens of neonatal sepsis; 70% of these isolates would not be eliminated by an empiric regimen of ampicillin and gentamicin. Many infections could be untreatable in resource-constrained environments, as a recent report demonstrated that 51% of *Klebsiella* spp. were extended-spectrum β-lactamase (ESBL) producers, 38% of *Staphylococcus aureus* strains were methicillin-resistant (MRSA) and 64% were resistant to co-trimoxazole [8].

The most serious, life-threatening infections are caused by a group of drug-resistant bacteria are named by the Infectious Diseases Society of America (IDSA) as *ESKAPE* pathogens, as they effectively escape the effects of antibacterial drugs. The six *ESKAPE* bacteria are *Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii*(A), *Pseudomonas aeruginosa* (P) and *Enterobacter* species/*Escherichia coli* (E). These bugs are responsible for two third of all healthcare associated infections (HAIs) [9]. They form the most common multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria [10], representing a serious threat to the patients who are frequently in an immunocompromised state (e.g. those undergoing transplantation, cancer and critically ill patients [5].

The objective of the present review is to consider the clinical importance of emerging Gram negative members of the *ESKAPE* pathogens and to investigate the possibility of using marine invertebrate derived antimicrobial peptides (AMPs) conjugated with silver nanoparticles (AgNPs) as a promising tool in controlling theses 'bad bugs'.

2. The *ESKAPE* pathogens

The *ESKAPE* pathogens will be of increasing relevance to antimicrobial chemotherapy in the coming years, not only due to the clinical threat they pose, but the anticipated honing of academic and industrial interests towards them[11]. The *ESKAPE* pathogens are known to 'escape' the effects of currently marketed antimicrobial agents. They are frequently multidrug-resistant (MDR) and are associated with poor patient outcomes because the patients infected with *ESKAPE* pathogens often receive inappropriate empirical antimicrobial therapy that leads to unfavorable clinical outcomes, high case fatality rates and opportunities for pathogen spread to other patients [12]. *ESKAPE* pathogens frequently present clinicians with serious therapeutic dilemmas because of their complex resistance profiles [13]. The following is a brief introduction to the group members.

2.1. Enterococcus faecium

Enterococci are gram positive, facultatively anaerobic, opportunistic pathogens frequently involved in Healthcare Associated Infections (HAIs) or nosocomial infections and can cause severe infectious diseases especially among immunocompromised patients [7, 14]. *Enterococcus* species were formerly classified as part of the genus *Streptococcus* [7]. They are distribute extensively in nature such as grounds, water, plants, food and are a part of the normal flora of human and animal enteric tract. [14]. Within the hospital environment, *Enterococci* have been described as 'triple-threat' pathogens displaying exemplary colonization of the gut and skin - with Enterobacteriaceae and *Staphylococcus aureus* respectively – alongside an environmental persistence characteristic of *Clostridium difficile*[15]

Some strains of the genus *Enterococci* are resistance to a broad number of antimicrobial drugs such as erythromycin, glycopeptides, tetracycline, vancomycin (vancomycin - resistant *Enterococci* - VRE, which have nine vancomycin resistance genes as *van* A, B, C, D, E, G, L, M and *van* N. [14]), aminoglycosides (high-level resistance - HLR), gentamicin (high-level resistance gentamicin - HLGR) and streptomycin (high-level resistance streptomycin - HLSR. These strains show inherent and acquired antimicrobial resistance. [16]. Also, the transmission of vancomycin resistant gene from *Enterococcus* spp. to Staphylococcus *aureus* has been detected in the laboratory. Since vancomycin has been considered as a drug of choice for treatment of Enterococcal and MRSA infections, the emergence of vancomycin- intermediate (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) can produce important problems to the treatment of *Staphylococcus aureus* [14].

Apart from the world widely reported nosocomial infections, various reports have demonstrated that *Enterococcus faecalis* and *Enterococcus faecium* can cause several infections including septicemia with higher toxicity rates in patient such as nephrotoxicity than monotherapy diet [17].

2.2. Staphylococcus aureus

Staphylococcus aureus - a Gram positive coccus – is a common representative of the skin microbiota and is most commonly isolated from the moist areas such as the anterior nares and the axillae. This was the first microorganism in clinical history to be shown growing as a biofilm. Nearly 25 - 30% of skin or noses of healthy people are colonized by *Staphylococcus aureus* [14, 18].

They show a wide category of resistance characteristics described as methicillin-resistant *Staphylococcus aureus* (MRSA – which are resistant to certain antibiotics such as methicillin, dicloxacillin, oxacillin, cloxacillin, nafcillin and closely related class of drugs such as cephalosporins) [14, 18], healthcare associated (HA-MRSA) or community-associated (CA-MRSA), vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) and so on [14]. Infected patients and health care providers may act as carriers and play a significant role in spreading and transferring this bad bug in hospitals. *Staphylococcus aureus* strains isolates from the hospital are named as hospital acquired *Staphylococcus aureus* (HASA) [14, 19, 20].

Staphylococcus aureus causes atopic dermatitis (AD) and induces skin inflammation by secreting super antigens following a chronic relapsing course and defects in innate and acquired immune responses resulting in a heightened susceptibility to bacterial, viral and fungal infections [14]. The high rate of *Staphylococcus aureus* colonization in the nasal cavity and skin lesions of AD patients has been reported, especially in ranges between 76–100%, compared to 2–25% in healthy people [14, 21].

2.3. *Klebsiella pneumoniae*

Klebsiella pneumonia are non-fastidious, Gram negative bacillibelonging to the family Enterobacteriaceae. They are usually encapsulated and frequently cause lower respiratory tract infection and catheter-associated urinary tract infection [7]. Along with *Escherichia coli*, and together they lead a mounting pathological threat as they are globally prevalent in both the hospital and community sectors [22].

Klebsiella pneumoniae is one of the MDR organisms identified as an urgent threat to human health by the World Health Organization, the US Centers for Disease Control and Prevention and the UK Department of Health. *Klebsiella pneumoniae* infections are particularly a problem among neonates, elderly and immunocompromised individuals within the healthcare setting, but this organism is also responsible for a significant number of community-acquired infections including pneumonia and sepsis [23, 24].

2.4. Acinetobacter baumannii

Acinetobacter baumannii is a typically short, almost round, rod-shaped (coccobacillus), Gram negative bacterium. It can be an opportunistic pathogen in humanand is becoming increasingly common in intensive care units (ICUs), causing infections that include bacteremia, ventilator associated pneumonia, meningitis, urinary tract infections, central venous catheter-related infections and wound infections [22, 25].

The most important human pathogen belonging to this genus is *Acinetobacter baumannii*, which has a relatively long survival time on human hands, and can lead to high rates of cross contamination in nosocomial infections [26]. Many strains of *Acinetobacter baumannii*is highly resistant even to carbapenems and polymyxin/colistin in some instances and thushas arisen as a particularly worrisome threat for which there is no new antibiotic in development. Thus, the emergence of antibiotics-resistant *Acinetobacter baumannii* especially, multi-resistant strains seriously challenges the treatment of these infections [27].

2.5. Pseudomonas aeruginosa

This is a Gram negative, rod-shaped, facultatively anaerobic bacterium which is a part of the normal gut flora that has an intrinsic resistance characteristic. The outcomes of infection caused by MDR *Pseudomonas* spp. may be related to enlarge morbidity and mortality, which can cause narrow effective antimicrobial choices as resistance to at least three of the five classes of antibiotics, mainly carbapenems, antipseudomonalpenicillins, cephalosporins, aminoglycosides and fluoroquinolones [7]. *Pseudomonas aeruginosa* is responsible for 9% of all healthcare-associated infections and the resistance to *Pseudomonas aeruginosa* is increasing [28]. *Pseudomonas aeruginosa* that infects Cystic Fibrosis patients has serious implications for infection control in the hospitals [7].

2.6. Enterobacter species

Enterobacter spp. are Gram negative rods that are sometimes encapsulated and are significantly responsible for urinary and respiratory tract infections. The emergence of antimicrobial resistance among *Enterobacter* spp. is of great concern worldwide in human medicine [29, 30]. They are also known to cause bloodstream infections and are becoming increasingly responsible for serious nosocomial infections, displaying broad MDR [7]. The resistance of *Enterobacter* spp. to extended-spectrum cephalosporins (ESC) is of particular concern [31]. They can cause opportunistic infections in immunocompromised, usually hospitalized patients and contain a wide range of antibiotic resistance mechanisms [7].

3. Antibiotic resistance in Gram negative *ESKAPE* pathogens

Out of the above mentioned MDR pathogens, the Gram-negative *ESKAPE* bacteria act as important reservoirs and transmitters of resistance, and are responsible for increased reporting of antimicrobial resistant nosocomial infections worldwide [12].The increasing prevalence of carbapenem-resistance presents a significant challenge for physicians as carbapenems are conventionally used to treat persistent infections caused by Gram-negative bacteria [7, 32]. Even if several intensive infection control practices are used, outbreaks of carbapenemase-mediated multidrug resistant (MDR) strains are only reduced and cannot be completely eradicated [7].

3.1. *Klebsiella pneumonia*

Klebsiella pneumonia belongs to the extended-spectrum β-lactamase or ESBL strains and is increasingly multidrug-resistant (MDR) to a wide spectrum of antibiotics such as cephalosporin or ceftazidime [33]. In recent years, many *Klebsiella pneumoniae* strains have acquired a massive variety of β-lactamase enzymes which can destroy the chemical structure of β-lactam antibiotics such as penicillins, cephalosporins and carbapenems [7, 34]. The emergence of *Klebsiella pneumonia* isolates producing carbapenemases (KPCs) has become a major obstacle in the last 5 years. Carbapenemases are able to destroy the carbapenems and cause resistance against a wide spectrum of antibiotics [14].

In addition, the emergence of the *Klebsiella pneumoniae* super enzyme, known as NDM-1 has increased the proportion of carbapenem-resistant *Klebsiella pneumoniae* isolates and may pose a threat to other antibiotics such as β-lactams, aminoglycosides and fluoroquinolones [7, 35]. As the carbapenems are conventionally used to treat persistent infections caused by Gram-negative bacteria, the increasing prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP), with resistance encoded by *bla*KPC presents a significant challenge for physicians [7].

3.2. *Acinetobacter baumannii*

Acinetobacter baumannii is intrinsically resistant to antibiotics due to the protection afforded by a Gram-negative outer membrane, constitutively expressed active efflux pump systems and low-quantity expression of small-aperture outer membrane porins (which greatly reduce the permeability of antimicrobials) [7, 14, 22].The epidemic potential and the clinical severity of *Acinetobacter baumannii*infections are primarily related to the capability to survive and propagate in healthcare settings and to expand resistance to a diversity of antimicrobial agents containing fluoroquinolones, broad-spectrum β-lactams and carbapenems [14, 36]. MDR *Acinetobacter baumannii*is resistant to 3 or more several classes of antibiotics containing β-lactams, aminoglycosides, fluoroquinolones and 3rd generation of cephalosporin [36]. Pan-drug resistance (PDR) *Acinetobacter baumannii*was described as the strains that were resistant to all examined antibiotics excluding colistin and tigecycline [14].

Recently, the emergence of carbapenemase producing *Acinetobactebaumannii*strains carrying imipenemmetallo-β-lactamases, encoded by *bla*IMP and oxacillinase serine β- lactamases encoded by *blaOXA* has been reported. These strains show resistance to both colistinand imipenem, and the combination of resistance genes makes them capable of evading the action of most of the traditional antibiotic compounds [7, 37].

3.3. *Pseudomonas aeruginosa*

The reduced permeability of the Gram negative outer membrane,function of multidrug efflux pumps, mutations in targets of antibiotics, the horizontal gene transfer of antibiotic resistance determinants etc. *make* the *Pseudomonas aeruginosa*resistant to a range of antimicrobials[14, 38]. Many of the *Pseudomonas aeruginosa* strains show an intrinsic reduced susceptibility to several antibacterial agents as well as a propensity to develop resistance during therapy especially in carbapenem-resistant (chiefly imipenem) strains [14, 39]. The emergence of MDR in *Pseudomonas aeruginosa* containing resistance to β-lactams, aminoglycosides and fluoroquinolones is extremely problematic in the treatment of burn patient. Serious infections such as burn wounds due to *Pseudomonas aeruginosa* are treated through the combination of a β-lactam drugs and an aminoglycoside. The continuous increase of MDR isolates presents a complicated situation for antimicrobial therapy; however colistin is still effective in most cases [14, 7].

3.4. *Enterobacter* spp.

Many *Enterobacter* strains contain ESBLs and carbapenemases including VIM, OXA, metallo-β-lactamase-1 and KPC [7]. Furthermore, stable de-repression of the AmpCβ-lactamases that can be expressed at high levels by mutation in this bacterial group is also notable. These MDR strains are resistant to almost all available antimicrobial drugs except tigecycline and colistin [7, 40]. Extended-spectrum β-lactamases (ESBLs) and carbapenemases have been identified in *Enterobacter* spp., exacerbating the issue of extended-spectrum cephalosporins (ESC) resistance. An even greater concern is that most ESC-resistant *Enterobacteriaceae* exhibit multidrug resistance, including fluoroquinolone resistance, mainly due to chromosomal mutations in the enzymes targeted by the drug and plasmid-mediated quinolone resistance (PMQR) [14, 40]. In recent years, these resistance mechanisms have been well documented among *Enterobacter* spp. isolates across several countries [7, 22].

Although few antimicrobials such as colistin and tigecycline are influenced by these resistant bacteria (and also against many of the other *ESKAPE* pathogens) there is little or no drugs in the 'pipeline', being remarkably effective in addressing this mounting health crisis [14]. In short, nosocomial *ESKAPE* bacteria represent paradigms of resistance, pathogenesis and disease transmission.

4. Mechanisms of antimicrobial resistance in Gram negative *ESKAPE* pathogens

There is a wide range of antimicrobial resistance mechanisms used by the nosocomial *ESKAPE* pathogens including enzymatic inactivation, modification of drug targets, changing cell permeability and mechanical protection provided by biofilm formation. Antimicrobial resistance genes may be carried on the bacterial chromosome, plasmids or transposons [41]. Mechanisms of drug resistance may be one or a combination of two or more of the following categories.

4.1. *Drug inactivation or alteration*

Many bacteria produce enzymes (that irreversibly modify and inactivate the antibiotics) such as β-lactamases, aminoglycoside-modifying enzymes or chloramphenicol acetyl transferases. The β-lactamases are highly prevalent and act by hydrolyzing the β-lactam ring which is present in all β-lactams like penicillins, cephalosporins, monobactams and carbapenems [42]. β-lactamases are classified using two main classification systems: the Ambler scheme (molecular classification) and the Bush-Jacoby-Medeiros system [43], out of which the former is being discussed here briefly.

Ambler class A enzymes consist of penicillinase, cephalosporinase, broad-spectrum β-lactamases, extended-spectrum β- lactamases (ESBLs) and carbapenemases. They can inactivate penicillins (except temocillin), third-generation oxyiminocephalosporins (e.g: ceftazidime, cefotaxime and ceftriaxone), aztreonam, cefamandole, cefoperazone and methoxycephalosporins (e.g: cephamycins and carbapenems). Class A enzymes can also be inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam or tazobactam [7]. The Ambler class A group contains a number of significant enzymes including ESBLs (eg: TEM-1 which is widespread among the members of the family Enterobacteriaceae like *Klebsiella pneumoniae*, *Enterobacter* spp. and in non-fermentative bacteria such as *Pseudomonas aeruginosa*. Another set of enzymes, the CTX-Ms have been identified in *ESKAPE* pathogens including *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. Some of the highest prevalence and significant clinical impact are associated with the extended spectrum β-lactamases in *Klebsiella pneumoniae* [7, 44]) and KPCs (eg: KPC-1 which is prevalent in *K. pneumonia* that imparts resistance to imipenem, meropenem, amoxicillin/clavulanate, piperacillin/tazobactam, ceftazidime, aztreonam and ceftriaxone [7]. The fast propagation of *Klebsiella pneumonia* resistance is perhaps due to the *bla*- KPC gene carriage on plasmids. Plasmid-mediated imipenem-hydrolyzing enzyme (KPC-2) among multiple carbapenem resistant bacteria is increasing [45].

Ambler class B enzymes or group 3 enzymes include metallo- β-lactamases (MBLs), which require Zn^{2+} as a cofactor. Bacteria that produce these enzymes show resistance to all β-lactams including penicillins, cephalosporins, carbapenems and β-lactamase inhibitors except aztreonam. Genes encoding MBLs are found on plasmids; hence, they are easily transmitted to other microorganisms. The most common MBLs are imipenemasemetallo-β- lactamases (IMP - found in *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter cloacae*), veronaintegron encoded metallo-β-lactamases (VIM - mostly in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) and the newly described New Delhi metallo-β-lactamase-1 (NDM-1 identified in *Klebsiella pneumoniae* and *Enterobacter cloacae*) [7, 46].

The Ambler class C group consists of enzymes including penicillinase and cephalosporinase such as AmpCβ-lactamase, which results in low level resistance to narrow-spectrum cephalosporin drugs. Chromosomally encoded AmpC are usually identified in *Pseudomonas aeruginosa* and bacteria in the Enterobacteriaceae family such as *Enterobacter* species where their production is typically in very low level and does not elicit any clinically relevant resistance but can be inducible during drug therapy. In *Enterobacter* spp., the extended-spectrum cephalosporins (ESC) resistance is most typically caused by the overproduction of AmpC β-lactamases, which is due to the de-repression of a chromosomal gene or the acquisition of a transferable AmpC β-lactamase [47].

Ambler class D consists of a variety of enzymes like oxacillin hydrolyzing enzymes (OXA). The most common members of this class such as OXA-11, OXA-14 and OXA- 16 demonstrate ESBL properties and are normally found in *Pseudomonas aeruginosa* [7, 48]. Almost all of the OXA enzymes except OXA-18, are resistant to β-lactamase inhibitors [20]. Furthermore, OXA-type carbapenemases are commonly found in *Acinetobacter* spp. [7].

4.2. *Modification of drug binding sites*

Some resistant bacteria avoid recognition by antimicrobial agents by modifying their target sites. The mutation of gene encoding for penicillin-binding proteins (PBPs) results in the expression of unique penicillin-binding proteins. By changing the peptidoglycan crosslink target (D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser) encoded by a complex gene cluster (*Van*-A, *Van*-B, *Van*-D, *Van*-C, *Van*-E and *Van*-G), *Enterococcus faecium* and *Enterococcus faecalis* can increase their resistance to many of the glycopeptides in current clinical use [41].

4.3. *Reduced intracellular drug accumulation*

Reducing the amount of antibiotic able to pass through the bacterial cell membrane is one of the strategis used by bacteria to develop antibiotic resistance. This may be achieved by either of the following methods.

4.3.1.Porin Loss.

The outer membranes of Gram-negative bacteria contain proteins called porins that form channels which allow the passage of many hydrophilic substances including antibiotics. *Pseudomonas aeruginosa, Acinetobacter baumannii* and MDR *Klebsiella pneumonia* make use of this mechanism in particular cases [7, 49].

4.3.2. *Efflux Pumps.*

To increase the removal of antibiotics from the intracellular compartment some bacteria contain membrane proteins that function as exporters, called efflux pumps for certain antimicrobial agents. These pumps expel the drug from the cell at a higher rate and most efflux pumps are multidrug transporters that efficiently pump a wide range of antibiotics and thus contributing to multidrug resistance. This has been reported in different strains of *Pseudomonas aeruginosa, Enterobacter aerogenes* and *Klebsiella pneumoniae* [7, 50].

4.4. *Biofilm formation*

Biofilms are complex microbial communities living as a thin layer on biotic or abiotic surfaces in a matrix of extracellular polymeric substances created by the members themselves [51]. The matrix of biofilms seems to provide a mechanical and biochemical shield that provides the conditions needed to attenuate the activity of the drugs (e.g. low O_2, low pH, high CO_2 and low water availability). Under these conditions it is difficult to eliminate bacteria using conventional antibiotics. Moreover, when the bacteria experience nutrient scarcity, they could become tolerant to antibiotics [7]. The most common Gram negative *ESKAPE* pathogens found in biofilms in a healthcare setting are *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumonia* [52].

As mentioned elsewhere, members of the *ESKAPE* pathogenic group may achieve the drug resistance through a combination of two or more mechanisms described above. For example, the most common mechanism of imipenem resistance in *Pseudomonas aeruginosa* is a combination of chromosomal AmpC production and porin change [7, 53]. Also, *Pseudomonas aeruginosa* produces ESBLs and can harbor other antibiotic resistance enzymes such as *Klebsiella pneumoniae* carbapenemases (KPC), VIM encoded by *bla*VIM gene and imipenemmetallo-β-lactamases. The combination of these enzymes leads to high rates of carbapenem resistance amongst *Pseudomonas aeruginosa* isolates and also to the emergence of fluoroquinolone-resistant strains as the corresponding mechanisms of resistance may be carried by the same plasmid [7].

5. Need for new antibiotics

The growing number of bacteria resistant to conventional antibiotics, reemergence of bacterial diseases, identification of new infectious agents, nosocomial and other dreaded infections by *ESKAPE* pathogens, limited number of available antibiotics, the similarities in the activity spectrum of the antibiotics [5, 54] etc. pose a major threat to the human life on earth [1] and has resulted in a major crisis in modern medicine which has stimulated major research efforts to develop novel antibiotic therapeutics [1, 55]. Unfortunately, the development of novel classes of antibiotics has been limited in the last 4 decades [5, 56]. While the development of new antibiotic drugs has been dramatically slow, the resistance rate has increased rapidly. The diversity of bacterial resistance mechanisms has largely outperformed our current classes of antibiotics [5]. Antimicrobial resistance causes major limits to treatment options in infected patients, particularly in isolates with carbapenems resistant [14, 57]. There is no current therapy that consistently provides bactericidal activity for critical infections due to vancomycin - resistant *Enterococci* (VRE) [14].

Nosocomial *ESKAPE* bacteria represent paradigms of resistance, pathogenesis and disease transmission. Antimicrobial resistance in these pathogens is a major menace to public health systems worldwide and seems likely to increase in the near future as resistance profiles change. Thus it remains as a challenge as there is no new antimicrobial agents with spectra of activity that reliably encompass multidrug-resistant and pan-resistant Gram negative *ESKAPE* isolates have not appeared in as a timely manner as hoped, and nosocomial infections remain a constant concern for patient health, particularly for critically ill inpatients as well as for patients requiring placement of invasive devices or surgical procedures [13, 37].There are very few new antimicrobial agents in development to treat gram-negative *ESKAPE* pathogens despite the well-recognized need [12, 37].

As the rise of new diseases is outnumbering, the goal of the pharmaceutical research is to find a new drug or a novel therapeutic compound to fight against deadly diseases. Though there are an umpteen number of drugs commercially available in the market, their use could be restricted due to the limitations such as side effects, no selectivity, low specificity and toxicity etc. among others [58]. There is an urgent

need to develop novel antibiotics with less chance of resistance development to combat the threat from multidrug-resistant (MDR) pathogens [1]. The Infectious Diseases Society of America (IDSA) supported a program called 'Bad Bugs, Need Drugs; 10 × '20 Initiative', with the aim of developing ten new systemic antibacterial drugs by 2020 [37].

A lack of new antibiotics for treatment together with the appearance and persistence of multidrug-resistant bacterial strains, has led to demands that new antimicrobial strategies be developed and explored [59]. Thus, intensive nonclinical and clinical research is now invested into the identification of new and non-conventional anti-infective therapies, including adjunctive or preventive approaches such as antibodies targeting a virulence factor, probiotics and vaccines [5, 54, 60]. This results in the dearth of potential therapeutic agents in the pipeline that causes real concerns but should trigger research and development of new antibiotics or new approaches to control the infections they cause [7].

6. The importance of marine source

The ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity with estimates ranging between 3 and 500 × 10^6 different species [61]. They constitute almost 80% of the world biota and the marine macro fauna alone comprise 0.5 to 10 × 10^6 species [62]. It is a wide and largely unexplored environment[59] with genetic uniqueness and diversity (in the tropical zones there are almost 1000 different species per square meter) [63]and has the presence of large chemical diversity structures (which is only rarely seen in terrestrials)[64].Out of the 33 known animal phyla, 32 are present in the aquatic environment& 21 are exclusively marine[61, 65].

Since marine organisms live in complex habitats and are exposed to extreme conditions such as salinity, pressure, temperature and illumination, they produce a wide variety of secondary metabolites that cannot be found elsewhere [66]. The marine organisms also have special structures that imparts different bioactivities ike antioxidant activity, antimicrobial activity, anticancer activity, antihypertensive activity, anti-inflammatory activity and so forth [67].

The marine environment is a potentially prolific source of highly bioactive secondary metabolites (in organisms such as fish, shellfish, molluscs, univalves, cephalopods, crustaceans and echinoderms, which significantly contribute to economic and research development [66] that might represent useful leads in the development of new pharmaceutical agents [61]. The first attempt to locate antimicrobial activity in marine organisms was initiated in 1950s. Since then, a large number of marine organisms from a wide range of phyla have been screened for antimicrobial activity. Many of these organisms have antimicrobial properties although most of the antibacterial agents that have been isolated from marine sources have not been active enough to compete with the classical antimicrobial activity[68].

However, utilization of marine organisms as sources of bioactive metabolites started seriously at the end of 1960s [69] with the isolation of prostaglandin derivatives from the Caribbean Gorgonian - *Plexaurahomomalla*. In the 1980s effective collaborations were established between marine chemists and pharmacologists and the investigations were focused on central nervous system membrane active toxins, ion channel effectors, anticancer and anti-viral agents, tumor promoters and anti-inflammatory agents [70].

7. Drugs from marine invertebrates

Invertebrates comprise approximately 60% of all marine animal diversity [62] and most of them belong to the phyla porifera (sponges), annelida, arthropoda, bryozoa, cnidaria, echinodermata, mollusca and chordata. Several studies addressing marine invertebrates also include these groups of organisms [70].

Marine invertebrates have proven to be the rich sources of bioactive compounds with activities ranging from antimicrobial to antitumor. [72]. This is better explained by the fact that, they lack an acquired, memory-type immunity based on T-lymphocyte subsets and clonally derived immunoglobulins, which means that their defense relies solely on innate immune mechanisms that include both humoral and cellular responses which differs from the vertebrate immune system [61].

The marine invertebratesare known for the presence of potential antimicrobial compounds in the blood and plasma. Their survival in an environment with invading microorganisms, numbering up to 10^6 bacteria/ml and 10^9 virus/ml of seawater, some of which are also present in anthropic environments [73] suggests that their innate immune system is effective and robust [74].

Marine natural sources as potential anticancer agents were reviewed in 2011 which mentioned 39 marine-derived potential anticancer agents, and among them 18 compounds were from sponges with different mechanisms of action. Interestingly, from the 16 marine natural products that are currently under preclinical trials as new drug candidates, most are derived from invertebrates [70]. Invertebrates - mainly sponges, tunicates, bryozoans or mollusks provided the majority of the marine natural products involved in clinical or preclinical trials [75]

The discovery of marine natural products has accelerated over the last two decades with the number of new compounds discovered annually increasing from 20 to more than 200 [76]. It has been estimated that by 2010 more than 15,000 new marine natural products (NMNP) had been discovered with 8368 new compounds recorded for the decade between 2001 and 2010. This constitutes over half of all the compounds discovered since 1951. [70]. Out of the NMNPs from invertebrates, 33% from sponges, 18% from coelenterates (sea whips, sea fans and soft corals) and 24% from the representatives of other invertebrate phyla such as ascidians (also called tunicates), opistho-branch mollusks (nudibranchs, sea hares, etc), echinoderms (starfish, sea cucumbers etc.) and bryozoans (moss animals) [77].

TABLE 1. The most important bioactive compounds isolated from marine non-chordates.

Marine sources (Major phylum)	Major bioactive compounds	Disease prevention
Porifera	Manzamine, phenolic or quinoid, alkaloids , terpenoids, brominated tryptamines,	Antimalarial, antiviral especially AIDS, antibacterial, antifungal, anticancer
Coelenterata	Postaglandins, proteins, enzymes, steroids, terpenoids, brominated alkaloids, macrolides and ceramides	antibacterial, antifungal, antialgal, cariac and nerve muscle relaxation, antitumour, anticancer, antineoplastic
Annelida	Peptides, Arenecins, hedistins, antimicrobial peptide (AMP),	Arthritis, oesteoporesis, bone cancer, antimicrobial, antibacterial, antifungal
Arthropoda	Lectin viz. limulin and carcinoscorpin, thiol ester protein, fatty acids, triglycerides, carotenoids and lipids,	Antibacterial, anti cancer, antioxidant, antiproliferative, antimutagenic, antiinflamatory, immune response
Mollusca	Dolostatin, lectin, steroid, terpenoids, acetylenic compounds, dollstains, polysaccharides	Antleukemic, immune response, hypotension, relaxation smooth muscle, antinicotinic activity, antiviral especially HIV virus inhibiting compound
Echinodermata	Saponinsans sterol derivatives, tarpenoids, glycoproteins, cerebrosides, pyrimidine nucleaosides, thymine deoxyriboside and uracil deoxyribose, polysaccharides, β-carotene	Hemolytic, antibacterial, antifungal, antineoplastic, antitumor, antiviral especially anti HIV activity, antiinflamatory, anti-cancer, anti-allergic

8. Antimicrobial peptides (AMPs) – the 'natural antibiotics'

AMPs are themajor component of the innate immune defense system in marine invertebrates with a broad spectrum of antimicrobial activities against bacteria, viruses and fungi [77]. They are also termed as "natural antibiotics"and areevolutionarily conserved [78].Alternative terms for AMPs have also appeared which include better descriptive terms such as ''host defense peptides,'' ''alarmins'' and even ''defensins'' (used in a broad context instead of the gene family)[79]

AMPs are relatively short (commonly consist of 6–100 amino acid residues, display an overall positive charge ranging from +2 to +11 and contain a substantial proportion (typically 50%) of hydrophobic residues [80]. They are mostly ribosomally produced peptides with less than 10 kDa in mass and provide an immediate and rapid response to invading microorganisms and are post-translationally activated by proteolytic cleavage [61].

AMPs have been found virtually in all the organisms including bacteria, fungi, protozoa, parasites and in vertebrates as a host defense mechanism against invading microorganisms and they display remarkable structural and functional diversity [81]. Even the human skin secrets some AMPs that act as a barrier against the invasion of microorganisms.[82]. Besides the direct antimicrobial activity, AMPs carry immunomodulatory properties [83], which make them especially interesting compounds for the development of novel therapeutics or food additives [59]. The ubiquitous presence of AMPs (AMPs) in nature attests to their overall importance in building the defense strategies of most organisms. They are

considered part of the humoral natural defense of invertebrates against infections. Their enhanced expression, due in part to various stress factors such as infective organisms, has been directly linked to the quick and efficient innate immune response of their hosts [59].

Sources of AMPs range from single celled microorganisms such as bacteria (bacteriocins) to invertebrates. Many AMPs exhibit relatively non-specific bactericidal activity against both Gram-positive and Gram-negative species and selectively kill prokaryotic rather than eukaryotic cells. Their value in innate immunity lies in their ability to function without either high specificity or memory. Moreover, they are synthesized without dedicated cells or tissues and they can rapidly diffuse to the point of infection[84, 85].

Since their discovery in the 1980s, AMPs have been considered to be promising candidates for therapeutic uses in humans, animals and plant health. During, more than the last 30 years, AMPs have been considered as a potential source for the development of new therapeutic molecules to control infectious diseases owing to their significant specificity for micro-organisms with low toxicity for mammal cells [58].Due to the fact that they exhibit broad spectrum antimicrobial activity, possess selective toxicities and are less prone to microbial resistance [2], AMPs represent an exciting class of bioactive compounds that could potentially provide major reprieve for mankind in the efforts to curb/control infections [59].

The biological activities of the amino acid residues of AMPs are based on their composition and sequence [67]. Recently, much attention has been paid to unravelling the structural, compositional, and sequential properties of bioactive peptides [86]. Many marine organisms are exposed to more extreme conditions than that on land, which make the marine bioactive peptides have significant different amino acid compositions and sequences from land bioactive peptides; besides, the species and amounts of marine bioactive peptides are more than that of land bioactive peptides. Moreover, marine bioactive peptides can be obtained from various marine animals, plants and lower organisms. Each is unique as a species, considering its great taxonomic diversity and special characteristics, marine bioactive peptides have better bioactivity in some areas than land bioactive peptides [67]. Despite these responses, additional care has to be taken when assessing the origin of the antimicrobial content in marine organisms [59]

The cationic charge of the AMPs promotes selectivity for negatively charged microbial cytoplasmic membranes over zwitterionic mammalian membranes and hydrophobic residues facilitate interactions with the fatty acyl chains. Many eukaryotic peptides act on bacterial membranes or other generalized targets, in contrast to most antibiotics, which usually target specific proteins. This creates an advantage for AMPs as the development of microbial resistance by gene mutation is less likely to occur[59, 87]. New vistas on AMPs suggest that they are capable of playing multifunctional roles that extend beyond their capacity to act as gene-encoded antibiotics. Thus, some of these peptides have not only been shown to display anticancer activity [88], but also act effectively to stimulate the immune system by favoring cytokine release, chemotaxis, antigen presentation, angiogenesis, inflammatory responses and adaptive immune induction [89, 59]

AMPs are a potential source of novel antimicrobial agents currently being extensively investigated and are considered attractive alternatives to conventional antibiotics in the fight against MDR pathogens [2]. The combination of a broad spectrum antimicrobial activities targeted at non-protein cellular components with localized, high-level expression at the site of infection, makes AMPs highly effective antimicrobial agents with significant potential as a source of new antimicrobial drugs such as new more effective anti-tubercular agents active against multidrug resistant (MDR) and extensively drug resistant (XDR) Mycobacterium tuberculosis complex pathogens [90, 91].

In many organisms AMPs are produced as inactive precursors requiring proteolytic cleavage to become active [92]. Their regulation is therefore not only dependent on their own expression but also on the abundance of appropriate proteases [93]. In multicellular organisms, some AMPs are constitutively expressed, stored at high concentrations as inactive precursors in granules and released locally at infection and inflammation sites, whereas the expression of others is induced in response to pathogen- associated molecular patterns (PAMPs) or cytokines [3, 93]. Criticality emerges in the case of deeper skin burn patients prone to nosocomial bacterial infections, their medical treatment becoming a major problem. [82]

Only a few AMPs have reached phases of clinical and preclinical pipelines [82, 95]. They have limitations in therapeutic development due to their poor enzymatic stability and low permeability across biological barriers [2, 96]. Chemical modifications such as peptide cyclization, the use of non-protein amino acids, peptidomimetics, lipidation, etc. are often used to overcome these drawbacks, particularly to enhance enzymatic stability [2].

There are encouraging examples of AMPs already introduced into the market, and many AMPs are currently being tested in clinical trials [97], which provide are as on for optimism for introduction of novel AMP-based drugs in several indication areas [60]. An updated database (Jan. 2012) shows 1773 AMPs and

are distributed as: antiviral (5.8%), antibacterial (78.56%), antifungal (31.19%) and antitumor (6.14%).Means, some of them function against more than one type of pathogens [58], in short, as a class, natural and synthetic AMPs exhibit a very high potential as new therapeutic agents because of their novel mechanism of antimicrobial activity, coupled with the difficulty of bacteria to develop resistance to them. 19–21 AMPs have also been shown to be key components of the innate immune response [60, 98]

Considering the diverse functions that AMPs exert, much of the research in this field has been focused over the past decade on the bioprospecting of novel antimicrobial compounds capable of exhibiting a broad spectrum of activity against a wide range of microorganisms, which include Gram-positive and Gram-negative bacteria, yeasts, fungi, viruses, protozoa and parasites such as nematodes [77]. More recently, bioinformatics tools have also helped to develop and/or remodel preexisting AMPs, driving their synthesis toward more selective and effective drugs [59]. Several databases exist for natural AMPs, today covering more than 2000 peptides [99]. Thus, interestingly, the AMPs (AMPs) have rapidly captured attention as novel drug candidates**[60]** and are currently being used for drug development due to their activity as immune modulators, which give them clinical potential beyond the treatment of antibiotic-resistant strains [100].

9. Marine invertebrate derived AMPs (AMPs) - a new promise

When considering marine invertebrates which are thought to account for 30% of all animal species, comprising 20 different phyla, it is noteworthy that they should have retained an ancestral, nonspecific innate immune-defense system mainly composed of AMPs. In such animals, critical elements such as antibodies and lymphocytes, immunological memory and true self *vs.* non-self-discrimination are absent. [59]. The cellular component of marine invertebrate immunity – as mentioned elsewhere in this article, is mediated by hemocytes, motile cells that phagocytose microbes, secreting soluble antimicrobial and cytotoxic substances in the hemolymph. For this reason, most of the AMPs reported in different groups of invertebrates have been isolated from hemocytes, which represent an interesting natural source of AMPs [59, 65].

AMPs with potential interest for biopharmaceutical companies have been isolated from marine invertebrates. Some AMPs, such as molluscdefensins, have very low MICs in the nanomolar range against Gram-positive bacteria [101]. Their fungal homologue – plectasin - is considered to be a major candidate for therapeutic use [100]. The AMPs characterized in marine invertebrates include those from arthropods, molluscs and cnidarians. They are cationic and hydrophobic, and target essential components of microbial cell walls and membranes, which determines their spectrum of activity [102]. In recent years, researchers have isolated AMPs from mud crab *(Scylla paramamosain)*, oyster *(Crassostreagigas),* sponge *(Trichoderma*sp.), the marine snail *(Cenchritismuricatus)* etc.[66]. Among AMPs from marine invertebrates, ALF-derived peptides have been shown to modulate the inflammatory response in murine macrophage cell lines and display anti-tumour activity against HeLa cells through the alteration of the cell membrane. Those novel activities may open the way to future drug developments [100]. LL37, a cationic AMP produced by the innate immune system exhibits antimicrobial activity and has a direct effect in wound healing, neovascularization and angiogenesis [103].

Approximately 75% of AMPs contained in the different databases are of animal origin [16], among which invertebrates represent over 80% of the Animal Kingdom and a huge majority of them are marine derived AMPs from invertebrates have been extensively studied with particular interest in marine invertebrates in the context of bioprospecting research for natural products [58]. Likewise, the value of invertebrate AMP research is not only focused on their capacity to kill microorganisms but also on their potential as insecticides, as in the case of peptides found in toxins and venoms of insects and their natural enemies [58, 104]. Thus, the marine invertebrates represent a potentially rich source for pharmacologically useful AMPs as presumably share antimicrobial tools effective against related pathogenic microorganisms present in vertebrate and invertebrate organisms [59]. Thus, the AMPs from marine organisms have safe, natural, inexpensive and high bioactivity properties [66].

10. Classification of AMPs

AMPs can be classified based on different criteria. Based on biosynthesis they can be grouped into i) non-ribosomally synthesized peptides (that contain at least two moieties acquired from amino acids are mostly produced by bacteria and significantly modified and ii) ribosomally synthesized peptides are produced by both prokaryotic and eukaryotic organisms [60].

Based on their structure they belong to any one of the four following classes.

(i) α-helices peptides: This group is composed of α-helical peptides such as magainins, cecropins and LL-37 and are often unstructured in aqueous solution, but adopt anamphipathic helical structure in contact with a biological membrane [80]. Two of the most studied peptides in this group are LL-37, which is present in neutrophils and epithelial cells and the human lactoferricin, which is derived from the proteolytic cleavage of the antimicrobial and immunomodulatory iron-binding glycoprotein lactoferrin, present in milk and exocrine secretions [105].

(ii) β-sheet peptides

The second group includes cationic peptides that contain two to four disulfide bridges that form β-sheet structures including β- defensins, plectasin and protegrins [2]. Due to their rigid structure the β-sheet peptides are more ordered in aqueous solution and do not undergo as drastic conformational change as helicalpeptides up on membrane interaction [60]. The best-studied β-sheet peptides are the defensins - a large group of AMPs, which are produced as inactive precursors in neutrophils, macrophages and epithelial cells [80, 89].

(iii) Loop peptides: They are the smallest group of AMPs that form loop structures due to interlinking by at least one disulfide bridge such as the dodecapeptides59 and tachyplesins.[60, 106]

(iv) Extended peptides: This group contains cationic peptides which are rich in proline, tryptophan, arginine or histidine.[106]

Based on the general mechanisms of action, the AMPs can be divided in two classes - the membrane disruptors and non-membrane disruptors. Based on the biological activity AMPs are classified as antibacterial, antifungal, antiviral, antitumoral, antiparasitic, spermicidal, insecticidal etc. They have also been classified on the basis of charge, length and hydrophic residue contents. [106, 107]

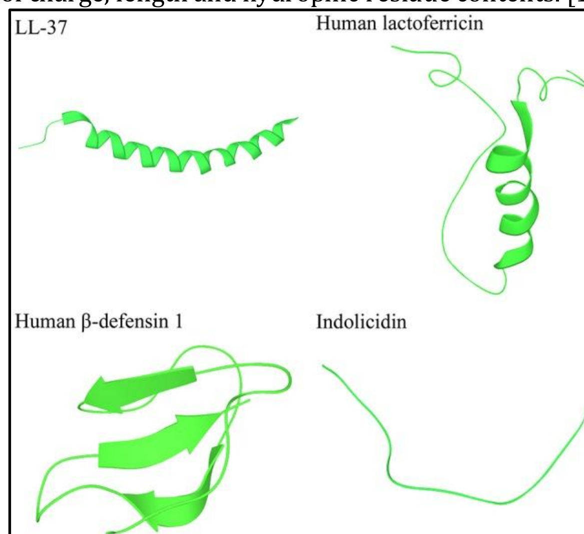


FIGURE 1.Peptides representing the three main categories of the secondary structures of AMPs. LL-37and human lactoferricin represent a-helicalpeptides, human b-defensin1 represents b-sheet peptides, and indolocidin represents extended/random-coil structures. Structures are from Protein Data Bankin Europe

11. The role of AMPs on the innate immune response

AMPs have been vastly studied with a special importance to marine invertebrate organism that lack an adaptive immune system. AMPs are a part of the innate immune response with a relevant role in the first line of defense against microorganisms. In addition to their direct antimicrobial activity, some AMPs possess different immunomodulatory functions [106]. Some of the most relevant are:

i) Chemotactic activity: In this case, they act as chemo-attractants capable of recruiting immune cells to the site of infection. in other words, AMPs induce the expression of a broad range of chemokines [106, 108]

ii) Anti-endotoxin activity: AMPs possess the capacity to dampen production of endotoxin-induced pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α) by blocking or modulating toll like receptor (TLR) signaling pathways [106].

iii) Immune cell differentiation: AMPs appear to have a direct link inducing cell differentiation and activation thereby bridging the innate and adaptive immune responses [106, 109]

iv) Wound healing and angiogenesis: Wound healing involves the re-growth of epithelial layers and the formation of new blood vessels (angiogenesis). AMPs act directly on the epithelial and endothelial cells

inducing promoting re-epithelialization and angiogenesis. AMPs also induce wound healing indirectly through their chemotactic effects.[109]

Due to these immunomodulatory and antibacterial properties, AMPs are excellent candidates for infection treatment as they can also control inflammation at the site of infection.

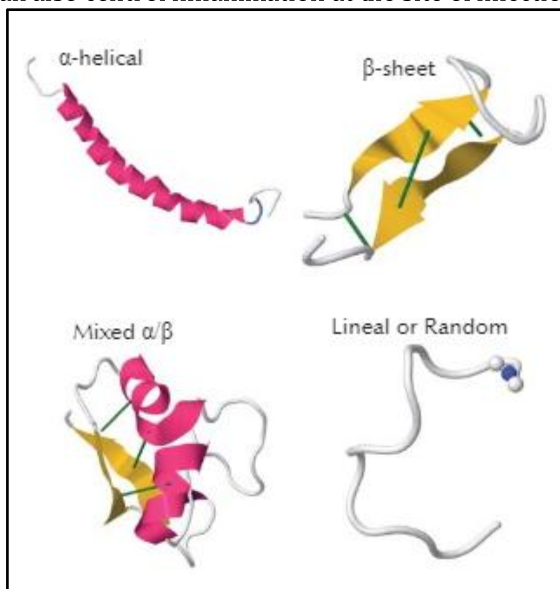


FIGURE 2.Representative secondary structure of AMPs.α-helix (Moricin), β-sheet (Hepcidin-20), mixed α-helix/ β-sheet (Hydramacin-1) and lineal or random (Lf11). α-helix structures are shown in magenta, β-sheet in yellow, and green lines represent the disulfide bridges.

12. Mechanisms of AMPs action

The mechanism of action of the AMPs is completely different to common antibiotics. These are very primitive molecules which have been functioning as a chemical shield of defense over millions of years in plants and animals [110]. Now, at this time of escalation in multi-drug resistant pathogens or superbugs, the scientific world is enthusiastically analyzing the possibilities to make these molecules an alternative for combating diseases, including those by Gram negative ESKAPE pathogens. The mechanism by which these peptides act make them very difficult to generate bacterial resistance, becoming a promising future as more durable therapeutic agents. Besides, knowing the role of peptides as modulator of the innate immune response, they can be helpful as guide for developing immunomodulatory therapies [110]. The mechanism of action of APMs may be by one or a combination of two or more of the following.

12.1. *Interaction with bacterial membrane*

The primary step in the direct antimicrobial activity of AMPs is the membrane interaction [60]. Many AMPs display a direct and rapid antimicrobial activity by causing disruption of the physical integrity of the microbial membrane and/ or by translocating across the membrane in to the cytoplasm of bacteria to act on intracellular targets [111]. Electrostatic forces between the cationic AMPs and the negatively charged bacterial surface are critical determinants for this interaction between peptides and microbial membrane [60].

The cytoplasmic membranes of both Gram-positive and Gram-negative bacteria are rich in the phospholipids phosphatidylglycerol, cardiolipin and phosphatidylserine, which have negatively charged head groups, highly attractive for positively charged AMPs. The teichoic acids which is present in the cell wall of Gram-positive bacteria and lipopolysaccharides (LPS) in the outer membrane of Gram- negative bacteria provide additional electronegative charge to the bacterial surface [60, 89]

12.2. *Membrane disruption and intracellular targets in bacterial cells*

As the outer membrane of Gram-negative bacteria constitute permeability barriers to the cytoplasmic membrane by cations like Ca^{2+} and Mg^{2+} (which bind with the inner layer of LPS), AMPs have to pass through the outer membrane and are proposed to be translocated through this outer membrane via so called self-promoted uptake [60]. This model suggests that, due to greater affinity for the LPS, AMPs displace the divalent cations and bind to the LPS. By being bulky, the AMPs then cause transient cracks and permeabilize the outer membrane, thereby permitting passage of the peptide itself across the membrane. In

contact with the cytoplasmic membrane, the AMPs form an amphipathic secondary structure essential for interaction with the cell membrane. The charged domains of the peptide allow for interaction with the hydrophilic head groups of the phospholipids, while the hydrophobic domains of the peptide interact with the hydrophobic core of the lipid bilayer, thereby driving the AMP deeper into the membrane [112]

Several models have been proposed describing the next events occurring at the bacterial cytoplasmic membrane, which ultimately lead to membrane permeabilization. According to the "barrel-stave model," the peptides insert perpendicularly into the bilayer while recruitment of additional peptides subsequently results in formation of a peptide-lined transmembrane pore. In this pore, the peptides are aligned with the hydrophobic side facing the lipid core of the membrane and the hydrophilic regions facing the interior region of the pore. According to the "toroidal-pore model," insertion of peptides forces the phospholipid to bend continuously from one leaflet to the other, resulting in a pore lined by both peptides and the head groups of the phospholipids. Finally, in the "carpet model," accumulation of peptides on the membrane surface causes tension in the bilayer that ultimately leads to disruption of the membrane and formation of micelles. [60, 113]

Membrane permeabilization by AMPs is suggested to initially lead to leakage of ions and metabolites, depolarization of the transmembrane potential with subsequent membrane dysfunction (e.g., impaired osmotic regulation and inhibition of respiration) and ultimately, membrane rupture and rapid lysis of microbial cells [60, 112]. Besides leading to membrane dysfunction and disruption, membrane permeabilization is important for translocation of certain AMPs into the cytoplasm, where they target key cellular processes including DNA/RNA and protein synthesis, protein folding, enzymatic activity, and/ or cell wall synthesis **[** 60, 114, 115].

The microbial death caused by AMPs could be a result of multiple and complementary actions, referred to as multi-hit mechanism. This strategy helps to increase the efficiency of AMPs and to evade resistance development [60]. It is likely that the mode of action of individual AMPs varies depending on parameters such as peptide concentration, target bacterial species, as well as tissue localization and growth phase of the bacteria [60, 116]. The membrane disruptor peptides are the most predominant and much of them have α-helix structures, which directly act at the plasma membrane level, altering the cell permeability or lysing cells through pores formation [100].

The non-membrane disruptor AMPs use show a variety of mechanisms like binding to nucleic acids, interference with the synthesis of nucleic acids, inhibition of the synthesis of proteins, inhibition of the enzymatic activity, inhibition of the synthesis of cell wall, blocking some virulence factors, such as flagella, proteases, secretion systems, effector proteins etc. [100, 117]

12.3. Immunomodulatory activities

Along with the anti-bacterial activities, the immunomodulatory actions of AMPs are also well documented [60, 117]. They include stimulation of chemotaxis, modulation of immune cell differentiation and initiation of adaptive immunity. All these contribute to the bacterial clearance of the host. The activities under this mechanism also include suppression of toll-like receptors (TLR), cytokine-mediated production of pro-inflammatory cytokines and anti-endotoxin activity, together preventing excessive and harmful pro-inflammatory responses including sepsis [60].

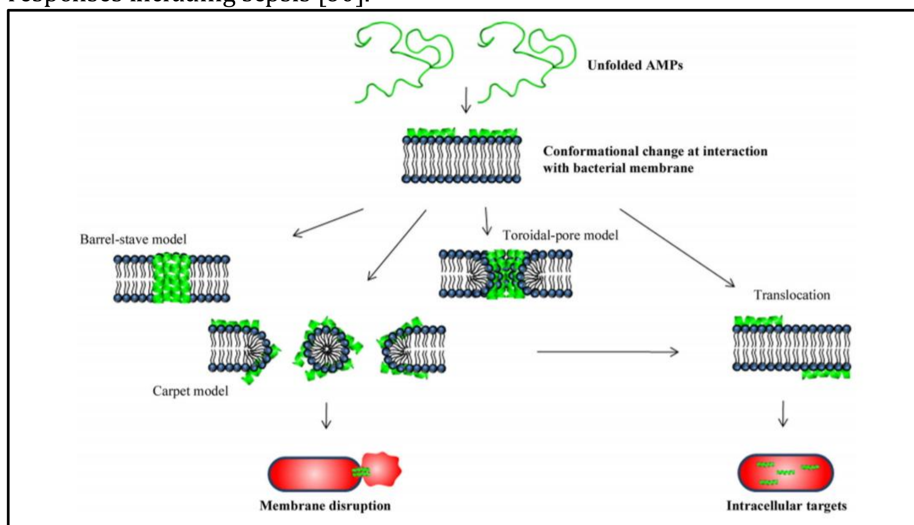


FIGURE 3.Schematic illustration of bacterial killing mechanisms by AMPs.

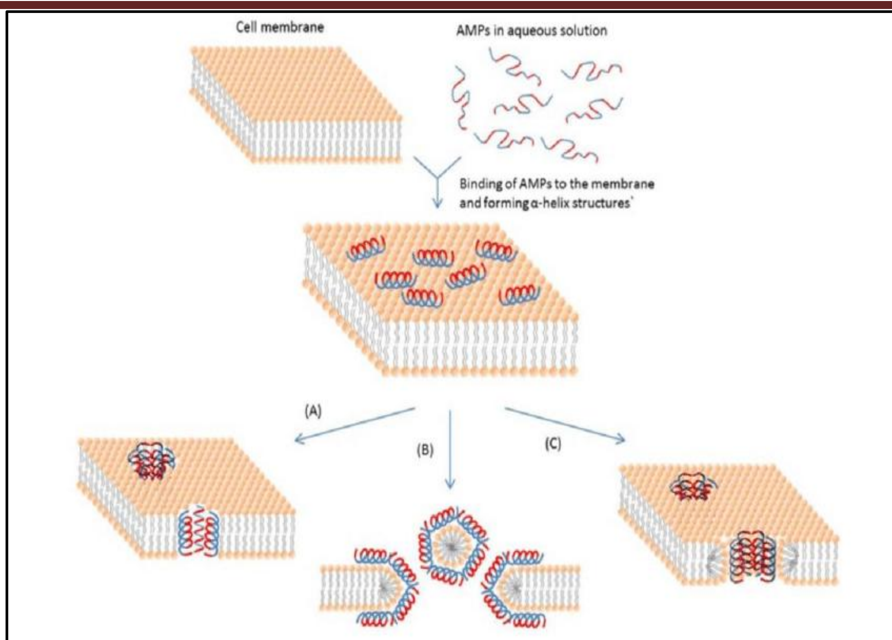


FIGURE 4.Schematic models of action mechanisms of membrane disruption by AMPs. A. Barrel stave pore. Peptides on the membrane surface are aggregated and inserted inside the membrane; the hydrophobic regions are aligned with the central lipid region of the bilayer, while the hydrophilic regions form the pore interior. B. Toroidal pore. Peptides are aggregated and induce that the lipid monolayer are continuously curved forming a pore; the pore interior is covered of peptides and also of polar heads of lipids. C. Carpet or detergent type. Peptides cover the membrane surface forming a carpet; toroidal pore are transitorily formed, which permit the entry of peptides and the membrane is starting to be disaggregated by the formation of micelles. Hydrophobic regions are represented in black and the hydrophilic in blue.

12.4. Differentiation between mammalian and bacterial cells

The basic differences in the net charge and chemical constitution between the microbial and mammalian membranes protect the latter from the action of AMPs [60]. The cytoplasmic membrane of mammalian cells is rich in the zwitterionic phospholipids phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, providing a membrane with a neutral net charge [60]. There is also an asymmetric distribution of phospholipids in mammalian membranes, with the zwitterionic phospholipids being present in the outer leaflet, while phospholipids with negatively charged head groups, if present, are localized in the inner leaflet facing the cytoplasm [60, 93]. So, the interactions between AMPs and mammalian cell membrane occur mainly via hydrophobic interactions, which are relatively weak compared to the electrostatic interactions taking place between AMPs and bacterial membranes. The high cholesterol content of mammalian cell membranes is proposed to reduce the activity of AMPs via stabilization of the phospholipid bilayer [60]

The differences in the mechanisms of action of AMPs, in many cases, determine the type of cells on which they act. For example, molluscdefensins, which are essentially active against Gram-positive bacteria, bind to lipid II, the precursor of peptidoglycan [37]. Arthropod anti-lipopolysaccharide factors (ALFs) and mollusc bactericidal/permeability-increasing protein (BPI), which are essentially active against Gram negative bacteria, bind to lipopolysaccharide (LPS) [38–40]. Finally, crustacean PvHCt, which is strictly antifungal, permeabilizes the fungal plasma membrane [41].

