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Red Rose Pigments Utilities as Dyes at Different pH but Poor Anti-Bacterial

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Abstract

Active plant extracts for drug development was studied as described in Charaka Samhita and Atharva Veda, and further being illustrated in 11 volumes of Bengali book, Chiranchib Bonoushadhi. Previously, we detected 40% ampicillin and amoxicillin resistant bacteria species but most of them were imipenem sensitive. Further, we described that Suregada multiflora, Cassia fistula as well as extracts of *Labanga and Derchini* spices are better than *Azidirachita indica* and *Chenopodium album*. Because rose and marigold cultivation are abundant in West Bengal, we have checked the possible anti-bacterial activities of flower petals extract. Petals ethanol extracts of rose (*Rosa indica*) and marigold (*Tagetes patula*) flowers have some anti-bacterial activities. Marigold ethanol extract seems more active than rose while chloroform extract is less effective. Both water soluble red and yellow pigments are inactive in Kirby-Bauer assay. Thus, antibacterial potential was not associated with color coded phyto-chemicals. However, we have found that water soluble red rose pigments are non-toxic and such abundant chemicals have utility as food and medicine color additives and in paint industry. We made organic abir and painted cotton fabrics at different pH giving beautiful different colors like red, blue and green. Unique acid-base property of water-soluble red rose dyes was presented which were likely terpenoids or flavo-alkaloids.

Keywords: ***Aminoglycosides; Carbapenems; Multi-resistance; Superbugs; Phyto-extracts; Ancient hindu civilization***

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Introduction

Ten thousand years old, Atharva Veda illustrates that plants are God carrying many medicines to cure diseases that are due to imbalance of different forces of our body [1]. But there was no concept of microorganisms those we could not see by naked eye [2]. Hippocrates' ethical code, known as the Hippocratic Oath, was guided the practice of western medicine to unfold. By 100 BC, China had become a major centre of medical research and its best doctors wrote the Neijing, a book about Chinese medicine [3]. Major breakthrough came when microscope was developed to see bacteria and fungi by Anton Van Leeuwenhoek

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(1676), development of pure culture and in vivo animal experiment to show that bacteria as culprit of diseases from the pioneering work of Louis Pasteur (1760) and David Koch (1856). Modern era of medicine was started in 1928, when Alexander Flaming discovered penicillin that could stop bacterial cell wall peptidoglycan biosynthesis. After World War II, United States of America came into power to make drug companies to commercialize antibiotics as they hold patents of those drugs. Thus, in 1950s, people's life expectancy was increased as 20 different antibiotics (ampicillin, oxacillin, tetracycline, chloramphenicol, trimethoprim, streptomycin, erythromycin, sulfadiazine etc.) were available to cure diseases like tuberculosis, typhoid, dysentery, cholera, tetanus and other viral diseases like small pox due to vaccination developed by Dr. Edward Jenner [4]. In 1953 after the discovery of DNA structure by Watson and Crick was major breakthrough in medicine followed by discovery of biochemical pathways using radioactive bio-molecules, DNA sequencing, recombinant DNA technology, genetic code, and purification of bio-molecules by chromatography as well as chemical structure determination by Mass, FT-IR and NMR spectrometry techniques. The concentration of active chemicals in different ayurvedic medicine as well as its utility has been questioned as extraction procedures vary during preparation. So, we have forgotten the old herbal medicine. In present days herbal pharmacopoeias like pH Eur 6, USP XXXI, and BP 2007 proscribed plant drugs of real medicinal value. This is similar in Charaka Samhita, Satruta Samhita and Atharva Veda written in Sanskrit during ancient Hindu Civilization in India (Bharat at that time). Due to antibiotic void, spread of multi-drug resistant bacteria, heavy toxicity of new drugs and very high cost of antibiotics, herbal research has given again priority to unfold traditional medicine [5].

First report of drug resistance in *Escherichia coli* appeared in 1940 and in 1965 sequence of the plasmid pBR322 was presented to discover two *mdr* genes *amp* and *tet* responsible for penicillin and tetracycline resistance respectively. Due to advancement of recombinant DNA technology and DNA sequencing, we have now million plasmids DNA sequences were deposited onto GenBank (www.ncbi.nlm.nih.gov/genbank). However, such sequencing programs confirmed that small R-plasmids were gradually combining with F'-plasmid (62.5kb) generating different large MDR conjugative plasmid that were continually donating the *mdr* genes to other gut and environmental bacteria by conjugation [6].

Amikacin was patented in 1971 and came into commercial use in 1976. It could be synthesized from Kanamycin-A, N-(benzylooxycarbonyloxy)-succinamide and (S)N-carbobenzyloxy-4-amino-2-hydroxy butyric acid. It is on the World Health Organization's List of Essential Medicines (Figure 1 and Figure 2) and was used against streptomycin resistant *Mycobacterium tuberculosis and Klebsiella pneumoniae*. The wholesale cost in the developing world is very high (50-130$ for a month). Amikacin irreversibly binds to 16S rRNA and the RNA-binding S12 protein of the 30S subunit of prokaryotic ribosome and inhibits protein synthesis. It changes the ribosome's shape so that it cannot read the mRNA codons correctly and likely also interact with the wobble base of the tRNA anticodon [7].

