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GANGA ACTION PLAN, HETEROGENEOUS PHYTO-ANTIBIOTICS AND PHAGE THERAPY ARE THE BEST HOPE FOR INDIA TACKLING SUPERBUG SPREAD AND CONTROL

Asit Kumar Chakraborty
Associate Professor of Biochemistry and Sr Research Officer,
Department of Biotechnology and Biochemistry, Genetic Engineering Laboratory,
Oriental Institute of Science & Technology (OIST), West Bengal, India

ABSTRACT ■ Life forms change with environmental toxicities. Most bacteria became multiple drug resistant due to use of high doses of antibiotics since 1940s. WHO and G-20 Leaders issued action plan to discover alternate to antibiotics. India needs to monitor spread of MDR bacteria in Ganga River which spans 2550 KM. India has also vast resources of medicinal plants to study as depicted in Sanskrit book like Charaka Samhita. In the earlier communications, we described the identification of many MDR bacteria from Kolkata Ganga River water, Rain water and Sewage water and many common West Bengal plants have no antibacterial activities but *Suregada multiflora* and *Cassia fistula*. Here, we have extended such studies to show the complex drug sensitivities, molecular identification of *mdr* genes as well as purification of active chemicals by TLC and HPLC followed MASS, NMR, and FT-IR technologies. Kolkata Ganga River water and Bay of Bengal water were polluted with MDR bacteria and *mdr* genes like beta-lactamases (*blaTEM*, *blaCTX-M1/2/9,* and *blaNDM-1*), acetyl-, phospho- transferases (*aacC1, aacA4, aphA2*) as well as *mcr, tet, acr* and *mex* types drug efflux genes are activated giving resistant to advance drug derivatives like imipenem, colistin, amikacin, linezolid, vancomycin, cefotaxime, ceftriaxone and lomofloxacin. Ganga Action Plan has been initiated by Indian Government with estimated >40,000 crores with mission 2020, when all industrial and domestic effluents will be treated before reach river and such effort will reduce MDR bacterial load in water. Phage therapy is another method of superbug control which is under process for FDA approval. We have also detected *Pseudomonas* specific bacteriophages in Kolkata sewage and Midnapore city pond. However, Ganga River water has less lytic bacteriophages indicating a symbiotic relation is exist. This implies that phage resistant factor is very important obstacle using bacteriophages in clinical therapy. Thus, our work has initiated to unfold new methods of superbug therapy following WHO and Indian Government guidelines.

Key words: gut microbiota, mdr genes, phyto-antibiotics, phage therapy, superbug control

INTRODUCTION

Penicillin antibiotics were discovered in 1928 by Nobel Laureate Alexander Flaming and thereafter by Dr. Selman Waksman discovered over twenty antibiotics including streptomycin (Yocum et al., 1980). In truth, sulphonamides was introduced in 1930, sulfa-drugs in 1940, penicillins in 1943, tetracycline in 1945, streptomycin and chloramphenicol in 1949, erythromycin in 1952, and ciprofloxacin in 1965 (Blackwood, 1985; Finch, 1997). Antibiotics are used by every

* **Corresponding author** : e-mail: chakraakc@gmail.com

7000 million people of this Earth to remove bacterial infections. Antibiotics are also aberrantly used in agricultural land and livestock growth and in viral infections. Multi-resistance has created panic in drug industry as new drug cost two billion dollar to market but only viable for six months to get resistant strain bacteria to appear. The molecular mechanism of drug resistance is creation of mdr genes in bacterial plasmids and such mdr genes code for enzymes that degrade or modify antibiotics causing resistance. Plasmid pBR322 was first sequenced in 1965 to know the sequence of two mdr genes, amp and tet (McNicholas et al., 1992). In fact, whole world were safe after World War II taking antibiotics in any bacterial infections and most reputed Pharmaceutical Companies were generated with great capital and share market at that time. However, since 1960 MIC of drugs were started increasing and to keep the capital market safe, drug companies were also produced 100 new derivatives of penicillin like ampicillin, oxacillin, methicillin, cefotaxime, ceftriaxone and many strong carbapenems (imipenem, dorripenem, meropenem) that target bacterial peptidoglycan biosynthesis (Chaw & Shlaes, 1991; Chakraborty, 2016a, 2017b) (see figure-1). Streptomycin and tetracycline resistance appeared between 1958-1962 followed by aminoglycosides resistance in 1980 (Brown & Ames, 2005; Blackwood, 1985; Finch, 1987). Ciprofloxacin resistance became prominent in 1985 and azithromycin resistance was vibrant between 1995-1999 (Nikaido, 1998). Most horror reported in 2009 that blaNDM1 enzyme cleaves all penicillins, cephalosporins and carbapenems and lastly Mcr-1 enzyme was reported in 2016 that make resistant to colistin drug by transferring phosphoethanolamine to lipid A of bacterial membrane (Di Pilato et al., 2016) (see, figure-1). A great horror appeared in Pharmaceutical Industry and many companies were sold or combined to accelerate the research on novel therapeutics like gene medicines, nano-carriers, phage therapy and enzybiotics (Chakraborty et al., 2016a, Chakraborty, 2017f). India uses 8 billion units antibiotics in 2001 to 12.9 billion units in 2010 with overall 36% increase in 10 years worldwide. This suggests that it is a habit for Indian peoples to take >10 antibiotic pill per year even the infection is viral one. Sadly, US peoples take average >20 antibiotic pill per year. So, poor as well as rich countries are equally affected although superbug infections like NDM-1 *Escherichia coli* or MRSA *Staphylococcus aureus* must be treated in the US or UK due to advancement in treatment protocol. Clinical bacteria from human are now >95% ampicillin and tetracycline resistant and 300 mdr genes have been detected during Human Microbiome Project in normal human. This led to conclusion that human gut is the main source of *mdr* genes creation and multi-resistance (Chakraborty, 2017d; Dickgiesser et al., 1982). Small plasmids (R-plasmids) were also combined with F'-plasmid generating super conjugative plasmids (50-500kb) that donated mdr genes to environmental bacteria creating AMR (Recchia & Hall, 1995; Projan & Novick, 1988; Chakraborty, 2016a).