13. AMP databases

As the number of bacterial strains and other pathogens developing resistance against conventional antibiotics increase, the search for natural compounds with novel modes of action reached in extraction and purification of many new AMPs [91]. The AMPs have achieved scientific and medical relevance by the identification of cecropins (in insects), magainins (in amphibians) and defensins (in humans) in 1980s. As an outcome of the extensive studies, various groups of AMPs have been described such as the cecropins and

defensins in insects, the clavanins in tunicates, the penaeidins and crustins in crustaceans, the caenopores in nematodes [58] etc.

As mentioned, thousands of AMPs have been isolated from plants, animals, fungi and several micro-organisms [58, 119] and obtained through a conventional chemistry approach or designed using AMPs as models to produce peptides with improved selectivity and potency [58]. This expanse of data has been compiled in approximately 23 specialized databases that facilitate extraction of information and provide bioinformatics tools to rationalize the design of new AMPs [120]. To mention few examples for such databases are CAMP, APD , YADAMP, DRAMP, PhytAMP (for plant AMPs), BACTIBASE (for bacterial AMPs), DADP (for amphibian AMPs), MilkAMP (for AMPs of dairy origin), InverPep (for AMPs from invertebrates) [58, 91] etc. AMSdb, Defensin knowledgebase, Peptaibol Database, SAPD, AMPer, CyBase, BAGEL, Minicope (the Innate immunity defense peptides MiniCOPE Dictionary) CAMP, RAPD, Dragon Antimicrobial Peptide Database (DAMPD, http://apps.sanbi.ac.za/dampd) etc. are other examples [58, 91, 121, 122]. Each of them contains details of hundreds and thousands of AMPs arranged based on different criteria such as taxonomy, species, AMP family, citation, keywords and a combination of search terms and advanced search fields.

14. Nanoparticles: candidates for conjugation to AMPs

Particles that are having at least one dimension ranging from 1nm to 100 nm are considered as nanoparticles (NPs). Richard P. Feynman (1959) introduced the concept, and it was Norio Taniguchi who coined the term “nanotechnology” in 1974 [2]. Feynman’s idea about the possibilities of manipulating matter at the atomic scale led to numerous revolutionary developments in multidisciplinary science. Thus, nanotechnology is emerging as a useful tool for various applications in biomedical devices, waste management, material science, and electronics [123]

Nanoparticles provide another potential solution to combat multidrug resistant pathogens. The large surface area to volume ratio of Nanoparticles provides a high loading of coated molecules.97 Nanoparticles by themselves (e.g. silver, other metal oxides such as titanium, copper, zinc and iron etc.) have been known to possess antimicrobial activities and work through numerous modes of action [2]. Nanoparticles can disrupt the bacterial cell membrane, causing cell penetration, react with intracellular target and cause toxicity [2, 124].

The area of bioconjugation in general and the conjugation of peptides and nanoparticles in particular is of growing interest and would attain great heights in the near future.90. When peptides are conjugated to nanoparticles, the resulting conjugate will have new critical properties like enhanced potency, site of action targeting capability, less toxicity, etc. that are directly acquired from the peptides but not previously possessed by the nanoparticles.[82] Nanoparticles are excellent candidates for transporting drugs to their targets [2]

The use of nanoparticles in combination with antibiotics makes it possible to decrease the toxicity from both agents toward human cells because of the synergistically enhanced antimicrobial activities and the reduced requirement for high dosages [125]. Nanoparticles as antibiotic carriers to the site of infection are emerging as a promising strategy in antibiotic therapy. The results from recent research in which nanoparticles have been combined with AMPs are promising and show an increasing trend toward a safer profile of the conjugates toward mammalian cells [2].

The linkage between nanoparticles and peptides should be stable and be responsive to other foreign agents and physical factors 99 for the effective release of drugs (peptides) from nanoparticles to the site of action. The density or ratio of the peptide to nanoparticles is also important for efficient uptake. The four strategies that are proposed to conjugate peptides and nanoparticles100 are, (a) the electrostatic interaction (between oppositely charged nanoparticles and peptides), (b) the direct interaction (binding of the peptides with the nanoparticle surface with high affinity), (c) the secondary interactions (like the biotinylation where the biotin on the peptide would mediate directional assembly) and (d) covalent attachment linkages (which utilize EDC-based coupling of amines to carboxylic acids and N-hydroxysuccinimide- and maleimide-mediated couplings to amines and thiols) [2, 82]

The examples for some peptides commonly used for the therapeutic delivery of nanoparticles are TAT and TAT-like peptides, RGD peptides, the pep-I peptide, organelle-specific peptides, neuropeptides, the rabies virus-derived peptide etc. Several recent studies have shown that the nanoparticles provide an efficient way to kill bacterial pathogens, including drug-resistant bacteria and have low probability for resistance development by the pathogens [2, 126]. The peptide conjugated nanoparticles have found numerous applications in various fields including intracellular delivery, drug delivery, cancer therapy, neurology and many others.

15. Silver nanoparticle – antimicrobial peptide (AgNPs - AMPs) conjugation

The interaction between proteins and silver nanoparticles (AgNPs) has been demonstrated to play a pivotal role in the nanomaterial's biocompatibility and ultimately, its antimicrobial performance. Proteins, peptides and free amino acids can be used to control the structure of AgNPs during synthesis and improve their stability under a variety of conditions. However, the mechanism(s) that underlie such stabilization as well as the exact role of various amino acid moieties remains elusive [127]. **I**t is being increasingly recognized that nanoparticles, especially silver nanoparticles have good antimicrobial properties and their conjugation with AMPs have been proposed to enhance the activity . The bio-functionalized AgNPs could remove endotoxin thus maximizing their potential in various applications [82].

Silver nanoparticles are known to be toxic to mammalian cells above a certain concentration and one way to reduce its toxicity is to conjugate it with peptides [128]. The conjugate is less toxic to mammalian cells as revealed by the MTT assay compared to AgNP alone, up to a concentration of 96 lg/mL concentration. The conjugation through weak interactions between the AMPs and the nanoparticles is sufficient to reduce the toxicity of the AgNP, which in turn may be due to controlled release of Ag+ [82].

The combination of AgNPs with the CSG-LL37 peptide (LL37@AgNP) has been used as the antimicrobial and anti-biofilm agent by [129] and they found that the LL37@AgNP exhibited very low MIC values against both Gram-positive and -negative bacteria. Also, LL37 capped AgNPs were not cytotoxic at the MIC levels or even at double the MIC level and they did not hinder cell proliferation at this level. The conjugate was found to be more biocompatible and more stable. Apart from that, the conjugate the ability of this conjugate to was able to prevent the biofilm formation by *Pseudomonas aeruginosa* [82, 129]

The activity of the cell penetrating peptide GGGRRRRRRYGRKKRRQRR (G3R6TAT) as the stabilizer and reductant to produce AgNPs and the screening of the same against Gram positive *Bacillus subtilis*, Gram negative *Escherichia coli* and the yeast *Candida albicans* showed that he conjugate dramatically improved the biocide efficacy as much as 50 times against *Bacillus subtilis*, at least 10 times against *Escherichia coli* and 3.3 times against *Candida albicans* with a very low MIC value. The SEM results have also shown the breakdown of bacteria and the conjugate showed low hemolytic activity at effective concentrations. Hence, this platform could provide an alternative therapy for topical or systemic infections [82, 130]. Also, it has been shown that the AgNPs with 20 nm diameter exhibit more cytotoxicity than the 40 nm diameter. Also, the AgNP-based hydrogels showed high antimicrobial efficiency against MDR *Pseudomonas aeruginosa* [131] It is expected that this Ag releasing biomaterial offers great potential for application in wound healing, particularly after surgery, and in the treatment of chronic and large surface wounds, such as diabetic ulcers or severe burns, due to its potential to reduce inflammation and prevent infection **[82]**. The antimicrobial and hemolytic properties of AgNPs in combination with different AMPs found that the need for high dosages can be reduced by the synergistic action of antimicrobial agents that can also bring down the side effects [82, 132]**.** Thus the silver nanoparticle – antimicrobial peptides (AgNPs - AMPs) conjugates bring a new hope of ray to the ongoing war against MDR pathogens including the Gram negative ESKAPE members.

The peptide exhibits a dynamic exchange from the surface of the AgNPs without undergoing a significant conformational change. The weak interaction is useful particularly in the case of AMPs where the charge and structure of the peptide is important for their activity [133] and hence too strong an interaction with Ag nanoparticles through the positively charged residues will reduce their activity. Very strong interactions can also perturb the structure of the AMPs resulting in reduced activity. Thus, the balance of strong interaction/stability vis-à-vis the activity is a key feature to be considered for design of new conjugates [133, 134]. Simulations predict high affinities of Arg, Cys, and Met residues for the Ag facet. However, cooperative effects over larger portions of the peptide sequence have long been known to be essential in determining affinity for metal surfaces and peptides selective for Ag surfaces [135] further demonstrate that the affinity is not determined solely by individual amino acids but also by the conformational statistics of the peptide as determined by its sequence [127].

The stability of the AgNP-AMP conjugate originates from steric repulsion. It has been shown that stability is enhanced if AgNPs are conjugated with polypeptides, where the steric repulsion between proteins prevent Nanoparticles from approaching each other at close distance, thereby preventing their aggregation [136]. The nature of interaction is closely associated with stability of the system. As the AgNPs are generally unstable in solution an AgNP-AMP system having long-term stability with enhanced activity is preferable. Conjugating a cysteine containing peptide with AgNP is expected to have a favorable effect on the strength and nature of nanoparticle-peptide interactions. However, for designing AgNP-AMP conjugates with increased stability and antimicrobial activity, it is important to understand the nature of interactions between the AMP and AgNP. [134].

16. Conclusion

It has been more than two decades that spread of the 'bad bugs', which are resistant to a wide spectrum of conventional antibiotics bothering the healthcare systems globally. The emergence of new infectious agents, infections that emerge out of surgical devices pose, the never lasting threat of nosocomial infections and other dreaded incidents by bacteria including the Gram negative members of *ESKAPE* group are serious threats which account for very high morbidity in hospitals [6]. In 2014, the World Health Organization (WHO) first looked at the antimicrobial resistant data globally; the data on the issue also revealed the serious and worldwide threat on the public health [137]. It is at these circumstances that the scientific world turned back to the nature in search for new antibiotics with novel modes of action.

Because the ocean occupies almost 70% of Earth's surface, it offers unlimited potential for biological and chemical diversity. Marine ecosystems comprise a continuous resource of immeasurable biological activities and vast chemical entities. The invertebrates, as the biggest group of the animal kingdom, are the principal source of Antimicrobial peptides with validated antimicrobial activity. Especially the marine invertebrates that rely solely on innate immune mechanisms for host defense is a spectacular resource for the development of new antimicrobial compounds [61] and is mainly composed of AMPs, which are endogenous and are exciting candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to these peptides [58]. Thus the AMPs offer promising alternatives to standard therapies as anti-infectives and immunomodulatory agents with mechanisms of action which are less prone to resistance induction compared to conventional antibiotics. Although challenges in translating nonclinical candidate AMPs into successful clinical products are well recognized, the discovery and commercial development of next-generation therapeutic peptides and peptide mimeticsis predicted to be accelerated by recent advances in overall understanding of their mechanism of action, resistance patterns ,and smart formulation strategies. With several AMPs currently undergoing latest age clinical development in different therapeutic areas, the next years hold a promise to confirm the therapeutic benefit of these novel candidates and lead to market authorization of several new AMP-based drugs [60]. It is apparent that many peptide drugs have entered the market and several are in the pipeline and few are in various stages of preclinical and clinical trials [82] Thus, peptide-based molecules present a promising future in drug discovery programmes.

It is at this stage that, the nanoparticles emerge as the bridge to fill this gap since these are endowed with unique properties and potential therapeutic applications. Their small size and high surface-to-volume ratio make them attractive both in therapeutic and biomedical fields. Further, these materials can also be used in theranostics, which incorporate both therapeutic and diagnostic moieties in a single species. This is possible because nanoparticles can be tethered with differently functionalized entities. Taking the advantage of this unique feature, there are pieces of evidence wherein nanoparticles are conjugated to different biologically active molecules and one among them is the peptides. These are considered as the "value added" constructs that will have the properties from both the moieties in-connection [58].

A wide variety of nano-technological devices for the treatment of infectious diseases have been developed, including microemulsions, vaccines and metallic, inorganic, lipid and polymeric-based Nanoparticles (Nanoparticles) [87]. Metallic Nanoparticles such as silver (AgNP) and gold (AuNP) show unique and considerably distinct physical, chemical and biological properties due to their high surface-to-volume ratio, with which surfaces can be modified with ligands containing functional groups, providing an electrostatic or steric stabilization [138].

The antibacterial activity of Ag nanoparticles against a broad spectrum of bacteria is well-known. Ag nanoparticles have been conjugated to different molecules with antibacterial activity in order to obtain synergic effects, such as poly (ethyleneimine), amoxicillin, polysaccharides, peptides, surfactants and polymers [87, 128]. Thus, the silver nanoparticles conjugated antimicrobial peptides open a window to the new horizon of hope in the fight against the 'bad bugs'

Conflict of Interest

Authors declare no conflict of interest.

References

1. R. P. Hicks, J. J. Abercrombie, R. K. Wong, K. P. Leung. Antimicrobial peptides containing unnatural amino acid exhibit potent bactericidal activity against ESKAPE pathogens, Bioorganic & Medicinal Chemistry 21 (2013) 205–214. DOI: 10.1016/j.bmc.2012.10.039
2. UrawadeeRajchakit and VijayalekshmiSarojini. Recent Developments in Antimicrobial-Peptide-Conjugated Gold Nanoparticles, Bioconjugate Chem. 2017, 28, 2673-2686, DOI: 10.1021/acs.bioconjchem.7b00368

3. **World Health Organization** Antibiotic resistance Fact sheet. Updated November 2017. **http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/**
4. O'Neill, J. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, HM Government: London, United Kingdom 2014
5. Deslouches B, Steckbeck JD, Craigo JK, Doi Y, Burns JL, Montelaro RC. 2015. Engineered cationic antimicrobial peptides to overcome multidrug resistance by ESKAPE pathogens. Antimicrob Agents Chemother 59:1329 – 1333. doi:10.1128/AAC.03937-14
6. S. S.Magill, J. R. Edwards, W. Bamberg et al., "Multistate pointprevalence survey of health care-associated infections,"TheNewEngland Journal ofMedicine, vol. 370, no. 13, pp. 1198–1208, 2014.
7. SirijanSantajit and NitayaIndrawattana, Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens, BioMed Research International Volume 2016, Article ID 2475067, 1- 8 http://dx.doi.org/10.1155/2016/2475067
8. Lubell Y, Ashley EA, Turner C, Turner P, White NJ. Susceptibility of community-acquired pathogens to antibiotics in Africa and Asia in neonates—an alarmingly short review.Tropical Medicine & International Health.2011; 16: 145–51.
9. Anuradha S De, Baveja S, D'Souza D and Patwegar S, Antimicrobial Resistance among Commonly Encountered Bacteria Isolated in 2013 – The ESKAPE Menace, Intern Med 2015, 5:3 Page 1 – 6 http://dx.doi.org/10.4172/2165-8048.1000193
10. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. ClinMicrobiol Infect 18: 268 –281. http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x.
11. Jack N Pendleton, Sean P Gorman, Brendan F Gilmore (2014). Clinical Relevance of the ESKAPE Pathogens Journal ofExpert Review of Anti-Infective Therapy 11(3):297-308. https://doi.org/10.1586/eri.13.12
12. Lob SH, Hackel MA, Kazmierczak KM, Young K, Motyl MR, Karlowsky JA, Sahm DF. 2017. In vitro activity of imipenem-relebactam against Gram-negative ESKAPE pathogens isolated by clinical laboratories in the United States in 2015 (results from the SMART global surveillance program). Antimicrob Agents Chemother 61:e02209-16. https://doi.org/10 .1128/AAC.02209-16.
13. James A. Karlowskya, Daryl J. Hobana,b, Meredith A. Hackel b, Sibylle H. Lob,∗, Daniel F. Sahmb , Brief communication Resistance among Gram-negative ESKAPE pathogens isolated from hospitalized patients with intra-abdominal and urinary tract infections in Latin American countries: SMART 2013–2015, braz j infect dis 2017; 21 (3) : 343 – 348 http://dx.doi.org/10.1016/j.bjid.2017.03.006
14. MasoumehNavidinia. The clinical importance of emerging ESKAPE pathogens in nosocomial infections, Journal of Paramedical Sciences (JPS) Summer2016 Vol 7, No3. ISSN 2008-4978)
15. G. D. Wright, "Bacterial resistance to antibiotics: enzymatic degradation and modification," Advanced Drug Delivery Reviews, vol. 57, no. 10, pp. 1451–1470, 2005. doi:10.1016/j.addr.2005.04.002
16. Franz CMAP, Stiles ME, Schleifer KH, Holzapfel WH. Enterococci in foods -A conundrum for food safety. Int J Food Microbiol 2003; 88:105–122.
17. Mouton JW, Van Ogtrop ML, Andes D, Craig WA. Use of pharmacodynamic indices to predict efficacy of combination therapy in vivo.Antimicrob Agents Chemother1999; 43 (10): 2473–2478.
18. Sasirekha B. Prevalence of ESBL, AmpC B- lactamases and MRSA among uropathogens and its antibiogram.Excli J 2013; 12(1):81–88.
19. Navidinia M. Detection of inducible clindamycin resistance (MLSBi) among methicillin-resistant Staphylococcus aureus (MRSA) isolated from health care providers. J ParamedSci 2015; 6(1): 91 -96.
20. Jindal N, Singh S, Grover P, Malhotra. Prevalence of Inducible Clindamycin Resistance among Clinical Isolates of MRSA in Malwa Region of Punjab (North India).Indian J Res 2013; 2 (10).DOI : 10.15373/22501991
21. Higaki S, Morohashi M, Yamagishi T, Hasegawa Y. Comparative study of staphylococci from the skin of atopic dermatitis patients and from healthy subjects. Int J Dermatol 1999; 38(4):265-259.
22. MARGARET CHAN, PAGE 4—IDSA Letter to World Health Organization RE Prioritizing AR Pathogens oct 4, 2016)
23. Quan TP, Fawcett NJ, Wrightson JM, Finney J, Wyllie D, Jeffery K, Jones N, Shine B, Clarke L, Crook D et al (2016) Increasing burden of community-acquired pneumonia leading to hospitalisation, 1998–2014. Thorax 71: 535 – 542
24. Timothy J Kidd, Grant Mills, Joana Sá-Pessoa, Amy Dumigan, Christian G Frank, José L Insua, Rebecca Ingram, Laura Hobley& José A Bengoechea. A Klebsiella pneumoniae antibiotic resistance mechanism that subdues host defences and promotes virulence EMBO Molecular MedicineVol 9. No 4 2017, 430 – 447
25. Michalopoulos, A, Falagas ME. Treatment of Acinetobacter Infections.Expert Opinion on Pharmacotherapy. 2010 April, 11(5): 779-88.
26. E. T. S. Houang, R. T. Sormunen, L. Lai, C. Y. Chan, and A. S.-Y. Leong, "Effect of desiccation on the ultrastructural appearances of Acinetobacter baumanniiand Acinetobacter lwoffii," Journal of Clinical Pathology, vol. 51, no. 10, pp. 786–788, 1998

27. Zahra Shamsizadeh, MahnazNikaeen, Bahram Nasr Esfahani, SeyedHamedMirhoseini, Maryam Hatamzadeh and Akbar Hassanzadeh, Detection of antibiotic resistant Acinetobacter baumannii in various hospital environments: potential sources for transmission of Acinetobacter infections. Environmental Health and Preventive Medicine (2017) 22:44, page 1 – 7. DOI 10.1186/s12199-017-0653-4
28. Erlangga Yusuf, Bruno Van Herendael, Walter Verbrugghe, Margareta Ieven, EmielGoovaerts, Kristof Bergs, KristienWouters, Philippe G. Jorens and Herman Goossens. Emergence of antimicrobial resistance to Pseudomonas aeruginosa in the intensive care unit: association with the duration of antibiotic exposure and mode of administration Ann. Intensive Care (2017) 7:72 Page: 1-7 DOI 10.1186/s13613-017-0296-z
29. Davin-Regli A, Pagès JM. Enterobacter aerogenes and Enterobacter cloacae; versatile bacterial pathogens confronting antibiotic treatment.Front Microbiol. 2015; 6: Article 392. pmid:26042091
30. Mezzatesta ML, Gona F, Stefani S. Enterobacter cloacae complex: clinical impact and emerging antibiotic resistance. Future Microbiol.2012; 7: 887–902.pmid:22827309
31. Paterson DL. Resistance in gram-negative bacteria: Enterobacteriaceae. Am J Infect Control 2006; 34: S20–28. pmid:16813978
32. A. M. Queenan and K. Bush, “Carbapenemases: the versatile β-lactamases,” Clinical Microbiology Reviews, vol. 20, no. 3, pp. 440–458, 2007.
33. Petrosillo N, Capone A, Di Bella S, Taglietti F. Management of antibiotic resistance in the intensive care unit setting. Expert Rev Anti Infect Ther 2010; 8(3): 289–302.
34. A. M. Queenan and K. Bush, “Carbapenemases: the versatile β-lactamases,” Clinical Microbiology Reviews, vol. 20, no. 3, pp. 440–458, 2007.
35. K. K. Kumarasamy,M. A. Toleman, T. R.Walsh et al., “Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study,” The Lancet Infectious Diseases, vol. 10, no. 9, pp. 597–602, 2010.
36. Maragakis LL, Perl TM: Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options. Clin Infect Dis 2008; 46(8): 1254-1263
37. H. W. Boucher, G. H. Talbot, J. S. Bradley et al., “Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America,” Clinical Infectious Diseases, vol. 48, no. 1, pp. 1–12, 2009.https://doi.org/10.1086/595011.
38. Strateva T, Yordanov D. Pseudomonas aeruginosa a phenomenon of bacteria resistance. J Med Microb 2009; 58(9): 1133-1148.
39. Nateghian A, GhasemiAhari SM, Harahdashti AL, Navidnia M, Mehrazma M. Prevalence of Vancomycin-resistant Enterococci Colonization, and Susceptibility to linezolid in Pediatric Intensive Care Units of a Referral Pediatric Center in Tehran, Iran .Arch Pediatr Infect Dis 2014; 2(2): e16970.
40. Gibsona JS , Cobbolda RN, Heisigb P, Sidjabatc HE, Kyaw-Tannera MT , Trotta DJ . Identification of Qnr and AAC (6′)-1b-cr plasmid-mediated fluoroquinolone resistance determinants in multidrug-resistant Enterobacter spp. isolated from extraintestinal infections in companion animals.Vet Microbial 2010; 143(14):329–336.
41. A. Giedraitien˙e, A. Vitkauskien˙e, R. Naginien˙e, and A. Pavilonis, “Antibiotic resistance mechanisms of clinically important bacteria,” Medicina, vol. 47, no. 3, pp. 137–146, 2011.
42. G. A. Jacoby and L. S.Munoz-Price, “Thenew beta-lactamases,” The New England journal of medicine, vol. 352, no. 4, pp. 380– 391, 2005.
43. K. Bush and G. A. Jacoby, “Updated functional classification of β-lactamases,” Antimicrobial Agents and Chemotherapy, vol. 54, no. 3, pp. 969–976, 2010.
44. W.-H. Zhao and Z.-Q. Hu, “Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria,” Critical Reviews inMicrobiology, vol. 39, no. 1, pp. 79– 101, 2013.
45. Leavitt A, Navon-Venezia S, Chmelnitsky I, Schwaber MJ, Carmeli Y. Emergence of KPC-2 and KPC-3 in carbapenem-resistant Klebsiella pneumoniae strains. Antimicrob Agents Chemother2007; 51(8): 3026–3029.
46. D. Yong, M. A. Toleman, C. G. Giske et al., “Characterization of a new metallo-β-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India,” Antimicrobial Agents and Chemotherapy, vol. 53, no. 12, pp. 5046–5054, 2009.
47. Harada K, Shimizu T, Mukai Y, Kuwajima K, Sato T, Kajino A, et al. (2017) Phenotypic and molecular characterization of antimicrobial resistance in Enterobacterspp. isolates from companion animals in Japan. PLoS ONE 12(3): e0174178. https://doi.org/10.1371/journal.pone.0174178
48. S. Dˇzidi´c, J. ˇSuˇskovi´c, and B. Kos, “Antibiotic resistance mechanisms in bacteria: biochemical and genetic aspects,” Food Technology and Biotechnology, vol. 46, no. 1, pp. 11–21, 2008.
49. J. M. Thomson and R. A. Bonomo, “The threat of antibiotic resistance in Gram-negative pathogenic bacteria: β-lactams in peril!,” Current Opinion in Microbiology, vol. 8, no. 5, pp. 518– 524, 2005.
50. H. Vaez, J. Faghri, B. N. Isfahani et al., “Efflux pump regulatory genes mutations in multidrug resistance Pseudomonas aeruginosa isolated from wound infections in Isfahan hospitals,” Advanced Biomedical Research, vol. 3, article 117, 2014.
51. G. Sharma, S. Rao, A. Bansal, S.Dang, S. Gupta, andR.Gabrani, “Pseudomonas aeruginosa biofilm: potential therapeutic targets,” Biologicals, vol. 42, no. 1, pp. 1–7, 2014.
52. N. Hoiby, T. Bjarnsholt, M. Givskov, S. Molin, and O. Ciofu, “Antibiotic resistance of bacterial biofilms,” International Journal of Antimicrobial Agents, vol. 35, no. 4, pp. 322–332, 2010.

53. H.-A. Elsner, I. Sobottka, D. Mack, M. Claussen, R. Laufs, and R. Wirth, "Virulence factors of Enterococcus faecalis and Enterococcus faecium blood culture isolates," European Journal of ClinicalMicrobiology and Infectious Diseases, vol. 19, no. 1, pp. 39–42, 2000.
54. Czaplewski L, et al . Alternatives to antibiotics—a pipeline portfolio review. The Lancet Infectious Diseases 2016; published online January 12. Pages 1-23 http://dx.doi.org/10.1016/S1473-3099(15)00466-1
55. Godballe, T.; Nilsson, L. L.; Petersen, P. D.; Jenssen, H. Chem. Biol. Drug Des.2011, 77, 107.
56. Alemayehu D, Quinn J, Cook J, Kunkel M, Knirsch CA. 2012. A paradigm shift in drug development for treatment of rare multidrugresistant Gram-negative pathogens.Clin Infect Dis 55:562–567. http://dx.doi.org/10.1093/cid/cis503
57. MatteoBassetti; Elda Righi; Silvano Esposito; Nicola Petrosillo; Laura Nicolini .Drug Treatment for Multidrug-resistant Acinetobacter baumannii Infections. Future Microbiol 2008; 3(6):649-660.
58. Esteban A. Gómez, Paula Giraldo, Sergio Orduz. InverPep: A database of invertebrate antimicrobial peptides, Journal of Global Antimicrobial Resistance, 8 (2017) 13–17, http://dx.doi.org/10.1016/j.jgar.2016.10.003)
59. Anselmo Jesus Otero-Gonza´lez, Beatriz SimasMagalha˜es, Monica Garcia-Villarino, Carlos Lo´pez-Abarrategui, Daniel Amaro Sousa, Simoni Campos Dias, and Octa´vioLuiz Franco, Antimicrobial peptides from marine invertebrates as a new frontier for microbial infection control, The FASEB Journal, Vol. 24 May 2010 1320 - 1334 •doi: 10.1096/fj.09-143388
60. Mahlapuu M, Håkansson J, Ringstad L and Björn C (2016) Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. Front. Cell. Infect. Microbiol. 6:194. doi: 10.3389/fcimb.2016.00194)
61. J. Andy Tincu and Steven W. Taylor, Antimicrobial Peptides from Marine Invertebrates,Antimicrob. Agents Chemother.2004, 48(10):3645. DOI: 10.1128/AAC.48.10.3645-3654.2004.
62. De Vries, D. J. and Hall, M. R. (1994), Marine biodiversity as a source of chemical diversity. Drug Dev. Res., 33: 161–173. doi:10.1002/ddr.430330213
63. D. Datta, S. NathTalapatra S. Swarnakar. Bioactive compounds from marine invertebrates for potential medicines – An overview, International Letters of Natural Sciences 20157 ISSN: 2300-9675, Vol. 34, pp 42-61)
64. Boman, H. 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. 13:61-92. DOI: 10.1146/annurev.iy.13.040195.000425
65. Mitta, G., Hubert, F., Dyrynda, E. A., Boudry, P., and Roch, P. (2000). Mytilin B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. Dev.Comp. Immunol. 24, 381–393 https://doi.org/10.1016/S0145-305X(99)00084-1
66. Xueqin Wang, Huahua Yu, Ronge Xing, and Pengcheng Li, Review Article Characterization, Preparation, and Purification of Marine Bioactive Peptides, BioMed Research International Volume 2017, Article ID 9746720, 1-16 pages https://doi.org/10.1155/2017/9746720
67. S. Lordan, R. P. Ross, and C. Stanton, "Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases," Marine Drugs, vol. 9, no. 6, pp. 1056–1100, 2011. doi: 10.3390/md9061056
68. **Charlet, M., S. Chernysh, H. Philippe, C. Hetru, J. A. Hoffmann, and P. Bulet.** 1996. Innate immunity: isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, Mytilusedulis. J. Biol. Chem.271:21808-21813 doi: 10.1074/jbc.271.36.21808
69. Proksch, P.; Edrada, R.; Ebel, R. Drugs from the seas—Current status and microbiological implications. Appl. Microbiol. Biotechnol.2002, 59, 125–134. DOI: 10.1007/s00253-002-1006-8
70. Mohammad FerdousMehbub 1,2,3, Jie Lei 1,2, Christopher Franco 1,2,* and Wei Zhang 1,2,*, Marine Sponge Derived Natural Products between 2001 and 2010: Trends and Opportunities for Discovery of Bioactives, Mar. Drugs 2014, 12, 4539-4577; doi:10.3390/md12084539
71. Blunt, J.W.; Copp, B.R.; Munro, M.H.G.; Northcote, P.T.; Prinsep, M.R. Marine natural products. Nat. Prod. Rep. 2006, 23, 26–78. DOI: 10.1039/b502792f
72. Borquaye LS, Darko G, Ocansey E, Ankomah E. Antimicrobial and antioxidant properties of the crude peptide extracts of Galatea paradoxa and Patella rustica. SpringerPlus. 2015;4:500. doi:10.1186/s40064-015-1266-2.1-6
73. David W. Hoskin and AyyalusamyRamamoorthy.Studies on Anticancer Activities of Antimicrobial Peptides BiochimBiophysActa. 2008 1778(2) : 357–375. doi: 10.1016/j.bbamem.2007.11.008
74. Menzel, L., Lee, B. Sjostrand, and R. Lehrer. 2002. Immunolocalization of clavanins in Styelaclava hemocytes. Dev. Comp. Immunol. 26:505-515. https://doi.org/10.1016/S0145-305X(02)00010-1
75. Alonso, D.; Khalil, Z.; Satkunanthan, N.; Livett, B. Drugs from the sea: Conotoxins as drug leads for neuropathic pain and other neurological conditions. Mini Rev. Med. Chem. 2003, 3, 785–787. DOI: 10.2174/1389557033487746
76. Hu, G.P.; Yuan, J.; Sun, L.; She, Z.G.; Wu, J.H.; Lan, X.J.; Zhu, X.; Lin, Y.C.; Chen, S.P. Statistical research on marine natural products based on data obtained between 1985 and 2008. Mar. Drugs 2011, 9, 514–525. doi: 10.3390/md9040514.
77. Hancock, R. E. W., Brown, K. L., and Mookherjee, N. (2006). Host defence peptides from invertebrates: emerging antimicrobial strategies. Immunobiology211, 315–322 DOI:10.1016/j.imbio.2005.10.017

78. MałgorzataMarcinkiewicz, SławomirMajewski The role of antimicrobial peptides in chronic inflammatory skin diseases AdvDermatolAllergol 2016; XXXIII (1): 6–12 doi: 10.5114/pdia.2015.48066
79. Jorge M.O.F., Nathalie S., Graham D.K and Valerie J.S (2005) .Oncorhyncin III. A potent Antimicrobial peptides derived from the non-histone chromosomal protein H6 of Rainbow Trout, Oncorhynchusmykiss. Biochemical Journal Immediate Publication.Biochem. J. (2003) 373, 621–628
80. Pasupuleti M[1], Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system Crit Rev Biotechnol. 2012 32 (2) : 143-71. doi: 10.3109/07388551.2011.594423.
81. Avila EE Functions of Antimicrobial Peptides in Vertebrates Curr Protein Pept Sci. 2017; 18 (11):1098-1119. doi: 10.2174/1389203717666160813162629.
82. Suhas Ramesh, Marcelo Grijalva, Alexis Debut, Beatriz G. de la Torre, Fernando Albericio and Luis H. Cumbal. Peptides conjugated to silver nanoparticles in biomedicine – a “value-added” phenomenon, Biomater. Sci., 2016, 4, 1713- 1725, 10.1039/c6bm00688d)
83. Fjell, C. D., Hiss, J. A., Hancock, R. E., and Schneider, G. (2012). Designing antimicrobialpeptides: form follows function. Nat. Rev. Drug Discov. 11,37–51. doi:10.1038/nrd3591
84. HåvardJenssen, Pamela Hamill, and Robert E. W. Hancock Peptide Antimicrobial Agents clinical microbiology reviews, July 2006, p. 491–511 Vol. 19, No. 3 doi:10.1128/CMR.00056-05
85. Olli S, Nagaraj R, Motukupally SR. 2015. A hybrid cationic peptide composed of human -defensin-1 and humanized -defensin sequences exhibits salt-resistant antimicrobial activity. Antimicrob Agents Chemother59:217–225. doi:10.1128/AAC.03901-14. A
86. S. Lordan, R. P. Ross, and C. Stanton, “Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases,” Marine Drugs, vol. 9, no. 6, pp. 1056–1100, 2011.
87. Enrique Morales-Avila, Guillermina Ferro-Flores, Blanca E. Ocampo-García, Gustavo López-Téllez, Johnny López-Ortega, Diana G. Rogel-Ayala, and Diego Sánchez-Padilla, Antibacterial Efficacy of Gold and Silver Nanoparticles Functionalized with the Ubiquicidin (29–41) Antimicrobial Peptide, Journal of Nanomaterials 2017, Article ID 5831959, 1- 10 page https://doi.org/10.1155/2017/5831959
88. Hoskin, D. W., and Ramamoorthy, A. (2008) Studies on anticancer activities of antimicrobial peptides. Biochim.Biophys.Acta1778, 357–375
89. Lai, Y., and Gallo, R. L. (2009) AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. Trends Immunol.30, 131–141
90. Yew,W.W., Lange,C. and Leung,C.C. (2011) Treatment of tuberculosis: update 2010. Eur. Respir. J. 37, 441–32.
91. VijayaraghavaSeshadriSundararajan, Musa NurGabere, Ashley Pretorius, Saleem Adam, Alan Christoffels, MinnaLehva¨ slaiho, John A. C. Archer and Vladimir B. Baji. DAMPD: a manually curated antimicrobial peptide database. Nucleic Acids Research, 2012, Vol. 40, Database issue, D1108–D1112, doi:10.1093/nar/gkr1063
92. Robert Bals. Epithelial antimicrobial peptides in host defense against infection. Respiratory Research 2000 1 : 3 DOI:10.1186/rr25
93. Yuping Lai and Richard L. Gallo.AMPed Up immunity: how antimicrobial peptides have multiple roles in immune defense Trends Immunol. 2009 March ; 30(3): 131–141. doi:10.1016/j.it.2008.12.003.
94. A. Lakshmanan, D. W. Cheong, A. Accardo, E. Di Fabrizio, C. Riekel and C. A. E. Hauser, Proc. Natl. Acad. Sci. 2013, 110, 519–524.
95. Fosgerau, K., and Hoffmann, T. (2015) Peptide therapeutics: current status and future directions. Drug Discovery Today 20, 122– 128.
96. Fox, J. L. (2013). Antimicrobial peptides stage a comeback. Nat. Biotechnol. 31, 379–382. doi: 10.1038/nbt.2572
97. Hancock, R. E., Nijnik, A., and Philpott, D. J. (2012). Modulating immunity as a therapy for bacterial infections. Nat. Rev. Microbiol. 10, 243–254. doi: 10.1038/nrmicro2745
98. Wang, G. (2015). Improved methods for classification, prediction and design of antimicrobial peptides. Methods Mol. Biol. 1268,43–66. doi:10.1007/978-1-4939-2285-7_3
99. Destoumieux-Garzo´n D, Rosa RD, Schmitt P, Barreto C, Vidal-Dupiol J, Mitta G, Gueguen Y, Bache`re E. 2016 Antimicrobial peptides in marine invertebrate health and disease. Phil. Trans. R. Soc. B 371: 20150300. http://dx.doi.org/10.1098/rstb.2015.0300
100. Schmitt P, Wilmes M, Pugnie`re M, Aumelas A, Bache`re E, Sahl H-G, Schneider T, DestoumieuxGarzo´n D. Insight into invertebrate defensin mechanism of action: oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II. J. Biol. Chem. 2010 285, 29 208– 29 216.doi:10.1074/ jbc.M110.143388
101. Schmitt P, Rosa RD, Destoumieux-Garzo´n D. 2015 An intimate link between antimicrobial peptide sequence diversity and binding to essential components of bacterial membranes. BBA Biomembr. 1858, 958–970. (doi:10.1016/j.bbamem.2015.10.011)
102. L. Steinstraesser, T. Koehler, F. Jacobsen, A. Daigeler, O. Goertz, S. Langer, M. Kesting, H. Steinau, E. Eriksson and T. Hirsch, Mol. Med., 2008, 14, 528–537.
103. Vassilevski AA, Kozlov SA, Samsonova OV, Egorova NS, Karpunin DV, Pluzhnikov KA, et al. Cyto-insectotoxins, a novel class of cytolytic and insecticidal peptides from spider venom. Biochem J 2008;411:687–96.
104. Hunter,H.N., Demcoe,A.R., Jenssen,H., Gutteberg,T.J. and Vogel,H.J. (2005). Human lactoferricin is partially folded in aqueous solution and is better stabilized in a membrane mimetic solvent. Antimicrob.AgentsChemother. 49, 3387–3395.doi:10.1128/aac.49.8.3387-3395.2005

105. Pooi Yin Chung, Ramona Khanum , Antimicrobial peptides as potential anti-biofilm agents against multidrugresistant bacteria. Journal of Microbiology, Immunology and Infection (2017) 50, 405-410 .http://dx.doi.org/10.1016/j.jmii.2016.12.005
106. Nakatsuji T, Gallo RL. Antimicrobial peptides: old molecules with new ideas. J Invest Dermatol 2012;132:887-95.
107. Dean SN, Bishop BM, van Hoek ML. Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against Staphylococcus aureus. BMC Microbiol 2011;11: 114e25.
108. Jorge P, Lourenc¸o A, Pereira MO. New trends in peptide-based anti-biofilm strategies: a review of recent achievements and bioinformatic approaches. Biofouling2012; 26:1033e61.
109. Jazmín Huerta-Cantillo, Fernando Navarro-García. Properties and design of antimicrobial peptides as potential tools against pathogens and malignant cells.Investigación en DiscapacidadVol. 5. 2 2016 pp 96-115
110. Hancock, R.E. and Sahl, H.G.(2006).Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. 24,1551–1557. doi:10.1038/nbt1267
111. Ebenhan, T., Gheysens, O., Kruger, H.G., Zeevaart, J.R. and Sathekge, M. M. (2014). Antimicrobial peptides: their role as infection selective tracers for molecular imaging. BioMedRes.Int. 2014:867381.doi:10.1155/2014/ 867381
112. Brogden, K.A.(2005).Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat.Rev. Microbiol. 3,238–250.doi:10.1038/ nrmicro1098
113. Toke,O.(2005).Antimicrobialpeptides:newcandidatesinthefightagainst bacterial infections. Biopolymers 80,717–735.doi:10.1002/bip.20286
114. Nguyen, L. T., Haney, E. F., and Vogel, H. J. (2011). The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol.29,464–472.doi:10.1016/j.tibtech.2011.05.001
115. Yeaman, M. R. and Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance.Pharmacol.Rev. 55,27–55.doi:10.1124/pr. 55.1.2
116. Smith VJ, Dyrynda EA. 2015 Antimicrobial proteins: from old proteins, new tricks. Mol. Immunol. 68, 383–398. (doi:10.1016/j.molimm. 2015.08.009)
117. Kosikowska,P., and Lesner,A. (2016). Antimicrobial peptides (AMPs) as drug candidates: a patent review (2003-2015). Expert Opin.Ther. Pat. 26, 689–702. doi:10.1080/13543776.2016.1176149
118. Wang G, Li X, Zasloff M. A database view of naturally occurring antimicrobial peptides: nomenclature, classification and amino acid sequence analysis. In: Wang G, editor. Antimicrobial peptides: discovery, design and novel therapeutic strategies. Wallingford, UK: CAB International; 2010. p. 1–21.
119. Wang G. Database resources dedicated to antimicrobial peptides. In: Chen CY, Yan X, Jackson CR, editors. Antimicrobial resistance and food safety. San Diego, CA: Academic Press; 2015. p. 365–84.
120. Waghu FH, Gopi L, Barai RS, Ramteke P, Nizami B, Idicula-Thomas S. CAMP: collection of sequences and structures of antimicrobial peptides. Nucleic Acids Res 2014;42 (Database issue):D1154–8.
121. Thomas,S., Karnik,S., Barai,R.S., Jayaraman,V.K. and Idicula-Thomas,S. (2010) CAMP: a useful resource for research on antimicrobial peptides. Nucleic Acids Res., 38, D774–D780.
122. Galiano, K., Pleifer, C., Engelhardt, K., Brossner, G., Lackner, P.,Huck, C., Lass-Florl, C., and Obwegeser, A. (2008) Silver segregation and bacterial growth of intraventricular catheters impregnated with silver nanoparticles in cerebrospinal fluid drainages. Neurol. Res. 30, 285−287.
123. Zhao, Y. Y., Tian, Y., Cui, Y., Liu, W. W., Ma, W. S., and Jiang, X. Y. (2010) Small Molecule-Capped Gold Nanoparticles as Potent Antibacterial Agents That Target Gram-Negative Bacteria. J. Am. Chem. Soc. 132, 12349−12356.
124. Allahverdiyev, A. M., Kon, K. V., Abamor, E. S., Bagirova, M., and Rafailovich, M. (2011) Coping with antibiotic resistance: combining nanoparticles with antibiotics and other antimicrobial agents. Expert Rev. Anti-Infect. Ther.9, 1035−1052.
125. Wang, L., Hu, C., and Shao, L. (2017) The antimicrobial activity of nanoparticles: present situation and prospects for the future. Int. J. Nanomed.12, 1227−1249.
126. HoracioPoblete,AnirudhAgarwal,Suma S. Thomas, Cornelia Bohne, RanjithkumarRavichandran, JaywantPhopase, Jeffrey Comerand Emilio I. Alarcon. New Insights into Peptide−Silver Nanoparticle Interaction: Deciphering the Role of Cysteine and Lysine in the Peptide Sequence, Langmuir 2016, 32, 265−273, DOI: 10.1021/acs.langmuir.5b03601
127. L. Liu, J. Yang, J. Xie, Z. Luo, J. Jiang, Y.Y. Yang, S. Liu, The potent antimicrobial properties of cell penetrating peptide-conjugated silver nanoparticles with excellent selectivity for gram-positive bacteria over erythrocytes, Nanoscale 5 (2013) 3834–3840.
128. M. Vignoni, H. de A. Weerasekera, M. J. Simpson, J. Phopase, T.-F.Mah, M. Griffith, E. I. Alarcon and J. C. Scaiano, Nanoscale, 2014, 6, 5725–5728.
129. L. Liu, J. Yang, J. Xie, Z. Luo, J. Jiang, Y. Y. Yang and S. Liu, Nanoscale, 2013, 5, 3834–3840.
130. A. Haase, J. Tentschert, H. Jungnickel, P. Graf, A. Mantion, F. Draude, J. Plendl, M. E. Goetz, S. Galla, A. Masic, A. F. Thuenemann, A. Taubert, H. F. Arlinghaus and A. Luch, J. Phys.: Conf. Ser., 2011, 304, 012030.
131. G. N. Serrano, G. G. Zhanel and F. Schweizer, Antimicrob. Agents Chemother., 2009, 53, 2215–2217.

132. C. SherlinaDaphny, M. ArputhaBibiana, R. Vengatesan, P. Selvamani, S. Latha, Antimicrobial peptides-A milestone for developing antibiotics against drug resistant infectious pathogens, J. Pharm. Sci. Res. 7 (2015) 226–230.
133. Indrani Pal, Varsha P. Brahmkhatri, SwapnaBera, Dipita Bhattacharyya, YasribQuirishi, AnirbanBhunia, Hanudatta S. Atreya.Enhanced stability and activity of an antimicrobial peptide in conjugation with silver nanoparticle.Journal of Colloid and Interface Science 483 (2016) 385–393, http://dx.doi.org/10.1016/j.jcis.2016.08.043
134. Hnilova, M.; Liu, X.; Yuca, E.; Jia, C.; Wilson, B.; Karatas, A. Y.; Gresswell, C.; Ohuchi, F.; Kitamura, K.; Tamerler, C. Multifunctional Protein-Enabled Patterning on Arrayed Ferroelectric Materials. ACS Appl. Mater. Interfaces 2012, 4 (4), 1865−1871.
135. A. Ceccon, V. Tugarinov, A. Bax, G.M. Clore, Global dynamics and exchange kinetics of a protein on the surface of nanoparticles revealed by relaxationbased solution NMR spectroscopy, J. Am. Chem. Soc. 138 (2016) 5789–5792.
136. Juan Liua ,Jee H. Jungb , and YonghongLiua, Antimicrobial Compounds from Marine Invertebrates-Derived Microorganisms, Current Medicinal Chemistry, 2016, 23, 1-14, DOI: 10.2174/09298673236661605251 13837
137. Q. H. Tran, V. Q. Nguyen, and A. Le, "Silver nanoparticles: synthesis, properties, toxicology, applications and perspectives," Advances in Natural Sciences: Nanoscience and Nanotechnology, vol. 4, no. 3, article 20, 2013

Beneficial Role of Probiotics in Oral Health

R.RENUGA DEVI[1], THAILA MUTHUSAMY[1], MANOHAR MURUGAN[2]
VIVEKANANDHA COLLEGE OF ARTS AND SCIENCES FOR WOMEN (AUTONOMOUS)
ELAYAMPALAYAM, TIRUCHENGODE.
[2]SADAKATHULLAH APPA COLLEGE, TIRUNELVELI.
Email: gunsun1@gmail.com

Abstract: The Micro Organisms found in the human oral cavity have been refered to as the oral micro flora, oral micro biota or more recently as the oral micro biome. The mouth acts as a window to lot of systemic diseases and serves as a part of entry of the various infections that can alter and affect the immune status of the person, probiotics are dietary supplements containing potentially beneficial bacteria or yeasts. These products help in stimulating health by promoting flora and also suppressing the pathologic colonization and disease spread. Probiotics are living microbes that have a beneficial influence on health. The advent of probiotics into the field of periodontics would possibly open new horizons to address the attractive alternative to antibiotics and to target particular periodontal pathogen, thus increasing the long – term of periodontal therapy.

KEY WORDS: Probiotics, Periodontal Therapy, Oral Microbiota, Oral Microflora & Periodontal Pathogens.

HISTORY OF PROBIOTICS

Gibson and Roberfroid introduce the term "prebiotic"[1,2]. In 1965 lilley and still well introduced the term probiotics the antonym of the term "Antibiotics". Fermented yoghurt is discovered by Mann and Spooering in 1974, fermented yoghurt reduced blood serum cholesterol. Hull identified the first probiotic species, the *Lactobacillus acidophilus* in 1984. Holcomph in 1991 identified *Bifidobacterium bifidum,* in 1994 world health organization [WHO] described the probiotics as the next most important in the immune defense system following antibiotic resistance [3,4]. In 1984 Hull *et al.,* introduce first probiotic species in research was *Lactobacillus acidophilus,* followed by *Bifidobacterium bifidum* by Holcombh *et al.,* in 1991[5-11].

INTRODUCTION

Probiotics are live microorganisms when consumed may confer a health benefit to the host. The term probiotic is currently used to name ingested microorganisms associated with benefits for humans and animals. Probiotics are live microorganisms that resemble the beneficial microorganisms found in the human gut. Most probiotics are small single celled bacteria that provide beneficial health effects. They are also known as "good" or "friendly" bacteria.

Probiotic microorganisms are *Lactobacillus, Lactococci, Bifidobacteria* and *Saccharomyces.* Various dairy products available in the *Lactobacillus* strain [cheese, yoghurt and curd][12]. Probiotic are capable to promote effects on host health, they must tolerate environments with high concentrations of bile salts and low pH and display high antimicrobial activities [13]. Lactic acid bacteria [LAB] are a group of Gram – positive, non – spore forming, cocci or rod shaped, catalase negative and fastidious organisms frequently isolated from milk and dairy products[14]. Yogurt is a food product produced by bacterial fermentation of milk. Traditional probiotic yogurt is made from dairy that's fermented into a creamy food packed with beneficial probiotics and is a balanced source of protein, fats, carbohydrates, vitamins and minerals. Yogurt is considered as a super food cows or goats.

The yogurt nutrition is maximized supply of Omega – 3 fatty acid, protein, calcium, magnesium, potassium, vitamin D, K2, enzymes and probiotics. There are numerous claimed benefits of using commercial probiotics such as reducing gastrointestinal discomfort, improving immune health, relieving constipation, avoiding the common cold. Probiotics are considered generally safe, but may cause bacteria – host interactions and unwanted side effects in rare cases.

Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other[15]. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. Biofilms are associated with many medical conditions including medical devices, dental plaque, upper respiratory tract infections, peritonitis and urogenital infections[16]. Biofilm producing bacteria not only for gram – positive but both gram positive and gram negative bacteria have the capability to form biofilms[17].

TYPES OF MICROORGANISMS

Health benefits have mainly been demonstrated for specific probiotic strains of the following genera: *Lactobacillus, Bifidobacterium, Saccharomyces, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus, E.coli. Lactobacillus acidophilus* play a role in treatment of travellers diarrhea[18] and reduction of hospital stay of children with acute diarrhea[19]. Reduction of irritable bowel syndrome[20]. *Lactobacillus plantarum* used to prevent the endotoxin production[21]. *Lactobacillus casei* used to treat the functional constipation in adults[22]. *Lactobacillus brevis* used to protect the bile salt tolerance[23].

PROBIOTIC FORMULATION

CAPSULE FORM: In 2004 Montalto *et al.,* administered probiotic mix both in capsules and liquid form without observing statistically significant difference, however, in the *S.mutans* counts between the two test groups[24].

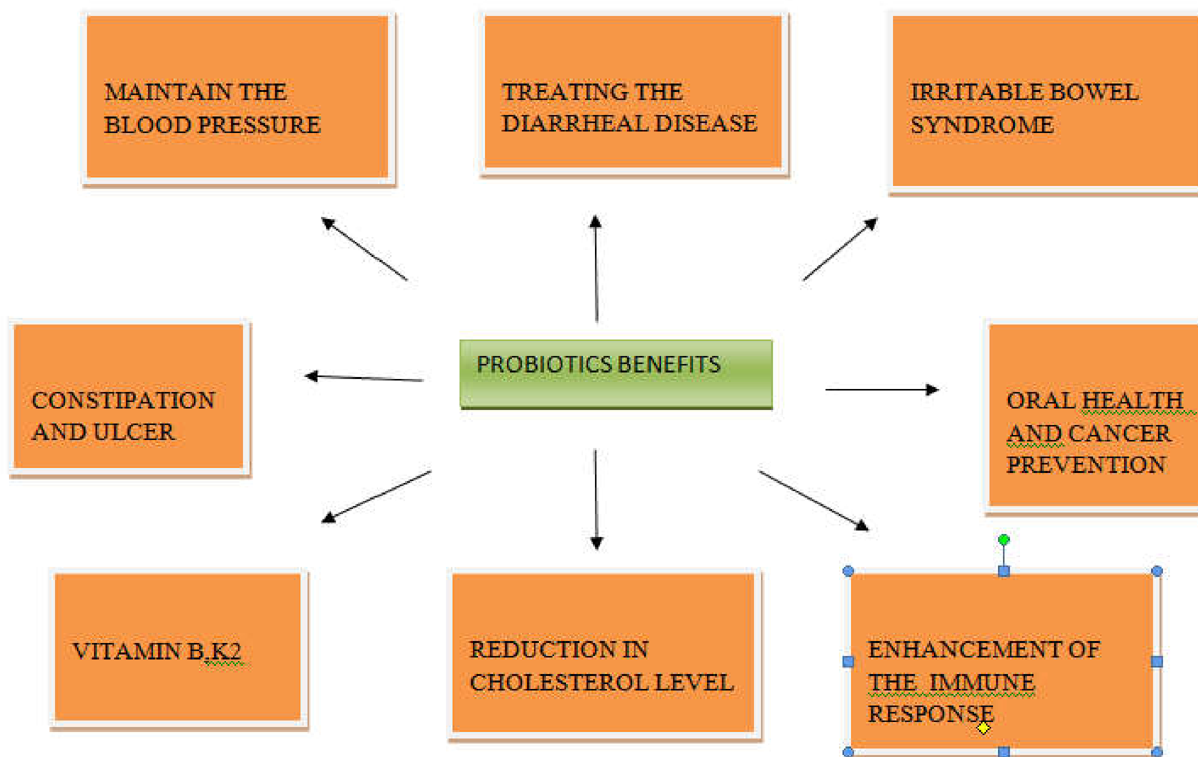
CHEWING GUM: The recent invention for caries prophylaxis is a chewing gum containing *Lactobacillus reuteri* prodentis consumed twice daily this was marketed to regulate *S.mutants* counts in the oral cavity, the average content of *Lactobacillus reuteri* was 10^8 CFU/ml. So however, the most suitable means of delivery and dosages of probiotics for various oral health purposes have not been defined[24].

TABLET FORM: In 1994 Pozharitskaia *et al., Lactobacillus acidophilus* contained in a tablet named the Acilact. It was first clinically tested and improved clinical parameters in periodontitis patients and shifts in local microflora towards gram positive cocci and *Lactobacilli.* In 2002 Grudianov *et al.,* carried out a clinical study where they obtained a probiotic mix in the tablrt forms viz, Acilact and Bifidumbacterin and found normalization of microflora and reduction of signs of gingivitis and periodontitis[25].

TOOTH PASTE: Tooth paste is available in the probiotic formulation. In 2017 F.Basak *et al.,* The *in vivo* study a new innovational aspect has been reached in the use of probiotics to ensure the balance between bacterial flora in the oral cavity. In daily routine the administration of probiotics to children is difficult. The toothpaste can be used as a vehicle to transport probiotics to children's mouth [26].

HEALTH BENEFITS OF PROBIOTICS

There is effective in favour of the claims of the beneficial effects of probiotics. It plays an effective role in treating diarrheal disease, brain function disorder, reduction of cholesterol level, to maintain the blood pressure, irritable bowel syndrome, enhancement of the immune response and cancer prevention.



CONCLUSION

Probiotics are dietary supplements containing potentially beneficial bacteria or yeasts. Which helps in stimulating health by promoting flora and suppressing the pathologic colonization and disease spread? It plays an effective role treating diarrheal disease, brain function disorder, reduction of cholesterol level, to maintain the blood pressure, irritable bowel syndrome, enhancement of the immune response and cancer prevention. In recent years there has been an upgrade in research in probiotics as well as growing commercial interest in the probiotic food concept. The role of probiotics in nutrition and medicine shows a significant increase of interest for upcoming generation. Which should be considered by medical provisional and food industries?

REFERENCES

1. *Bhardwaj A, Bhardwaj SV. Role of probiotics in dental caries and periodontal Disease. Arch ClinExp Surg. 2012; 1(1): 49 – 9.*
2. *Bhushan J, Chachra S. Probiotics – Their role in prevention of dental caries. JOHCD. 2010;4(3): 78 – 82.*
3. *Narwal A. Probiotics in Dentistry – A Review. J Nutr Food Sci.2011; 1(5):1 – 4.*
4. *Nagaraj T, Ravi B, Sankara SN, Madhu K. Probiotics and Oral Health. JIAOMR.2012;24(2):146 – 8.*
5. *Munjal S, Munjal S. Probiotics in Oral Health Promotion: The New Approach. IJBAMS. 2014;4(1):204 – 9.*
6. *Lawande S.. Probiotics for management of periodontal disease: A novel therapeutic strategy?. IOSRPHR. 2012;2(4):41 – 6.*
7. *Reddy SR, Swapna LA, Ramesh T, Singh TR, Vijayalaxmi N, Lavanya R.Bacteria in oral health – probiotics and prebiotics A review. Int J Bio Med Res. 2011;2(4): 1226 – 33.*
8. *Gupta G. Probiotics and periodontal health. J Med Life. 2011;4(4):387 – 94.*
9. *Tandon V, Arora V, Yadav V, Singh V, Punia H, Agrawal S. Concept of probiotics in Dentistry. Int J Dent Med Res. 2015;1(6)206 – 9.*

10. *Joshi SS, Pandharbale AA. Probiotics and periodontal disease – A review. Indian J Dent Adv. 2015;7(2):136 – 41.*
11. *Chitra N. Bactermia associated with probiotic use in Medicine and Dentistry. Ijirset. 2013;2(12):7322 – 25.*
12. *Kumar D, Kumar D. Isolation and characterization of bacteria from dairy samples of solan in Himachal Pradesh for identification of Lactobacillus sps., Int J Pharm Sci Rev Res 25: 2014: 110 – 114.*
13. *Biradar SS,Bahagvati and Shegunshi. Probiotics and Antibiotics: A brief overview. Int.J. Nutr. 2005: Wellness http://ispub.com/IJNW/2/1/7058.*
14. *Tasneem Chowdhury and Saiful Islam. Isolation, Identification and Determination of probiotic potential of Latic Acid Bacteria from local Curd. International Journal of Scientific and Engineering Research. 2016; vol 7;Issue 4, issn 2229 – 5518.*
15. *Donlan RM, Costerton W. Biofilms: Survival mechanisms of clinically relevant Microorganisms. Clin Microbiol Rev. 2002; 15(2):167 – 93.*
16. *Reid G. Biofilms in infectious disease and on medical devices. Int. J. Antimic Ag. 1999; 11:223 – 6.*
17. *Donlan RM. Biofilms and device – associated infections. Emerg Infect Dis. 2001; 7(2): 277 – 81.*
18. *McFarland L.V. Meta-analysis of probiotics for the prevention of traveler's diarrhea. Travel Med. Infect. Dis.2007,5, 97–105.*
19. *Phavichitr N, Puwdee P, Tantibhaedhyangkul R. Cost-benefit analysis of the probiotic treatment of children hospitalized for acute diarrhea in Bangkok, Thailand. Southeast Asian J. Trop. Med. Public Health.2013, 44, 1065–1071.*
20. *Ortiz L, Ruiz F, Pascual L, Barberis L. Effect of two probiotic strains of Lactobacillus on in vitro adherence of Listeria monocytogenes, Streptococcus agalactiae, and Staphylococcus aureus to vaginal epithelial cells. Curr. Microbiol.2014, doi:10.1007/s00284-014-0524-9.*
21. *Lee S.J, Bose S, Seo J.G, Chung W.S, Lim C.Y, Kim H. The effects of co-administration of probiotics with herbal medicine on obesity, metabolic endotoxemia and dysbiosis: A randomized double-blind controlled clinical trial. Clin. Nutr.2013, doi:10.1016/j.clnu.2013.12.006.*
22. *Chmielewska A, Szajewska H. Systematic review of randomised controlled trials: Probiotics for functional constipation. World J. Gastroenterol. 2010, 16, 69–75.*
23. *Suzuki S, Yakabe T, Suganuma H, Fukao M, Saito T, Yajima N. Cell-bound exopolysaccharides of Lactobacillus brevis KB290: protective role and monosaccharide composition. Can. J. Microbiol. 2013, 59, 549–555.*
24. *Anuj Singh Parihar, Vartika Katoch. Probiotics in Oral Health: A review. Journal of Advanced Medical and Dental sciences Research. 2015;vol.3; Issue 1.*
25. *Volozhin A.I. Development and use of periodontal dressing of collagen and Lactobacillus casei 37 cell suspension in combined treatment of periodontal disease of inflammatory origin (a microbiological study). Stomatologiia 2004; 83: 6 – 8.*
26. *Maden EA, Altun C, Ozmen B, Basak F. Antimicrobial Effect of Toothpastes Containing Fluoride,Xylitol or Xylitol – Probiotic on Salivary Streptococcus mutans and Lactobacillus in children. 2017; vol 21; Issue 2;134 – 138.*

BIOMEDICAL POTENTIAL OF MEDICINAL PLANTS EXTRACTS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

M.Manohar[a*]., P.Pandiselvi[a], Dr.R.Jannet Rani[a], K,Chitra[a] and Feroz Khan[a]
PG and Research Department of Microbiology,
Sadakathullah Appa College (Autonomous) Tirunelveli – 627006
***Corresponding Author:** gansun1@gmail.com

Abstract - The major problem existing in the field of medicine is drug resistance. The organisms were developing resistance to almost all the existing drugs. Among such organisms MRSA pose a major threat. The prevalence of MRSA infection is also increasing steadily. So we are in need of alternative therapy to cure such infections. One such remedy could be the use of extracts from medicinal plants. In the present study we used the extracts of some herbal plants such as Punica granatum, Solanum trilobatum, Ocimum tenuiflorum, Ripes uva-crispa, Psidium guajava, Cajanus cajan, Euphorbia hirta, Phyllanthus niruri, Phyllanthus amarus against MRSA. Among these plant methanol extract of P. granatum (inhibition zone 20mm) showed remarkable results against MRSA. We also checked the synergistic effect of some antibiotics with Punica granatum. They showed synergistic effect with Amoxicillin Cephalothin and Cefepime.

Keywords: MRSA, Solanum trilobatum, Psidium guajava. Antibacterial activity

INTRODUCTION

All over the ages, humans have relied on nature for their basic needs, such as food, shelter, clothing, transportation, fertilizers, flavors , medicine etc, (Critchley, A.T. (1983).). For thousands of years, plants formed the basis of traditional medicine systems that have been existing and continue with new remedies to mankind. Eventhough some of the therapeutic properties of the plants have been proven. It is based on the empirical findings of thousands of years (Heinrich *et al*., 2004). Various types of plants used in herbalism and many of these plants have medicinal properties. 3.3 billion Peoples in the developing countries utilize the medicinal plants which are the "backbone" of traditional medicine (Davidson-Hunt I *et al*., 2000).

Fruits and vegetables are most popular due to their dietetic value worldwide and rich sources of beneficial vitamins and fibers, anti-oxidants, minerals. The standard consumption of fresh fruits and vegetables may diminish the risk of cardiovascular diseases, stroke and certain cancers. Usually fruits are processed into beverage, squash juice and syrups. However by-products can be used as functional food ingredients such as phytochemicals, pharmaceuticals, essential oils, seed oil, pectin, food products, and dietary fibers (Azad *et al*., 2014). Therefore, fruits by-products not only superior source of bioactive compounds but also could be used as several value-added products (Noor *et al*., 2014).

In conventional chemistry and pharmacology plants might provide a useful source for the production of new effective medicines and this may be used to replace existing drugs . In general traditional medicine is turned out to be very useful in the discovery of natural products such as pharmaceutical drugs (Patwardhan *et al*., 2004). The shrub *Prosopsis juliflora* (Sw.) DC. (*P. juliflora*) commonly identified as mesquite has been used as a traditional medicine on various continents for curing catarrh, inflammation, colds, sore throat, excrescences, flu, measles, diarrhea, dysentery and hoarseness, for the healing of wounds (Hartwell *et al*., 1971).

METHODOLOGY

Isolation of *Staphylococcus aureus*

For the isolation of *Staphylococcus aureus*, pus samples were collected from wounds. The specimens were collected from Bose Clinical Lab, Madurai. Fluid Thioglycollate medium was used as an enriched medium for *Staphylococcus aureus*. The specimen was inoculated on Mannitol salt agar, Mac Conkey agar and Blood agar plates. The plates were incubated aerobically at 37^0C overnight. The test organisms further confirmed by their physiological and biochemical characters.

Detection of MRSA

Disk Susceptibility Test (Acar and Goldstein, 1991). The Kirby-Bauer disk diffusion test was developed for this study.

The sensitivity pattern of MRSA strains to β-lactam antiboiotics

Disk susceptibility test (Acar and Goldstein., 1991).

The Kirby –Bauer disk diffusion test was developed for these studies as described above with the antibiotic disks methicillin, amoxyclav, amoxicillin, cefdin, cephalothin, cefepime, cefaclor, vancomycin and teicoplanin. The sensitivity pattern of the test organism was recorded.

Determination of the degree of methicillin resistance

The Kirby-Bauer disk diffusion test was developed for this study. MRSA strains were individually tested for their degree of methicillin resistance with methicillin 5 µg and 10 µg.

Collection of herbal plants

The following fresh plant materials and sea weeds were collected from Vilathikulam, Thoothukudi district. Plants such as *Punica granatum, Prosopis juliflora, Ocimum tenuiflorum,* and sea weeds such as *Sargassum muticum* and *Jania . rubens.*

Preparation of plant extract

Water extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of sterile distilled water. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no:1 filter paper.

Methanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of methanol. They were placed in shaker for 24 hours. The methanol extract was filtered by using Whatmann No:1 filter paper.

Ethanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of ethanol. They were placed in shaker for 24 hours. The ethanol extract was filtered by using Whatmann no:1 filter paper.

Petroleum ether

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of petroleum ether. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no: 1 filter paper.

Determination of antibacterial activities of plant extract against MRSA strain

Loading sterile disks with plant extract

Antibacterial activity was measured using disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI). Briefly petriplates containing approximately 25-30 ml of Mueller-Hinton agar medium were inoculated using a cotton swab with a 4-6 fold culture of the bacteria. (Acar and Goldstein, 1991).

Disk susceptibility test

The Kirby- Bauer disk diffusion test was developed for these studies as described above with the disks of plant extract. The sensitivity pattern of the test organism was recorded.

Determination of synergetic effect of plant extract with some antibiotics against MRSA

The Kirby- Bauer disk diffusion test was developed for these studies as described above. To access possible interactions between the extract of plant materials and β-lactam antibiotics, disk containing the extract of plant materials and β-lactam antibiotics were placed on a plate that had been inoculated with MRSA strain. The distance between the disks was equal to the sum of the radii of their zones of inhibition when examined alone.

Checkerboard method to determine the antimicrobial combinations against MRSA

The checkerboard (or chessboard) method is the technique used most frequently to assess antimicrobial combinations in vitro, presumably because its rationale is easy to understand, the mathematics necessary to calculate and interpret the results are simple, it can readily be performed in the clinical laboratory using microdilution systems that are obtain able commercially, and it has been the technique most frequently used in studies that have suggested an advantage of synergistic therapy in the treatment of neutropenic patients with Gram-negative septicemia. The term "checkerboard" refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested in concentration equal to, above, and below their minimal inhibitory concentrations for the organisms being tested.

RESULTS

Isolation and characterization of *staphylococcus aureus*

Eight numbers of MRSA strains were isolated from pus. Various media (Table 3) were used to identify the pathogenic strain. Mannitol salt agar was used as a differential and selective medium for recovering strain from the specimen. *Staphylococcus aureus* produced yellow colour colonies in Mannitol Salt agar.

Table 1.Cultural characterization of *Staphylococcus aureus*

S. No	Medium	Colony Morphology	Interpretation
1	MacConkey Agar	Pink colour colonies	Lactose fermentation positive
2	Blood Agar	Yellow to cream colonies or white colonies with clear zone	β-haemolytic colonies
3	Mannitol Salt Agar	Yellow colonies	Mannitol fermentation positive

Table.2. Degree of Methicillin Resistance of MRSA strains (Fig. 20)

S. No	Methicillin Disk Content	Zone diameter of inhibition (mm)	Reaction of the Organism
1	Methicillin (5 μg)	-	Resistant
2	Methicillin (10 μg)	-	Resistant
3	Methicillin (30 μg)	-	Resistant

Determination of antibacterial activities of plant extract against MRSA strain

The antimicrobial activity of various extracts of *Punica granatum* and *Prosopis juliflora* were tabulated (Table 7 -9). (Fig. 5-12).

Table.3. Antimicrobial activity of various extracts of *Punica granatum*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	10	15	15	_
MRSA 1	Partial	10	15	_
MRSA 2	Partial	10	20	_
MRSA 3	Partial	15	20	_
MRSA 4	10	15	21	_
MRSA 6	10	15	20	_
MRSA 7	Partial	10	15	_
MRSA 8	10	15	20	_

Table 4.Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Table 5. Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Fig. 1. *P. granatum* (pomegranate)

Fig.2. *P. juliflora* (karuvelam)

Fig. 3 *S. muticum*

Fig. 4. *J.rubens*

Plate 1. Synergistic effect between *P. granatum* and methicillin 10 µg

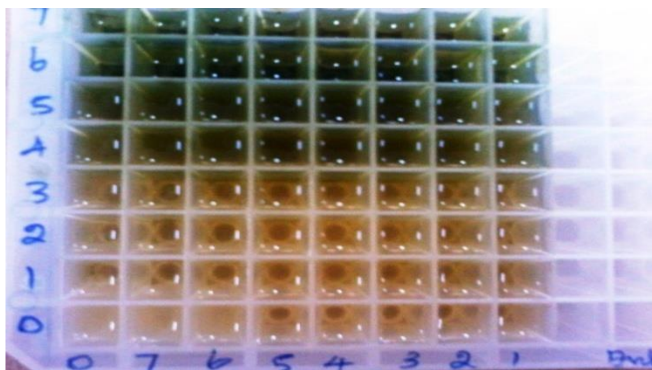
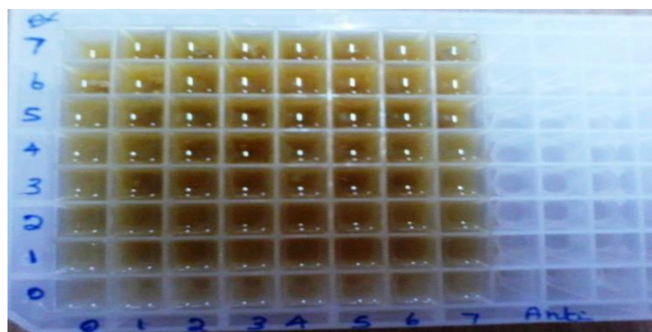
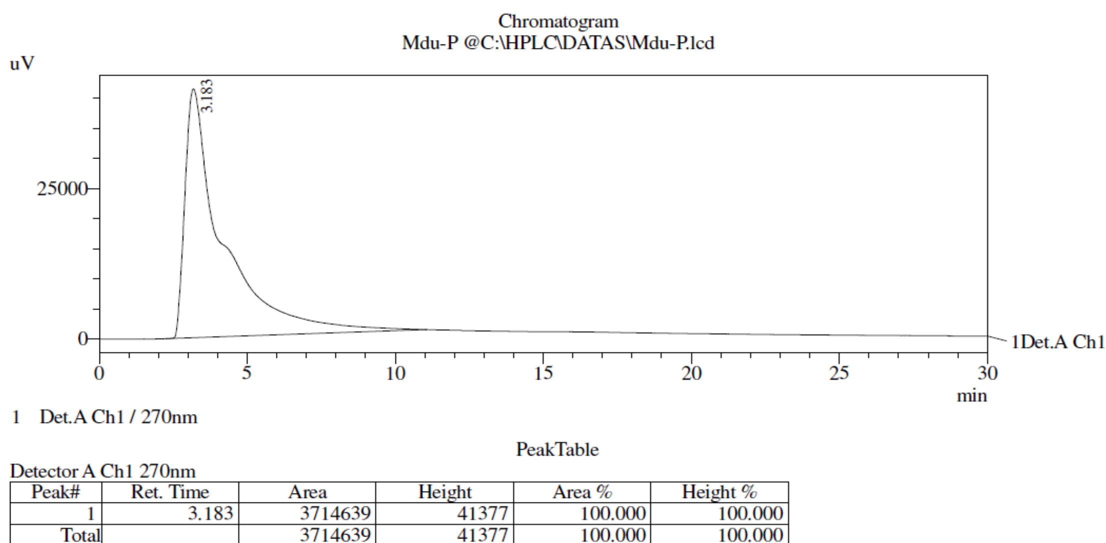


Plate 2. Synergistic of *P.juliflora* with Amoxyclav





PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.183	3714639	41377	100.000	100.000
Total		3714639	41377	100.000	100.000

Fig. 6. HPLC analysis of *P. granatum*

DISCUSSION

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years, the World Health Organization advocated that countries should interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origin (WHO. 1978).

The increasing occurrence, particularly in hospitals, of *S. aureus* resistant not only to methicillin but to a wide range of antimicrobial agents, including all kinds of β-lactams, has made therapy more difficult (Unci Ney *et al.,*2006).. Although strategies have been proposed in an attempt to control the spread, the search for new ways to treat MRSA infections stimulates the investigation of natural compounds as an alternative treatment of these infections.In the present study, the analysis of the growth inhibition activity by the disk diffusion method showed

that 10 medicinal plants (*P. juliflora*, *P. granatum*, *S.trilobatum*, *O.tenuiflorum*, *R.uva-crispa*, *P.guajava*, *C. cajan*, *E. hirta*,*P.niruri* and *P. amarus*),were active against hospital strains of MRSA under test conditions with crude extract concentrations as high as 5g/20ml.(Williams, et al.,2007)

In the present study, 2 plant extracts (*P. juliflora*, *P. granatum*) were effective against MRSA, MRSA1, MRSA2, MRSA 3, MRSA 4, MRSA 6, MRSA 7, MRSA 8 strains. We were using water, ethanol, methanol, petroleum ether extracts for all the 8 strains. Methanol extract inhibit the growth of 8strains of MRSA. The synergistic method were also performed for the methonal extracts with 10 antibiotics of methicillin (5µg), methicillin (10µg), amoxyclav (30 µg 10 µg), amoxicillin (30 µg 10µg), cefdin (5µg), cephalothin (30µg), cefepime (30µg), cefaclor (30µg), vancomycin (30 µg) and teicoplanin (30µg) in MRSA strain.

References:

1. Critchley, A.T. (1983). *Sargassum muticum*: a taxonomic history including world-wide and western pacific distributions. *Journal of the Marine Biological Association of the United Kingdom* 63: 617–625.
2. Davidson-Hunt. I. (2000). Ecological ethno botany: stumbling toward new practices and paradigms. MASA J 16:1–13, 2000.
3. Azad, A.K.M., M. A. Ali, M.S. Akter, M.J. Rahman and M. Maruf Ahmed. (2014). Isolation and Characterization of Pectin Extracted from Lemon Pomace during Ripening. *Journal of Food and Nutrition Sciences* Vol. 2, No. 2, pp. 30-35.
4. Noor, F., M.J. Rahman, M.S. Mahomud, M.S. Akter, M.A.I. Md. Aminul Islam Talukder and M. Ahmed. (2014). Physicochemical Properties of Flour and Extraction of Starch from Jackfruit Seed. *International Journal of Nutrition and Food Sciences* Vol. 3, No. 4, pp. 347-354.
5. Patwardhan, B., A. D. Vaidya and M. Chorghade. (2004). Ayurveda and natural products drug discovery. *Curr Sci* 86: 789-799.
6. Hartwell, J.L. (1971). Plants used against cancer. A survey. *Lloydia* 34: 386-425.
7. Acar, J.F. and F. W. Goldstein, (1991). Disk Susceptibility Test. In: Lorian V. (Ed). Antibiotics in Laboratory Medicine. Williams and Wilkins, London.
8. WHO. (2002). Traditional medicine. Report, EB111/9, World Health Organization, Geneva.
9. Williams, S. L. and J. E. Smith. (2007). A Global Review of the Distribution, Taxonomy, and Impacts of Introduced Seaweeds. *The Annual Review of Ecology, Evolution and Systematics*, 38, 327-59.
10. Unci Ney, J.Y., W.Y. Shieh, Jeng and W.L. Huang. (2006). Antibiotic activity of lectins from marine algae against marine Vibrio. *Journal of International Microbiology and Biotechnology* 30: 433-439.

ANTIBACTERIAL ACTIVITY OF HOLY MEDICINAL PLANTS AGAINST RESPIRATORY PATHOGENS

M.Abdul Salam[a*] , S.Muthu Raj[a] , S.Mohammed Lathif[a] , Renuga Devi[a] , and M.Manohar[a]

PG and Research Department of Microbiology Sadakathullah Appa College (Autonomous)

*__Corresponding Author__:abdulsalammas1102@gmail.com

Abstract:** The Holy aromatic medicinal plants such as Ocimum tenuiflorum (Tulsi), Solanum trilobatum (Thuthuvalai) and Coleus aromaticus (Karpooravalli) was chosen for the antibacterial activity against respiratory pathogens such as MRSA and K.pneumoniae .This medicinal plants was studied against two different pathogens identified from clinical samples. The phytochemical test for three medicinal plants Tannins, Flavanoids, Trepenoids are present in three plants. From the phytochemical analysis plant contains some components. These components play a main role in antibacterial activity. We compared our finding with standard antibiotic disc against respiratory pathogens. Our finding shows the maximum zone of inhibition observed in Tetracycline antibiotic. Then antibiotic sensitivity of respiratory pathogens Methicillin Resistant Staphylococcus aureus (MRSA) against Vancomycin, Bacitracin, Tetracyclin, Kanamycin were used. Among the 4 antibiotics Tetracycline was more effective against MRSA. Klebsiella pneumoniae against the antibiotic for Tetracycline, Azithromycin, Erythromycin were used. Among the 3 antibiotics Tetracycline was more effective against Klebsiella pneumoniae . The antibacterial activity of aromatic medicinal plants against respiratory pathogens. In our present study analysed by four different concentration of plant extract 10mg,7.5mg,5mg,2.5mg both disc and well diffusion methods.

***Keywords:** Klebsiella pneumonia, Azithromycin, Methicillin, Ocimum tenuiflorum, Coleus aromaticus, Solanum trilobatum*

Introduction:

A country like India is very much suited for the development of drugs from the medicinal plants. Because of its vase and wide variations in soil and some types of climatic conditions, the Indian sub-continent is suitable for cultivation of large number of medicinal and aromatic plant which can be used as raw plant materials for pharmaceuticals, perfumery, cosmetics, food and agrochemical industries. Many of the plant materials used in traditional medicine are readily available in rural areas at low cost than modern medicine (Milin Agarwal *et al.,* 2012). *Solanun trilobatum* playing an important role in health of all people's life in many villages of India in their day to day life by its traditional usage. It has been widely used to treat the respiratory disorders, diabetes, cholera, bronchitis, high blood pressure, asthma, tuberculosis *etc* ., (Daunay &Chadha 2004). This Tulsi's different plant parts are used to the Ayurveda and Siddha systems of medicine for prevention and cure some illnesses and common cold, head ache, cough, sore throat, bronchitis, asthma, flu, earache, fever, colic pain, hepatic diseases, malaria fever, snake bite, scorpion sting, migraine headaches, fatigue, digestive disorders, wound, arthritis, skin diseases, night blindness and diarrhea. Also the Tulsi leaves are good for the nerves system sharpen memory and cure the ulcers and infections of mouth (Prajapati *et al.,* 2003). The leaves of Thuthuvalai are specific to treat the fever and common cold. During the rainy season some of viral and bacterial infections are spread and cause infections

Materials and Method:

In the present study, three important medicinal plants namely *Ocimum tenuiflorum, Solanum trilobatum* and *Coleus aromaticus* assessed the phytochemical components and antibacterial activity against respiratory pathogens with different concentration and combination.

The following objectives were carried out in this study:

- The organic solvents viz., Petroleum ether, Chloroform, Methanol, Acetone, Ethyl acetate, water were used for the extraction of plant material.
- The extracts were screened for the presence of phytochemical compounds and also test antibacterial activity against respiratory pathogens such as *Klebsiella pneumoniae,* Methicillin Resistant *Staphylococcus aureus* (MRSA).
- The selected medicinal plants used in different concentration with different combinations to analyze the antibiogram activity.

Collection of plant materials:

Healthy leafs of *Ocimum tenuiflorum, Solanum trilobatum* and *Coleus aromaticus* were collected from Kandiyaperi near by Villages, Tirunelveli Dt. The leaves were washed thoroughly with normal tap water followed by the sterile distilled water . Then the leaves were dried under shadow condition at room temperature. Dried leaves were crushed to powder using grinding machine. Powder was stored at 4^0C in tight air container bottle.

Test Microorganisms :

Microorganisms choosen were obtained from the Laboratory of Micro Lab, Tirunelveli. The organisms used for this study were; Methicillin Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae.* The bacterial isolates were confirmed by using Gram staining, biochemical tests and plates on selective medium via of MSA (Mannitol Salt Agar) medium, Mac Conkey agar.

Preparation of leaf extract:

The preparations of different leaves extract was done through the standard method . We choose the solvent low polar to high polar concentrations. Solvents are 1.Petroleum ether 2.Chloroform 3.Methanol 4. Acetone 5.Ethyl acetate 6.Water.

Petroleum ether extraction method:

The shaded dried leaf materials were used to treat the petroleum ether solvent.50gms of each powder were weighed and mix the petroleum ether (1:3w/v) 150ml. This was incubated for 24 hours. After 24hrs the extract was filtered through the gauze cloth and Wattman No.1 filter paper in a beaker and allow to dry it for evaporation. The plant powder was further treated with chloroform.

Chloroform extraction method:

The petroleum ether treated the each plant powder was again dry it, and weigh the each plant powder. The powder were mixed with the chloroform solvent (1:3 w/v) which was incubated for 24hours. After 24hrs, the extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allow to dry it for evaporation. The plant powder was further treated with methanol.

Methanol extraction method:

The chloroform treated with the each plant powder was dry it. And weigh the each powder. The powder were mixed with the methanol solvent (1:3 w/v) which was incubated for 24hours. After 24hrs, the extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allows to dry it for evaporation. The plant powder was further treated with acetone.

Acetone extraction method:

The methanol treated with the each plant powder was dry it. And weigh the each powder. The powder were mixed with the acetone solvent (1:3 w/v) which was incubated for 24hours. After 24hrs, the extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allow to dry it for evaporation. The plant powder was further treated with Ethyl acetate.

Ethyl acetate extraction method:

The acetone treated with the each plant powder was dry it. And weigh the each powder. The powder were mixed with the ethyl acetate solvent (1:3 w/v) which was incubated for 24hours. After 24hrs, the extract was filtered through the gauze cloth and Whattman No.1filter paper in a beaker, and allow to dry it for evaporation. The plant powder was further treated with distilled water.

Water extraction method:

The ethyl acetate treated with the each plant powder was dry it. And weigh the each plant powder. The powder were mixed with the Distilled water (1:3 w/v) which was incubated for 24hours. After 24hrs, the extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allow to dry it .

Phytochemical screening:

The various solvent extracts of the powder of leaves of *Ocimum tenuiflorum, Solanum trilobatum* and *Coleus aromaticus* were subjected to the phytochemical test for the identification of various active constituents, using the method followed (Malcom and Sofowora ,1969).

Test for Tannins:

About 2ml of the aqueous extract was mixed with 2ml of distilled water and few drops of $FeCl_3$ solution were added.

Test for Saponins:

3ml of the aqueous extract shaken vigorously with 3ml of distilled water in a test tube and warmed.

Test for Phlobatannins:

2ml of aqueous extract was added and 2ml of 1%HCl and the mixture was boiled.

Test for Flavonoids:

5ml of aqueous extract was added and 1ml of H_2SO_4 added. Few minutes wait and observe the result.

Test for Terpenoids:

2ml of aqueous extract was dissolved in 2ml of chloroform and evaporate it. Then 2ml of concentrated H_2SO_4 was added and heated for about 2min.

Test for Glycosides:

2ml of aqueous extract was dissolved in 2ml of chloroform and 2ml of acetic acid was added in it. The solution was cooled in ice. Then H_2SO_4 was added carefully. Then observe the result.

Test for Steroids:

2ml of extract was dissolved in 2ml of chloroform and 2ml of concentrated H_2SO_4 was added and observe the result.

Test for Phenols:

1ml of aqueous extract were dissolved in 5ml of alcohol and treated with few drops of $FeCl_3$ solution.

Test for protein and Aminoacids:

2ml of plant extract dissolved in 2ml of water. Change the colour indicates the presence of protein.

Test for Alkaloids:

5ml of plant extract was mixed with 1% HCl .The solution obtained was filtered and then 1ml of filtrate was treated with few drops of Mayer's reagent. Formation of turbidity or cream precipitate indicates presence of alkaloids.

Antibacterial Activity:

Well Diffusion Method:

The modified Antibacterial test was performed using agar well diffusion method. (Collins *et al,* 1995).The test microorganisms were inoculated on Muller Hinton agar (MHA) and spread uniformly using the organism swab. Wells are 8mm in diameter were made on MHA using a sterile well puncher. The agar blocks were carefully removed by the use of sterilized forceps. The various concentration of each plant extracts were transferred to the wells. Then the one of the well were filled with solvents are used to take the extract, so that solvent was adding the one well. The plates were allowed to stand for 1hour at room temperature for diffusion of the substances before the growth of the microorganism commenced. The plates were incubated at 37^0C for 24hours and the zone of the inhibition was recorded.

Disc Diffusion Method:

The Muller Hinton agar was poured into sterile petriplates separately which were labeled appropriately using aseptic technique. The broth culture of the microorganisms was spread over the surface of agar plate. And the standard disc was placed . And another petriplate , solvent control was added. Each solvent was added to each disc and finally each plant solvent was treated with Dimethyl sulphoxide (DMSO) dissolve it. Then different concentrated each extracts were added to the discs. The petriplate were incubated at 37^0C for 24hours in an inverted position. The inhibition zone of microbes was observed and were measured and control disc were observed.

Result and Discussion:

In the present investigation the respiratory pathogens were isolated from the clinical samples. The organisms were identified by the preliminary and biochemical test. Based on the results we isolated two respiratory pathogens namely Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae.* these two pathogens selected for antibacterial studies.

Table :1. Preliminary Bacterial Identification Test

S.NO.	Name of the organisms	Gram'staining	Motility	Oxidase	Catalase
1.	**Methicillin Resistant *Staphylococcus aureus*(MRSA)**	Gram positive cocci (Grape like clusters)	Non-motile	Negative	Positive

2.	***Klebsiella pneumonia***	Gram negative bacilli (rod)	Non-motile	Negative	Positive

Table : 2. Biochemical reactions of Respiratory pathogens

Organism Name	**Indole**	**MR**	**VP**	**Citrate**	**Urease**	**TSI**	**G**	**S**	**L**	**M**
Methicillin Resistant ***Staphylococcus aureus***	-	+	+	-	-	A/A G(-) H_2S (-)	A/G	A/G	A/G	A/G
Klebsiella pneumoniae	-	-	+	+	+	A/A G(+) H_2S (-)	A/G	A/G	A/G	A/G

Table: 3. Phytochemical results for *Ocimum tenuiflorum, Solanum trilobatum* and *Coleus aromaticus* (Aqueous extract)

S.NO	**Chemical constituents**	***Ocimum tenuiflorum***	***Solanum trilobatum***	***Coleus aromaticus***
1	**Tannins**	+	+	+
2	**Saponins**	**-**	+	**-**
3	**Phobatannins**	+	**-**	**-**
4	**Flavanoids**	+	**-**	+
5	**Terpenoids**	+	**-**	+

6	**Glycosides**	+	-	+
7	**Steroids**	+	+	-
8	**Phenols**	-	-	+
9	**Protein,Amino acid**	-	-	+
10	**Alkaloids**	-	-	+

Figure1: Antibacterial activity of *Coleus aromaticus* by disc diffusion method against MRSA

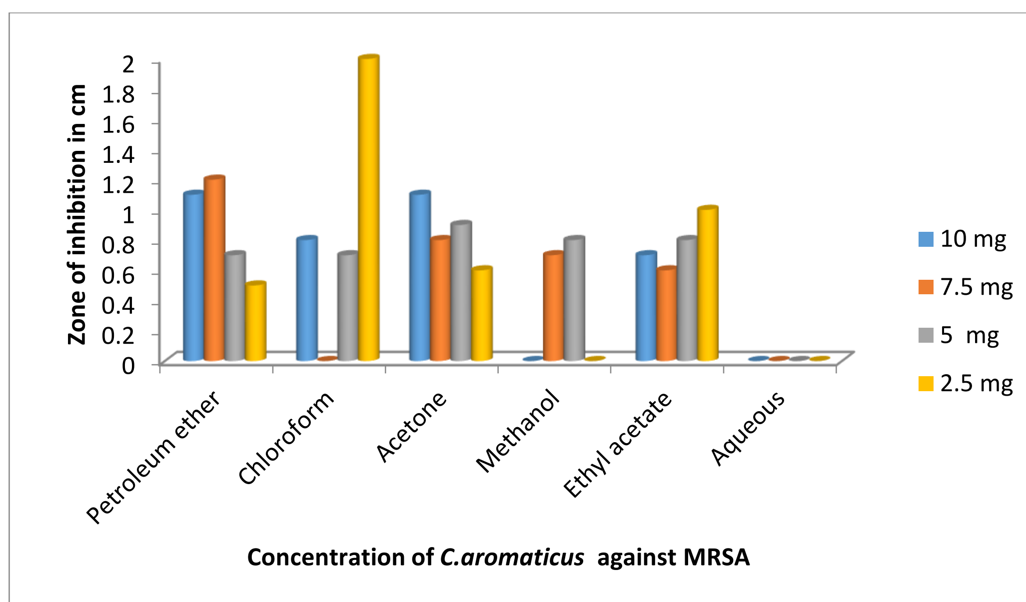
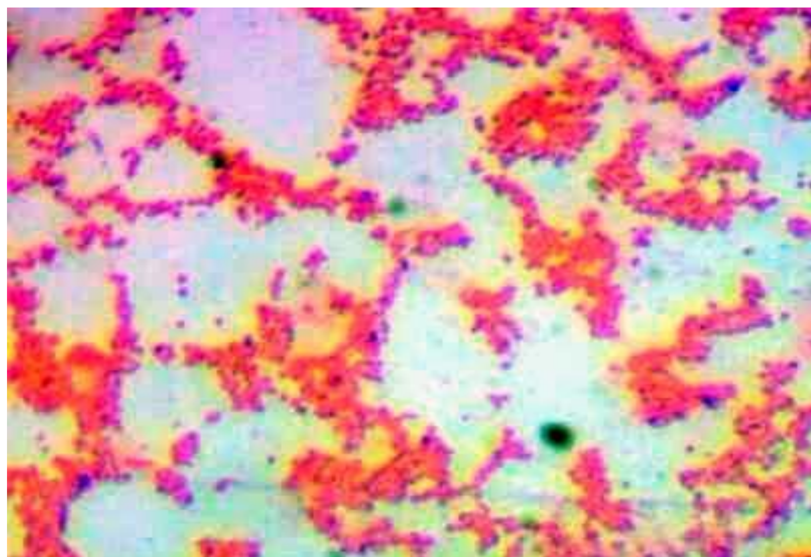


Table 4: Antibacterial activity of *Ocimum tenuiflorum &Solanum trilobatum* (OT&ST) against *Klebsiella pneumoniae*

S.No	Solvent	Well diffusion method (mm)			
		10mg	7.5mg	5mg	2.5mg
1.	**Methanol**	1.2±0.15	1.0±0.05	0.8±0.05	0.8±0.05

Sensitive: ≥ 10 mm Resistant: ≤ 10 mm

Picture.1. Methicillin Resistant *Staphylococcus aureus*



References:

1. Abdollahzadeh S.H., Mashouf R., Mortazavi H., Moghaddam M., Roozbahani N., Vahedi M. Antibacterial and antifungal activities of *Punica granatum* peel extracts against oral pathogens. Journal of Dent Tehran. (2011); 8: 1 – 6. [PMC free article] [pubMed.
2. Annapurani S., and Priya R. Antimutagenic, antitumourogenic and antigenotoxic effects of polyphenol extracts of selected medicinal plants. Indian J. of Nutrition and Dietetics. (1999); 36 (10) : 431 – 35.
3. Ashish Ranjan Singh., Vijay kumar Bajaj., Pritam Singh Sekhawat., and Kapil Singh.Journal of Natural Product and Plant Research. (2013); Vol 3 (1) : 51 – 58.
4. Auil F., Khan MS., Owais M., Ahmed I. Effect of certain bioactive plant extracts on clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus.* Journal Basic Microbiol. (2005); 45 (2) : 106 – 114.
5. Bhakuni D.S., Dhar M.L., Dhar M.M., Dhawan B.N.,and Mehrotra B.N. Screening of Indian plants for biological activity. Part II. Indian Journal of Exp. Bio. (1969); 7 : 250 – 262.
6. Brieskorn C.H., and Riedel W., Flavonoids from *Coleus amboinicus*, Planta Medica. (1977); 31 (4) : 308 – 10.
7. Chinthana P., Ananthi T. Journal of Chem. Pharma. Res. (2012); 4 (1) : 72 – 74.
8. Chaudhri R.D., Herbal Drugs Industry . Eastern Publisher, New Delhi, 1st Edn, (1996).
9. Das S., Khan M.L., Rabha A., Bhattacharjya D.K. Ethnomedicinal plants of Manas National Park, Asam, Northeast India. Indian Jtrad Know.(2009); 8 : 514 - 517.
10. Das S.K., and Vasudevan D.M., Tulsi: The Indian holy power plant. Natural Product Radiance. (2006); 5 : 279 - 83.
11. Danuay M.C., and Chadha M.L. *Solanum melongena* L. In: Gruben GJH & Denton OA (eds). Plant Resources of Tropical Africa 2. Vegetables. Wageningen : PROTA Foundations/Backhuys Publishers /CTA. (2004); 21 - 23.

12. Dutta S. Essential oil of *Coleus aromaticus* of Indian origin. Indian oil and Soap J. (1959); 25 : 120.
13. Durgadevi R., and Ponmozhi P. Preliminary Phytochemical Screening of Solanum trilobatum(L) Leaves on Steamed Processing. Indo-Asian Journal of Multidisciplinary Research (IAJMR). (2016); Vol (2). 593 - 597 .
14. Geeta Vasudevan D.M., Kedlaya R., Deepa S., Ballal M. Activity of *Ocimum sanctum* (the traditional meditional plant) against the enteric pathogens. Indian Journal of Med Sci. (2001); 55 (8) :434 - 438.
15. Gomathinayagam Subramanian., Brij B.Tewari., Rekha Gomathinayagam. Studies of Antimicrobial Properties of Different Leaf Extract of Tulsi *(Ocimum teuniflorum)* against Human Pathogens. American International Journal of Contemporary Research. (2014); 4 No.8 .
16. Gupta S., Lakshmi A.J., Manjunath M.N., and Prakash Journal Analysis of nutrient and antinutrient content of underutilized green leafy vegetables LWT. (2005); 38: 339 – 345.
17. Gurgel A.P.A.D., da Silva J.G., Grangeiroa A.R.S., Oliveira D.C., Limaa C.M.P., da Silvaa A.C.P., Oliveira R.A.G., and Souzac I.A. In vivo study of the anti-inflammatory and antitumor activities of leaves from *Plectranthus amboinicus* (Lour). Spreng (Lamiaceae), Journal of Ethnopharmacol. (2009); 125: 361 – 363.
18. Gurgel A.P.A.D., da Silva J.G, Grangeiro A.R.S., Xavier H.S., Oliveira R.A.G., Pereira M.S.V., de Souza I.A. Antibacterial Effects of *Plectranthus amboinicus* (Lour.) Spreng (Lamiaceae) in Methicillin Resistant *Staphylococcus aureus* (MRSA). Lat. Am. Journal of Pharm. (2009); 28 (3): 460 - 4
19. Haniffa M.A., and Kavitha K. Antibacterial activity of medicinal herbs against the Fish pathogens *Aeromonas hydrophila*. Journal of Agricultural Technology. (2012); 8 (1): 205 -211,
20. IDMA. Indian Herbal Pharmacopoeia. Mumbai, India. (2002);272.
21. Harborne J.B., Baxter H. Phytochemical Dictionary, Taylor and Francis Washington DC. (1993); 1765.
22. Jawahar M., Amalan Rabert G., and Jeyaseelan M .Rapid Proliferation of multiple shoots in *Solanum trilobatum* L. Plant tissue culture. (2004); 14 (2): 107 - 112.
23. Jayachithra A., and Krithiga N. Antioxidant and antibacterial activity on *Coleus aromaticus* and Lawsonia inermis. International Journal of Pharmacy & Life Sciences . (2012).
24. Jayachitra J., and Chitra M. Antibacterial activity of *Coleus aromaticus* L. world journal of pharmacy and pharmaceutical sciences. (2015); 4.
25. John de Britto A., Herin Sheeba Gracelin D., Benjamin Jeya Rathna Kumar P. Antimicrobial activity of few medicinal plants against Gram negative bacteria. International Journal of Applied Biology and Pharmaceutical Technology. (2011); Vol.2..
26. Juhi sahu., Bhawana Rathi., Sameksha Koul., Khosa R.L. *Solanum trilobatum* solanaceae family An Overview. Journal of Natural Remedies. July (2013); Vol 13 (2).
27. Kannabiran K., and Swapna LathaP. Antimicrobial activity and Phytochemicals of *Solanum trilobatum* Linn. African Journal of Biotechnology. (2006); Vol.5 (23): 2402 - 2404.
28. Krishnan K., Ramalingam R.T., Venkatesan G. J. App. Bio. Sci. (2008); 2(3):109 – 112.
29. Kaliappan., Nirmala Devi., and Periyanayagam Kasi viswanathan. 'Pharmacognostical studies on the leaves of *Plectranthus amboinicus* (Lour) Spreng', International Journal of Green Pharmacy. (2008); 2 (3): 182 – 4. http://www.greenpharmacy.info/article.asp?issn=0973-8258;year=2008;volume=2;issue=3;spage=182;epage=184;aulast=Kaliappan
30. Loeraa J.A., Reyes-Ortib C., kuoa Y.F. Predictor’s of complementary and alternative medicine use among older Mexican Americans. Complement Therap Clin Prac. (2007); 13: 224 - 231.
31. Livingston R.N.R., Pooshan G.V., Venkatachalam V.V., Kumar M.V. Pharmacologyonline. (2009); 3:758 – 765.
32. Milin K.Agrawal., and Alka Varma. *Ocimum santum* (Tulsi)- In-vitro anti-bacterial activity against Dermatophytes. IJSR- INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH. (2012).

33. Marganingsih S.C., Triana H., Fak. Kedokteran Hewan. Phytochemical compounds and the effect water extract of Bangun-bangun leaves *(Coleus aromaticus,*L.) on phagocytosis activity of rat (Rattus norvegicus) neutrophil cells. Majalah Farmasi Indonesia. (2005); 16 (3): 141 -148.
34. Odebiyi O.O., and Sofowora E.A. Phytochemical screening of Nigerian Medical Plants II Lloydia. (1978); 41: 2234 - 2246.
35. Poonam Mishra., and Sanjay Mishra. Antibacterial activity of *Ocimum sanctum* Extract Against Gram positive and Gram negative Bacteria. American Journal of Food Technology. (2011); Vol 6 (4): 336 – 341.
36. Prakash P., Gupta N. Therapeutic uses of *Ocimum sanctum* Linn (Tulsi) with a note on eugenol and its pharmacological actions: a short review Indian J. Physiol Pharmacol. (2005); 49 (2): 125 - 131.
37. Prajapati N.D., Purohit S.S,. Sharma A.K., and Kumar T. A. Hand Book of Medicinal Plant. Agrobios, India.(2003); 367.
38. Pritima R.A., and R.S. Pandian. Antimicrobial activity of *Coleus aromaticus* (benth) against microbes of Reprodutive tract infectious among women Afr.Journal of Infect. Diseases. (2008); 1 (1): 18 - 24.

ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/341622065

PHYTOCHEMICAL ANALYSIS OF Prosopis juliflora AND ITS ANTIBACTERIAL ACTIVITY AGAINST HUMAN PATHOGENS

Article · May 2020

DOI: 10.22192/lsa.2019.5.6.3

CITATIONS
0

READS
72

6 authors, including:

S Muthu Raj
K L University

9 PUBLICATIONS **2** CITATIONS

SEE PROFILE

All content following this page was uploaded by S Muthu Raj on 25 May 2020.

The user has requested enhancement of the downloaded file.

Available online at www.jpsscientificpublications.com

Life Science Archives (LSA)
ISSN: 2454-1354
Volume – 5; Issue - 6; Year – 2019; Page: 1720 – 1725
DOI: 10.22192/lsa.2019.5.6.3

Research Article

PHYTOCHEMICAL ANALYSIS OF *Prosopis juliflora* AND ITS ANTIBACTERIAL ACTIVITY AGAINST HUMAN PATHOGENS

S. Muthu Raj*, M. Abdul Salam, S. Mohamed Lathieef Laseem, S. Kalaivani and M. Manohar

PG and Research Department of Microbiology, Sadakathullah Appa College (Autonomous), Tirunelveli, Tamil Nadu, India.

Abstract

Plants and plant products have been used as medicine from the ancient. It is estimated by the World Health Organization that approximately 75 – 80 % of the world's population uses plant medicines either party entirely as medicine. Recently there been a shift inuniversal trend from synthetic to herbal medicine, which we can say Return to Nature Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich oftherapeutic agents for the prevention of diseases and ailments. Nature has bestowed our country withan enormous wealth of medicinal plants, therefore India has often been referred to as the Medicinal Garden of the world Medicinal plants are the important source for the new chemical substances withpotential therapeutic effects (Sharma *et al.,* 2012).

Article History

Received : 19.09.2019
Revised : 31.10.2019
Accepted: 22.11.2019

Key words: *Prosopis julifora,* Phytochemical analysis, Bacteria, Antibacterial activity and Well diffusion assay.

1. Introduction

Research in herbal medicine has increased in developing countriesway to restore ancient traditions as well as an alternative solution to health problems. Therefore with the increasing acceptance of traditional medicine as an alternative form of health care, the screening of medicinal plants for active compound has become very important (Deepa Chandra, 2013). *Prosopis juliflora* has been used as a folk remedy for catarrh cold, diant dysentery, excrescences, Nu, inflammation, measles, sore throat and in healing of wound from *Prosopis juliflora* is one of the most economically and ecologically important tree species in arid and semi-atidzones of the world. It is an important species because of its high nitrogen fixing potential in very dryareas and in drought seasons and also because of it provides shelter and food to many species of animals on its nectar, pollen, leaves and fruits. The shrubs of *Prosopis juliflora* are highly esteemedfor windbreaks, soil binders, sand stabilizers, living fences, fuel wood, bee plants and animal feed. These uses together with fast growth, drought resistance and salt tolerance love lead to its introduction in many arid ones. The genera *Prosopis* and each contain some of the mostwidespread and important tree species in the arid and semi-arid zones of the tropical and sub-tropical world species of these two genera have

Corresponding author*: **S. Muthu Raj
E.mail: muthurajmicrobiology@gmail.com

©2019 Published by JPS Scientific Publications Ltd. All Rights Reserved

been estimated to occupy some 3.1 million square kilometers. *Prosopis juliflora* grows abundantly in Indian sub-continent and commonly it is known as Mosquito (English), Algarroba (Spanish), Vilayati babul, Vilayati khan, Gando baval and Vilayati kikar (India). The genus *Prosopis* is thought to be evolved approximately 70 million years ago, before the African and south American continents separated. *Prosopis* genus cropped up in the American sub continent with two centers of diversity, the Texan-Mexican and the Argentinean center having a large number of sympatric species. Laves are bipinnate, with 1 to 10 leaves per node and petiole plus rachis 5 to 20 cm long. While trees are generally evergreen, *Prosopis juliflora*is occasionally deciduous, possibly due to drought or cold temperature.

2. Materials and Methods

Collection of plant materials

Healthy leafs of *Prosopis juliflora* were collected from Tirunelveli, Tamil Nadu, India. The leaves were washed thoroughly with normal tap water followed by the sterile distilled water. Then, the leaves were dried under shadow condition at room temperature. Dried leaves were crushed to powder using grinding machine. Powder was stored at 4˚C in tight air container bottle for further use.

Test microorganisms

Test sample were collected form reputed laboratory in Tirunelveli, Tamil Nadu, India. Pathogens were isolated from wound infections. The test pathogens identified based on their physiological and biochemical characters. The bacterial isolates were confirmed by using Gram staining, Biochemical tests and Platting on selective medium like MSA (Mannitol Salt Agar) medium, MacConkey agar and King's Medium. The organisms used for the current study were *Staphylococcus* sp., *Bacillus* sp. and *Pseudomonas* sp.

Preparation of Leaf extracts

The preparation of different leaves extract was done through the Standard method. The solvent with low polar concentrations like Petroleum ether, Chloroform, Methanol, Ethanol, Ethyl Acetate and Water were used for the present research.

Petroleum ether extraction method

The shaded dried leaf materials were used to transfer to the Petroleum ether solvent. Fifty grams of each powder were weighed and mixed with the 150 ml Petroleum ether (1:3 w/v) and incubated for 24 hours. After 24 hrs, the extract was filtered through the gauze cloth and Wattman No.1 filter paper in a beaker and allows drying it for evaporation. The plant powder was further treated with chloroform.

Chloroform extraction method

The Petroleum ether treated each plant powder was again dried and weighed. The powder was mixed with the Chloroform solvent (1:3 w/v) which was incubated for 24 hrs. The extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allow to dry it for evaporation. The plant powder was further treated with methanol.

Methanol extraction method

The chloroform treated with each plant powder was dried and weighed. The powder was mixed with the methanol solvent (1:3 w/v) which was incubated for 24 hours. After 24 hrs, the extract was filtered through the gauze cloth and whattman No.1 filter paper in a beaker, and allowed to dry it for evaporation. The plant powder was further treated with ethanol.

Ethanol extraction method

The methanol treated with each plant powder was dried and weighed. The powder was mixed with the ethanol solvent (1:3 w/v) which was incubated for 24 hours. After 24 hrs, the extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allows drying it for evaporation. The plant powder was further treated with ethyl acetate.

Ethyl acetate extraction method

The ethanol treated with each plant powder was dried and weighed. The powder was mixed

©2019 Published by JPS Scientific Publications Ltd. All Rights Reserved

with the Ethyl acetate solvent (1:3 w/v) which was incubated for 24 hours. After 24 hrs, the extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allowed to dry it for evaporation. The plant powder was further treated with distilled water.

Aqueous extraction method

The ethyl acetate treated with each plant powder was dried and weighed. The powder was mixed with the distilled water (1:3 w/v) which was incubated for 24 hours. After 24 hrs, the extract was filtered through the gauze cloth and Whattman No.1 filter paper in a beaker, and allows drying it.

Phytochemical screening

The various solvent extracts of powder of leaves of *Prosopis juliflora* were subjected to the phytochemical test for the identification of various active constituents, using the method followed by Cowan (1999).

Test for Tannins

About 2 ml of the aqueous extract was mixed with 2 ml of distilled water and few drops of $FeCl_3$ solution were added.

Test for Saponins

About 3 ml of the aqueous extract shaken vigorously with 3 ml of distilled water in a test tube and warmed.

Test for Phlobatannins

About 2 ml of aqueous extract was added and 2 ml of 1 % HCl and the mixture was boiled.

Test for Flavonoids

About 5 ml of aqueous extract was added and 1 ml of H_2SO_4 was added. Few minutes wait and observe the result.

Test for Terpenoids

About 2 ml of aqueous extract was dissolved in 2 ml of chloroform and evaporate it. Then 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes.

Test for Glycosides

About 2 ml of aqueous extract was dissolved in 2 ml of chloroform and 2 ml of acetic acid was added in it. The solution was cooled in ice. Then, H_2SO_4 was added carefully and results observed.

Test for Steroids

About 2 ml of extract was dissolved in 2 ml of chloroform and 2 ml of Concentrated $H_2SO_4$was added and observe the result.

Test for Phenols

About 1 ml of aqueous extract were dissolved in 5 ml of alcohol and treaded with few drops of $FeCl_3$ solution.

Test for Protein and Aminoacids

About 2 ml of plant extract dissolved in 2 ml of water. Change the colour indicates the presence of protein.

Test for Alkaloids

About 5 ml of plant extract was mixed with 1 % HCl. The solution obtained was filtered and then 1ml of filtrate was treated with few drops of Mayer's reagent. Formation of turbidity or cream precipitate indicates presence of alkaloids.

Test for Carbohydrates

About 1 ml of aqueous extract was added and few drops of Molisch's reagent were added, and then few minutes wait. Then, few drops of concentrated H_2SO_4 was added and heated for about 2 minutes.

Test for Quinins

About 1 ml of aqueous extract was added and 1 ml of $H_2 SO_4$ was added after few minutes result was observed.

Test for Glycosides

About 1 ml of aqueous extract was added with 2 ml of Glacial acetic acid and waited for few minutes. Then, few drops of 5 % Ferric chloride were added. Then, 1 ml of H_2SO_4 was added, waited for few minutes to observe the result.

©2019 Published by JPS Scientific Publications Ltd. All Rights Reserved

Test for Coumarin

About 1 ml of aqueous extract was added and 1 ml of 10 % of NaOH was added. After few minutes, result was observed.

Test for Triterpeniods

About 1.5 ml of aqueous extract was added and 1ml of Libeman buchard reagent was added. Then, 1 ml of concentrated H_2SO_4 was added and the results were observed after few minutes.

Test for Anthraquinones

About 1 ml of aqueous extract was added and few drops of 10 % Ammonia solution were added.

Determination of Antibacterial activity by Well diffusion method

The modified antibacterial test was performed using agar Well diffusion method. The test microorganisms were inoculated on Muller Hinton agar (MHA) and spread uniformly using the swab. Wells are 8 mm in diameter were made on MHA using a sterile well puncher. The agar blocks were carefully removed by the use of sterilized forceps. The various concentrations of each plant extracts were transferred to the wells. Then, the one of the well were filled with solvents are used to take the extract, so that solvent was adding the one well. The plates were allowed to stand for 1 hour at room temperature for diffusion of the substances before the growth of the microorganisms commenced. The plates were incubated at 37 ˚C for 24 hours and the zone of inhibition was recorded.

3. Results and Discussion

The microorganisms were identified by the preliminary and then biochemical test. Based on the biochemical characterization, three respiratory pathogens were isolated namely *Staphylococcus* sp., *Bacillus* sp. and *Pseudomonas* sp. were identified and the results were given in Table – 1.

Table – 1: Biochemical characterization of identified bacteria

Name of the bacteria	Indole	MR	VP	Citrate	Urease	TSI
Staphylococcus sp.	-	+	+	+	+	-
Bacillus sp.	-	-	+	+	-	-
Streptococcus sp.	-	+	-	-	-	-

Table – 2: Phytochemical character for *Prosopis juliflora*

Chemical Constituents	Acetone	Benzene	Chloroform	Water
Carbohydrates	+	+	+	+
Tannins	-	+	+	+
Saponins	-	-	-	-
Flavonoids	-	-	+	+
Alkaloids	-	-	+	+
Reducing sugar	-	-	+	+
Phytosterols	-	-	-	-
Xantho proteins	-	-	-	-
Phenols	-	-	+	+
Coumarins	-	-	+	-
Steroids	-	-	-	-
Triterpenoids	-	-	-	-
Anthra quinines	-	-	+	+

©2019 Published by JPS Scientific Publications Ltd. All Rights Reserved

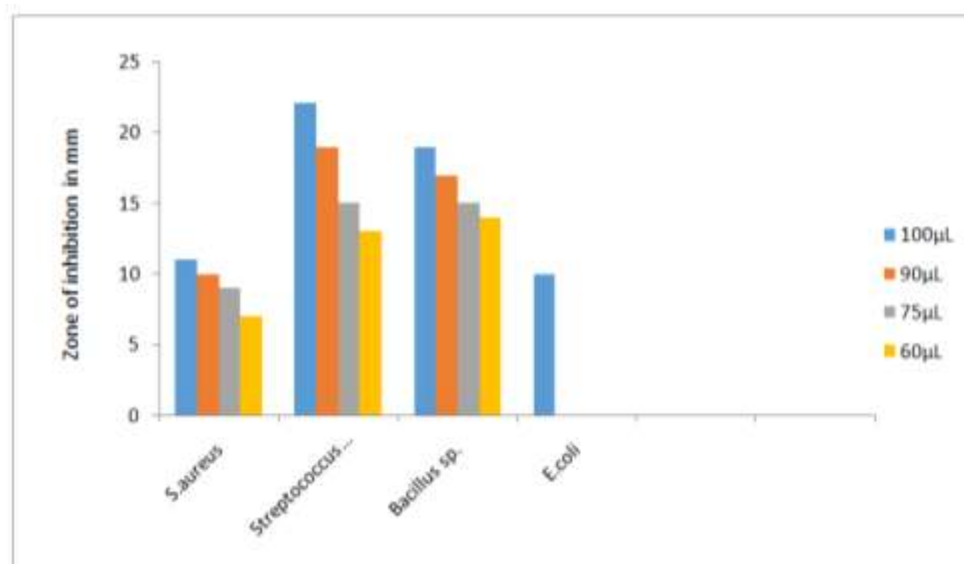


Figure – 1: Screening of *Prosopis juliflora* crude fresh green extract as assayed by Well diffusion method against different pathogenic bacteria

4. References

1) Basis, H. P., Vepachedu, R., Gilory, S., Callaway, R. M and Vivanco, J. M. (2003). Allelopathy and exotic plant invasion: From molecules and genes to species interactions. *Science,* 301: 1377 – 1380.
2) Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinic Microbiology Reviews*, 12: 564 - 582
3) Deepa, R., Chandra, S., Nayaka, K and Girish, M. P. (2013). Synergistic effect of *Prosopis juliflora* extract and chemical fungicides against seed borne toxigenic fungi. *International Journal of Advanced Life Science*, 6(4): 312 – 331.
4) Ismail Saadoun, Lina Mhd Al Challah, Fatima M. Aldhuhouri, Aisha A. Hamoudi and Ban AL Joubori. (2014). Antagonistic effect of the exotic plant "*Prosopis juliflora*" extract on different bacterial pathogens. *International Journal of Biological Sciences*, 3(7): 115 – 121.
5) Kumar, A., Yadav, S. K and Singh, S. (2011). Analgesic activity of ethanolic extract of roots of *Prosopis cineraria*. *Journal of Applied Pharmaceutical Science,* 1(8): 158 – 160.
6) Sharma, R., Jodhawar, N., Purohit, S and Kaur, S. (2012). Antibacterial activity and phytochemical activity. *Journal of Medicinal Plants Research*, 3(8): 550 – 514.

©2019 Published by JPS Scientific Publications Ltd. All Rights Reserved

Access this Article in Online

Quick Response Code	
Website	**www.jpsscientificpublications.com**
DOI Number	**DOI: 10.22192/lsa.2019.5.6.3**
Thomson Reuters Researcher ID	**L – 5547 – 2016**
ISI Impact Factor	**4.206**

How to Cite this Article:

S. Muthu Raj, M. Abdul Salam, S. Mohammed Lathieef Laseem, S. Kalaivani and M. Manohar. 2019. Phytochemical analysis of *Prosopis juliflora* and its Antibacterial activity against Human pathogens. *Life Science Archives*, 5(6): 1720 – 1725.

DOI: 10.22192/lsa.2019.5.6.3

©2019 Published by JPS Scientific Publications Ltd. All Rights Reserved

View publication stats

APPLICATION OF PROTEOMICS IN POTENTIAL DIAGNOSIS OF DISEASES AND ITS APPROPRIATE THERAPEUTICS

Mohan M[1]*, Manohar M[2] and Ganesh P[3]

1. Mahendra Engineering College (Autonomous), Namakkal, mohanrtt@gmail.com
2. Sadakathullah Appa College (Autonomous), Rahmath Nagar, Tirunelveli, gansun1@gmail.com
3. Department of Microbiology, Faculty of Science, Annamalai University, Chithamparam, drpganesh@gmail.com.

* Corresponding author: mohanrtt@gmail.com

Abstract - Proteomics is a recently emerging technology which includes gel-based profiling, quantitative mass-spectrometry and array-based high-throughput proteomics. This provides considerable outputs in differential proteomic expression analysis for identification of suitable biomarkers with promising diagnostic and prognostic implications. Recent development in mass spectrometric and other viable analytical techniques enabled the organization and characterization of proteome. Main focus of proteomics on cancer studies and more biomarkers for infectious diseases, autoimmune disorders and cardiovascular diseases are also been developed and evaluated. Mass spectrometry and protein micro-arrays have been emphasized the proteins to enable the characteristics and functional features by qualitatively and quantitatively. Bio-specimens of human proteome studies revealed the path of disease by their level of expression, different forms of protein variation and post-translational modifications (PTM). Proteomics have been stimulated the researchers to perform effective diagnosis and prognosis followed by the genomic era. It is important to elaborate the clinical proteomics to form a strong platform in terms of technological development, protein chemistry and pathways.

Keywords: proteomics, post translational modifications (PTM), diagnosis, therapeutics, mass spectrometry

I. Introduction

Proteomics is a recently emerged field of research which has more effective complementary approach to genomics for the better diagnosis of diseases. Proteomics decipher characterization of protein by using the process and parameters like isoforms, modifications of protein and its interactions related to their functional and structural modalities. The identification of biomarkers for diseases is one of the main objective of proteomics. A proteome of a cell represents a subset of possible gene products. Modifications of protein is observed through translational, post-translational, regulatory and degradative processes of protein which impact on structure and function of protein. Proteomic studies provide insight into the proteomic functionalities observed in a tissue, cell or organism at a particular moment. Data procured from proteomics experiments are more significant to clinical observation and it is creating way to identify the effective diagnostic tools and suitable therapeutic methods. This attempt is to made possible outcomes observed in the field of proteomics and their potential in diagnosis and therapeutics of diseases.

II. Proteomics techniques

Proteomics studies can be carried out by diverse field of techniques which make platform for separation and identification of protein and to determine the bio molecular interactions, function, regulation and many other processes for distributing protein information (Figure 1)

III. Microarray techniques

High-throughput detection from meagre quantity of sample is performed by protein microarray methods which are classified into three types viz., analytical protein microarray, functional protein microarray and reverse-phase protein microarray. Antibody microarray is one of the representative type of analytical protein microarray. Protein samples are detected by direct protein labeling after antibody capture. Protein expression level and its binding affinities are typically used to measure cancer cells through antibody microarray (Ebhardt et.al., 2015). Differential protein expression in tissues derived from squamous carcinoma cells of oral cavity was carried out through antibody microarray by Knezevic et.al., (2001). Functional protein microarray method is created by using purified protein thereby permitted to observe various interactions like protein–DNA, protein–RNA and protein–protein, protein–drug, protein–lipid and enzyme–substrate. The reverse-phase protein array (RPPA) is used analyze signaling pathways by using tissue samples. In RPPA, the cell lysates prepared from different cells and tissues are arrayed on nitrocellulose coated slide that are probed with antibodies. The antibodies are detected by fluorescent, chemi-luminescent and colorimetric assays. For quantification of protein, reference peptides are printed on slides. The altered or dysfunction protein are indicating the particular disease can be determined by using microarrays.

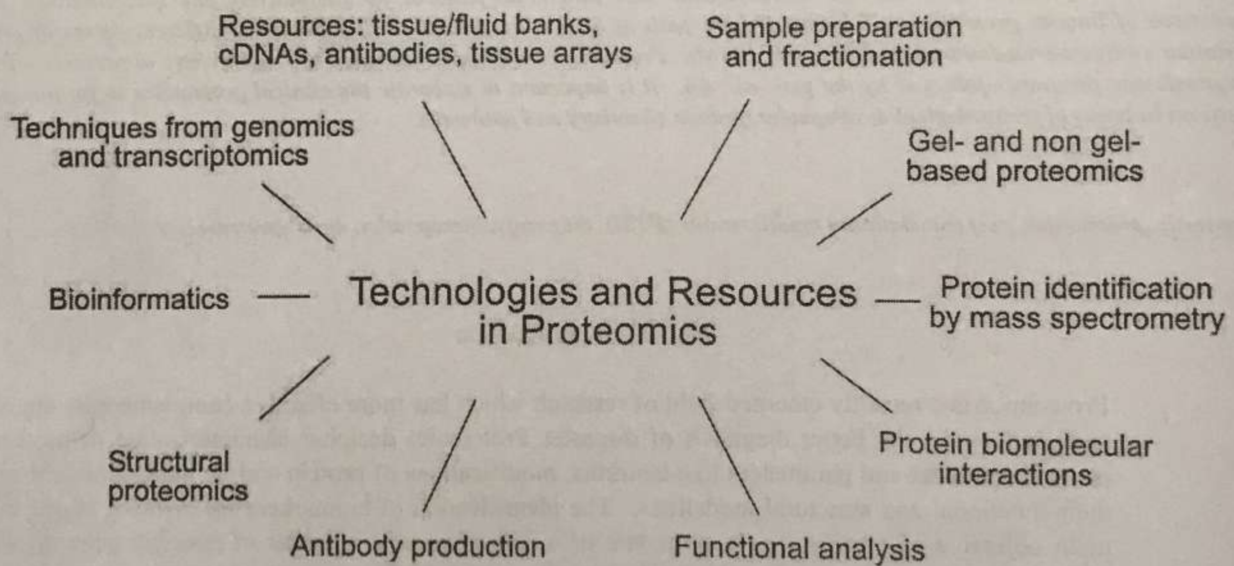


Fig.1. Techniques and resources in proteomics (Celis and Gromov. 2003)

IV. Gel-based proteomics techniques

Analysis of proteins expressed in different manner is carried out by two dimensional gel electrophoresis (2-DE). followed by MS serves as a classical approach in [70-72]. In 2-DE method, separation is done by two steps

viz., isoelectric focusing (IEF) and Sodium Dodecyl Sulphae – Poly Acrylamide Gel Electrophoresis (SDS-PAGE). In the first step, proteins are separated based on their charge in an immobilized pH gradient and followed by the method based on their molecular mass reflected in the polyacrylamide gel. Then, the protein is visualized and the intensity of signal generated is used for analysis. According Chevalier (2010), 2-DE supported for separation of up to 10000 proteins.

V. Mass-Spectrometry Based Proteomics

2-DE is widely used method for the separation of proteins and many novel techniques are emerged during recent years to complement and improve the proteomic analysis includes post-translational modifications (PTM) including phosphorylation and glycosylation need more focusing. Mass spectrometry (MS) is considered to be a powerful tool to observe extremely lower mass particles to get highly accurate results. Mass spectrometers are highly sensitive up to fragment level and it is automated to detect peptides and enzymatic fragments. MS-based proteomics method includes matrix-assisted laser desorption and ionization (MALDI). MALDI can be performed by using the peptides linked with acidic matrix and applied to the plate made up of stainless steel or injected through a needle in liquid state using electrospray and are ionized in the MS intrument. MALDI is used for new biomarker discovery and can identify nano scale to pico scale amounts of protein.

VI. Proteomics in diagnostics

Proteomics technology is emerged as a powerful tool to understand molecular basis of disease by the way to identify active biomarker to find suitable drug discovery. This novel technique is widely used worldwide in the field of cancer research and other diseases threating the human population. Analysis of protein sample can be done by 2 DE followed by MS can be quantitatively measured by label based or label free methods. Disease markers are to be identified for the identification and examination of protein sample. Latest development noticed in proteomics technology pave the way for the simultaneous analysis of thousands of low molecular weight proteins. This envisages the patterns of disease and early detection and assessing prognosis of a disease. Thus, the biomarkers are used as the potential diagnostic markers to identify the state of disease to perform precision translational medicine. Many proteomics studies are focused on cancer and earlier diagnosis of cancer is possible by using modern proteomic approach. Early diagnosis of cancer can be done by MALDI. Ovarian cancer is highly lethal due to lack of specific screening tool for early detection. Identification of biomarkers using proteomic techniques resulted improved positive predictive value (Bast. et.al., 2005). Drug resistant *Mycobacterium tuberculosis* strain is affecting millions of population. Proteomic study enable the detection of protein secreted by Mycobacterium *tuberculosis* during in vitro clinical trials (Bahk.*et.al.*,. 2004).

VII. Proteomics in therapeutics

Proteomics is an efficient tool for identification of genes responsible for the disease. A sequence of study carried out using autopsy brain tissue of late infantile neuronal ceroid lipo fuscinosis (LINCL) patients by 2-DE and affinity chromatography. A team of researchers observed the absence of protein in LINCL patients compared to

normal persons. Mutations were identified during sequence analysis of the gene in LINCL patients (Sleat *et. al.*,1999). Innumerable proteomic markers were identified for other diseases followed by the research using the genes responsible for the disease. The reverse-phase protein array has established a distinctive ability to analyze signaling pathways using tiny amount of human cells collected from biopsy specimens obtained during clinical trials (Paweletz et.al, 2001). Molecular profiling using gene arrays is considerably important for the classification of patients based on the disease stage or clinical outcome (Fig.2).

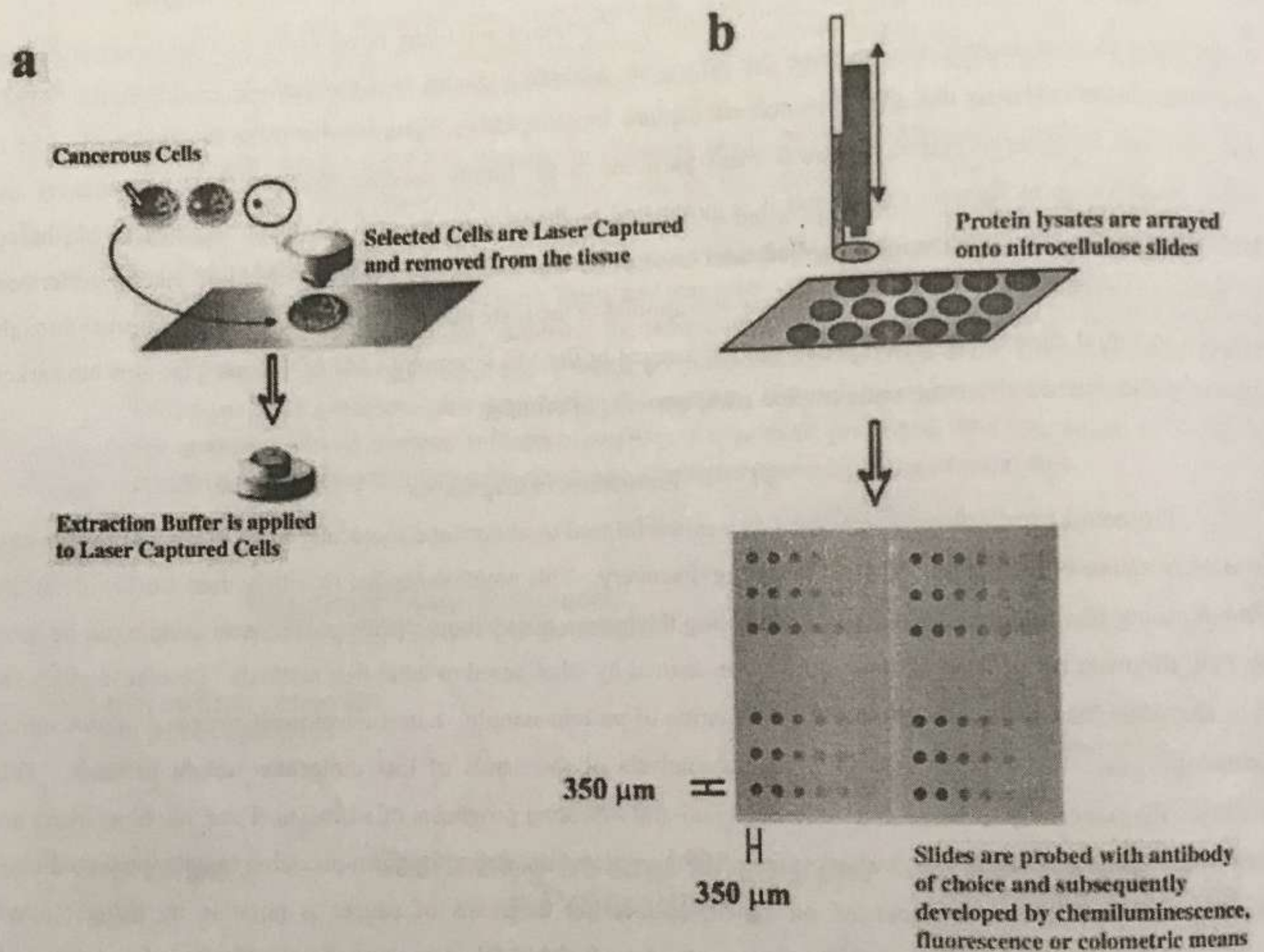


Fig. 2. Reverse-phase protein arrays a. Isolation of cells b. protein array (Petricoin and Liotta ., 2003)

Blood or urine samples are used for proteomic studies by many researchers. Serum profiling has been efficient to predict response to cyclooxygenase-2 inhibitor, celecoxib, for prevention and treatment of cancer (Xiao. *et.al.*, 2004). A proteomic experiment performed to examine Cisd2 protein, a key regulator of lifespan in mice. This is a disease gene for Wolfram syndrome in humans. 70% decrease in Cisd2 protein resulted in gastrocnemius aging animal model (Huang et.al, 2018). Colon cancer incidence happened by the Cdc42BPA protein and Cdc42 signaling cycle. By blocking Cdc42BPA and Cdc42 signaling of colon cancer. The expression Cdc42BPA protein leads to metastasis and even death may occur to the colon cancer patients (Hu et.al., 2018).

ISSN NO: 1076-5131

VIII. Conclusion

It is important to deal the diagnosis and therapeutics by integration of biochemical, genetic and proteomic data to overcome the threats faced by the population. Latest years there is a tremendous development observed in the field of proteomics. This is observed to be a rapid, accurate and efficient to solve clinical problems. Combination of emerging techniques to further more development of new insights and probable solutions for the potential diseases. High throughput proteomic methods and novel approaches enable us to provide appropriate remedy for the diseases and betterment of the society.

Acknowledgement

The authors are extending their immense pleasure to thank Dr.G.S.Vijayalalakshmi, former Professor of Environmental Biotechnology, Manonmaniam Sundaranar University, Tirunelveli for the support and valuable suggestions given during the preparation of the manuscript.

References

Bahk YY, Kim SA, Kim JS, Euh HJ, Bai GH, Cho SN and Kim YS. (2004). Antigens secreted from Mycobacterium tuberculosis: identification by proteomics approach and test for diagnostic marker. Proteomics 2004; 4: 3299-3307.

Bast RC Jr, Badgwell D, Lu Z, Marquez R, Rosen D, et al. (2005) New tumor markers: CA125 and beyond. International Journal of Gynecological Cancer 15 (3): 274-281.

Celis. J.E., and Gromov.P., (2003). Proteomics in translational cancer research: Toward an integrated approach. Cancer Cell. 3: 9-15.
Chevalier F: Highlights on the capacities of "Gel-based" proteomics. Proteome Science 2010, 8:23

Ebhardt, H.A., Root, A., Sander, C., Aebersold, R.; Applications of targeted proteomics in systems biology and translational medicine; Proteomics, (2015); 15(18): 3193–3208.
Rosenberg, J.M., Utz, P.J.; Protein microarrays: a new tool for the study of autoantibodies in immunodeficiency; Frontiers in Immunology, (2015); 6: 138.

Gillespie, J. W., Emmert-Buck, M. R., Roth, M. J., Petricoin, E. F., 3rd. and Liotta, L. A. (2001) Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front.
Oncogene 20: 1981–1989
Huang YL, Shen ZQ, Wu CY, Teng YC, Liao CC, Kao CH, et al. Comparative proteomic profiling reveals a role for Cisd2 in skeletal muscle aging. Aging Cell 2018; 17(1): e12705.

Hu HF, Xu WW, Wang Y, Zheng CC, Zhang WX, Li B, et al.Comparative proteomics analysis identifies Cdc42-Cdc42BPA signaling as prognostic biomarker and therapeutic target for colon cancer invasion. J Proteome Res 2018; 17(1): 265-275.

Knezevic, V., Leethanakul, C., Bichsel, V.E., Worth, J.M., Prabhu, V.V.,Gutkind, J.S., et al.; Proteomic profiling of the cancer microenvironment by antibody arrays; Proteomics, (2001); 1(10): 1271–1278.

Paulo. J.A., Kadiyala, V., Banks. PA., Steen. H. and Conwell. DL. (2012). Yale Journal of Biology and Medicine 85 (2012), pp.59-73.

Paweletz, C. P., Charboneau, L., Bichsel, V. E., Simone, N. L., Chen, T., Paweletz, C. P., Charboneau, L., Bichsel, V. E., Simone, N. L., Chen, T.,Gillespie, J. W., Emmert-Buck, M. R., Roth, M. J., Petricoin, E. F., 3rd. & Liotta,L. A. (2001) Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front.Oncogene 20: 1981–1989

Petricoin. E.F., and Liotta. L.A., (2003). Clinical Applications of Proteomics. Journal of Nutrition. 133: 2476S–2484S

Available online at www.jpsscientificpublications.com

Life Science Archives (LSA)

ISSN: 2454-1354

Volume – 4; Issue - 1; Year – 2018; Page: 1239 – 1247

DOI: 10.22192/lsa.2018.4.1.1

Research Article

RESPONSE OF DIFFERENT FORMULATIONS OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) DEAMINASE POSITIVE WILD AND DEFICIENT MUTANT STRAINS OF *Pseudomonas* AND *Paenibacillus* ON PLANT GROWTH PROMOTING CHARACTERISTICS IN MAIZE (*Zea mays* L.) *cv*. CO1

S. Esath Natheer[1*], R. Haseena[2], M. Syed Abdul Rahman[3] and K. Feroz Khan[3]

[1]*Department of Microbiology, Nehru Arts and Science College, Coimbatore – 641 105. Tamil Nadu, India.*
[2]*Department of Food and Agricultural Process Engineering, Tami Nadu Agricultural University, Coimbatore – 641 003, Tamil Nadu, India.*
[3]*Department of Marine Microbiology, Faculty of Marine Sciences, Centre of Advance Study in Marine Biology, Annamalai University, Parangipettai – 608 502, Tamil Nadu, India.*

Abstract

The response of different formulations of 1-aminocyclopropane-1-carboxylate (ACC) deaminase positive wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on plant growth promoting characteristics in maize was studies under *in vitro* condition. The bioinoculation effect of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* as co-flocs, augmented the seed vigour index and adhesion to maize roots to a higher level and also decreased the incidence of *Helminthosporium turcicum*, followed by co-inoculation and single strain inoculation. Between the two wild and mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*, wild strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* recorded the highest value for plant growth promoting characteristics when compared to their respective mutant strains. When comparing among two wild strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*, the wild strain *Pseudomonas fluorescens* were found to produce better performance than wild strains of *Paenibacillus polymyxa*. The results of the present study clearly envisaged the positive effect of co-flocs formulation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* wild strains, exhibiting ACC deaminase activity in augmenting the plant growth promoting characteristics.

Article History

Received : 15.10.2017
Revised : 12.11.2017
Accepted: 30.12.2017

Key words: Maize, *Pseudomonas fluorescens*, *Paenibacillus polymyxa*, ACC deaminase, Co-flocs, Wild, *acd* deficient mutant and Plant growth promotion.

1. Introduction

Impact of rhizobacteria generally on plant growth and health may be classified as neutral, deleterious or beneficial (Kloepper, 1989). However, plant growth promoting rhizobacteria (PGPR) specifically are beneficial and the beneficial effects have been utilized in many areas including biofertilizer, disease control, microbe-rhizoremediation, biopesticide, in forestry (Kloepper, 2003; Lucy, 2004), as well as probiotics. PGPR are naturally occurring soil

* *Corresponding author*: **Dr. S. Esath Natheer**
E.mail: esathnatheer@gmail.com

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

bacteria that aggressively colonize plant roots and enhance the plant growth directly by eliciting root metabolic activities by supplying biologically fixed nitrogen (Glick *et al*., 1999), hormonal interaction, improvement in root growth, solubilization of nutrients and indirectly by acting against phytopathogens (Lugtenberg *et al*., 2002; Morrissey *et al*., 2004; Ehlers, 2006). The well known PGPR include genera, *viz*., *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Bacillus*, *Azoarcus*, *Klebsiella*, *Arthrobacter*, *Enterobacter*, *Serratia* and *Rhizobium* on non-legumes (Burdman *et al*., 2000; Berg, 2009). Among these bacteria *Pseudomonas* and *Bacillus* are the most widely reported PGPR.

Maize (*Zea mays* L.) is the third major crop of the world after wheat and rice which provides more nutrients for humans and animals than any other cereals and the same is grown in many countries, including India. The desire of every grain producer is to obtain high yields at reduced production costs so as to optimize net returns on investments. Crop establishment and plant vigour represent key factors that influence the success of grain crop production (Hammermeister *et al*., 2008). Certain bacteria possess an enzyme *viz*., ACC-deaminase (*acd*) that hydrolyzes 1-aminocyclopropane-1-carboxylate into ammonia and α-ketobutyrate (Mayak *et al*., 1999). PGPR strain containing ACC-deaminase activity could suppress the accelerated endogenous ethylene synthesis, thus facilitate the root elongation and nutrient absorption of host plant which resulted in improved growth and yield of crop plant improved growth and yield of crop plant. The inoculant formulations play a critical role in determining the success of bioinoculant. Among the various novel agricultural bioinoculant technologies, the EPS rich "Intergeneric PGPR co-flocs", containing ACC-deaminase positive rhizobacterial strains, seems to be a better one, in terms of, high cell titre, increased adhesion to plant roots, enriched in encysted cells and spores with EPS rich network, longer shelf life and Induced systemic resistance (ISR) mediated biocontrol against phytopathogens.

Seed inoculation of PGPR strains containing *acd* activity reduces the inhibitory effect of ethylene at spermosphere and leads to the improvement plant growth promotion (Penrose *et al*., 2001; Mayak *et al*., 2004). *Pseudomonas* and *Paenibacillus* are the two important PGPR genera which are frequently encountered from the rhizosphere of maize and the PGPR characteristics of the same have been described by many authors in different crop plants (Sheng *et al*., 2008; Timmusk *et al*., 2005). Among the various novel agricultural bioinoculant technologies, the EPS rich "Intergeneric PGPR co-flocs", containing ACC-deaminase positive rhizobacterial strains, seems to be a better one, in terms of, high cell titre, increased adhesion to plant roots, enriched in encysted cells and spores with EPS rich network and longer shelf life. However, there were no reports on the response of different formulations of *Pseudomonas* and *Paenibacillus* ACC-deaminase wild and deficient mutants on different plant growth promoting attributes in maize crop.

Hence, the present study has been undertaken with an aim to exploit the response of *acd* positive (wild) and *acd* deficient mutant strain of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* along with "intergeneric co-flocs" on enhancement of plant growth promotion and biocontrol of leaf blight disease.

2. Materials and Methods

Culture condition

Pseudomonas fluorescens and *Paenibacillus polymyxa*, isolated from the rhizosphere of maize grown at Kadampuliyur, Chidambaram taluk, Cuddalore district, Tamil Nadu state, India were used in the present study. The *P. fluorescens* and *P. polymyxa* isolates were positive for their ACC-deaminase (*acd*) activity, maintained in King's medium B (King *et al*., 1954) and Nutrient Glucose agar (Englesberg and Ingraham, 1957) slants, respectively, and incubated at 28±2ºC, with monthly transfer. *Helminthosporium turcicum* (AU-1), obtained from the Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, India, was used as a reference strain for the biocontrol study and the same was maintained in

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

Potato Dextrose agar (PDA) slants and examined periodically for its virulence.

P. fluorescens and *P. polymyxa* was also grown on DF salts minimal medium [(composition in g per litre) KH_2PO_4, 4.0; Na_2HPO_4, 6.0; glucose, 2.0; gluconic acid, 2.0; citric acid, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.0001; H_3BO_3, 0.0010; $ZnSO_4 \cdot 7H_2O$, 0.0070; CuSO4·5H20, 0.0050; MoO_3, 0.0010] (Dworkin and Foster, 1958). The medium was supplemented with either 2.0 g of ammonium sulphate or 3.0 mM ACC (Clearsynth, Mumbai).

Chemical mutagenesis and selection

The construction of ACC-deaminase deficient mutant (*acd*) of *P. fluorescens* and *P. polymyxa* isolates was carried out according to Miller (1972) and selection was carried out according to Glick *et al*. (1994).

Preparation of *P. fluorescens* and *P. polymyxa* co-flocs

The co-flocculation of *P. fluorescens* and *P. polymyxa* isolates were prepared in co-floc buffer as described by Grimaudo and Nesbitt (1997). One ml aliquot of each PGPR isolates *viz*., *P. fluorescens* and *P. polymyxa* strains were mixed together in 10 ml co-floc buffer. The mixtures were vortexed for 10 sec, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for 24 hrs. All co-floc reactions were performed in triplicate and uninoculated buffer served as control. After incubation co-flocculation percentage was measured as described by Madi and Henis (1989).

Preparation of different bioformulation

One ml culture of each PGPR isolate (1×10^7 CFU per ml) under different formulations were prepared *viz*., control, single strain inoculation of either wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa*, co-inoculation of wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* and co-flocs application of wild and *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* and used to evaluate plant growth promoting characteristics of maize *cv*. CO1 on the enhancement of seed vigour index, adhesion to maize roots and reduction in *Helminthosporium turcicum* incidence was studied under *in vitro* condition.

Response of different formulations on plant growth promoting characteristics

Seed vigour index of maize

Maize (*Zea mays* L.) *cv*. CO1 maize seeds were surface sterilized by immersion in 95% ethanol for 1 min, followed by 20 min in 1% NaOCl. After rising three times with sterile distilled water, the sterilized seeds were placed on the surface of 1 per cent water agar in petriplates (9 cm dia, at five seeds per plate) were treated with the different bioformulations as mentioned above. The maize seeds were subjected to the above treatments, dried in shade for 30 min. Then, the inoculated maize seeds were arranged in two rows on a sheet of blotting paper dipped in sterile water. Then, they were covered with another blotting paper dipped in sterile water, rolled and placed vertically in a moist chamber at 20 °C. Uninoculated seeds with distilled water treatment served as control. After the incubation for 5 days, each roll was opened and the vigour indices of germinated maize seeds were calculated by the method of Abdul-Baki and Anderson (1973).

Vigour index = (mean root length + mean hypocotyl length) × % germination

Adhesion to maize roots

Surface sterilized seeds were incubated in an inverted position for 3 days at room temperature to allow germination. The plates were sealed with wax to avoid agar dryness during germination. After germination, the three day old seedlings were transferred to slopes of Fahraeus solution (Fahraeus, 1957) solidified with 1.5 per cent agar in test tubes. Sterile Fahraeus solution was added to fill the empty portion of the agar slopes and the tubes were incubated for three more days (24ºC day / 22ºC night). After the incubation period, the roots were collected from each tube separately, washed first with sterile water and later three times in 0.1 M phosphate buffer (pH 6.8),

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

cut into 5 cm pieces and used for adsorption study as described by Gafni *et al*. (1986).

Preparation of growth chamber and challenge inoculation

The growth chamber was a desiccator (12 x 10 cm) consisting of two parts. The lower part was filled with weaver's medium (Weaver *et al*., 1975) and upper part contained stainless steel wire mesh (mesh size 3 mm) supports. The lid was placed over the cotton and the chamber was closed before sterilization. The growth chamber was sterilized by autoclaving. Fifty germinated maize seeds with coleoptile (2 cm high) were transferred aseptically onto the stainless steel wire mesh, incubated for 10 days in the growth chamber with 14 hrs day and 10 hrs night cycle and temperature ranging from 24°C at night to 32°C around noon. By this time, the maize roots yielded many lateral roots, well spread in the Weaver's medium maintained at the lower part of the growth chamber.

Maize plants were challenge inoculated by spraying the spore suspension of *Helminthosporium turcicum* at a spore concentration of 50,000 spores per ml inoculum level on 10th DAS with an atomizer and control plant was sprayed with sterile Weaver's medium. High humidity was created by sprinkling the water frequently in the polyhouse.

After one week of challenge inoculation, three plants from each treatment were carefully removed and rinsed with sterile distilled water. The leaf blight disease incidence was enumerated with a score chart of 0 to 9 grades devised by International Rice Research Institute (1980).

Statistics analysis

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure described by Gomez and Gomez (1984).

3. Result and Discussion

Pseudomonas fluorescens and *Paenibacillus polymyxa* wild strains registered a normal growth in the DF salts medium amended with ACC and suggested the ability of the wild strains to utilize ACC, as a sole 'N' source whereas the ACC deaminase (*acd*) deficient strains could not able to metabolize ACC and resulted in poor or no growth (data not shown). Glick *et al*. (1994) constructed an *acd* deficient mutant strain of *Pseudomonas putida* GR-12-2 by chemical mutagenesis (nitrosoguanidine) and reported the inability of the respective mutants to utilize ACC. The results of the present study also clearly in accordance with the earlier findings of Glick *et al*. (1994).

The response of different formulations of wild and *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* cells *viz*., control, single strain inoculation of either wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa*, co-inoculation of wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* and co-flocs application of wild and *acd* deficient mutants of *P. fluorescens* and *P. polymyxa*, on plant growth promoting characteristics of maize *cv*. CO1 *viz*., enhancement of seed vigour index, adhesion to maize roots and reduction in *Helminthosporium turcicum* was studied under *in vitro* condition. Among the different formulations of *P. fluorescens* and *P. polymyxa* cells, the application of co-flocs of *P. fluorescens* and *P. polymyxa* wild cells augmented the highest level of seed vigour index and adhesion to maize roots, followed by the co-flocs of *P. fluorescens* and *P. polymyxa acd* mutant strains, coinoculation of *P. fluorescens* and *P. polymyxa* wild strains, coinoculation of *P. fluorescens* and *P. polymyxa* mutant strains, single wild strain inoculation of *P. fluorescens* and *P. polymyxa*, single mutant strain inoculation of *P. fluorescens* and *P. polymyxa* and control. Moreover, the application effect of *P. fluorescens* and *P. polymyxa* wild strains as co-flocs positively reduced the incidence of leaf blight disease to a higher level when compared to other treatments (Table - 1).

The improved plant growth promoting of seed vigour index is due to application of co-flocs containing *acd* positive *P. fluorescens* and *P. polymyxa* suggested the positive role of *acd* in

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

modifying the ethylene level during germination of maize seeds as suggested earlier by Glick *et al*. (1994). Phytostimulatory effect of *Pseudomonas* cells has been already reported by Ahmad *et al*. (2005) and Glick *et al*. (2007). Neyra *et al*. (1999) described the phytostimulatory effect of "Intergeneric coaggregates" containing *Azospirillum and Rhizobium*, on the enhancement of growth parameters in faba bean. Further, the positive role of rhizobacterial ACC-deaminase activity on the enhancement of seed germination and plant growth stimulation has been reported by many authors (Glick *et al*., 1994; Contesto *et al*., 2008; Ma *et al*., 2008; Barnawal *et al*., 2017). The production of ACC-deaminase by *P. fluorescens* and *P. polymyxa* has been confirmed (Glick *et al*., 1994; Belimov *et al*., 2007).

Table – 1: Response of different formulations of *P. fluorescens* and *P. polymyxa* wild and *acd* deficient mutant strains of different plant growth promoting (PGP) characteristics in maize *cv*. CO1

Treatment[a]	Seed vigour index[b,c]	No. of adhered cells (10^4 CFU g^{-1} dry wt. of root h^{-1})[b,c]	Percentage of disease incidence[b,c]
Control	10320 ± 19.18[i]	-	82.58 ± 1.27[i]
P. fluorescens (W)	12980 ± 9.72[e]	272.81 ± 2.97[e]	59.85 ± 0.93[e]
P. fluorescens (M)	11850 ± 17.27[g]	198.16 ± 2.07[g]	70.21 ± 0.52[g]
P. polymyxa (W)	12420 ± 16.83[f]	248.72 ± 2.60[f]	64.63 ± 0.34[f]
P. polymyxa (M)	11350 ± 20.08[h]	183.31 ± 1.81[h]	76.48 ± 0.83[h]
Co-I-*P. fluorescens* + *P. polymyxa* (W)	14462 ± 15.37[c]	331. 45 ± 3.97[c]	48.07 ± 0.67[c]
Co-I-*P. fluorescens* + *P. polymyxa* (M)	13625 ± 12.43[d]	300.23 ± 3.32[d]	52.31 ± 0.56[d]
Co-F-*P. fluorescens* + *P. polymyxa* (W)	16030 ±14.06[a]	408.62 ± 5.11[a]	22.72 ± 0.31[a]
Co-F-*P. fluorescens* + *P. polymyxa* (M)	15370 ± 11.16[b]	385.19 ± 4.27[b]	32.11 ± 0.35[b]
LSD (P<0.05)	0.99	0.98	0.98

W – Wild; M – Mutant; Co-I – Coinoculation; Co-F – Co-floc
a - At $1x10^7$ CFU per ml inoculum level
b - Values are mean of three replication ± SD
c - Values followed by different letters are significantly differed at 5 % level according to student 't' test

The adhesion mechanism of *Pseudomonas* and EPS rich *Paenibacillus* cells has already been reported in many crop plants by many authors (O'Sullivan and O'Gara, 1992; Wei *et al*., 1996; Puente *et al*., 2009). The results of the present study clearly revealed the improved adhesion of EPS rich PGPR co-flocs, containing ACC-deaminase positive strains of *P. fluorescens* and *P. polymyxa* to maize roots when compared to PGPR co-flocs, containing *acd* deficient mutant strains of *P. fluorescens* and *P. polymyxa* and individual application of either *acd* positive wild or *acd* deficient PGPR cells and emphasized the positive role of EPS rich, ACC-deaminase positive rhizobacterial strains in the early events of adhesion to maize roots.

The application effect of different formulations of PGPR cells on the incidence of *Helminthosporium turcicum* in maize, the co-flocs, consisting of ACC-deaminase positive wild strains of *P. fluorescens* and *P. polymyxa* reduced the leaf blight disease incidence to a higher level when compared to others formulations. Pieterse *et al*. (2000) reported the positive role of ACC-deaminase containing rhizobacterial strains on the modulation of ethylene in crop rhizosphere which leads to induction of systemic resistance (ISR) in *Arabidopsis thaliana*. The biocontrol of maize leaf blight disease due to *Pseudomonas* and *Paenibacillus* bioinoculation has already been reported by Belimov *et al*. (2007), Haggag (2007) and Jalili *et al*. (2009) in various plant crops and also it is believed to alleviate drought and salt tolerance (Ann Maxton *et al*., 2017; Saikia *et al*., 2018).

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

4. Conclusion

The results of present study envisaged the positive effect of PGPR strains, containing ACC deaminase positive wild strains of *P. fluorescens* and *P. polymyxa*, in augmenting the seed vigour index, adhesion to maize root and biocontrol against *H. turcicum* in semiarid maize *cv*. CO1 when compared to *acd* deficient strains of *P. fluorescens* and *P. polymyxa*. The mechanism regarding the improved PGPR characteristics by *acd* positive wild strains of *P. fluorescens* and *P. polymyxa* needs further exploitation.

Acknowledgement

We are thankful to University Grants Commission (UGC) of India for providing funds under the scheme of Maulana Azad National Fellowship (MANF) for the Minority students in support of this research for providing necessary facilities to carry out this work.

5. References

1) Abdul-Baki, A.A. and J.D. Anderson, 1973. Vigour determination in soybean seed and multiple criteria. *Crop Science,* 13: 630 - 633.
2) Ahmad, F., L. Ahmad and M. Saghir, 2005. Indolacetic acid production by the indigenous isolates of *Azotobacter* and *Pseudomonas fluorescens* in the presence and absence of tryptophan. *Turkish Journal of Biology,* 29 - 34.
3) Ann Maxton, S. Poonam and S.A. Masih, 2017. ACC deaminase-producing bacteria mediated drought and salt tolerance in *Capsicum annuum*, Journal of Plant Nutrition, 41(5): 574 - 583.
4) Barnawal, D., S.S. Pandey, N. Bharti, A. Pandey, T. Ray, S. Singh, C.S. Chanotiya and A. Kalra, 2017. ACC deaminase-containing plant growth-promoting rhizobacteria protect *Papaver somniferum* from downy mildew, *Journal of Applied Microbiology,* 122(5): 1286 - 1298.
5) Belimov, A.A., I.C. Dodd, V.I. Safronova, N. Hontzeas and W.J. Davies, 2007. *Pseudomonas brassicacearum* strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato. *Journal of Experimental Botany,* 58: 1485 - 1495.
6) Berg, G, 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology,* 84: 11 - 18.
7) Burdman, S., Y. Okon and E. Jurkevitch, 2000. Surface characteristics of *Azospirillum* brasilense in relation to cell aggregation and attachment to plant roots. *Critical Reviews on Microbiology,* 26: 91 - 110.
8) Contesto, C., G. Desbrosses, C. Lefoulon, B. Bena, F. Borel, M. Galland, L. Gamet, F. Varoquaux and B. Touraine, 2008. Effects of rhizobacterial ACC deaminase activity on *Arabidopsis* indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. *Plant Science,* 175: 178 - 189.
9) Dworkin, M. and J. Foster, 1958. Experiments with some microorganisms which utilize ethane and hydrogen. *Journal of Bacteriology,* 75: 592 - 601.
10) Ehlers, R.U. 2006. Einsatz der Biotechnologie im biologischen. *Pflanzenschutz. Schr. Dtsch. Phytomed. Ges*, 8: 17 - 31.
11) Englesberg, G.J. and P. Ingraham, 1957. Glucose broth for the production of Exopolysaccharide by *Paenibacillus polymyxa* isolates. *Journal of Microbiology,* 14: 56 - 58.
12) Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a single glass slide technique. *Journal of General Microbiology,* 16: 374 - 381.
13) Gafni, R., Y. Okon, Y. Kapulnik and M. Fischer, 1986. Adsorption of *Azospirillum brasilense* to corn roots. *Soil Biology and Biochemistry,* 18: 69 - 75.
14) Glick, B.R., C.B. Jacobson, M.M.K. Schwarze and J.J. Pasternak, 1994. 1-

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

Aminocyclopropane-1-carboxylic acid deaminase mutants of the plant growth promoting rhizobacterium *Pseudomonas putida* GR 12-2 do not stimulate canola root elongation. *Canadian Journal of Microbiology,* 40: 911 - 915.

15) Glick, B.R., C.L. Patten, G. Holguin and D.M. Penrose, 1999. Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London, p 267.
16) Glick, B.R., Z. Cheng, J. Czarny and J. Duan, 2007. Promotion of plant growth by ACC deaminase-containing soil bacteria. *Europen Journal of Plant Patholology,* 119: 329 - 339.
17) Gomez, K.A. and A.A. Gomez, 1984. Statistical procedures for agricultural research, John Wiley and Sons, New York, USA. p.20-29.
18) Grimaudo, N.J. and W.E. Nesbitt, 1997. Coaggregation of *Candida albicans* with oral *Fusobacterium sp*. *Oral Microbiology and Immunology,* 12: 168 - 173.
19) Haggag, W.M. 2007. Colonization of exopolysaccharide - producing *Paenibacillus polymyxa* on peanut roots for enhancing resistance against crown rot disease. *African Journal of Biotechnology,* 6: 1568 - 1577.
20) Hammermeister, A., M. Bernard, J. Mackenzie and R. Beavers, 2000. Crop establishment and soil fertility on maritime organic grain farms. Organic Agriculture Centre of Canada, Final Research report, 2008 - 2041.
21) IRRI. 1980. International Rice Research Institute. Annual Report for 1979. Los Banos, Philippines, pp. 171 - 182.
22) Jalili, F., K. Khavazi, E. Pazira, A. Nejati, A.H. Rahmani, R.H., Sadaghiani and M. Miransari. 2009. Isolation and characterization of ACC deaminase producing fluorescent pseudomonads, to alleviate salinity stress on canola (*Brassica napus* L.) growth. *Journal of Plant Physiology,* 166: 667 - 674.
23) King's, E.O., M.K. Ward and D.E. Raney, 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine,* 44: 301 - 307.
24) Kloepper, J.W. 2003. A review of mechanisms for plant growth promotion by PGPR. Presented at the 6th international PGPR Workshop, 5 – 10 October 2003, Calicut, India.
25) Kloepper, J.W., R. Lifshitz and R.M. Zablotwicz, 1989. Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnology,* 7: 39 - 43.
26) Lucy, M., E. Reed and B.R. Glick, 2004. Application of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek,* 86: 1 - 25.
27) Lugtenberg, B.J.J., T.F.C. Chin-A-Woeng and G.V. Bloemberg, 2002. Microbe–plant interactions: principles and mechanisms. *Antonie van Leeuwenhoek,* 81: 373-383.
28) Ma, Y., M. Rajkumar and H. Fritas, 2008. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *Journal of Environmental Management,* 90: 831 - 837.
29) Madi, L. and Y. Henis, 1989. Aggregation in *Azospirillum brasilense* cd. conditions and factors involved in cell-to-cell adhesion. *Plant and Soil,* 115: 89 - 98.
30) Mayak, S., T. Tirosh and B.R. Glick, 1999. Effect of wild type and mutant plant growth promoting rhizobacteria on the rooting of mung bean cuttings. *Journal of Plant Growth Regulation,* 18: 49 - 53.
31) Mayak, S., T. Tirosh and B.R. Glick, 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiology and Biochemistry,* 42: 565 - 572.
32) Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33) Mohammadhossein, R., R. Sasidharan, L.A.C.J. Voesenek, G.A. Kowalchuk and A. Jousset, 2017. ACC deaminase-producing rhizosphere bacteria

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

modulate plant responses to flooding, *Journal of Ecology,* 105(4): 978 - 986.

34) Morrissey, J.P., J.M. Dow, L. Mark and F. O'Gara, 2004. Are microbes at the root of a solution to world food production? *EMBO Rep.,* 5: 922 - 926.
35) Neyra, C.A., L.A. Atkison, O. Olubayi, L. Sadasivam, D. Zaurov and R. Zappi, 1999. Novel microbial technologies for the enhancement of plant growth and biocontrol of fungal diseases in crops. *Cahiers Options Mediterranean,* 31: 447 - 455.
36) O'Sullivan, D.B. and F. O'Gara, 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiology Reviews,* 56: 662 - 676.
37) Penrose, D.M. and B.R. Glick, 2001. Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. *Canadian Journal of Microbiology,* 47: 368 - 372.
38) Pieterse, C.M.J., J.A. Van Pelt, J. Ton, S. Parchmann, M.J. Mueller, A.J. Buchala, J.P. Metraux and L.C. van Loon, 2000. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiology and Molecular Plant Pathology,* 57: 123 - 134.
39) Puente, M.E., C.Y. Li and Y. Bashan, 2009. Rock-degrading endophytic bacteria in cacti. *Environmental Experimental Botany,* 66: 389 - 401.
40) Sheng, X.F., J.J. Xia, C.Y. Jiang, L.Y. He and M. Qian, 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting growth and lead accumulation of rape. *Environmental Pollution,* 156: 1164 - 1170.
41) Timmusk, S., N. Grantcharova and G.H. Wagner, 2005. *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Applied Environmental Microbiology,* 71: 7292 - 7300.
42) Weaver, P.K., J.D. Wall and H. Gest, 1975. Characterization of *Rhodopseudomonas capsulate*. *Archives in Microbiology,* 105: 207 - 216.
43) Wei, L., J.W. Kloepper and S. Tuzun, 1996. Induced systemic resistance to cucumber diseases and increased plant growth promoting rhizobacteria under field conditions. *Phytopathololology,* 86: 221 - 224.

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

Access this Article in Online

Quick Response Code	
Website	**www.jpsscientificpublications.com**
DOI Number	**DOI: 10.22192/lsa.2018.4.1.1**

How to Cite this Article:

S. Esath Natheer, R. Haseena, M. Syed Abdul Rahman and K. Feroz Khan. 2018. Response of different formulations of 1-Aminocyclopropane-Carboxylate (ACC) Deaminase positive Wild and Deficient strains of *Pseudomonas* and *Paenibacillus* on plant growth promoting characteristics in Maize (Zea mays L.) *cv*. CO1. *Life Science Archives*, 4(1): 1239 – 1247.

DOI: 10.22192/lsa.2018.4.1.1

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

Available online at www.jpsscientificpublications.com

Life Science Archives (LSA)

ISSN: 2454-1354

Volume – 4; Issue - 1; Year – 2018; Page: 1239 – 1247

DOI: 10.22192/lsa.2018.4.1.1

Research Article

RESPONSE OF DIFFERENT FORMULATIONS OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) DEAMINASE POSITIVE WILD AND DEFICIENT MUTANT STRAINS OF *Pseudomonas* AND *Paenibacillus* ON PLANT GROWTH PROMOTING CHARACTERISTICS IN MAIZE (*Zea mays* L.) *cv*. CO1

S. Esath Natheer[1*], R. Haseena[2], M. Syed Abdul Rahman[3] and K. Feroz Khan[3]

[1]*Department of Microbiology, Nehru Arts and Science College, Coimbatore – 641 105. Tamil Nadu, India.*

[2]*Department of Food and Agricultural Process Engineering, Tami Nadu Agricultural University, Coimbatore – 641 003, Tamil Nadu, India.*

[3]*Department of Marine Microbiology, Faculty of Marine Sciences, Centre of Advance Study in Marine Biology, Annamalai University, Parangipettai – 608 502, Tamil Nadu, India.*

Abstract

The response of different formulations of 1-aminocyclopropane-1-carboxylate (ACC) deaminase positive wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on plant growth promoting characteristics in maize was studies under *in vitro* condition. The bioinoculation effect of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* as co-flocs, augmented the seed vigour index and adhesion to maize roots to a higher level and also decreased the incidence of *Helminthosporium turcicum*, followed by co-inoculation and single strain inoculation. Between the two wild and mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*, wild strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* recorded the highest value for plant growth promoting characteristics when compared to their respective mutant strains. When comparing among two wild strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*, the wild strain *Pseudomonas fluorescens* were found to produce better performance than wild strains of *Paenibacillus polymyxa*. The results of the present study clearly envisaged the positive effect of co-flocs formulation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* wild strains, exhibiting ACC deaminase activity in augmenting the plant growth promoting characteristics.

Article History

Received : 15.10.2017

Revised : 12.11.2017

Accepted: 30.12.2017

Key words: Maize, *Pseudomonas fluorescens*, *Paenibacillus polymyxa*, ACC deaminase, Co-flocs, Wild, *acd* deficient mutant and Plant growth promotion.

1. Introduction

Impact of rhizobacteria generally on plant growth and health may be classified as neutral, deleterious or beneficial (Kloepper, 1989). However, plant growth promoting rhizobacteria (PGPR) specifically are beneficial and the beneficial effects have been utilized in many areas including biofertilizer, disease control, microbe-rhizoremediation, biopesticide, in forestry (Kloepper, 2003; Lucy, 2004), as well as probiotics. PGPR are naturally occurring soil

* *Corresponding author*: **Dr. S. Esath Natheer**

E.mail: esathnatheer@gmail.com

LSA ISSN: 2454-1354

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

bacteria that aggressively colonize plant roots and enhance the plant growth directly by eliciting root metabolic activities by supplying biologically fixed nitrogen (Glick *et al*., 1999), hormonal interaction, improvement in root growth, solubilization of nutrients and indirectly by acting against phytopathogens (Lugtenberg *et al*., 2002; Morrissey *et al*., 2004; Ehlers, 2006). The well known PGPR include genera, *viz*., *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Bacillus*, *Azoarcus*, *Klebsiella*, *Arthrobacter*, *Enterobacter*, *Serratia* and *Rhizobium* on non-legumes (Burdman *et al*., 2000; Berg, 2009). Among these bacteria *Pseudomonas* and *Bacillus* are the most widely reported PGPR.

Maize (*Zea mays* L.) is the third major crop of the world after wheat and rice which provides more nutrients for humans and animals than any other cereals and the same is grown in many countries, including India. The desire of every grain producer is to obtain high yields at reduced production costs so as to optimize net returns on investments. Crop establishment and plant vigour represent key factors that influence the success of grain crop production (Hammermeister *et al*., 2008). Certain bacteria possess an enzyme *viz*., ACC-deaminase (*acd*) that hydrolyzes 1-aminocyclopropane-1-carboxylate into ammonia and α-ketobutyrate (Mayak *et al*., 1999). PGPR strain containing ACC-deaminase activity could suppress the accelerated endogenous ethylene synthesis, thus facilitate the root elongation and nutrient absorption of host plant which resulted in improved growth and yield of crop plant improved growth and yield of crop plant. The inoculant formulations play a critical role in determining the success of bioinoculant. Among the various novel agricultural bioinoculant technologies, the EPS rich "Intergeneric PGPR co-flocs", containing ACC-deaminase positive rhizobacterial strains, seems to be a better one, in terms of, high cell titre, increased adhesion to plant roots, enriched in encysted cells and spores with EPS rich network, longer shelf life and Induced systemic resistance (ISR) mediated biocontrol against phytopathogens.

Seed inoculation of PGPR strains containing *acd* activity reduces the inhibitory effect of ethylene at spermosphere and leads to the improvement plant growth promotion (Penrose *et al*., 2001; Mayak *et al*., 2004). *Pseudomonas* and *Paenibacillus* are the two important PGPR genera which are frequently encountered from the rhizosphere of maize and the PGPR characteristics of the same have been described by many authors in different crop plants (Sheng *et al*., 2008; Timmusk *et al*., 2005). Among the various novel agricultural bioinoculant technologies, the EPS rich "Intergeneric PGPR co-flocs", containing ACC-deaminase positive rhizobacterial strains, seems to be a better one, in terms of, high cell titre, increased adhesion to plant roots, enriched in encysted cells and spores with EPS rich network and longer shelf life. However, there were no reports on the response of different formulations of *Pseudomonas* and *Paenibacillus* ACC-deaminase wild and deficient mutants on different plant growth promoting attributes in maize crop.

Hence, the present study has been undertaken with an aim to exploit the response of *acd* positive (wild) and *acd* deficient mutant strain of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* along with "intergeneric co-flocs" on enhancement of plant growth promotion and biocontrol of leaf blight disease.

2. Materials and Methods

Culture condition

Pseudomonas fluorescens and *Paenibacillus polymyxa*, isolated from the rhizosphere of maize grown at Kadampuliyur, Chidambaram taluk, Cuddalore district, Tamil Nadu state, India were used in the present study. The *P. fluorescens* and *P. polymyxa* isolates were positive for their ACC-deaminase (*acd*) activity, maintained in King's medium B (King *et al*., 1954) and Nutrient Glucose agar (Englesberg and Ingraham, 1957) slants, respectively, and incubated at 28±2ºC, with monthly transfer. *Helminthosporium turcicum* (AU-1), obtained from the Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, India, was used as a reference strain for the biocontrol study and the same was maintained in

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

Potato Dextrose agar (PDA) slants and examined periodically for its virulence.

P. fluorescens and *P. polymyxa* was also grown on DF salts minimal medium [(composition in g per litre) KH_2PO_4, 4.0; Na_2HPO_4, 6.0; glucose, 2.0; gluconic acid, 2.0; citric acid, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.0001; H_3BO_3, 0.0010; $ZnSO_4 \cdot 7H_2O$, 0.0070; CuSO4·5H20, 0.0050; MoO_3, 0.0010] (Dworkin and Foster, 1958). The medium was supplemented with either 2.0 g of ammonium sulphate or 3.0 mM ACC (Clearsynth, Mumbai).

Chemical mutagenesis and selection

The construction of ACC-deaminase deficient mutant (*acd*) of *P. fluorescens* and *P. polymyxa* isolates was carried out according to Miller (1972) and selection was carried out according to Glick *et al*. (1994).

Preparation of *P. fluorescens* and *P. polymyxa* co-flocs

The co-flocculation of *P. fluorescens* and *P. polymyxa* isolates were prepared in co-floc buffer as described by Grimaudo and Nesbitt (1997). One ml aliquot of each PGPR isolates *viz.*, *P. fluorescens* and *P. polymyxa* strains were mixed together in 10 ml co-floc buffer. The mixtures were vortexed for 10 sec, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for 24 hrs. All co-floc reactions were performed in triplicate and uninoculated buffer served as control. After incubation co-flocculation percentage was measured as described by Madi and Henis (1989).

Preparation of different bioformulation

One ml culture of each PGPR isolate (1×10^7 CFU per ml) under different formulations were prepared *viz.*, control, single strain inoculation of either wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa*, co-inoculation of wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* and co-flocs application of wild and *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* and used to evaluate plant growth promoting characteristics of maize *cv*. CO1 on the enhancement of seed vigour index, adhesion to maize roots and reduction in *Helminthosporium turcicum* incidence was studied under *in vitro* condition.

Response of different formulations on plant growth promoting characteristics

Seed vigour index of maize

Maize (*Zea mays* L.) *cv*. CO1 maize seeds were surface sterilized by immersion in 95% ethanol for 1 min, followed by 20 min in 1% NaOCl. After rising three times with sterile distilled water, the sterilized seeds were placed on the surface of 1 per cent water agar in petriplates (9 cm dia, at five seeds per plate) were treated with the different bioformulations as mentioned above. The maize seeds were subjected to the above treatments, dried in shade for 30 min. Then, the inoculated maize seeds were arranged in two rows on a sheet of blotting paper dipped in sterile water. Then, they were covered with another blotting paper dipped in sterile water, rolled and placed vertically in a moist chamber at 20 °C. Uninoculated seeds with distilled water treatment served as control. After the incubation for 5 days, each roll was opened and the vigour indices of germinated maize seeds were calculated by the method of Abdul-Baki and Anderson (1973).

Vigour index = (mean root length + mean hypocotyl length) × % germination

Adhesion to maize roots

Surface sterilized seeds were incubated in an inverted position for 3 days at room temperature to allow germination. The plates were sealed with wax to avoid agar dryness during germination. After germination, the three day old seedlings were transferred to slopes of Fahraeus solution (Fahraeus, 1957) solidified with 1.5 per cent agar in test tubes. Sterile Fahraeus solution was added to fill the empty portion of the agar slopes and the tubes were incubated for three more days (24ºC day / 22ºC night). After the incubation period, the roots were collected from each tube separately, washed first with sterile water and later three times in 0.1 M phosphate buffer (pH 6.8),

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

cut into 5 cm pieces and used for adsorption study as described by Gafni *et al*. (1986).

Preparation of growth chamber and challenge inoculation

The growth chamber was a desiccator (12 x 10 cm) consisting of two parts. The lower part was filled with weaver's medium (Weaver *et al*., 1975) and upper part contained stainless steel wire mesh (mesh size 3 mm) supports. The lid was placed over the cotton and the chamber was closed before sterilization. The growth chamber was sterilized by autoclaving. Fifty germinated maize seeds with coleoptile (2 cm high) were transferred aseptically onto the stainless steel wire mesh, incubated for 10 days in the growth chamber with 14 hrs day and 10 hrs night cycle and temperature ranging from 24°C at night to 32°C around noon. By this time, the maize roots yielded many lateral roots, well spread in the Weaver's medium maintained at the lower part of the growth chamber.

Maize plants were challenge inoculated by spraying the spore suspension of *Helminthosporium turcicum* at a spore concentration of 50,000 spores per ml inoculum level on 10th DAS with an atomizer and control plant was sprayed with sterile Weaver's medium. High humidity was created by sprinkling the water frequently in the polyhouse.

After one week of challenge inoculation, three plants from each treatment were carefully removed and rinsed with sterile distilled water. The leaf blight disease incidence was enumerated with a score chart of 0 to 9 grades devised by International Rice Research Institute (1980).

Statistics analysis

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure described by Gomez and Gomez (1984).

3. Result and Discussion

Pseudomonas fluorescens and *Paenibacillus polymyxa* wild strains registered a normal growth in the DF salts medium amended with ACC and suggested the ability of the wild strains to utilize ACC, as a sole 'N' source whereas the ACC deaminase (*acd*) deficient strains could not able to metabolize ACC and resulted in poor or no growth (data not shown). Glick *et al*. (1994) constructed an *acd* deficient mutant strain of *Pseudomonas putida* GR-12-2 by chemical mutagenesis (nitrosoguanidine) and reported the inability of the respective mutants to utilize ACC. The results of the present study also clearly in accordance with the earlier findings of Glick *et al*. (1994).

The response of different formulations of wild and *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* cells *viz*., control, single strain inoculation of either wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa*, co-inoculation of wild or *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* and co-flocs application of wild and *acd* deficient mutants of *P. fluorescens* and *P. polymyxa*, on plant growth promoting characteristics of maize *cv*. CO1 *viz*., enhancement of seed vigour index, adhesion to maize roots and reduction in *Helminthosporium turcicum* was studied under *in vitro* condition. Among the different formulations of *P. fluorescens* and *P. polymyxa* cells, the application of co-flocs of *P. fluorescens* and *P. polymyxa* wild cells augmented the highest level of seed vigour index and adhesion to maize roots, followed by the co-flocs of *P. fluorescens* and *P. polymyxa acd* mutant strains, coinoculation of *P. fluorescens* and *P. polymyxa* wild strains, coinoculation of *P. fluorescens* and *P. polymyxa* mutant strains, single wild strain inoculation of *P. fluorescens* and *P. polymyxa*, single mutant strain inoculation of *P. fluorescens* and *P. polymyxa* and control. Moreover, the application effect of *P. fluorescens* and *P. polymyxa* wild strains as co-flocs positively reduced the incidence of leaf blight disease to a higher level when compared to other treatments (Table - 1).

The improved plant growth promoting of seed vigour index is due to application of co-flocs containing *acd* positive *P. fluorescens* and *P. polymyxa* suggested the positive role of *acd* in

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

modifying the ethylene level during germination of maize seeds as suggested earlier by Glick *et al*. (1994). Phytostimulatory effect of *Pseudomonas* cells has been already reported by Ahmad *et al*. (2005) and Glick *et al*. (2007). Neyra *et al*. (1999) described the phytostimulatory effect of "Intergeneric coaggregates" containing *Azospirillum and Rhizobium*, on the enhancement of growth parameters in faba bean. Further, the positive role of rhizobacterial ACC-deaminase activity on the enhancement of seed germination and plant growth stimulation has been reported by many authors (Glick *et al*., 1994; Contesto *et al*., 2008; Ma *et al*., 2008; Barnawal *et al*., 2017). The production of ACC-deaminase by *P. fluorescens* and *P. polymyxa* has been confirmed (Glick *et al*., 1994; Belimov *et al*., 2007).

Table – 1: Response of different formulations of *P. fluorescens* and *P. polymyxa* wild and *acd* deficient mutant strains of different plant growth promoting (PGP) characteristics in maize *cv*. CO1

Treatment[a]	Seed vigour index[b,c]	No. of adhered cells (10^4 CFU g^{-1} dry wt. of root h^{-1})[b,c]	Percentage of disease incidence[b,c]
Control	10320 ± 19.18[i]	-	82.58 ± 1.27[i]
P. fluorescens (W)	12980 ± 9.72[e]	272.81 ± 2.97[e]	59.85 ± 0.93[e]
P. fluorescens (M)	11850 ± 17.27[g]	198.16 ± 2.07[g]	70.21 ± 0.52[g]
P. polymyxa (W)	12420 ± 16.83[f]	248.72 ± 2.60[f]	64.63 ± 0.34[f]
P. polymyxa (M)	11350 ± 20.08[h]	183.31 ± 1.81[h]	76.48 ± 0.83[h]
Co-I-*P. fluorescens* + *P. polymyxa* (W)	14462 ± 15.37[c]	331. 45 ± 3.97[c]	48.07 ± 0.67[c]
Co-I-*P. fluorescens* + *P. polymyxa* (M)	13625 ± 12.43[d]	300.23 ± 3.32[d]	52.31 ± 0.56[d]
Co-F-*P. fluorescens* + *P. polymyxa* (W)	16030 ±14.06[a]	408.62 ± 5.11[a]	22.72 ± 0.31[a]
Co-F-*P. fluorescens* + *P. polymyxa* (M)	15370 ± 11.16[b]	385.19 ± 4.27[b]	32.11 ± 0.35[b]
LSD (P<0.05)	0.99	0.98	0.98

W – Wild; M – Mutant; Co-I – Coinoculation; Co-F – Co-floc
a - At $1x10^7$ CFU per ml inoculum level
b - Values are mean of three replication ± SD
c - Values followed by different letters are significantly differed at 5 % level according to student 't' test

The adhesion mechanism of *Pseudomonas* and EPS rich *Paenibacillus* cells has already been reported in many crop plants by many authors (O'Sullivan and O'Gara, 1992; Wei *et al*., 1996; Puente *et al*., 2009). The results of the present study clearly revealed the improved adhesion of EPS rich PGPR co-flocs, containing ACC-deaminase positive strains of *P. fluorescens* and *P. polymyxa* to maize roots when compared to PGPR co-flocs, containing *acd* deficient mutant strains of *P. fluorescens* and *P. polymyxa* and individual application of either *acd* positive wild or *acd* deficient PGPR cells and emphasized the positive role of EPS rich, ACC-deaminase positive rhizobacterial strains in the early events of adhesion to maize roots.

The application effect of different formulations of PGPR cells on the incidence of *Helminthosporium turcicum* in maize, the co-flocs, consisting of ACC-deaminase positive wild strains of *P. fluorescens* and *P. polymyxa* reduced the leaf blight disease incidence to a higher level when compared to others formulations. Pieterse *et al*. (2000) reported the positive role of ACC-deaminase containing rhizobacterial strains on the modulation of ethylene in crop rhizosphere which leads to induction of systemic resistance (ISR) in *Arabidopsis thaliana*. The biocontrol of maize leaf blight disease due to *Pseudomonas* and *Paenibacillus* bioinoculation has already been reported by Belimov *et al*. (2007), Haggag (2007) and Jalili *et al*. (2009) in various plant crops and also it is believed to alleviate drought and salt tolerance (Ann Maxton *et al*., 2017; Saikia *et al*., 2018).

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

4. Conclusion

The results of present study envisaged the positive effect of PGPR strains, containing ACC deaminase positive wild strains of *P. fluorescens* and *P. polymyxa*, in augmenting the seed vigour index, adhesion to maize root and biocontrol against *H. turcicum* in semiarid maize *cv*. CO1 when compared to *acd* deficient strains of *P. fluorescens* and *P. polymyxa*. The mechanism regarding the improved PGPR characteristics by *acd* positive wild strains of *P. fluorescens* and *P. polymyxa* needs further exploitation.

Acknowledgement

We are thankful to University Grants Commission (UGC) of India for providing funds under the scheme of Maulana Azad National Fellowship (MANF) for the Minority students in support of this research for providing necessary facilities to carry out this work.

5. References

1) Abdul-Baki, A.A. and J.D. Anderson, 1973. Vigour determination in soybean seed and multiple criteria. *Crop Science,* 13: 630 - 633.
2) Ahmad, F., L. Ahmad and M. Saghir, 2005. Indolacetic acid production by the indigenous isolates of *Azotobacter* and *Pseudomonas fluorescens* in the presence and absence of tryptophan. *Turkish Journal of Biology,* 29 - 34.
3) Ann Maxton, S. Poonam and S.A. Masih, 2017. ACC deaminase-producing bacteria mediated drought and salt tolerance in *Capsicum annuum*, Journal of Plant Nutrition, 41(5): 574 - 583.
4) Barnawal, D., S.S. Pandey, N. Bharti, A. Pandey, T. Ray, S. Singh, C.S. Chanotiya and A. Kalra, 2017. ACC deaminase-containing plant growth-promoting rhizobacteria protect *Papaver somniferum* from downy mildew, *Journal of Applied Microbiology,* 122(5): 1286 - 1298.
5) Belimov, A.A., I.C. Dodd, V.I. Safronova, N. Hontzeas and W.J. Davies, 2007. *Pseudomonas brassicacearum* strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato. *Journal of Experimental Botany,* 58: 1485 - 1495.
6) Berg, G, 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology,* 84: 11 - 18.
7) Burdman, S., Y. Okon and E. Jurkevitch, 2000. Surface characteristics of *Azospirillum* brasilense in relation to cell aggregation and attachment to plant roots. *Critical Reviews on Microbiology,* 26: 91 - 110.
8) Contesto, C., G. Desbrosses, C. Lefoulon, B. Bena, F. Borel, M. Galland, L. Gamet, F. Varoquaux and B. Touraine, 2008. Effects of rhizobacterial ACC deaminase activity on *Arabidopsis* indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. *Plant Science,* 175: 178 - 189.
9) Dworkin, M. and J. Foster, 1958. Experiments with some microorganisms which utilize ethane and hydrogen. *Journal of Bacteriology,* 75: 592 - 601.
10) Ehlers, R.U. 2006. Einsatz der Biotechnologie im biologischen. *Pflanzenschutz. Schr. Dtsch. Phytomed. Ges*, 8: 17 - 31.
11) Englesberg, G.J. and P. Ingraham, 1957. Glucose broth for the production of Exopolysaccharide by *Paenibacillus polymyxa* isolates. *Journal of Microbiology,* 14: 56 - 58.
12) Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a single glass slide technique. *Journal of General Microbiology,* 16: 374 - 381.
13) Gafni, R., Y. Okon, Y. Kapulnik and M. Fischer, 1986. Adsorption of *Azospirillum brasilense* to corn roots. *Soil Biology and Biochemistry,* 18: 69 - 75.
14) Glick, B.R., C.B. Jacobson, M.M.K. Schwarze and J.J. Pasternak, 1994. 1-

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

Aminocyclopropane-1-carboxylic acid deaminase mutants of the plant growth promoting rhizobacterium *Pseudomonas putida* GR 12-2 do not stimulate canola root elongation. *Canadian Journal of Microbiology,* 40: 911 - 915.

15) Glick, B.R., C.L. Patten, G. Holguin and D.M. Penrose, 1999. Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London, p 267.
16) Glick, B.R., Z. Cheng, J. Czarny and J. Duan, 2007. Promotion of plant growth by ACC deaminase-containing soil bacteria. *Europen Journal of Plant Patholology,* 119: 329 - 339.
17) Gomez, K.A. and A.A. Gomez, 1984. Statistical procedures for agricultural research, John Wiley and Sons, New York, USA. p.20-29.
18) Grimaudo, N.J. and W.E. Nesbitt, 1997. Coaggregation of *Candida albicans* with oral *Fusobacterium sp*. *Oral Microbiology and Immunology,* 12: 168 - 173.
19) Haggag, W.M. 2007. Colonization of exopolysaccharide - producing *Paenibacillus polymyxa* on peanut roots for enhancing resistance against crown rot disease. *African Journal of Biotechnology,* 6: 1568 - 1577.
20) Hammermeister, A., M. Bernard, J. Mackenzie and R. Beavers, 2000. Crop establishment and soil fertility on maritime organic grain farms. Organic Agriculture Centre of Canada, Final Research report, 2008 - 2041.
21) IRRI. 1980. International Rice Research Institute. Annual Report for 1979. Los Banos, Philippines, pp. 171 - 182.
22) Jalili, F., K. Khavazi, E. Pazira, A. Nejati, A.H. Rahmani, R.H., Sadaghiani and M. Miransari. 2009. Isolation and characterization of ACC deaminase producing fluorescent pseudomonads, to alleviate salinity stress on canola (*Brassica napus* L.) growth. *Journal of Plant Physiology,* 166: 667 - 674.
23) King's, E.O., M.K. Ward and D.E. Raney, 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine,* 44: 301 - 307.
24) Kloepper, J.W. 2003. A review of mechanisms for plant growth promotion by PGPR. Presented at the 6th international PGPR Workshop, 5 – 10 October 2003, Calicut, India.
25) Kloepper, J.W., R. Lifshitz and R.M. Zablotwicz, 1989. Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnology,* 7: 39 - 43.
26) Lucy, M., E. Reed and B.R. Glick, 2004. Application of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek,* 86: 1 - 25.
27) Lugtenberg, B.J.J., T.F.C. Chin-A-Woeng and G.V. Bloemberg, 2002. Microbe–plant interactions: principles and mechanisms. *Antonie van Leeuwenhoek,* 81: 373-383.
28) Ma, Y., M. Rajkumar and H. Fritas, 2008. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *Journal of Environmental Management,* 90: 831 - 837.
29) Madi, L. and Y. Henis, 1989. Aggregation in *Azospirillum brasilense* cd. conditions and factors involved in cell-to-cell adhesion. *Plant and Soil,* 115: 89 - 98.
30) Mayak, S., T. Tirosh and B.R. Glick, 1999. Effect of wild type and mutant plant growth promoting rhizobacteria on the rooting of mung bean cuttings. *Journal of Plant Growth Regulation,* 18: 49 - 53.
31) Mayak, S., T. Tirosh and B.R. Glick, 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiology and Biochemistry,* 42: 565 - 572.
32) Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33) Mohammadhossein, R., R. Sasidharan, L.A.C.J. Voesenek, G.A. Kowalchuk and A. Jousset, 2017. ACC deaminase-producing rhizosphere bacteria

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

modulate plant responses to flooding, *Journal of Ecology,* 105(4): 978 - 986.

34) Morrissey, J.P., J.M. Dow, L. Mark and F. O'Gara, 2004. Are microbes at the root of a solution to world food production? *EMBO Rep.,* 5: 922 - 926.
35) Neyra, C.A., L.A. Atkison, O. Olubayi, L. Sadasivam, D. Zaurov and R. Zappi, 1999. Novel microbial technologies for the enhancement of plant growth and biocontrol of fungal diseases in crops. *Cahiers Options Mediterranean,* 31: 447 - 455.
36) O'Sullivan, D.B. and F. O'Gara, 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiology Reviews,* 56: 662 - 676.
37) Penrose, D.M. and B.R. Glick, 2001. Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. *Canadian Journal of Microbiology,* 47: 368 - 372.
38) Pieterse, C.M.J., J.A. Van Pelt, J. Ton, S. Parchmann, M.J. Mueller, A.J. Buchala, J.P. Metraux and L.C. van Loon, 2000. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiology and Molecular Plant Pathology,* 57: 123 - 134.
39) Puente, M.E., C.Y. Li and Y. Bashan, 2009. Rock-degrading endophytic bacteria in cacti. *Environmental Experimental Botany,* 66: 389 - 401.
40) Sheng, X.F., J.J. Xia, C.Y. Jiang, L.Y. He and M. Qian, 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting growth and lead accumulation of rape. *Environmental Pollution,* 156: 1164 - 1170.
41) Timmusk, S., N. Grantcharova and G.H. Wagner, 2005. *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Applied Environmental Microbiology,* 71: 7292 - 7300.
42) Weaver, P.K., J.D. Wall and H. Gest, 1975. Characterization of *Rhodopseudomonas capsulate*. *Archives in Microbiology,* 105: 207 - 216.
43) Wei, L., J.W. Kloepper and S. Tuzun, 1996. Induced systemic resistance to cucumber diseases and increased plant growth promoting rhizobacteria under field conditions. *Phytopatholology,* 86: 221 - 224.

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

Access this Article in Online

Quick Response Code

Website www.jpsscientificpublications.com

DOI Number DOI: 10.22192/lsa.2018.4.1.1

How to Cite this Article:

S. Esath Natheer, R. Haseena, M. Syed Abdul Rahman and K. Feroz Khan. 2018. Response of different formulations of 1-Aminocyclopropane-Carboxylate (ACC) Deaminase positive Wild and Deficient strains of *Pseudomonas* and *Paenibacillus* on plant growth promoting characteristics in Maize (Zea mays L.) *cv*. CO1. *Life Science Archives*, 4(1): 1239 – 1247.

DOI: 10.22192/lsa.2018.4.1.1

©2018 Published by JPS Scientific Publications Ltd. All Rights Reserved

BIOMEDICAL POTENTIAL OF MEDICINAL PLANTS EXTRACTS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

M.Manohar[a*]., P.Pandiselvi[a], Dr.R.Jannet Rani[a], K,Chitra[a] and Feroz Khan[a]
PG and Research Department of Microbiology,
Sadakathullah Appa College (Autonomous) Tirunelveli – 627006
***Corresponding Author:** gansun1@gmail.com

Abstract - The major problem existing in the field of medicine is drug resistance. The organisms were developing resistance to almost all the existing drugs. Among such organisms MRSA pose a major threat. The prevalence of MRSA infection is also increasing steadily. So we are in need of alternative therapy to cure such infections. One such remedy could be the use of extracts from medicinal plants. In the present study we used the extracts of some herbal plants such as Punica granatum, Solanum trilobatum, Ocimum tenuiflorum, Ripes uva-crispa, Psidium guajava, Cajanus cajan, Euphorbia hirta, Phyllanthus niruri, Phyllanthus amarus against MRSA. Among these plant methanol extract of P. granatum (inhibition zone 20mm) showed remarkable results against MRSA. We also checked the synergistic effect of some antibiotics with Punica granatum. They showed synergistic effect with Amoxicillin Cephalothin and Cefepime.

Keywords: MRSA, Solanum trilobatum, Psidium guajava. Antibacterial activity

INTRODUCTION

All over the ages, humans have relied on nature for their basic needs, such as food, shelter, clothing, transportation, fertilizers, flavors , medicine etc, (Critchley, A.T. (1983).). For thousands of years, plants formed the basis of traditional medicine systems that have been existing and continue with new remedies to mankind. Eventhough some of the therapeutic properties of the plants have been proven. It is based on the empirical findings of thousands of years (Heinrich *et al*., 2004). Various types of plants used in herbalism and many of these plants have medicinal properties. 3.3 billion Peoples in the developing countries utilize the medicinal plants which are the "backbone" of traditional medicine (Davidson-Hunt I *et al*., 2000).

Fruits and vegetables are most popular due to their dietetic value worldwide and rich sources of beneficial vitamins and fibers, anti-oxidants, minerals. The standard consumption of fresh fruits and vegetables may diminish the risk of cardiovascular diseases, stroke and certain cancers. Usually fruits are processed into beverage, squash juice and syrups. However by-products can be used as functional food ingredients such as phytochemicals, pharmaceuticals, essential oils, seed oil, pectin, food products, and dietary fibers (Azad *et al*., 2014). Therefore, fruits by-products not only superior source of bioactive compounds but also could be used as several value-added products (Noor *et al*., 2014).

In conventional chemistry and pharmacology plants might provide a useful source for the production of new effective medicines and this may be used to replace existing drugs . In general traditional medicine is turned out to be very useful in the discovery of natural products such as pharmaceutical drugs (Patwardhan *et al*., 2004). The shrub *Prosopsis juliflora* (Sw.) DC. (*P. juliflora*) commonly identified as mesquite has been used as a traditional medicine on various continents for curing catarrh, inflammation, colds, sore throat, excrescences, flu, measles, diarrhea, dysentery and hoarseness, for the healing of wounds (Hartwell *et al*., 1971).

METHODOLOGY

Isolation of *Staphylococcus aureus*

For the isolation of *Staphylococcus aureus*, pus samples were collected from wounds. The specimens were collected from Bose Clinical Lab, Madurai. Fluid Thioglycollate medium was used as an enriched medium for *Staphylococcus aureus*. The specimen was inoculated on Mannitol salt agar, Mac Conkey agar and Blood agar plates. The plates were incubated aerobically at 37^{0}C overnight. The test organisms further confirmed by their physiological and biochemical characters.

Detection of MRSA

Disk Susceptibility Test (Acar and Goldstein, 1991). The Kirby-Bauer disk diffusion test was developed for this study.

The sensitivity pattern of MRSA strains to β-lactam antiboiotics

Disk susceptibility test (Acar and Goldstein., 1991).

The Kirby –Bauer disk diffusion test was developed for these studies as described above with the antibiotic disks methicillin, amoxyclav, amoxicillin, cefdin, cephalothin, cefepime, cefaclor, vancomycin and teicoplanin. The sensitivity pattern of the test organism was recorded.

Determination of the degree of methicillin resistance

The Kirby-Bauer disk diffusion test was developed for this study. MRSA strains were individually tested for their degree of methicillin resistance with methicillin 5 µg and 10 µg.

Collection of herbal plants

The following fresh plant materials and sea weeds were collected from Vilathikulam, Thoothukudi district. Plants such as *Punica granatum, Prosopis juliflora, Ocimum tenuiflorum,* and sea weeds such as *Sargassum muticum* and *Jania . rubens.*

Preparation of plant extract

Water extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of sterile distilled water. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no:1 filter paper.

Methanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of methanol. They were placed in shaker for 24 hours. The methanol extract was filtered by using Whatmann No:1 filter paper.

Ethanol extract

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of ethanol. They were placed in shaker for 24 hours. The ethanol extract was filtered by using Whatmann no:1 filter paper.

Petroleum ether

Fresh plant materials were collected and then dried at room temperature for 6 days. They were subsequently grounded into fine powder. 5g of plant materials were mixed with 25ml of petroleum ether. They were placed in shaker for 24 hours. The water extract was filtered by using Whatmann no: 1 filter paper.

Determination of antibacterial activities of plant extract against MRSA strain

Loading sterile disks with plant extract

Antibacterial activity was measured using disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI). Briefly petriplates containing approximately 25-30 ml of Mueller-Hinton agar medium were inoculated using a cotton swab with a 4-6 fold culture of the bacteria. (Acar and Goldstein, 1991).

Disk susceptibility test

The Kirby- Bauer disk diffusion test was developed for these studies as described above with the disks of plant extract. The sensitivity pattern of the test organism was recorded.

Determination of synergetic effect of plant extract with some antibiotics against MRSA

The Kirby- Bauer disk diffusion test was developed for these studies as described above. To access possible interactions between the extract of plant materials and β-lactam antibiotics, disk containing the extract of plant materials and β-lactam antibiotics were placed on a plate that had been inoculated with MRSA strain. The distance between the disks was equal to the sum of the radii of their zones of inhibition when examined alone.

Checkerboard method to determine the antimicrobial combinations against MRSA

The checkerboard (or chessboard) method is the technique used most frequently to assess antimicrobial combinations in vitro, presumably because its rationale is easy to understand, the mathematics necessary to calculate and interpret the results are simple, it can readily be performed in the clinical laboratory using microdilution systems that are obtain able commercially, and it has been the technique most frequently used in studies that have suggested an advantage of synergistic therapy in the treatment of neutropenic patients with Gram-negative septicemia. The term "checkerboard" refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested in concentration equal to, above, and below their minimal inhibitory concentrations for the organisms being tested.

RESULTS

Isolation and characterization of *staphylococcus aureus*

Eight numbers of MRSA strains were isolated from pus. Various media (Table 3) were used to identify the pathogenic strain. Mannitol salt agar was used as a differential and selective medium for recovering strain from the specimen. *Staphylococcus aureus* produced yellow colour colonies in Mannitol Salt agar.

Table 1.Cultural characterization of *Staphylococcus aureus*

S. No	Medium	Colony Morphology	Interpretation
1	MacConkey Agar	Pink colour colonies	Lactose fermentation positive
2	Blood Agar	Yellow to cream colonies or white colonies with clear zone	β-haemolytic colonies
3	Mannitol Salt Agar	Yellow colonies	Mannitol fermentation positive

Table.2. Degree of Methicillin Resistance of MRSA strains (Fig. 20)

S. No	Methicillin Disk Content	Zone diameter of inhibition (mm)	Reaction of the Organism
1	Methicillin (5 µg)	-	Resistant
2	Methicillin (10 µg)	-	Resistant
3	Methicillin (30 µg)	-	Resistant

Determination of antibacterial activities of plant extract against MRSA strain

The antimicrobial activity of various extracts of *Punica granatum* and *Prosopis juliflora* were tabulated (Table 7 -9). (Fig. 5-12).

Table.3. Antimicrobial activity of various extracts of *Punica granatum*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	10	15	15	_
MRSA 1	Partial	10	15	_
MRSA 2	Partial	10	20	_
MRSA 3	Partial	15	20	_
MRSA 4	10	15	21	_
MRSA 6	10	15	20	_
MRSA 7	Partial	10	15	_
MRSA 8	10	15	20	_

Table 4.Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Table 5. Antimicrobial activity of various extracts of *Prosopis juliflora*

Diameter of zone of inhibition (mm)				
Organism	**Water Extract**	**Ethanol Extract**	**Methanol Extract**	**Petroleum Ether Extract**
MRSA	15	20	28	_
MRSA 1	15	20	25	_
MRSA 2	15	22	23	_
MRSA 3	16	20	20	_
MRSA 4	18	23	24	_
MRSA 6	16	21	22	_
MRSA 7	15	20	23	_
MRSA 8	17	20	24	_

Fig. 1. ***P. granatum*** **(pomegranate)**

Fig.2. ***P. juliflora*** **(karuvelam)**

Fig. 3 ***S. muticum***

Fig. 4. ***J.rubens***

Plate 1. Synergistic effect between *P. granatum* and methicillin 10 µg

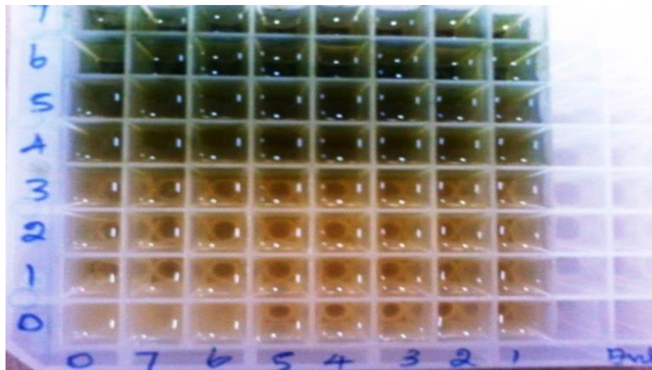
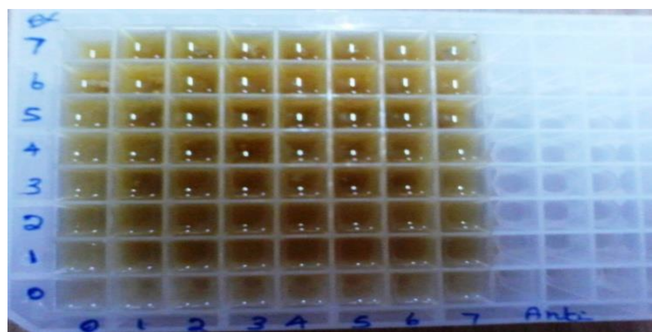
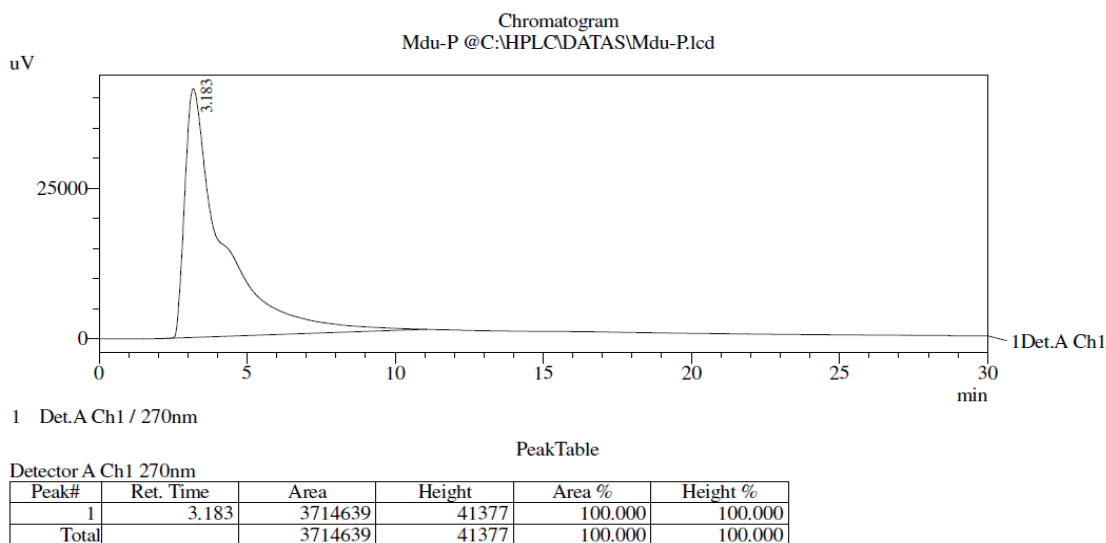


Plate 2. Synergistic of *P.juliflora* with Amoxyclav





PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.183	3714639	41377	100.000	100.000
Total		3714639	41377	100.000	100.000

Fig. 6. HPLC analysis of *P. granatum*

DISCUSSION

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years, the World Health Organization advocated that countries should interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origin (WHO. 1978).

The increasing occurrence, particularly in hospitals, of *S. aureus* resistant not only to methicillin but to a wide range of antimicrobial agents, including all kinds of β-lactams, has made therapy more difficult (Unci Ney *et al.,*2006).. Although strategies have been proposed in an attempt to control the spread, the search for new ways to treat MRSA infections stimulates the investigation of natural compounds as an alternative treatment of these infections.In the present study, the analysis of the growth inhibition activity by the disk diffusion method showed

that 10 medicinal plants (*P. juliflora*, *P. granatum*, *S.trilobatum*, *O.tenuiflorum*, *R.uva-crispa*, *P.guajava*, *C. cajan*, *E. hirta*,*P.niruri* and *P. amarus*),were active against hospital strains of MRSA under test conditions with crude extract concentrations as high as 5g/20ml.(Williams, et al.,2007)

In the present study, 2 plant extracts (*P. juliflora*, *P. granatum*) were effective against MRSA, MRSA1, MRSA2, MRSA 3, MRSA 4, MRSA 6, MRSA 7, MRSA 8 strains. We were using water, ethanol, methanol, petroleum ether extracts for all the 8 strains. Methanol extract inhibit the growth of 8strains of MRSA. The synergistic method were also performed for the methonal extracts with 10 antibiotics of methicillin (5µg), methicillin (10µg), amoxyclav (30 µg 10 µg), amoxicillin (30 µg 10µg), cefdin (5µg), cephalothin (30µg), cefepime (30µg), cefaclor (30µg), vancomycin (30 µg) and teicoplanin (30µg) in MRSA strain.

References:

1. Critchley, A.T. (1983). *Sargassum muticum*: a taxonomic history including world-wide and western pacific distributions. *Journal of the Marine Biological Association of the United Kingdom* 63: 617–625.
2. Davidson-Hunt. I. (2000). Ecological ethno botany: stumbling toward new practices and paradigms. MASA J 16:1–13, 2000.
3. Azad, A.K.M., M. A. Ali, M.S. Akter, M.J. Rahman and M. Maruf Ahmed. (2014). Isolation and Characterization of Pectin Extracted from Lemon Pomace during Ripening. *Journal of Food and Nutrition Sciences* Vol. 2, No. 2, pp. 30-35.
4. Noor, F., M.J. Rahman, M.S. Mahomud, M.S. Akter, M.A.I. Md. Aminul Islam Talukder and M. Ahmed. (2014). Physicochemical Properties of Flour and Extraction of Starch from Jackfruit Seed. *International Journal of Nutrition and Food Sciences* Vol. 3, No. 4, pp. 347-354.
5. Patwardhan, B., A. D. Vaidya and M. Chorghade. (2004). Ayurveda and natural products drug discovery. *Curr Sci* 86: 789-799.
6. Hartwell, J.L. (1971). Plants used against cancer. A survey. *Lloydia* 34: 386-425.
7. Acar, J.F. and F. W. Goldstein, (1991). Disk Susceptibility Test. In: Lorian V. (Ed). Antibiotics in Laboratory Medicine. Williams and Wilkins, London.
8. WHO. (2002). Traditional medicine. Report, EB111/9, World Health Organization, Geneva.
9. Williams, S. L. and J. E. Smith. (2007). A Global Review of the Distribution, Taxonomy, and Impacts of Introduced Seaweeds. *The Annual Review of Ecology, Evolution and Systematics*, 38, 327-59.
10. Unci Ney, J.Y., W.Y. Shieh, Jeng and W.L. Huang. (2006). Antibiotic activity of lectins from marine algae against marine Vibrio. *Journal of International Microbiology and Biotechnology* 30: 433-439.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Variability in the diet diversity of catfish highlighted through DNA barcoding

Chinnamani Prasannakumar[1,2*], Gunasekaran Iyyapparajanarasimapallavan[3,4], M. Ashiq Ur Rahman[5], P. Mohanchander[6], T. Sudhakar[7], K. Kadharsha[8], K. Feroz Khan[9], J. Vijaylaxmi[10], Narra Prasanthi[11], Kumaran Subramanian[12] and Seerangan Manokaran[13]

[1]Biological Oceanography Division, CSIR-National Institute of Oceanography, Dona Paula, Panaji, Goa-403 004, India.

[2]Institute of Marine Microbes and Ecosphere, State Key Laboratory for Marine Environmental Sciences, Xiamen University, Xiamen, Fujian, 361 102, China.

[3]Department of Fisheries Laboratory, Tamil Nadu Fisheries Department, Chetpet Ecopark Campus, Kilpauk, Chennai – 600010

[4]Marine Biotechnology Division, Central Marine Fisheries Research Institute, Ernakulam North P.O., Kochi - 682018

[5]Department of Zoology, Hajee Karutha Rowther Howdia College, Uthamapalayam, Theni District, Tamil Nadu- 625 533, India

[6]Department of Zoology, Madras Christian College, East Tambaram, Chennai, Tamil Nadu-600 059, India

[7]Department of Zoology, Kavitha's college of arts and science, Vaiyappamalai, Trinchangode, Tamil Nadu- 637 410, India

[8]PG & Research Department of Zoology, C. Abdul Hakeem College, Melvisharam, Vellore, Tamil Nadu- 632 509, India

[9]Research Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli Tamil Nadu -627011, India

[10]Department of Marine Sciences, Goa University, Taleigao Plateau, Goa-403206, India

[11]Centre of Advance studies in Marine Biology, Parangipettai, Tamil Nadu-608502, India

[12]Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Tamil Nadu-600119, India

[13]Center for Environment & Water, King Fahd University of Petroleum and Minerals, Dhahran-31261, Saudi Arabia

*Corresponding author's email id.: micropras@gmail.com

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Abstract

Identification and quantification of fish diet diversity was the first step in understanding the food web dynamics and ecosystem energetics, where the contribution of DNA barcoding technique has been important. We used DNA barcoding to identify the stomach contents of a euryhaline, benthophagous catfish *Ariius maculatus*. From 40 catfish stomach items sampled in two different seasons, we barcoded 67 piscine and macro-invertebrates prey items, identified as belonging to 13 species in 4 major phyla (viz., Chordate, Arthropod, Annelida and Mollusca). It is important to note that the mollusc taxa (*Meritrix meritrix* and *Perna viridis*) and a species of fish (*Stolephorus indicus*) could not be found among the gut contents of *A. maculatus* sampled during the pre- and post-monsoon season, respectively. Among the piscine diets of *A. maculatus*, *Eubleekeria splendens* (23.5%) and *Stolephorus indicus* (23.5%) were the major prey taxa during pre-monsoon season. The hermit crabs forms the major constituents of both pre- and post-monsoon seasons, among invertebrate taxa. Polychaete, *Capitella capitata* (25.92%) was abundantly consumed invertebrates next to hermit crabs. We noticed that in pre-monsoon *A. maculatus* was more piscivorous than post-monsoon. As revealed through Kimura-2 parametric pair-wise distance analysis, the diet diversity was relatively higher in post-monsoon. The accumulation curve estimated 57 haplotypes within 14 barcoded species (including the host *A. maculatus*). Majority of haplotypes were found among fishes (47.36%) followed by Arthropods (28.07%), Annelids (14.03%) and Mollusca (10.52%), respectively. This study also highlights that there is a growing concern about *A. maculatus*'s aggressive predation on commercially important stocks of fish and invertebrates. We will continue to expand the coverage of species barcoded in the reference database, which will become more significant as meta- and environmental DNA barcoding techniques become cheaper and prevalent.

Keywords: *Arius maculatus*, catfish, Stomach analysis, DNA barcoding, pairwise distance, diet diversity

1. Introduction

In Ichthyology, the first step towards identifying trophic levels of the fish species was the determination and quantification of fish stomach contents (Hynes, 1950; Hyslop, 1980; Cresson et al., 2014) and understanding of food web dynamics and energy of the ecosystem

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

(Beauchamp et al. 2007). The traditional visual survey of stomach content, however, did not provide data to be compared across studies (Cortés, 1997; Hernández, 2019), as level of taxonomic resolution at which a prey was classified (e.g. order, family, genus or species level) and the metrics used for quantification (e.g. count, volume) differed between the studies (Berg, 1979; Hyslop, 1980; Hansson, 1998; Hernández, 2019). While much work has been expended in standardizing dietary analysis (Pinkas, 1971; Cortés, 1997), it has not been universally accepted as a standard technique (Baker et al., 2014). Prey items that is present at different digestive stages when analysing the gut produces ambiguity in taxonomic resolution and identification. These findings differ through studies and rely on different factors, such as the methodology adapted for prey identification, prey condition, prior taxonomic knowledge of the prey species, and the objective of the gut analysis (example: Elliott, 1967; Baker and Sheaves, 2005; Saunders et al., 2012; Gray et al., 2015). In addition to complications due to unreported prey assumption, variable taxonomic resolutions compound the inconsistency and make the findings incomparable between studies.

DNA barcoding refers to sequencing the standard barcode region and matching them with archived sequences derived from validated species to facilitate species identification (Hebert et al., 2003; Joly et al., 2014; Kress et al., 2015). DNA barcoding has been used to study trophic dynamics (Valdez-Moreno et al. 2012; Wirta et al. 2014; Moran et al. 2015), environmental forensics (Dalton and Kotze 2011; Handy et al. 2011; Gonçalves et al. 2015), cryptic diversity and invasive species identification (Hebert et al. 2004; Conway et al. 2014; Bariche et al. 2015), ecosystem and evolutionary diversity evaluation (Ward et al. 2005; Baldwin et al. 2011; Weigt et al. 2012a; Leray and Knowlton 2015) and phylogenetic exploration (Nagy et al. 2012; Baeza and Fuentes 2013; Betancur-Ret al. 2013). While a number of markers available (e.g., 16sRNA, 18sRNA, matK, rbcL, ITS), a ~ 650 base pair (bp) region in the mitochondrial cytochrome c oxidase 1 (COI) gene is one of the most widely used in fish (and other animals) (Ward et al., 2005; Lakra et al., 2010; Khan et al., 2011; Weigt et al. 2012b). Species recognition is made by comparing the query sequence to that of archived sequences in reference databases, such as the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007) using the BOLD-Identification System (BOLD-IDs) and in GenBank (National Center for Biotechnology Information) using Basic Local Alignment Sequence Tool (BLAST) (Altschul et al. 1990). DNA barcoding has been widely applied in assessment of diversity and composition of fin and shell fishes (Teodoro et al., 2016; Sharawy et al., 2017; Zhang et al., 2017; Wang et al., 2017; Kuguru et al., 2018; Ran et al., 2020; Xu et al., 2021). DNA barcoding is currently commonly used in consumer markets

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

to identify sharks and their products (Holmes et al., 2009; Nijman et al., 2015; Hellberg et al., 2019). DNA barcodes acts as a potential forensic tool to track illegally traded and mislabeled endangered fishery products (Pappalardo & Ferrito, 2015; Di Pinto et al., 2015; Sembiring et al., 2015; Velez-Zuazo et al., 2015; Carvalho et al., 2017; Bunholi et al., 2018).

In recent years, the use of DNA barcoding to identify digested prey items has increased, especially in the identification of deepwater sharks prey items (Barnett et al. 2010; Dunn et al. 2010); Lake fish predators (Carreon-Martinez et al. 2011); invasive lionfish *Pterois* sp. (Valdez-Moreno et al. 2012; Cote et al. 2013; Rocha et al. 2015; Dahl et al., 2017; Ritger et al., 2020; Santamaria et al., 2020); groundfish (Paquin et al. 2014); pterygophagous (fin eating; Arroyave and Stiassny 2014) and lepidophagous (scale eating; Boileau et al. 2015) fishes; introduced largemouth Bass *Micropterus salmoides* (Jo et al. 2014); warm-water catfish (Moran et al. 2015); herbivorous juvenile Sandy Spinefoot *Siganus fuscescens* (Chelsky Budarf et al. 2011); gray seals *Halichoerus grypus* and harbor porpoises *Phocoena phocoena* (Méheust et al. 2015) and stranded Humboldt squid *Dosidicus gigas* (Braid et al. 2012). Nonetheless, few studies have investigated the effectiveness of DNA barcoding in relation to the digestive state of fish prey (Carreon-Martinez et al. 2011; Moran et al. 2015). Although few studies have successfully used DNA barcoding in the analysis of catfish piscine prey items of (Moran et al., 2015; Aguillar et al., 2016; Guillerault et al., 2017), studies are rare in elucidating both vertebrate and invertebrate prey content of benthophagous catfishes like *Arius maculatus* (Thunberg 1792). *A. maculatus* is a euryhaline, benthophagous species in tropical and sub-tropical waters, estuaries, rivers and coastal regions (Mazlan et al., 2008; Chu et al., 2011; Jumawan et al., 2020), whose economic importance and potential for aquaculture have recently been recognised (Jumawan et al., 2020). Commonly referred as spotted catfish (Chu et al., 2011), *A. maculatus* belongs to Ariidae family (Carpenter and Niem 1999), along with eight other species and is known for its pharmaceutical and nutraceutical values (Al-Bow et al., 1997; Osman et al., 2007). The present study aims to identify the species composition in the dietary items of *A. maculatus* occurring in the Vellar estuary (southeast coast of India).

The effectiveness of DNA barcoding for species identification largely depends on the establishment of broad and robust barcode reference databases of validated, verified species with vouchered specimens. A lack of vouchered or incorrectly identified sequences (Ekrem et al. 2007; Valdez-Moreno et al. 2012; Weigt et al. 2012a) would severely decrease the utility of reference databases. In addition, to capture potential genetic variation, including undocumented cryptic diversity, it is important to sequence an adequate number of

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

individuals from across a species range (Weigt et al. 2012b). We have made considerable efforts in the past decade as part of the Indian Census of Marine Life (ICoML) to recover barcodes in reasonable numbers of marine phyla including fin & shell fishes, invertebrates (Khan et al., 2010, 2011; PrasannaKumar et al., 2012; Thirumaraiselvi et al., 2015; Rajthilak et al., 2015; Rahman et al., 2013, 2019; Hemalatha et al., 2016; Palanisamy et al., 2020; PrasannaKumar et al., 2020a, b; Manikantan et al., 2020; Thangaraj et al., 2020) and plants (Sahu et al., 2016; Narra et al., 2020) occurring in and around the Vellar estuary. Hence we predict a high rate of success in identification of dietary items of *A. maculatus* occurring in this environment.

2. Material and methods

2.1. Catfish collection and stomach analysis

During April (pre-monsoon) and December (post-monsoon) 2011, catfish sampling was carried out in the Vellar estuary (latitude: 11° 29'N; longitude: 79° 46'E). Individuals were collected fresh from local fishery folks who used hand nets to fish regularly in Vellar estuary. A total of 40 catfishes were collected (18 in pre-monsoon; 22 in post-monsoon). Upon collection freshly captured fish were transported to DNA barcoding Lab, Centre of Advanced Study in Marine Biology, Annamalai University, within 1 km from collection site, in the saltwater ice slurry to slow down catfish digestion and prey DNA degradation (Baker et al., 2014). Each catfish has been measured (to nearest mm; total length (Lt)) and weighed (to nearest 0.1g).

After examination of the oesophagus and gills for prey, Catfish (*Arius maculatus* (Thunberg, 1792)) digestive tracts were removed. In order to remove digestive enzymes, excess chyme and particulate organic matter using 500 µm sieves, the digestive tracks were dissected and their contents were rinsed with RO water. Recognisable prey taxa were divided into two classes, namely fish and invertebrates, and into 2 digestive stages, namely whole animals (i.e., most of the biomass was present) and digestive remnants (in bits and pieces). The remnants were rinsed once in 100% molecular grade ethanol (Sigma) and approximately 3 mm^3 tissue plug was exercised under the ~1 mm top tissue layer (especially in fish preys) from all recognisable prey items (examined under binocular microscope whenever necessary) to avoid sample contamination by *A. maculatus* cells (Cote et al., 2013). Once again, the tissues exercised were rinsed and stored in microfuge tubes (Thermo Fisher Scientific) containing 100% ethanol at 4ºC until further analysis. Residual tissues in scalpel and forceps were removed with 95% ethanol and flame sterilized between each exercise. Samples of

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

lateral tissue samples were also taken from *A. maculatus* for DNA barcoding because if cannibalism was alleged in the diet, it would provide useful reference (Valdez-Moreno et al., 2012).

2.2. DNA extraction, PCR and sequencing the dietary contents

The DNA was extracted using the DNeasy Blood & Tissue Kits (Qiagen), following the manufacturer's instructions or standard protocols (Prasannakumar et al., 2020). Tissue was placed in an extraction buffer containing proteinase K and homogenized with polypropylene disposable sterile pestles (Thermo Fisher Scientific). The homogeneous was digested at 56 ºC until complete digestion of the sample (i.e, when most of the homogenate is more translucent) that was within 12 hours. Elution buffer was used as the negative control to test the purity of the reagents. The extracted DNA for quantification and purity estimation (i.e., using 260/280 nm ratio) was quantified using Nanodrop (Thermo Fisher Scientific). Elution buffer provided in the kit was used during Nanodrop measurements to calibrate the blank. Also, DNA concentration was checked in 1.2% Agarose gels and the high DNA yields (>85 ng/µl) were 10X diluted before PCR in ultrapure water.

DNA from fish-like prey samples was PCR amplified using COI primers; FishF1 and FishR1 (Ward et al., 2005) and invertebrate samples were using LCO1490 and HCO2198 (Folmer et al., 1994). A reaction mixture volume of 25µl was used to conduct PCR; 12.5µl Taq PCR Master Mix (Invitrogen, India), 11µl distilled water, 0.5µl forward primer (10 µM), 0.5µl reverse primer (10 µM), and 0.5µl DNA template (50–80 ng/µl) were used. Initial steps of 2 min at 95 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 54 ºC, and 1 min at 72 ºC, followed by 10 min at 72 ºC and kept at 4 ºC were used for FishF1/FishR1 primer amplified samples. Initial denaturation for 2 min at 95 ºC, followed by 5 cycles at 94 ºC for 30 s, 46 ºC for 45 s, 72 ºC for 45 s and 35 cycles at 94 ºC for 30 s, 51ºC for 45 s, 72 ºC for 45 s, and a final elongation stage at 72 ºC for 5 min were used for LCO1490/HCO2198 primers amplified samples. Positive and negative control were used each set of PCR sample runs. Ultrapure water, which replaces DNA templates, serves as a negative control and extracted DNA of *A. maculatus* was used as positive control. On 1.5% agarose gels, PCR products were visualized and positive reactions were verified by a clear band aligned parallel to 650 bp of the Invitrogen 100 bp DNA ladder (Thermo Fisher Scientific, MA) fragment. All positive PCR products were labelled with the acronym DADB1, DADB2,…DADB71, (Dietary Analysis using DNA Barcodes) (n=71) alongside positive and negative control and were commercially sequenced bi-directionally with the sequencing primers M13F and M13R

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

(Ivanova et al., 2007) and BigDye Terminator Cycle sequencing kit (Applied Biosystems) on an ABI 3730 capillary sequencer at Macrogen Inc. (South Korea).

2.3. DNA sequence analysis

Until at least every generated sequence was >600 bp in length, sequencing efforts were repeated. Forward and reverse sequences were trimmed using ChromasLite ver.2.1 to remove ambiguous and/or low quality bases and remnant primer from amplification or sequencing reactions. Sequences were compiled, and by translating DNA sequences into putative amino acid sequences in BioEdit ver. 7.9 (Hall, 1999) and aligned in Clustal X ver. 2.0.6 (Thompson, 1997), the gaps within the DNA sequences were tested. More than 600 bp length were all final contigs. The Barcode of Life Data Systems (BOLD) ID search engine (Ratnasingham and Hebert, 2007) and GenBank's BLAST tool (Altschul et al., 1990) have been used as a reference libraries to identify the barcode sequences generated. Molecular Evolutionary Genetic analysis (MEGA) ver. 4.1 (Kumar et al., 2018) using Kimura 2 parameters (K2P) (Kimura, 1980) was used to construct neighbour-joining (NJ) tree (Saitou et al., 1987). Bootstrap test (100 replicates) was used to test the reliability of the branches (Felsenstein, 1985). For tree based identification, sequences of statistically significant (highest query coverage (q) or lowest error (e) values) references were extracted from GenBank. For better representation of tree based identification, the NJ tree was redrawn using the Interactive Tree Of Life (iTOL) (Letunic and Bork, 2019). The K2P model and the Tajima's test for pair-wise distance and nucleotide diversity (Tajima, 1989) estimation were conducted in MEGA, respectively. The compositions of nucleotides and the diversity of haplotype were measured using the workbench tools delivered in BOLD. Less than 3% of the divergence between the unknown and the reference sequence was used to assign a specie level identity (Valdez-Moreno et al., 2012). That is, if the sequence matches reference GenBank sequence by at least 97%, the species identity has been accepted. To estimate the number of species and haplotypes present in the sample, we used the accumulation curve provisions provided in BOLD to visualise the taxonomic and sequence diversity. The also helps the user to monitor and compare the efficacy of sampling between groups or sites.

Sequence data generated in this study was released through GenBank and accessible via accession numbers JX676110-JX676180. Sequences along with their meta-data were also made available via BOLD (www.barcodeoflife.org) and could be accessed via project code DADB, and title; "Dietary analysis using DNA barcodes" or through http://dx.doi.org/10.5883/DS-DADB.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

3. Results

3.1. Morphometric characterization of *A. maculatus*

This is the first study that explores *A. maculatus*'s gut content that occurr in Indian water using DNA barcodes. Out of 40 *A. maculatus* sampled, average length of 21.15 cm and biomass of 130.27 g was recorded (**Table 1**). The total length varied from 11 and 30 cm, and biomass ranged between 112 and 151 g. In pre- and post-monsoons sampling, maximum (30 cm) and minimum (10.3 cm) lengths were recorded, respectively.

Table 1: Mean values and ranges of *Arius maculatus* total length (Lt) and biomass sampled from the velar estuary

Season	No. of guts	Lt (cm)		Biomass (g)	
		Mean ± SD	Range	Mean + SD	Range
Pre-monsoon	22	22.67 ± 6.7	11.67-30.6	134.23 + 14.28	114.56-151.23
Post-monsoon	18	19.28 ± 6.13	10.3-27.98	125.44 + 14.22	112.6-148.78
Over all	40	21.15 ± 6.6	11.67-30.6	130.27 ± 14.75	112.6-151.23

3.2. Dietary composition and sequence analysis

Of the 40 stomach contents examined (22 in pre-monsoon, 18 in post-monsoon), 32 stomachs (80%) had prey taxa that could be measured. 6 and 2 individuals collected in pre-monsoon and post-monsoon did not had measurable prey items, respectively. A total of 76 prey items were collected, of which 67 items were successfully sequenced (88.15%). The prey taxa could be classified into 4 major phyla, *viz*., Chordata, Arthropoda, Annelida and Mollusca. 4 barcodes of *A. maculatus* and 67 prey taxa constituted 71 sequences (DADB1 to DADB71) for BLAST analysis. DADB1 to DADB4 were *A. maculatus* tissue samples randomly sampled twice each time during pre-and post-monsoon. The pre-monsoon samplings of prey items were tagged from DADB5 to DADB33 (n=29) and the post-monsoon sampling were from DADB34 to DADB71 (n=38). **Table S1** list the respective PCR primer pairs (FishF1/FishR1 or LCO1490/HCO2198) used to barcode.

In GenBank database, all 71 sequences were matched with <3% cut off with that of reference sequences by identifying the taxa to species level. The prey taxa belonged to 13 species (12 genera in 11 families) under 4 phyla (viz., Chordate, Arthropod, Annelida and Mollusca) (**Table S1**). Pisces constituted the major prey items (43.28%) followed by Arthropod (29.85%), Annelida (17.91%) and Mollusca (8.95%). List of Pisces and

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

invertebrate species and their seasonal variability was represented in **Fig. 1.** Of 29 Pisces sequences, 7 species were recognized *viz.*, *Eubleekeria splendens*, *Stolephorus indicus*, *Photopectoralis bindus*, *Leptomelanosoma indicum*, *Lutjanus fulviflamma*, *Upeneus vittatus*, and *Gerres filamentosus*, respectively. In Annelida (*Capitella capitata*, *Perinereis vallata*), Arthropoda (*Clibanarius clibanarius*, *C. longitarsus*) and Mollusca (*Meretrix meretrix*, *Perna viridis*), the remaining taxa were made up of 2 species each.

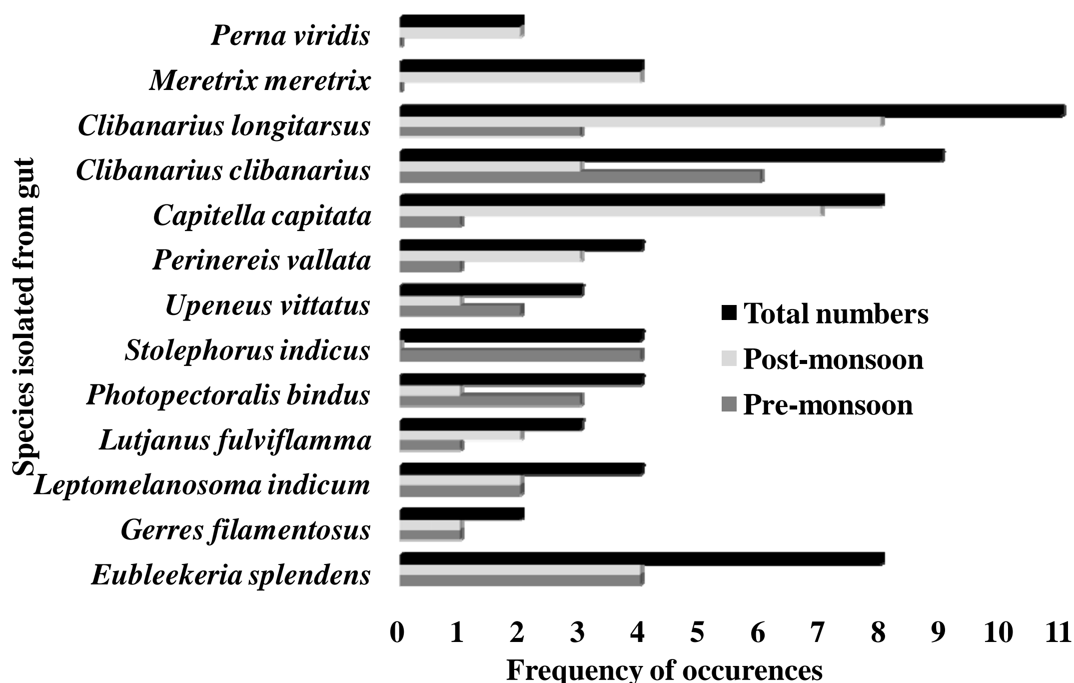


Fig. 1: Seasonal variation in diet diversity of *A. maculatus*. The isolated species and their frequency of occurrence were given in the X and Y axes, respectively.

The mean BLAST similarity score was 98.94% with standard deviation of ±0.99% (**Table S1**). The maximum and minimum similarity scores were respectively 100% and 97.01%. All 4 barcodes of *A. maculatus* was precisely identified with >99.3% identity (**Table S1**). All 71 barcodes produced in this study contained 43% average GC content (in the range of 35-50%) (**Table 2**).

Table 2: Summary statistics for nucleotide frequency distribution of dietary barcodes

Composition (%)	Minimum	Mean	Maximum	Standard Error
Guanine (G)	16.98	19.12	22.73	0.22
Cytosine (C)	13.49	23.91	30.08	0.64
Adenine (A)	19.16	24.10	29.84	0.35
Thymine (T)	27.76	32.87	42.68	0.59

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

GC	35.04	43.03	50.86	0.56
GC Codon position 1	40.47	52.15	59.45	0.64
GC Codon position 2	38.14	42.63	44.08	0.13
GC Codon position 3	19.52	34.31	52.58	1.08

3.3. NJ tree-based species identification

Identification of *A. macultus* specimens were also verified using the reference sequences extracted from GenBank via NJ tree construction. Other species in the *Arius* genera, such as *A. manillensis* (HQ682617), *A. dispar* (KF604635), *A. subrostratus* (MK348195), and *A. jella* (KU894613) was used as an out-group in the construction of NJ tree. All four sequences (DADB1-DADB4) have been placed in a single clade (**Fig. 2**). Presence of haplotypes within the 4 COI sequences of *A. maculatus* was also hinted.

For the construction of the NJ tree, all 7 piscine species with their reference sequences (2 to 5 reference sequences per species) constituting a total of 56 nucleotide sequences were used. All pisces prey taxa clustered in one clade with its respective species indicating the similarity between the sequenced COI and sequenced taxa in the database (**Fig. 3**). Even the cladding patterns of constructed NJ tree suggested the presence of haplotypes among the prey taxa; for example, 2 sub-clades were evident among the *Stolephorus indicus* clade (the top most clade of the NJ tree) (**Fig. 3**). One contained DADB11, DADB12 and DADB24 with other sequences of reference, and another contained DADB26 and DADB30 with other sequences of reference. Similar patterns were also observed in *U. vittatus*, *E. splendens* and *L. indicum* clades. For the NJ tree based invertebrate prey taxa identification, the 2 to 9 reference sequence per invertebrate prey taxa was similarly used. The final dataset had 68 nucleotide sequences. All invertebrate prey taxa in Arthropod, Annelid and Mollusca, clustered with its respective sequences of the reference species in single clade (**Fig. S1**), indicating the success of tree based identification.

3.4.Dietary diversity: Pre-monsoon versus Post-monsoon

The major difference between pre- and post-monsoon dietary composition is that, in pre-monsoon, the molluscan taxa was completely absent and a piscine species (*Stolephorus indicus*) was present only in pre-monsoon (**Fig. 1**). Among piscine taxa, *Eubleekeria splendens* (23.5%) and *Stolephorus indicus* (23.5%) were the major prey taxa followed by *Photopectoralis bindus* (17.6%) during pre-monsoon while *E. splendens* (36.36%) alone

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

forms the major prey taxa followed by *Leptomelanosoma indicum* (18.18%) and *Lutjanus fulviflamma* (18.18%) during the post-monsoon season.

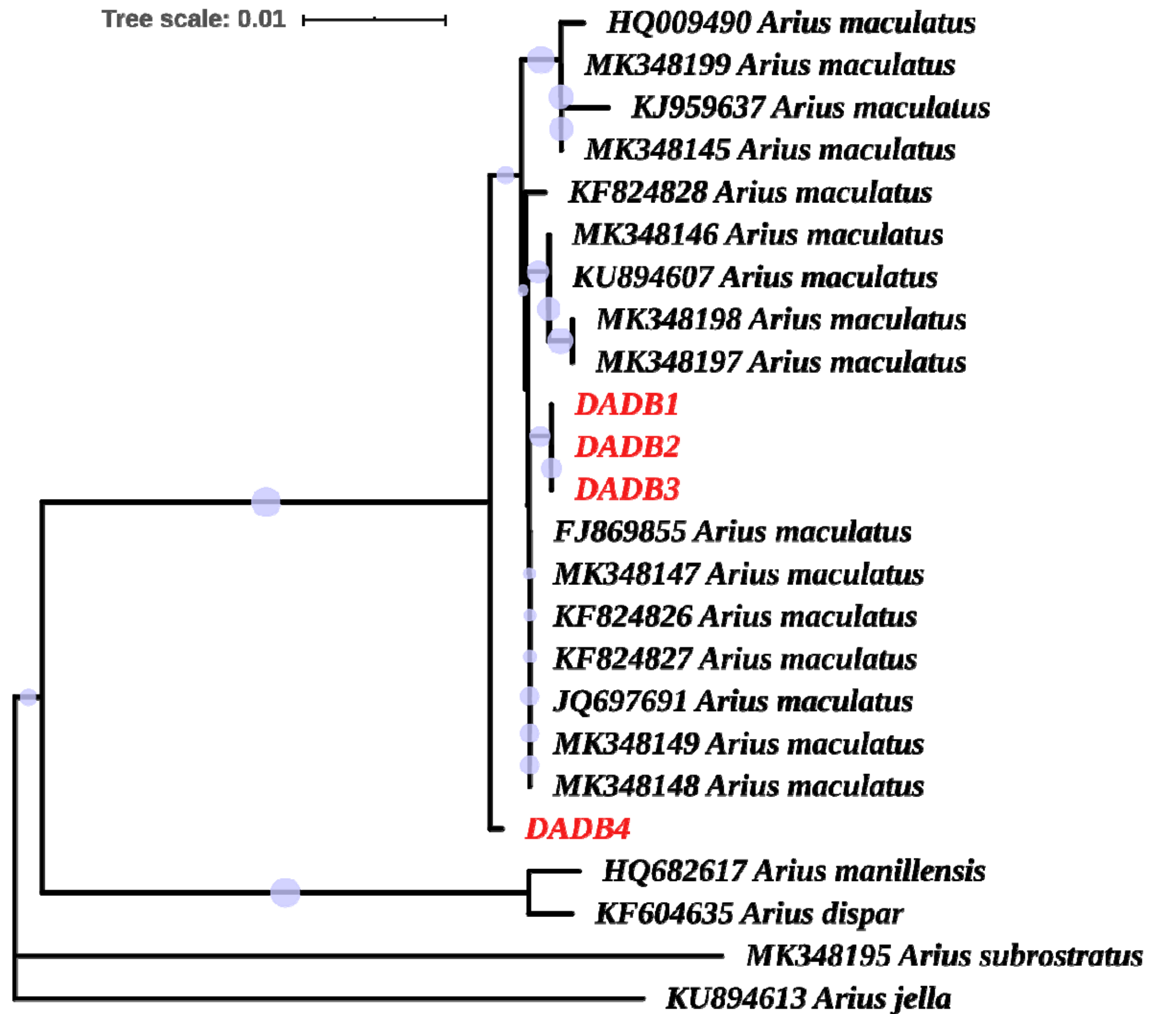


Fig. 2: COI-NJ tree based identification of *Arius maculatus*. DADB1-DADB4 were the sequences produced in this study. The alpha-numerical present prefix to species name represent the Genbank accession numbers. Different species of *Arius* genera was used as an out-group was successfully delineated.

The hermit crabs forms the major constituents of both pre- and post-monsoon seasons (81.81% & 40.74%, respectively) among invertebrate taxa. The dominant (54.54%) invertebrate prey taxa were the hermit crab, *Clibanarius clibanarius* was followed by *C. longitarsus* (27.27%) during pre-monsoon sampling. *Clibanarius longitarsus* were the dominant (33.33%) invertebrate prey taxa during post-monsoon sampling followed by a polychaete worm *Capitella capitata* (25.92%) (**Fig. 1**).

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

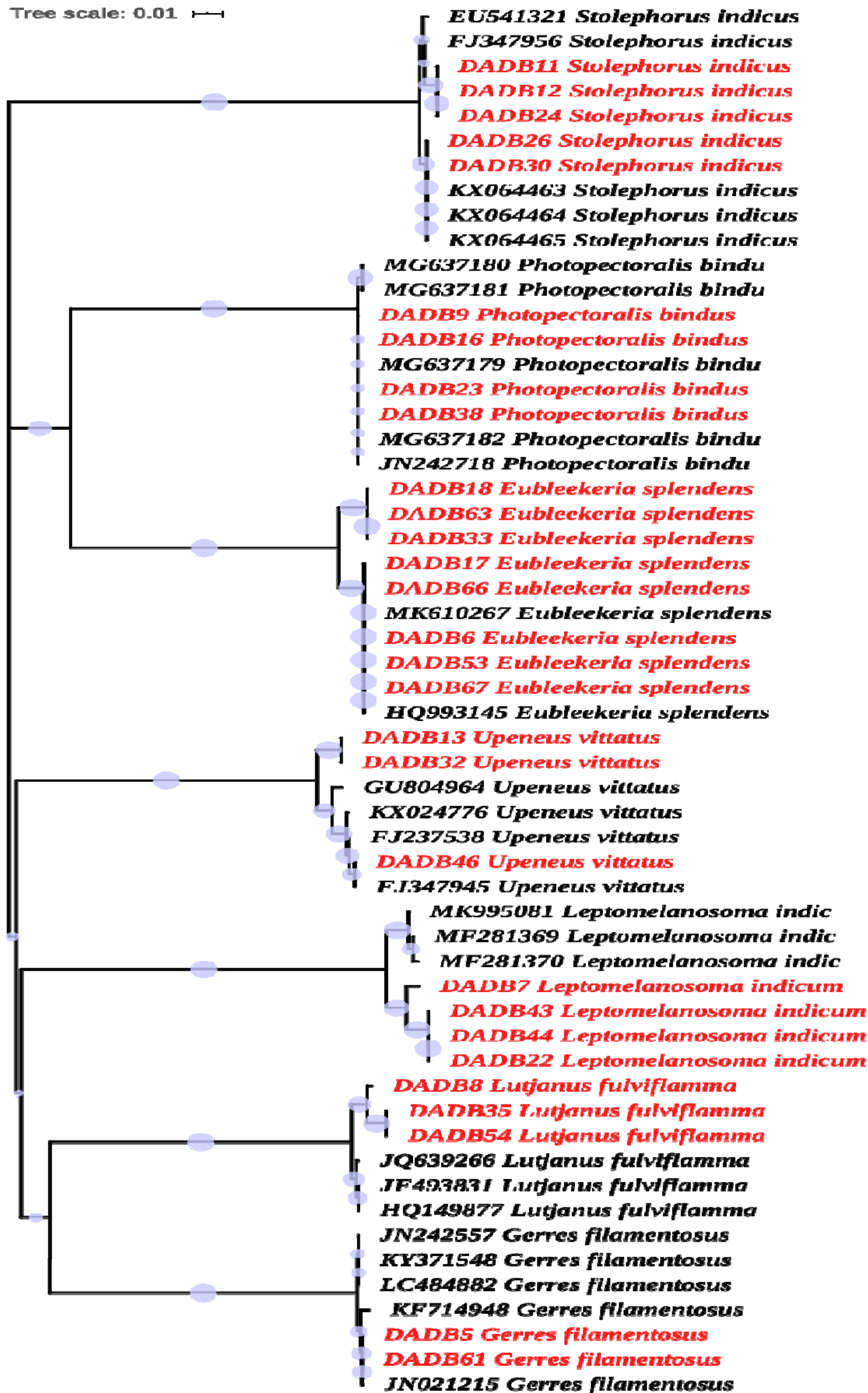


Fig. 3: NJ tree constructed for tree based identification of pisces prey taxa. The percentage of

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown as circles next to the branches. The number of base substitutions per site was indicated as tree scale given on the top left corner. The acronym DADB and alpha-numerical prefix to the species name indicates the sequences produced in this study and reference sequence, respectively.

During pre-monsoon, piscine gut items constituted the major prey taxa (58.62%) and invertebrate forms the major prey (71.05%) taxa in post-monsoon season. During the post-monsoon, consumption of piscine items dropped by 20.69% and invertebrate taxa increased by 42.11%. However, as the sample size was severely limited to draw such inference, it was possible to adjust further sampling efforts accommodated with next generation sequencing to verify such claims.

For invertebrate taxa, the average pair-wise distance (pwd) and nucleotide diversity were higher (0.35 and 0.26, respectively) than for pisces (0.22 and 0.18, respectively) (**Table 3**). During the post-monsoon season, the total higher pwd values (0.40) were observed than pre-monsoon values (0.31) suggesting higher dietary diversity consumed in the post-monsoon season. Similarly, the decrease in pisces's pwd and nucleotide diversity values and increased values of the same parameters in post-monsoon invertebrate prey taxa indicate that invertebrates were more preferred diet during the post-monsoon season. The overall pwd values for the barcoded prey taxa were 0.377.

Table 3: Pair-wise distance (Pwd) and nucleotide diversity (π) of diet-DNA barcodes

	Pre-monsoon		**Post-monsoon**		**Overall**	
	Pwd	π	Pwd	π	Pwd	π
Pisces	0.223	0.187	0.124	0.180	0.221	0.184
Invertebrates	0.206	0.164	0.386	0.282	0.346	0.257
Overall	0.314	0.246	0.405	0.299	0.377	0.283

3.5. Haplotype diversity

The analysis of the accumulation curve in BOLD reveals the presence of 57 haplotypes within 14 species barcoded (including the host *A. maculatus*) (**Fig. 4**). In Pisces (47.36%), the majority of haplotypes were found, followed by Arthropods (28.07%), Annelids (14.03%) and Mollusca (10.52%). Inthis analysis, the mean haplotype (n=57) richness per taxa barcoded taxa (n=14) was found to be 4.07.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

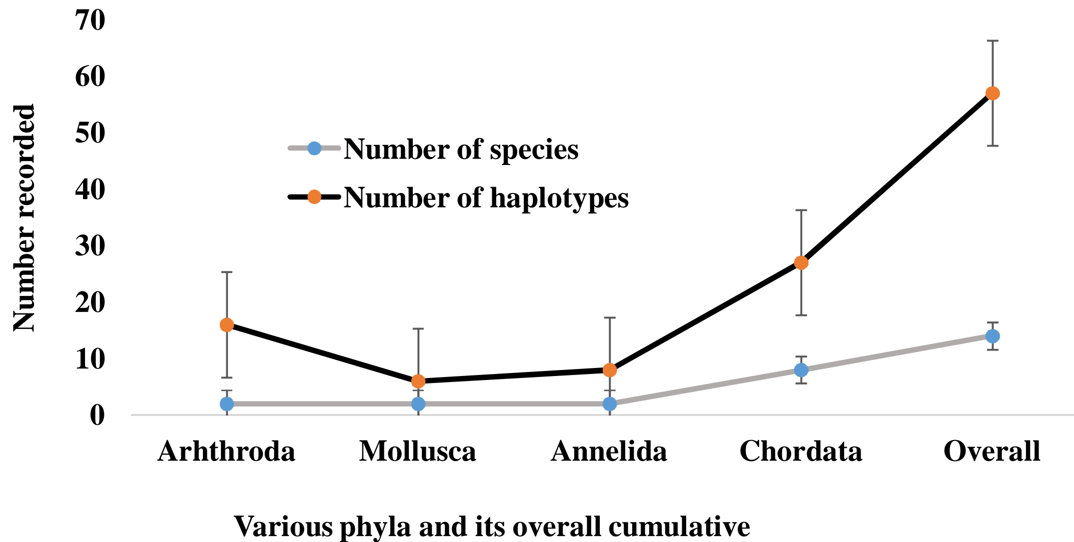


Fig. 4: Accumulation curve demonstrating the documentation of various haplotypes present among the taxa in dietary composition.

4. Discussion and conclusion

In this study, the mean length recorded was comparable to *A. maculatus* occurring on the west coast of India (Maitra et al., 2019), but comparatively smaller than those occurring on the coast of Philippines (Chu et al., 2011). Many studies have not been able to identify the stomach content (prey items) of fishes using conventional morphological analysis (Legler et al. 2010; Paquin et al. 2014; Moran et al. 2015), as the digestive process rapidly disintegrates the morphological characteristics of the prey item (Schooley et al. 2008; Legler et al. 2010; Carreon-Martinez et al. 2011), resulting in substantial losses of the valuable data for taxonomist and resource managers. While in this study, most prey items were in high digestive state and rendered morphological identification impossible, segregating and treating the individual stomach content for DNA barcoding resulted in a success rate of 88% sequencing with 100% species level identification. Using visual survey, previous analysis, which could only assign 10% of catfish gut content to species level, used DNA barcoding to witness the taxonomic assignments to species level identification of 90% of prey items (Aguilar et al., 2016). None of the specimen could be recognised in this study without DNA barcoding, which could lead to poor understanding of catfish and their diet diversity. Previous nationalised efforts to barcoding the marine diversity (Lakra et al., 2010; Bineesh et al., 2014; Bamaniya et al., 2015) along with localised efforts to barcode the diversity of

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Vellar estuary (Khan et al., 2010, 2011; PrasannaKumar et al., 2012; Thirumaraiselvi et al., 2015; Rajthilak et al., 2015; Rahman et al., 2013, 2019; Hemalatha et al., 2016; Sahu et al., 2016; Palanisamy et al., 2020; PrasannaKumar et al., 2020; Manikantan et al., 2020; Thangaraj et al., 2020; Narra et al., 2020) resulted in strengthening the reference library which insured that no ambiguous sequences were present in this study (as all sequences were identified to species level). Identification success was likely due to the use of previously generated references sequences from Indian waters from morphologically verified species and published through reference databases (such as GenBank and BOLD). This is significant because the ability of DNA barcoding to identify unknown specimens might be impeded by miss-identification of reference specimen, cryptic diversity, sharing of haplotype, and lack of reference sequences (i.e., species yet not barcoded) in the database.

Previously, cryptic diversity has been recorded among catfish prey (April et al., 2011) and Indian waters have vast marine fish diversity (2443 species) (Gopi and Mishra, 2015) with several cryptic families (Bamaniya et al., 2015). The application of DNA barcoding in this study, identified 57 haplotypes in 14 species barcoded. When we previously barcoded Vellar estuarine fishes (43 species), for the first time 58% (n=25 species) were sequenced (Khan et al., 2011). These first time barcodes have been useful to identify *Lutjanus fulviflamma*, *Stolephorus indicus*, *Upeneus vittatus*, and *Eubleekeria splendens* in this study. In this study, the wide diversity of marine fish in catfish diets (7 species in 5 families) was not surprising, as previous studies have witnessed 23-25 fish taxa (up to 11 families) in catfish diets (Moron et al., 2015; Aguilar et al., 2016). Previous studies using DNA barcoding to investigate the catfish diet were limited to fish prey items (Moron et al., 2015; Aguilar et al., 2016), considering the difficulties of segregating and sequencing invertebrates. This study is the first of its kind to investigate the diversity of invertebrate prey in the diet of catfish using DNA barcodes. The economically important clams (*Meritxix meritrix*) (Yeh et al., 2016; Desrita et al., 2019) and mussel (*Perna viridis*) (Sulvina et al., 2020) found in the diet of *A. maculatus* in this study is of concern, as local populations were known to depend on these resources for food. Since accurate identification of macro-benthic invertebrates from benthophagous fish could facilitate the assessment of human impact on the ecosystem (Tupinambás et al., 2015), further studies could be directed toward a detailed picture of trophic levels in DNA barcoding based gut analysis of other catfish and fin fish species inhabiting Vellar estuary (Khan et al., 2011; Sakthivel et al., 2012).

We found that in pre-monsoon *A. maculatus* was more piscivorous than post-monsoon. However such conclusions should be supported by multiple seasonal sampling and not with

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

single seasonal sampling like in this study. It was also appropriate to note that most prey items (almost all larvae and immature forms) identified in this study, were of commercially important fish species such as *Eubleekeria splendens*, which occurred abundantly in the East and West coast of India (Rawat et al., 2019). The diversity of diet estimated through DNA barcoding shows that, *A. maculatus* can feed at a range of depths and habitats (especially indicated by the high haplotype diversity), including shallow margins (such as mangrove habitats where immature forms seeks habitats) and open waters. The DNA sequencing mechanism and success of the detection of prey items might be disrupted by co-amplificatin of the predator's DNA along with its prey (Vestheim and Jarman 2008; Leray et al. 2015). We have not obtained any co-amplification of *A. maculatus*, however. Similar observations were also previously made (Carreon-Martinez et al., 2011). While contaminations may have triggered sequencing failures (since approximately 12% of the samples were not successfully sequenced), false positive detections did not result (as all prey barcodes matched the level of species cut off with that of the same species in the reference database).

We found that DNA barcoding was very effective in the identification of even highly digested prey items. In this and other studies, prey items have been digested to the point that even higher taxonomic ranks of the prey could not be given an indication by the visual exam (Carreon-Martinez et al. 2011; Schloesser et al. 2011; Moran et al. 2015; Rocha et al. 2015; Aguilar et al., 2016). Even though the present study involved the detection of fish and invertebrates through DNA barcoding, the success rates of barcoding were higher than previously reported ~65% by Moran et al. (2015), 70% by Cote et al. (2013), ~80% by Carreon-Martinez et al. (2011) and nearly equivalent to (93%) Aguilar et al. (2016). The variations in the success rate of barcoding may be due to the choice of sequencing techniques, predator/prey within each study, prey item acquisition, predator/prey handling, and prey condition (digestion resistant features) (Macdonald, 1982; Buckland et al., 2017). Although the DNA barcoding techniques for fish and invertebrate using universal robust primers have been well developed (Ivanova et al. 2007; Weigt et al. 2012, Ward et al., 2005, Prasannakumar et al., 2012, 2020), barcoding success rates may be more likely to be affected during prey acquisition and other upstream barcoding processes (storage, pre-processing, DNA extraction, etc.), as higher success rate was witnessed in processing freshly acquired guts rather than the ethanol preserved whole predator samples. Limited diffusion of preservative medium into gut contents was reported for decrease success rate (Valdez-Moreno et al., 2012). In this study, care was taken to process the gut samples as fresh as could and the individual prey items was stored in ethanol rather than the whole predator. We

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

also recommend that the extracted gut contents be stored in a deep freezing condition rather than in a preservative medium for a higher success rate of barcoding as previously observed (Aguilar et al., 2016).

We propose estimating the degree of generalist predation among the predators through pair-wise distance (pwd) and nucleotide diversity (π) estimation. For example, in post-monsoon in the diet of *A. maculatus*, the pwd and nucleotide values of prey items increases, indicating more post-monsoon generalist predation than pre-monsoon predation. However, it was beyond the scope of this study to investigate the factors responsible for such a rise. For now, the influence of pwd and π values on predator's biology, functioning of predator habitats is unexplored, as more studies may be directed towards it in the near future. These barcode data (along with the associated full diet analysis) will reveal *A. maculatus*' trophic dynamics in Vellar estuary and provide valuable data for the development of management strategies, particularly in relation to its predation of commercially important fish and invertebrates. Pisces and invertebrates from these ecosystems will continue to be collected and barcoded, as the coverage of species barcodes in the reference database will become more significant as meta- and environmental DNA barcoding is becoming cheaper and more prevalent in fishery surveys (Leray et al. 2013; Miya et al. 2015; Galal-Khallaf et al., 2016; Evans & Lamberti, 2018).

5. Acknowledgement

First author thanks the Department of Science and Technology’s INSPIRE Fellowship (IF10431), India and China Postdoctoral Research Foundation’s National Postdoctoral fellowship (0050-K83008), China for the financial assistance. The field help rendered my Mr. Karuna is acknowledged. Authors are grateful to anonymous reviewers for improving the quality of this manuscript.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

6. References

Aguilar, R., Ogburn, M. B., Driskell, A. C., Weigt, L. A., Groves, M. C., & Hines, A. H. (2016). Gutsy genetics: identification of digested piscine prey items in the stomach contents of sympatric native and introduced warmwater catfishes via DNA barcoding. Environmental Biology of Fishes, 100(4), 325–336. https://doi.org/10.1007/s10641-016-0523-8

Ajmal Khan S, Prasannakumar C, Lyla P. S., Murugan S (2011) Identifying Marine fin fishes using DNA barcodes. Current Science, vol. 101 (9): 1152-1154.

Al-Bow HA, Al-Hassan JM, Thomson M, Thulesius O, Elkhawad A. 1997. Multiple vasoactive factors in epidermal secretions of the Arabian Gulf catfish, Arius bilineatus (Valenciennes). General Pharmacology 28(5): 737-744.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

April J, Mayden RL, Hanner RH, Bernatchez L (2011) Genetic calibration of species diversity among NorthAmerica's freshwater fishes. Proc Natl Acad Sci U S A 108(26): 10602–10607

Arroyave J, Stiassny MLJ (2014) DNA barcoding reveals novel insights into pterygophagy and prey selection in distichodontid fishes (Characiformes: Distichodontidae. Ecol Evol 23(4):4534–4542

Ashiq Ur Rahman, M., PrasannaKumar, C., Mohanchander, P., Manikantan, G., Ajmal Khan, S. and Lyla, P.S. (2019) Identification of eggs, larva and adults of Scylla serrata (Forsskal, 1775) using DNA barcodes. Journal of Aquatic Biology & Fisheries. 7: 24-30.

Baeza JA, Fuentes MS (2013) Exploring phylogenetic informativeness and nuclear copies of mitochondrial DNA (numts) in three commonly used mitochondrial genes: mitochondrial phylogeny of peppermint, cleaner, and semi-terrestrial shrimps (Caridea: Lysmata, Exhippolysmata, and Merguia). Zoo J Linn Soc-Lond 168:699–722

Baker, R., Buckland, A., Sheaves, M., 2014. Fish gut content analysis: robust measures of diet composition. Fish Fish. 15 (1), 170–177. http://dx.doi.org/10.1111/faf.12026

Baker, R., Sheaves, M., (2005). Redefining the piscivore assemblage of shallow estuarine nursery habitats. Mar. Ecol. Prog. Ser. 291, 197–213. http://dx.doi.org/10.3354/meps291197

Baldwin CC, Brito BJ, Smith DG, Weigt LA, Escobar-Briones E (2011) Identification of early life-history stages of Caribbean Apogon (Perciformes: Apogonidae) through DNA barcoding. Zootaxa 3133:1–36

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Bamaniya, D. C., Pavan-Kumar, A., Gireesh-Babu, P., Sharma, N., Reang, D., Krishna, G., & Lakra, W. S. (2015). DNA barcoding of marine ornamental fishes from India. Mitochondrial DNA Part A, 27(5), 3093–3097. https://doi.org/10.3109/19401736.2014.1003923

Bariche M, Torres M, Smith C, Sayar N, Azzurro E, Baker R, Bernardi G (2015) Red Sea fishes in the Mediterranean Sea: a preliminary investigation of a biological invasion using DNA barcoding. J Biogeogr 42:2363–2373

Barnett A, Redd KS, Frusher SD, Stevens JD, Semmens JM (2010) Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. J Exp Mar Biol Ecol 393: 188–192

Beauchamp DA, Wahl DH, Johnson BM (2007) Predator-prey interactions. In: Guy CD, Brown ML (eds) Analysis and interpretation of freshwater fisheries data. American Fisheries Society, Bethesda, Maryland, pp. 765–842

Berg, J., 1979. Discussion of methods of investigating the food of fishes, with reference to a preliminary study of the prey of Gobiusculus flavescens (Gobiidae). Mar. Biol. 50 (3), 263–273. http://dx.doi.org/10.1007/bf00394208

Betancur-R R, Broughton RE,Wiley EO, Carpenter K, López JA, Li C, Holcroft NI, Arcila D, Sanciangco M, Cureton II JC, Zhang F, Buser T, Campbell MA, Ballesteros JA, Roa-Varon A, Willis S, Borden WC, Rowley T, Reneau PC, Hough DJ, Lu G, Grande T, Arratia G, Ortí G (2013) The Tree of Life and a New Classification of Bony Fishes. PLoS Curr Tree of Life. Apr 18. Edition 1. doi:10.1371/currents.tol.53ba26640df0ccaee75bb165c8c26288.

Bineesh K.K., Akhilesh K.V., Sajeela K.A., Abdussamad E.M., Gopalakrishnan A., Basheer V.S. and Jena J.K. (2014) DNA Barcoding Confirms the Occurrence Rare Elasmobranchs in the Arabian Sea of Indian EEZ. Middle-East Journal of Scientific Research 19 (9): 1266-1271.

Boileau N, Cortesi F, Egger B, Muschick M, Indermaur A, Theis A, Büscher HH, Salzburger W (2015) A complex mode of aggressive mimicry in a scale-eating cichlid fish. Biol Lett 11(9):20150521

Braid HE, Deeds J, DeGrasse SL, Wilson JJ, Osborne J, Hanner RH (2012) Preying on commercial fisheries and accumulating paralytic shellfish toxins: a dietary analysis of invasive Dosidicus gigas (Cephalopoda Ommastrephidae) stranded in Pacific Canada. Mar Biol 159:25–31

Buckland, A., Baker, R., Loneragan, N., & Sheaves, M. (2017). Standardising fish stomach content analysis: The importance of prey condition. Fisheries Research, 196, 126–140. https://doi.org/10.1016/j.fishres.2017.08.003

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Bunholi, I. V., Ferrette, B. L. da S., De Biasi, J. B., Magalhães, C. de O., Rotundo, M. M., Oliveira, C., Foresti, F., & Mendonça, F. F. (2018). The fishing and illegal trade of the angelshark: DNA barcoding against misleading identifications. Fisheries Research, 206, 193–197. https://doi.org/10.1016/j.fishres.2018.05.018

Carreon-Martinez L, Johnson TB, Ludsin SA, Heath DD (2011) Utilization of stomach content DNA to determine diet diversity in piscivorous fishes. J Fish Biol 78:1170–1182

Carvalho, D. C., Guedes, D., da Gloria Trindade, M., Coelho, R. M. S., & de Lima Araujo, P. H. (2017). Nationwide Brazilian governmental forensic programme reveals seafood mislabelling trends and rates using DNA barcoding. Fisheries Research, 191, 30–35. https://doi.org/10.1016/j.fishres.2017.02.021

Chelsky Budarf A, Burfeind DD, Loh WK, Tibbetts IR (2011) Identification of seagrasses in the gut of amarine herbivorous fish using DNA barcoding and visual inspection. J Fish Biol 79:112–121

Chu. (2011). Estimates of age, growth and mortality of spotted catfish, Arius maculatus (Thunberg, 1792), off the Coast of Yunlin, Southwestern Taiwan. African Journal of Biotechnology, 10(68). https://doi.org/10.5897/ajb11.1552

Conway KW, Baldwin C,White MD (2014) Cryptic diversity and venom glands in western Atlantic clingfishes of the genus Acyrtus (Teleostei: Gobiesocidae). PLoS One 9(5): e97664

Cortés, E., 1997. A critical review of methods of studying fish feeding based on analysis of stomach contents: application to elasmobranch fishes. Can. J. Fish. Aquat. Sci. 54 (3), 726–738. http://dx.doi.org/10.1139/f96-316

Cote IM, Green SJ, Morris JA, Akins JL, Steinke D (2013) Diet richness of indo-Pacific lionfish revealed byDNA barcoding. Mar Ecol Prog Ser 472:249–256

Cote L, Johnson TB, Ludsin SA, Heath DD (2011) Utilization of stomach content DNA to determine diet diversity in piscivorous fishes. J Fish Biol 78:1170–1182

Côté, I., Green, S., Morris, J., Jr, Akins, J., & Steinke, D. (2013). Diet richness of invasive Indo-Pacific lionfish revealed by DNA barcoding. Marine Ecology Progress Series, 472, 249–256. https://doi.org/10.3354/meps09992

Cresson, P., Ruitton, S., Ourgaud, M., & Harmelin-Vivien, M. (2014). Contrasting perception of fish trophic level from stomach content and stable isotope analyses: A Mediterranean artificial reef experience. Journal of Experimental Marine Biology and Ecology, 452, 54–62. https://doi.org/10.1016/j.jembe.2013.11.014

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Dahl, K. A., Patterson, W. F., III, Robertson, A., & Ortmann, A. C. (2017). DNA barcoding significantly improves resolution of invasive lionfish diet in the Northern Gulf of Mexico. Biological Invasions, 19(6), 1917–1933. https://doi.org/10.1007/s10530-017-1407-3

Dalton DI, Kotze A (2011) DNA barcoding as a tool for species identification in three forensic wildlife cases in South Africa. Forensic Sci Int 207:e51–e254

Desrita, Susetya, I. E., Suriani, M., & Rahman, A. (2019). Biology and growth of Asiatic Hard Clam (Meretrix meretrix) population in Tanjung Balai, North Sumatera. IOP Conference Series: Earth and Environmental Science, 260, 012108. https://doi.org/10.1088/1755-1315/260/1/012108

Dhanze J R and Jayaram K C (1982) Some biometric studies of certain closely related species of the genus Arius (Pises: Siluriformes: Ariidea). Proc. Indian Acad. Sci. 91(1): 79-98

Di Pinto, A., Marchetti, P., Mottola, A., Bozzo, G., Bonerba, E., Ceci, E., Bottaro, M., & Tantillo, G. (2015). Species identification in fish fillet products using DNA barcoding. Fisheries Research, 170, 9–13. https://doi.org/10.1016/j.fishres.2015.05.006

Dunn MR, Szabo A, McVeagh MS, Smith PJ (2010) The diet of deep water sharks and the benefits of using DNA identification of prey. Deep-Sea Res 57:923–930

Ekrem T, Willassen E, Stur E (2007) A comprehensive DNA sequence library is essential for identification with DNA barcodes. Mol Phylogenet Evol 43:530–542

Elliott, J., 1967. The food of trout (Salmo trutta) in a Dartmoor stream. J. Appl. Ecol. 59–71. http://dx.doi.org/10.2307/2401409

Evans, N. T., & Lamberti, G. A. (2018). Freshwater fisheries assessment using environmental DNA: A primer on the method, its potential, and shortcomings as a conservation tool. Fisheries Research, 197, 60–66. https://doi.org/10.1016/j.fishres.2017.09.013

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Folmer O, Hoeh W, Black M, Vrijenhoek R. (1994) Conserved primers for PCR amplification of mitochondrial DNA from different invertebrate phyla. Mol Mar Biol Biotechnol. \ 3:294-299.

Galal-Khallaf, A., Osman, A. G. M., Carleos, C. E., Garcia-Vazquez, E., & Borrell, Y. J. (2016). A case study for assessing fish traceability in Egyptian aquafeed formulations using pyrosequencing and metabarcoding. Fisheries Research, 174, 143–150. https://doi.org/10.1016/j.fishres.2015.09.009

Gonçalves PFM, Oliveira-Marques AR, Matsumoto TE, Miyaki CY (2015) DNA Barcoding identifies illegal parrot trade. J Hered 106(S1):560–564

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Gopi, K. C., & Mishra, S. S. (2015). Diversity of Marine Fish of India. In Marine Faunal Diversity in India (pp. 171–193). Elsevier. https://doi.org/10.1016/b978-0-12-801948-1.00012-4

Gray, B.P., Norcross, B.L., Blanchard, A.L., Beaudreau, A.H., Seitz, A.C., 2015. Variability in the summer diets of juvenile polar cod (Boreogadus saida) in the northeastern Chukchi and western Beaufort Seas. Polar Biol. 1–12. http://dx.doi.org/10.1007/s00300-015-1796-7

Guillerault, N., Bouletreau, S., Iribar, A., Valentini, A., & Santoul, F. (2017). Application of DNA metabarcoding on faeces to identify European catfish Silurus glanis diet. Journal of Fish Biology, 90(5), 2214–2219. https://doi.org/10.1111/jfb.13294

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Handy SM, Deeds JR, Ivanova NV, Hebert PDN, Hanner RH, Ormos A, Weigt LA, Moore MM, Yancy HF (2011) A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. J AOAC Int 94(1):201–210

Hansson, S., 1998. Methods of studying fish feeding: a comment. Can. J. Fish. Aquat. Sci. 55 (12), 2706–2707. http://dx.doi.org/10.1139/f98-158

Harms-Tuohy, C., Schizas, N., & Appeldoorn, R. (2016). Use of DNA metabarcoding for stomach content analysis in the invasive lionfish Pterois volitans in Puerto Rico. Marine Ecology Progress Series, 558, 181–191. https://doi.org/10.3354/meps11738

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical kipper butterfly Astraptes fulgerator. Proc Natl Acad Sci U S A 101(41):14812–14817

Hellberg, R. S., Isaacs, R. B., & Hernandez, E. L. (2019). Identification of shark species in commercial products using DNA barcoding. Fisheries Research, 210, 81–88. https://doi.org/10.1016/j.fishres.2018.10.010

Hemalatha, A., Mohammed Esa, S. A. R., Suresh, M., Thajuddin, N., & Anantharaman, P. (2016). Identification of Odontella aurita by rbcL gene sequence – a high antibacterial potential centric marine diatom. Mitochondrial DNA Part A, 28(5), 655–661. https://doi.org/10.3109/24701394.2016.1166222

Hernández J. (2019) Feeding studies take guts – critical review and recommendations of methods for stomach contents analysis in fish. J Fish Biol. 95: 1364–1373. https://doi.org/10.1111/jfb.14151

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Holmes, B. H., Steinke, D., & Ward, R. D. (2009). Identification of shark and ray fins using DNA barcoding. Fisheries Research, 95(2–3), 280–288. https://doi.org/10.1016/j.fishres.2008.09.036

Hubert N, Hanner R, Holm E, Mandrak NE Taylor E (2008) Identifying Canadian freshwater fishes through DNA barcodes. PLoS One 3(6): e2490

Hynes, H., 1950. The food of fresh-water sticklebacks (Gasterosteus aculeatus and Pygosteus pungitius), with a review of methods used in studies of the food of fishes. J. Anim. Ecol. 36–58. http://dx.doi.org/10.2307/1570.

Hyslop, E., 1980. Stomach contents analysis—a review of methods and their application. J. Fish Biol. 17 (4), 411–429. http://dx.doi.org/10.1111/j.1095-8649.1980.tb02775.x

Ivanova NV, Zemlak TS, Hanner R, Hebert PDN (2007) Universal primer cocktails for fish DNA barcoding. Mol Ecol Notes 7: 544–548.

Jo H, Gim JA, Jeong KS, Kim HS, Joo GJ (2014) Application of DNA barcoding for identification of freshwater carnivorous fish diets: is number of prey items dependent on size class for Micropterus salmoides? Ecol Evol 4:219–229

Joly S, Davies TJ, Bruneau A, Derry A, KembelSW, Peres-Neto P, Vamosi J, Wheeler TA (2014) Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. Mol Ecol Resour 14(2):221–232

Jumawan, C., Metillo, E., & Mutia, M. T. (2020). Stock Assessment of Arius maculatus (Thurnberg, 1792) (Ariidae, Siluriformes) in Panguil Bay, Northwestern Mindanao. The Philippine Journal of Fisheries, 27(1), 40–53. https://doi.org/10.31398/tpjf/27.1.2019a0013

Khan, S. A., Lyla, P. S., John, B. A., Kuamr, C. P., Murugan, S., & Jalal, K. C. A. (2010). DNA Barcoding of Stolephorus indicus, Stolephorus commersonnii and Terapon jarbua of Parangipettai Coastal Waters. Biotechnology (Faisalabad), 9(3), 373–377. https://doi.org/10.3923/biotech.2010.373.377

Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120.

Kress WJ, García-Robledo C, Uriarte M, Erickson DL (2015) DNA barcodes for ecology, evolution and conservation. Trends Ecol Evol 30(1):25–35

Kuguru, G., Maduna, S., da Silva, C., Gennari, E., Rhode, C., & Bester-van der Merwe, A. (2018). DNA barcoding of chondrichthyans in South African fisheries. Fisheries Research, 206, 292–295. https://doi.org/10.1016/j.fishres.2018.05.023

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.

Lakra, W. S., Verma, M. S., Goswami, M., Lal, K. K., Mohindra, V., Punia, P., Gopalakrishnan, A., Singh, K. V., Ward, R. D., & Hebert, P. (2010). DNA barcoding Indian marine fishes. Molecular Ecology Resources, 11(1), 60–71. https://doi.org/10.1111/j.1755-0998.2010.02894.x

Legler ND, Johnson TB, Heath DD, Ludsin S (2010) Water temperature and prey size effects on the rate of digestion of larval and early juvenile fish. Trans Am Fish Soc 139:868–875

Leray M, Knowlton N (2015a) DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. Proc Natl Acad Sci U S A 112(7):2076–2081

Leray M, Meyer CP, Mills SC (2015b) Metabarcoding dietary analysis of coral dwelling predatory fish demonstrates the minor contribution of coral mutualists to their highly partitioned, generalist diet. PeerJ 3: e1047

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ (2013) A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front Zool 10:34

Letunic, I., & Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Research, 47(W1), W256–W259. https://doi.org/10.1093/nar/gkz239

Macdonald, J.S., Waiwood, K.G., Green, R.H., (1982). Rates of digestion of different prey in atlantic cod (Gadus morhua), ocean pout (Macrozoarces americanus), winter flounder (Pseudopleuronectes americanus), and american plaice (Hippoglossoides platessoides). Can. J. Fish. Aquat. Sci. 39 (5), 651–659. http://dx.doi.org/10.1139/f82-094.

Maitra, S., Harikrishnan, M., & Nidhin, B. (2019). Feeding strategy, dietary overlap and resource partitioning among four mesopredatory catfishes of a tropical estuary. Journal of Fish Biology, 96(1), 130–139. https://doi.org/10.1111/jfb.14194

Manikantan, G., PrasannaKumar, C., Vijaylaxmi, J., Pugazhvendan, S. R., & Prasanthi, N. (2020). Diversity, phylogeny, and DNA barcoding of brachyuran crabs in artificially created mangrove environments. Cold Spring Harbor Laboratory. https://doi.org/10.1101/2020.09.07.286823

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Mazlan A. G, Abdullah S., Shariman M. G. and Arshad A. (2008) On the Biology and Bioacoustic Characteristic of Spotted Catfish Arius maculatus (Thunberg 1792) from the Malaysian Estuary Research Journal of Fisheries and Hydrobiology, 3(2): 63-70.

Méheust E, Alfonsi E, Le Ménec P, Hassani S, Jung J (2015) DNA barcoding for the identification of soft remains of prey in the stomach contents of grey seals (Halichoerus grypus) and harbour porpoises (Phocoena phocoena. Mar Biol Res 11(4):385–395

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, IwasakiW(2015)MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R Soc Open Sci 2: 150088

Moran Z, Orth DJ, Schmitt JD, Hallerman EM, Aguilar R (2015) Effectiveness of DNA barcoding for identifying piscine prey items in stomach contents of piscivorous catfishes. Environ Biol Fish 99(1):161–167

Moran, Z., Orth, D. J., Schmitt, J. D., Hallerman, E. M., & Aguilar, R. (2015). Effectiveness of DNA barcoding for identifying piscine prey items in stomach contents of piscivorous catfishes. Environmental Biology of Fishes, 99(1), 161–167. https://doi.org/10.1007/s10641-015-0448-7

Nagy ZT, Sonet G, Glaw F, Vences M (2012) First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. PLoS One 7(3): e34506

Narra, P., Prasannakumar, C., Annadurai, D., & Mahendran, S. (2020). Identifying seaweeds species of Chlorophyta, Ochrophyta and Rhodophyta using DNA barcodes. Cold Spring Harbor Laboratory. https://doi.org/10.1101/2020.08.30.274456

Nijman, V. (2015). Targeted shark fisheries, DNA barcoding and regulating shark fin trade in Indonesia. Fisheries Research, 168, 33–34. https://doi.org/10.1016/j.fishres.2015.03.016

Osman F, Jaswir I, Khaza-ai H, Hashim R. 2007. Fatty acid profiles of finfish in Langkawi Island, Malaysia. Journal of Oleo Science 56(3): 107-113.

Palanisamy, S. K., PrasannaKumar, C.*, Paramasivam, P., & Sundaresan, U. (2020). DNA barcoding of horn snail Telescopium telescopium (Linnaeus C, 1758) using mt-COI gene sequences. Regional Studies in Marine Science, 35, 101109. https://doi.org/10.1016/j.rsma.2020.101109

Pappalardo, A. M., & Ferrito, V. (2015). DNA barcoding species identification unveils mislabeling of processed flatfish products in southern Italy markets. Fisheries Research, 164, 153–158. https://doi.org/10.1016/j.fishres.2014.11.004

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Paquin MM, Buckley TW, Hibpshman RE, Canino MF (2014) DNA-based identification methods of prey fish from stomach contents of 12 species of eastern North Pacific groundfish.Deep-Sea Res 85:110–117

Pinkas, L., 1971. Food habits study. Fish Bull. 152 (5), 10.

PrasannaKumar C, Akbar John B, Ajmal Khan S, Lyla P S, and Jalal K C A (2012) Limit of DNA Barcode in Delineating Penaeus Monodon and in its Developing Stages. Sains Malaysiana 41(12): 1527-1533.

Prasannakumar, C., Manikantan, G., Vijaylaxmi, J., Gunalan, B., Manokaran, S., & Pugazhvendan, S. R. (2020). Strengthening of marine amphipod DNA barcode libraries for environmental monitoring. Cold Spring Harbor Laboratory. https://doi.org/10.1101/2020.08.26.268896

PrasannaKumar, C., Rethinavelu, S., & Balamurugan, S. (2020b). First barcodes of Bathynomus kensleyi (Lowry & Dempsey, 2006) and Bathynomus decemspinosus (Shih, 1972) from the Southeast coast of India. Regional Studies in Marine Science, 101489. https://doi.org/10.1016/j.rsma.2020.101489

Rahman A M, Khan S A, Lyla P S, PrasannaKumar C (2013) DNA Barcoding Resolves Taxonomic Ambiguity in Mugilidae of Parangipettai Waters (Southeast Coast of India). Turkish Journal of Fisheries and Aquatic Sciences 13: 321-330.

Rajthilak, C., Santhanam, P., Raja, M., Suman, T. Y., Rajasree, S. R. R., Ramkumar, R., & Perumal, P. (2015). First distributional record of Nitokra affinis Gurney, 1927 (Copepoda: Harpacticoida: Ameiridae) from Vellar estuary (south-east India): structural and molecular evidence. Marine Biodiversity Records, 8. https://doi.org/10.1017/s1755267215000391

Ran, K., Li, Q., Qi, L., Li, W., & Kong, L. (2020). DNA barcoding for identification of marine gastropod species from Hainan island, China. Fisheries Research, 225, 105504. https://doi.org/10.1016/j.fishres.2020.105504

Ratnasingham, S., & Hebert, P. D. N. (2007). Barcoding: BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes, 7(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Rawat S., Benakappa S., Deepak George Pazhayamadom, Jitendra Kumar, Chitra Soman, Venugopal R. (2019) Stock structure analysis of Splendid ponyfish Eubleekeria splendens (Cuvier, 1829) along Indian coast using truss network system. Indian Journal of Geo Marine Sciences, 48 (04): 434-443.

Ritger, A. L., Fountain, C. T., Bourne, K., Martín-Fernández, J. A., & Pierotti, M. E. R. (2020). Diet choice in a generalist predator, the invasive lionfish (Pterois volitans/miles).

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Journal of Experimental Marine Biology and Ecology, 524, 151311. https://doi.org/10.1016/j.jembe.2020.151311

Rocha LA, Rocha CR, Baldwin CC, Weigt LA (2015) Invasive lionfish preying on critically endangered reef fish. Coral Reefs 34:803–806.

Rocha, L. A., Rocha, C. R., Baldwin, C. C., Weigt, L. A., & McField, M. (2015). Invasive lionfish preying on critically endangered reef fish. Coral Reefs, 34(3), 803–806. https://doi.org/10.1007/s00338-015-1293-z

Sahu, S. K., Singh, R., & Kathiresan, K. (2016). Multi-gene phylogenetic analysis reveals the multiple origin and evolution of mangrove physiological traits through exaptation. Estuarine, Coastal and Shelf Science, 183, 41–51. https://doi.org/10.1016/j.ecss.2016.10.021

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sakthivel M., Deivasigamani B., Kumaran S., Balamurugan S., Rajasekar T. (2012) Seasonal variation in immune organs and immune response of catfish Arius maculatus in Parangipettai coastal area, J. Chem. Pharm. Res., 4:7: 3342–3348.

Santamaria CA, Locascio J, Greenan TM. 2020. First report of lionfish prey from Western Florida waters as identified by DNA barcoding. PeerJ 8:e9922 https://doi.org/10.7717/peerj.9922

Santos B S and Quilang J P (2011) DNA Barcoding of Arius Species (Siluriformes: Ariidae) in Laguna de Bay, Philippines Using the Cytochrome C Oxidase Subunit I Gene. Philipp. Agri. Scientist, 94(2): 205-210

Saunders, R.J., Fowler, A.J., Gillanders, B.M., 2012. The use of food resources by 0+snapper, Chrysophrys auratus, from northern Spencer Gulf, South Australia. Mar.Freshwater Res. 63 (8), 680–686. http://dx.doi.org/10.1071/MF11266

Schloesser RW, FabrizioMC, Latour RJ, Garman GC, Greenlee B, Groves M, Gartland J (2011) Ecological role of blue catfish in Chesapeake Bay communities and implications for management. In: Michaletz PH, Travnichek VH (ed.) conservation, ecology, and Management of Catfish: the second international symposium. Am Fish Soc Symp 77, Bethesda, Maryland. pp. 369–382.

Schooley JD, Karam AP, Kresner BR, Marsh PC, Pacey CA, Thornbrugh DJ (2008) Detection of larval remains after consumption by fishes. Trans Am Fish Soc 137:1044–1049

Sembiring, A., Pertiwi, N. P. D., Mahardini, A., Wulandari, R., Kurniasih, E. M., Kuncoro, A. W., Cahyani, N. K. D., Anggoro, A. W., Ulfa, M., Madduppa, H., Carpenter, K. E., Barber, P. H., & Mahardika, G. N. (2015). DNA barcoding reveals targeted fisheries for

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

endangered sharks in Indonesia. Fisheries Research, 164, 130–134. https://doi.org/10.1016/j.fishres.2014.11.003

Sharawy, Z. Z., Abbas, E. M., Khafage, A. R., Galal-Khallaf, A., Ismail, R. F., Ahmed, H. O., Mohammed-Geba, K., & Kato, M. (2017). Descriptive analysis, DNA barcoding and condition index of Penaeids (Crustacea: Decapoda) from the Egyptian Mediterranean coast. Fisheries Research, 188, 6–16. https://doi.org/10.1016/j.fishres.2016.12.007

Sulvina, S., Abidin, Z., & Supono, S. (2020). Production Analysis of Green Mussel (Perna viridis) in Lampung Province. E-Jurnal Rekayasa Dan Teknologi Budidaya Perairan, 8(2), 975. https://doi.org/10.23960/jrtbp.v8i2.p975-984

Tajima F. (1989). Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics 123:585-595.

Teodoro, S. S. A., Terossi, M., Mantelatto, F. L., & Costa, R. C. (2016). Discordance in the identification of juvenile pink shrimp (Farfantepenaeus brasiliensis and F. paulensis□: Family Penaeidae): An integrative approach using morphology, morphometry and barcoding. Fisheries Research, 183, 244–253. https://doi.org/10.1016/j.fishres.2016.06.009

Thangaraj M, Annadurai D, Ramesh T, Kumaran R & Ravitchandirane V (2020) DNA barcoding and preliminary phylogenetic analysis of few gastropods (Family: Potamididae and Nassariidae) in Vellar estuary mangroves, India by COI and 18S rRNA genes. Ind. J of Geo. Mar. Sci. 49(4): 596-600.

Thirumaraiselvi, R., Das, S., Ramanadevi, V., & Thangaraj, M. (2015). MtDNA Barcode Identification of Finfish Larvae from Vellar Estuary, Tamilnadu, India. Notulae Scientia Biologicae, 7(1). https://doi.org/10.15835/nsb.7.1.9478

Thompson,J.D., Gibson,T.J., Plewniak,F., Jeanmougin,F. and Higgins,D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 25:4876-4882.

Tupinambás, TH., Pompeu, PS., Gandini, CV., Hughes, RM., & Callisto, M. (2015). Fish stomach contents in benthic macroinvertebrate assemblage assessments. Brazilian Journal of Biology, 75(1), 157–164. https://doi.org/10.1590/1519-6984.09913

Valdez-Moreno M, Quintal-Lizama C, Go´ mez-Lozano R, Garcı´a-Rivas MdC (2012) Monitoring an Alien Invasion: DNA Barcoding and the Identification of Lionfish and Their Prey on Coral Reefs of the Mexican Caribbean. PLoS ONE 7(6): e36636. doi:10.1371/journal.pone.0036636

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Velez-Zuazo, X., Alfaro-Shigueto, J., Mangel, J., Papa, R., & Agnarsson, I. (2015). What barcode sequencing reveals about the shark fishery in Peru. Fisheries Research, 161, 34–41. https://doi.org/10.1016/j.fishres.2014.06.005

Vestheim H, Jarman SN (2008) Blocking primers to enhance PCR amplification of rare sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs. Front Zool 5:12

Wang, H.-Y., Dong, C. A., & Lin, H.-C. (2017). DNA barcoding of fisheries catch to reveal composition and distribution of cutlassfishes along the Taiwan coast. Fisheries Research, 187, 103–109. https://doi.org/10.1016/j.fishres.2016.11.015

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. Philos Trans R Soc Lond Ser B Biol Sci 360(1462):1847–1857

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. N. (2005). DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462), 1847–1857. https://doi.org/10.1098/rstb.2005.1716

Weigt LA, Baldwin CC, Driskell A, Smith DG, Ormos A, Reyier EA (2012a) Using DNA barcoding to assess Caribbean reef fish biodiversity: expanding taxonomic and geographic coverage. PLoS One 7(7): e41059

Weigt LA, Driskell AC, Baldwin CC, Ormos A (2012b) DNA barcoding fishes. In: Lopez EricksonDL (ed) DNA barcodes: Methods and Protocols. Humana Press, New York City, NY, pp. 109–126

Wirta HK, Hebert PDN, Kaartinena R, Prosser SW, Várkonyi G, Roslin T (2014) Complementary molecular information changes our perception of food web structure. Proc Natl Acad Sci U S A 111(5):1885–1890

Wong LL, Peatman E, Lu J, Kucuktas H, He S, et al. (2011) DNA Barcoding of Catfish: Species Authentication and Phylogenetic Assessment. PLoS ONE 6(3): e17812. doi:10.1371/journal.pone.0017812

Xu, L., Wang, X., Van Damme, K., Huang, D., Li, Y., Wang, L., Ning, J., & Du, F. (2021). Assessment of fish diversity in the South China Sea using DNA taxonomy. Fisheries Research, 233, 105771. https://doi.org/10.1016/j.fishres.2020.105771

Yeh, C.-Y., Huang, J.-F., Lee, J.-M., & Schafferer, C. (2016). An economic analysis of hard clam (Meretrix meretrix) farmer polyculture with milkfish (Chanos chanos), silver sea bream (Rhabdosargus sarba), and shrimps at different hard clam stocking densities: a case study of Yunlin County, Taiwan. Aquaculture International, 25(3), 1039–1055. https://doi.org/10.1007/s10499-016-0094-x

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.18.268888; this version posted October 17, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Zhang, Y.-H., Qin, G., Zhang, H.-X., Wang, X., & Lin, Q. (2017). DNA barcoding reflects the diversity and variety of brooding traits of fish species in the family Syngnathidae along China's coast. Fisheries Research, 185, 137–144. https://doi.org/10.1016/j.fishres.2016.09.015

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Previously unrecorded distribution of marine sediments derived yeast isolates revealed by DNA barcoding

Chinnamani PrasannaKumar*[1,2], Shanmugam Velmurugan[2,3], Kumaran Subramanian[4], S. R. Pugazhvendan[5], D. Senthil Nagaraj[3], K. Feroz Khan[2,6], Balamurugan Sadiappan[1,2], Seerangan Manokaran[7], Kaveripakam Raman Hemalatha[8], Wilson Aruni[9,10], Bhagavathi Sundaram Sivamaruthi[11], Chaiyavat Chaiyasut[11]

[1]Biological Oceanography Division, CSIR-National Institute of Oceanography, Dona Paula, Panaji, Goa-403004, India

[2]Centre of Advance studies in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu- 608502, India

[3]Madawalabu University, Bale, Robe, Ethiopia

[4]Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Tamil Nadu-600119, India

[5]Department of Zoology, Arignar Anna Government Arts College, Cheyyar, Tamil Nadu-604407, India

[6]Research Department of Microbiology, Sadakathullah Appa College, Rahmath Nagar, Tirunelveli Tamil Nadu -627011, India

[7]Center for Environment & Water, King Fahd University of Petroleum and Minerals, Dhahran-31261, Saudi Arabia

[8]Department of Microbiology, Annamalai University, Annamalai Nagar, Chidambaram, Tamil Nadu- 608002, India

[9]Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu-600119, India

[10]US Department of Veteran affairs, Loma Linda, CA, USA

[11]Innovation Center for Holistic Health, Nutraceuticals, and Cosmeceuticals, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand.

*Corresponding author email: micropras@gmail.com

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Abstract

For the yeast population and diversity, marine habitats are the least explored niches and the lack of validated database is considered to be a drawback for yeast research. The aim of the present study is to create a comprehensive DNA barcode library for marine derived yeast species isolated from organic burial hotspots such as coastal sediment in mangrove and continental shelf ecosystems. As we enriched, isolated and ITS gene sequenced 1017 marine derived yeast isolates belonging to 157 marine species in 55 genera, 28 families, 14 orders, 8 classes of 2 Phyla (*viz*., Ascomycota and Basidiomycota) of which 13 yeast species were first time barcoded. We witnessed yeast species of both terrestrial and marine endemic origin in the barcode datasets. Due to the large volume of sequencing trace files, the variable length of extracted sequences, and the lack of reference sequences in public databases, difficulties were faced in taxonomic sequence validation. The length of the majority of the sequences (99.42%) were more than or equal to 600 base pairs. BLAST analysis revealed that 13 yeast species were barcoded for the first time. The genus, *Candida* was the speciose genera isolated in this study. K2P intra-species distance analysis performed for selective groups yielded an average of 0.33%, well below the previously proposed yeast barcode gap. ITS gene NJ-tree based identification conducted for selective species in Ascomycota and Basidomycota, precisely clustered the same species into one group, indicating the efficacy of ITS gene in yeast species delineation. Besides isolating some of the common marine yeast species such as *Candida oceani*, *Bandonia marina* and *Yarrowia lipolytica*, we found approximately 60% of the yeast species isolates were previously unrecorded from the marine environment (example; *Cystobasidiopsis lactophilus*, *Slooffia cresolica*, *Udeniozyma ferulica*, *Colacogloea falcatus* and *Pichia guilliermondii*), of which 16.5% were recognised as potential human pathogens (example; *Candida orthopsilosis*, *C. rugosa*, *Debaryomyces fabryi* and *Yamadazyma triangularis*). Apart from releasing the barcode data in GenBank, provisions were made to access the entire dataset along with meta-data in the Barcode of life database (http://dx.doi.org/10.5883/DS-MYIC). This research constitutes the largest dataset to date for collecting marine yeast isolates and their barcodes. As meta- and environmental barcoding analysis were expanding its scope including environmental assessment and monitoring, the datasets such as ours will be more useful.

Key words: Marine yeast, DNA barcoding, unrecorded yeasts, yeast pathogens, ITS gene

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1. Introduction

In the tropical and subtropical geographies, mangroves and its associated mycobiota are vital components of the ecologically and economically significant food web (Lee et al., 2014). So far 69-77 mangrove species (including their associates and hybrids) have been documented (Alappatt, 2008; Statzell-Tallman et al., 2008). The mangroves constitutes 4 Gton of Carbon with global biomass of 8.7 Gton, distributed in 112 countries over 10-24 million hectares (Kathiresan & Bingham, 2001; Giri, 2011). The primary production rate in mangrove ecosystems is equal to the rates in evergreen tropical humid forests (Alongi et al., 2014). On the other hand, in the Bay of Bengal, one of the largest sediment inputs occurs among the world's ocean, receiving 2000 million tons of sediments annually (Mohanty et al., 2008) along with significant amount of terrestrial organic materials (Hedges and Keil, 1995; de Haas et al., 2002) contributed by various rivers including the Kaveri on the southeast coast of India (Khan et al., 2012). Yeast populations have rarely been explored in these sites besides an abundant occurrence of culturable filamentous fungal isolates in these large carbon burial sites (Das et al., 2009; Velmurugan et al., 2013; Godson et al., 2014).

In comparison to aquatic ecosystems, fungi in terrestrial and fresh water environments have been well studied (Gulis et al., 2009; Raja et al., 2018). Through decomposition plant material and nutrient replenishments (Schmidt & Shearer, 2004; Shearer et al., 2007), fungi plays a significant role in the mangrove ecosystems by providing food for fishes and invertebrates (Hogarth, 1999). The unicellular fungi, yeast (Kutzman and Fell, 2015) is a polyphyletic group (Kutty and Philip, 2008), and when requires seawater for its growth was described as marine yeast (Chi, 2012). Marine yeasts are known for their parasitic, mutualistic or saprophytic relationship with marine animals in addition to their significance in nutrient cycling, and may therefore be associated with various marine invertebrates, including crabs, clams, mussels, prawns, oysters or other substrates (Jones and Pang, 2012; de Araujo et al., 1995; Kosawa da Costa et al., 1991; Pagnocca et al., 1989). In two major phyla viz., Ascomycota and Basidiomycota, the diverse marine yeast is largely classified (Kurtzman 2011; Boekhout et al. 2011). Though basidiomycetous yeast species were known for high salt tolerance (Tekolo et al., 2010), most of marine derived Ascomycetous yeast species are of terrestrial origin with widespread phylogenetic diversity (Fell, 2012).

Since its first isolation (Fischer and Brebeck, 1894), which was recently overcome using molecular taxonomy (Fell, 2012), numerous difficulties have existed in marine yeast nomenclature and taxonomy. DNA barcoding has simplified the recognition of biological species using short DNA fragment sequencing and analysis (Hebert et al., 2003). One of the

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

vision behind DNA barcoding was the simple identification of biological species by non-experts for the advancement of biological, ecological and medical research. Internal transcribed spacer (ITS) gene has been recognised as DNA barcode which successfully delineates filamentous fungal species (Schoch et al., 2012; Velmurugan et al., 2013; Vu et al., 2019) and even works better for classification of yeast species (Vu et al., 2016), substituting the previously used laborious and frequently inaccurate phenotypic analysis (Kurtzman et al., 2015). Lack of validated data sets for yeast research is considered to be a drawback for its identification (Bidartondo, 2008). The goal of this study is to use DNA barcoding to explore the marine sediments in mangrove swamps along Indian coast and continental shelf off southeast coast of India for yeast diversity. We aim to synthesise and publish, in GenBank and BOLD, a sizable amount of marine derived yeast DNA barcodes. Besides evaluating such database for identification of yeast species, we suspect that even after the production of large yeast barcode dataset (8669 barcodes for 1351 yeast species) (Vu et al., 2016), marine habitats will still be able to incubate some yeast species yet to be barcoded. The purpose of the study was to gather as many marine derived yeast cultures as possible for a comprehensive DNA barcode library synthesis, as studies are uncommon in exploring such a large scale marine environments for yeast isolates.

2. Materials and methods

2.1. Study area and sample collection

Between Nov, 2008 and Jan 2013, sediment samples were extensively collected from two separate ecosystems, viz., 1) inter-tidal sediments flats under mangrove trees along Indian's coastline, 2) continental shelf sediments off India's southeast coast. The setails of sampling sites with geographical descriptions were given in **Table S1**.

Inter-tidal sediments under mangrove trees were collected along the Indian coastline from various mangrove environments ranging from Gujarat's Kachchh Mangroves in West coast of India to West Bengal's Sundraban ecosystem in East coast of India. Sampling of 14 mangrove station called M1 through M14 and details of collection date, geographical coordinates, and number of samples per station are provided in table S1**.** During pre-monsoon seasons, mangrove sediments were collected mainly at low tide. Approximately 50 to 100g of undisturbed sediments were collected (in triplicates) using sterile spatula, transferred to sterile polythene bags (Nasco bags, HiMedia), and transported to laboratory in ice. Samples were processed normally within 48 hours. In mangrove environment, sediments (n=52) were obtained from 13 mangrove species viz., *Avicennia* sp., *A. ilicifolius, A. alba, A. marina, A.*

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

officinalis, Bruguiera cylindrical, Ceriops decandra, Excoecaria agallocha, Pongamia pinnata, Rhizophora mucronata, Sonneratia apectala, S. caseolaris and *Xylocarpus mekongensis* in 14 sampling stations (named as M1 –M14) (table S1). Sediment pore water salinity was measured using hand-held refractometer (BEXCO, USA). Highest of 35ppt salinity was measured in Parangipettai and Pitchavaram mangroves (station, M9 and M10, respectively) whereas station M14 which is upstream mangroves infringe inland of Sundarbans, West Bengal, records the lowest salinity of 8ppt.

During cruise No. 260 (Lyla et al., 2012) of Fishery and Ocean Research Vessel (FORV) Sagar Sampada conducted from 06th to 28th December 2008 using Smith–McIntyre grab which covers an area of $0.2m^2$, sediment samples along the Southeast continental self of India were collected. On the Southeast continental shelf, the sampling station was fixed at the depth gradients of about 30, 50, 100, 150 and 200 meters at each transacts, constituting a total of 7 transects and 32 sampling stations, called from CS1 through CS32 **(table S1).** In-built with FORV-Sagar Sampada, the depths of sediment sampling were measured using multi-beam echo sounder (capable of measuring up to 1000m depth). Sterile spatula was used for sediment sub-sampling (trice per station) from the grab. The cruise collected a total of 96 sediment samples. Salinity of continental shelf sediments ranged from 33 to 35ppt. All culture media were prepared using the ambient seawater (AS) obtained from respective collection sites, as it provides closer to natural environmental conditions (Zaky et al., 2014). AS was filtered through 0.22µ cellulose filter membrane (Miilipore) and autoclaved, before being used to prepare culture media.

2.2. Enrichment and isolation of yeast-like cells

Two types of media were used for enrichment, as enrichments were known to increase the number of yeast species isolates (Gadanho & Sampaio, 2004). Either one or both of the media (for most samples collected after 2010) was used for enrichment of yeast cells before plate culture. Briefly, after homogenization of the sediment samples in the collection container, yeast cells were enriched by adding one gram (g) of sediments to 100ml of Yeast/Malt extract (YM) broth (3 g malt extract, 3 g yeast extract, 10 g dextrose, and 5 g peptone, in 1L sterile-AS) and/or GPY broth (2% glucose, 1% peptone, 0.5% yeast extract in 100ml sterile-AS) supplemented with an antibiotic cocktails (300 mg L^{-1} penicillin, 300 mg L^{-1} streptomycin, 250 mg L^{-1} sodium propionate, and 0.02% of chloramphenicol) to inhibit bacterial growth. We used both enrichment media for sediments sampled after 2010, to increase the number of yeast species being isolated. At 150 rpm, the enrichment broth with

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

sediment samples in a 250 mL Erlenmeyer flasks was shaken on a rotary shaker, incubated for 2-3 days at 17-20 ºC (temperature >20 ºC was found to accelerate filamentous fungal growth in plate cultures). Autoclaved AS has been used as control. After incubation from corresponding broth cultures, 100µl to 1000µl (based on the turbidity of the broth) was spread over (in triplicates) YM and/or GYP agar plates (composition as same for broth preparation with addition of 1.5% agar). Culture media was autoclaved twice (at 100ºC for 30min) during two consecutive days to reduce mould contaminations (Gadanho and Sampaio, 2005). The remaining broth was conserved at 4ºC with the over lay of mineral oil for future use, just in case the incubated plates did not produce any colonies or over production of filamentous fungi. The inoculated plates were incubated for 10-20 days or until colonies appeared and continuously monitored at every 24 hours. In order to promote the full recovery of yeast-like species including slow growing colonies, prolonged incubation period with concurrent removal of fast growing colonies were adopted. Also care was taken to stop the incubation when there was a high probability of mould over growth.

Microscopic analyses of yeast-like colonies were started from the minimum of 5 days of incubation with methylene blue staining (checked for single type forms and to ensure no association of bacterial cells) and purified by streaking onto fresh agar (YM or GYP) plates (to prevent growth over other colonies or to save from rapidly growing moulds). Gradually one representative morphotype of each colony per sample (i.e., when the yeast colonies were <50 numbers or when the mould was entirely absent or scarce in the agar plates) was streaked twice on corresponding agar plates for purification. The representative number of colonies (40-70 colonies) is randomly selected and purified twice in other cases (i.e., when yeast colonies is >50 or dense mould growth in the agar plates).

Enrichments cultures as mentioned above were done for continental shelf sediments on the ship board Microbiology lab, FORV-Sagar Sampada. After 2 sub-culturing of yeast-like cells, the isolates were grown for 2-3days in the shaker (at 120 rpm) 1mL of GPY broth (in duplicates) prepared in 2mL microfuge tubes. The culture in one tube was used for DNA isolation and other cryopreserved (culture increased to 1.5ml volume using 2% GPY broth, 10% glycerine) in the Marine Microbial Culture Facility, Centre of Advanced Study in Marine Biology, Annamalai University. There were a total of 1398 colonies for molecular analysis (916 colonies from mangrove sediments, and 482 colonies from continental shelf sediments). Each purified cultures were numbered under DBMY (DNA Barcoding Marine Yeast) acronym. The key features described in Yarrow (1998) and Kurtzman et al. (2010) were used for the macro- and micro-morphological analysis for identification.

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

2.3.DNA extraction, polymerase chain reaction and DNA sequencing

In order to recover the yeast-like cells grown in the 2mL microfuge, the tubes were centrifuged at 8000 X g for 5 minutes. Following manufacturer's instructions, DNA was extracted from the pellets using GeneiPure Yeast DNA preparation kit (GeNei) or DNeasy blood and tissue kit (Qiagen). The DNA was eluted in the elution buffer (provided with the kit) and stored at -20ºC. The ITS primers; ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) (White et al., 1990) was used for amplification. The primers targets the DNA fragments containing, partial 18S ribosomal RNA gene; complete sequences of internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2 and partial sequences of 28S ribosomal RNA gene. PCR was performed on a thermal cycler 130045GB (GeNei) under following conditions: 4 min at 94 ºC, followed by 30 cycles of 30 s at 94 ºC, 40 s at 48 ºC for annealing and 90 s at 72 ºC, with a final extension at 72 ºC for 7 minutes. PCR amplicon were separated by 1.5% agarose gel electrophoresis. Amplicons were sequenced two ways using commercial sequencing services of Macrogen (South Korea) or Bioserve Biotechnologies Pvt. Ltd. (India).

2.4.DNA sequence analysis

Following sequencing, the forward and reverse sequences were assembled using BioEdit ver. 5.0 (Hall, 1999). Sequences were aligned using CLUSTAL X (Larkin et al., 2007) and manually adjusted in MEGA X (Kumar et al., 2018). DNA sequences were compared with GenBank sequences using BLAST algorithms (Altschul et al., 1997) and a cut-off species threshold of 98.41% (Vu et al., 2016) was used for delamination of yeast species. The species that were first time barcoded (i.e., when species threshold is <98%) were confirmed by double checking BLAST search similarity values and by searching for ITS gene sequences of the species in GenBank.

Using the reference sequences extracted from GenBank, the Neighbor-Joining method (Saitou and Nei, 1987) was used for tree based yeast species identification. In the bootstrap test, the percentage of replicate trees in which the associated taxa clustered together (100 replicates) (Felsenstein, 1985) is indicated as circles next to the branches. The tree is drawn to scale, with branch lengths in the same units as those used to infer the phylogenetic tree from evolutionary distances. The evolutionary distances have been computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

(complete deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The NJ trees were manipulated in interactive Tree of Life (iTOL) database (Letunic and Bork, 2019) for better representation.

DNA sequences generated in the present study were release to GenBank and could be accessed through accession numbers: KJ706221-KJ707237. Entire dataset produced in this study could also be accessed through Barcode of life database under the project title "DNA barcoding marine yeast" with a tag, "DBMY" or through a digital object identifier; http://dx.doi.org/10.5883/DS-MYIC .

3. Results and discussion

3.1. Composition of marine yeast species revealed through BLAST analysis

Out of the 1398 isolates selected for molecular analysis, 1017 (72.74%) were successfully sequenced. The BLAST analysis shows that ITS gene sequences belonged to 157 yeast species in 55 genera, 28 families, 14 orders, 8 classes (viz., Agaricostilbomycetess, Cystobasidiomycetes, Dothideomycetes, Exobasidiomycetes, Microbotryomycetes, Saccharomycetes, Tremellomycetes, Ustilaginomycetes) in 2 Phyla (*viz*., Ascomycota and Basidiomycota). Two species (*viz*., *Curvibasidium cygneicollum* (J.P. Sampaio, 2004), and *Hasegawazyma lactosa* (T. Hasegawa, 1959)) could not be classified into any defined family, and were therefore placed under *incertae sedis*. Of the total number of sequences (n=1017) generated, 52.01% (n=529) of the sequences were the representatives of Ascomycota and the remaining 47.99% (n=488) of the sequences were the representatives of Basidiomycota. List of recovered species and their respective sampling stations were given in **Table 1.**

Table 1: List of marine derived yeast species and its respective sampling station. Colonies that could not be identified to species level was indicated by "sp."

No. of Sp.	Family	Genera	Species	Sampling source
1	Agaricostilbaceae	*Sterigmatomyces*	*elviae*	CS23, M5, CS25, CS5, CS6.
2			*halophilus*	M5, M6, M7, CS28, CS26.
3	Buckleyzymaceae	*Buckleyzyma*	*kluyveri-nielii*	CS4, M6, M7, M8, M9.
4			*salicina*	CS25, M7, M10, M11, M12, M13.
5	Chionosphaeraceae	*Cystobasidiopsis*	*lactophilus*	M3, CS31, M2, CS19.
6	Chrysozymaceae	*Chrysozyma*	*griseoflava*	M9, CS13, M9, CS26, M1.
7		*Fellozyma*	*inositophila*	M4, M5, CS5, CS30, CS31.
8		*Hamamotoa*	*lignophila*	M2, CS3, CS13, M10, M11.
9			*singularis*	M1, CS16, M2, CS29, M4.

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

10		*Oberwinklerozyma*	*yarrowii*	M10, CS14, CS27, M2.
11		*Pseudohyphozyma*	*bogoriensis*	M11, CS15, CS28, M3.
12			*buffonii*	M14, CS17, M13, CS28.
13			*pustula*	CS27, M14, CS11, M8, M9.
14		*Slooffia*	*cresolica*	CS28, M10, CS18, CS26, CS27.
15			*pilati*	M12, M13, M14, M8., CS8
16			*tsugae*	M13, CS27, CS15, CS16.
17		*Trigonosporomyces*	*hylophilus*	CS25, CS26, CS27, M8, M9.
18		*Udeniozyma*	*ferulica*	M4, CS32, CS20, M5, M6.
19		*Vonarxula*	*javanica*	M3, CS4, CS14, M11.
20	Cystobasidiaceae	*Cystobasidium*	*pallidum*	M12, CS26, CS14, CS15.
21			*pinicola*	M13, M14, CS25, CS26, CS27.
22			*slooffiae*	M13, M14, CS25, CS26, CS27.
23	Cystofilobasidiaceae	*Cystofilobasidium*	*macerans*	M5, M9, CS28, CS29.
24		*Filobasidium*	*globisporum*	CS19, M1, M11, CS16, M14.
25			*stepposum*	M5, CS7, M3, CS31, M6.
26			*uniguttulatum*	M4, M5, CS26, CS27, CS28.
27	Debaryomycetaceae	*Debaryomyces*	*fabryi*	M9, CS29, CS30.
28			*hansenii*	M10, CS11, CS12, CS13.
29			*mycophilus*	M13, CS13, M10, CS11, CS12, CS29, M11, CS19, CS27, CS28, M8.
30			*nepalensis*	M1, CS30, M2, CS18, M5.
31			*pseudopolymorphus*	CS27, CS28, CS32, M12, CS29, M10.
32			*subglobosus*	M14, CS28, CS16, CS15.
33		*Millerozyma*	*acaciae*	M1, CS30, CS31.
34		*Scheffersomyces*	*coipomoensis*	M13, CS29, CS11, CS25, CS26.
35			*shehatae*	M12, M1, CS31, CS30, CS30, CS29, CS28, CS27, CS20, CS19, CS11.
36		*Yamadazyma*	*scolyti*	M11, CS27, CS13, CS14, CS15.
37			*triangularis*	CS20, CS6, M1, M2, M3.
38	Dipodascaceae	*Galactomyces*	*geotrichum*	M1, M2, M11, CS11.
39		*Yarrowia*	*lipolytica*	CS18, CS4, M9, M10, M11.
40	Erythrobasidiaceae	*Erythrobasidium*	*elongatum*	M13, CS29, CS3, CS13.
41	Filobasidiaceae	*Cryptococcus*	*adeliensis*	M12, M7, CS7, M3.
42			*albidus*	M11, CS25, CS13, CS14.
43			*diffluens*	CS24, CS26, M8, CS25.
44			*magnus*	CS25, CS28, CS29, M10, CS26.
45			*oeirensis*	CS4, CS27, M9, CS25.
46			*randhawai*	M1, M2, M3, M4, CS22, CS20.
47			sp.1	M6, CS31, CS21, M3.
48			*uzbekistanensis*	M14, M9, CS26, CS27, CS28.
49		*Naganishia*	*globosa*	CS20, CS13, M4, M5, M10.
50			*liquefaciens*	M2, M3, CS31, CS32, CS3.

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

51			*vishniacii*	CS25, CS28, M10, CS26, CS25, CS27, M14, M3, M4, M5, M6, CS11, CS29, CS30, M8, M9.
52	Heterogastridiaceae	*Colacogloea*	*diffluens*	CS29, CS18, CS19, M13, M14.
53			*eucalyptica*	CS27, CS28, M12, M13.
54			*falcata*	CS24, CS25, CS26, M11, M12.
55			*foliorum*	CS31, CS32, M10, M11.
56			*philyla*	CS5, CS6, CS11, M9, M10.
57	incertae sedis	*Curvibasidium*	*cygneicollum*	CS28, CS31, CS32, M8, M9.
58		*Hasegawazyma*	*lactosa*	M1, M2, M3, CS30, CS12.
59	Kondoaceae	*Kondoa*	*aeria*	M12, M13, M11, CS18, CS19.
60			*malvinella*	CS28, CS29, CS30, M7, M8.
61	Kriegeriaceae	*Phenoliferia*	*glacialis*	CS28, CS29, CS18, CS19, M6, M5.
62			*psychrophenolica*	CS5, CS26, CS27, M5, M6.
63	Leucosporidiaceae	*Leucosporidium*	*fellii*	CS6, CS31, CS32, M1.
64			*scottii*	CS18, CS15, CS16, M5, M6.
65	Metschnikowiaceae	*Metschnikowia*	*noctiluminum*	CS15, CS16, CS30, M4, M5.
66	Microsporomycetaceae	*Microsporomyces*	*pini*	CS28, CS29, CS5, CS14, M3, M4.
67	Microstromataceae	*Microstroma*	*bacarum*	CS29, CS30, M3, M2.
68	Phaffomycetaceae	*Wickerhamomyces*	*anomalus*	CS31, CS17, CS18, M2, M3.
69			*subpelliculosus*	CS29, CS6, M13, M14.
70	Pichiaceae	*Nakazawaea*	*pomicola*	CS22, M6, CS31, CS21, M3.
71		*Pichia*	*cecembensis*	CS32, M14, CS31, CS18.
72			*farinosa*	CS11, CS27, CS28, M9.
73			*fermentans*	CS25, CS29, CS30, CS31, M9.
74			*guilliermondii*	CS6, CS11, CS25, M14.
75			*jadinii*	CS30, CS31, CS32, M1, M2.
76			*kluyveri*	CS27, CS28, CS29, M14, M1.
77			*kudriavzevii*	M8, M5, M11, M10, CS6, CS5, CS29, CS28.
78			*subpelliculosa*	CS28, CS29, CS30, M3, M12.
79	Ruineniaceae	*Ruinenia*	*dracophylli*	CS26, CS27, M4, M11.
80			*rubra*	CS11, CS28, M5, M10.
81	Saccharomycetaceae	*Candida*	*albicans*	CS19, M14, M1, CS16, M2.
82			*boidinii*	M12, CS28, M10, CS26.
83			*boleticola*	CS26, CS31, M12, CS23.
84			*carpophila*	CS27, M14, M3, M4, M5.
85			*catenulata*	CS5, CS6, M14, M13, CS31.
86			*carvajalis*	M3, M14, M11, M10, CS31, CS28, CS27, CS26, CS26, CS25.
87			*dubliniensis*	CS27, CS28, M5, M9, M10.
88			*duobushaemulonii*	CS6, CS7, CS26, M6, M8.
89			*ergatensis*	CS5, CS25, M7, CS32, CS22, M4, CS24, CS4, M6, M8, M9, M10, CS18, CS19, CS20, CS21.
90			*fermenticarens*	CS28, CS29, CS18, M7, M8.
91			*fragi*	CS28, CS26, CS27, M14, M13.

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

92			*glaebosa*	CS31, CS26, CS27, M10, M11.
93			*haemulonii*	CS29, CS30, M5, M6.
94			*humilis*	CS26, CS27, CS28, M8, M9.
95			*hungchunana*	CS11, CS25, M9, M10, M11.
96			*insectamans*	M8, M5, M2, M13, M10, CS32, CS31, CS30, CS28.
97			*intermedia*	CS4, CS32, CS6, M11, M12.
98			*magnoliae*	CS22, CS32, CS3, M13, M14.
99			*molendinolei*	M12, M11, CS26, CS27.
100			*nivariensis*	CS30, CS31, CS32, M1, M3.
101			*oceani*	CS28, CS29, M6, M7.
102			*orthopsilosis*	CS26, CS27, M3, M7.
103			*palmioleophila*	M13, CS21, M5, CS30.
104			*parapsilosis*	M2, M3, M5, CS29, CS31.
105			*quercitrusa*	M14, M9, M1, CS31, CS32.
106			*railenensis*	CS28, CS2, M10, CS26.
107			*rugosa*	M7, CS31, M8, CS27, CS32.
108			*santamariae*	CS6, CS26, CS26, M8.
109			sp.1	CS3, M14, CS22, M6.
110			*spencermartinsiae*	CS3, CS25, CS26, M9.
111			*viswanathii*	CS1, CS2, M12, CS28.
112			*xylopsoci*	CS3, M13, CS21, M5.
113			*zeylanoides*	CS2, CS5, CS13, M7, M9.
114		*Hanseniaspora*	*guilliermondii*	CS3, CS4, CS6, M14.
115			*occidentalis*	M2, CS30, M3, CS31, CS9.
116			*opuntiae*	CS6, CS29, CS30, M6.
117			*uvarum*	CS10, CS25, CS28, CS29, M10.
118		*Issatchenkia*	*orientalis*	CS11, CS12, M3, CS19.
119		*Kazachstania*	*aerobia*	CS5, CS2, CS25, M7.
120			*exigua*	CS32, CS22, M4, CS24.
121			*humilis*	CS1, CS4, CS5, M14.
122		*Kluyveromyces*	*dobzhanskii*	CS6, CS26, CS27, CS31, M1.
123			*lactis*	M4, CS24, CS4, M6.
124			*marxianus*	M7, M8, M9, CS5.
125		*Saccharomyces*	*bayanus*	M12, CS28, M14, CS22.
126			*cerevisiae*	M6, CS31, CS21, M3.
127			*kudriavzevii*	M8, CS2, CS23, M5.
128		*Torulaspora*	*delbrueckii*	CS2, M10, CS26, CS25.
129			*franciscae*	CS23, M5, CS25, CS5.
130		*Williopsis*	*saturnus*	M1, M2, M3, M4, CS25, M7.
131	Saccharomycopsidaceae	*Saccharomycopsis*	*fibuligera*	M13, M14, M9, CS32.
132	Saccotheciaceae	*Aureobasidium*	*pullulans*	M12, M13, CS22, M4.
133			*mansoni*	M6, M14, M13, M11, M10, CS32, CS31, CS28, CS27, CS26, CS25.
134			sp.1	CS16, M13, CS27, CS15, M14, CS22, M6, M11, CS28, CS25, M10,

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

				M7, CS7.
135	Sakaguchiaceae	*Sakaguchia*	*dacryoidea*	M6, CS27, CS20, M7.
136	Sporidiobolaceae	*Rhodosporidiobolus*	*fluvialis*	M7, CS7, M3, CS31.
137		*Rhodosporidium*	*diobovatum*	M2, M3, M4, CS7.
138		*Rhodotorula*	*araucariae*	M14, M9, M1, CS3, CS4, CS5.
139			*babjevae*	M12, M13, CS1, CS4.
140			*dairenensis*	M12, CS28, M10, M11.
141			*glutinis*	M13, M14, M9, CS32, CS26.
142			*graminis*	M5, CS3, CS4, CS5, CS6.
143			*ingeniosa*	M7, M8, M9, CS8, CS28.
144			*kratochvilovae*	CS3, M7, CS24, M6, CS26.
145			*mucilaginosa*	CS22, CS11, CS25, M6, M7.
146			*nothofagi*	M3, M4, M5, CS7, CS8.
147			*paludigena*	CS1, M9, CS28, CS25.
148			*sphaerocarpa*	M1, CS1, CS2.
149			*toruloides*	CS22, M6, CS31, CS21.
150		*Sporobolomyces*	*beijingensis*	M3, CS2, CS31, CS18.
151			*koalae*	CS9, CS27, CS28, M8.
152			*oryzicola*	M12, CS20, CS28, CS29.
153			*roseus*	CS29, CS6, M7, M8.
154	Symmetrosporaceae	*Symmetrospora*	*foliicola*	M6, M7, CS26, CS11.
155			*gracilis*	M1, CS16, M3, CS29.
156	Tetragoniomycetaceae	*Bandonia*	*marina*	M9, M4, M3, M2, M13, M12, M11, M10, M1, CS28, CS25.
157	Ustilentylomataceae	*Ustilentyloma*	*graminis*	M11, CS19, CS27.

Minimum of 6 isolates was sequenced from each yeast species isolated in this study. The isolates of *Scheffersomyces ergatensis* (Santa María, 1971), *Naganishia vishniacii* (Vishniac & Hempfling, 1979) and *Aureobasidium* sp. (Viala & G. Boyer, 1891) provided a maximum of 19 sequences each. Each of the yeast species; *viz*., *Aureobasidium mansonii* (Cooke W.B., 1962), *Bandonia marina* (Uden & Zobell, 1962), *Candida carvajalis* (James S. A., 2009), *Candida insectamans* (D.B. Scott, Van der Walt & Klift, 1972), *Debaryomyces mycophilus* (Thanh, Van Dyk & M.J. Wingf., 2002), *Scheffersomyces shehatae* (H.R. Buckley & Uden, 1964) was sequenced for 13 times and the *Pichia kudriavzevii* (Boidin, Pignal & Besson, 1965) was sequenced for 12 times. *Candida* spp. was the most speciose genera (n=33 species) followed by *Rhodotorula* spp. (n=13 species). Largely, we did not find clear pattern of differentiation in yeast species isolated from mangrove and continental shelf sediments.

3.2.Character based identification and BLAST analysis

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

The length of the ITS sequence recovered varied from 542bps to 891bps (**Fig. 1**). The majority of the sequences (62.24%; n=633 sequences belonging to 81 species) were between 600 and 649 bps. Minimum length of 552 - 599 bps was only recovered only for 6 sequences (0.58%). All remaining recovered sequences (99.42%) were larger than or equivalent to 600bp length. The list of 1017 barcodes with their respective species match was given in **Table S2** containing details of the GenBank reference sequence (with percentage of similarity, its accession numbers and its taxonomy)**.**

BLAST analysis revealed that 13 yeast species were barcoded for the first time (**Table 2**) and individual search of the ITS gene of those species in GenBank did not yield any results, re-confirming that those species were first time barcoded. Despite *Candida* spp. was speciose genera reported and DNA barcoding, the ITS gene sequences of *Candida carvajalis* (n=15), C. *duobushaemulonii* (n=6) and *C. haemulonis* (n=6) was barcoded for the first time, as they were absent in the reference database until now. Even though previous extensive study, barcoded 1351 yeast species producing 8669 barcodes (Vu et al., 2016), the above mentioned 13 *Candida* spp. species was not included in their collection, as the previous study did not explore marine environments.

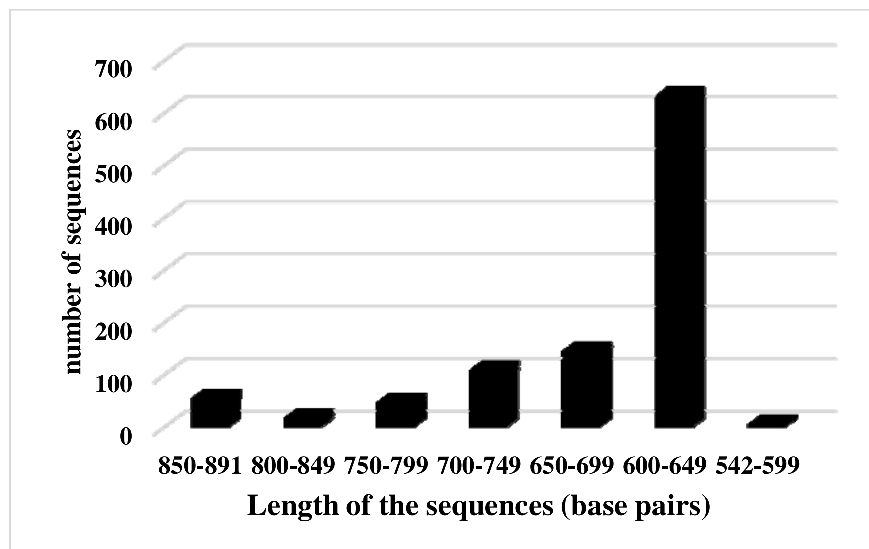


Fig. 1: ITS gene sequence length (bps) variations distributed among 1017 sequences. More than 60% of the sequences were in between 600 and 649 bps lengths.

Of the 13 first time barcoded species, a minimum of 0.25 K2P distance and 0.024 nucleotide diversity were reported for *Candida carvajalis* (n = 13) (fig. 2). Maximum K2P distance of 0.37 was recorded for *Buckleyzyma salicina* (n=6), *Trigonosporomyces hylophilus* (n=6) and *Rhodosporidiobolus sphaerocarpa* (n=6) (where maximum of nucleotide diversity of 0.036 was also recorded).

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

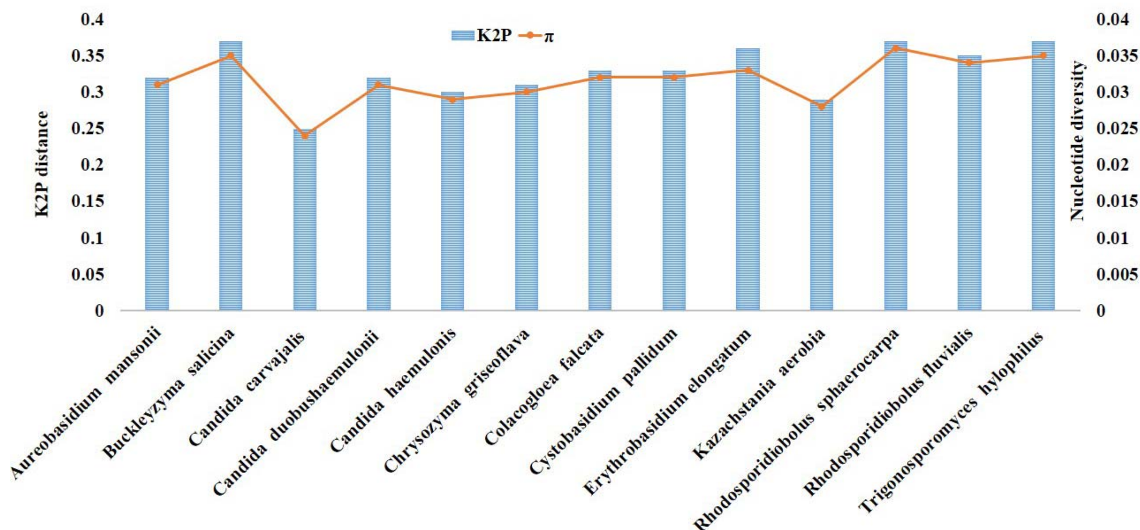


Fig. 2: Intra-species average K2P distance and nucleotide diversity (π) of ITS gene sequences of 13 species barcoded for the first time.

The overall intra-species K2P distance average was 0.33% which was well below the threshold proposed for the yeast species (1.59%) (Vu et al., 2016). Nucleotide diversity values directly proportionated the K2P distances (Fig. 2).

3.3.Tree based identification

Since the tree based identification was not feasible for all 157 species and requires the construction of mega tress which will be difficult to curate. Randomly selected genera in phylum Ascomycota (*Cryptococcus* spp.) and Basidomycota (*Colacogloea* spp.) were thus used to evaluate the tree based identification. We used a maximum of 3 sequences per species generated in this study belonging to 5 selective species against the corresponding available GenBank reference sequences to create NJ tree of *Cryptococcus* spp. All selected species in the *Cryptococcus* spp. genera, precisely clustered its corresponding reference sequences in one clade (**Fig. 3**). This indicates the efficacy of ITS gene sequences in delineating yeast species. The overall mean K2P pairwise distance of *Cryptococcus* spp. genera was 0.7% which is well within the proposed yeast species cut off (1.59%) (Vu et al., 2016). Tree based *Colacogloea* spp. identification reveals that the individual species clusters the in one clade along with its corresponding GenBank reference sequence (**Fig. 4**). The overall kimura-2 parametric distance was 1.4% which is well within the cut-off value proposed by Vu et al. (2016).

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Table 2: List of marine derived yeast species first time barcoded with their respective species authority and corresponding accession numbers in GenBank

No.	Family and species	Authority	Acc. Nos.
1	Family: Buckleyzymaceae *Buckleyzyma salicina*	B.N. Johri & Bandoni	KJ706369, KJ706528, J706687, KJ706846, KJ707005, KJ707163
2	Family: Chrysozymaceae *Chrysozyma griseoflava*	Nakase & M. Suzuki	KJ706363, KJ706522, J706681, KJ706840, KJ706999, KJ707157
3	*Trigonosporomyces hylophilus*	Van der Walt, D.B. Scott & Klift	KJ706323, KJ706482, J706641, KJ706800, KJ706959, KJ707117
4	Family: Cystobasidiaceae *Cystobasidium pallidum*	Lodder	KJ706324, KJ706483, J706642, KJ706801, KJ706960, KJ707118
5	Family: Erythrobasidiaceae *Erythrobasidium elongatum*	R.G. Shivas & Rodr. Mir.	KJ706362, KJ706521,KJ706680, KJ706839, KJ706998, KJ707156
6	Family: Heterogastridiaceae *Colacogloea falcata*	Nakase, Itoh & M. Suzuki	KJ706367, KJ706526,KJ706685, KJ706844, KJ707003, KJ707161
7	Family: Saccharomycetaceae *Candida carvajalis*	S.A. James, E.J. Carvajal, C.J. Bond, K. Cross, N.C. Núñez	KJ706236, KJ706395,KJ706554, KJ706713, KJ706872,KJ707031, KJ707176, KJ707185,KJ707194, KJ707203, KJ707212,KJ707221, KJ707230
8	*Candida duobushaemulonii*	Cend.-Bueno, Kolecka, Alastr.-Izq., Gómez-López, Cuenc.-Estr. & Boekhout	KJ706233, KJ706392,KJ706551, KJ706710, KJ706869, KJ707028
9	*Candida haemulonis*	Uden & Kolip.	KJ706229, KJ706388,KJ706547, KJ706706, KJ706865, KJ707024
10	*Kazachstania aerobia*	F.Y. Bai & Y.M. Cai	KJ706291, KJ706450,KJ706609, KJ706768, KJ706927, KJ707085
11	Family: Saccotheciaceae *Aureobasidium mansonii*	Castell.	KJ706380, KJ706539,KJ706698, KJ706857, KJ707016,KJ707174, KJ707183, KJ707192,KJ707201, KJ707210, KJ707219,KJ707228
12	Family: Sporidiobolaceae *Rhodosporidiobolus fluvialis*	Fell, Kurtzman, Tallman & J.D. Buck	KJ706317, KJ706476,KJ706635, KJ706794, KJ706953, KJ707111
13	*Rhodosporidiobolus sphaerocarpa*	S.Y. Newell & Fell	KJ706316, KJ706475,KJ706634, KJ706793, KJ706952, KJ707110

3.4. New occurrences of marine derived yeast species

Only 25.5% (n=40) of the isolated marine yeast species in this study were previously isolated form marine environment. Example; *Sterigmatomyces halophilus* was known to improve marine fish immunity (Reyes-Becerril et al., 2017). *Yarrowia lipolytica* has been known for

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

its crude oil degradation capability (Hassanshahian et al., 2012) and for its dimorphic growth when it is especially isolated from oil polluted seawater (Zinjarde et al., 1998).

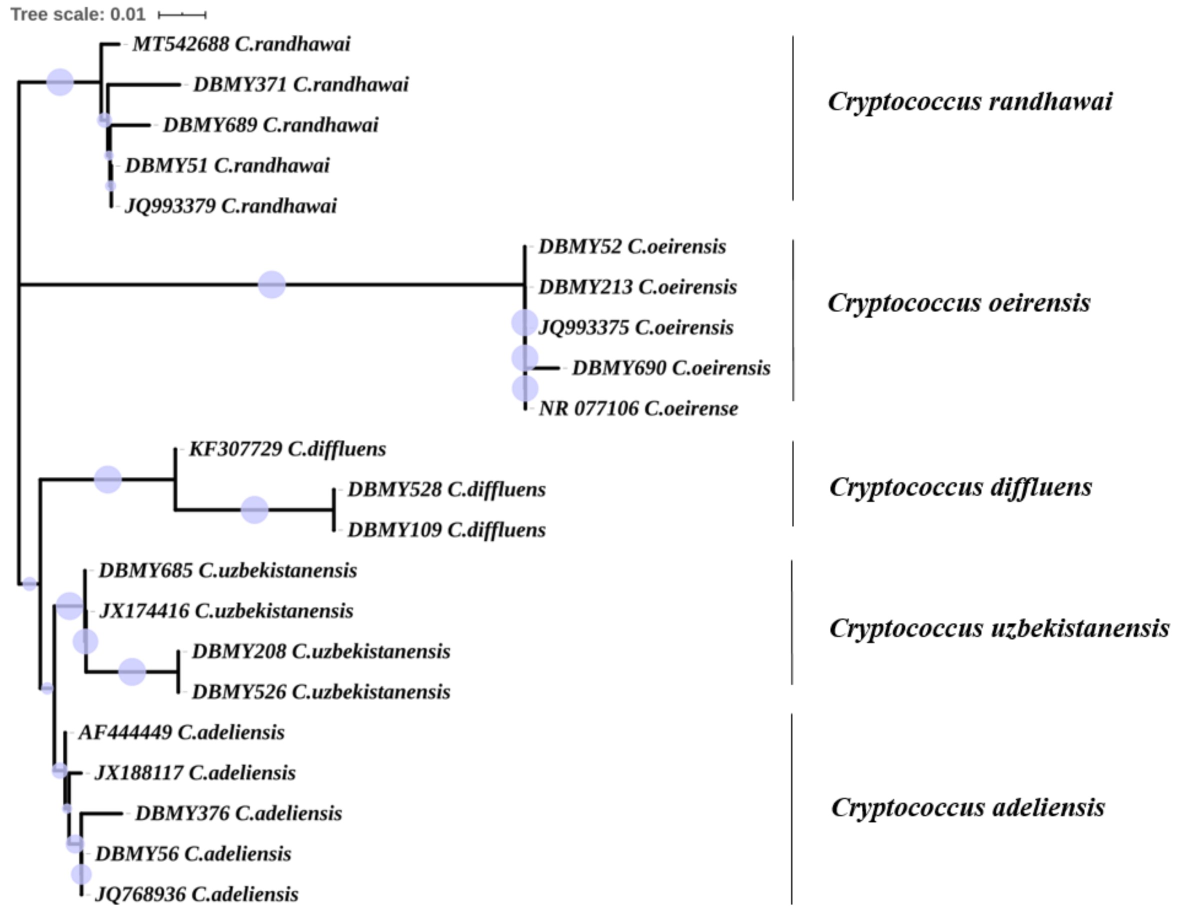


Fig. 3: NJ tree based identification of *Cryptococcus* spp. spp. (phylum: Ascomycota, class: Saccharomycetes). The acronym "DBMY" denotes the sequences produced in the present study. The acronym other "DBMY" denotes the accession number of GenBank reference sequences.

Another species isolated in this study, *Candida oceani*, first isolated from hydrothermal vents in Atlantic (Burgaud et al., 2011), was noted for its ability to withstand high hydro-static pressure (Burgaud et al., 2015). *Bandonia marina* reclassified from *Candida marina* (Liu et al., 2015), first isolated from the marine environment (Van Uden and Zobell, 1962), was also recognized for its hydrocarbonoclastic potential (Itah and Essien, 2005), and was previously isolated from tar balls obtained from the northwest coastal waters of India (Shinde et al., 2017). Other species (~58%; n=91) recorded in this study were either reported to occur in soil, plants and animals (including insects) and their presence in the

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

marine environment was previously unrecorded. Approximately 16.6% of the yeast species isolated in this study were previously reported as potential human pathogens (n=26 species).

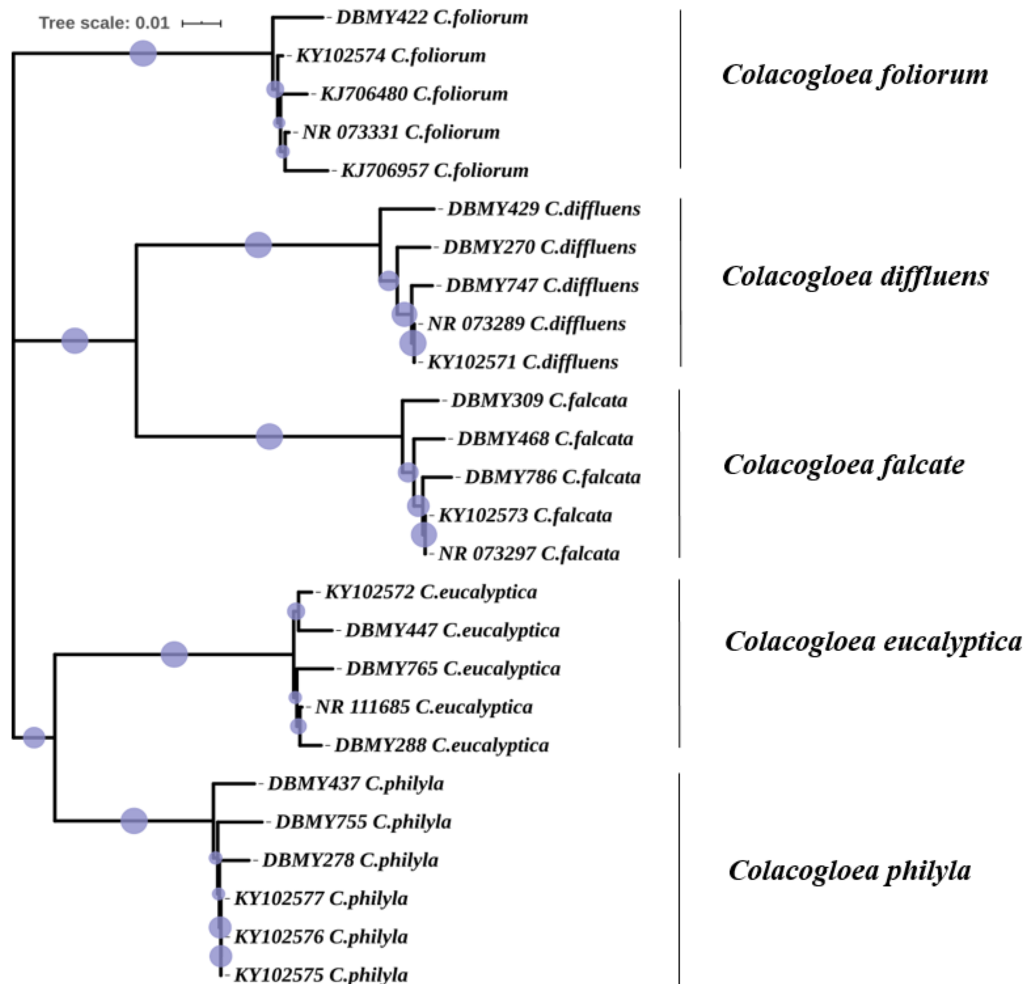


Fig. 4: NJ tree based identification of *Colacogloea* spp. (phylum: Basidiomycota, class: Microbotryomycetes). The acronym "DMBY" denotes the sequences produced in the present study.

3.4.1. **Yeast species previously unrecorded from marine environment**

Approximately 60% of the yeast species (n=94) reported in this study were previously unrecorded in the marine environment. Since it would be exhaustive to describe the previous source of occurrences of 94 yeast species, we present a few examples as follows. *Cystobasidiopsis lactophilus* has been reclassified from *Sporobolomyces lactophilus* (Wang et al., 2015), previously isolated from phyllosphere of the coniferous trees (Nakase et al., 1990). Previously known to occur in forest soils (Mašínová et al., 2018), *Oberwinklerozyma yarrowii* reclassified from *Rhodotorula silvestris* (Wang et al., 2015) was first recorded to

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

occur in mangrove sediments. River run off could be an important medium of transport to occur in mangrove sediment. *Slooffia cresolica* reclassified from *Rhodotorula cresolica* (Wang et al., 2015) was considered to be a part of soil microbiome (Middelhoven and Spaaij, 1997) and its abundances was also correlated with soils with high oil contaminations (Csutak et al., 2005). Formerly known for high hydrocarbon levels (Lyla et al., 2012) are the continental shelf sediments from which these strains were isolated in the present study. Similarly, in the present study *Candida catenulata* previously isolated from polluted sites with hydrocarbon (Habibi et al., 2017; Babaei et al., 2018) was also isolated.

First isolated from tree associated beetles (van der Walt et al., 1971), is *Trigonosporomyces hylophilus* reclassified from *Candida hylophila* (Wang et al., 2015) and their isolation from the mangrove sediments in this study suggests their potential occurrences in mangrove habitat related insects. *Udeniozyma ferulica* and *Vonarxula javanica*, reclassified from *Rhodotorula ferulica* and *Rhodotorula ferulica*, respectively (Wang et al., 2015) were known to occur in polluted river waters (Sampaio and Van Uden, 1991). River run offs could be the reason for these species to occur in mangrove and continental shelf sediments.

The fact that *Debaryomyces mycophilus* was first isolated from wood lice (Thanh et al., 2002) opens the possibility that this species may also occur in mangrove habitat related insects, as the genetic materials of insect could be obtained and studied from the sediment of its habitat (Thomsen et al., 2009). *Debaryomyces pseudopolymorphus* has been extensively involved in the wine fermentation and associated processes (Potgieter, 2004; Villena et al., 2006; Arevalo-Villena et al., 2007). *D. pseudopolymorphus* isolation and its function in the mangrove environment is new and unknown. *Scheffersomyces shehatae* has been known to occur in degrading woods (Kordowska-Wiater et al., 2017) and in wood digesting insects (Suh et al., 2013) was also commonly used for the production of bio-ethanol (Tanimura et al., 2015; Kordowska-Wiater et al., 2017). The association of *S. shehatae* with mangroves and its associated insects could be further explored. *Colacogloea falcatus* reclassified from *Sporobolomyces falcatus* (Wang et al., 2015) were first isolated from dead plant leaves (Nakase et al., 1987). Also, they were isolated from plants phylosphere (Nakase et al., 2003; Takashima and Nakase, 2000) and acidic soils (Delavat et al., 2013). Though ubiquitous distribution trends of certain yeast taxa in decomposing leaves were known (Sampaio et al., 2007), their presence in sediments of mangrove and continental shelf was unknown until now.

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Pichia guilliermondii has widespread occurrences such as plant endophytes (Zhao et al., 2010), citrus fruit flora (Arras et al., 1998), beetle associated (Suh et al., 1998), and in sewage sludge (de Silóniz et al., 2002). Therefore their isolation in this study may be correlated with multiple sources. *Kazachstania aerobia*, first isolated from plants (Magalhaes et al., 2011) and latter recognised as plant associated yeast (Lu et al., 2004) was unknown to occur in mangrove related habitats until now. *Sporobolomyces koalae* was first isolated from koalas bear (Satoh and Makimura, 2008), and found in other animals such as horses (Fomina et al., 2016) were unknown to occur in marine related habitats until now.

3.4.2. Potential human pathogenic yeasts

Of 157 species isolated in this study, 26 yeast species were recognised as potential human pathogens (**Table S3**). *Candida* spp. contributes 42.3% (n=11 species), followed by *Cryptococcus* spp. (19.2%; n=5). While few human pathogens (such as *Candida orthopsilosis, C. viswanathii, Rhodotorula mucilaginosa* and *Sterigmatomyces elviae*) isolated from this study was previously reported in marine environments (Li et al., 2010; Zaky et al., 2016; Pinheiro et al., 2018; Rasmey et al., 2020), species such as *Candida dubliniensis, C. duobushaemulonii , C. haemulonii, C. nivariensis, C. parapsilosis, C. rugosa, Debaryomyces fabryi* and *Yamadazyma triangularis* were obligate human pathogens (Gasparoto et al., 2009; Ramos et al., 2018; Gade et al., 2020; Ben-Ami et al., 2017; KLi et al., 2014; Mesini et al., 2020; Mloka et al., 2020; Tafer et al., 2016; Kurtzman et al., 2011) with previously unknown occurrences outside of the human host.

4. Conclusion

Although the definition of species was not widely applicable (Wheeler and Meier, 2000), in particular for non-obligatory sexually reproductive species such as fungi, the identification of species is a key step for various biological fields such as ecology, agriculture, biotechnology and medicine to identify biological interactions, for example; biodiversity assessment, bioremediation and pathology (de Queiroz, 2007). There could be ~3.8 million unknown fungal species (Hawksworth and Lücking 2017) and the environmental selection pressure plays a crucial role in new species evolution (Handelsman, 2004, Hibbett, 2016; Gabaldón, 2020). The present study was first of its kind in exploring large scale of marine environments for culturable species of yeast. As a result, 1017 barcodes of 157 marine yeast species were produced, of which 91 barcodes of 13 species was barcoded for the first time. This study recorded terrestrial yeast species introduced into the marine environment

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

(ex., *Cystobasidiopsis lactophilus, Oberwinklerozyma yarrowii*) and marine endemic species whose occurrences was restricted to specific marine ecosystem (Ex., *Bandonia marina, Candida oceani*). The DNA barcodes have been published via GenBank and BOLD databases for public use, which will also improve the yeast species barcode coverage and taxonomy in the public databases. The barcodes published in this study (on 2014, in GenBank), have already proved worthy in identifying majority yeast species in previous studies (Vu et al., 2016). These DNA barcodes can also help identify and estimate marine yeast diversity from environmental samples, as many metagenomic diversity studies suffers from lack of local species barcode library (Hawksworth, 2001; Handelsman, 2004; Hibbett, 2016). The entire DNA barcode set produced from this study could be exclusively accessed at http://dx.doi.org/10.5883/DS-MYIC.

Our next challenge will be to explore the synthetic biology, biochemistry, clinical and industrial potential of the isolated strains by venturing into new marine environments with continuous expansion of the barcode databases, as marine derived yeast species were known for many unique features (Guaragnella et al., 2013; Zaky et al., 2014; Dai et al., 2014; Deparis et al., 2017; Zhang et al., 2017; Yashiroda & Yoshida, 2019; Kumar & Kumar, 2019). This may be the largest DNA barcode dataset for culturable marine yeast species. The yeast barcode data produced may be used to explore taxonomic distribution of specific physiological traits (ex., theromotolerance), species of climate and pathological significance (Robert et al. 2015). Correlation of the yeast barcode data with other traits such as the ability to produce various metabolites and industrial products of biotechnological significance (example, antibiotics) would be a valuable resource for yeast researchers willing to apply DNA barcoding technology beyond taxonomic and identification applications.

Acknowledgement

First author thanks the Department of Science and Technology's INSPIRE Fellowship (IF10431), India and China Postdoctoral Research Foundation's National Postdoctoral fellowship (0050-K83008), China for the financial assistance. The authors are thankful for the ship time (FORV-Sagar Sampada) provided by the Centre for Marine Living Resources and Ecology, Ministry of Earth Sciences, Kochi, Government of India for sampling continental shelf sediments under Benthic Productivity program. The encouragement and support provided by Lyla P S and Ajmal Khan S is acknowledged. The authors gratefully

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

acknowledges the Faculty of Pharmacy, and Chiang Mai University, Chiang Mai, Thailand. The research was partially supported by Chiang Mai University.

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

5. References

Alappatt, J. P. (2008). Structure and Species Diversity of Mangrove Ecosystem. In Biodiversity and Climate Change Adaptation in Tropical Islands (pp. 127–144). Elsevier. https://doi.org/10.1016/b978-0-12-813064-3.00005-3

Alongi, D. M. Carbon Cycling and Storage in Mangrove Forests. Annu. Rev. Mar. Sci. 6, 195–219 (2014).

Altschul SF, Madden TL, Sch€affer AA, et al. (1997). Gapped blast and psi-blast: a new generation of protein database search programs. Nucleic Acids Research 25: 3389–3402.

Arevalo-Villena, M., Ubeda Iranzo, J., & Briones Perez, A. (2007). Enhancement of aroma in white wines using a β-glucosidase preparation from Debaryomyces pseudopolymorphus (A-77). Food Biotechnology, 21(2), 181-194.

Arras, G., Cicco, V. D., Arru, S., & Lima, G. (1998). Biocontrol by yeasts of blue mould of citrus fruits and the mode of action of an isolate of Pichia guilliermondii. The Journal of Horticultural Science and Biotechnology, 73(3), 413-418.

Babaei, F., & Habibi, A. (2018). Fast biodegradation of diesel hydrocarbons at high concentration by the sophorolipid-producing yeast candida catenulata KP324968. Journal of molecular microbiology and biotechnology, 28(5), 240-254.

Bidartondo MI (2008). Preserving accuracy in Genbank. Science 319: 1616.

Burgaud, G., Arzur, D., Sampaio, J. P., & Barbier, G. (2011). Candida oceani sp. nov., a novel yeast isolated from a Mid-Atlantic Ridge hydrothermal vent (– 2300 meters). Antonie Van Leeuwenhoek, 100(1), 75-82.

Burgaud, G., Hué, N. T. M., Arzur, D., Coton, M., Perrier-Cornet, J. M., Jebbar, M., & Barbier, G. (2015). Effects of hydrostatic pressure on yeasts isolated from deep-sea hydrothermal vents. Research in microbiology, 166(9), 700-709.

Chi, E.A., 2012. Production, characterization and gene cloning of the extracellular enzymes from the marine-derived yeasts and their potential applications. Anton. Leeuw. Int. J. Gen. Mol. Microbiol. 3, 411–417.

Csutak, O., Ghindea, R., Stoica, I., Soare, S., Ionescu, R., Creangã, O., & Vassu, T. (2005). Preliminary identification and classification of five new yeast strains isolated from oil-polluted environment. Romanian Archives, 10, 65.

Dai, Z., Liu, Y., Guo, J., Huang, L., & Zhang, X. (2014). Yeast synthetic biology for high-value metabolites. FEMS Yeast Research, n/a-n/a. https://doi.org/10.1111/1567-1364.12187

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Das, S., Lyla, P. S., & Khan, S. A. (2009). Filamentous fungal population and species diversity from the continental slope of Bay of Bengal, India. Acta Oecologica, 35(2), 269–279. https://doi.org/10.1016/j.actao.2008.11.003

de Haas, H., van Weering, T. C. E., & de Stigter, H. (2002). Organic carbon in shelf seas: sinks or sources, processes and products. Continental Shelf Research, 22(5), 691–717. https://doi.org/10.1016/s0278-4343(01)00093-0

de Queiroz K (2007). Species concepts and species delimitation. Systematic Biology 56: 879–886.

de Silóniz, M. I., Balsalobre, L., Alba, C., Valderrama, M. J., & Peinado, J. M. (2002). Feasibility of copper uptake by the yeast Pichia guilliermondii isolated from sewage sludge. Research in microbiology, 153(3), 173-180.

Delavat, F., Lett, M. C., & Lièvremont, D. (2013). Yeast and bacterial diversity along a transect in an acidic, As–Fe rich environment revealed by cultural approaches. Science of the total environment, 463, 823-828.

Deparis, Q., Claes, A., Foulquié-Moreno, M. R., & Thevelein, J. M. (2017). Engineering tolerance to industrially relevant stress factors in yeast cell factories. FEMS Yeast Research, 17(4). https://doi.org/10.1093/femsyr/fox036

Fell, J. W. (2012). Yeasts in marine environments. In Marine Fungi and Fungal-like Organisms. DE GRUYTER. https://doi.org/10.1515/9783110264067.91

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Fischer B, Brebeck C. Zur Morphologie, Biologie und Systematik der Kahmpilze, der Monilia candida Hansen und des Soorerregers C. Jena: Fischer, 1894 :1 – 52

Fomina, M. A., Polishchuk, L. V., Tkachenko, K. S., Hong, J. W., Zelena, L. B., Ianieva, O. D., & Pidgorskyi, V. S. (2016). Complex identification of red yeast isolate from gastrointestinal tract of Hucul long-liver (Carpathians, Ukraine). Мікробіологічний журнал, (78,№ 5), 2-11.

Gabaldón, T. (2020). Hybridization and the origin of new yeast lineages. FEMS Yeast Research, 20(5). https://doi.org/10.1093/femsyr/foaa040

Gadanho, M., & Sampaio, J. (2004). Application of temperature gradient gel electrophoresis to the study of yeast diversity in the estuary of the Tagus river, Portugal. FEMS Yeast Research, 5(3), 253–261. https://doi.org/10.1016/j.femsyr.2004.09.001

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Gadanho, M., & Sampaio, J. P. (2005). Occurrence and Diversity of Yeasts in the Mid-Atlantic Ridge Hydrothermal Fields Near the Azores Archipelago. Microbial Ecology, 50(3), 408–417. https://doi.org/10.1007/s00248-005-0195-y

Giri, C. Status and distribution of mangrove forests of the world using earth observation satellite data. Global Ecol. Biogeogr. 20, 154–159 (2011).

Godson, P.S., Chandrasekar, N., Kumar, S.K. et al. Microbial diversity in coastal sediments during pre- and post-tsunami periods in the south east coast of India. Front. Biol. 9, 161–167 (2014). https://doi.org/10.1007/s11515-014-1296-0

Guaragnella, N., Palermo, V., Galli, A., Moro, L., Mazzoni, C., & Giannattasio, S. (2013). The expanding role of yeast in cancer research and diagnosis: insights into the function of the oncosuppressors p53 and BRCA1/2. FEMS Yeast Research, 14(1), 2–16. https://doi.org/10.1111/1567-1364.12094

Gulis, V., Kuehn, K. A., & Suberkropp, K. (2009). Fungi. In Encyclopedia of Inland Waters (pp. 233–243). Elsevier. https://doi.org/10.1016/b978-012370626-3.00129-0

Habibi, A., & Babaei, F. (2017). Biological Treatment of Real Oilfield-Produced Water by Bioaugmentation with Sophorolipid-Producing Candida catenulata. Environmental Processes, 4(4), 891-906.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Handelsman J (2004). Metagenomics: Application of Genomics to Uncultured Microorganisms. Microbiology and Molecular Biology Reviews 68: 669–685.

Hassanshahian, M., Tebyanian, H., & Cappello, S. (2012). Isolation and characterization of two crude oil-degrading yeast strains, Yarrowia lipolytica PG-20 and PG-32, from the Persian Gulf. Marine pollution bulletin, 64(7), 1386-1391.

Hawksworth DL (2001). The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycological Research 105: 1422–1432.

Hawksworth DL, Lücking R (2017). Fungal diversity revisited: 2.2 to 3.8 million species. Microbiology Spectrum 5: 1–2.

Hebert PH, Cywinska A, Ball DL, et al. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences 270: 313–321.

Hedges, J. I., & Keil, R. G. (1995). Sedimentary organic matter preservation: an assessment and speculative synthesis. Marine Chemistry, 49(2–3), 81–115. https://doi.org/10.1016/0304-4203(95)00008-f

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Hibbett D (2016). The invisible dimension of fungal diversity. Science 351(6278): 1150–1151.

Hogarth PJ (1999) The Biology of Mangroves. Oxford University Press, Oxford.

Itah, A. Y., & Essien, J. P. (2005). Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the Bight of Bonny, Nigeria. World Journal of Microbiology and Biotechnology, 21(6-7), 1317-1322.

Jones, E.B.G., Alias, S., Pang, K.L., 2013. Distribution of marine fungi and fungus-like organisms in the South China Sea and their potential use in industry and pharmaceutical application. Malaysian Journal of Science 32, 95–106.

Jones, E.B.G., Pang, K.L., 2012. Tropical aquatic fungi. Biodivers. Conserv. 21, 2403–2423.

Kathiresan K Bingham BL (2001) Biology of mangroves and mangrove ecosystems. Adv Mar Bio40: 81–251.

Khan, S. A., Ansari, K. G. M. T., & Lyla, P. S. (2012). Organic matter content of sediments in continental shelf area of southeast coast of India. Environmental Monitoring and Assessment, 184(12), 7247–7256. https://doi.org/10.1007/s10661-011-2494-8

Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120.

Kordowska-Wiater, M., Kuzdraliński, A., Czernecki, T., Targoński, Z., Frąc, M., & Oszust, K. (2017). The ability of a novel strain Scheffersomyces (syn. Candida) shehatae isolated from rotten wood to produce arabitol. Polish Journal of Microbiology, 66(3), 335-343.

Kosawa da Costa, A.N., Sanchez-Nunez, M.L., van Weerelt, M.D.M., Fonseca, G.V., Mendoca-Hagler, L.C., Hagler, A.N., 1991. Microbial quality of the mussel Perna perna (linne, 1758) extracted from Guanabara bay, Rio de Janeiro. Brazil. Coastal Zone 4, 3259–3267.

Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.

Kumar, R., & Kumar, P. (2019). Yeast-based vaccines: New perspective in vaccine development and application. FEMS Yeast Research, 19(2). https://doi.org/10.1093/femsyr/foz007

Kurtzman, C. P., Mateo, R. Q., Kolecka, A., Theelen, B., Robert, V., & Boekhout, T. (2015). Advances in yeast systematics and phylogeny and their use as predictors of

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

biotechnologically important metabolic pathways. FEMS Yeast Research, 15(6), fov050. https://doi.org/10.1093/femsyr/fov050

Kurtzman, C., Fell, J. W., & Boekhout, T. (Eds.). (2011). The yeasts: a taxonomic study. Elsevier.

Kutty, S.N., Philip, R., 2008. Marine Yeasts - A Review. pp. 465–483.

Kutzman, C.P., Fell, J.W., 2015. The Yeasts. Statewide Agricultural Land Use Baseline 2015 1.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). CLUSTAL W and CLUSTAL X version 2.0. Bioinformatics 23, 2947–2948.

Lee, S. Y., Primavera, J. H., Dahdouh-Guebas, F., McKee, K., Bosire, J. O., Cannicci, S., Diele, K., Fromard, F., Koedam, N., Marchand, C., Mendelssohn, I., Mukherjee, N., & Record, S. (2014). Ecological role and services of tropical mangrove ecosystems: a reassessment. Global Ecology and Biogeography, 23(7), 726–743. https://doi.org/10.1111/geb.12155

Letunic, I., and Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Research, 47(W1), W256–W259. https://doi.org/10.1093/nar/gkz239

Li, J., Shan, Y., Fan, S., & Liu, X. (2014). Prevalence of Candida nivariensis and Candida bracarensis in vulvovaginal candidiasis. Mycopathologia, 178(3-4), 279-283.

Liu, X.-Z., Wang, Q.-M., Göker, M., Groenewald, M., Kachalkin, A. V., Lumbsch, H. T., Millanes, A. M., Wedin, M., Yurkov, A. M., Boekhout, T., & Bai, F.-Y. (2015). Towards an integrated phylogenetic classification of the Tremellomycetes. Studies in Mycology, 81, 85–147. https://doi.org/10.1016/j.simyco.2015.12.001

Lu, H. Z., Cai, Y., Wu, Z. W., Jia, J. H., & Bai, F. Y. (2004). Kazachstania aerobia sp. nov., an ascomycetous yeast species from aerobically deteriorating corn silage. International journal of systematic and evolutionary microbiology, 54(6), 2431-2435.

Lyla, S., Manokaran, S., & Khan, A. (2012). Petroleum hydrocarbon distribution in continental shelf region of southeast coast of India. International Journal of Sediment Research, 27(1), 73–83. https://doi.org/10.1016/s1001-6279(12)60017-1

M Rasmey, A. H., A Aboseidah, A., M Khalaf, E. S., H Ahmed, M., & Hawary, H. (2020). Kinetic models application on glycerol production from glucose by the marine yeast Candida orthopsilosis. Egyptian Journal of Aquatic Biology and Fisheries, 24(1), 1-12.

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Magalhaes, K.T., Pereira, G.V.M., Campos, C.R., Dragone, G. & Schwan, R.F. (2011). Brazilian kefir: structure, microbial communities and chemical composition. Brazilian Journal of Microbiology. 42: 693-702.

Mašínová, T., Yurkov, A., & Baldrian, P. (2018). Forest soil yeasts: Decomposition potential and the utilization of carbon sources. Fungal Ecology, 34, 10-19.

Middelhoven, W. J., & Spaaij, F. (1997). Rhodotorula cresolica sp. nov., a cresol-assimilating yeast species isolated from soil. International Journal of Systematic and Evolutionary Microbiology, 47(2), 324-327.

Mohanty P.K., Pradhan Y., Nayak S.R., Panda U.S., Mohapatra G.N. (2008) Sediment Dispersion in the Bay of Bengal. In: Mohanty P.K. (eds) Monitoring and Modelling Lakes and Coastal Environments. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-6646-7_5

Mokhtarnejad, L., Arzanlou, M., Babai-Ahari, A., Di Mauro, S., Onofri, A., Buzzini, P., & Turchetti, B. (2016). Characterization of basidiomycetous yeasts in hypersaline soils of the Urmia Lake National Park, Iran. Extremophiles, 20(6), 915-928.

Nakase, T., Itoh, M., & Suzuki, M. (1987). Sporobolomyces falcatus sp. nov., isolated from a dead leaf of Miscanthus sinensis in Japan. Transactions of the Mycological Society of Japan (Japan).

Nakase, T., Itoh, M., Suzuki, M., & Bandoni, R. J. (1990). Sporobolomyces lactophilus sp. nov., a ballistospore-forming yeast isolated from Abies firma collected in Japan. Transactions of the Mycological Society of Japan (Japan).

Nakase, T., Tsuzuki, S., Lee, F. L., Sugita, T., Jindamorakot, S., Jan-ngam, H., ... & Takashima, M. (2003). Sporobolomyces magnisporus sp. nov., a new yeast species in the Erythrobasidium cluster isolated from plants in Taiwan. The Journal of general and applied microbiology, 49(6), 337-344.

Pagnocca, F.G., Mendonça-Hagler, L.C., Hagler, A.N., 1989. Yeasts associated with the white shrimp Penaeus schmitti, sediment, and water of Sepetiba Bay, Rio de Janeiro, Brasil. Yeast 5 (Spec No, S479–483).

Potgieter, N. (2004). The evaluation of β-glucosidase activity produced by wine-isolated yeasts (Doctoral dissertation, Stellenbosch: Stellenbosch University).

Raja, H. A., Shearer, C. A., & Tsui, C. K.-M. (2018). Freshwater Fungi. In eLS (pp. 1–13). John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470015902.a0027210

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Reyes-Becerril, M., Guluarte, C., Ceballos-Francisco, D., Angulo, C., & Esteban, M. Á. (2017). Dietary yeast Sterigmatomyces halophilus enhances mucosal immunity of gilthead seabream (Sparus aurata L.). Fish & Shellfish Immunology, 64, 165-175.

Robert V, Cardinali G, Casadevall A (2015). Distribution and impact of yeast thermal tolerance permissive for mammalian infection. BMC Biology 13: 1–14. http://dx.doi.org/10.1186/s12915-015-0127-3.

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sampaio, A., Sampaio, J. P., & LeÃ£o, C. (2007). Dynamics of yeast populations recovered from decaying leaves in a nonpolluted stream: a 2-year study on the effects of leaf litter type and decomposition time. FEMS Yeast Research, 7(4), 595–603. https://doi.org/10.1111/j.1567-1364.2007.00218.x

Sampaio, J. P., & Van Uden, N. (1991). Rhodotorula ferulica sp. nov., a yeast that degrades ferulic acid and other phenolic compounds. Systematic and applied microbiology, 14(2), 146-149.

Satoh, K., & Makimura, K. (2008). Sporobolomyces koalae sp. nov., a basidiomycetous yeast isolated from nasal smears of Queensland koalas kept in a Japanese zoological park. International journal of systematic and evolutionary microbiology, 58(12), 2983-2986.

Schmidt JP, and Shearer CA (2004) Geographic and host distribution of lignicolous mangrove microfungi. Bot Mar47: 496–500.

Schoch C, Seifert KA, Huhndorf S, et al. (2012). The nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proceedings of the National Academy of Sciences of the United States of America 109: 6241–6246.

Shearer C.A., Descals E., Kohlmeyer B., Kohlmeyer J., Marvanová L., Padgett D., Porter D., Raja H.A., Schmit J.P., Thorton H.A., et al. Fuangal diversity in aquatic habitats. Biodivers. Conserv. 2007; 16:49–67. doi: 10.1007/s10531-006-9120-z.

Shinde, V. L., Suneel, V., & Shenoy, B. D. (2017). Diversity of bacteria and fungi associated with tarballs: recent developments and future prospects. Marine Pollution Bulletin, 117(1-2), 28-33.

Spencer, J. F. T., & Spencer, D. M. (1997). Ecology: Where Yeasts Live. Yeasts in Natural and Artificial Habitats, 33–58. doi:10.1007/978-3-662-03370-8_4

Statzell-Tallman, A., Belloch, C., & Fell, J. W. (2008). Kwoniella mangroviensisgen. nov., sp. nov. (Tremellales,Basidiomycota), a teleomorphic yeast from mangrove habitats in the

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Florida Everglades and Bahamas. FEMS Yeast Research, 8(1), 103–113. https://doi.org/10.1111/j.1567-1364.2007.00314.x

Suh, S. O., & Blackwell, M. (2004). Three new beetle-associated yeast species in the Pichia guilliermondii clade. FEMS Yeast Research, 5(1), 87-95.

Suh, S. O., Houseknecht, J. L., Gujjari, P., & Zhou, J. J. (2013). Scheffersomyces parashehatae fa, sp. nov., Scheffersomyces xylosifermentans fa, sp. nov., Candida broadrunensis sp. nov. and Candida manassasensis sp. nov., novel yeasts associated with wood-ingesting insects, and their ecological and biofuel implications. International journal of systematic and evolutionary microbiology, 63(11), 4330-4339.

Takashima, M., & Nakase, T. (2000). Four new species of the genusSporobolomyces isolated from leaves in Thailand. Mycoscience, 41(4), 357-369.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30, 2725–2729.

Tanimura, A., Kikukawa, M., Yamaguchi, S., Kishino, S., Ogawa, J., & Shima, J. (2015). Direct ethanol production from starch using a natural isolate, Scheffersomyces shehatae: toward consolidated bioprocessing. Scientific reports, 5, 9593.

Tekolo, O. M., Mckenzie, J., Botha, A., & Prior, B. A. (2010). The osmotic stress tolerance of basidiomycetous yeasts. FEMS Yeast Research, 10(4), 482–491. https://doi.org/10.1111/j.1567-1364.2010.00612.x

Thanh, V. N., Van Dyk, M. S., & Wingfield, M. J. (2002). Debaryomyces mycophilus sp. nov., a siderophore-dependent yeast isolated from woodlice. FEMS yeast research, 2(3), 415-427.

Thomsen, P. F., Elias, S., Gilbert, M. T. P., Haile, J., Munch, K., Kuzmina, S., Froese, D. G., Sher, A., Holdaway, R. N., & Willerslev, E. (2009). Non-Destructive Sampling of Ancient Insect DNA. PLoS ONE, 4(4), e5048. https://doi.org/10.1371/journal.pone.0005048

van der Walt JP, Scott DB, van der Klift WC (1971) Four new, related Candida species from South African insect sources. Antonie Van Leeuwenhoek 37:449–460

Van Uden, N., & Zobell, C. E. (1962). Candida Marina nov. spec., Torulopsis torresii nov. spec. andT. maris nov. spec., three yeasts from the Torres Strait. Antonie van Leeuwenhoek, 28(1), 275-283.

Velmurugan, S., Prasannakumar, C., Manokaran, S., Ajith Kumar, T., Samkamaleson, A., & Palavesam, A. (2013). DNA barcodes for marine fungal identification and discovery. Fungal Ecology, 6(5), 408–418. https://doi.org/10.1016/j.funeco.2013.05.003

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Villena, M. A., Iranzo, J. Ú., & Briones Pérez, A. (2006). Relationship between Debaryomyces pseudopolymorphus enzymatic extracts and release of terpenes in wine. Biotechnology progress, 22(2), 375-381.

Vu, D., Groenewald, M., de Vries, M., Gehrmann, T., Stielow, B., Eberhardt, U., Al-Hatmi, A., Groenewald, J. Z., Cardinali, G., Houbraken, J., Boekhout, T., Crous, P. W., Robert, V., & Verkley, G. J. M. (2019). Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. Studies in Mycology, 92, 135–154. https://doi.org/10.1016/j.simyco.2018.05.001

Vu, D., Groenewald, M., Szöke, S., Cardinali, G., Eberhardt, U., Stielow, B., de Vries, M., Verkleij, G. J. M., Crous, P. W., Boekhout, T., & Robert, V. (2016). DNA barcoding analysis of more than 9□000 yeast isolates contributes to quantitative thresholds for yeast species and genera delimitation. Studies in Mycology, 85, 91–105. https://doi.org/10.1016/j.simyco.2016.11.007

Wang, Q. M., Yurkov, A. M., Lumbsch, H. T., Leavitt, S. D., Groenewald, M., Theelen, B., ... & Bai, F. Y. (2015). Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. Studies in mycology, 81, 149-189.

Wheeler DQ, Meier R (2000). Species concepts and phylogenetic theory: a debate. Columbia University Press.

White, T. J., Bruns, T. D., Lee, S. & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: a Guide to Methods and Applications, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. Sninsky & T. J. White. San Diego: Academic press.

Yarrow D (1998) Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman CP, Fell JW (eds) The yeasts, a taxonomic study, 4th edn. Elsevier, Amsterdam, pp 77–100

Yashiroda, Y., & Yoshida, M. (2019). Intraspecies cell–cell communication in yeast. FEMS Yeast Research, 19(7). https://doi.org/10.1093/femsyr/foz071

Zaky, A. S., Tucker, G. A., Daw, Z. Y., & Du, C. (2014). Marine yeast isolation and industrial application. FEMS Yeast Research, 14(6), 813–825. https://doi.org/10.1111/1567-1364.12158

Zhang, Y., Nielsen, J., & Liu, Z. (2017). Engineering yeast metabolism for production of terpenoids for use as perfume ingredients, pharmaceuticals and biofuels. FEMS Yeast Research, 17(8). https://doi.org/10.1093/femsyr/fox080

bioRxiv preprint doi: https://doi.org/10.1101/2020.08.29.273490; this version posted October 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Zhao, J., Mou, Y., Shan, T., Li, Y., Zhou, L., Wang, M., & Wang, J. (2010). Antimicrobial metabolites from the endophytic fungus Pichia guilliermondii isolated from Paris polyphylla var. yunnanensis. Molecules, 15(11), 7961-7970.

Zinjarde, S. S., Pant, A., & Deshpande, M. V. (1998). Dimorphic transition in Yarrowia lipolytica isolated from oil-polluted sea water. Mycological research, 102(5), 553-558.

ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/341622119

EFFICACY OF ALLIUM CEPA AND ALLIUM SATIVUM AGAINST DERMATOPHYTES

Article · May 2020

CITATION
1

READS
506

4 authors, including:

S Muthu Raj
K L University

9 PUBLICATIONS **2** CITATIONS

SEE PROFILE

All content following this page was uploaded by S Muthu Raj on 25 May 2020.

The user has requested enhancement of the downloaded file.

EFFICACY OF ALLIUM CEPA AND ALLIUM SATIVUM AGAINST DERMATOPHYTES

M.Mohammed Latheef[a] , S.Muthu Raj[a*] , MAbdul Salam[a] , Ragavi[a] , and K.Chitra[a]

PG and Research Department of Microbiology Sadakathullah Appa College(Autonomous)

Tirunelveli-627011

***Corresponding Author:muthurajmicrobiology@gmail.com**

Abstract: Medicinal plants such as Allium cepa L. (Onion) and Allium sativum (Garlic) have become the focus of intense study regarding to gather conservation and potential pharmacological effects. Allium cepa L. (Onion) which belongs to the family Alliaceae is also known as 'garden onion' or 'bulb' onion (Yin & Cheng, 1998). Allium sativum (Garlic) has been used as herbal medicine in world wide and it help prevent various diseases and disorders. Allicin is the main biologically active component present in freshly crushed garlic cloves it is an antioxidant compounds found to have been health-protecting factor. Primary sources of antioxidants Allicin compounds are naturally present in whole grains, fruits and vegetables (Lawson et al., 1991). In the present study, two important medicinal plants namely Allium cepa (Onion) and Allium sativum (Garlic), assessed the phytochemical components and antifungal activity against skin pathogens with different concentration and combination.

Keywords: Allium cepa, Allium sativum, Allicin, Alliaceae

I. Introduction:

Allium cepa L. (Onion):

About 80% of individuals developed countries used traditional medicine, which has compound derived being medicinal plants. Medicinal plants such as *Allium cepa* L. (Onion) and *Allium sativum* (Garlic) have become the focus of intense study regarding to gather conservation and potential pharmacological effects. *Allium cepa* L. (Onion) which belongs to the family *Alliaceae* is also known as 'garden onion' or 'bulb' onion (Yin & Cheng, 1998). It is an oldest cultivated vegetables in history. The bulbs from in onion family have being utilized as a food source for millennia. The bulb grows underground and is used for energy storage. The leaves are bluish green and hollow, the bulbs are large, fleshly and firm. Three main varieties of onion are available red, white and purple skinned. Onions are easily propagated, transported and stored (Nivas et al., 2006).

Allium sativum (Garlic):

Allium sativum (Garlic) has been used as herbal medicine in world wide and it help prevent various diseases and disorders. Allicin is the main biologically active component present in freshly crushed garlic cloves it is an antioxidant compounds found to have been health-protecting factor.

Primary sources of antioxidants Allicin compounds are naturally present in whole grains, fruits and vegetables (Lawson et al ., 1991). All of the background research involving allicin has been done by either garlic powder from different manufactures or processing the garlic cloves through the many different chemicals in order to obtain allicin. It is produced by the interaction of non-protein amino acid allin with used enzyme alliinase (Ponnulakshmi *et al.*, 2013).

Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Garlic has traditional dietary and medicinal applications as anti-infective agent.

In vitro evidence of the antimicrobial activity of fresh and freeze-dried garlic extract against many bacteria , fungi, virus (Rahman M.M et al., 2012).

II. Materials and Method:

In the present study, two important medicinal plants namely *Allium cepa* (Onion) and *Allium sativum*(Garlic), assessed the phytochemical components and antifungal activity against skin pathogens with different concentration and combination.

The following objectives were carried out in this study:

- The organic solvents viz., Petroleum ether, Chloroform, Methanol, Acetone, Ethyl acetate, water was used for the extraction of plant material.
- The extracts were screened for the presence of phytochemical compounds and also test antifungal activity against *Aspergillus* spp, *Mucor* spp *Altertaria* spp, and *Culvularia* sp

The selected medicinal plants used in different concentration with different combinations to analyze the antibiogram activity.

Collection plant materials:

The plant materials, red onion and white garlic were selected for the investigation of antimicrobial activities. Fresh Onion and Garlic were procured from the local market in Tirunelveli Dt, Tamil Nadu, India.

III. Extraction Plant Materials:

i) Aqueous extract:

Fresh onions and garlic were peeled of the outer layer and 1 kg of onion and garlic washed thoroughly a distilled water and the bulb has cut the pieces and was made into a crude paste. The paste was soaked in 1litre of sterile distilled water for 24 hours at 4ºC and it was then filtered thrice using a sterile muslin cloth. The filtrate was poured into a beaker and concentrated on a water bath at 100°c to obtain semi-solid residues and they aqueous extract was weighted and this was immediately subjected to preliminary phyto-chemical and antifungal analysis using standard method (Ponnulakshmi *et al*., 2013).

ii) **Ethanolic extract :**

After cleaning 1kg of onion and garlic as described earlier they were made into a paste was soaked in 500ml of ethanol for 15 days at room temperature than it were filtered using sterile muslin cloth and the filtrate was poured into a beaker and concentrated on a water bath at 70-80ºC to obtain semi-solid residue. The weight of the yield was noted and this was subjected in preliminary phyto-chemical and antifungal analysis using standard method.(Mazhood Khan *et al.,* 2012)

iii) **Chloroform extract**:

After making a paste of 1 kg onion and garlic of different varieties as described earlier, they were separately soaked in 300 ml of chloroform for a week at room temperature the filtered using sterile muslin cloth and filtrate was concentrated in a beaker at 60-62°C to obtain semi-solid residue. This have weighted and subjected to preliminary phyto-chemical and antifungal analysis.(Prakash s. et al., 2016)

iv) **Petroleum ether extract:**

Following the earlier procedure, onion and garlic bulb of different varieties was prepared and soaked in 200ml of petroleum ether for 15 days at room temperature. It was filtered and the filtrate we concentrated at 40-60ºc. The extract were weighted and subjected to preliminary phyto-chemical and antifungal analysis standard method. (Gulsen Gonagul *et al* 2010)

Test Pathogens:

Antifungal activities of aqueous, ethanol, chloroform, petroleum ether extracts of various *A.cepa* and *A.sativum* varieties small onion and garlic were studied. Ketoconazole was used as standard drug. The microorganisms, maintained on Potato Dextrose agar. (Four species of fungi, *Aspergillus* spp, *Mucor* spp *Altertaria spp,* and *Culvularia* sp were used in study.

Processing of Clinical Sample:

The skin scraping is collected from wound infected patients. The sample was emulsed in saline water.

Isolation:

The skin scrapping was further transfer to Nutrient agar plate for isolation of bacteria. The skin scrapping was inoculated with Potato Dextrose agar for the isolation of Fungi.

Selective Medium:

The isolated organisms of *Staphylococcus* sp, were streaked on the selective medium of Mannitol Salt agar. The isolated organisms of *Bacillus* sp, were streaked on the selective medium of Nutrient agar. The isolated organisms of *Pseudomonas* sp, were streaked on the selective medium of King's medium.

IV. Phytochemical Anasysis

a. **Determination of Water content:**

Water content of ***A.cepa*** and *A.sativum* determined using a method of a Thermal drying method was used in a determination in water content of the samples. 10g bulbs of different ***A.cepa*** and *A.sativum* varieties were weighted crucible. This was placed in oven and dried at 105ºC (Hot air oven) for 3 hours. The samples were allowed to cool in a desiccators and then reweighted**.** The percentage of water content has calculated can be expressing he loss the weight on drying as a fraction of the initial weight of sample used and multiple by 100.

b.**Determination of Total ash:**

A known weight of varieties dry bulb which to has and the weight of there by obtained a expressed can be terms of percentage. In a clean crucible, three varieties of *A.cepa* and *A.sativum* dry bulb as taken and weighted. Weighted dish was placed over the tripod stand carefully. The crucible were opened partially and directed to the tip of the flame for gradual heating at 500°c. The onion and garlic samples were heated gently to avoid catching fire. When the smoke subsides the burner was placed the dish. Then the dish was cooled to room temperature and weighted with the contents. Again the sample was heated to effect for any possible and weighted. The process was repeated three consecutive weighing and complete combustion was taken. The total was then determined and recorded. The percentage of an content has calculated using the formula:

$$Ash(\%) = Ma/Ms \times 100$$

Where Má = mass of ash (g) and Mś =Mass of sample used (g)

c.**Determination of pH**:

pH of different *A.cepa* and *A.sativum* varieties were determined per the method of park and the pH values of *A.cepa* were measured. *A.cepa* and *A.sativum* varieties were homogenized with 90 ml of sterile double distilled water, after which, pH values were, measured five times for its concurrency and expressed in average values.

d. **Phytochemical screening**:

The various solvent extracts of powder of leaves of *Leucas asper* were subjected to the phytochemical test for the identification of various active constituents, using the method followed by Malcom and Sofowora ,1969.

i) Test for tannis:

About 2ml of the aqueous extract was mixed with 2ml of distilled water and few drops of Fe Cl_3 solution were added.

ii) Test for saponins:

3ml of the aqueous extract shaken vigorously with 3ml of distilled water in a test tube and warmed.

ii) Test for phlobatannins:

2ml of aqueous extract was added and 2ml of 1%HCl and the mixture was boiled.

iii) Test for flavonoids:

5ml of aqueous extract was added and 1ml of $H_2 SO_4$ added. Few minutes wait and observe the result.

iv) Test for Terpenoids:

2ml of aqueous extract was dissolved in 2ml of chloroform and evaporate it. Then 2ml of concentrated $H_2 SO_4$ was added and heated for about 2min.

v) Test for Glycosides:

2ml of aqueous extract was dissolved in 2ml of chloroform and 2ml of acetic acid was added in it. The solution was cooled in ice. Then $H_2 SO_4$ was added carefully. Then observe the result.

vi) Test for Steroids:

2ml of extract was dissolved in 2ml of chloroform and 2ml of concentrated $H_2 SO_4$ was added and observe the result.

vii) Test for Phenols:

1ml of aqueous extract were dissolved in 5ml of alcohol and treaded with few drops of $Fecl_3$ solution.

viii) Test for Protein and Aminoacids:

2ml of plant extract dissolved in 2ml of water. Change the colour indicates the presence of protein.

ix) Test for Alkaloids:

5ml of plant extract was mixed with 1% HCl. The solution obtained was filtered and then 1ml of filtrate was treated with few drops of Mayer's reagent. Formation of turbidity or cream precipitate indicates presence of alkaloids.

x) Test for Carbohydrates:

1ml of aqueous extract was added and few drops of molischs reagent was added, and then few minutes wait. Then few drops of concentrated $H_2 SO_4$ was added and heated for about 2minutes.

xi) Test for Quinins:

1ml of aqueous extract was added and 1ml of $H_2 SO_4$ $_4$ added, few minutes wait and observe the result.

xii) Test for Glycosides:

1ml of aqueous extract was added and 2ml of glacial acetic acid wait for few minutes. Then few drops of 5% ferric chloride was added. Then added 1ml of $H_2 SO_4$ added. Few minutes wait and observe the result.

xiii) Test for coumarin:

1ml of aqueous extract was added and 1ml of 10% of NAOH added, few minutes wait and observe the result.

xiv) Test for Triterpeniods:

1.5ml of aqueous extract was added and 1ml of libeman buchard reagent was added. Then added 1ml of concentrated $H_2 SO_4$ added. And then added few minutes.

xv) Test for anthraquinones:

1ml of aqueous extract was added and few drops of 10% Ammonia solution.

V. RESULTS:

In the present investigation *Allium cepa* and *A.sativum* collected from the Local Markets Tirunelveli Dt., Collected samples processed and analysed their Phytochemical characters. Table 1 show that Flavonoids. Coumanis are positive for the five extracts and Anthrequinones is negative.

Table:1 Phytochemical results for *Allium cepa*

S.No	Chemical Constituents	Petroleum ether	Chloroform	Methanol (H_2O)	Ethyl acetate	Aqueous
1	Carbohydrates	-	+	+	-	-
2	Tannins	-	-	+	_	_
3	Saponins	-	-	_	_	_
4	Flavonoids	+	+	+	+	+
5	Alkaloids	+	+	+	_	+
6	Quinins	-	+	+	+	_
7	Glycosides	-	-	_	_	_
8	Cardiac glycosides	+	+	+	+	+
9	Terpenoids	+	-	+	_	_
10	Phenols	+	-	_	_	_
11	Coumanis	+	+	+	+	+
12	Steroids	-	-	-	+	_
13	Phlobatannins	-	-	-	+	-
14	Triterpenoids	+	+	+	+	-
15	Proteins	+	+	-	+	-
16	Anthrequinones	-	-	-	-	-

Table 2 shows that phytochemical characters of *Allium sativum* from that Coumanis and Cardiac glycosides and Flavonoids shows positive results followed by Triterpenoids, proteins and Anthroquinones showed negative results

Table: 2. Phytochemical results for *Allium sativum*

S.No	Chemical Constituents	Petroleum ether	chloroform	Methanol (H_2O)	Ethyl acetate	Aqueous
1	Carbohydrates	-	+	+	+	+
2	Tannins	-	-	+	_	_
3	Saponins	-	-	_	_	_
4	Flavonoids	+	+	+	+	+
5	Alkaloids	+	+	+	_	+
6	Quinins	-	+	+	+	_
7	Glycosides	-	-	_	_	_
8	Cardiac glycosides	+	+	+	+	+

9	**Terpenoids**	+	-	+	_	_
10	**Phenols**	+	-	_	_	_
11	**Coumanis**	+	+	+	+	+
12	**Steroids**	-	-	-	+	_
13	**Phlobatannins**	-	-	-	+	-
14	**Triterpenoids**	+	+	+	+	-
15	**Proteins**	+	+	-	+	-
16	**Anthrequinones**	-	-	-	-	-

Table: 3 Antifungal activities of *Allium cepa* against *Aspergillus* sp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method (mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 4 Antifungal activities of *Allium cepa* against *Mucor* spp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 5 Antifungal activities of *Allium cepa* against *Altertaria* spp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9

3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 6 Antifungal activities of *Allium cepa* against *Culvularia* sp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 7 Antifungal activities of *Allium sativum* against *Aspergillus* sp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 8 Antifungal activities of *Allium sativum* against *Mucor* spp

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Table: 9 Antifungal activities of *Allium sativum* gainst *Altertaria spp*

S.NO	Solvents	Well diffusion method(mm)				Disc diffusion method(mm)			
		25µl	50µl	75µl	150µl	2.5µl	5µl	7.5µl	10µl
1	Petroleum ether	1.4	2	3.7	3.2	0.1	0.3	0.5	0.7
2	Chloroform	3.4	3.7	4	4.2	0.3	0.5	0.7	0.9
3	Ethanol	3.5	3.7	3.9	4.1	-	0.1	0.3	0.4
4	Aqueous	1.8	2.5	2.7	3	-	0.1	0.3	0.5

Figure 1: Phytochemical Analysis of *A.cep*

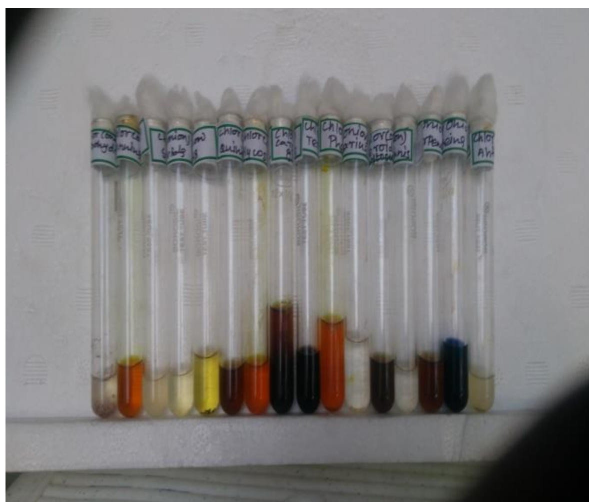
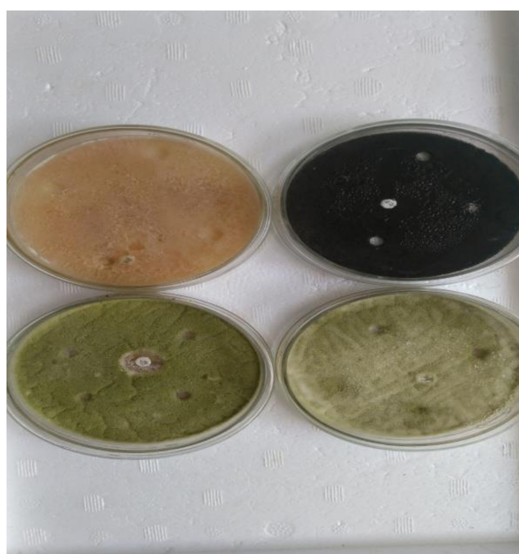


Figure 2: Phytochemical Analysis of *A.sativum*

Figure 3: Fungal pathogens



VI. DISCUSSION:

A.cepa and *A.sativum* extracts has been extensively studied for its antimicrobial activity against a wide range of fungal, and parasitic organisms. However, a limited data is available so far regarding its efficacy against *Aspergillus* spp. Both the extracts exhibited antifungal activity against aspergiius spp. The antifungal activity of purple type of *A.cepa* and *A.sativum* extracts was found to be better as compared to yellow type *of A.cepa* and *A.sativum* extract. Agar well diffusion method showed that EEP both the tested extracts had antifungal activity against *Aspergillus* spp than EEY demonstrated at 12. 5 % and showed no activity at 6.25%. our results are comparable with a study conducted by Nelson at al revealed that ethanolic extract of onion and garlic gave 11 mm zone of inhibition with MIC 0.8 mg /ml against *Mucor* spp and 9 mm of inhibition with MIC 0.8 mg/ml.8.9.In a study conducted by N.Benkebila in Algeria, red/ purple onion and garlic exhibited better antifungal activity as compared to yellow onion and garlic alternoria spp. The zone of inhibition of extracts. In study conducted by Mahash in India, antimicrobial activity of certain plants was evaluated using the disc diffusion method against certain fungi.The onion and garlic bulbs contain numerous organic sulphur compounds including sulfoxide, flavinoides, phenolic acids, sterols including cholesterol, saponins, sugars and a trace of volatile oil compounds may explain the anti - microbial activity of this plants.The phytochemical screening in the present, study, has revealed the presence of triterpenoids,

steroids, glycosides, flavonoids, tannins, carbohydrates and vitamin C in the seeds extract. Further the presence of different phytoconstituents in the four different extracts may be responsible for the therapeutic properties of onion and garlic.The preliminary phyto chemical screening tests may be useful in the detection of the bioactive components and subsequently may lead to the drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacological active chemical compounds.

References:

1. Adetumbi., G.T. Javor, and B.H .S. Lau.(1986). *Allium sativum* (garlic) inhibits lipid synthesis by *Candida albicans''*, *Antimicrob. Agents Chemother*, 30: 499 -501 .

2. Augusti K, (1996). Therapeutic values of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) *Indian J exp Biol*, 34: 634-640.

3. Azu NC, onyeagba RA, Nworie O, kalu J. (2007). Antibacterial Activity of Allium Cepa (onion) and zinger officinale (Ginger) on *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from high vaginal swab. Interent J Trop Med, 3(2) 234-239.

4. C.J. Cavallito, and J.H. Bailey (1994) 'Antibacterial principal of *Allium sativum*'' *J. Am. Chem, Soc*. 66: 1950,

5. EI-meleig, M., Ahme, R., Arafa, N. Ebrahim and EI Kholany., (2010) ''Cytotoxicity of four essential oils on some human and bacterial cells,'' *J. Appl, Sci. in Environ Sanit*., 5, 143 - 159.

6. Griffiths. L., Trueman,T. Crowther,B. Thomas and Smith, B (2002) ''Onions: a global benefit to health'', *Phyto Ther Res*., 16, 603 -615.

7. Hannan, T. Humayun, M. Yasir and Sikandar, S(2010) ''In vitro antibacterial activity of onion (*Allium cepa*) against clinical isolates of *vibro cholera*,'' *J Ayu. Med Coll* , 22 (2) 160 – 163.

8. Helen. K. Krishnakumar,P, Vijayammal. A and Augusti, K. (2000) ''Antioxidant effect of onion oil (*Allium cepa*. Linn) on the damages induced by nicotine in rats as compared to alpha tocopherol'', Toxicol *Lett*., 116, 61 - 68.

9. K.Augusti, (1996) ''Therapeutic values of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.)'', *Indian*. J. Exp. Boil, 34, 634 -640.

10. Lampe.J .(1999) "Health effects of vegetables and fruits: assessing mechanisms of action in human experimental studies,'' *J. Clin. Nutr* 70, 475, 90.

11. Lawson, L.D., Wang, Z. J and Hughes, B. (1991). Identification and HPLC quantization of the sulphides and thiosulpfinates in commercial garlic products. *Planta medica* 57 (4): 363 -70.

12. Lawson, L.D., Wood, S.G and Hughes, B. (1991). HPLC analysis of Allicin and other thiosulfinates in Garlic clove Homogenates. *Planta Medica* 57(3): 263 – 270.

13. Nemeth K, Piskula MK, (2007). Food content, processing, absorption and metabolism of onion flavonoids, Crit . Rev. Food. Sci.Nutr, 47(4): 397 – 409.

14. Parekhm P.J. and Chandam S.V. (2007) " In vitro antimicrobial activity and phytochemical analysis of some Indian medicinal plants'', *Turk J boil*., 31: 53 -58.

15. Rees, S.F. Minney, N. T.plummer, J.H. Slater, and Skyrme. D.A. (1993)'' A quantitative assessment of the antimicrobial activity of garlic'' (*Allium sativum*). *World J. Microbiol. Biotechnol*., 9: 303 – 307.

16. Rose. P Whiteman, M. Moore P. and Zhu Y.(2005). Bioactive cysteine sulfoxide metabolites in the genus *Allium* : the chemistry of potential therapeutic agent, Natural Product Rep, 22, 351 - 368.

17. Wang L, Lee IM, Zhang SM, (2009). Dietary intake of selected flavonoids, flavones, and flavonoid – rich foods and risk of cancer in middle aged and older women, *Am J Clin Nutr*, 89(3): 905 – 912.

18. Weber, D.O. Anderson, J.A. North, B.K. Murry, L. and Hughes, B.G. (1992) ''In vitro viricidal effects of *Allium sativum* (garlic) extract and compounds''. *Planta Med*., 58: 417 – 423.

19. Wilson E A, Dermming - Adams B,, (2007). Antioxidant, anti-inflammatory and antimicrobial properties of garlic and onions, Nutr Food Sci, 37(3): 178 -183.

20. Ying MC, Chang WS, (1998). Antioxidant activity of general *Allium* members. *J. Agri Food Chem*, 46: 4097-4101.

21. Zohri, K. Gwad, and Saber S (1995) ‘’Antibacterial, antidermatophytic and antioxigenic activities of onion (*Allium cepa*) oil’’, *Microbial*. *Res*., 150 , 167 -172.

View publication stats