The literature search demonstrated that rose extracts had poor antibacterial activities but had strong anti-oxidant activities [8]. *Rosa canina L* extract showed the high methoxy cinnamic content (0.546 mg/g DW but *Rosa officinalis L* had 0.836 mg/g DW. Gallic acid, procyanidin-B2, catechin, chlorogenic, t-caffeic, ferulic, p-coumaric and sinapic acids were principal for all rose hip species [8]. *Rosa rubiginosa* and *Rosa canina* leaves have high flavonoids (2.5 mg/g DW), phenolics (4-5 mg/g DW) and antioxidant activities. Kazaz et al. [9] have shown that different roses have different chemical composition. So, studies on different color of roses and marigolds are justified [9,10]. Study indicated that *Rosa canina* had 0.006, 3.44, 1.52, 0.15, 0.55 mg/g DW of ferulic acid, p-anisic acid, salicylic acid, cinnamic acid and methoxy-cinnamic acid respectively and less amount

of those chemicals in *Rosa rubiginosa* except cinnamic acid which is little bit higher (Figure 3). Thus, chemical analysis of rose organic compounds varies from Author to Author.

Politi et al. [11] also have demonstrated recently that *Tagetes patula* has a complex chemical composition containing pigments, glycosylated flavonoids and thiophenes but less inhibitory to *Beauveria bassiana* or *Metarhizium anisopliae* and also has some effect on *Microsporum canis* and *Trichophyton rubrum* at 193.3 µg/mL and 253.9 µg/mL, respectively. *Tagetes* species have shown as natural pesticides due to presence of thiophenes, and polyacetylenic compounds to suppress nematode populations in the soil [12,13]. Mainly, 5-(3-buten-1-ynyl)-2, 2'-bithienyl (BBT) and 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (BBTOAc) are major thophanes in *Tagetes* species (*T. erecta, T. filifolia, T. lucida, T. minuta, T. patula and T. Tenuifolia*) and *T. minuta* had the highest total thiophene yield (518.8 mg/ml) (Figure 3). Thus, our search for antibacterial activities of rose and marigold extracts is justified. We have thus described here the effects of rose and marigold extracts on MDR bacteria as well as utility of red rose pigments.

Materials and Methods

Media and Antibiotics

LB media was prepared by adding 10 g NaCl, 10 g Tryptone and 5 g Peptone in 1000ml water and pH was adjusted with NaOH to pH 7.4. Autoclaved (15 psi/15min) LB+1.5% agar media was cooled to 45˚C, each 25 ml was taken in a sterilized 50 ml tube, antibiotic was added, mixed well and plated onto 10cm autoclaved plate. Antibiotic papers were purchased from HiMedia according to CLSI standard. Antibiotic papers are: Met-10 µg (methicillin), CAZ-30 µg (ceftazidime), AT-50 (aztreonam), COT-25 µg (Cotrimoxazole), LOM-15 µg (lomofloxacin), VA-10 µg (vancomycin), AK-10 µg (Amakacin), LZ-10 µg (linezolid), TGC-15 µg (tigecycline) and IMP-10 µg (imipenem). The antibiotic solutions were made as follows: ampicillin 50 mg/ml in water, tetracycline 20 mg/ml in ethanol, chloramphenicol 34 mg/ml in ethanol, ciprofloxacin 50 mg/ml in water, amikacin 10 mg/ml in water, cefotaxime 25 mg/ml in water, streptomycin 50 mg/ml in water and meropenem 10 mg/ml. All antibiotic solutions are stored at -20˚C [16].

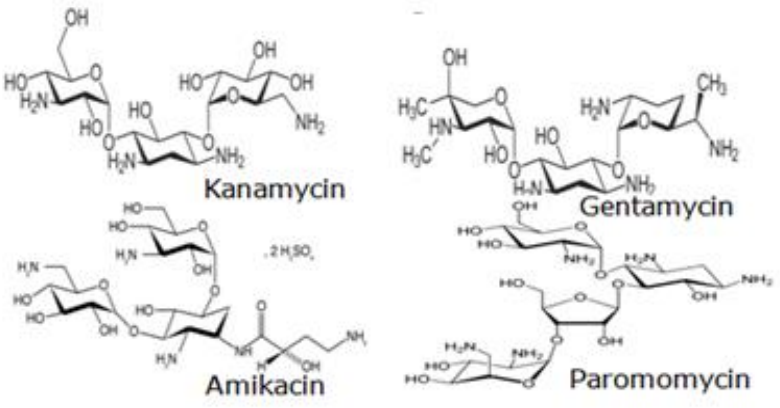

Figure 1: Structure of aminoglycoside antibiotics used to kill bacteria.

Isolation of superbugs from Ganga River Water

Ganga River water was collected in the day 3rd December, 2018 at 7AM during Lower Tide from Babughat, Kolkata-700001. 100 µl water was spread onto 10 cm LB + 1.5% Agar plates in presence of 2, 5, 10, 50 µg/ml amikacin. The plates are incubated

overnight at 37˚C and 1mm colonies are counted. 10 ml LB media + 10 µg/ml amikacin was inoculated with individual colony to grow for further work. Superbugs are isolated by further selection in 10 µg/ml meropenem and then further selection with each tetracycline (20 µg/ml), chloramphenicol (34 µg/ml), azithromycin (50 µg/ml), ofloxacin (50 µg/ml) [14].

Preparation of petal extracts and antibacterial assay

Fresh rose and marigold flowers were purchased from local market of Kolaghat (near Mechada Thermal Power Station at the bank of Rupnarayan River). The four-color codes were presented in (Figure 3). The procedure of extraction was shown below in a diagram. Chloroform extraction gave good solubility yellow extract with marigold but rose red color chemical appeared insoluble in chloroform and overnight extraction at room temperature without agitation give red oil and rest extract is white 0.1 ml overnight culture of MDR bacteria was spread onto 10 cm agar plate and 6 mm hole was done and extract was applied into hole and was incubated at 37˚C for overnight to see the lysis zone. Below 10 mm diameter zone was taken as insignificant [15,22].