Presently, *Salmonella typhi, Mycobacterium tuberculosis, Siegella sonni, Escherichia coli, Acinetobacter baumannii,* and *Staphylococcus aureus* etc. are now drug resistant (Fosberg et al., 2012; Jenson et al., 2015). Over use of antibiotics indeed has killed vital gut microbiota (since 1943) which produce vitamins needed for metabolism. Such defects now compensated by taking multivitamins (B1, B2, B6, B12, A, C, D) and probiotic capsules containing Lactobacillius,

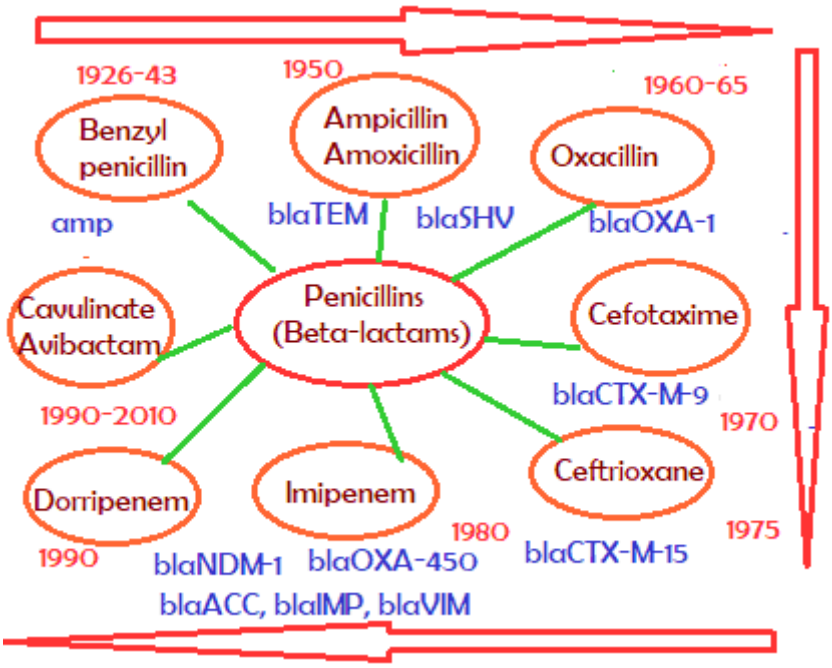

Fig.1. History of penicillin antibiotics development commensurate with mdr genes (Beta-lactamases) creation. Beta-lactamases inactivate most Beta-lactam antibiotics creating an acute crisis in the treatment of infectious bacterial pathogenesis like cholera, TB, fever, skin infections, UTI and gonorrhoea (Ambler, 1980; Bassetti et al., 2009; Drawz & Bonomo, 2010; McArthur et al., 2013).

Bifidobacteria and Streptococcus (D'Costa et al, 2011; Ceccarelli et al., 2017). India has grown considerably since its independence and now 125 cores peoples need much attention than 35 cores in 1947. In 2011 Indian Government issued action plan to reduce spread of MDR bacterial pathogenesis and control. WHO Action Plan suggested that intestinal MDR bacteria are contaminated the water resources of pond, riverand sea as rain water carry MDR-bacterial spores. The situation of water quality in Ganga River of India is worse. About 40% of peoples reside at the bank of Ganga River and many big industries are made nearby releasing many pollutants and excretory matters which support lower life forms but also toxicity present (Chakraborty, 2017e). In Ganga River, we see number of bacteria is increasing with 40% resistant to semi-synthetic drugs ampicillin and amoxicillin. Such drugs have cured most infections between1940-1990 although gradual increase in drug resistance was detected in many continents as early as 1960 (Davis & Davis, 2010). Ganga River during the course of its journey (2525KM) from the hills to the sea, municipal sewage from large urban centres, trade effluents from industries and polluting waste from several other non-point sources are discharged into river resulting its pollutions (Rahaman, 2009). The Ganga basin lies between East longitudes 73°02' - 89°05' and North latitudes of 21°06' - 31°21', covering an area of 1,086,000 sq km. The pollution level in the holy Ganga at Kumbh (Sangam) has risen 7.4 mg/L but BOD limit is 3mg/L indicating Ganga River water is polluted (Rai, 2011). The Government of India started the "Mission Clean Ganga" project and "Namami Ganga" project with

the objective that by 2020, no municipal sewage and industrial waste would be released in the river without treatment which will reduce toxicities and also MDR bacterial load (Jain et al., 2007; Suvedi, 2005; Das & Tamminga, 2012).

Phyto-therapy: Plant History, Nomenclature and Folk use

Identity of plants were done by Prof. N. Paria, Department of Botany, Calcutta University. However, very uncommon plant like *Suregada multiflora* was checked by Central National Herbarium, Botanical Survey of India (No. CNH/41/2013/Tech.II/1036). *Cassia fistula* Linn. (Caesalpiniaceae) is known as the Golden Shower and is distributed in various countries including Asia, South Africa, Mexico, China, West Indies, East Africa, and Brazil as an ornamental tree for its beautiful branches of yellow flowers. It is used as purgative, antipyretic, analgesic and anti-bacterial agents (Kumar et al., 2006; Bhalodia et al., 2012). The whole plant is also used to treat diarrhea; fruits are used to treat skin diseases, fever, abdominal pain, leprosy by traditional people due to presence of high aloin and phenol compounds (Chakraborty, 2015).

Cinnamomum zeylanicum and *Cinnamon cassia* (Lauraceae, also known as *Cinnamomum aromaticum*). Ceylon cinnamon (*Cinnamomum zeylanicum*) or 'true cinnamon' is indigenous to Sri Lanka and southern parts of India. Three of the main components of the essential oils obtained from the bark are trans-cinnamaldehyde, eugenol, and linalool, which represent 82.5% of the total composition with variety antimicrobial activities. Clove (*Syzygium aromaticum*, Myrtaaceae) is one of the most valuable spices that have been used for centuries as food preservative and for many medicinal purposes. Clove is native of Indonesia but nowadays is cultured in several parts of the world including Brazil in the state of Bahia and Kerala of India. The essential oil extracted from the dried flower buds of clove is used as a topical application to relieve pain and to promote healing and also finds use in the fragrance and flavouring industries. The main constituents of the essential oil are phenylpropanoids such as carvacrol, thymol, eugenol and cinnamaldehyde. The phyto-chemicals are strong antibacterial agents and used in cosmetics as shown in figure-2.

