FINAL REPORT
19th January, 2017 to 19th July, 2020

Project Title:

"Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India and cattle, poultry and ducks of West Bengal"

(BT/PR16149/NER/95/85/2015 dated 19/01/2017)

Implementing NER Centres

PI: Dr. T. K. Dutta, Professor & Head
Department of Veterinary Microbiology
CVSc&AH, Central Agricultural University
Selesih, Aizawl, Mizoram - 796014

PI: Dr. Manabendra Mandal, Professor
Molecular Biology and Biotechnology
Tezpur University,
Napam, Tezpur, Assam-784028

Collaborating Centres

PI: Dr. S. N. Joardar, Professor
Department of Veterinary Microbiology
West Bengal University of Animal & Fishery Sciences
68, K. B. Sarani, Kolkata- 700037

PI: Dr. Asifa Qureshi, Senior Scientist
Environmental Biotechnology and Genomics Division (EBGD)
CSIR-NEERI, Nagpur 440020

ANNUAL PROGRESS REPORT FOR R&D PROJECTS
(19/01/2017 to 31/03/2019)
Section-A: Project Details

A1. Project Title:

"Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India and cattle, poultry and ducks of West Bengal**"**

A2. DBT Sanction Order No. & Date:

BT/PR16149/NER/95/85/2015 dated 19/01/2017

A3. Name of Principal Investigator:

NER Institute-1
Dr. T. K. Dutta, Professor & Head (PI)

Name of Co-PI/Co-Investigator:
Dr. A. K. Samanta, Professor & Head
Dr. Gunjan Das, Associate professor
Dr. P. Roychoudhury, Associate Profesosr
Dr. T. K. Rajkhowa, Professor & Head

NER Institute-2
Dr. Manabendra Mandal, Professor (PI)

Collaborating Institute-1
Dr. S. N. Joardar, Professor (PI)

Name of Co-PI/Co-Investigator:
Dr. Indranil Samanta, Assistant Professor
Dr. Tapas Kumar Sar, Assistant Professor(SS)
Dr. Samir Dey, Assistant Professor

Collaborating Institute-2
Dr. Asifa Qureshi, Senior Scientist (PI)

Name of Co-PI/Co-Investigator:
Dr. Hemant J Purohit, Scientist

A4. Institute:

1. College of Veterinary Sciences & Animal Husbandry, CAU, Mizoram (NER Institute)
2. Tezpur University, Napam, Tezpur, Assam (NER Institute)
3. West Bengal University of Animal and Fishery Sciences, 68 K. B. Sarani, Kolkata-700037, West Bengal (Collaborating Institute)
4. Environmental Biotechnology and Genomics Division, CSIR-NEERI, Nagpur, Maharasthra (Collaborating Institute)

A5. Address with Contact Nos. (Landline & Mobile) & Email:

(NER Institute-1)
Department of Veterinary Microbiology
College of Veterinary Sciences & Animal Husbandry
Central Agricultural University

Selesih, Aizawl, Mizoram-796014
tapandutta@rediffmail.com
0389-2361748(O), 09862335294(M)

(NER Institute-2)
Department of Molecular Biology and Biotechnology
Tezpur University, Napam, Tezpur, Assam-784028
+919864181445; mandal@tezu.ernet.in

(Collaborating Institute-1)
Department of Veterinary Microbiology
West Bengal University of Animal and Fishery Sciences, 68 K. B. Sarani, Kolkata 700037, West Bengal
Phone: 09007836307, 09231533335
Email: joardar69@gmail.com

(Collaborating Institute-2)
Department of Environmental Genomics Division
Environmental Biotechnology & Genomics Division,
CSIR-National Environmental Engineering Research Institute (NEERI)
Nehru Marg, Nagpur PIN: 440020
0712-2249883; a_qureshi@neeri.res.in

A6. Total Cost:
Rs. 150.00 Lakhs (for four collaborating partners)
(Funds for **CSIR-NEERI –**Rs **27.23 lakhs**)

A7. Duration:
03 years from the date of issue of the sanction order No. BT/PR16149/NER/95/85/2015 dated 19/01/2017.
(got Six months extension till July 2020)

A8. Approved Objectives of the Project:

- To study the prevalence of biofilm producing bacteria (*Staphylococcus*, *Escherichia coli*, *Salmonella* and *Pseudomonas*) in pigs, cattle and poultry in NER and in cattle, poultry and ducks in West Bengal (CAU and WBUAFS).
- To detect the linkage between presence of biofilm-associated genes and *in vitro* biofilm production capacity of the isolates (CAU and WBUAFS).
- To detect the correlation between biofilm-associated genes, virulence genes, antimicrobial resistance genes of the isolates (CAU and WBUAFS).
- To detect antigenic characteristics and to identify immunodominant proteins of biofilm producing bacterial isolates (WBUAFS).
- To screen the traditionally used medicinal plants from North East India for microbial biofilm inhibition and quorum sensing inhibition (model organism: *Pseudomonas aeruginosa*) (Tezpur University).
- To purify and characterize bioactive molecules from active plant sources (Tezpur University).
- **To study the mechanism of action of bioactive molecule on quorum sensing inhibition and/or anti-biofilm property (NEERI,** Tezpur University).

- **To study the gene expression analysis of targeted biofilm forming genes (*pel, alg, bdlA*) and transcriptional profile in model biofilm bacterial culture in presence of bioactive molecules (NEERI)**

A9. Specific Recommendations made by the Task Force (if any): NA

Section-B: Scientific and Technical Progress

Lead Centre-1 at CAU, Aizawl, Mizoram

B1. Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period (1000-1500 words for interim reports; 2500-3500 words for final report; data must be included in the form of up to 3 figures and/or tables for interim reports; up to 7 figures and/or tables for final reports):

Sample collection

A total of 789 fresh samples were collected randomly from cattle (milk=301), pigs (feces=215) and chicken (cloacal and oropharyangeal swabs= 273) of five North eastern states of India (Table-1). All the samples were collected from the animal maintanined under organized as well as unorganized farming system irrespective age, sex and breed during the study. All the samples were collected using a sterilized adsorbent cotton swab. However, for collection of samples from distant locations, a sterilized swab dipped in brain heart infusion broth was used as transport medium and transported to the laboratory under cold chain for further processing. Details are given in Table 1-7.

Table-1: Details of sample collection from five North eastern states of India for isolation of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* from cattle, pigs and chickens

Sl	Host	Specimen	Number of samples collected					
			Arunachal Pradesh	Assam	Manipur	Meghalaya	Mizoram	Total
1.	Cattle	Milk	40	46	28	37	150	301
2.	Pig	Fecal sample	30	25	30	30	100	215
3.	Chicken	Oropharyngeal and cloacal swabs	40	50	40	43	100	273
Total			110	121	98	110	350	789

Table-2: Isolation of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* from cattle, pigs and chickens of five North eastern states

Sl. No.	Source	Sample	No. of samples	Isolates			
				E. coli	*Salmonella* spp.	*S. aureus*	*P. aeruginosa*
1.	Cattle	Milk	301	_	_	183	_
2.	Pig	Fecal sample	215	291	29	_	29
3.	Chicken	Oropharyngeal and cloacal swabs	273	173	24	_	19
Total			789	464	53	183	48

Table-3: Isolation of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* from cattle, pigs and chickens of Arunachal Pradesh

Sl. No.	Source	Sample	No. of samples	Isolates			
				E. coli	*Salmonella* spp.	*S. aureus*	*P. aeruginosa*
1.	Cattle	Milk	40	_	_	19	_
2.	Pig	Fecal sample	30	22	2	_	1
3.	Chicken	oropharyngeal and cloacal swabs	40	13	-	_	-
		Total	110	35	2	19	1

Table-4: Isolation of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* from cattle, pigs and chickens of Assam

Sl. No.	Source	Sample	No. of samples	Isolates			
				E. coli	*Salmonella* spp.	*S. aureus*	*P. aeruginosa*
1.	Cattle	Milk	46	_	_	29	_
2.	Pig	Fecal sample	25	46	11	_	8
3.	Chicken	oropharyngeal and cloacal swabs	50	39	12	_	2
		Total	121	95	23	29	10

Table-5: Isolation of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* from cattle, pigs and chickens of Manipur

Sl. No.	Source	Sample	No. of samples	Isolates			
				E. coli	*Salmonella* spp.	*S. aureus*	*P. aeruginosa*
1.	Cattle	Milk	28	_	_	25	_
2.	Pig	Fecal sample	30	29	6	_	11
3.	Chicken	oropharyngeal and cloacal swabs	40	37	4	_	11
		Total	98	66	10	25	22

Table-6: Isolation of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* from cattle, pigs and chickens of Meghalaya

Sl. No.	Source	Sample	No. of samples	Isolates			
				E. coli	*Salmonella* spp.	*S. aureus*	*P. aeruginosa*
1.	Cattle	Milk	37	_	_	30	_
2.	Pig	Fecal sample	30	33	1	_	3
3.	Chicken	oropharyngeal and cloacal swabs	43	16	2	_	1
		Total	110	49	3	30	4

Table-7: Isolation of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* from cattle, pigs and chickens of Mizoram

Sl. No.	Source	Sample	No. of samples	Isolates			
				E. coli	***Salmonella* spp.**	***S. aureus***	***P. aeruginosa***
1.	Cattle	Milk	150	_	_	80	_
2.	Pig	Fecal sample	100	161	9	_	6
3.	Chicken	oropharyngeal and cloacal swabs	100	68	6	_	5
		Total	350	229	15	80	11

Isolation and identification of *E. coli*, *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Escherichia coli

The collected faecal samples and cloacal swabs were directly inoculated on MacConkey's Agar (HiMedia) plates and incubated at 37ºC overnight. After incubation, at least 5 pink coloured colonies were randomly selected from each plate and streaked on Eosin Methylene Blue (EMB) agar (HiMedia) plates and incubated overnight at 37ºC. Colonies with characteristic metallic sheen on EMB agar were studied for their morphological characteristics (Quinn *et al.* 2004).

A total of 464 *E. coli* was isolated and identified, of which 291 and 173 were from feaces of pigs and cloacal swabs of chicken, respectively (Table-2).

Morphologically, all the *E. coli* isolates showed small, pink colonies on MacConkey's (MLA) agar medium. A characteristics metallic sheen was also recorded on Eosin Methylene Blue (EMB) agar. On Gram's staining, all the isolates were Gram negative coccobacilli under 100 X light microscope. Biochemically, all were positive for indole and methyl red tests and negative for oxidase, Voges-Proskauer and citrate utilization tests. All the isolates also fermented glucose, sucrose and lactose with production of gas. All the phenotypically confirmed isolates were further subjected for the conformation by BD Phoenix™ and genotypically confirmed by 16S-rRNA species specific PCR.

***Salmonella spp*.**

Isolation of *Salmonella* spp. from collected samples was done as per standard bacteriological method. About 1 to 2 gm of faecal sample was inoculated in 5 ml Rappaport Vassiliadis broth (Hi Media) and incubated at 37ºC overnight for enrichment. After incubation, they were streaked on Hektoen Enteric agar (HiMedia) and Xylose lysine deoxycholate agar (HiMedia) and incubated at 37ºC overnight. Typical black centred with bright edged colonies were selected randomly and at least 5 colonies were streaked on Brilliant green agar (BGA) plates (HiMedia) followed by overnight incubation at 37ºC. Colonies with pink colour on BGA agar were studied for their morphological characteristics (Quinn *et al.* 2004).

A total of 53 *Salmonella* spp. were isolated and identified, of which 29 and 24 were from feces of pigs and cloacal swabs of chicken, respectively (Table-2).

Morphologically, all the *Salmonella* spp. isolates showed typical black centred colonies on Hektoen Enteric agar (HEA) and Xylose lysine deoxycholate agar (XLD) medium. The suspected colonies from HEA and XLD were further streaked on Brilliant green agar (BGA) plates followed by overnight incubation at 37ºC. Colonies with pink colour on BGA agar were studied for Gram's staining. All the isolates were Gram negative coccobacilli under 100 X light microscope. Biochemically, all were positive for methyl red tests and citrate utilization test and negative for oxidase, Voges-Proskauer and indole test. All the isolates also fermented glucose, maltose and mannitol. All the phenotypically confirmed isolates were further subjected for the conformation by BD Phoenix™ and genotypically confirmed by 16S- rRNA species specific PCR.

Staphylococcus aureus

The collected milk samples were directly inoculated on Nutrient Agar (HiMedia) plates and incubated at 37ºC overnight. After incubation, at least 5 golden yellow coloured colonies were randomly selected from each plate and streaked on Baired Parker agar (HiMedia) plates and incubated overnight at 37ºC. Colonies with characteristic black colour from BP agar were studied for their morphological characteristics (Quinn *et al.* 2004).

A total of 183 *Staphylococcus aureus* was isolated and identified from 301 milk samples (Table-2).

Morphologically, all the *S. aureus* isolates showed golden yellow colonies on Nutrient agar medium. A characteristic black coloured colony was also recorded on Baired Parker agar. On Gram's staining, all the isolates were Gram positive coccai with characteristics branch of grapes appearance under 100 X light microscope. Biochemically, all were positive for catalase, methyl red test and Voges-Proskauer test and negative for oxidase and indole test. All the isolates also fermented glucose, sucrose, maltose and mannitol without gas. All the phenotypically confirmed isolates were further subjected for the conformation by BD Phoenix™ and genotypically confirmed by 16S- rRNA species specific PCR.

Pseudomonas aeruginosa

The collected milk samples, fecal samples, cloacal swabs and oropharyngeal swabs were directly inoculated on Cetrimide Agar (HiMedia) plates and incubated at 37ºC overnight. After incubation, at least 5 yellow green coloured colonies were randomly selected from each plate and were studied for their morphological characteristics (Quinn *et al.* 2004).

A total of 48 *P. aeruginosa* was isolated and identified, of which 29 and 19 were from pigs and chicken, respectively (Table-2).

Morphologically, all the *P. aeruginosa* showed yellow green coloured mucoid or non-mucoid colonies on Cetrimide Agar (CMA). On Gram's staining, all the isolates were Gram

negative bacilli under 100 X light microscope. Biochemically, all were positive for catalase, oxidase and citrate utilization test and negative for indole, methyl red and Voges-Proskauer test. All the isolates ferment mannitol. All the phenotypically confirmed isolates were further subjected for the conformation by BD Phoenix™ and genotypically confirmed by 16S- rRNA species specific PCR.

Detection of *in vitro* biofilm production ability of the bacterial isolates

Escherichia coli

Biofilm production ability of *E. coli* isolates were performed by microtiter plate biofilm assay as described by Weiss-Muzkat *et al.* (2010) with some modification. The detailed method applied was as follows-

1. Isolates were grown overnight at 37°C in LB broth.
2. The optical density at 600 nm (OD_{600}) of the culture was adjusted to 1.0 (corresponding to ca. 10^8 CFU/ml).
3. The culture was then diluted at 1:10 dilution in fresh LB broth and 20 µl inoculated in three consecutive wells of 96 well tissue culture plate, which was already filled with 200 µl fresh LB broth.
4. Three parallel wells containing 200 µl fresh LB broth were marked as negative control for each isolates.
5. Plates were incubated at 37°C for 24 hours.
6. After incubation, plates were washed three times with 250 µl/well of sterile phosphate buffered saline (PBS) (pH-7.4) to remove the unattached bacteria.
7. The plates were dried at 26°C for 15 min, and each well was stained with 200 µl 0.1% crystal violet (in water) for 20 min.
8. Excess stain was gently rinsed off with tap water and the plates were air dried.
9. The OD_{595} values was determined in a microplate reader after solubilization of the dye with 95% ethanol (200 µl per well).
10. Bacterial biofilms were classified based on a OD cut-off ODc (OD of negative control) as described by Stepanovic *et al*. (2004). The ODc was defined as three standard deviations from the OD mean of the negative control. No biofilm formation was OD < ODc; a weak biofilm former was ODc < OD < (2 X ODc); a moderate was biofilm former (2 X ODc) < OD < (4 X ODc); and, a strong biofilm former was (4 X ODc) < OD (Stepanovic *et al*. 2004).

A total 55 (18.90%) *E. coli* isolates from pigs were biofilm producer, of which 29 (9.96%), 15 (5.15%) and 11 (3.78%) were recorded as weak, moderate and strong biofilm producer, respectively (Table-8, Fig-5). Similarly, a total of 74 (42.77%) *E. coli* isolates from chicken were biofilm producer, of which 23 (13.29%), 27 (15.61%) and 24 (13.87%) isolates were recorded as weak, moderate and strong biofilm producer, respectively (Table-8, Fig-5).

***Salmonella* spp.**

Biofilm assay was performed by microtiter plate biofilm assay as described by Vetsby *et al.* (2009) with some modification.