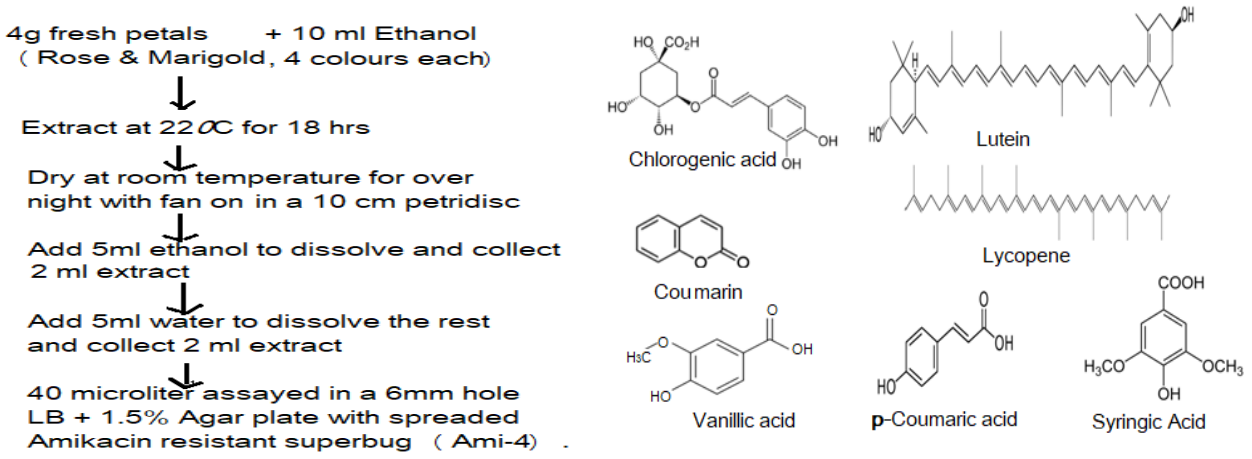

Figure 2: Structure of pigments (xanthocyanins) and chemicals (polyphenols and flavones).

Figure 3: Flowers used in the study for detection of anti-bacterial activities and color pigments.

Isolation of genomic DNA and plasmid DNA

Genomic DNA was isolated by Proteinase-K-SDS method following extraction with phenol-$CHCl_3$-isoamyl alcohol (25:24:1) and ethanol precipitation. 1.5 ml over-night culture was spun at 5,000 rpm and the bacterial pellet was dissolved in 50 µl TE

buffer (10 mM TrisHCl pH 8.0 + 1 mM EDTA) and 25µl 10% SDS and 5 µl of 20 mg/ml proteinase-K were added, incubated for 2-4 hrs and extracted with 25µl 5M sodium chloride and 100 µl $CHCl_3$: isoamyl alcohol (25:1). Then centrifuged at 8,000 rpm for 10 min. and the DNA pellet was dissolved in 50 µl TE buffer, treated 1µl RNase A and extracted with phenol-$CHCl_3$-isomyl alcohol (25:24:1) and was precipitated with 1/9 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol. The plasmid DNA was isolated from overnight culture using Alkaline-Lysis Method. Simply, to bacterial pellet 100 solution-I was added and vortexed. Then 200 µl of cold Solution-II added to make transparent solution and then 150 µl cold of Solution-III was added and mixed well and centrifuged at 10,000 rpm for 10 min [16]. To clear solution then added 1 ml 99% ethanol and centrifuged at 10,000 rpm for 10 min at 4˚C. Plasmid DNAs from four such preparation were combined and the RNA were removed by RNase-A treatment as above and finally plasmid DNA was dissolved in 50 µl TE buffer and was stored at -20˚C. 0.8% agarose gel electrophoresis in 1x TAE buffer at 50V for 4-6 hrs was performed to see the plasmid DNAs after staining in 0.5 µg/ml ethidium bromide and UV illumination [17].

Result

Isolation of Amikacin resistant bacteria from Ganga River water

100 µl of water was spread onto LB + 1.5% agar plate in presence of 0, 2, 5, 10, 50 µg/ml of amikacin giving 1.2×10^4, 0.34×10^3, $0.14x10^3$, $0.03x10^3$ colonies/ml water (Figure 4). This indicated that 0.0022% bacteria were amikacin resistant and which was very similar to meropenem, determined as 0.002%.

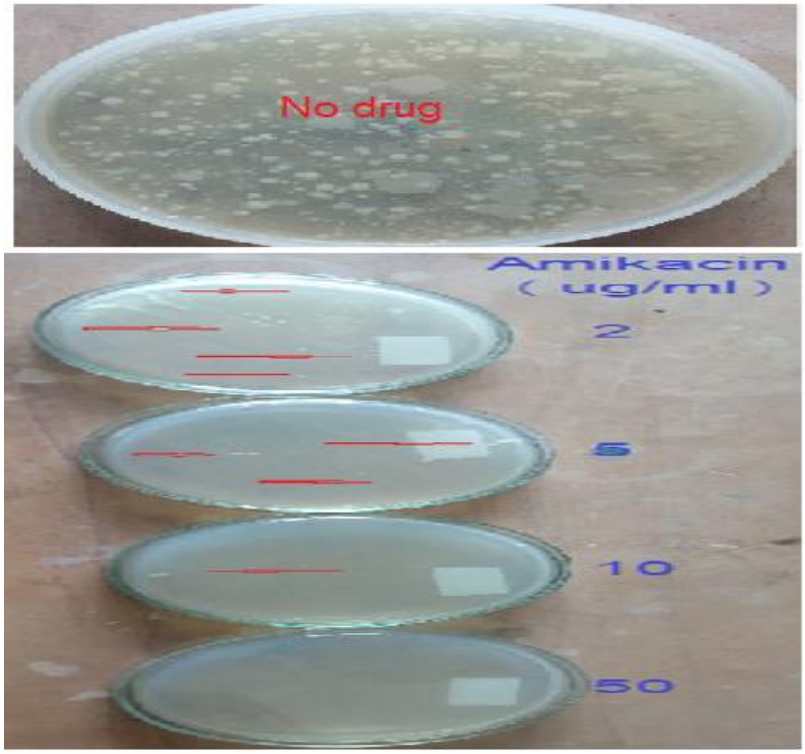

Figure 4: Selection of amikacin resistant bacteria from Ganga River water.