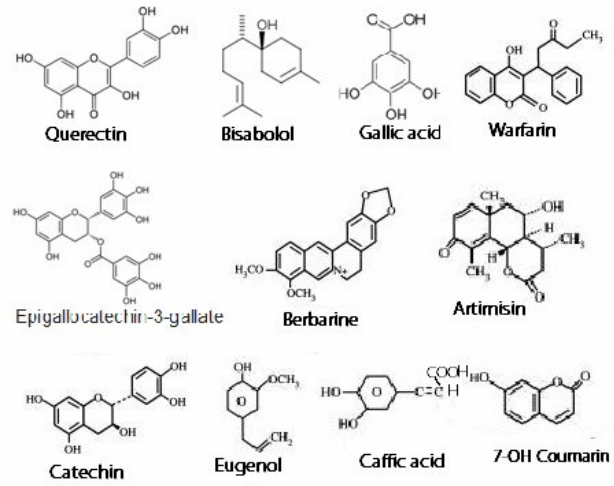

Fig.2. Structure of plant phyto-chemicals as antibacterial agents. The compounds are polyphenol, flavonoids, hetorocyclics and quinines those inhibit drug efflux, RNA polymerase and membrane transport.

Bacteriophages as potential for controlling MDR pathogenesis

Bacteriophages are viruses that infect bacteria and stay as lysogen after integrating into chromosome or stay as lytic which replicate rapidly inside bacteria and ultimately lyses the bacteria releasing millions of new phages ready to infect new bacterial cells. As MDR bacterial infections are alarming where no antibiotic work, WHO advices for new therapeutics development. Now lytic bacteriophages are in centre stage for MDR therapy as pioneered Frederick Twort and Félix d´Herelle (Twort, 1915; Abedon et al., 2011; Chan et al., 2013). Phages are the most abundant and ubiquitous microorganisms on Earth, ten times more abundant in the environment than bacteria, with numbers estimated to be in the order of 10^{31}. Phages are ubiquitous, found in sites such as water, soil, humans and animals (skin, feces, gut) and even in our food (Hermoso et al., 2007). One gram of fresh or processed meat contains about 10^{8} viable phages. In a general way, one might say that a phage can be found in any environment where bacteria or archaea are present. Phages present an extensive diversity of shape, size, capsid symmetry and structure. Phages can have double-stranded (ds) or single-stranded (ss) DNA or RNA. Phages are classified by the policies of the International Committee for Taxonomy of Viruses (ICTV) under a myriad of properties, but most important are the type of nucleic acid, morphology, physiochemical properties and genomic composition. Figure-3 has demonstrated that the bacteriophage research in India has not get momentam yet as only 1.5% phages are isolated and reported but in Russia, France, Germany, Georgia and South Korea are far ahead (6-12% of reported) . We have discussed here horror of superbugs in Kolkata water and their possible remedy by Ganga Action Plan, Phyto-research and phage-therapy. We just want to mention our first report of bacteriophages detection in West Bengal water where lytic phages are hard to isolate for phage therapy. This is a warning to Indian Government as the available phage medicine likely very genetically engineered as thus may be toxic as contain unwanted cis-acting regulatory DNA sequences.

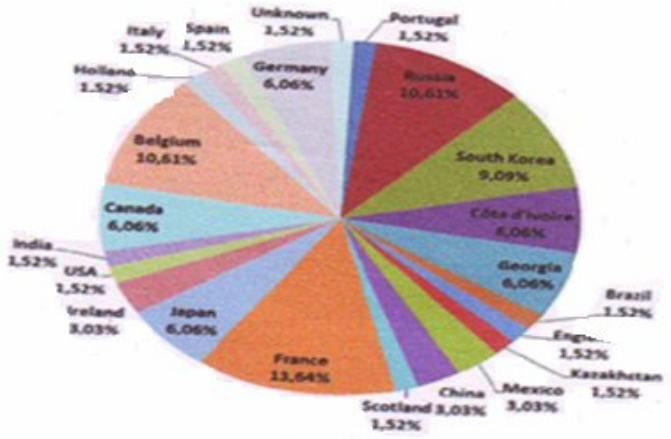

Fig. 3. Worldwide distribution of isolated bacteriophages. India is far behind in phage therapy research (1.52%) than Russia, Georgia, South Korea, Germany and France (6-13%). (see, US patents US82822920B2; US2011000873A1 and US20130122549A1 for phage therapy development).

MATERIALS AND METHODS

Purification of superbugs from Ganga River water and Digha Sea water

Water from Ganga River was collected at the morning from Babu Ghat (Kolkata-700001) and Howrah Station River (Chakraborty, 2017a). About 100μl of water was spread onto 1.5% Luria Barton-agar plate containing different concentration of antibiotics at 2-50μg/ml. MDR bacteria were selected in agar-plate containing ampicillin, streptomycin, chloramphenicol, tetracycline or ciprofloxacin at 50, 50, 34 and 20 μg/ml respectively. As imipenem and meropenem resistant bacteria were present low (0.08-0.2 cfu/ml water), a modified method was followed. 2 ml 5x LB media was added into 10 ml River/Sea water at 2-10μg/ml concentration and was incubated 24 hrs to get drug resistant bacteria population. Meropenem resistant bacteria were further selected on tetracycline, chloramphenicol and streptomycin to get the superbugs. Antibiotics were purchased from HiMedia and stored at 2-50mg/ml at -20^{O}C (Chakraborty, 2015; Chakraborty & Hodgson, 1998). Antibiotic papers were also purchased from HiMedia according to CLSI standard. Antibiotic papers are: A-25 (ampicillin), T-10 (tetracycline), AT-50 (aztreonam), COT-25μg (Cotrimoxazole), Met-10μg (methicillin), CAZ-30μg (ceftazidime), LOM-15μg (lomofloxacin), VA-10μg (vancomycin), AK-10μg (Amakacin), TGC-15μg (tigecycline), LZ-10μg (linezolid), and IMP-2μg (imipenem).