1. Isolates were inoculated in LB broth without Nacl supplemented with 2% glucose and incubated at 37°C for 12-16 hours under constant shaking.
2. The optical density at 595 nm (OD_{595}) of the culture was adjusted to 0.2 (corresponding to ca. 10^8 CFU/ml).
3. 30 µl bacterial suspension was inoculated in three consecutive wells of 96 well tissue culture plate, which was already filled with100 µl LB broth without Nacl supplemented with 2% glucose.
4. Three parallel wells containing 100 µl fresh LB broth without Nacl supplemented with 2% glucose were marked as negative control for each isolates.
5. Plates were incubated at 20.0 ± 1°C for two days without shaking.
6. Following incubation, plates were gently washed once with sterile distilled water (SDW) to remove unattached bacteria and dried at room temperature.
7. Each well was stained with 130 µl of 1% crystal violet and incubated at room temperature for 30 minutes.
8. The plates were washed three times with SDW.
9. Then 130 µl ethanol: acetone mixture (70:30 v/v) was added in each well and incubated at room temperature for 10 minutes.
10. The OD_{595} values were determined in a microplate reader after solubilization of the dye with ethanol: acetone.
11. Bacterial biofilms were classified based on a OD cut-off ODc (OD of negative control) as described by Stepanovic *et al*. (2004). The ODc was defined as three standard deviations from the OD mean of the negative control. No biofilm formation was OD < ODc; a weak biofilm former was ODc < OD < (2 X ODc); a moderate was biofilm former (2 X ODc) < OD < (4 X ODc); and, a strong biofilm former was (4 X ODc) < OD (Stepanovic *et al*. 2004).

A total 17 (58.62%) *Salmonella* spp. isolates from pigs were recorded as biofilm producer, of which 13 (44.82%), 3 (10.34%) and 2 (6.90%) were recorded as weak, moderate and strong biofilm producer, respectively (Table-8, Fig-5). Similarly, a total of 22 (91.67%) *Salmonella* spp. from chickens were found to be positive for biofilm production, of which 9 (37.50%), 8 (33.33%) and 5 (20.83%) isolates were recorded as weak, moderate and strong biofilm producer, respectively (Table-8, Fig-5).

Staphylococcus aureus

Biofilm assay was performed by microtiter plate biofilm assay as described by Emel Mataraci (2012) with some modification.

1. Isolates were inoculated in LB broth supplemented with 0.9% Nacl and 1% glucose and incubated at 37°C for 24 hours under constant shaking @ 130 rpm.
2. The culture was diluted at 1:50 in fresh LB broth supplemented with 0.9% Nacl and 1% glucose to make a final concentration of approximately 1×10^7 CFU/200 µl and inoculated in three consecutive wells of 96 well tissue culture plate.
3. Three parallel wells containing 200 µl fresh LB broth supplemented with 0.9% Nacl and 1% glucose were marked as negative control for each isolates.
4. Plates were incubated at 37°C for 24 hours without shaking.
5. After incubation, plates were washed three times with 200 µl/well of sterile phosphate buffered saline (PBS) (pH-7.4) to remove the unattached bacteria and air dried.
6. A 200 µl volume of 99% methanol was added in each well for 15 min and incubated at room temperature for fixation.
7. Then contents were aspirated gently by pipette and plates were air dried.
8. Each well was stained with 200 µl 0.1% crystal violet (in water) for 5 minutes.
9. Excess stain was gently rinsed off with tap water and plates were air dried.
10. Stain was resolubilized in 200 µl of 95% ethanol with shaking in an orbital shaker for 30 minutes.
11. The OD_{595} values were determined in a microplate reader.

Bacterial biofilms were classified based on a OD cut-off ODc (OD of negative control) as described by Stepanovic *et al*. (2004). The ODc was defined as three standard deviations from the OD mean of the negative control. No biofilm formation was OD < ODc; a weak biofilm former was ODc < OD < (2 X ODc); a moderate was biofilm former (2 X ODc) < OD < (4 X ODc); and, a strong biofilm former was (4 X ODc) < OD (Stepanovic *et al*. 2004).

A total of 83 (45.36%) isolates from cattle milk were recorded as biofilm positive, of which 27 (14.75%), 25 (13.66%) and 31 (16.94%) were recorded as weak, moderate and strong biofilm producers, respectively (Table-8, Fig-5).

Pseudomonas aeruginosa

Biofilm assay was performed by microtiter plate biofilm assay as described by Perez *et al.* (2011) with some modification.

1. Isolates were inoculated in LB broth supplemented with 1% glucose and incubated overnight at 35±1°C for 18 h without shaking.
2. The stationary phase culture was vortexed and diluted at 1:10 in fresh LB broth supplemented with 1% glucose.
3. 200 µl of diluted culture were inoculated in three consecutive wells of 96 well tissue culture plate.
4. Three parallel wells containing 200 µl fresh LB broth supplemented with 1% glucose were marked as negative control for each isolates.
5. Plates were incubated at 35±1°C for 18h without shaking.

6. After incubation, plates were washed three times with 200 µl/well of sterile phosphate buffered saline (PBS) to remove the unattached bacteria and air dried.
7. Each well was stained with 200 µl 0.9% crystal violet (in water) for 15 min.
8. Excess stain was gently rinsed off with tap water and plates were air dried.
9. Stain was resolubilized in 200 µl of 95% ethanol for 15 min.
10. The OD595 values were determined in a microplate reader.
11. Bacterial biofilms were classified based on a OD cut-off ODc (OD of negative control) as described by Stepanovic et al. (2004). The ODc was defined as three standard deviations from the OD mean of the negative control. No biofilm formation was OD < ODc; a weak biofilm former was ODc < OD < (2 X ODc); a moderate was biofilm former (2 X ODc) < OD < (4 X ODc); and, a strong biofilm former was (4 X ODc) < OD (Stepanovic et al. 2004).

All *P. aeruginosa* isolates from pigs were recorded as biofilm positive, among them 5 (17.24%) isolate was moderate and 24 (82.76%) were recorded as strong biofilm producer (Table-8, Fig-5). Similarly, a total of 14 (73.68%) of *P. aseruginosa* isolates from chickens were found to be biofilm positive, of which 11 (57.89%) and 3 (15.79%) were recorded as weak and moderate biofilm producers (Table-8, Fig-5).

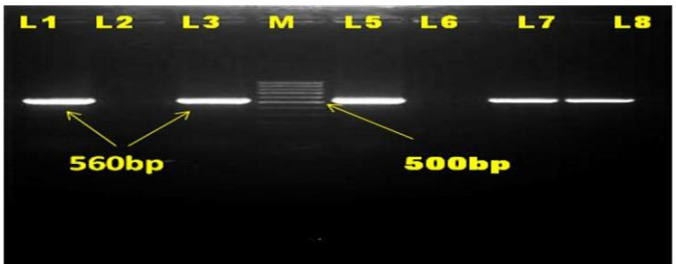


Figure-1: Agarose gel electrophoresis showing the PCR amplicons of *E. coli* 16S rRNA gene (560bp) obtained from *E. coli* isolated from pig and chicken of Mizoram. M: 100bp DNA ladder; L1: Positive control; L2: Negative control; L6: Negative sample; L3, L5, L7 & L8: Representative samples showing *E. coli* 16S rRNA gene amplicons.

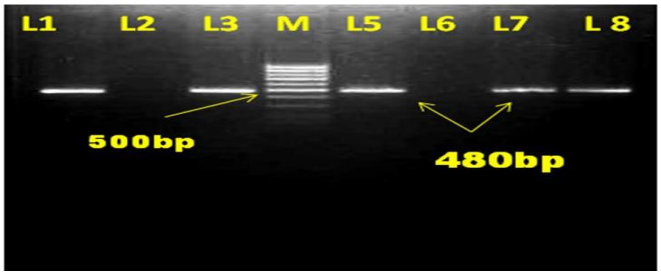


Figure-2: Agarose gel electrophoresis showing the PCR amplicons of *Salmonella* spp. *16s rRNA* gene (480bp) obtained from *Salmonella* spp. isolated from pig and chicken of Mizoram. M: 100bp DNA ladder; L1: Positive control; L2: Negative control; L6: Negative sample; L3, L5, L7, L8: Representative samples showing *Salmonella* spp. 16S rRNA gene amplicons.

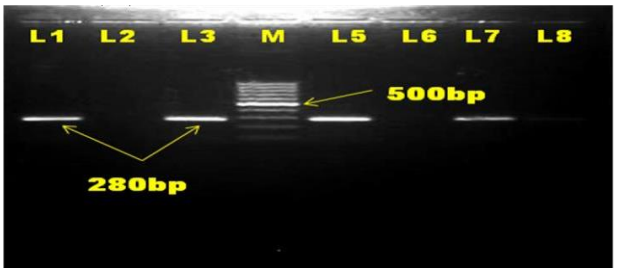


Figure-3: Agarose gel electrophoresis showing the PCR amplicons of *S. aureus nuc* gene (280bp) obtained from *S. aureus* isolated from cattle of Mizoram. M: 100bp DNA ladder; L1: Positive control; L2: Negative control; L6: Negative sample; L3, L5, L7, L8: Representative samples showing *S. aureus nuc* gene amplicons.

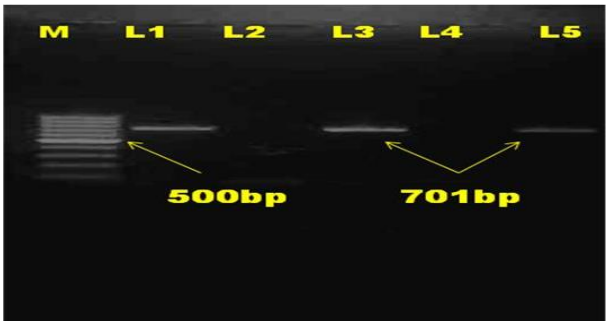


Figure-4: Agarose gel electrophoresis showing the PCR amplicons of *P. aeruginosa 16s rRNA* (701bp) obtained from *P. aeruginosa* isolated from pig and poultry of Mizoram. M: 100bp DNA ladder; L1: Positive control; L2: Negative control; L4: Negative sample; L3, L5: Representative samples showing *S. aureus nuc* gene amplicons.

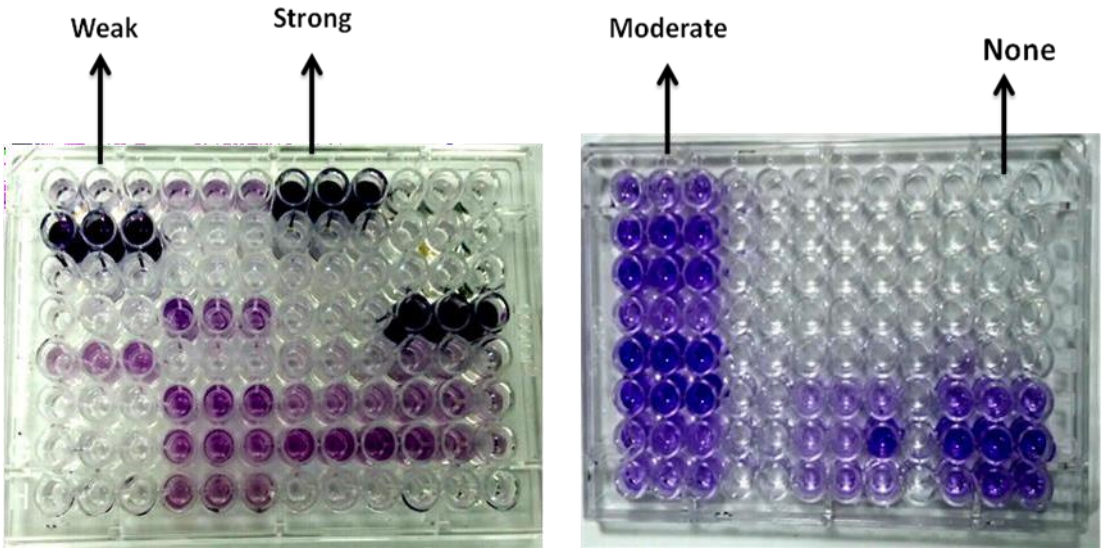


Fig-5: *In vitro* biofilm production by microtiter plate assay showing none, strong, moderate and weak biofilm producers differentiated by crystal violet stain in 96-well tissue culture plate.

Table-8: Phenotypic detection of biofilm producing ability of *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* isolated from cattle, pigs and chickens.

Biofilm producing ability of *Staphylococcus aureus*					
Source	**No. Of isolates tested**	**Weak**	**Moderate**	**Strong**	**Total**
Cattle	183	27 (14.75%)	25 (13.66%)	31 (16.94%)	83 (45.36%)
Subtotal	183	27 (14.75%)	25 (13.66%)	31 (16.94%)	83 (45.36%)
Biofilm producing ability of *Escherichia coli*					
Pig	291	29 (9.96%)	15 (5.15%)	11 (3.78%)	55 (18.90%)
Chicken	173	23 (13.29%)	27 (15.61%)	24 (13.87%)	74 (42.77%)
Subtotal	464	52 (11.21%)	42 (9.05%)	35 (7.54%)	129 (27.80%)
Biofilm producing ability of *Salmonella* spp.					
Pig	29	13 (44.82%)	3 (10.34%)	2 (6.90%)	17 (58.62%)
Chicken (Cloacal swabs)	24	9 (37.50%)	8 (33.33%)	5 (20.83%)	22 (91.67%)
Subtotal	53	22 (41.51%)	11 (20.75%)	7 (13.21%)	40 (75.47%)
Biofilm producing ability of *Pseudomonas aeruginosa*					
Pig	29	_	5 (17.24%)	24 (82.76%)	29 (100%)
Chicken	19	11 (57.89%)	3 (15.79%)	_	14 (73.68%)
Subtotal	48	11 (22.92%)	86 (16.67%)	24 (50.00%)	43 (89.58%)
Grand Total	748	112 (14.97%)	164 (21.93%)	97 (12.97%)	295 (39.44%)

Table-9: List of oligonucleotide primers to be used for species specific PCR for confirmation of bacterial isolates.

Stages	*nuc*	*E. coli*	*Salmonella*	*Pseudomonas aeruginosa*
Initial denaturation	95°C for 5 min	95°C for 3 min	95°C for 5 min	94°C for 4min
Denaturation	95°C for 30 sec	95°C for 30 sec	94°C for 45 sec	94°C for 45 sec
Annealing	59 °C for 45 sec	57 °C for 30 sec	59 °C for 45 sec	59 °C for 45 sec
Elongation	72 °C for 45 sec	72°C for 1min	72 °C for 45 sec	72 °C for 45 sec
Final extension for 1 cycle	72 °C for 6 min	72°C for 5min	72 °C for 6 min	72 °C for 6 min
No. of cycles	30	30	30	30

Table-10: Thermal cycling conditions for detection of genus and species specific (*nuc* and *16S rRNA*) genes carried out in this study

Gene specific for	Primer sequence (5'- 3')	Expected amplicon size (bp)	Reference
nuc (S.aureus)	F:GCGATTGATGGTGATACGGTT R:AGCCAAGCCTTGACGAACTAAAC	280	Othman *et al.* (2014)
E. coli	F:GACCTCGGTTTAGTTCACAGA R:CACACGCTGACGCTGACCA	585	Candrian *et al.* (1991)
Salmonella	F:TATCTGGCTATCGCTGGCAGTG R:TCCGCTAATCTTTTGGCAACC	480	Whyte et al. (2002)
Pseudomonas aeruginosa	F:GGCAGTAAGTTAATACCTTGCT R:CCTTAGAGTGCCCACCCGAG	701	Finnan *et al.* (2004)

Screening of biofilm producing isolates for biofilm associated gene(s), antimicrobial resistance gene(s) and virulence gene(s)

Table-11: List of oligonucleotide primers to be used for detection of biofilm associated gene(s)

Primer name	Primer sequence (5'- 3')	Expected amplicon size (bp)	Reference
icaA	F:CCTAACTAACGAAAGGTAG R:AAGATATAGCGATAAGTGC	1315	Dhanawade *et al.* (2010)
icaD	F: AAACGTAAGAGAGGTGG R:GGCAATATGATCAAGATAC	381	Dhanawade *et al.* (2010)
bap	F:CCCTATATCGAAGGTGTAGAATTGCAC R:GCTGTTGAAGTTAATACTGTACCTGC	971	Cucarella *et al.* (2004)
IS257	F: TTGGGTTCAAGAATATGCCC R: CTTCGTTGAAGGTGCCTGAT	271	Sidhu *et al.* (2002)
agr	F: TATGCTCCTGCAGCAACTAA R: CTTGCGCATTTCGTTGTTGA	1073	Gilot and Van Leeuwen (2002)
algD	F:AAGGCGGAAATGCCATCTCC	445	Fazeli and Momtaz

	R:AGGGAAGTTCCGGGCGTTTG		(2014)
algU	F:CGCGAACCGCACCATCGCTC R:GCCGCACGTCACGAGC	705	Fazeli and Momtaz (2014)
phzI	F:CATCAGCTTAGCAATCCC R:CGGAGAAACTTTTCCCTC	392	Finnan *et al.* (2004)
csgA	F:GCGGTAATGGTGCAGATGTTG R:GAAGCCACGTTGGGTCAGA	68	Fink *et al.* (2012)
csgB	F:CATAATTGGTCAACGTGGGACTAA R:GCAACAACCGCCAAAAGTTT	75	Fink *et al.* (2012)
papC	F: GTGGCAGTATGAGTAATGACCGTTA R: ATATCCTTTCTGCAGGGATGCAATA	202	Melchior *et al*. (2009)
csgA	F:ATTGCAGCAATCGTAGTTTCTGG R:ATWGAYCTGTCATCAGAGCCCTGG	245	Akbari *et al.* (2015)
csgD	F:TGAAARYTGGCCGCATATCAATG R:ACGCCTGAGGTTATCGTTTGCC	355	Akbari *et al.* (2015)
adrA	F:GGCCATTAAATTAGCGGAAC R:AATAAAATTTCCCAGTGGCG	99	Grantcharova et al. (2010)

Table-12: List of oligonucleotide primers used for detection antimicrobial resistance genes.

