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## Characterization of Drug Resistant Bacteria, Conjugal Transfer Efficiency and their Growth Kinetics Against *Cassia* Plants Leaf Extract

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### ABSTRACT

In this present investigation, two Multi Antibiotics Resistant (MAR) bacterial strains namely SR2 and SR4 were isolated from clinical waste. The bacterial isolate SR2 was resistant to most of the antibiotics tested, but sensitive to levofloxacin. While, the other strain SR4 was sensitive to Cefixime and levofloxacin. Morphological, biochemical and 16S rRNA sequence analysis identified these 2 bacterial strains SR2 and SR4 as *Acinetobacter* sp. (GenBank Acc. No. KJ879241) and *Aeromonas hydrophila* (GenBank Acc. No. KJ879242), respectively. The conjugal transfer efficiency of antibiotics resistant gene to another bacterial strain was also tested, using *Corynebacterium alkanolyticum* ATH3 as a recipient. In order to control these pathogenic bacterial strains, leaf extracts obtained from three *Cassia* plants named *C. siamea*, *C. allata* and *C. occidentalis* were used at different concentration. *Cassia siamea* was recorded to be most effective against both of these bacterial strains. Growth kinetics of these two bacterial strains revealed that growth was decreased with gradual increase of concentration of leaf extract, obtained from *C. siamea* and ultimately negligible growth at 30 mg mL<sup>-1</sup> (3%).

**Key words:** Drug resistant bacteria, 16S rRNA, conjugal transfer efficiency, growth inhibition, *Cassia*, leaf extract, growth kinetics

### INTRODUCTION

Emergence of multiple drug resistance bacteria in last few years are major concern for world health issues. Over use of antibiotics in different sectors leading bacteria made adaptation to got resistance against antibiotics (Snell, 2008). Not only clinical sector, random use of antibiotics in different animal farm is the another way to make Multi Drug Resistant (MDR) bacteria, which infect human through food chain (McEwen and Fedorka-Cray, 2002; Fraise, 2002). Mostly, antibiotics resistance bacteria are found in soil sample of clinical and other hospital areas (Montelli and Levy, 1991). Many researchers have isolated and characterized the multiple drug resistance bacteria from different sources like edible snow crab (Kim *et al.*, 2013), retail ground meat (White *et al.*, 2001) and waste water (Pandey *et al.*, 2011). In a similar study carried out by Chapin *et al.* (2005), reported the existence of multidrug resistant bacteria in swine feeding materials. Random usage of antibiotics serves as an environmental pressure to select for strains

with elevated drug tolerance. The WHO (2000) reported the world wide prevalence of MDR. Aminov (2010) stated that in the year 1992 US Government spent about \$ 1.3 billion for treatment against bacterial infection and in the year 2006 it became \$ 1.87 billion. Bacteria possess several antibiotics resistance mechanism such as enzymatic inactivation of drug (Davies, 1994), acquired resistance through mutation in genetic material (Seward *et al.*, 1998) and protein channel, which efflux the antibiotics out of the cell (Nikaido, 1994).

For a long period, plants are used as traditional medicine against different disease in all over the world. It is already established that plants are the good source of phenolic compound, vitamins and minerals that have been used in various field like cancer, tumor, illness as well as microbial infection (Malini *et al.*, 2013). World Health Organization considered the medicinal plants to be the best source for drug in different countries (Borde *et al.*, 2013). Previously, a research team in Argentina tested 122 plants for their antimicrobial activity against 2 well-known pathogenic bacterial species, *Staphylococcus aureus* and *Escherichia coli* (Nascimento *et al.*, 2000). Replacement of antibiotics with plant extracts might be the solution for controlling MDR bacteria, which is a burning issue for environment and animal health. Till date the medical importance of different species of *Cassia* plants have not been explored. Therefore, the aim of the present research work was (1) Characterization and identification of multi drug resistant (2) Conjugal transfer efficiency of antibiotic resistant property and (3) effect of *Cassia* plant leaf extract in growth regulation of the MAR bacteria.