The plasmid DNA was isolated from overnight culture using Alkaline-Lysis Method (Sambrook et al., 1989; Chakraborty & Das, 2003). 16S rDNA gene colour Sanger's di-deoxy sequencing was performed by SciGenom Limited, Kerala, India (Sanger et al., 1977). PCR amplification was performed using 1 unit Taq DNA polymerase, 20ng DNA template, 0.25mM dXTPs, 1.5mM MgCl2, for 35 cycles at 95^{O}C/30" (denaturation)-52^{O}C/50"(annealing)-72^{O}C/1.5' (Chakraborty et al., 1993; 1991; Ausubel et al., 1989). The product was resolved on a 1% agarose gel in 1X TAE buffer at 50V for 2-4 hrs and visualized under UV light and photograph was taken. NCBI BLAST analysis was performed for bacterial specific gene analysis (www.ncbi.nlm.nih.gov/blast) and data was submitted to GenBank. NCBI databases were retrieved using the BLAST programmes (Sambrook et al., 1989; Jhonson et al., 2008; Chakraborty & Das, 2003).

The complete genes are sequenced in plasmids and were analyzed by Seq-2 programme of BLAST (Sanger et al., 1977). Multalin protein sequence software was used

Table-1. Primers used in this study (Chakraborty AK, 2015)

Name	Sequence of the primers	Tm	size
P27F	5'-AGA GTT TGA TCC GAA CGC T-3'	62^{O}C	1.4kb
P1392R	5'-TAC GGC TAC CTT GTT ACG ACT TCA-3'	65^{O}C	
cmrF	5'-TTC GTT AGT CTG CCG TTG CT-3'	56^{O}C	323bp
cmrR	5'-ATC GCT GGC AAA CAG GGT TA-3'	57^{O}C	
tem-sF1U	5'-ATGATGAGCACYTTTAAAGT-3' Y=C/T	56^{O}C	312bp
tem-sR1U	5'-TCATTCAGYTCCGKTTCCCA-3'Y=C/T; K=G/T	58^{O}C	
tetF	5'-CTT CGC TAC TTG GAG CCA CT-3'	57^{O}C	910bp
tetR	5'-GCA GAC AAG GTA TAG GGC GG-3'	57^{O}C	
acrAB-F	5'-ATG CTC TCA GGC AGC TTA GCC-3'	59^{O}C	.1kb
acrAB-R	5'-TGT CAC CAG CCA CTT ATC GCC-3'	59^{O}C	
ctxF1U	5'.-AACACMGCMGATAATTCACA-3' M=A/C	59^{O}C	586bp

to get the nature of conserved sequences among metallo-class B â-lactamases (Johnson et al., 2008). Sometime, diverged sequences are manually cut and paste into align position in MS word so that it is appeared both sequences have similarity. For retrieving any nucleotide, we type the same at the NCBI port (www.ncbi.nlm.nih.gov/nucleotide *or Protein) and to BLAST search to type the accession number for protein or DNA into BLAST port (*https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch*) (*Bairoch and Apweiler, 2000; Altschul, 1997; Chakraborty, 2016b).

Preparation of organic phyto-extract (MDR-Cure)

The barks of *Suregada multiflora* (Ban-Naranga), *Cassia fistula* (Bandhorlathi) were collected on July 2016 from medium sized tree at Midnapore district of West Bengal. *Syzygium aromaticum*-flowerbuds (labanga spice) and *Cinnamomum zeynalium*-Bark (darchini) were purcheshed from grocery stores at Kolkata and Midnapore respectively, Each 10gms dried and grinded plant and spice parts (*Suregada multiflora*, *Cassia fistula*, *Syzygium aromaticum*, *Chenopodium album*, *Cinnamomum zeynalicum*) was suspended in 40 ml ethanol or ethanol-ethylacetate for overnight. Then concentrated 5-10 times (MDR-Cure) at room temperature and 50μl used for Kirby-Bauer agar hole assay (Sastri, 1956; Dubey et al., 2005; Duglia, 2011). The crude plant extract was purified by Thin Layer Chromatography (TLC) was performed using Methanol, water and Acetic acid as mobile phase (60:30;10) for 0.8hr-1.2 hrs. Organic molecules were seen and recovered by UV shadowing and was eluted in ethanol from silica-gel.

High Performance Liquid Chromatography and Furier Transformed Infra Red Spectroscopy

5mg TLC-purified active sample dissolved in 0.5ml methanol, filtered through a membrane filter and 0.1ml sample was loaded onto a HPLC C-18 column equilibrated with methanol (Cesari et al., 2016; Naidoo et al., 2018). 0.5ml fractions were collected and major active peak (retention time-10.5min) was collected and vacuum dried. 5mg HPLC-purified dry active chemical was mixed with 200mg IR-grade KBr and the tablet was prepared at 13mm Die SET (Kimaya Engineers) at 10Kg/cm^2. Spectra were taken with a Perkin Elmer Spectrum 100 FT-IR Spectrometer (Serial no. 80944) for 10 min (Griffiths & de Hasseth, 2007; Chau et al., 2016)).

Bacteriophage isolation and purification

We made 2% LB-agar plate (10g Tryptone + 10g NaCl + 5g peptone +15g bacto-agar/L water) and 0.9% LB-top agar. 0.2 ml bacteria (*Pseudomonas aeruginosa* DB-2_mdr) and 0.2 ml water (as phage source) was incubated in phage absorption buffer for 15 min, mixed with 3 ml top-agar and poured into 2% base LB-agar plate. Incubated for 48 hrs and lysis zones are transferred by tips to newly growing *Escherichia coli* or *Pseudomonas aeruginosa* resistant to ampicillin and tetracycline. Each plaque was re-suspended in 300 ìl of SM buffer (5x 5 M NaCl, 1 M MgSO4, 1 M Tris–HCl pH 7.5, gelatine solution distilled water). Dilutions were then prepared and plating performed to allow the purification and isolation of a single phage (Merabishvili et al., 2009). Consecutive rounds of plaque purification were carried out, by repeated subculture on the respective host strain. Purified phages are stored in 50 % glycerol (v/v) in LB at -20 ºC for long term use and 4 °C short term use (Schooley et al, 2017).