Thus, amikacin and meropenem are good drug whereas ampicillin, amoxicillin, tetracycline, streptomycin, ciprofloxacin, erythromycin, chloramphenicol and sulfamethozazole resistant species were determined previously as 40%, 35%, 26%, 33%,

22%, 18%, 12% and 16% respectively [18,22]. But single amikacin resistant is not a measure of multi-resistance. Individual colonies (Ami-1 to Ami-6) were picked up and grown in 10 µg/ml amikacin and such overnight culture was assayed for drug sensitivities using Hi-Media antibiotic papers according to CSLA guidelines. The result was demonstrated in (Figure 5A-5B).

Drug sensitivities of Amikacin resistant bacteria from Ganga River

We checked the drug sensitivity of isolated amikacin-resistant clones ami-1, ami-2 and ami-3 using antibiotics disc according to the CSLA standard. It concluded that all three clones are amikacin resistant but imipenem, aztreonam and Tigecycline sensitive where as ceftriaxone or sulfamethoxazole or prulifloxacin resistant (Figure 5A-5B). Thus, such study indicated that further selection is necessary to get ESBL and MBL-type superbugs.

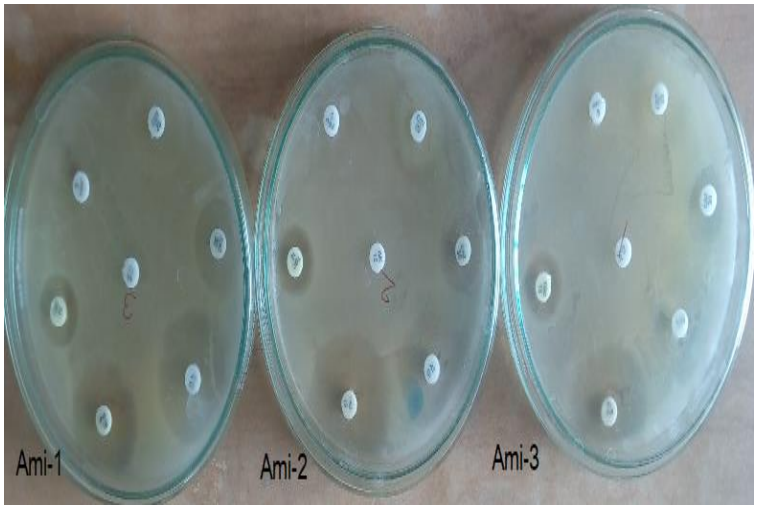

Figure 5A: Drug sensitivity of three amikacin resistant bacteria isolates.

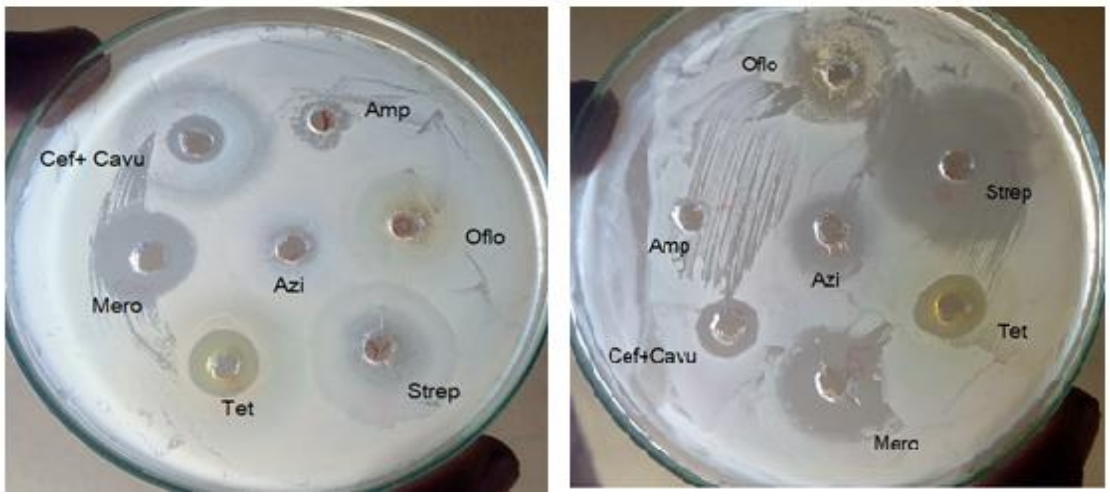

Figure 5B: Drug sensitivity of newly isolate amikacin resistant bacteria by agar hole assay. It was resistant to ampicillin, ceftriaxone, azithromycin, and ofloxacin but sensitive to meropenem and to moderate resistant to streptomycin and tetracycline (ami-4).

Isolation of multi-resistant superbugs from amikacin-selected bacteria

As we hardly detected the XRD amakacin-resistant superbugs during single amikacin-selection, we further selected amikacin-resistant pool of bacteria using meropenem. The meropenem resistant pool bacteria were further selected with each tetracycline,

chloramphenicol, ofloxacin and azithromycin. Such study is important to check how severely XRD and PDR clones are contaminated in Ganga River water which still carries most excreta of human, animals, birds and fishes. We isolated plasmids DNA and single colony purified for 16S rRNA sequencing (Figure 6). We also tested old extracts of *Suregada multiflora* roots extracted separately each with ethanol, methanol, ethyl acetate, chloroform, acetone, and benzene. This anti-bacterial study was conducted because our previous study confirmed that *E. coli* KT-1_mdr and *P. aeruginosa* PB-1_mdr (accession nos. KY769881, KY769875) were inhibited by all extracts. Amikacin + meropenem + tetracycline, amikacin + meropenem + chloramphenicol, amikacin + meropenem + ofloxacin, amikacin + meropenem + azithromycin are superbugs as resistant to three un-related advanced drug derivatives. Result indicated that all four superbugs populations were equally inhibited by six extracts (data not shown). We were excited about the result because such extracts were made on 12th May, 2018 and were kept at 4°C. We have checked the plasmid content and were detected few light plasmid bands as shown in (Figure 6). However, few bands are high molecular weight and needs further purification, PCR and sequence analysis to check Mdr genes.

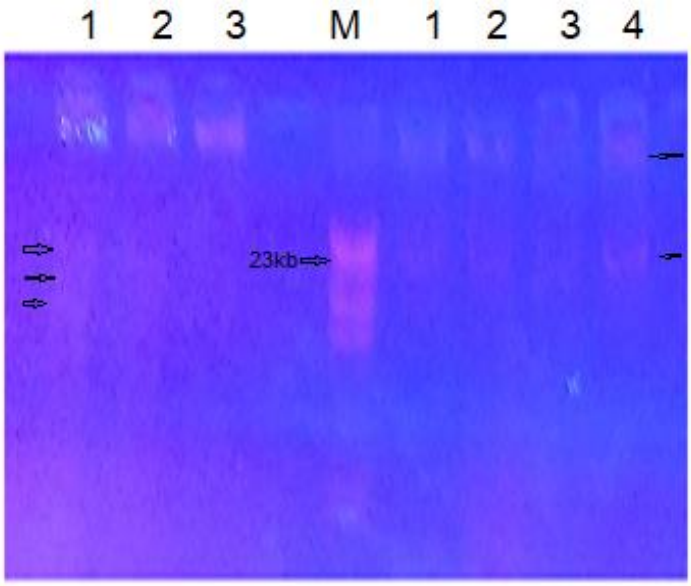

Figure 6: Isolation of plasmid and chromosomal DNA from amikacin resistant bacteria.

Color bleaching of Rose and Marigold ethanol extracts

The extracted chemicals and pigments gave differential color of ethanol solution extracts as shown in (Figure 7). While red rose gave violet color, rose color bleached into yellow as also yellow and white rose extracts. The red marigold gave brick color extract and brick or brown gave yellow extract while yellow marigold gave brick color extract. Interestingly, when we added ethanol to soluble the pigments, some parts were insoluble in ethanol. When we added water into residual pigments, all turned out very soluble in water, not only that red rose and marigold regained their red color indicating a oxidation-reduction process during such color bleaching. Chloroform extractions also induce such color bleaching in red rose or red marigold but yellow pigments are resistant to color bleaching (data not shown).

When red rose and red marigold were extracted with chloroform, color of marigold extract was yellow and color of residue petals remained red; but the color of rose extract was whitish and residue petals were red, although both cases a less amount red color oils were evident. Both chemicals dissolved in ethanol, gave differential anti-bacterial activities and chloroform

marigold extract was always better inhibitor than rose (data not shown). Red rose acetone and methanol extract gave bleached color but when dried, it gave beautiful huge magenta pigments suitable for painting of brick wall or coloring of fabrics while red marigold pigments in such extracts were insignificant but bioactive.

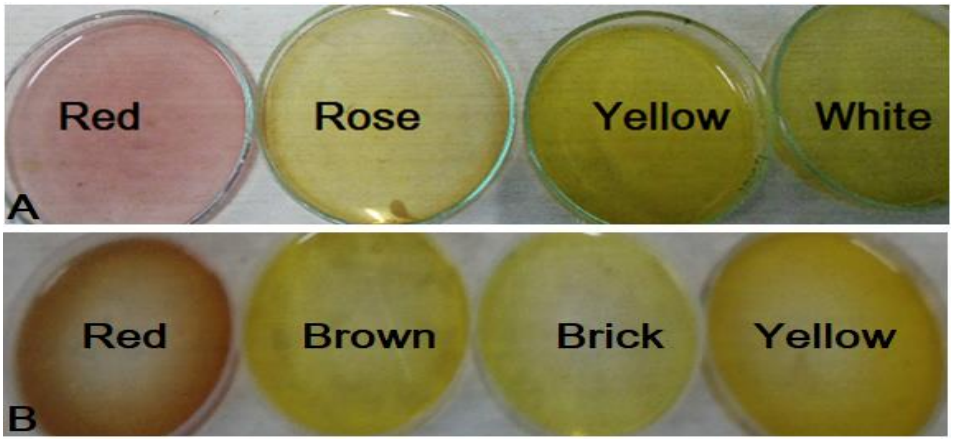

Figure 7: Color of different Rose and Marigold ethanol extracts after drying.

Effects of rose and marigold extracts on MDR bacteria from Ganga River water

We add 1ml Ganga River water to 10 ml LB media + 10 µg/ml amikacin to get overnight drug resistant bacteria population which will be further characterized biochemically and by 16S rRNA gene sequencing (in progress). (Figure 8A) showed that both rose and marigold alcohol extracts were inhibitory whereas rose color was best (21 mm zone). When all extract were dried and dissolved in 1st water and then residual un-dissolved chemicals in ethanol, it gave some valuable result. As for example, overall ethanol fractions gave better result than water fractions as demonstrated in (Figure 8B) for rose extracts. However, study confirmed that marigold dyes were more soluble in chloroform than ethanol, whereas such extract with red rose produced color oil which was inactive and rest extract was colorless but had anti-bacterial activity. Comparative anti-bacterial assays of all fractions of rose color for rose were presented in (Figure 8B). It was found that chloroform extract of rose two fractions gave distinct different anti-bacterial activities, white extract was strong than red oil fraction but total rose ethanol extract has better anti-bacterial activity. On the contrary, many phyto-oils were demonstrated for their potential anti-bacterial activity. While we have demonstrated a method of separating oil from rose petals and it was also a method to separate active ingredients from major color pigments of red rose and obvious may have a good industrial application. Similarly, when we extracted yellow petals of marigold with ethanol first, followed by chloroform, major yellow pigments were separated from other active chemicals in ethanol extract. In other words, differential solubility exists separating color pigments from rose and marigold. To differentiate the active chemicals, red rose ethanol fraction (bleached brick color) and ethanol-solubilized red oils of red rose subjected to preparative thin layer silica gel chromatography. While color pigment was cut from the TLC plate by naked eye, the colorless chemicals best separated by UV-shadowing. Other region of the TLC plate just scratched and solubilized in ethanol to get active chemical if any.

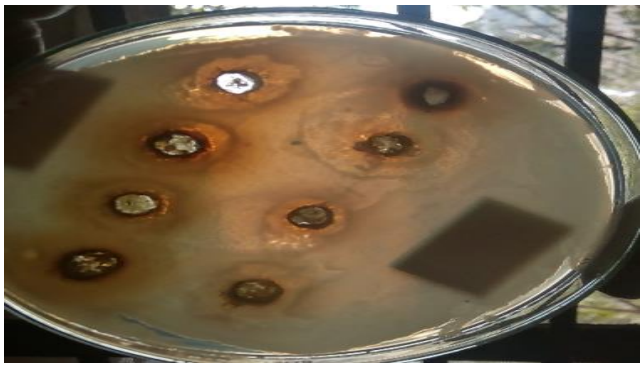

Figure 8A: Effects of crude ethanol extracts of different colors of rose and marigold on amikacin-resistant bacteria populations from Ganga River water.

Figure 8B: Differential bio-activities of fractionated rose extracts on MDR-bacteria.

Purification of active chemicals and their identification

Modern approach in pharmaceutical science is to elucidate the structure of active chemical by MASS, FT-IR and NMR spectrometry. We did preparative Thin Layer Chromatography to separate dyes and other colorless chemicals as presented in (Figure 9) illustrating that the water-soluble pigments of red rose could be significant commodity and yellow pigment in marigold was another. The ethanol soluble part however bio-activity against MDR-bacteria had and gave UV-shadowed band on TLC. Such purified chemicals were found potent to inhibit amikacin resistant superbugs. We checked the type of chemical in red rose water soluble pigment and as shown in (Figure 10) that triterpenoids was major product but trace amount of alkaloids and flavonoids were also detected. It needs NMR and FT-IR to address chemical structure more carefully.

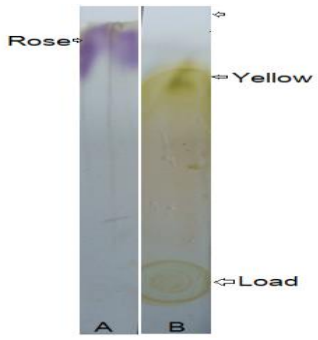

Figure 9: TLC of color chemicals from red rose and red marigold.

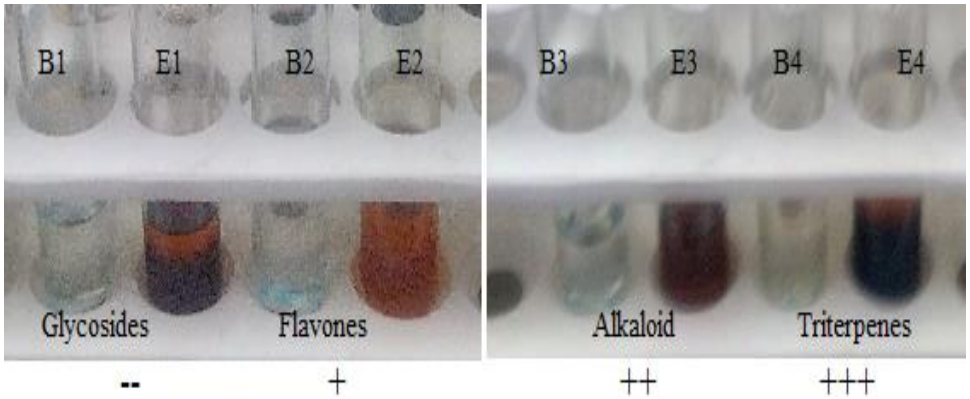

Figure 10: Chemical Assays of red rose water soluble pigments. It was indicated triterpenes and flavo-alkaloids are major chemical types.

Utility of flower pigments as organic paint for cotton fabrics and to make organic abir

As the extracted pigments were bleached in ethanol, we had tested the probability of such pigments to make organic paints. When we removed ethanol, the air oxidation was evident indicating electronic shift of organic pigments were possibly happening (Figure 11). So, when we added the 6N acid (HCl) into water soluble pigment, a dense red color solution was produced (Figure 11A). Similarly, when we added 6N alkali (NaOH), a dense bluish green color was produced which was abolished by addition of excess alkali and we got green color (Figure 11A). To get blue color, we would be added alkali less as demonstrated in (Figure 11B) and the compare with pH paper was shown in (Figure 11C). We make organic abir suitable for dole celebration in India as seen in (Figure 12A). The dye also used to stain the cotton cloth as demonstrated in (Figure 12B). However, for wall painting, such color bleaches due to reaction with lime present in wall we tested but it would be working for new cement wall or clay wall likely used by poor tribes of West Bengal.

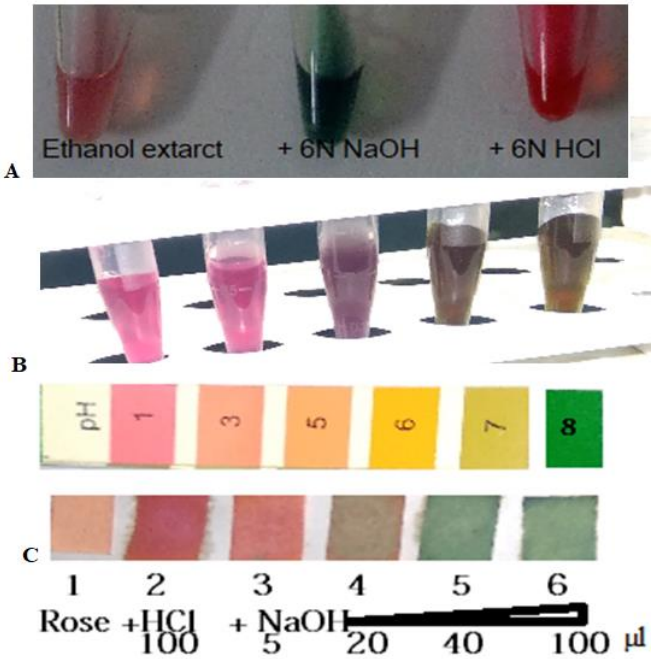

Figure 11: Acid-Base property of red rose water soluble pigments.

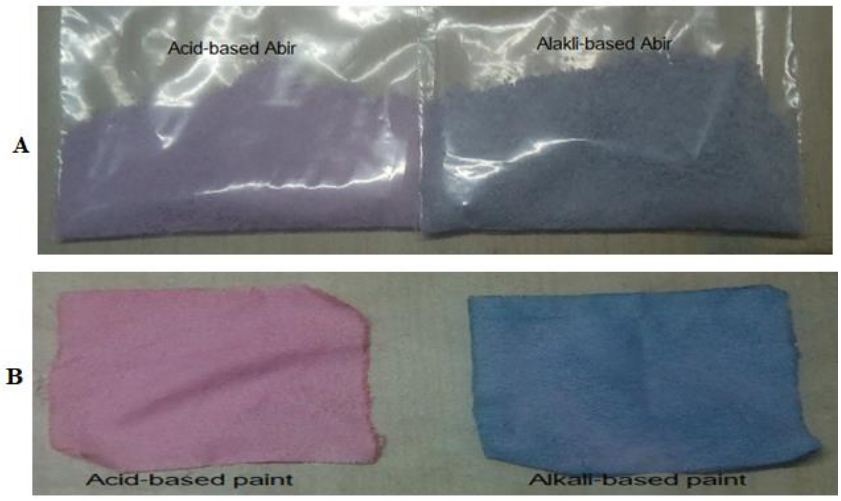

Figure 12: (A) Preparation of organic abir using rice powder + flour + talcum powder colored with acid-alkali treated water soluble red rose petals. **(B)** Staining of cotton fabrics using acid-alkali based rose pigments. Acid gave red color fabrics and alkali gave blue fabrics.

Discussion

The first paper of our laboratory was appeared in 2015 to demonstrate that Naringa bark extract had good activity to inhibit MDR-bacteria isolated from rain water and Kolkata street water [14]. The work was extended in 2017 to demonstrate that *Cassia fistula* and *Chenopodium album* had some activities [17]. In 2017, we clearly demonstrated the multi-resistance in *Escherichia coli, Pseudomonas aeruginosa and Stenotrophomonas sp.*, characterized by 16S-rRNA sequencing and BLAST. We characterized the effects of Derchini and Labanga on isolated superbugs from Ganga River and Digha Sea [18]. Now we detected very potent superbugs resistant to most advanced drugs like amikacin and meropenem. In this project, we have extensively studied the effect of Rose and Marigold flowers extracts on multidrug-resistant bacteria from Ganga River water collected during Lower Tide at 7 AM on 03-12-2018.

Tet and *cat* are early *mdr* genes and frequently associated with same plasmids close to *amp* gene or *bla* genes. Because we selected with meropenem, likely such clones may contain blaTEM, blaOXA, blaCTX-M and blaNDM1 types early beta-lactamases but surely gave ampicillin and cefotaxime resistant with respect to meropenem marker (likely blaVIM, blaKPC, blaIMP, blaNDM1 genes) are activated [19-21]. We have isolated single colony bacteria and again tested the effect of rose and marigold water and ethanol or chloroform soluble chemicals. We detected the amikacin resistant bacteria as contaminant in Ganga River water as 0.0022% which was very similar to 0.002% as in case of meropenem using 10 µg/ml drug concentration [18]. Such concentration is very high as compare to overall MIC used to characterize anti-bacteria activity of drug during pharmacokinetics analysis. Most bacteria with no MDR plasmid or chromosomal MDR island have estimated to die at 0.05µg/ml of those advanced derivatives. We determined that our isolated MDR bacteria were resistant to ampicillin and ceftriaxone but cavilinic acid has some additive effect in some but not in all. CSLA standard amikacin paper disc did not gave clear zone and indicated that Ami-1 to Ami-6 were real multi-drug resistant (Figure 5). But few potent drugs are inhibitory and thus such strains are not PAN drug resistant. Plasmids profiles in agarose gel did not give no clear band neither small plasmid (3-9 kb) band hardly have seen in all studied and again indicated heterogeneous high molecular conjugative plasmids (>50 kb). However, we have not tested *Tra* and *mdr* genes in our plasmids. We extensively reviewed *mdr* genes and sequenced *mdr*

plasmids which also have *Tra* genes and also many *IS*-elements with resolvases, DNA topoisomerases, DNA polymerases, integrases and recombinases scattered all over the plasmids [18,22,23]. Such plasmids are very bad in the sense, they could able to donate the MDR Genes to non-pathogenic as well as pathogenic bacteria amplifying the *mdr* genes in million bacteria present in the human and animal gut and water of river and sea [22]. Apart for multi-resistance study, we also made a method to isolate rose oil with chloroform. Further another method we have developed to isolate yellow pigment from marigold using differential extraction, first with ethanol and then with chloroform to get very good yellow pigment free of other chemicals (data not shown). Sadly, such red and yellow pigments have no anti-bacterial activities and may be utilized as food or medicine color additives [24,25].

Multi-drug resistance is worry some and affects million people worldwide. We must use vitamins and probiotics during each antibiotic use [18,26]. We must try alternate ways to develop basis for new drug development. As suggested by WHO and Antimicrobial Action Plan was established by G20 Nations. Antibiotics are good as long as R-plasmids cause resistance as in pSc101 and pMB plasmids those are used to make pBR322 containing *amp* and *tet* mdr genes. Now bacteria developed recombinant large conjugative F' plasmids (62.5 kb) that codes 20 TRA or TbrABC proteins and easily can donate the mdr genes by conjugation to all household bacteria in the intestine and water [37]. Large industry like mineral industry, paint industry, drug industry, paper industry, petroleum industry and excreta from 100 million peoples in many big cities (Mumbai, Delhi, Kanpur and Kolkata) release tons of chemicals, antibiotics and heavy metals into river water that are very harmful to bacterial central dogma enzymes like those are involved in replication, transcription and translation. Thus, bacteria also made drug efflux genes (known as tetA, acrAB, mexAB/CD/EF, and ABC genes) that could remove drugs and chemicals from cytoplasm of bacteria into outside keeping safe its cellular enzymes and nucleic acids as no enough drug concentration achieved above the drug MIC [19,20,24]. The research has also proved that taking excessively and repeatedly different prescription antibiotics cause genetic changes among the gut microbiota causing widespread multi-resistance. *Staphylococcus aureus* and *Acinetobacter baumannii* and also many household bacteria like *Escherichia coli* genome-MDR-islands were sequenced confirming the calamity (see accession nos. LK054503; KM921776). That is not the end, porin membrane proteins were also mutated in such a way that antibiotics receptors were altered and no drug could enter into bacteria at low drug concentration giving MDR. Further, ribosomal ribonucleic acids (16S and 23S rRNA genes) and ribosomal proteins (*rps*L and *rrs* genes) were mutated [26]. On one word, bacteria have achieved many shrouds against antibiotics and drug companies did not know where to start. So study the superbugs of Ganga River is important and herbal extract research must be accelerated. Marmol et al. [27] recently have showed some anti-bacterial and anti-cancer activities of rose extract as nanoparticles. Interestingly, we showed less activity in rose extract as compare to marigold using amikacin resistant bacteria. Amikacin is a potent drug and widely prescribed against multi-resistant infections. Amikacin has limited access to nucledyl transferase and aminoacetyl transferase but most -OH phosphorylating and acetylating enzymes could inactivate the drug more easily. AAC6'-IV, AAD (4, 4') and APH6'-1b-cr of *Pseudomonas aeruginosa* and *Staphylococcus aureus* may be resistant to amikacin, tobramycin, neomycin and kanamycin. Thus, such *mdr* genes likely accumulated in isolated superbugs. The rose and marigold ethanol extract are less potent as compare to MDR-Cure and water soluble pigment have growth promoting effects on bacteria. However, purified chemicals (flavones, thiophanes and alkaloids) may be useful in combating MDR-infections.

Rosa canina chemical, tellimagrandin was shown a good inhibitor of MDR bacteria by interacting penicillin binding protein of MRSA [29]. Zhao et al. [30] tested the effect of *Rosa laevigata* flavonoids on rat models and confirmed their nephroprotective

effect and also *Rosa laevigata* extract reduced reactive oxygen species levels via increasing superoxide dismutase [31-33]. Rose arotinoids like lutein, neochrome, zeaxanthin, rubixanthin, lycopene was cytotoxic to *Helicobacter pyroli*. However, the amount of chemicals is not enough for drug development. The estimated phyto-chemicals in methanol extract of Tagetes species were determined by Sytar et al. recently. 4-hydroxy benzoic acid, vanillic acid, chlorogenic acid, syringic acid, and pcoumaric acid content were estimated as 0.279, 0.258, 0.032, 0.132, and 0.078 mg/g respectively [33]. Data indicated that specific antibacterial components (UV-deflective) are less in both flowers. However, higher thiophene content in Tagetes minuta in regulating pests and nematodes well described as its utility [11]. We described the utility of red rose and marigold in dye industry as rose color chemicals were converted into intense blue and red pigments that colored the cotton fabrics and cement wall very well. We also made color nontoxic organic abir from grounded rice and wheat mix which may be very useful during Dole Festival of India. It is very disappointed that we are unable to add rose and marigold extracts to prepare MDR Cure extract to prevent superbug infections [14]. Notably, other than heterogeneous phyto-antibiotics, various efforts are underway to develop phage therapy and gene therapy technologies against MDR infections [34,35]. Nanotechnology applications have advanced to kill superbugs at low toxic drug concentration and gold nanoparticles with plant extract are also beneficial to cure MDR infections [36]. However, multi-resistance is becoming worse day by day as more and more genetic changes in bacterial plasmids and chromosome [21,37-40].

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