## **MATERIALS AND METHODS**

**Collection of soil sample:** In this present investigation, soil samples were collected from several hospitals located at New Delhi, India. One gram of soil sample was dissolved in 10 mL of distilled water and serial dilution was made up to  $10^{-5}$  times. For isolation of antibiotics resistant bacteria, 100  $\mu$ L solution from each dilution was spread on nutrient agar plates (pH 7.0) containing ampicillin ( $100 \mu\text{g mL}^{-1}$ ) and incubated for 24 h at  $37^\circ\text{C}$ . Separate bacterial colony appeared on the plates were collected and pure culture was done by repeated streaking method. Primarily twenty isolates were taken for minimum inhibitory concentration experiment.

**Determination of minimum inhibitory concentration:** Minimum Inhibitory Concentration (MIC) of seven different antibiotics named ampicillin, amoxicillin, penicillin-G, levofloxacin, tetracycline, streptomycin and cefixime were determined in Mueller-Hinton agar plates (pH 7.0) by well diffusion assay. Each antibiotic was used in several concentrations, ranging from  $2.5\text{-}10 \mu\text{g mL}^{-1}$  and MIC was determined visually by appearance of transparent zone surrounding the well. On the basis of MIC experiment, two bacterial strains designated as SR2 and SR4 isolated from Lal Bahadur Sastri Govt. Hospital Mayur vihar phase III New Delhi were selected for further studies due to their multiple drug resistant property.

**Phenotypic and biochemical characterization of the selected strains:** Colony morphology (pigment, surface, margin and elevation) of these selected bacterial strains were visually determined in nutrient agar plates. Gram's staining was done following standard Hans Christian Gram protocol. Physiological characteristic i.e temperature and pH tolerance of these selected strains were checked in tryptone soya broth medium (pH 7.0). In order to characterize these bacterial strains, various biochemical tests viz., catalase, oxidase, MR, VP, indole, citrate and urease were done manually. Acid production from various carbohydrates were checked in HiMedia kit.

**Extraction of genomic DNA:** Genomic DNA was extracted using standard protocols. In brief, 14 h cultured bacterial broth were transferred in a 1.5 mL tube and centrifuged at 10,000×g for 10 min. Supernatant was discarded and to the pellet 500 µL of lysis buffer and 100 µL SDS (10%) were added, mixed carefully and kept for incubation (50°C) for 30 min. Thereafter, equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added, mixed thoroughly and again centrifuged for 10 min at 10,000×g. Upper aqueous layer containing the DNA was collected carefully and mix with equal volume of isopropanol and kept at -20°C for 1 h. In order to collect the precipitated DNA, it was again centrifuged for 10 min at 10,000 ×g, followed by 70% ethanol wash (twice). Finally, ethanol was evaporated and DNA was dissolved in appropriate TE buffer and stored it at -20°C.

**Polymerase chain reaction amplification and 16S rDNA analysis:** PCR was carried out in standard condition in Eppendorf Thermal Cycler. The PCR products were bi-directionally sequenced using forward AGAGTTTGATCMTGGCTCAG (B27 F) and reverse GGTTACCTTGTTACGACTT (1491 R) primer. Finally, sequenced data were edited, aligned and analyzed using bioinformatics tool (Codon-code and Mega 4.0) for finding the closest homolog of the microbes using a combination of NCBI (National Centre for Biotechnology Information) Gen Bank and RDP (Ribosomal Database Project) database.

**Conjugal transfer of antibiotics resistant property:** In order to study the transfer of antibiotic resistant property, these two bacterial isolates SR2 (white colony) and SR4 (creamy white colony) were cultured separately in TSA broth (pH 7.0) along with an antibiotic sensitive (ampicillin and penicillin-G) bacterial strain *alkanoalyticum* ATH3 (Gen Bank Acc. No. JX656749). After 14 h of incubation, 100 µL of samples were taken from each conical flask, diluted 5 times, plated on TSA plate and incubated for 24 h at 37°C. Single colony (Yellow colour) of *Corynebacterium alkanolyticum* ATH3 obtained after incubation was again cultured in nutrient broth and checked its antibiotic resistant property in Mueller-Hinton agar plates against different concentration of ampicillin and penicillin-G.

**Preparation of plant extract:** Mature fresh leaves of *C. allata*, *C. occidentalis* and *C. siamea* were collected from local area and washed with distilled water. Leaves were dried in shade area and finally in hot air oven at 50°C. Dried leaves were then powdered with the help of liquid nitrogen and methanolic extract, was prepared using Soxhlet extractor. Methanol was evaporated by rotary evaporator and crude extract was collected and stored at 4°C for future use.

**Inhibition of MAR bacteria using plant extract:** Effect of leaves extract against MAR bacteria were carried out on Mueller-Hinton agar plates (pH 7.0) using well diffusion assay. Several concentrations (5-30 mg mL<sup>-1</sup>) of leaf extracts were prepared in distilled water and dissolved using 10 µL of DMSO. Hundred microliter of log phase culture from each bacterial samples were spread on agar plates. Five millimeter wells were made using cork brooder and filled with plant extract and incubated for 24 h at 37°C. Transparent zone around the well indicated the inhibition of growth.

**Growth kinetics study against *Cassia siamea* leaf extract:** The leaf extract, which showed highest growth inhibition in plate assay, was chosen for growth kinetics studies. These 2 bacterial strains SR2 and SR4 were cultured separately in 200 mL of tryptone soya broth (pH 7.0) along with

different percentage of *C. siamea* leaf extract and incubated at 37°C at 100 rpm. Optical density was measured at 600 nm using spectrophotometer (Beckman DU730) at every one hour of interval for 16 h, considering extract free bacterial culture as control.

## RESULTS

In the present study, two antibiotic resistant bacterial strains namely SR2 and SR4 were isolated from hospital soil samples. Minimum Inhibitory Concentration (MIC) of these two bacterial strains SR2 and SR4 were determined and tabulated in Table 1 and 2, respectively. The bacterial strain SR2 can tolerate amoxicillin and penicillin-G at higher concentration (1 mg mL<sup>-1</sup>), followed by cefixime, ampicillin and tetracycline (100, 100 and 50 µg mL<sup>-1</sup>, respectively). While, levofloxacin was detected to be highly sensitive for SR2. Similarly, the bacterial strain SR4 can grow on amoxicillin and penicillin-G containing media upto 1 mg mL<sup>-1</sup> but highly sensitive to cefixime and levofloxacin (Table 2).

Primarily, these bacterial strains were identified by morphological and biochemical characterization. Morphologically both the strains SR2 and SR4 are smooth, convex and regular but differ in their surface pigment (Table 3). Both the strains were able to grow at 20°C and can tolerate upto 50°C. The bacterial strain SR2 can grow at pH 6-9, whereas SR4 can grow at pH 5-9. Biochemical and carbohydrate utilization ability of these two strains SR2 and SR4 were given in the Table 4. Both the strains showed positive reaction in citrate utilization, catalase production, indole production and gelatin utilization, whereas, negative for methyl red and oxidase production. The bacterial strain SR2 was negative for Voges-Proskauer test and unable to utilize glucose, fructose and sucrose but can hydrolyze starch and lactose. Whereas, the bacterial strain SR4 showed positive reaction for Voges-Proskauer test and can actively use glucose, fructose and sucrose as their carbon source. These 2 MAR bacterial strains were further identified by 16S rDNA sequence analysis (Fig. 1). The bacterial strain SR2 was identified as *Acinetobacter* sp. (Acc No. KJ879241), while SR4 was identified as *Aeromonas hydrophila* (Acc No. KJ879242).

Table 1: Minimum inhibitory concentration for the selected bacterial strain SR2 against seven antibiotics

Concentration used	Amoxicillin	Penicillin-G	Tetracycline	Levofloxacin	Streptomycin	Cefixime	Ampicillin
2.5 (µg mL <sup>-1</sup> )	R	R	R	R	R	R	R
5 (µg mL <sup>-1</sup> )	R	R	R	R	R	R	R
10 (µg mL <sup>-1</sup> )	R	R	R	S	R	R	R
25 (µg mL <sup>-1</sup> )	R	R	R	S	R	R	R
50 (µg mL <sup>-1</sup> )	R	R	R	S	S	R	R
100 (µg mL <sup>-1</sup> )	R	R	S	S	S	R	R
250 (µg mL <sup>-1</sup> )	R	R	S	S	S	S	S
500 (µg mL <sup>-1</sup> )	R	R	S	S	S	S	S
1 (mg mL <sup>-1</sup> )	R	R	S	S	S	S	S
10 (mg mL <sup>-1</sup> )	S	S	S	S	S	S	S

R: Resistant, S: Sensitive

Table 2: Minimum inhibitory concentration of the selected bacterial strain SR4 against seven antibiotics

Concentration used	Amoxicillin	Penicillin-G	Tetracycline	Levofloxacin	Streptomycin	Cefixime	Ampicillin
2.5 (µg mL <sup>-1</sup> )	R	R	R	R	R	R	R
5 (µg mL <sup>-1</sup> )	R	R	R	R	R	R	R
10 (µg mL <sup>-1</sup> )	R	R	R	S	R	S	R
25 (µg mL <sup>-1</sup> )	R	R	R	S	R	S	R
50 (µg mL <sup>-1</sup> )	R	R	S	S	R	S	R
100 (µg mL <sup>-1</sup> )	R	R	S	S	R	S	R
250 (µg mL <sup>-1</sup> )	R	R	S	S	S	S	S
500 (µg mL <sup>-1</sup> )	R	R	S	S	S	S	S
1 (mg mL <sup>-1</sup> )	R	R	S	S	S	S	S
10 (mg mL <sup>-1</sup> )	S	S	S	S	S	S	S

R: Resistant, S: Sensitive

Table 3: Morphological and physiological characteristics of these selected bacterial strain

Characteristics	Bacterial strain	
	SR2	SR4
Morphology		
Pigment	White	Creamy white
Surface	Smooth	Smooth
Margin	Regular	Regular
Elevation	Convex	Convex
Gram stain	- Rod	- Rod
<b>Temperature (°C)</b>		
10	-	-
20-40	+	+
50	+	+
60	-	-
<b>pH</b>		
4	-	-
5	-	+
8-June	+	+
9	+	+
10	-	-

+: Growth, -: No growth

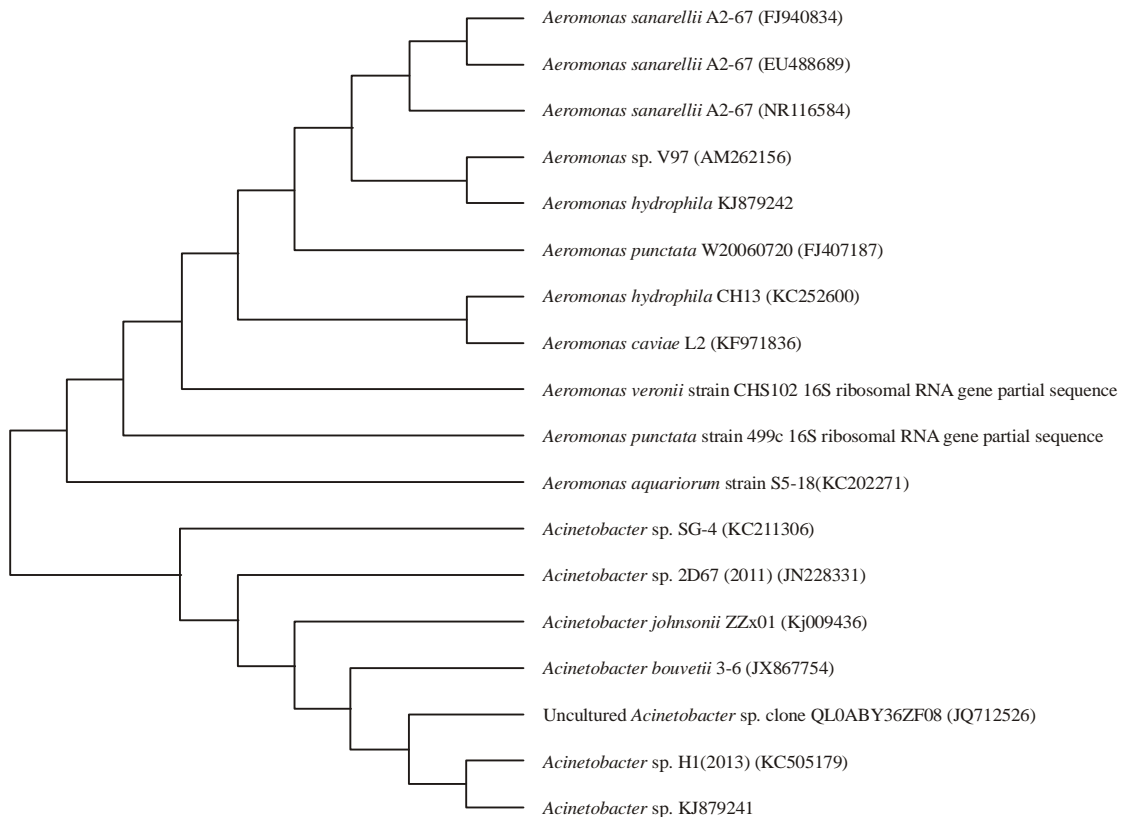


Fig. 1: Phylogenetic analysis of these two bacterial strains with their close homologous available in NCBI database

Conjugal transfer of antibiotic resistant gene to an another antibiotic sensitive bacterium, *C. alkanolyticum* ATH3 was tabulated in Table 5. It was observed that the recipient

Table 4: Biochemical characteristics of these two selected bacterial strains

Biochemical test	Strain SR2	Strain SR4
Citrate	+	+
Methyl red	-	-
Voges-proskauer	-	+
Indole	+	+
Catalase	+	+
Oxidase	-	-
Gelatin	+	+
Urease	+	-
Glucose	-	+
Fructose	-	+
Starch	+	-
Sucrose	-	+
Lactose	+	-

+: Positive, -: Negative

Table 5: Conjugal transfer of antibiotic resistant gene

<i>Corynebacterium alkanolyticum</i> ATH3				
Concentration used	Before transfer		After transfer	
	Ampicillin	Penicillin-G	Ampicillin	Penicillin-G
10 ( $\mu\text{g mL}^{-1}$ )	S	S	R	R
25 ( $\mu\text{g mL}^{-1}$ )	S	S	R	R
50 ( $\mu\text{g mL}^{-1}$ )	S	S	R	R
100 ( $\mu\text{g mL}^{-1}$ )	S	S	R	R
250 ( $\mu\text{g mL}^{-1}$ )	S	S	S	R
500 ( $\mu\text{g mL}^{-1}$ )	S	S	S	R
1 ( $\text{mg mL}^{-1}$ )	S	S	S	S

R: Resistance, S: Sensitive

bacterial strain *C. alkanolyticum* ATH3 became resistant to ampicillin ( $100 \mu\text{g mL}^{-1}$ ) and penicillin-G ( $500 \mu\text{g mL}^{-1}$ ) after conjugal transfer experiment.

In the present study, leaf extracts obtained from three *Cassia* plants, namely *Cassia siamea*, *Cassia allata* and *Cassia occidentalis* were used at different concentration to control the growth of these isolated antibiotics resistance bacterial strains (Plate 1). Leaf extract from *C. siamea* showed highest inhibitory action against both of these isolated bacterial strains (Fig. 2a and b). Whereas, leaf extract obtained from *C. occidentalis* was not effective against SR2 and SR4 (Fig. 2e and f). While, extract obtained from *C. allata*, was effective at higher concentration (Fig. 2c and d). Growth kinetics of these selected bacterial isolates SR2 and SR4 were presented in Fig. 3 and 4, respectively. In both cases, it was observed that bacterial growth was decreased with gradual increase of concentration of leaf extract obtained from *C. siamea* and ultimately negligible growth at  $30 \text{ mg mL}^{-1}$  (3%).

## DISCUSSION

Antibiotics are chemotherapeutic compound that are widely used against different microbial infections. Since the discovery of penicillin, antibiotics were considered as “magic bullets” in curing infectious diseases. But in course of time it has been misused and abused in the clinical treatment. Say for example, antibacterial drugs were inappropriately prescribed to patients with viral infection due to misdiagnosis. In this present study, we have isolated 2 bacteria, namely SR2 and SR4 that showed resistant against few broad spectrum antibiotics. Based on morphological, biochemical and 16S rRNA sequence analysis these two bacterial strains SR2 and SR4 were

identified as *Acinetobacter* sp. and *Aeromonas hydrophila*, respectively. The 16S rDNA gene sequence analysis provides a detailed phylogenetic placement of the bacterial isolates and can be

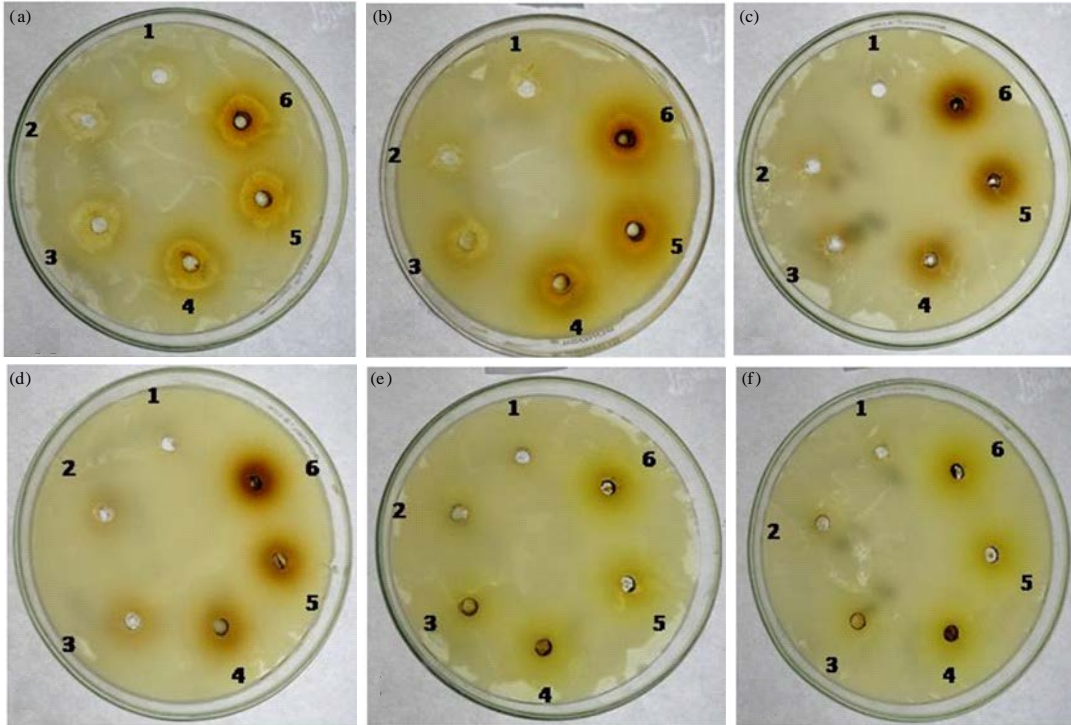


Fig. 2(a-f): Antimicrobial activity of three *Cassia* sp. leaf extract against these two bacterial strains SR2 and SR4, (a, c and e), effect of leaf extract obtained from *C. Siamea*, *C. allata* and *C. occidentalis* against SR2, respectively, (b, d and f), effect of leaf extract obtained from *C. Siamea*, *C. allata* and *C. occidentalis* on SR4, respectively. 1:5, 2:10, 3:15, 4:20, 5:20 and 6:30 mg mL<sup>-1</sup>

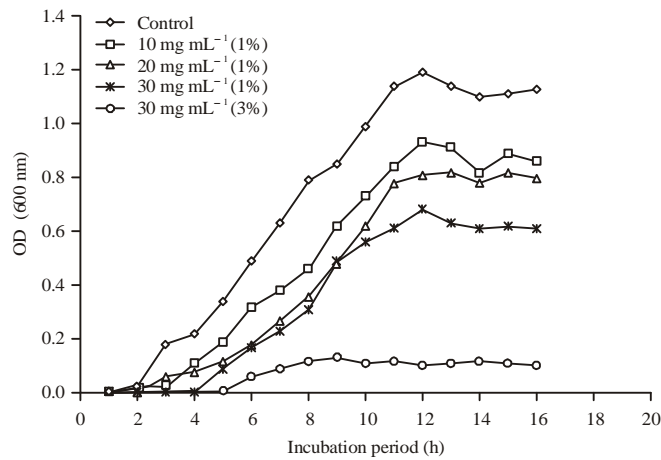


Fig. 3: Growth kinetics of the bacterial strain SR2 against *C. siamea* leaf extract. Here 1 and 3% means 1 and 3 mL of plant extract in 100 mL of culture broth



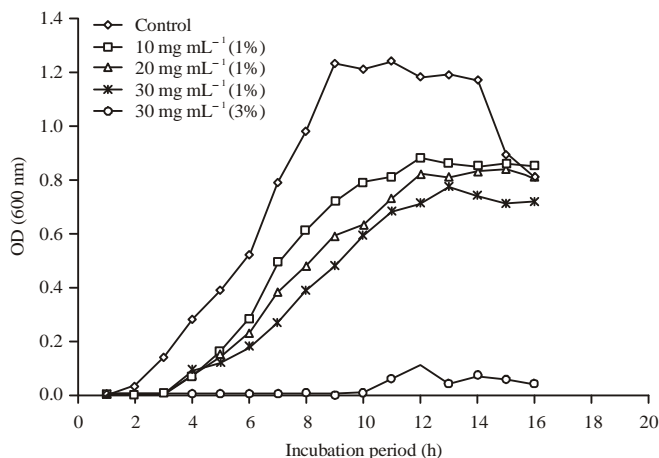


Fig. 4: Growth kinetics of the bacterial strain SR4 against *C. siamea* leaf extract. Here 1 and 3% means 1 and 3 mL of plant extract in 100 mL of culture broth

used for the construction of a set of oligonucleotide probes for a rapid detection of bacterial community. Emergence and spread of *Acinetobacter* sp. against available antimicrobial agent is a major concern and often reflects an evolutionary processes. Infections caused by *Acinetobacter* sp. are broad and mainly found in tropical region (Leung *et al.*, 2006). Hanberger *et al.* (1999) isolated *Acinetobacter* sp. from different human health care units and reported their antibiotics resistance level. Existence of drug resistance *Acinetobacter* associated with hospital waste was also reported (Turner *et al.*, 2003). While, Disease associated with *Aeromonas hydrophila* are commonly found in aquatic animal, mainly in fish (Vijayabaskar and Somasundaram, 2008). *Aeromonas hydrophila* was also reported to cause septicemia and wound in the gastrointestinal tract of human (Bi *et al.*, 2007). The development and persistence of multi-drug resistant bacteria increasing the challenges to public health and the use of antibiotics in animal farm has markedly contributed to this critical problem as well.

Conjugation is the genetic material exchanging process found in bacteria. Antibiotic resistant gene or R-plasmid is an autonomously replicating extra-chromosomal material that confers antibiotic resistant property in bacteria. Bacteria can transfer their R-plasmid to other bacterial strain through lateral gene transfer mechanism (Kruse and Sorum, 1994). In our investigation, it was detected that both of these isolated bacterial strains were capable of transfer their drug resistant property. Previously researchers have investigated the plasmid transfer in bacteria in several natural conditions such as, wastewater (Gealt *et al.*, 1985), soil (Trevors *et al.*, 1987) and gastrointestinal tract (Linton, 1986). Due to this property, antibiotic resistant bacteria make other bacterial species to be resistant against a wide range of antibiotics, which are a great threat for human being. If this is going on for a long period of time, the antibiotics available in market will be useless.

The control of MAR bacteria is a big challenge for environment, as well as for human health. It was found that plant extract, which is cost effective, might be useful to control the MAR bacteria. *Cassia* plants are widely distributed in India and people from ancient period are using this against skin disease such as eczema and scabies (Singh *et al.*, 2013). Powder made up from *Cassia* plants are also used as natural pesticides in India (Linton, 1986). Due to great potential of antimicrobial

activity and availability, *C. siamea* leaf extract might be useful in treatment of various infections caused by these drug resistant bacteria. Further details study should be conducted to purify and characterize the active compound.

## CONCLUSION

In recent year, multiple drug resistance bacteria are the major threat for environment, as well as for animal health. In this present investigation, 2 multidrug resistant pathogenic bacterial strains were isolated from clinical waste, characterized and identified as *A. hydrophila* and *Acinetobacter* sp. Due to conjugal transfer efficiency of antibiotic resistant property, these bacteria are considered to be dangerous for environment. In order to control of these isolated bacterial strains, we have used leaf extracts obtained from three *Cassia* plants. It was observed that *Cassia siamea* was most effective and it can completely inhibit these bacterial growth at lower concentration (30 mg mL<sup>-1</sup>). So further research should be conducted to purify and characterize the active compound that can be used against these bacterial strains in future.

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