RESULT

The water from Ganga River, Kolkata streets and Digha sea gave very distinct drug resistant colonies in presence of 50μg/ml of ampicillin and amoxicillin with 3900-5800 cfu/ml of

water. The cfu/ml of water were reduced to five fold in case of tetracycline and azithromycin at 20μg/ml and 50μ/ml repectively, where as reduced to 49 fold with 34μg/ml chloramphenocol and 50μg/ml streptomycin. In presence of beta-lactamase inhibitors cavulinic acid and sulbactam, cfu/ml further reduced to ~50 cfu /ml of water. Further, imipenem and meropenem resistant species were found rare with only 0.2-0.5 cfu/ml of Ganga River water and Digha sea water (Belotti et al., 2015). The results indicated that everywhere had MDR-bacteria and 30-40% was ampicillin and amoxycillin resistant as well as <1-2% were superbugs (MDR but % XDR was low and no PDR was detected). As for example, meropenem resistant bacteria species were present extremely low or ~ 0.006%. According to law, MDR bacteria must be resistant to at least three different groups of antibiotics. So the percentage of MDR-bacteria like resistant to three drugs, ampicillin, streptomycin and tetracycline was also as low as only 0.2%. Then we have tested the pure rain water (collected on 4th floor roof keeping a 50 ml plastic tube inside a 500 ml beaker) and it has also very similar numbers of bacteria indicating as the major source of bacterial contamination on streets, ponds and sea. This means if any superbug got escape from clinics to environment by physical calamity like storm, tide, flood or earth quake and then bacterial spore could be spread to anywhere by wind and would fall during rain affecting mass populations. The old city like Kolkata has damage sewage system and floods everywhere of the city during monsoon causing nail or skin infections (Chakraborty, 2015). The experiment indicated the rain water contains a mixer of bacterial

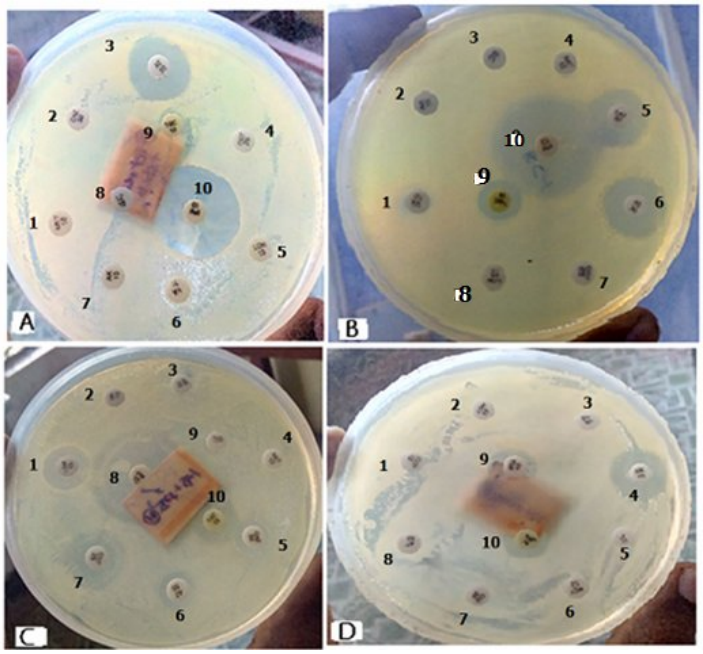

Fig.4. Antibiotic paper disk assay of few superbugs. Those are KT-1. KC-2 and KG-12 and DMT-1 strains. In (A) KG-12 isolate: 1=MET-10, 2=CAZ-30, 3=AT-50, 4=COT-25, 5=LOM-15, 6=VA-10, 7=AK-10, 8=LZ-10, 9=TGC-10 and 10=IMP-10. In (B) KC-1 isolate: 1=VA-10, 2=AK-10, 3=LN-10, 4=MET-10, 5=CAZ-30, 6=AT-50, 7=COT-25, 8=LOM-15, 9=TGC-15, 1and 10=IMAP-10.

populations having different genotypes and drug sensitivities. We have isolated few strains of gram-negative superbugs (KA1, KR1, DG1, KC1, KT1, KG1, DM1 that are resistant to at least three different groups of drug (e.g. ampicillin, streptomycin, cefotaxime, azithromycin, ciprofloxacin, tetracycline or chloramphenicol). The data presented in figure-4 where potent drug resistant species like KG-12 (panel-A) , KC-1 (panel-B). KR-1 (panel-C) and DM-1 (panel-D) were detected. This suggests 3rd generation cephalosporins still could be used in Kolkata for common bacterial infection. Most of the bacteria were *Escherichia* rod and flagellates as demonstrated by electron microscopy and also could form circular spores (data not given). Surprisingly, KG-1, KT-1 or KC-1 strains are resistant to 12 Hi-Media antibiotic strips according to CLSI standard and also highly resistant to vancomycin, co-tromoxazole and methicillin (Chakraborty, 2015).

We checked the presence of the drug resistance mdr genes in our environmental superbugs. Figure-5 showed the amp gene amplification where all 10 isolates had blaTEM genes which was confirmed by sequence. Similarly, a blaCTX-M universal proble detected the cefotaxaminases in most isolates (panel-B) and surprisingly, we also detected the acetyl transferases gene (aac6'-1b). DB-1 and DB-2 strains isolated from Bay of Bengal may contain multiple genes for blaCTX-M beta-lactamases and aac6'-1b acetyl transferases (lanes 7, 8). It appeared KT-2 strain had no blaCTX-M gene and KC-2 strain had no aac6'-1b gene.

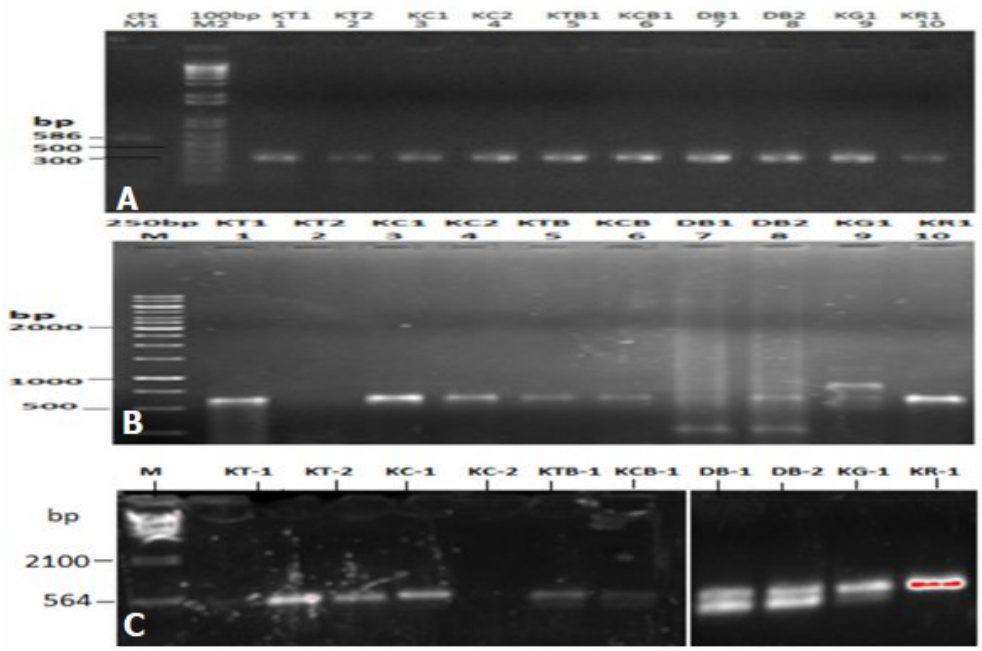

Fig.5. Detection of *mdr* genes in MDR bacteria. The MDR bacteria were from rain water of Kolkata, Ganga River water of Kolkata and Bay of Bengal (Digha). Panel A, blaTEM/SHV common primers (Amp-type beta-lactamase) , panel B, blaCTX-M1/2/9 types universal common primer (CTX-M beta-lactamases) and panel C, aac6'-1b (drug acetyl transferase) primers. Interestingly, the three panels experiments were done separately and different DNA makers were used: (A) lane 1, 586bp CTX-M PCR as in (B) and lane 2, 100bp DNA Ladder; (B) 100bpDNA Ladder but were ran longer and (C) LamdaHind3 DNA marker. Further, in (C) mini gel with two panels was ran and thus two panels were joined.

As our superbugs are authentic and determined as *Escherichia coli* and *Pseudomonas aeruginosa* or *Stenotrophomonas sp* (16S rRNA sequencing), we planned to study the heterogenous phyto-antibiotics from Indian medicinal plants as described previously (Chakraborty, 2015, 2017). Bark ethanol extracts of two medicinal plants are chosen due to high antibacterial activities as shown in figure-6. We then purified the phytochemicals by Thin layer chromatography as shown in figure-7. It appeared in methanol : acetic acid : water at a ratio 60:10:30 separated an active chemical that had antibacterial activity on superbugs (Lanes, 3, 8). The active TLC band was excised from gel and eluted in ethanol, concentrated and 100μl was loaded onto C18 HPLC column. The elution profile was shown in figure-8. The FT-IR absorption spectra of the HPLC purified chemical was shown in figure-9 where -NH2, –OH and un-saturation carbon were detected. The work is in progress to determine the chemical structure of the active principles.

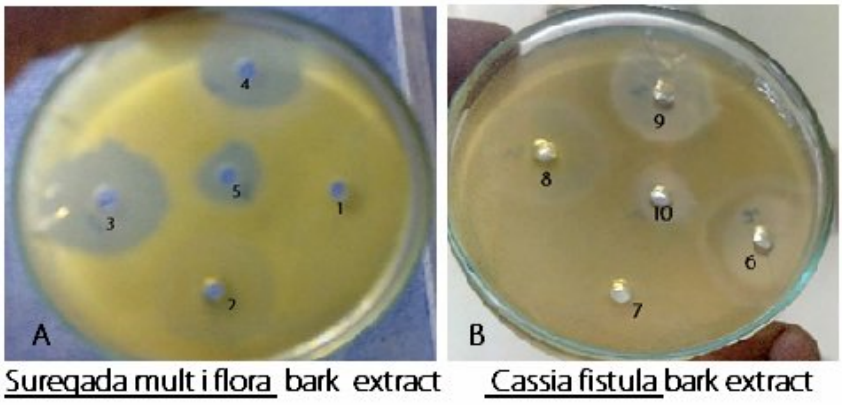

Fig.6. Demonstration of high anti-bacterial activities of organic plant extracts. Naringa bark extract (A) and Bandorlathi bark extract (B) on *Pseudomonas aeruginosa* DB-2_mdr (accession no. KY769876). (A) Lane 1, ampicillin 10μl (50mg/ml), lane 2, tetracycline 10μl (20mg/ml), lane 3 50 μl Naringa bark extract; lane 4, 10μl (2mg/ml imipenem) and lane 5, 10μl (5mg/ml feropenem). (B) Lane 6, 50 μl Naringa extract, lane 7, ampicillin 10μl (50mg/ml), lane 8, 50 μl Bandhorlathi ethanol extract, lane 9, 10μl (2mg/ml imipenem) and lane 10, 10μl (5mg/ml feropenem).

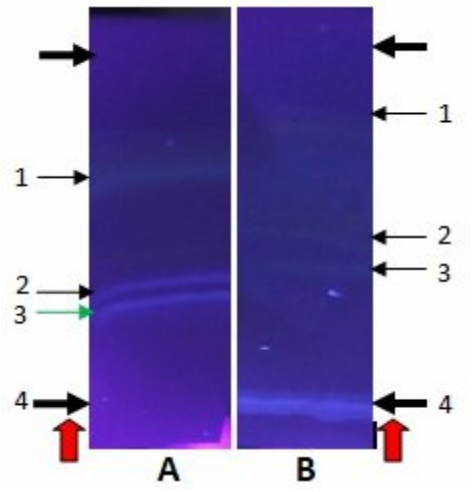

Fig.7. TLC purification of active phyto-chemicals by UV shadow method. Organic extract of *Suregada multiflora* was separated by Thin Layer Chromatography (A) Methanol: Acetic acid: water (60:10:30) and (B) Methanol:Ammonium hydroxide:water (60:10:30). The active principle was shown by green arrow. No activity recovered during TLC with NH4OH. The active means at least 15mm zone diameter with 7mm hole as also shown in figure-6. In (A), there was no detectable band in position 4 which indicated in alkali the active principles were ionized and were retained in the lane being hydrophilic. Rf values were determined as (A) 0.68, 0.38. 0.36 and (B) 0.80, 0.52, 0.43. and 0.10.

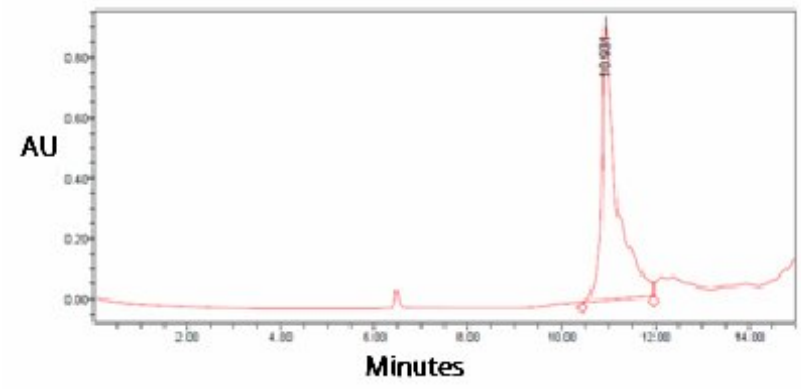

Fig.8. Purification of TLC purified (green arrow band in figure-6) phyto-chemical by HPLC. C18 column was used at 254nm scan. The data indicated that during 4 time TLC purification, the activity was almost purified giving a single major band. The retention time of the molecule about 10.5 min

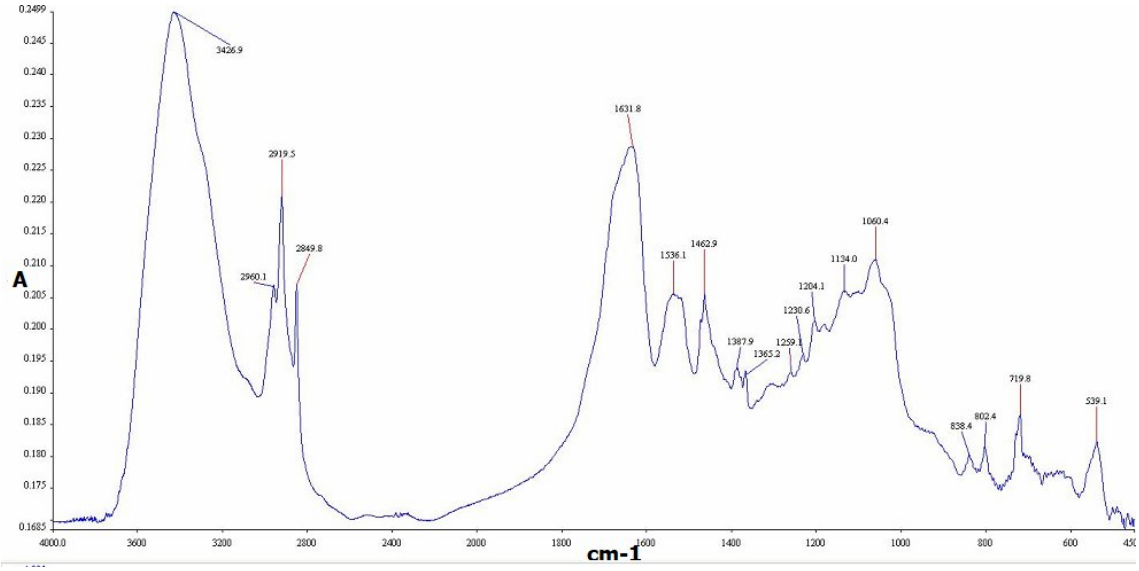

Fig.9. Detection of function groups of the active compound by FT-IR. Peak at 3426.9 is for –N-H streaching and –O-H streaching; 2960.1, 2849.8 cm-1 are for –CH3 streaching; 1631.8 and 1536.1 cm-1 are for CO-NH2 scissoring; 1462.9, 1387.9 cm-1 represent –O-H bending likely phenolics; 1259.1 and 1230.6 may represent C-NH2; 11.34.0cm-1 for C-C-C bending; 802.4 cm-1 for –NH2 wagging; 719.8 may represents -CH2- rocking.

Finally, We have explored the abundance of bacteriophages in Ganga River water (Ceyssen & Lavigne, 2010). Our result indicated that very poor quantity of bacteriophages were present in Ganga River water as compared to multidrug-resistant bacteria. We collected many water samples from Midnapore city and first detected bacteriophages in a local pond near Rangamati, Midnapore as demonstrated in figure-10. Similarly, we detected abundant bacteriophages in Kolkata sewage collected from Kolkata open drain near Cosba-Garfa area (Kolkata-700078).

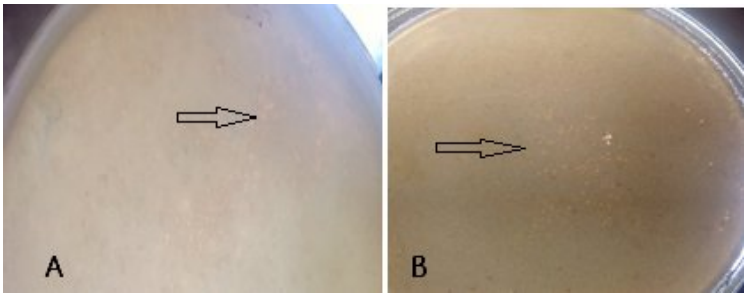

Fig.10. Demonstration of MDR *Pseudomonas aeruginosa* specific bacteriophages. Kolkata sewage has abundant phages (B) but not in Ganga River water (A). Limitation of this experiment is we have not able to make an phage extract with high titre due to complete lysis of bacteria which has signalled a great symbiotic relation between bacteria and bacteriophages (Clark & March, 2006).

DISCUSSION

Antibiotic void is serious threat to human and animal. MDR creation is so rapid and instrumental that any new antibiotic over use will create new mdr gene to inactivate the drug (Ceccarelli et al., 2017; Cecchini et al., 2017). We our detection of abundant superbugs in Ganga River water is not surprising as all domestic sewages are released into Ganga water. We used MDR-Cure organic phyto-extract to kill MDR bacteria similar to ancient time (before 1928) and we are quite success using ethanol extract of bark and root of *Suregada multiflora* and *Cassia fistula*. Now we are characterizing the active chemicals to determine the chemical structure for new drug development and to test such chemicals on molly fishes and rats for toxicological studies like hypersensitivity, tumour genesis and liver toxicity (Cowan, 1999). We are also started bacteriophages research (Chan et al., 2013). Phages are easily isolated from nature, e.g. sewage samples, and have a relatively low cost of development. Phages have narrow host range and have a minimal risk of normal intestinal flora disruption when compared to antibiotics. Phage treatment would only require low doses because of its self-replicating property – as long as the target cells are present the lytic cycle takes place. Phages can be used in liquids, impregnated into hydrogels and are suitable for use by most delivery routes, such as oral and parenteral, but also local and by inhalation. Phage infection has a completely different mode of action to antibiotics and therefore antibiotic resistant bacteria present no special challenge to therapy (Schooley et al., 2017). Phage proteins are also used as enzybiotics and recombinant lysins are great for combating multidrug-resistant infections (Manohardas et al., 2009; Dethlefsen et al., 2015; Chakraborty, 2017d).

Artiminisin, taxol, quinine and etoposide are plant derived pure chemicals were used as drugs against malaria and cancer. So we have shown that MDR-Cure phyto-extracts are good in curing multidrug-resistant infections. But we have not yet able to sequence the full length plasmid from our *Escherichia coli* KT-1/KC-1/KR-1/KG-12 superbug isolates. All isolated superbugs were completely inhibited by purified plant extracts –alone or combination. Thus MDR-cure phyto-extract will overcome multi-resistance. It is very non-toxic being plant-derived. Recently, we also got active principles from Labanga and Derchini widely used in food. Thus we want to inject OIST-developed MDR-Cure during acute superbug sepsis in patients. There were

abundant reports of large MDR conjugative plasmids and fully sequenced. IncA/C2 plasmid (pMRV150, accession no. EU116442) of *Vibrio cholera* strain 2012EL-2176 contains *blaCMY-2, blaCTX-M-2, blaTEM-1, floR, aac(3)-IIa , strA/B, sul1/2, dfrA1, dfrA27, tetA, mphA, mdr-genes and also resistant to ciprofloxacin due to mutation in gyrA*(S83I)/*parC*(S85L). *Bacillus thuringiensis* plasmid pBMB293 (Accession no. CP007615, 294kb) has no *mdr* gene but genes for enterotoxins (protein id. AIM34697), dipterans toxin (protein id. AIM34741) and reverse transcriptase, DNA polymerase â, DNA topoisomerase III and type II secretion system. Similarly, *Bacillus anthrus* plasmid pX01 (accession no. CM002399; 171kb) has toxin gene (protein id. AFL55645, 809aa) and also in pBMB293 plasmid. More and more genes in plasmids will be evident and likely phage therapy comes of age as alternate to antibiotics (Finch, 1997; Rodríguez-Rubio et al., 2016). Small non-coding RNAs (miRNA) have potential to therapeutic applications as in cancer but its role in MDR bacteria remains elusive (Yang et al., 2014; Slaby et al., 2017). Drug efflux pumps (Mcr, NorA, AcrABC, MexAB-EF) in plasmids are serious threat in developing therapeutics (Delmer et al., 2014). We believe that *mdr* genes are created to save gut bacteria which are in tight symbiosis with intestinal cells for vitamin synthesis (Hill, 1997; Jandhyala et al., 2015). So until we stop oral antibiotics, mdr genes formation will be rapid where any drug within few days oral intake will be inactivated by the creation of new deadly *mdr* gene like *blaNDM-1* and *mcr-1* (Huang et al., 2013; Liu et al., 2016).

CONCLUSION

Diversified MDR genes are created highly in bacterial plasmids and it appears most bacteria have received transferable small, medium and or large plasmids with 5-15 *mdr* genes and 10-60 transposons and IS-elements (McArthur et al., 2013). Indian NAP-AMR (The National Action Plan on Antimicrobial Resistance- 2017-2021) has pinpointed five main areas to curve superbug horror: (i) improving awareness and understanding of AMR through effective education and surveillance, (ii) reducing infection by increasing preventive measures, (iv) reducing the use of antimicrobials in health, food animals and agriculture (v) promoting for AMR research and drug innovations and (v) strengthening India's leadership on AMR and International collaboration (assessed on October, 2017).

WHO advocates that no antibiotic without probiotics and vitamins (Timson et al., 2017). Interestingly, many vitamin synthesizing gene are mobilized into MDR plasmids indicating a crisis of vitamin (Carattoli, 2009). Raw vegetables or fruits are great source of vitamins but most poor peoples of Africa, Asia and Latin America hardly take those foods regularly. The civilization we made is deadly as we have large excreta of 7000 millions global peoples and similar quantity of animals and avians., combined with organic chemicals from paper, lather, fertilizer and paint industries as well as heavy metal leaching from mineral and electronic industries (9 billion pounds treated but 2.5 millions untreated released, 2012 US-EPA report). Air is totally unhealthy due to petroleum and travel industries (10x PM1, PM2.5 and PM10 labels). Research activity has increased but we have no data how serious to perform experiments with recombinant DNA expression vectors, radioactive chemicals, florescence drugs or ddXTPs used in DNA sequencing. Toxic chemicals were incinerated in developed countries but were thrown into river side in most countries polluting world river and sea water (Timson et al., 2017). Indian Government has initiated Ganga Action

Plan 2020 to treat sewage water before reach river. We are totally busy with growth but not safety of this Earth as habitat (Costerton et al., 1987). We need phyto-antibiotics back to effects as in 1900s and beyond so that cost effective remedies available to poor. Sadly, high cost of modern therapeutics drags few millions peoples of this Earth into poverty line each year which is very catastrophic. India had 125 cores population and every year forests are converted into agricultural land and housing destroying valuable medicinal plants (In 1985 74% forest, in 2005 was 50%, in 2020 will be 33%). We slogan, "Come back to nature. Save plants and use as medicine" (Daglia, 2011). But we see tremendous activities in phage therapy, enzybiotics and gene therapy (Rashel et al., 2007; Donovan et al., 2009; Fernandes et al, 2012; Yang et al., 2013; Kutter et al., 2015; Dalmasso et al., 2016; Yu et al., 2016; Chakraborty, 2017d; Dunbar et al., 2018). We are very ahead in phyto-chemicals discovery (figures, 6, 7, 8, 9), and has started bacteriophage isolation (figure-10) but enzybiotic screening from Indian flowers and herbs just has initiated. Perhaps Ganga Action Plan has its own merit to reduce MDR infections in India.

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