Primer name	Primer sequence (5'-3')	Expected amplicon size (bp)	Reference
bla$_{TEM}$	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC	1081	Dutta *et al.* (2013)
bla$_{SHV}$	F: TTATCTCCCTGTTAGCCACC R: GATTTGCTGATTTCGCTCGG	795	Dutta *et al.* (2013)
bla$_{CTX-M-Universal}$	F: ATGTGCAGYACCAGTAARGTKATGGC R: TGGGTRAARTARGTSACCAGAAYCAGCGG	593	Dutta *et al.* (2013)
bla$_{CTX-M-9}$	F: CAATGTGCAGCACCAAGTAA R: CGCGATATCGTTGGTGGTG	540	Dutta *et al.* (2013)
mecA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	533	Louie *et al.* (2002)

Table-13: List of oligonucleotide primers to be used for detection of virulence gene(s).

Primer name	Primer sequence (5'- 3')	Expected amplicon size (bp)	Reference
papC	F: GTGGCAGTATGAGTAATGACCGTTA R: ATATCCTTTCTGCAGGGATGCAATA	202	Melchior *et al*. (2009)
tsh	F:AAGTCTGTCAGACGTCTGTGTT R:GGATAGCGCTCCTTATCCAGAT	478	Melchior *et al*. (2009)
iucC	F:GTGGCAGTATGAGTAATGACCGTTA R:ATATCCTTTCTGCAGGGATGCAATA	541	Melchior *et al*. (2009)
stx₁	F: ATAAATCGCCATTCGTTGACTAC R: AGAACGCCCACTGAGATCATC	180	Paton & Paton (1998)
stx₂	F: GGCACTGTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	255	
eaeA	F: GACCCGGCACAAGCATAAGC R: CCACCTGCAGCAACAAGAGG	384	
ehxA	F: GCATCATCAAGCGTACGTTCC R: AATGAGCCAAGCTGGTTAAGCT	534	
invA	F:GTGAAATTATCGCCACGTTCGGGGCAA R: TCATCGCACCGTCAAAGGAACC	284	Rahn *et al.* (1992)
sefA	F: GATACTGCTGAACGTAGAAGG R: GCGTAAATCAGCATCTGCAGTAGC	488	Melchior *et al*. (2009)
lasA	F: GCAGCACAAAAGATCCC R: GAAATGCAGGTGCGGTC	1075	Finnan *et al*. (2004)
lasB	F: ACAGGTAGAACGCACGGTTG R: GATCGACGTGTCCAAACTCC	284	Wolska *et al*. (2009)
exoS	F: CGTCGTGTTCAAGCAGATGGTGCTG R: CCGAACCGCTTCACCAGGC	444	
exoT	F: CAATCATCTCACCACAACCC R: TGTCGTAGAGGATCTCCTG	1159	Finnan *et al*. (2004)
apr	F: TGTCCAGCAATTCTCTTGC R: CGTTTTCCACGGTGACC	1017	
plcH	F: GCACGTGGTCATCCTGATGC R: TCCGTAGGCGTCGACGTAC	608	Wolska *et al*. (2009)
plcN	F: TCCGTTATCGCAACCAGCCCTACG R: TCGCTGTCGAGCAGGTCCAAC	481	
phzI	F:CATCAGCTTAGCAATCCC R:CGGAGAAACTTTTCCCTC	392	Finnan *et al*. (2004)
toxA	F: CTGCGCGGGTCTATGTGCC R: GATGCTGGACGGGTCGAC	270	Wolska *et al*. (2009)
tsst1	F: GCTTGCGACAACTGCTACAG R: TGGATCCGTCATTCATTGTTAT	559	Jonas *et al.* (2002)
coa	F: AACAAAGCGGCCCATCATTAAG R:TAAGAAATATGCTCCGATTGTCG	850	Montesinos *et al*. (2002)
nuc	F:GCGATTGATGGTGATACGGTT R:AGCCAAGCCTTGACGAACTAAAC	280	Othman *et al.*(2014)
ETA	F: CTATTTACTGTAGGAGCTAG R: ATTTATTTGATGCTCTCTAT	741	Ruzikova *et al*. (2005)
ETB	F: ACGGCTATATACATTCAATT R: TCCATCGATAATATACCTAA	200	Ruzikova *et al*. (2005)
ETD	F: AACTATCATGTATCAAGG R: CAGAATTTCCCGACTCAG	376	Ruzikova *et al*. (2005)

Table-14: Thermal cycling conditions for detection of various biofilm associated genes carried out in this study

Stages	*icaA*	*icaD*	*IS257*	*bap*	*agr*	*algD*	*algU*	*papC*	*phzI*	*csgA(EC)*	*csgB(EC)*	*csgA(Sal)*	*csgD(Sal)*	*adrA*
Initial denaturation	94°C for 5 min	94°C for 5 min	95°C for 5 min	94°C for 5 min	95°C for 5 min	95°C for 2 min	95°C for 2 min	95°C for 5 min	95°C for 5 min	94°C for 10 min	94°C for 10 min	95°C for 5 min	95°C for 5 min	95°C for 15 min
Denaturation	94°C for 45 sec	94°C for 45 sec	95°C for 1 min	94°C for 30 sec	95°C for 45 sec	94°C for 30 sec	94°C for 30 sec	94°C for 45 sec	95°C for 30 sec	94°C for 15 sec	94°C for 30 sec	95°C for 15 sec	95°C for 45 sec	95°C for 15 sec
Annealing	49°C for 45 sec	49°C for 45 sec	54°C for 1 min	55°C for 45 sec	58°C for 45 sec	58°C for 30 sec	58°C for 30 sec	58°C for 45 sec	49°C for 45 sec	59°C for 1 min	59°C for 1 min	59°C for 30 sec	59°C for 30 sec	59°C for 45 sec
Elongation	72 °C for 45 sec	72 °C for 45 sec	72 °C for 72 min	72 °C for 45 sec	72 °C for 45 sec	72 °C for 1 min	72 °C for 1 min	72 °C for 45 sec	72 °C for 45 sec	72 °C for 30 sec	72 °C for 45 sec	72 °C for 30 sec	72 °C for 30 sec	72 °C for 30 sec
Final extension for 1 cycle	72 °C for 6 min	72 °C for 6 min	72 °C for 7 min	72 °C for 6 min	72 °C for 6 min	72 °C for 7 min	72 °C for 7 min	72 °C for 7 min	72 °C for 6 min	72 °C for 10 min	72 °C for 10 min	72 °C for 7 min	72 °C for 6 min	72 °C for 6 min
No. Of cycle	32	30	32	32	30	30	30	30	40	40	40	35	35	40

Table-15: Thermal cycling conditions for detection of various virulence associated genes carried out in this study

Stages	*papC*	*tsh*	*iucC*	*Stx₁, stx₂, eaeA* and *ehxA*	*invA*	*sefA*	*TSST1*	*coa*	*nuc*	*ETA, ETB* and *ETD*
Initial denaturation	95°C for 5 min	94°C for 5 min	94°C for 5 min	95°C for 5 min	95°C for 2 min	95°C for 5 min	95°C for 4 min	95°C for 5 min	95°C for 5 min	95°C for 2 min
Denaturation	94°C for 45 sec	94°C for 45 sec	94°C for 45 sec	95°C for 1 min	94°C for 30 sec	94°C for 45 sec	95°C for 45 sec	95°C for 30 sec	95°C for 30 sec	95°C for 45 sec
Annealing	58°C for 45 sec	49°C for 45 sec	55°C for 45 sec	61°C for 45 sec	58°C for 30 sec	58°C for 45 sec	47.6°C for 45 sec	47.5°C for 30 sec	59 °C for 45 sec	54°C for 45 sec
Elongation	72 °C for 45 sec	72 °C for 45 sec	72 °C for 45 sec	72 °C for 45 sec	72 °C for 1 min	72 °C for 45 sec	72 °C for 1 min	72 °C for 30 sec	72 °C for 45 sec	72 °C for 1 min
Final extension for 1 cycle	72 °C for 7 min	72 °C for 6 min	72 °C for 6 min	72 °C for 7 min	72 °C for 7 min	72 °C for 7 min	72 °C for 10 min	72 °C for 6 min	72 °C for 6 min	72 °C for 10 min
No. Of cycle	30	30	35	30	35	30	30	30	30	30

Table-16: Thermal cycling conditions for detection of various virulence associated genes carried out in this study

Stages	*lasA*	*lasB*	*toxA*	*phzI*	*plcH*	*plcN*	*exoS*	*exoT*	*apr*
Initial denaturation	95°C for 5 min	95°C for 5 min	95°C for 5 min	95°C for 5 min	95°C for 5 min	95°C for 5 min	94°C for 3 min	95°C for 5 min	95°C for 5 min
Denaturation	95°C for 45 sec	95°C for 45 sec	95°C for 45 sec	95°C for 30 sec	95°C for 45 sec	95°C for 45 sec	94°C for 30 sec	95°C for 45 sec	95°C for 45 sec
Annealing	47°C for 45 sec	55°C for 1 min	55°C for 45 sec	49°C for 45 sec	56°C for 45 sec	55°C for 45 sec	55°C for 1 min	54°C for 45 sec	51°C for 45 sec
Elongation	72 °C for 45 sec	72 °C for 1.5 min	72 °C for 1 min	72 °C for 45 sec	72 °C for 1 min	72 °C for 1 min	72 °C for 45 sec	72 °C for 45 sec	72 °C for 45 sec
Final extension for 1 cycle	72 °C for 6 min	72 °C for 5 min	72 °C for 6 min	72 °C for 6 min	72 °C for 6 min	72 °C for 6 min	72 °C for 6 min	72 °C for 6 min	72 °C for 6 min
No. Of cycle	30	30	30	40	30	30	30	30	30

Table-17: Thermal cycling conditions for detection of various antimicrobial resistance genes carried out in this study

Stages	bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M\text{-}Universal}$	$bla_{CTX\text{-}M\text{-}9}$	*mecA*
Initial denaturation	95°C for 5 min	95°C for 5 min	95°C for 5 min	95°C for 10 min	94°C for 5 min
denaturation	94°C for 30 sec	94°C for 45 sec	95°C for 30 sec	95°C for 30 sec	94°C for 30 sec
Annealing	58°C for 30 sec	57°C for 45 sec	60°C for 45 sec	58°C for 45 sec	55°C for 30 sec
Elongation	72°C for 40 sec	72°C for 45 sec	72°C for 45 sec	72°C for 1 min	72°C for 30 sec
Final extension for 1 cycle	72°C for 5 min	72°C for 5 min	72°C for 7 min	72°C for 8 min	72°C for 10 min
No. Of cycle	40	30	30	35	30

Table-18: Distribution of Biofilm associated genes in *E. coli* isolated from pigs and chickens

SL. No.	Biofilm associated genes of Escherichia coli	No. of isolates
1.	*csgA, csgB and papC*	17
2.	*csgA* and *papC*	3
3.	*csgB* and *papC*	3
4.	*csgA* and *csgB*	22
5.	*csgA*	1
6.	*csgB*	9
7.	*papC*	2
	Total	57

Table-19: PCR based detection of biofilm associated genes in *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* isolated from cattle, pigs and chickens

Biofilm associated genes of Staphylococcus aureus						
Source	**No. Of isolates tested**	***icaA***	***icaD***	***IS257***	***bap***	***agr***
Cattle	38	7 (18.42%)	29 (76.32%)	32 (84.21%)	5 (13.16%)	13 (34.21%)
Biofilm associated genes of *Escherichia coli*						
Source	**No. of isolates tested**	***csgA***	***csgB***		***papC***	
Pig	27	22^{NS} (81.48%)	21^{NS} (77.78%)		22** (81.48%)	
Chicken	35	22^{NS} (62.86%)	31^{NS} (88.57%)		3** (8.57%)	
Subtotal	62	44 (70.97%)	52 (83.87%)		25 (40.32%)	
Biofilm associated genes of *Salmonella* spp.						
Source	**No. of isolates tested**	***csgA***	***csgD***		***adrA***	
Pig	5	_	_		5 (100%)	
Chicken	5	_	_		5 (100%)	
Subtotal	10	_	_		10 (100.0%)	
Biofilm associated genes of *Pseudomonas aeruginosa*						
Source	**No. of isolates tested**	***algD***	***algU***		***phzI***	
Pig	6	_	_		6 (100%)	
Chicken (oropharyngeal swabs)	1	_	_		_	
Chicken (Cloacal swabs)	4	_	_		_	
Subtotal	11	_	_		6 (54.55%)	

Table-20: Distribution of Biofilm associated genes in *S. aureus* isolated from cattle

SL. No.	Biofilm associated genes of Staphylococcus aureus	No. of isolates
1.	*icaA, icaD,agr* and *IS257*	1
2.	*icaA, icaD, bap* and *IS257*	3
3	*icaA, icaD* and *IS257*	3
4.	*icaD, agr* and *IS257*	9
5.	*icaD* and *agr*	3
6.	*IS257*	6
7.	*icaD* and *bap*	1
8.	*icaD* and *IS257*	9
9.	*icaD*	1
10.	*bap, agr* and *IS257*	1
	Total	37

Table-21: Distribution of virulence genes in *E. coli* isolated from pigs

SL. No.	Virulence genes of Escherichia coli isolated from pig faecal samples	No. of isolates
1.	*papC, stx*$_1$ and *ehxA* (STEC)	1
2.	*papC* and *stx*$_2$(STEC)	1
3.	*papC* and *ehxA* (EHEC)	2
4.	*papC* and *eaeA* (EPEC)	1
5.	*ehxA* (EHEC)	2
6.	*papC*	18
	Total	25

Table-22: Distribution of virulence genes in *E. coli* isolated from chickens

SL. No.	Virulence genes of Escherichia coli isolated from poultry cloacal swabs	No. of isolates
1.	*iucC, stx*$_2$ and *ehxA* (STEC)	1
2.	*tsh, papC* and i*ucC* (APEC)	1
3.	*tsh* and *iucC* (APEC)	4
4.	*iucC* and *stx*$_2$ (STEC)	1
5.	*papC*	1
6.	*tsh*	2
7.	*iucC* (APEC)	5
8.	*ehxA* (EHEC)	2
9.	*stx*$_2$ (STEC)	1
	Total	18

Table-23: PCR based detection of virulence genes in *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* isolated from cattle, pigs and chickens

Virulence genes of biofilm producing *Staphylococcus aureus*							
Source	**isolates No.**	***nuc***	***TSST1***	***coa***	***ETA***	***ETB***	***ETD***
Cattle	38	38 (100%)	1 (2.63%)	2 (5.26%)	–	–	–

Virulence genes of biofilm producing *Escherichia coli*							
Source	**Isolate No.**	***papC***	***stx₁***	***stx₂***	***eaeA***	***ehxA***	
Pig	27	22 (81.48%)	1 (3.70%)	1 (3.70%)	1 (3.70%)	4 (14.81%)	
Source	**Isolate No**	***papC***	***tsh***	***iucC***	***stx₁***	***stx₂***	***eaeA***	***ehxA***
Chicken	35	3 (8.57%)	7 (20%)	11 (31.43%)	–	2 (5.71%)	–	3 (8.57%)
Subtotal	62	25 (40.32%)	7 (11.29%)	11 (17.74%)	1 (1.61%)	3 (4.84%)	1 (1.61%)	7 (11.29%)

Virulence genes of biofilm producing *Salmonella* spp.			
Source	**isolates No.**	***invA***	***sefA***
Pig	5	5 (100%)	–
Chicken	5	5 (100%)	3 (60%)
Subtotal	10	10 (100.00%)	3 (30.00%)

Virulence genes of biofilm producing *Pseudomonas aeruginosa*										
Source	**Isolates No.**	***lasA***	***lasB***	***exoS***	***exoT***	***apr***	***plcH***	***plcN***	***phzI***	***toxA***
Pig	6	3 (50%)	–	4 (66.67%)	–	4 (66.67%)	4 (66.67%)	5 (83.33%)	6 (100%)	1 (16.67%)
Chicken (oropharyngeal swabs)	1	1 (100%)	1 (100%)	–	–	–	–	–	–	1 (100%)
Chicken (Cloacal swabs)	4	3 (75%)	1 (25%)	2 (50%)	1 (25%)	1 (25%)	1 (25%)	2 (50%)	–	1 (25%)
Subtotal	11	7 (63.64%)	2 (18.18%)	6 (54.55%)	1 (9.1%)	5 (45.45%)	5 (45.45%)	7 (63.64%)	6 (54.55%)	3 (27.27%)

Table-24: Distribution of virulence genes in *S. aureus* isolated from cattle

SL. No.	Virulence genes of Staphylococcus aureus	No. Of isolates
1.	*nuc and coa*	3
2.	*nuc and TSST*1	1
3.	*nuc*	34
	Total	38

Table-25: Association of biofilm associated genes and virulence genes in *S. aureus* isolated from cattle

SL. No.	Biofilm genes	Virulence repetoires	No. of isolates
1.	*icaA, icaD,agr* and *IS257*	*nuc*	1
2.	*icaA, icaD, bap* and *IS257*	*nuc*	3
3.	*icaA, icaD* and *IS257*	*nuc*	3
4.	*icaD, agr* and *IS257*	*coa* and *nuc*	1
5.	*icaD, agr* and *IS257*	*nuc*	8
6.	*IS257*	*nuc*	6
7.	*icaD* and *bap*	*TSST1* and *nuc*	1
8.	*icaD* and *IS257*	*nuc*	1
9.	*icaD* and *IS257*	*nuc*	8
10.	*bap, agr* and *IS257*	*nuc*	1
11.	*icaD*	*nuc*	1
12.	*icaD* and *agr*	*nuc*	3
	Total		37

Table-26: Distribution of virulence genes in *P. aeruginosa* isolated from pigs

Sl. No.	Virulence genes of *P. aeruginosa* isolated from pig faecal samples	No. Of isolates
1.	*phzI, lasA, exoS, apr, plcH* and *plcN*	1
2.	*phzI, toxA, exoS, apr, plcH* and *plcN*	1
3.	*phzI*, *exoS, apr, plcH* and *plcN*	2
4.	*phzI, lasA* and *plcN*	1
5.	*phzI* and *lasA*	1
Total		6

Table-27: Distribution of virulence genes in *P. aeruginosa* isolated from chickens

Sl. No.	Virulence genes of *P. aeruginosa* isolated from chicken cloacal and oropharyngeal swabs	No. Of isolates
1.	*lasA, exoS, apr, plcH and plcN*	1
2.	*lasA, exoS, exoT and apr*	1
3.	*lasB, plcH, plcN and toxA*	1
4.	*lasA, lasB and toxA*	1
5.	*lasA*	1
Total		5

Table-28: Distribution of AMR genes in *E. coli* isolated from pigs and chickens

SL. No.	AMR genes of Escherichia coli isolated from pig faecal samples	No. of isolates
1.	*bla_{TEM} and $bla_{CTX-M-U}$*	1
2.	*bla_{TEM}*	4
3.	*$bla_{CTX-M-U}$*	1
Total		6

Table-29: Association of biofilm genes, virulence genes and AMR genes in *E. coli* isolated from pigs and chickens

SL. No.	Biofilm genes	Virulence repetoires	AMR genes	No. of isolates	Source
1.	*papC, csgB*	*papC and eaeA*	*bla_{TEM}*	1	Pig
2.	*csgA* and *csgB*	*ehxA*	*bla_{TEM}*	1	Pig
3.	*papC, csgA* and *csgB*	*papC and stx_2*	*bla_{TEM}*	1	Pig
4.	*papC, csgA* and *csgB*	*papC*	*$bla_{CTX-M-U}$*	1	Pig
5.	*papC, csgA* and *csgB*	*papC*	*bla_{TEM}*	1	Pig
Subtotal				5	
6.	*csgB*	*iucC*	*bla_{TEM} and $bla_{CTX-M-U}$*	1	Chicken
7.	*papC, csgA* and *csgB*	*papC*	*bla_{TEM}*	1	Chicken
8.	*papC* and *csgB*	*tsh, papC, iucC*	*bla_{TEM}*	1	Chicken
9.	*csgA*	*tsh, iucC*	*bla_{TEM}*	1	Chicken
10.	*papC, csgA* and *csgB*	*papC, ehxA and stx_2*	*bla_{TEM}*	1	Chicken
11.	*csgB*	*tsh, iucC*	*bla_{TEM}*	2	Chicken
Subtotal				7	
Grand Total				12	

Table-30: Association of biofilm genes, virulence genes and AMR genes in *Salmonella* spp. isolated from pigs and chickens

SL. No.	Biofilm genes	Virulence repetoires	AMR genes	No. of isolates	Source
1.	*adrA*	*invA*	*bla_{TEM}*	4	pig
2.	*adrA*	*invA*	*bla_{TEM} and $bla_{CTX-M-9}$*	1	pig
Sub Total				5	
1.	*adrA*	*invA*	*bla_{SHV}*	1	Chicken
2.	*adrA*	*invA* and *sefA*	*bla_{SHV}*	1	Chicken
Sub Total				2	
Grand Total				7	

Table-31: PCR based detection of antimicrobial resistance genes in *E. coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* isolated from cattle, pigs and chickens

Antimicrobial resistance genes of biofilm producing *Escherichia coli*					
Source	**isolates No.**	***bla*$_{TEM}$**	***bla*$_{SHV}$**	***bla*$_{CTX-M-Universal}$**	***bla*$_{CTX-M-9}$**
Pig	27	4 (14.81%)	_	1 (3.70%)	_
Chicken	35	8 (22.86%)	_	1 (2.86%)	_
Subtotal	62	12 (19.35%)	_	2 (3.23%)	_
Antimicrobial resistance genes of biofilm producing *Salmonella* spp.					
Pig	5	5 (100%)	_	_	1 (20%)
Chicken	5	1 (20%)	2 (40%)	2 (40%)	_
Subtotal	10	6 (60.0%)	2 (20.0%)	2 (20.0%)	1 (10.0%)
Antimicrobial resistance genes of biofilm producing *Pseudomonas aeruginosa*					
Pig	6	5 (83.33%)	_	_	
Chicken (oropharyngeal swabs)	1	1 (100%)	_	_	_
Chicken (Cloacal swabs)	4	2 (50%)	_	_	1 (25%)
Subtotal	11	8 (72.73%)	_	_	1 (9.09%)
Grand Total	83	26 (31.33%)	2 (2.41%)	4 (4.81%)	2 (2.41%)

Table-32: Association of biofilm genes, virulence genes and AMR genes in *P. aeruginosa* isolated from pigs

SL. No.	**Biofilm genes**	**Virulence repetoires**	**AMR genes**	**No. of isolates**
1	*phzI*	*toxA, exoS, apr, plcH* and *plcN*	*bla*$_{TEM}$	1
2	*phzI*	*exoS, apr, plcH* and *plcN*	*bla*$_{TEM}$	2
3	*phzI*	*lasA* and *plcN*	*bla*$_{TEM}$	1
4	*phzI*	*lasA*	*bla*$_{TEM}$	1

B2. Summary and Conclusions of the Progress made so far (minimum 100 words, maximum 200 words):

A total of 789 fresh samples were collected randomly from cattle (milk=301), pigs (feces=215) and chicken (cloacal and oropharyangeal swabs= 273) of five North eastern states of India. All the bacterial isolates were confirmed by BD Phoenix automated bacterial identification system as well as 16S rRNA based PCR. The confirmed isolates were subjected to determine the biofilm production ability by 96 well microtiter plate assay. Of the 789 samples, 183 *S. aerues,* 464 *E. coli,* 53 *Salmonella* spp. and 48 *P. aeruginosa* were isolated. All the isolates were screened phenotypically to detect the biofilm production ability by microtiter plate assay

and categorized as strong, moderate and weak biofilm producer. A total 55 (18.90%) *E. coli* isolates from pigs were biofilm producer, of which 29 (9.96%), 15 (5.15%) and 11 (3.78%) were recorded as weak, moderate and strong biofilm producer, respectively (Table-8, Fig-5). Similarly, a total of 74 (42.77%) *E. coli* isolates from chicken were biofilm producer, of which 23 (13.29%), 27 (15.61%) and 24 (13.87%) isolates were recorded as weak, moderate and strong biofilm producer, respectively. A total 17 (58.62%) *Salmonella* spp. isolates from pigs were recorded as biofilm producer, of which 13 (44.82%), 3 (10.34%) and 2 (6.90%) were recorded as weak, moderate and strong biofilm producer, respectively (Table-8, Fig-5). Similarly, a total of 22 (91.67%) *Salmonella* spp. from chickens were found to be positive for biofilm production, of which 9 (37.50%), 8 (33.33%) and 5 (20.83%) isolates were recorded as weak, moderate and strong biofilm producer, respectively. A total of 83 (45.36%) isolates from cattle milk were recorded as biofilm positive, of which 27 (14.75%), 25 (13.66%) and 31 (16.94%) were recorded as weak, moderate and strong biofilm producers, respectively. All *P. aeruginosa* isolates from pigs were recorded as biofilm positive, among them 5 (17.24%) isolate was moderate and 24 (82.76%) were recorded as strong biofilm producer (Table-8, Fig-5). Similarly, a total of 14 (73.68%) of *P. aseruginosa* isolates from chickens were found to be biofilm positive, of which 11 (57.89%) and 3 (15.79%) were recorded as weak and moderate biofilm producers. Majority of *S. aureus* were positive for *IS257* (84.21%), followed by *icaD* (76.32%), *agr* (34.21%), *icaA* (18.42%) and *bap* (13.16%) genes. All the isolates were positive for *nuc* gene followed by *coa* (5.26%) and *TSST1* (2.63%) but were negative for *mecA* gene. In case of *E. coli,* most of them were positive for *csgB* (83.87%) gene followed by *csgA* (70.97%) and *papC*(40.32%) genes. In the present study, it was found that the biofilm producing *E. coli* were also harbouring different virulence genes, among them *papC* (40.32%) gene was highest followed by *iucC* (17.74%), *tsh* and *ehxA* (11.29%), stx_2 (4.84%), stx_1 (1.61%) and *eaeA* (1.61%) genes. The biofilm associated gene *adrA* was recorded in all *Salmonella* spp. isolates but were negative for *csgA* and *csgD* genes. All the isolates were positive for *invA* genes and 30% of the isolates were also positive for *sefA* gene. All the *P. aeruginosa* were as biofilm producers and 54.55% isolates were positive for *phzI* gene but all were negative for *algD* and *algU* genes. All the isolates were harbouring multiple virulence genes, of which majority of the isolates were found to be positive for *lasA* and *plcN* (63.64%) genes followed by *exoS* and *phzI* (54.55%), *apr* and *plcH* (45.45%), *toxA* (27.27%), *lasB* (18.18%) and *exoT* (9.1%). Majority of the biofilm producing isolates were carrying bla_{TEM} (31.33%) genes followed by $bla_{CTX\text{-}M\text{-}Universal}$ (4.81%) and bla_{SHV} (2.41%) and $bla_{CTX\text{-}M\text{-}9}$ (2.41%)

Lead Centre-2 at Tezpur University, Assam

- To screen the traditionally used medicinal plants from North East India for microbial biofilm inhibition and quorum sensing inhibition (model organism: *Pseudomonas aeruginosa*) (Tezpur University).
- To purify and characterize bioactive molecules from active plant sources (Tezpur University).
- **To study the mechanism of action of bioactive molecule on quorum sensing inhibition and/or anti-biofilm property (NEERI,** Tezpur University).

1. Collection of plants from Arunachal Pradesh, Meghalaya, Manipur, Assam, Mizoram and preparation of extracts

Ethno-medicinal plants were collected from different geographical places of Arunachal Pradesh, Meghalaya, Manipur, Assam, and Mizoram till March 2019. The collected samples included both fresh and dry plant parts including leaves, flowers, branches and roots. Photographs, and pressed vouchers were made and unique sample ID was allotted to each sample. The plant herbarium file was prepared and samples have been sent for the identification. A total of 33 ethno medicinal plant samples were collected and aqueous and/ or methanol extracts were prepared from dry powder and/or fresh samples. The fresh samples were washed with distilled water, surface sterilized by 70% ethanol, and bleached with 5% aqueous sodium hypochlorite. The samples were again washed with double distilled water to make the samples chemical free. Thereafter, the samples were air dried at room temperature and finally crushed to powder in grinder/mixer. The powdered samples were stored at -20°C till further use. Both fresh and dry extracts were prepared using water and Methanol as solvents. 10% aqueous and methanolic extracts were prepared by following the method of Ahmad et al (11). 10 g of powdered samples were soaked in 100 ml of solvent and kept for 24 hrs under continuous shaking followed by centrifugation at 10000 rpm for 10 minutes. The supernatants were filtered by Whatman filter paper No. 1, dried by Rotary evaporator and lyophilised to obtain crude extracts and stored at -20°C for further use.

Table 1: Sample collected till March 2018

S. No.	Common name	Scientific name	Date of collection	Code	Extract (A or M)	Part used (D or F)
1	Green tea	*Camellia sinensis*	06-11-2017	CS	A , M	Leaves (F, D)
2	Manimuni	*Centella asiatica*	08-11-2017	CA	A , M	Leaves(F, D)
3	Curry patta	*Murraya koenigii*	13-11-2017	CA	A , M	Leaves, (F, D)
4	Soratpatta	*Dendrocnida sinuate*	15-11-17	DS	Aqueous	Leaves
5	Robabtenga	*Citrus maximus*	16-11-17	CM	Aqueous	Leaves
6	Star fruit	*Averrhoa carambola*	20-11-17	AC	Aqueous	Leaves
7	Gullmohar	*Delonix regia*	20-11-2017	DR	Aqueous	Leaves
8	Neem	*Azadirachta indica*	22-11-2017	AI	A , M	Leaves (F, D)
9	Otenga	*Dilenia indica*	23-11-2017	DI	Aqueous	Leaves

10	Oorihingchabi	*Mikania micrantha*	14-12-17	MM	Aqueous	Leaves (F)
11	Kapou phool	*Rhynchostylis retusa*	14-12-17	RR	Aqueous	Leaves (F)
12	Bander kekwa	*Mucuna pruriens*	14-12-17	MP	Aqueous	Seeds (F)
13	Ashoka tree	*Saraca ashoka*	28-12-17	SA	Aqueous	Leaves(F, D)

14	Cotton tree	*Bombax ceiba*	28-12-17	BC	Aqueous	Leaves
15	Tengesai	*Oxalis corniculata*	05-11-18	OC	A, M	Leaves (F)
16	Naharu	*Allium sativum*	28-2-18	AS	A, M	Roots (F)
17	Orange like	Yet to be identified	28-2-18	OL	A,M	Peel(D)
18	Tomato like	Yet to be identified	28-2-18	TL	A,M	Fruit(D)
19	Flower like	Yet to be identified	28-2-18	FL	A,M	Flower (D)
20	Green grass	Yet to be identified	28-2-18	GG	A,M	Shoot(F)
21	Masala like	Yet to be identified	28-2-18	ML	M	Shoot (D)
22	Bor thekera	*Garcinia pedunculata*	13-3-18	GP	A, M	Fruit (F)
23	Wild ginger	*Zingiber officinale*	13-3-18	GL	A,M	Rhizome (F)
24	Rupahi thekera	*Garcinia lanceaefolia*	21-3-18	RT	A, M	Leaves (F)
25	Tez patta	*Cinamomum tamata*	21-3-18	CT	A,M	Leaves(F)

Table 2: Sample collected till March 2019

S. No.	**Scientific name**	**Common name**	**Place of collection**	**Date of Collection**	**Code**	**Extract (A or M)**	**Part used (D or F)**
1	*Hottuynia cordata*		Meghalaya	04-08-18	HA	D(W,	Leaves(D)
2	*Erigreon karvinskianus*		Meghalaya	04-08-18	EK	D(W, M)	Leaves(D)
3	*Eupatorium riparium*		Meghalaya	04-08-18	ER	D(W, M)	Leaves(D)
4	*Crotalaria pallid*		Meghalaya	04-08-18	CP	D(W, M)	Leaves(D)
5	*Ocimum canum*		Meghalaya	04-08-18	OC5	D(W, M)	Leaves(D)
6	*Allium tuberesum*	Nakuppi	Manipur	10-08-18	AT	D(M) F(W, M)	Chive(F,D)
7	*Diplazium escalatum*	Dhekia saag	Assam	20-07-18	DE	D(W)	Whole plant (D)
89	*Ipomea aquatic*	Kalmou saag	Assam	20-07-18	IA	D(W)	Leaves
10	*Spondias pinnata*	Amora	Arunachal Pradesh	10-04-18	Spin	W	Leaves(F)
11	*Spilanthes uliginosa*	Marsh para cress (marsing)	Arunachal Pradesh	10-04-18	SU	W	Leaves(F) Flowers(F)
12	*Clerodendron colebrookianum*	Nephaphu	Arunachal Pradesh	10-04-18	CC	W	Leaves(F,D)
13	*Eupatorium odoratum*	Bonesets	Arunachal Pradesh	10-04-18	EO	W	Leaves(F,D)

14	*Zanthoxylum oxyphyllum*	Ongear	Arunachal Pradesh	10-04-18	ZO	W	Leaves(F,D)
15	*Paederia foetida*	Skunkvine (bhedailata)	Arunachal Pradesh	10-04-18	PF	W	Leaves(F)
16	*Pouzolgia viminalis*	Oyik	Arunachal Pradesh	10-04-18	PV	W	Leaves(F,D)
17	*Solanum torvum*	Teeta guti	Arunachal Pradesh	10-04-18	ST	W	Leaves(F,D)
18	*Terminalia chebula*	Hilikha	Arunachal Pradesh	10-04-18	TC	W	Leaves(F,D)
19	*Ageratum conyzoides*	Billygoat weed (hanuman patta)	Arunachal Pradesh	10-04-18	AC	W	Leaves(F,D)
20	*Terminalia bellerica*	Bhumora	Arunachal Pradesh	10-04-18	TB	W	Leaves(D)
21	*Elaeocarpus floribundus*	Indian olive (jalphai)	Arunachal Pradesh	10-04-18	EF	W	Leaves(D)
22	Yet to be identified	Large leaves	Dibrugarh Assam	21-03-18	LL	D(M)	Leaves(D)
23	Yet to be identified	Wild tea	Dibrugarh Assam	21-03-18	WT	D(M)	Leaves(D)
24	*Eupatorium odoratum*	Pohu	Tezpur Assam	10-07-18	EO	D(W,M)	Leaves(D)
25	*Justica gendarussa*		Tezpur Assam	10-07-18	JD	D(W,M)	Leaves(D)
26	*Garcinia acuminate*		Tezpur Assam	10-07-18	GA	D(W,M)	Leaves(D)
27	*Bryophyllum pinnatum*	Duper tenga	Guwahati Assam	10-08-18	BP	F(M,W)	Leaves(F)
28	*Spondia dulchis*	Amra	Tezpur Assam	20-10-18	SD	F(W,M)	Fruit(F)
29	Yet to be identified	Broad leaves	Tezpur Assam	23-10-18	BL	F(W,M)	Leaves(F)
30	Yet to be identified	Loong type	Tezpur Assam	23-10-18	LT	F(W,M)	Leaves(F)
31	Yet to be identified	Hathkora	Tezpur Assam	20-10-18	H	F(M)	Fruit peel(F)
32		TC	Tezpur Assam	23-10-18	TC	F(W,M)	Green bark(F)
33	*Datura stramonium*		Mizoram	28-02-18	DtS	F(W)	Leaves(F)

W=Water, **M**= Methanolic, **F**= Fresh, D=Dry

2. Microbial strains and culture condition

All reference strains were obtained from the Microbial Type Culture Collection, CSIR-Institute of Microbial Technology, Chandigarh, India.

3. Antimicrobial activity:

Agar well diffusion method was used. Cultural bacterial strains of *Staphylococcus aureus* MTCC 430 (SA), *Listeria monocytogenes* MTCC 839 (LM), *Salmonella* (Sal), *Bacillus cereus* MTCC 430 (BC), *Yersinia pestis* (YP), *Pseudomonas aeruginosa* MTCC 2297 (PA), *Pseudomonas aeruginosa* 01 (PA01), *Yersinia enercolitica* (YE), *Chromobacterium violaceum*026 (CV026) and *Chromobacterium violaceum*12472 (CV12472) were grown overnight. LBA was prepared, poured in Petri plates and allowed to solidify. 100 µl of the

culture were spread in corresponding plates. Wells were made by well borer, 100 µl sample was put in each well, and incubated overnight at appropriate temperatures (37and 28°C). 33 different plant samples were collected from different geographical isolates of North East India and 10% (w/v) extracts were prepared using water and methanol as solvents. Identification of plant samples was done in BSI Shillong. We checked antibacterial activity of extracts against *P. aeruginosa* and other strains and found that methanolic extracts of *Spondia dulchis* (SDMF)*, Garcinia acuminate* (GAMD)*, Bryophyllum pinnatum* (BPMF)*,* showed antibacterial activity against all strains. *Solanum torvum* (STWF) and *Terminalia chebula* (TCWF) showed activity against *P. aeruginosa.* Zone of inhibition was measured to access the results. Gentamicin (2.5mg/ml) was used as positive control.

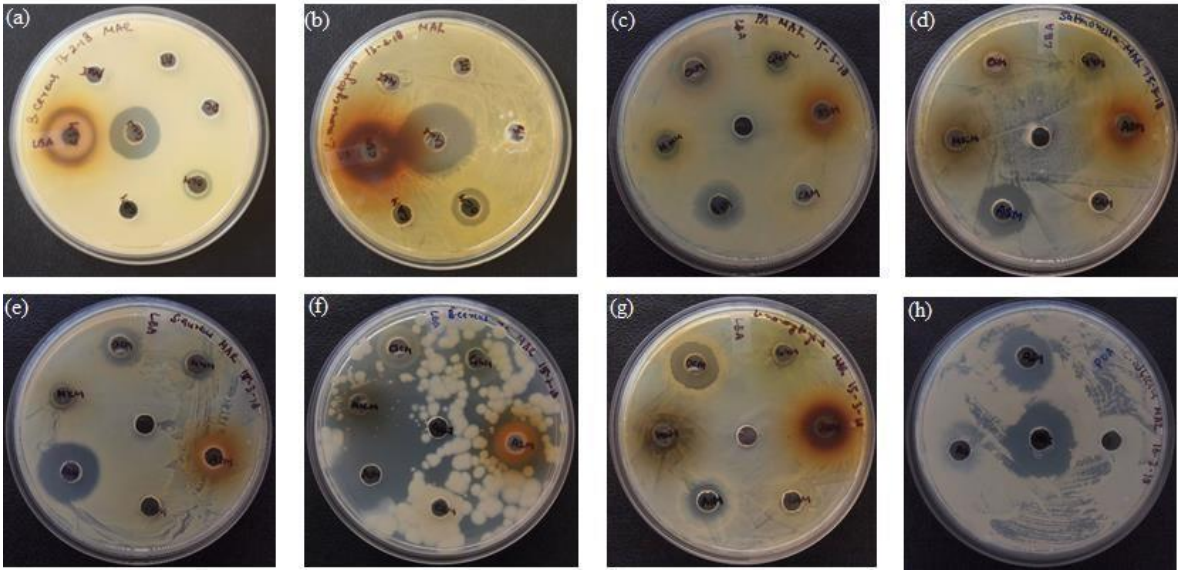


Fig.1: Antibacterial activity of CSM, OCM against *S. aureus* (a) and *L. monocytogenes* (b); OCM, MKM, ASM, CAM, AIM, & GGM against *P. aeruginosa* (c), *Salmonella* (d), *S. aureus* (e), *B. cereus* (f), *L. monocytogenes* (g); ASM, ASW against *C. albicans* (h)

Table 3: Zone of Inhibition of CSM, OCM, ASM, ASW

S. No.	Bacteria	Zone of inhibition									
		CSM	**Gent**	**OCM**	**MKM**	**CAM**	**AIM**	**GGM**	**ASM**	**ASW**	**Nys.**
1	*Bacillus cereus*	18±1	20±1	13±1	ND	ND	ND	ND	15±1	ND	ND
2	*Listeria monocytogenes*	12±1	24±1	16±1	ND	ND	ND	ND	13±1	ND	ND
3	*Pseudomonas aeruginosa*	ND	ND	13±1	ND	ND	ND	ND	15±1	ND	ND
4	*Salmonella*	ND	ND	11±1	ND	ND	ND	ND	16±1	ND	ND
5	*Staphylococcus aureus*	ND	ND	13±1	ND	ND	ND	ND	20±1	ND	ND

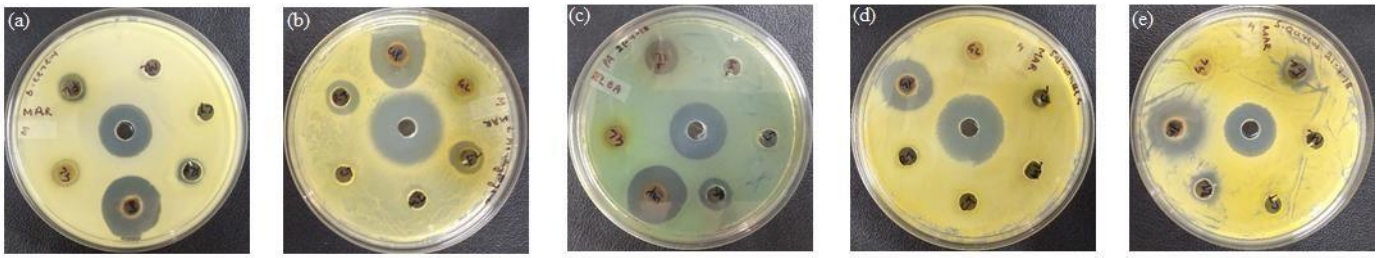


Fig.2: Antibacterial activity of GLM, GPM, RTM, CTM, MLM, and FLM against *B. cereus* (a), *L. monocytogenes* (b), *P. aeruginosa* (c), *Salmonella* (d), *S. aureus*

Table 4: Zone of Inhibition of GPM, RTM, FLM

S. No.	Bacteria	Zone of inhibition						
		GPM	**RTM**	**FLM**	**GLM**	**CTM**	**MLM**	**Gent**
1	*Bacillus cereus*	18±1	ND	10±1	ND	ND	ND	20±1
2	*Listeria monocytogenes*	21±1	12±1	12±1	ND	ND	ND	25±1
3	*Pseudomonas aeruginosa*	21±1	12±1	12±1	ND	ND	ND	20±1
5	*Salmonella*	16±1	ND	ND	ND	ND	ND	25±1
6	*Staphylococcus aureus*	18±1	10±1	10±1	ND	ND	ND	19±1

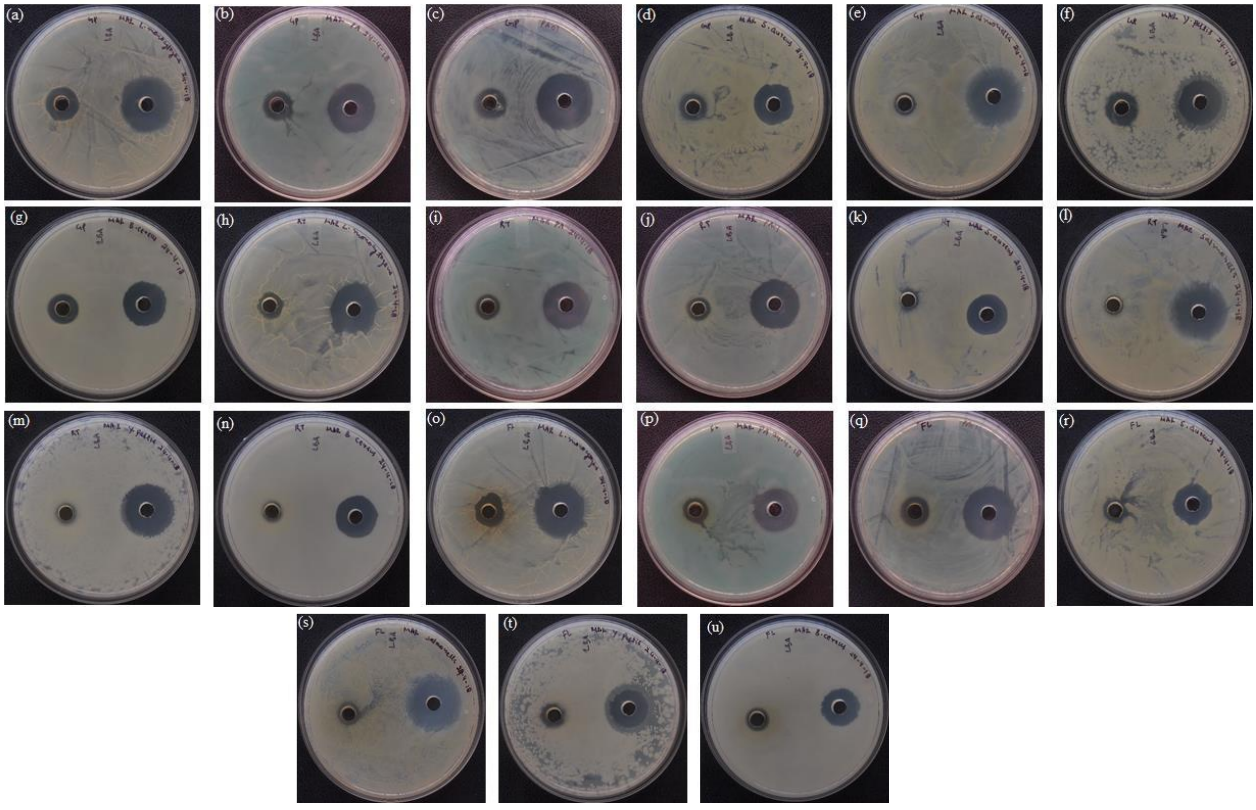


Fig.3: GPM, RTM, FLM Antibacterial activity of GPM (a-g), RTM (h-n), FLM (o-u) against *L. monocytogene* (a, h, o), *P. aeruginosa* (b, I, p), PA01 (c, j, q), *S. aureus* (d, k, r), *Salmonella* (e, l, s), *Y. pestis* (f, m, t), *B. cereus* (g, n, u),

Table 5: Zone of inhibition of GPM, RTM, FLM

S. No.	Bacteria	Zone of inhibition					
		GPM	Gent.	RTM	Gent.	FLM	Gent.
1	*Bacillus cereus*	13±1	20±1	8±1	19±1	11±1	18±1
2	*Listeria monocytogenes*	15±1	25±1	11±1	22±1	13±1	24±1
3	*Pseudomonas aeruginosa*	13±1	22±1	11±1	21±1	11±1	20±1
4	*Pseudomonas aeruginosa* 01	15±1	25±1	11±1	21±1	13±1	24±1
5	*Salmonella*	10±1	25±1	8±1	25±1	10±1	25±1
6	*Staphylococcus aureus*	12±1	18±1	10±1	19±1	10±1	17±1
7	*Yersenia pestis*	14±1	22±1	8±1	24±1	10±1	20±1

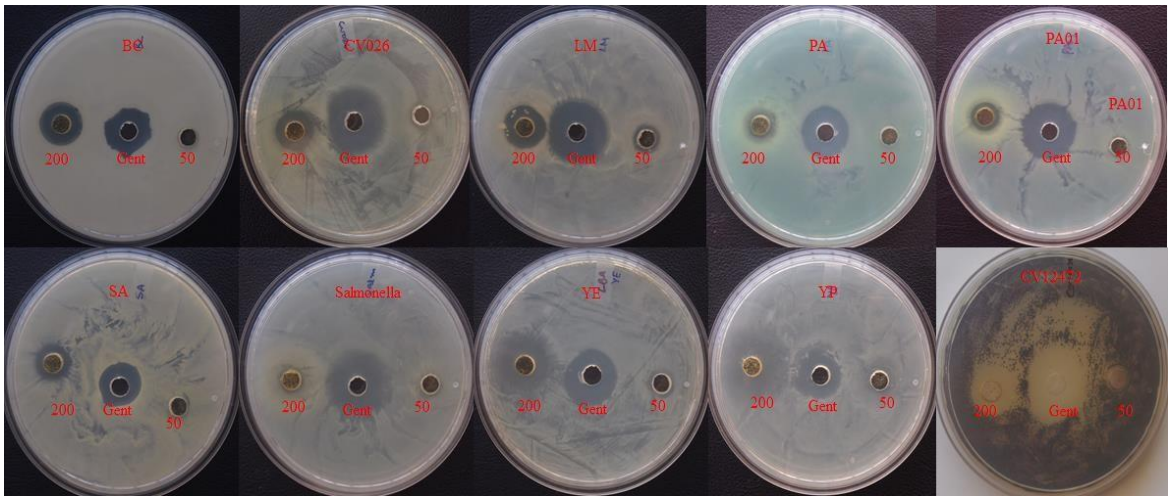


Fig.4: Antibacterial activity of SDMF at concentration 200 and 50mg/ml against BC, CV026, CV12472, LM, PA, PA01, Sal, SA, YE, YP

Table 6: Zone of inhibition of SDMF at 50mg/ml and 200mg/ml

S. No	Bacteria	ZOI		
		SDMF 50mg/ml	SDMF 200mg/ml	Gent 2.5mg/ml
1	*Bacillus cereus* (BC)		16	17
2	*Chromobacterium violaceum*026 (CV026)	10	18	20
3	*Chromobacterium violaceum*12472 (CV12472)		18	19
4	*Listeria monocytogenes* (LM)	11	15	22
5	*Pseudomonas aeruginosa* (PA)		13	18
6	*Pseudomonas aeruginosa* 01 (PA01)		11	20
7	*Salmonella* (Sal)	ND	ND	23
8	*Staphylococcus aureus* (SA)		13	15
9	*Yersinia enterocolitica* (YE)	10	17	20
10	*Yersinia pestis* (YP)		15	17

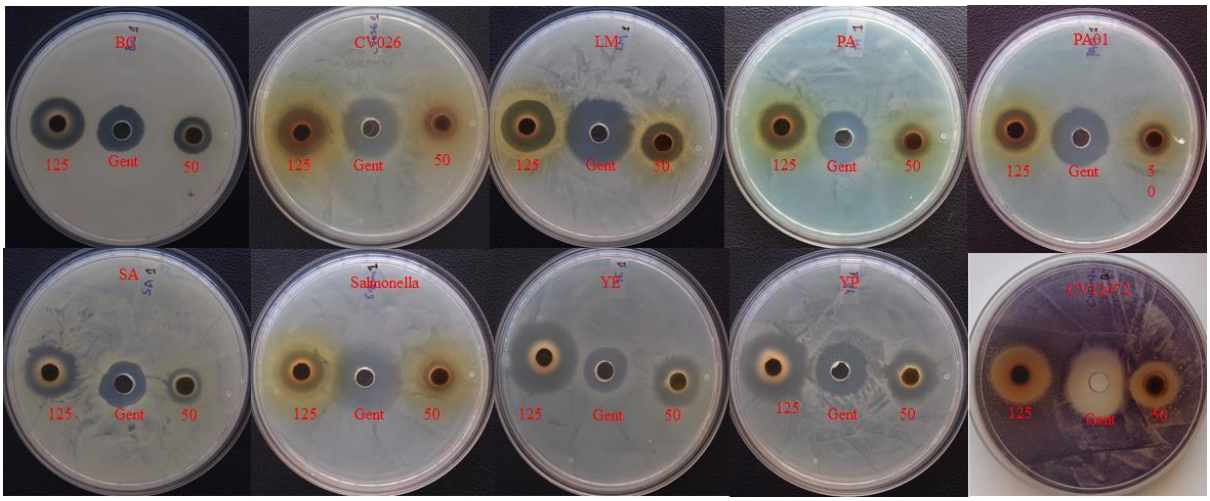


Fig.5: Antibacterial activity of GAMD at concentration 50 and 125mg/ml against BC, CV026, CV12472, LM, PA, PA01, Sal, SA, YE, YP

Table 7: Zone of inhibition of GAMD at 50mg/ml and 125mg/ml

S. No.	Bacteria	ZOI		
		GAMD 50mg/ml	GAMD 125mg/ml	Gent 2.5mg/ml
1	*Bacillus cereus* (BC)	13±1	19±1	16±1
2	*Chromobacterium violaceum*026 (CV026)	15±1	21±1	20±1
3	*Chromobacterium violaceum*12472 (CV12472)	17±1	23±1	20±1
4	*Listeria monocytogenes* (LM)	14±1	19±1	24±1
5	*Pseudomonas aeruginosa* (PA)	12±1	16±1	19±1
6	*Pseudomonas aeruginosa* 01 (PA01)	12±1	14±1	21±1
7	*Salmonella* (Sal)	11±1	15±1	17±1
8	*Staphylococcus aureus* (SA)	11±1	17±1	16±1
9	*Yersinia enterocolitica* (YE)	17±1	24±1	16±1
10	*Yersinia pestis* (YP)	18±1	23±1	18±1

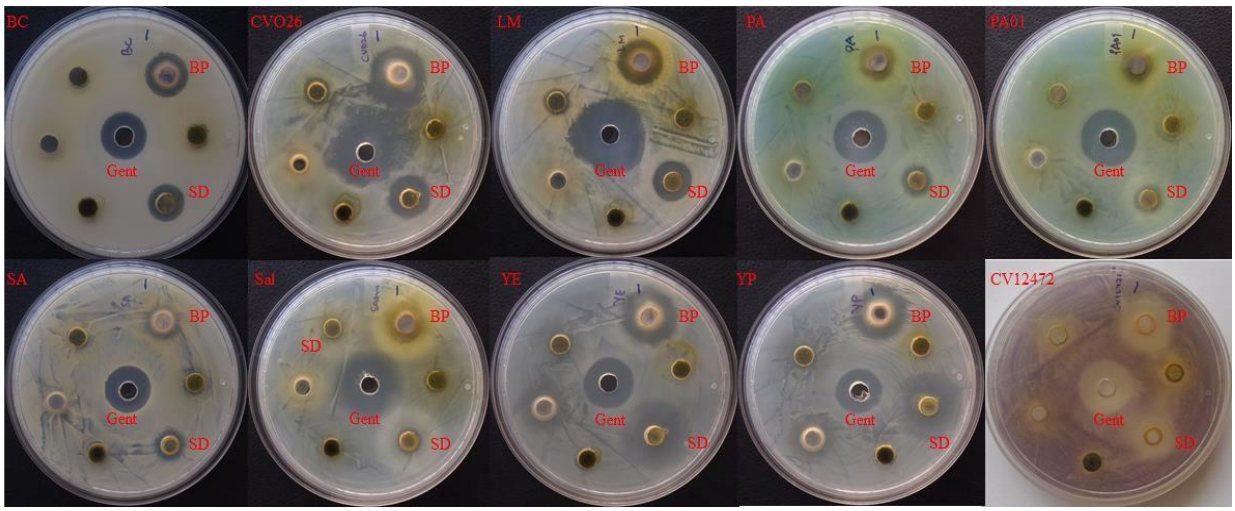


Fig.6: Antibacterial activity of SDMF and BPMF against BC, CV026, CV12472, LM, PA, PA01, Sal, SA, YE, YP

Table 8: Zone of inhibition of SDMF and BPMF

S. No.	Bacteria	Zone of inhibition		
		SDMF	BPMF	Gen
1	*Bacillus cereus* (BC)	12±1	15±1	16±
2	*Chromobacterium violaceum*026 (CV026)	15±1	19±1	20±1
3	*Chromobacterium violaceum*12472 (CV12472)	14±1	16±1	18±1
4	*Listeria monocytogenes* (LM)	15±1	16±1	27±1
5	*Pseudomonas aeruginosa* (PA)	11±1	13±1	18±1
6	*Pseudomonas aeruginosa* 01 (PA01)	10±1	10±1	20±1
7	*Salmonella* (Sal)	10±1	ND	25±1
	Staphylococcus aureus (SA)	12±1	11±1	18±1
9	*Yersinia enterocolitica* (YE)	21±1	20±1	17±1
10	*Yersinia pestis* (YP)	15±1	18±1	17±1

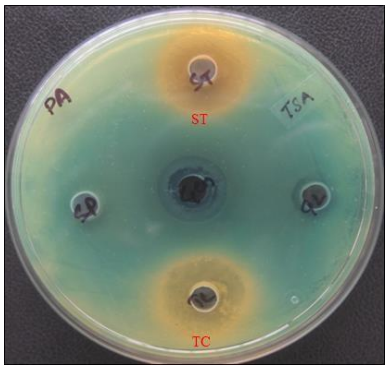


Fig.7: Antibacterial activity of STWF and TCWF against PA

Table 9: Zone of inhibition of STWF and TCWF

S.No.	Bacteria	ST	TC
1	*Pseudomonas aeruginosa* (PA)	14±1	15±1

4. Anti-biofilm activity of plant extracts

For biofilm inhibition assay, Tissue Culture Plate method was used as described elsewhere. Briefly, 100 µL of 10^6 to 10^7 CFU/ml microbial culture was added in 1 ml of appropriate media in 96 well plate. To the culture, plant extracts at 100 mg/ml concentration was added in corresponding wells. The plate was incubated at 37 $^{\circ}$C for 24 h. After incubation the content of each well was gently removed and the wells were washed three times with phosphate buffer saline (PBS pH 7.4) to remove free-floating planktonic cells. Adherent biofilms on the surface of the well were stained with crystal violet (0.1%, w/v) followed by removal of excess stain and thoroughly rinsed by deionised water. The plate was kept for drying and after drying, 100% methanol was added to the wells to solubilise the bound strain and the optical densities (OD) of stained adherent bacteria were determined with a microplate reader (Thermo Scientific Multiskan GO) at 570 nm. The biofilm inhibition was calculated based on the solubility of the retained dye in wells by using the formula-

$$\%\ Biofilm\ inhibition = \frac{ODcontrol - ODtest}{ODcontrol} \times 100$$

Where control $OD_{Control}$ is the absorbance without addition of plant extracts and $0D_{test}$ is the absorbance of treated sample.

Antibiofilm activity of both water and methanolic extracts was checked against *P. aeruginosa* at 10% (v/v) and 1mg/ml respectively. Out of all extracts 15 methanolic extracts GGMF, AHMF, GPMF, GLMF, CTMF, FLMD, CSMD, CAMD, CpAMD, SDMF, BPMF, ATMD, JGMD, EOWD, OC5WD and 15 water extracts namely GGWF, AHWF, GPWF, FLWD, EOWD, GAWD, DEWD, EKWD, CPWD, OC5WD, BPWF, CSWD, CAWD, MLWD, AIWD, SUWF (flower), CCWF, TCWF showed anti-biofilm activity in the range of 50-80% against *P. aeruginosa.*

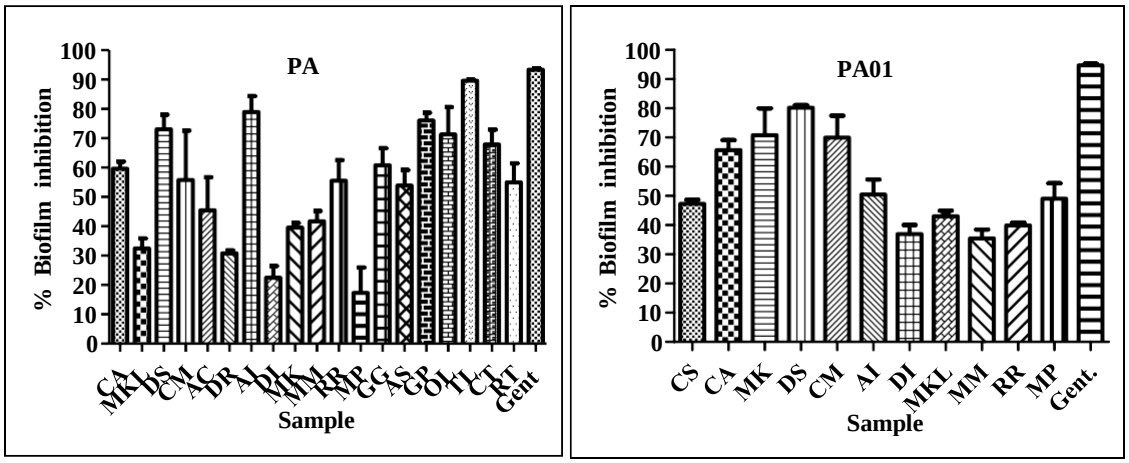
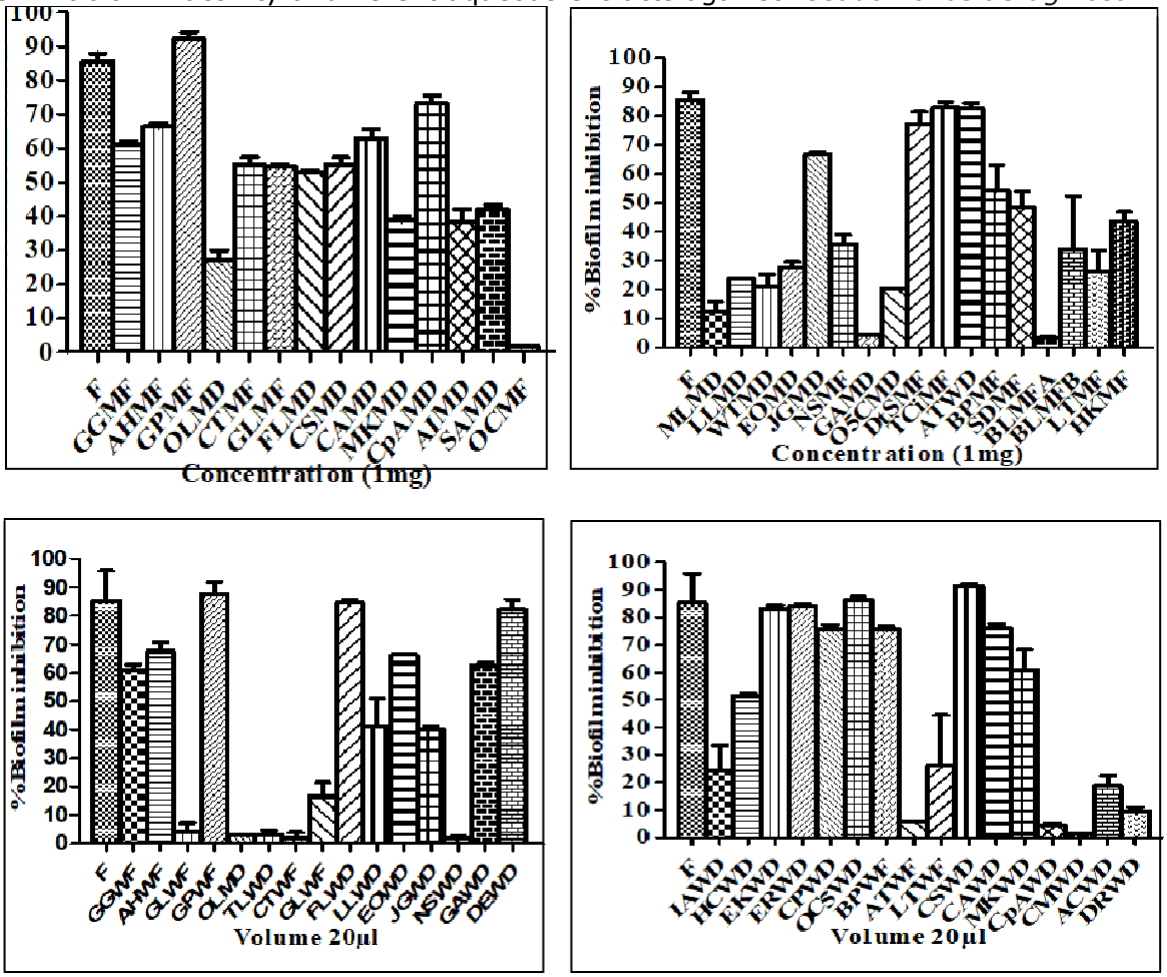


Fig.8: Antibiofilm activity of different aqueous extracts against *Pseudomonas aeruginosa*



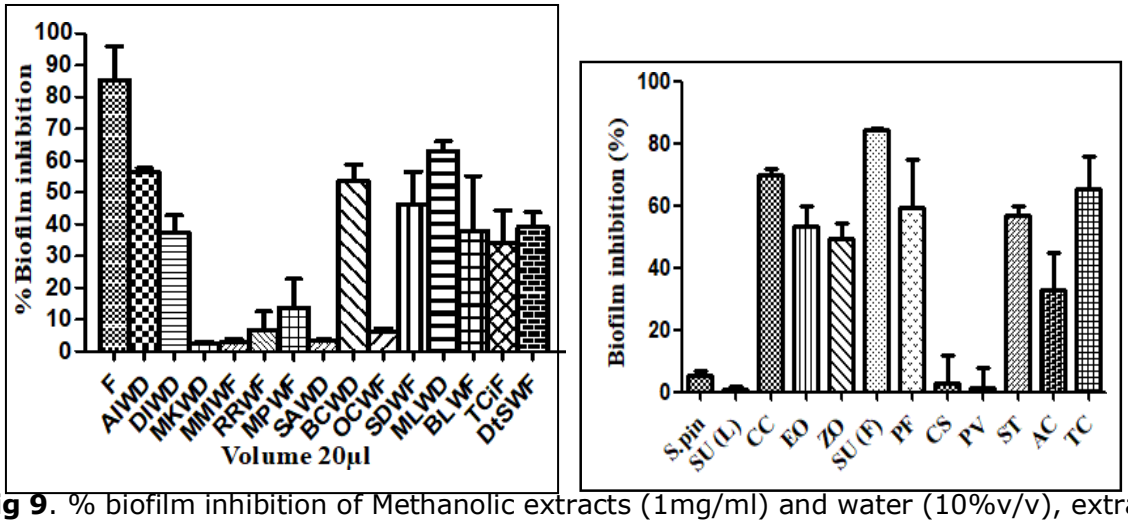


Fig 9. % biofilm inhibition of Methanolic extracts (1mg/ml) and water (10%v/v), extracts against *P. aeruginosa,* Furanone as positive control

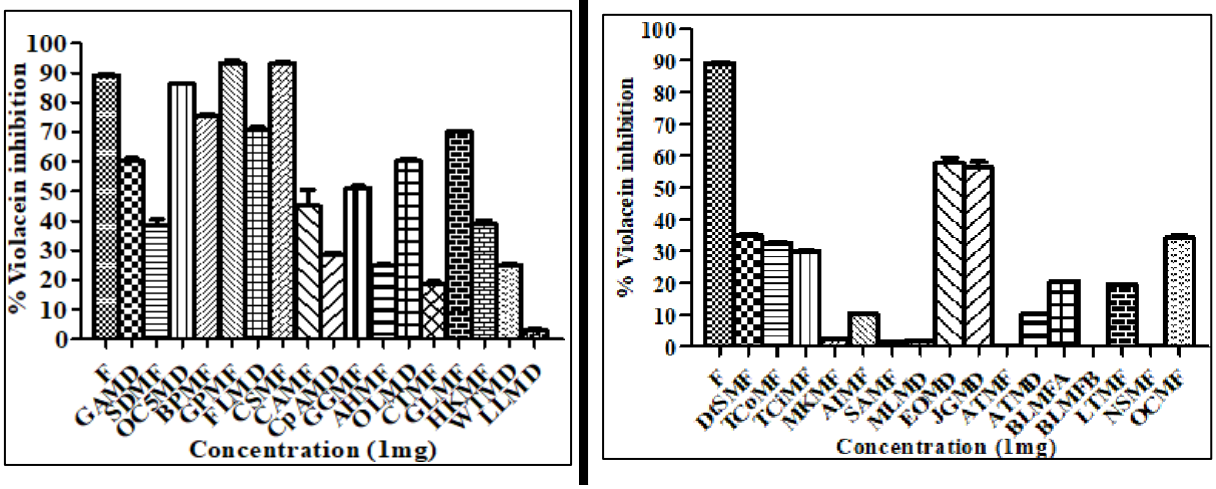
5. Quorum sensing inhibition bioassay by plant extracts

Violacein inhibition by plant extracts was carried out quantitatively by the flask incubation method. *Chromobacterium violaceum* ATCC 1472 was inoculated in LB broth supplemented with 10 mg/ml concentrations of plant extracts and incubated for 24 h at 30 ºC. Violacein was extracted. Briefly 1 ml of treated and untreated culture from each flask was centrifuged at 10,000 rpm for 10 min to precipitate violacein. The obtained pellet was resuspended in 1 ml of dimethyl sulfoxide (DMSO) and vortexed for 5 min to completely solubilize the violacein. Obtained mixture was centrifuged again to precipitate the cells violacein present in the supernatant was quantified by measuring the optical density at 585 nm. The experiment was carried out in triplicate and the percentage inhibition of violacein was calculated by the formula:

$$\%\ Of\ violacein\ inhibtion = \frac{Control\ OD - Test\ OD}{Control\ OD} \times 100$$

Where control $OD_{Control}$ is the absorbance without addition of plant extracts and $0D_{test}$ is the absorbance of treated sample

Out of all the extracts 12 methanolic extracts at a concentration of 1mg/ml showed anti-quorum sensing activity against *Chromobacterium violaceum* MTCC 2656.



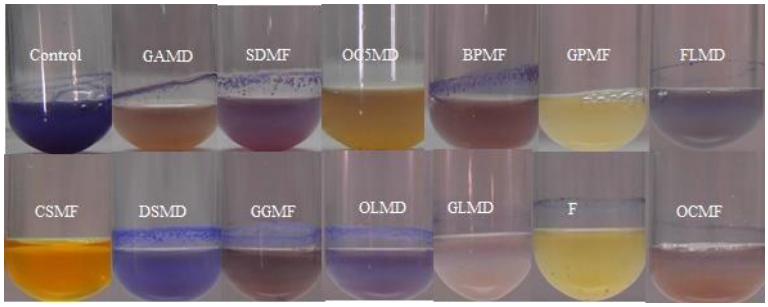


Fig 10. % Violacein inhibition of *C. violaceum* by different plant methanolic extracts (1mg/ml), Furanone as positive control.

6. Effect of plant extracts on pyocyanin production by *P. aeruginosa*:

The effect of plant extracts on the pyocyanin production of *P. aeruginosa* was determined by pyocyanin quantification assay given by Essar *et.al.* Briefly, 100µl of 0.4 OD overnight grown culture was inoculated in 10ml of LB broth. Samples in different concentrations were added into test tubes and incubated at 37°C under shaking conditions. After 24 hours of incubation 10ml culture was centrifuged at 8000 rpm for 10 minutes. Supernatant was collected and the pellets were discarded. 3.5ml of chloroform was added to the supernatant in each test tube. Test tubes were vortexed for 5minutes. The solvent phase was collected separately and 1.5ml of 0.2M HCl was added into each test tube. Test tubes were vortexed again and OD was the measured at 595nm.

Pyocyanin production was quantified for untreated and treated samples

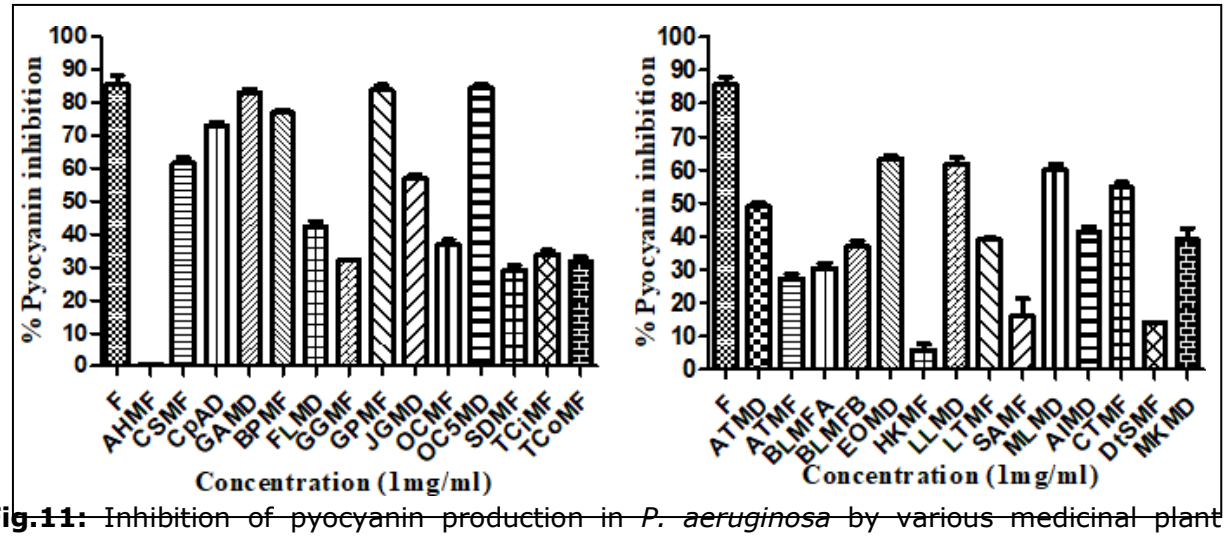


Fig.11: Inhibition of pyocyanin production in *P. aeruginosa* by various medicinal plant extracts

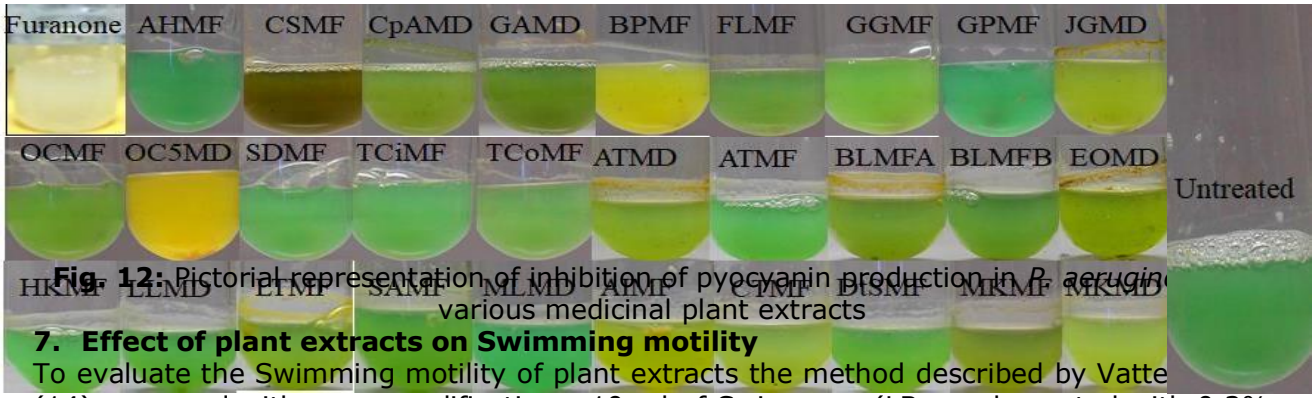


Fig. 12: Pictorial representation of inhibition of pyocyanin production in *P. aeruginosa* by various medicinal plant extracts

7. Effect of plant extracts on Swimming motility

To evaluate the Swimming motility of plant extracts the method described by Vatte (14) was used with some modifications. 10 ml of Swim agar (LB supplemented with 0.3% w/v agar) mixed with suitable amount of plant extract was poured in 60mm petri-dish and allowed to dry. 5µl of *P. aeruginosa* was point inoculated in each petri-dish and incubated 37°C for 24 h. To determine the extent of swimming motility, the motility diameter of treated ones was compared with untreated control.

Dose dependent swimming motility inhibition was shown by FLMD, SDMF, GAMD, GPMF at concentrations of 0.5 mg/ml, 0.75mg/ml, 1 mg/ml against *P. aeruginosa.*

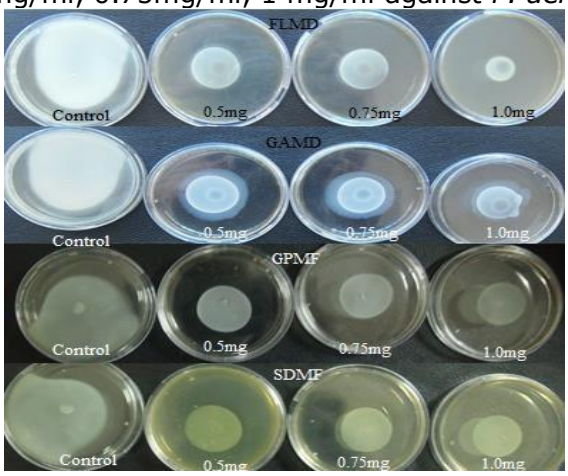


Fig 13. % Swimming motility of Methanolic extracts of FLMD, GAMD, GPMF, SDMF 0.5 mg/ml, 0.75mg/ml, 1 mg/ml against *P. aeruginosa.*

8. Detailed study of *Spilanthes uliginosa*

8.1. Effect of plant extracts on pyocyanin production by *P. aeruginosa*:

The effect of plant extracts on the pyocyanin production of *P. aeruginosa* was determined by pyocyanin quantification assay given by Essar *et.al.* Briefly, 100µl of 0.4 OD overnight grown culture was inoculated in 10ml of LB broth. Samples in different concentrations were added into test tubes and incubated at 37ºC under shaking conditions. After 24 hours of incubation 10ml culture was centrifuged at 8000 rpm for 10 minutes. Supernatant was collected and the pellets were discarded. 3.5ml of chloroform was added to the supernatant in each test tube. Test tubes were vortexed for 5minutes. The solvent phase was collected separately and 1.5ml of 0.2M HCl was added into each test tube. Test tubes were vortexed again and OD was the measured at 595nm (Fig. 14a).

8.2. Effect of *S. uliginosa* flower extract on biofilm of *P. aeruginosa*:

Dose dependent antibiofilm activity of *S. uliginosa* extract was checked as per the same procedure mentioned earlier (Fig. 14b).

8.3. Azocasein degrading proteolytic activity of *S. uliginosa* flower extract:

The proteolytic activity of *P. aeruginosa* in the presence of different concentrations of the plant extracts was determined according to the method of Kessler *et al.* with minor modifications. 100 µl of overnight grown bacterial culture was inoculated in 10ml LB broth with different concentrations of *S. uliginosa* flower extract in test tube and incubated for 48hours at 37 ˚C. After incubation, the solutions were centrifuged at 10000 rpm for 5 min. Supernatant was collected and 150µl supernatant was added to 1 ml of 0.3% azocasein in 0.05M Tris-HCl and incubated at 37 ˚C for 15minutes. Reaction was stopped by adding 0.5 ml of 10% trichloro acetic acid and then the tubes were centrifuged at 10000rpm for 5minutes. Absorbance was measured at 400nm (Fig. 14c).

8.4. Inhibition of Las B elastase activity of *P. aeruginosa* by *S. uliginosa* flower extract:

The elastolytic activity of the cell-free culture supernatant of *P. aeruginosa* was determined by following the method of Ohman *et al.* 100µl of cell-free supernatant of *P. aeruginosa* cultured with different concentration of plant extracts was mixed with 900µl of ECR buffer (100mM Tris and 1mM $CaCl_2$ at pH 7.5) with 20mg of ECR (Elastin Congo Red). The samples were incubated at 37 ˚C for 3hours. Then 1ml of 0.7 M Sodium Phosphate Buffer (pH 6) was added to stop the reaction. Samples were given a cold water bath and then the insoluble ECR was removed by centrifugation at 10000rpm for 10minutes. Absorbance was measured at 495nm (Fig. 14d).

8.5. Effect of *S. uliginosa* flower extract on EPS production of *P. aeruginosa*:

EPS was isolated and characterized by the method described by Tribedi and Sil, with minor modifications. 100 µl of 0.4 OD *P. aeruginosa* bacterial culture was inoculated in 10ml LB broth in separate test tubes having different concentrations of plant extracts and incubated at 37°C for 48 hours in static condition. After 48 hours of incubation, the samples were centrifuged at 10000rpm for 15minutes. The pellets obtained were used for extraction of bound EPS and the supernatant was used to extract free EPS (Fig. 14e).

8.6 Effect on Bound EPS:
The obtained cell pellets were washed twice (in PBS at 7.4pH or 0.9% NaCl) and resuspended in 1:1 solution of 0.9% NaCl and 2% EDTA. Samples were then incubated at 4°C for 60 minutes. After 60 minutes, the samples were centrifuged at 10000rpm for 30 minutes. The obtained supernatant was filtered through nitro cellulose membrane filter and stored in -20°C until further used (Fig. 14e).

8.7. Effect on Free EPS:
The cell-free supernatant was again centrifuged at 10000rpm for 15 minutes. Then each tube was precipitated with 1:3 volume of chilled absolute ethanol and stored at -20°C for 18 hours. After 18hours of incubation, again the tubes were centrifuged at 10000rpm for 15minutes at 4°C. The now obtained pellets were resuspended in autoclaved distilled water and stored in -20°C until further used. Further, the bound and free EPS thus extracted were used to estimate carbohydrate by phenol-sulphuric acid method (Fig. 14e).

8.8. Estimation of total protein concentration of the extracted EPS:
The EPS extracted in the previous protocol was precipitated with 1:5 ice-cooled accetone at -20°C for 18 hours. After 18hours of incubation, the EPS solution was centrifuged at 10000rpm for 15minutes at 4°C. The supernatant was discarded and again 2ml acetone was added for precipitation. Again the tubes were centrifuged at 10000rpm for 15 minutes at 4°C. The obtained pellets were resuspended in 1ml distilled water and protein content was estimated by lowry's method of protein estimation (Fig. 14f).

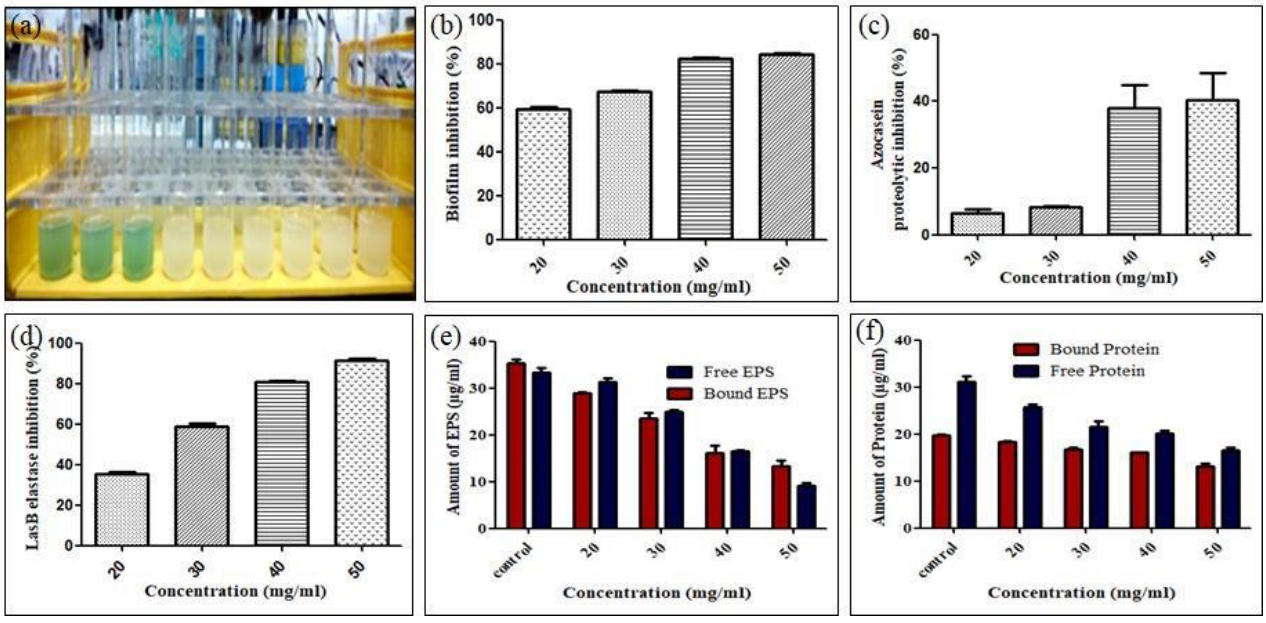


Fig.14: (a) Inhibition of pyocyanin production in *P. aeruginosa* by *Spilanthes uliginosa* at different concentrations (b) Dose dependent biofilm inhibition against *P. aeruginosa.* (c) % Azocasein proteolytic inhibition (d) % LasB elastase inhibition (e) Dose dependent decrease in EPS production in *P. aeruginosa* (f) Dose dependent decrease in production of Bound and Free proteins in *P. aeruginosa*

9. Detailed study of *Allium hookeri* (AHMF)

9.1 Antifungal activity of AHMF

Fungal culture of *Candida albicans* was grown overnight PDB. PDA plate was prepared the next day and 100µl of the cultures were spread on the plate. Wells were made in the plates and plant extract was added into each well, Nystatin was used as positive control. The plate was incubated overnight at 28°C. The plate was later screened for the presence of inhibition zones whose diameters were further measured accordingly (Fig.15a).

9.2 Time kill assay of *Allium hookeri* against *C. albicans*

To check the time dependent microbial reduction by AHMF Time kill assay was performed. 100 µl of freshly prepared 0.4OD *C. albicans* culture was added to PDB to which AHMF extract was added in different concentrations. The glass tubes were incubated at 30°C and after every 2hrs 100µl culture at a dilution of 10^{-5} was spread on PDA plates. The plates were incubated at 30°C for 24hrs. The number of colonies formed were counted and CFU/ml was calculated (Fig. 15b).

9.3 Growth Curve of *C. albicans* in the presence of AHMF:

Growth curve of *C. albicans* in presence of different concentrations of AHMF was determined and compared to the growth of the untreated. The Fig.15(c) shows that the extract restricted the growth of the strain efficiently and concentration dependently. This suggests that growth was not inhibited, but the virulence characters were inhibited.

9.4 Dose dependent biofilm inhibition of *C. albicans* biofilm

Effect of AHMF at different concentrations on biofilm inhibition was evaluated using the same protocol as mention earlier. The only modification is the use of RPMI+ 10%FBS in place of TSB (Fig. 15d).

9.5 Effect on Cell Surface Hydrophobicity

The hydrophobicity of *C. albicans* after the treatment with AHMF was determined. Cells were grown in PDB medium containing different concentrations of AHMF and incubated for 24hrs. Cells grown in PDB medium without AHMF was used as control(untreated). After incubation cells were centrifuged and resuspended in PBS (Ph7.4) and OD_{600} was measured. Equal volume of cell suspension was mixed with ethylacetate and n-hexadecane seperately and vortexed for 2 min and left undisturbed (Fig. 15e, f). After 1hr the aqueous layer was carefully pipetted out and OD_{600} was measured. CSH was calculated using the formula

$$Hydrophobicity(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 = OD_{600} at 0hr and A_1 = OD_{600} at 1hr

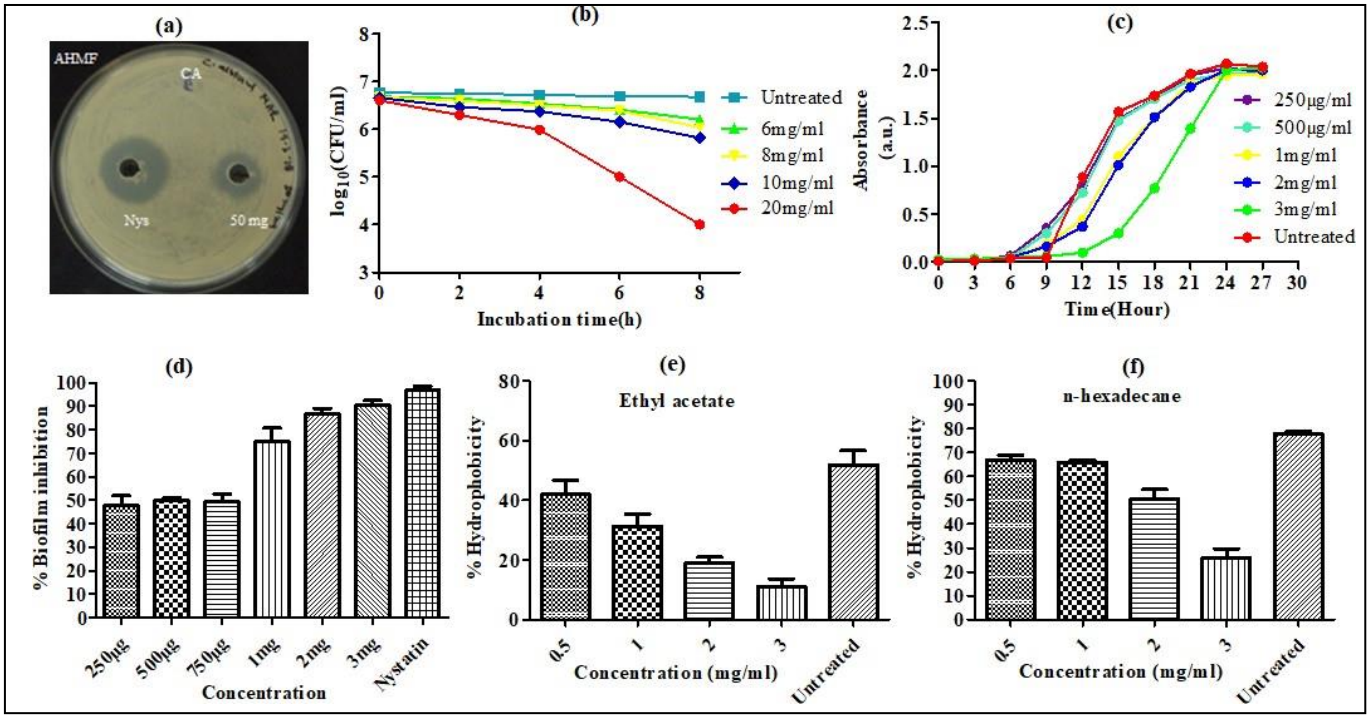


Fig. 15: Different assays for AHMF (a) Antifungal activity (b) Time kill Assay (c) Growth curve analysis (d) Biofilm inhibition (e,f) Hydrophobicity

10. Inhibition of *C. albicans* yeast to hyphal transition

Effect of AHMF extract on *C.albicans* yeast to hyphal transition was estimated by the method Marlene Chevalier et al., (13) with slight modifications. In brief *C.albicans* (10^6 to 10^7 CFU/ml) was grown on RPMI medium supplemented with Fetal Bovine Serum (10%) containing AHMF at different concentrations for 24hrs at 30°C. After incubation period the hyphal inhibition was visualized under Scanning Electron Microscope (Fig. 16).

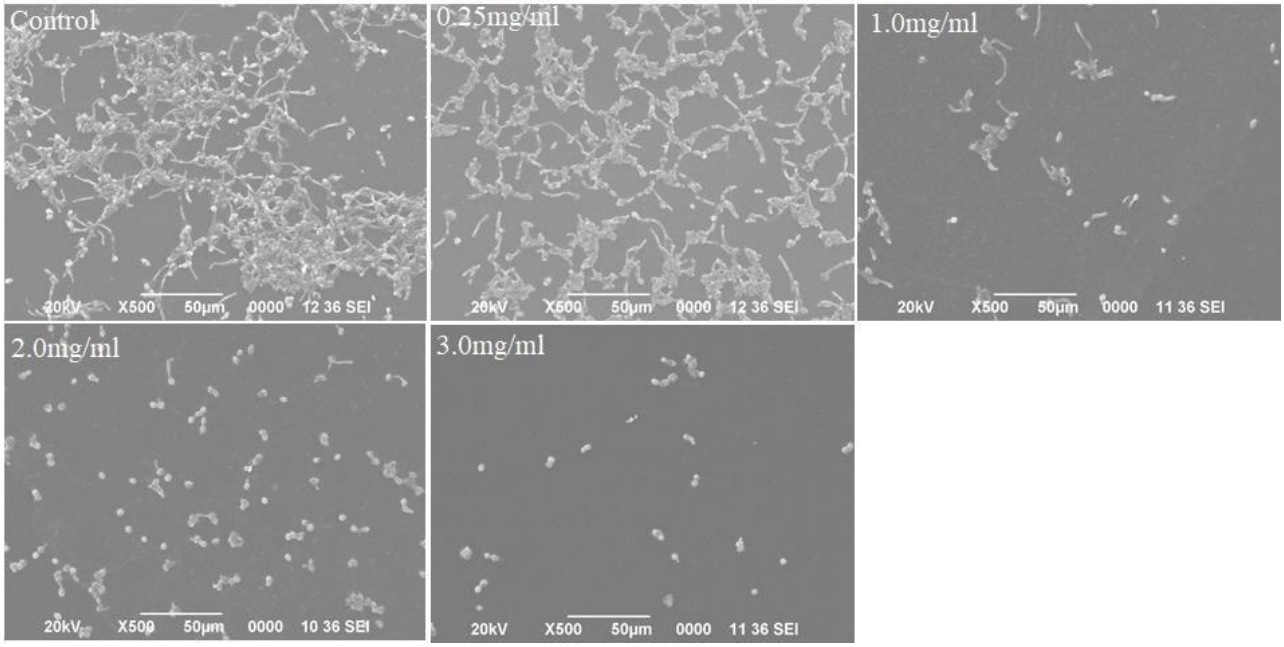


Fig. 16: SEM (Yeast to hyphal transition)

Summary:

1. 58 plants were selected from different places of Arunachal Pradesh, Meghalaya, Manipur, Assam, and Mizoram between October 2017 to March 2019 of North East India
2. Using methanol and/or water as solvents various plant extracts were prepared either from their dry powder or fresh sample.
3. Antibacterial activity was found in that methanolic extracts of *Spondia dulchis* (SDMF)*, Garcinia acuminate* (GAMD)*, Bryophyllum pinnatum* (BPMF)*, Camellia sinensis, Oxalis corniculata, Garcinia pedunculata, Garcinia lanceaefolia* and Flower like.
   showed antibacterial activity against all strains. Water extracts of *Solanum torvum* (STWF) and *Terminalia chebula* (TCWF) showed activity against *P. aeruginosa* while *Allium hookeri* extracts show antifungal activity against *C. albicans.*
4. Good antifungal activity was seen in aqueous and methanolic extracts of *Allium hookeri* (AHMF).
5. Ant-biofilm activity in the range of 60-80% was found in 15 methanolic extracts GGMF, AHMF, GPMF, GLMF, CTMF, FLMD, CSMD, CAMD, CpAMD, SDMF, BPMF, ATMD, JGMD, EOWD, OC5WD and 18 water extracts namely GGWF, AHWF, GPWF, FLWD, EOWD, GAWD, DEWD, EKWD, CPWD, OC5WD, BPWF, CSWD, CAWD, MLWD, AIWD, SUWF (flower), CCWF, TCWF showed anti-biofilm activity in the range of 50-80% against *P. aeruginosa.*
6. Anti-quorum sensing activity was exhibited by GAMD, SDMF, OC5MD, BPMF, GPMF, FLMD, CSMF, GGMF, OLMD, GLMF, EOMD, JGMD against *Chromobacterium violaceum*
7. FLMD, SDMF, GAMD, GPMF inhibited swimming motility of *P. aeruginosa*.
8. *Spilanthes uliginosa* exibited inhibition of biofilm formation, pyocyanin production, inhibition of protease and Las B elastase activity. The extract showed the decrease in EPS production and production of Bound and Free proteins in *P. aeruginosa.*
9. *Allium hookeri* showed antifungal activity, dose dependent biofilm inhibition, exhibited decline in hydrophobicity and inhibited yeast to hyphal transition in *C. albicans.*

B2. Summary and Conclusions of the Progress made so far (minimum 100 words, maximum 200 words):

58 different plants have been collected and the prepared extracts were screened for the antibiofilm activity against *P. aeruginosa.* Considereing the potential activity of more than 50% biofilm inhibition, we have found that 15 methanol extracts and 18 water extracts show promising antibiofilm activity at sub-MIC. The extracts were checked for other virulence factors associated with biofilm formation and we found that our extracts inhibited the biofilm related virulence factors as well, thus, validate our results.

Section-C: Details of Grant Utilization#

C1. Equipment Acquired or Placed Order with Actual Cost:
Sanctioned equipments procurred.

C2. Manpower Staffing and Expenditure Details: Statement enclosed.

The sanctioned post of RA was filled on 14-07-2017 and JRFs was filled on 13-10-2017. The newly recruited Project JRF (Project Assistant III) is carrying out all the activities as per the objectives of the project including maintaining laboratory records, log book and official works.

Detail of the JRF recruited:

Sl. No.	Name of the Fellow	Position	Date of recruitment
1.	Mr. Muzamil Ahmad Rather	JRF/SRF	13-10-2017
2.	Dr. Kuldeep Gupta	RA	14-07-2017

C3. Details of Recurring Expenditure: Expenditure statement enclosed.

Consolidated Statement of Expenditure of Tezpur University, Tezpur (2017 to 2022)

Rs. in Lakhs

Details of grant, expenditure and balance

S. No.	Heads	Sanctioned Cost	Year-wise Releases made						Year-wise Expenditure incurred					
			1st yr	2nd yr	3rd Yr	4th Yr	Total		1st yr	2nd yr	3rd yr	4th yr	Total	Balance
A.	**Non-recurring**													
	Equipments	19.93	19.93	0.00	0.00	0.00	19.93		0.00	0.00	19.90510	0.00	19.90510	0.02490
B.	**Recurring**													
1.	Manpower	24.54	8.05	4.07	0.00	0.00	12.12		4.07147	6.19200	1.75090	0.00	12.01437	0.10563
2.	Consumables	5.00	2.00	1.99	0.00	0.00	3.99		1.99283	0.00	1.98610	0.00	3.97893	0.01107
3.	Travel	1.25	0.50	0.42	0.00	0.00	0.92		0.42222	0.19703	0.32008	0.00	0.93933	-0.01933
4.	Contingency	1.50	0.50	0.02+0.47[a]	0.00	0.00	0.99		0.48589	0.36479	0.12497	0.00	0.97565	0.01435
5.	Overhead	0.75	0.30	0.0+0.25 [a]	0.00	0.00	0.55		0.30	0.00	0.15625	0.00	0.45625	0.09375
	Total	**33.04**	**11.35**	**6.5+0.72 [a]**	**0.00**	**0.00**	**18.57**		**7.27241**	**6.75382**	**4.3383**	**0.00**	**18.36453**	**0.20547**
	Grand Total (A+B)	**52.97**	**31.28**	**7.22**	**0.00**	**0.00**	**38.5**		**7.27241**	**6.75382**	**24.24340**	**0.00**	**38.26963**	**0.23037[b]**

Note: [a]- amount (0.72) reappropriated from interest generated during 2017-2018; **b**- add an amount of 0.56254 (interest generated during 2018-2019 & 2019-2020) to the balance amount i,e 0.23037+0.56254=0.79291**. So, total unspent balance =0.79291 lakh**

Note: The unspent balance amounting 0.79291 lakhs has been refunded in the account of Refund of Unspent Grant (PAO DBT) through bharatkosh.gov.in with transaction Ref. No. 2807220007549 (INR 23037.00) and 2807220007664 (INR 56254.00) dated Jul 30, 2022

C4. Financial Requirements for the Next Year with Justifications:

Fund may be sanctioned as per the sanction order No. BT/PR16149/NER/95/85/2015 dated 19/01/2017 for smooth execution of the project work.

Collaborating Centre-1 at WBUAFS, Kolkata

Objective 1

To study the prevalence of biofilm producing bacteria (*Escherichia coli*, *Salmonella, Staphylococcus* and *Pseudomonas*) in cattle, poultry and ducks in West Bengal

Timeline: 0-30 months

1. Materials and methods

1.1. Collection of samples from cattle, poultry and ducks of any age group, either sex

The cloacal swabs of the poultry (broilers, layers, backyard), ducks (indigenous/ Khaki Campbell) and rectal swabs / milk samples of the cattle were collected from different districts of West Bengal (Kolkata, Nadia, Hooghly, Howrah, North 24 Parganas, Jalpaiguri, South Dinajpur) throughout the study period as described in Table-1. The samples were collected in sterile cotton swabs (HiMedia, India) and transported to the laboratory maintaining the cold chain.

1.2. Isolation and identification of *E. coli*

In the laboratory the samples were kept in the nutrient broth (HiMedia, India) & incubated at 37^0C for overnight. It was transferred to MacConkey's agar (HiMedia, India) and again incubated at 37^0C for overnight. Next day 2-3 rose pink colonies were randomly picked and transferred to EMB agar (HiMedia, India) followed by an overnight incubation at 37^0C. Colonies were observed for metallic sheen and single colony was streaked into nutrient agar (HiMedia, India) slant for further biochemical confirmation. All the pure cultures obtained from nutrient agar slant were subjected to Gram's staining and standard biochemical tests like Catalase, Oxidase, Indole, Methyl red, Voges Proskauer, Citrate, Urease tests as described earlier (Quinn *et al*., 1994).

1.3. Isolation and identification of *Salmonella spp.*

The samples were enriched in Selenite-F broth (HiMedia, India) by incubating overnight at 37ºC. On the next day reddish turbidity of the Selenite-F broth in selected samples were observed. