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Isolation of Antimicrobial Compounds using Marine Bacteria from Nagapattinam Coast

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Abstract

At present situation in the pharma field, only some numbers of effective drugs are exist. The emergence of multidrug resistant pathogens must be necessary of novel antibiotics. Currently opportunistic pathogens like V.cholerae, S.typhi were resistant against vancomycin, ampicillin, methicillin, tetracycline etc. Marine resources are hopeful environment for new drug discoveries. Especially, marine microbes are played main role on discovery of antibiotics. In this study, marine sediment samples were used for isolation of marine bacterial colonies from Nagapattinam coast. Distinct colonies noted as DC and DN were isolated using nutrient agar and actinomycetes isolation agar and morphology, biochemical properties were performed. Antimicrobial susceptibility test were done against common clinical pathogens and multidrug resistant (MDR) pathogens. Among isolated colonies, DC5 has better bactericidal activity. It showed average results against all opportunistic pathogens. Used ethyl acetate extraction, it has more functional groups and analysed by Fourier Transformed Infrared spectroscopy (FT-IR).

Keywords: *Drugs, Vancomycin, Methicillin, MDR, FT-IR.*

Introduction

Due to increasing of multidrug resistant (MDR) pathogens, became crisis problem in the field of drug discovery and drug development. The potential for a major antibiotic healthcare crisis is best summarized by the Infectious Diseases Society of America (IDSA) and the European Centre for Disease Prevention and Control, both of which report that there are only a few potential drugs in clinical development (Butler *et al.*,, 2011). Common clinical pathogens are mutated their genetic structure and leads to change the protein productions against applicable antibiotics so it became to resistant (Maragakis *et al.*, 2008, Raghunath, 2008). Normally, Pro and Eukaryotic pathogenic organisms cause common diseases, among them fungi and yeast cause mycoses and nosocomial infection in human beings whereas bacterial pathogens cause typhoid, cholera etc. In other conditions, pathogens are resistant against major antibiotics such as cephalosporin, vancomycin, fluconazole, tetracyclin (Kumarasamy *et al.*, 2010; Messai *et al.*, 2008; Sekhsokh *et al.*, 2008). Tetracycline has been widely used in human and veterinary medicine, in food animal production for growth promotion and prophylaxis, and in horticulture. One of the derivatives, oxytetracycline is widely used in aquaculture systems to treat and prevent bacterial diseases of fish and other marine animals. This wide use of oxytetracycline, however, has increased the occurrence of tetracycline resistant fish pathogens. Presently, more than 40 different tetracycline resistance determinants have been reported (Seok-Ryel Kim *et al.*, 2004). So, there is a need for most potent novel antibiotics against MDR pathogens throughout the world.

Microbial natural products are having more applications for human health which includes organ transplantation, cancer treatment while, helping agriculture like biocontrol agents against plant pathogenic fungi (Doumbou *et al.*, 2002) and pharma industries like antibiotics (Stewart, 2012). Around 70% of earth is covered by marine environment (Valli *et al.*, 2012) and unexploited drugs and pharmacologically active substances still exist **(**Sivasubramanian *et al*, 2011). Marine habitat play a major role in human medicine especially marine microbes which synthesize numerous potent novel compounds or drugs (Waters *et al*., 2010; Simmons *et al*., 2008).

Bacterial pathogens such as gram positive and gram negative are present everywhere. Gram positive pathogens are resistant to some potent antibiotics in last two decades such as vancomycin and methicillin-resistant *Staphylococcus aureus*, the Gram negatives, underlined, in the list of ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter species*), are in many cases more of a pressing treatment challenge (Christopher *et al*., 2014).

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The International Centre for Diarrhoeal Disease Research, Bangladesh reported antimicrobial susceptibility patterns for *Shigella* and *Vibrio*, which are determined to inform clinicians about appropriate antibiotic treatment options. Underscoring the need for antimicrobial sensitivity testing is the emergence of MDR, defined here as isolates resistant to multiple drugs (Das *et al.,* 2013). *V. cholerae* has been reported to contain a distinct class of integrons, which permit it to acquire open reading frames and convert them to new functional genes. This implies that not only non-toxigenic strains can acquire virulence genes from the environment but acquisition of antibiotic resistance genes is also possible. Given that the massive use of antibiotics in prophylaxis during previous cholera outbreaks in Douala resulted in the selection of multidrug resistant strains of *V. cholerae.* Current data on antibiotic susceptibility pattern of isolates will update knowledge on appropriate antibiotics for use in empiric treatment in case of an outbreak (Akoachere *et al.*, 2013). Multidrug-resistant typhoid fever (MDRTF) is defined as typhoid fever caused by *Salmonella enterica serovar Typhi* strains (*S. typhi*), which are resistant to the first-line recommended drugs for treatment such as chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole. In Asia, almost 80% of people were suffered because of typhoid. Overall around 20 million people were affected (Rajiv kumar *et al,* 2007).

In vitro studies of marine bacterial pharmaceutical compounds, especially actinomycetes (Gram positive and filamentous bacteria) showed sufficient results against MDR strains and new diseases (Xiong *et al*., 2012). Marine bacteria produce numerous secondary metabolites, which are having distinct chemical structures with well biological activities (Spizek *et al.,* 2010). In Yellow sea - China, actinomycetes have produced efficient compounds against some clinical pathogens and marine sediment is the best way for invitro studies (Xiong *et al.,* 2014). In 2011 survey of five antibiotic candidates in phase III trials, the one synthetic molecule was a second-generation oxazolidinone torezolid phosphate. Another second-generation oxazolidinone, radezolid, is progressing through late stage trials (Christopher *et al*., 2014). At present situation, there is a need for high potent and multi functional novel antibiotics. The conceptual of this study is exploitation of effective marine drugs from marine bacterial colonies against clinical and MDR strains.

Materials and Methods

Sampling site

The sediment sample was collected from Nagapattinam coastal area using PVC pipe at two different distances. The samples were transferred to zip lock cover, kept in an icebox, and transferred to laboratory for further study.

Analysis of Habitat Conditions

Physico-chemical parameters such as pH (Digital pH Meter – ELICO -111), Electro Conductivity (Deluxe Conductivity Meter 601 - ELICO), Total alkalinity (Titration), Phosphate (Spectrophotometer), Nitrate (Titration), Sodium (Flame photometer), Potassium (Flame photometer), Calcium (Titration), Magnesium (Titration), Salinity (Refractometer), Total nitrogen (Titration) and Chloride (Titration) were analysed. For the measurement of pH, salinity and electro conductivity the dried sediment was mixed with sterile deionised water in the ratio of 1:5 (Trivedy *et al.,* 1987). All other parameters were analysed by the book of Practical methods in Ecology and Environmental science, Trivedy *et al.,* 1987.

Isolation

1g of sediment sample was ten-fold serially diluted using sterile deionised water. Prior to serial dilution, the collected samples were autoclaved @ 70^0C for 15 minutes to prevent any contaminants. The spread plate technique was applied for the isolation of marine bacterial colonies using Nutrient agar (HiMedia) and Actinomycetes isolation agar (HiMedia) by maintaining a neutral pH in the medium. Inoculated plates were incubated @ 30°C for 2-6 days. After proper incubation, different defined colonies were observed and separated from agar.

Biochemical Tests

Isolated colonies were further assigned to check the biochemical properties such as Gram's Staining – Slide Technique, Indole test – Broth Method, Triple Sugar Iron Test – Slant Technique and Citrate test – Broth Culture and MR-VP Test – Broth method.

Growth curve

After proper incubation, 100 ml conical flasks were taken which consisted of Nutrient broth and Actinomycetes isolation broth and 1ml of inoculum was added to each flasks. An extra flask containing two different broths without inoculum was noted as control. Control tube reading was taken using Spectrophotometer (ELICO -SL171 MINI SPEC) @ 600 nm. All the flask culture readings were taken at every four hours interval and OD values were noted. When the values started decreasing, the readings were stopped. Finally, using all values growth curve (Lag phase, Log phase, Stationary phase and Decline phase) was plotted (Figure.1 and 2). When the decline phase reached, sometime OD values repeated.

Antimicrobial Susceptibility Test

Isolated colonies were tested against pathogenic bacterial colonies such as *S.aureus* (NCIM 5021), *P. aeroginisa* (NCIM 5029), *B.subtilis* (NCIM 2920) and MDR *V.cholerae* (MTCC 3906), *S.typhi* (NCIM 2501). This screening test was done by well diffusion method using Mueller hinton agar (Bushra Uzair *et al.,* 2007).

These pathogens were resistant to Fluconazole antibiotic. This antibiotic test was carried out using commercial discs.

Mass Production of antimicrobial compounds

Five percentage of inoculum was prepared and transferred into the optimized fermentation medium (composition: D-glucose-20g; malt extract-40g; yeast extract-4g; Dipotassium hydrogen phosphate-5g; Sodium Chloride-2.5g; Zinc Sulphate-.04g; Calcium Carbonate-.4g; 1000ml sterile deionised water with pH 6.0) (Dasari *et al*., 2011) and then kept on shaker for 2-4 days. After that, fermented broth was centrifuged at 4000 rpm for 10 minutes and extracted twice-using equal amount of ethyl acetate.

Functional Group Analysis

The extracted compounds were lyophilized. The powder state compounds were checked functional groups by Fourier-Transform Infra-Red spectroscopy (Perkinelmer Spectrum – 10.03.09) and used KBr pelletizer. All measurements were carried out in the range of 400 – 4,000 cm-1 at a resolution of 4 cm-1.

Results

pH, Electrical conductivity and Salinity values were observed to be little high in DN site compared to DC site. Sodium, Potassium and Total Nitrogen values were low in DC site compared to DN site while the other parameters were high in DN site (Table.1). Ten distinct colonies were isolated from two different distances and noted as DC1 to 5 and DN1 to 5. In the order of DC1 to 5, yellow with white shadowed, unclear white, fragmented white, fragmented white and orange were their physical nature and in the order of DN1 to 5, yellow with white shadowed, pure yellow, unclear yellow with white shadowed, unclear yellow and unclear yellow with white shadowed were their physical nature. Most of the colonies were gram negative, indole test, and MR-VP test negative. DC5 was tentatively Streptomyces sp.

Among these colonies, the growth curve was measured using spectrophotometer. Each four hours time interval OD values were noted. On DC colonies, log and lag phase took 24.8 hours, stationary phase 16.8 hours and decline phase took 22.4 hours. DN colonies log and lag phase reached 28.8 hours, stationary phase 13.6 hours and decline phase 21.6 hours (Table.2 and Figure. 1,2). Some of the colonies cell divisions were found to be varied because of some environmental factors like temperature, humidity etc. Antimicrobial susceptibility test were done against clinical pathogens such as *S.aureus*, *B.subtilis* and *P.aeruginosa* and MDR *V.cholrae* and *S.typhi*. DN1,2,5 and DC1,5 showed moderate result against overall pathogens for the concentration of 150µg/ml. DC5 showed excellent result against *S.aureus.* DN2,5 and DC1,5 showed better results against *P.aeruginosa.* (Figure.3) DN1,4,5 and DC5 showed minimum result against *B.subtilis.* DN and DC5 having optimized bactericidal activity compared to other isolated colonies against common clinical pathogens. In the part of MDR, DN4, DC3 and 5 showed positive result against MDR *S.typhi* and DN4,5 and DC3,4,5 showed positive result against MDR *V.cholerae* (Figure.4). When compared to all other colonies, DC5 was found to have good activity against MDR colonies (Table.3,4). FT-IR absorption spectrum of the DC5 compound was shown in Figure.5. The compound has more functional groups with strong and medium level peaks. It consisted of more alkenes groups and remained aliphatic amines, aromatics etc.

Discussion

At current stage, multidrug resistant pathogens, action has increased due to insufficient and ineffective antibiotics. Some of the secondary metabolites such as antibiotics, enzymes from marine bacterial colonies are helpful to control the virulent of common clinical pathogens and almost having good results against MDR pathogens. In the last era, chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole were most usable drugs against bacterial infectious diseases. Nowadays numerous opportunistic pathogens became resistant against important drugs. Therefore, multi functional antibiotics are emergency needed. Due to this situation, *invivo* studies such as compound prediction, drug development are must be necessary for pharmaceutical applications. Once again proved, marine resources drugs were applicable against most common and MDR pathogens. Among isolated bacterial colonies, some of these only has inhibited the pathogenicity of opportunistic clinical pathogens especially DC5 strain. While observing habitat conditions, coastal area contamination was found to be high, thus leading to diseases. Therefore, awareness of coastal area pollution is also necessary.

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References

1. Bushra Uzair, Nuzhat Ahmed, Viqar Uddin Ahmad, Faryal Vali Mohammad & David H. Edwards (2008), The isolation, puri¢cationand biological activityofa novel antibacterial compound produced by *Pseudomonas stutzeri*, *FEMS Microbiol Lett* 279: 243–250.
2. Christopher T Walsh &Timothy A Wencewicz (2014), Prospects for new antibiotics: a molecule-centered perspective, *The Journal of Antibiotics* 67: 7–22.
3. Doumbou CL, Hamby Salove MK, Crawford DL & Beaulieu C (2001) Actinomycetes, promising tools to control plant diseases and to promote plant growth. *Phytoprotection* 82: 85–102.

4. Jane-Francis Tatah Kihla Akoachere, Thomas Njinuwoh Masalla & Henry Akum Njom (2013), Multi-drug resistant toxigenic *Vibrio cholera* O1 is persistent in water sources in New Bell-Douala, Cameroon, Akoachere et al. BMC Infectious Diseases 13:366.
5. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F & Balakrishnan R (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* 10: 597—602.
6. Maragakis LL, Perencevich EN & Cosgrove SE (2008) Clinical and economic burden of antimicrobial resistance. *Expert Rev Anti Infect Ther* 6: 751-763.
7. Mark S Butler & Matthew A Cooper (2011) Antibiotics in the clinical pipeline in 2011, *The Journal of Antibiotics* 64: 413–425.
8. Messai Y, Iabadene H, Benhassine T, Alouache S, Tazir M & Gautier V (2008) Prevalence and characterization of extended spectrum b-lactamases in Klebsiella pneumoniae in Algiers hospitals (Algeria). *Pathol Biol* 56:319—25.
9. Raghunath D (2008) Emerging antibiotic resistance in bacteria with special reference to India. *J Biosci* 33: 593-603.
10. Rajiv Kumar, Nomeeta Gupta & Shalini (2007), Multidrug – Resistant Typhoid Fever, *Indian journal of Pediatrics* 74, 39-42.
11. Seok-Ryel Kim, Lisa Nonaka & Satoru Suzuki (2004), Occurrence of tetracycline resistance genes tet (M) and tet (S) in bacteria from marine aquaculture sites *FEMS Microbiol Lett* 237: 147–156.
12. Shuvankar Ballav, Savita Kerkar, Sabu Thomas & Nimmy Augustine (2014), Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites, *Journal of Bioscience and Bioengineering*, 1-8.
13. Simmons TL, Coates RC, Clark BR, Engene N, Gonzalez D, Esquenazi E, Dorrestein PC & Gerwick WH (2008) Biosynthetic origin of natural products isolated from marine microorganism–invertebrate assemblages. *Proc Natl Acad Sci USA* 105:4587–4594.
14. Sivasubramanian K, Ravichandran S & Vijayapriya M (2011) Antagonistic activity of marine bacteria Pseudoalteromonas tunicata against microbial pathogens. *Afr J Microbiol Res* 5: 562-567.
15. Spízek J, Novotn J, Rezanka T & Demain LA (2010) Do we need new antibiotics? The search for new targets. *J Ind Microbiol Biotechnol*; 37:1241-1248.
16. Stewart EJ (2012) Growing unculturable bacteria. *J Bacteriol* 194:4151–4160.
17. Sumon Kumar Das, Erik H. Klontz, Ishrat J. Azmi, Abu I. M. S. Ud-Din, Mohammod Jobayer Chisti, Mokibul Hassan Afrad, Mohammad AbdulMalek, Shahnawaz Ahmed, Jui Das, Kaisar Ali Talukder *et al.*, (2008) Characteristics of Multidrug Resistant Shigella and Vibrio cholerae O1 Infections in Patients Treated at an Urban and a Rural Hospital in Bangladesh, *Hindawi Publishing Corporation*, 8.
18. Trivedy RK, Goel PK & Trisal CL (1987) Practical methods in Ecology and Environmental Science. *Enviro Media Publications*, Series in methodology – 2.
19. Valli S, Suvathi Sugasini S, Aysha OS, Nirmala P, Vinoth Kumar P & Reena A. (2012) Antimicrobial potential of Actinomycetes species isolated from marine environment. *Asian Pac J Trop Biomed* 2: 469-473.
20. Waters AL, Hill RT, Place AR & Hamann MT (2010) The expanding role of marine microbes in pharmaceutical development. *Curr Opin Biotechnol* 21:780–786.
21. Xiong ZQ, Zhang ZP, Li JH, Wei SJ & Tu GQ (2012) Characterization of Streptomyces padanus JAU4234, a producer of actinomycin X2, fungichromin, and a new polyene macrolide antibiotic. *Appl Environ Microbiol* 78:589–592.
22. Zhi Qiang Xiong, Qiao Xia Liu, Zhao Long Pan, Na Zhao, Zhi Xiang Feng & Yong Wang (2014), Diversity and bioprospecting of culturable actinomycetes from marine sediment of the Yellow Sea, China, *Arch Microbiol*.

Tables

Table.1. Physico-chemical parameters analysis from sediment sample.

Colonies	**Log and Lag Phase (Hrs)**	**Stationary Phase (Hrs)**	**Decline Phase (Hrs)**
DC1	28	16	20
DC2	36	16	12
DC3	24	16	24
DC4	12	20	32
DC5	24	16	24
Average	**24.8**	**16.8**	**22.4**
DN1	36	12	16
DN2	28	16	20
DN3	36	12	16
DN4	28	12	24
DN5	16	16	32
Average	**28.8**	**13.6**	**21.6**

Table.2. Incubation analysis of isolated colonies at 600nm.

Parameters (Units)	**DC**	**DN**
pH	8.07	8.12
EC (µmhos.cm-1)	86	89
Salinity (ppt)	35	37
PO4 (%)	0.7	0.3
NO3 (%)	4.5	3.3
Ca (%)	0.6	0.5
Mg (%)	0.3	0.2
Na (%)	92.9	128.1
K (%)	6.6	8.1
Tot. Nitrogen (%)	0.72	0.84
Tot. Alkalinity (meq/100g)	0.09	0.05
Chloride (%)	0.73	0.52

Table.3. Antimicrobial susceptibility test against clinical pathogens

Isolated colonies	**Common**			**MDR**	
	S.aureus	***P.aeruginosa***	***B.subtilis***	***V.cholerae***	***S.typhi***
DN 1	10	≤10	≤10	-	-
DN 2	12	12	-	-	-
DN 3	-	-	-	-	-
DN 4	10	≤10	≤10	≤10	≤10
DN 5	12	12	≤10	≤10	-
DC 1	11	12	-	-	-
DC 2	-	-	-	-	-
DC 3	12	≤10	-	≤10	**13**
DC 4	≤10	11	-	**13**	-
DC 5	**16**	**12**	**≤10**	**≤10**	**11**

Table 4. Status of antibiotic resistant clinical pathogens

Pathogens Antibiotics/mcg	***S.aureus***	***P.aeruginosa***	***V.cholerae***	***S.typhi***
Penicillin (10)	+	+	+	+
Methicillin (10)	+	+	-	-
Vancomycin (10)	+	+	-	-
Cloxacilin (10)	+	+	-	-
Tetracycline (10)	+	-	-	+

Figure. 1. Growth curve of DN1-5 colonies.

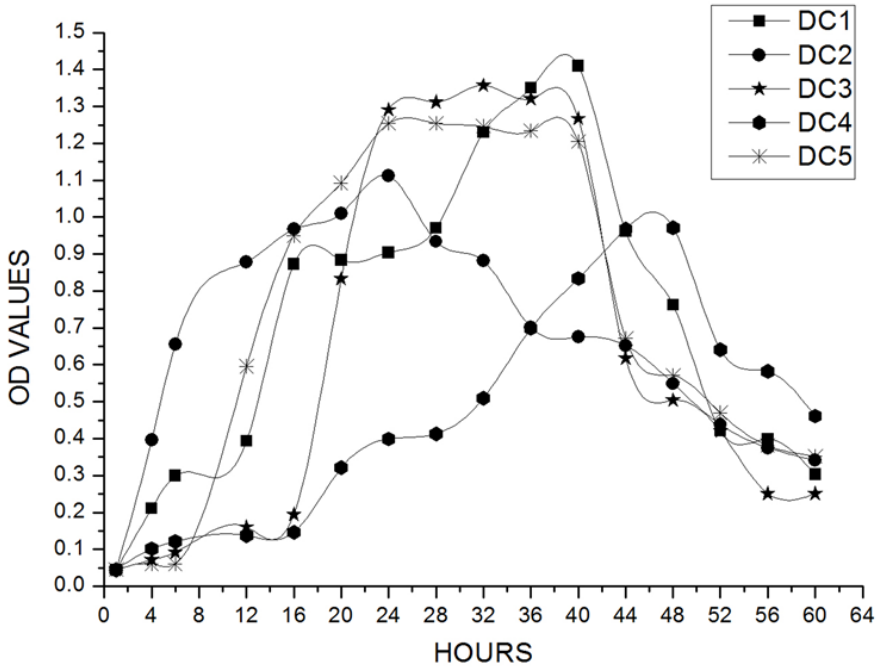

Figure.2. Growth curve of DN1-5 colonies.

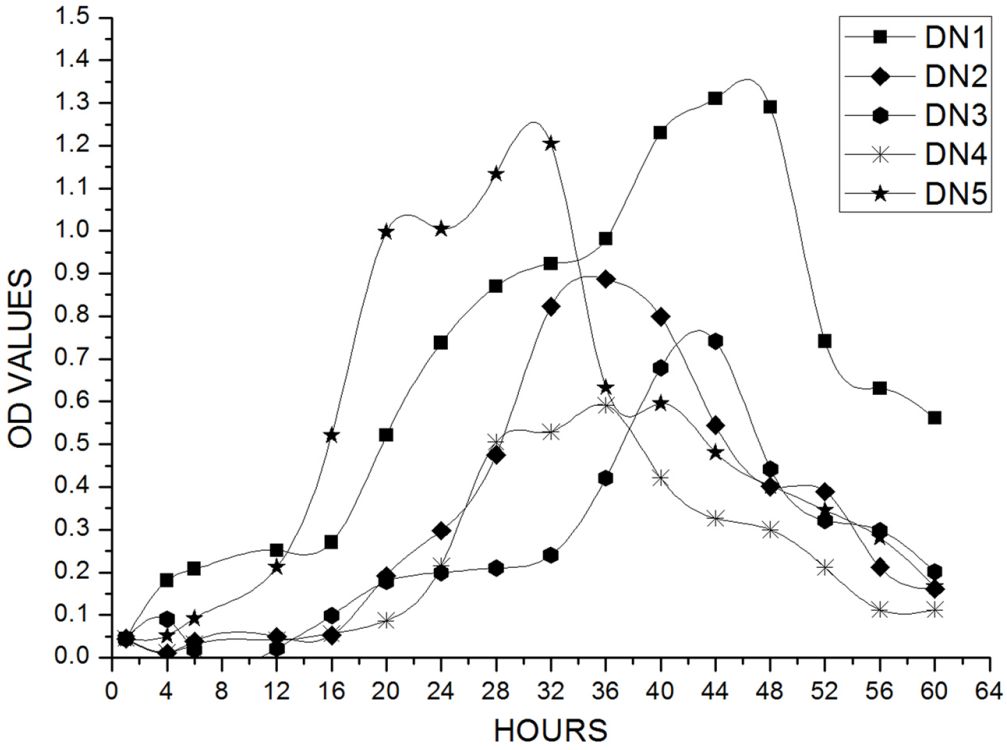

Figure3. Antimicrobial Susceptibility Test against *S.aureus*

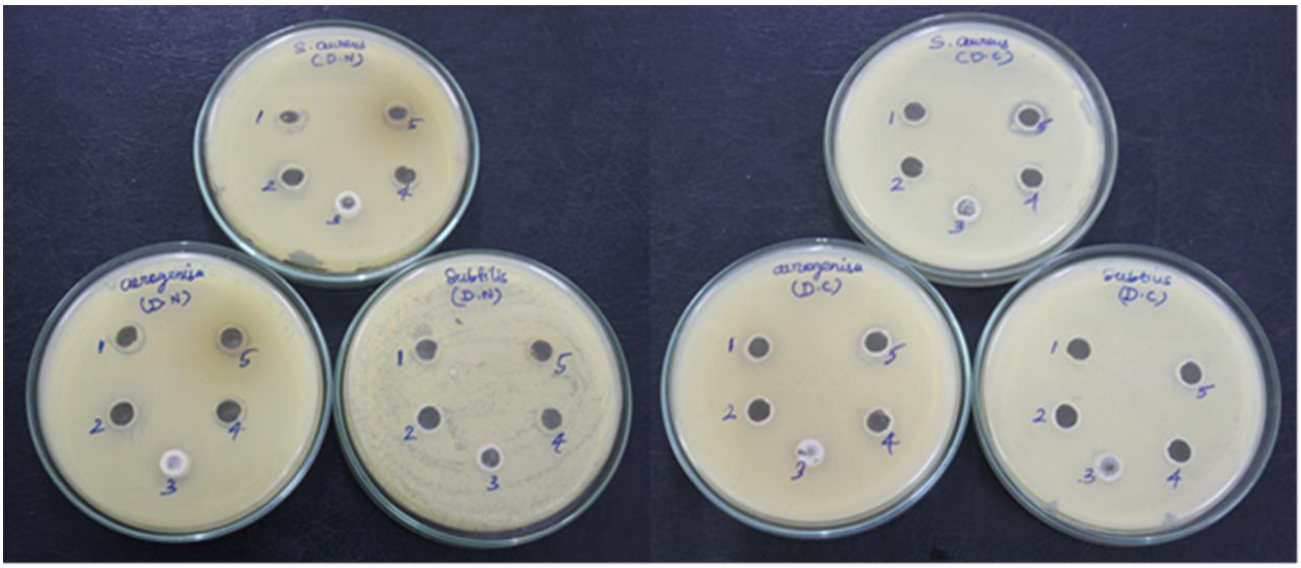

Figure4. Isolated colonies against MDR Strains.

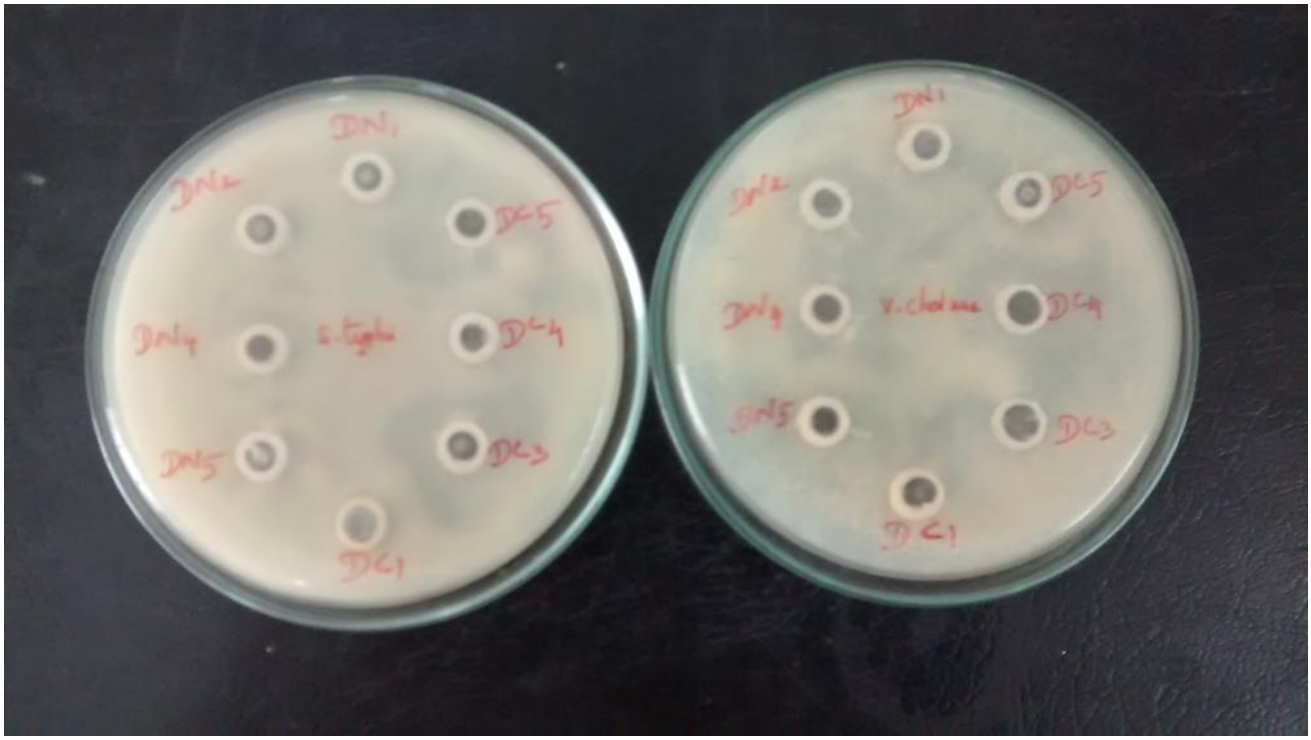

Figure 5. FT-IR absorption spectrum of the DC5 compound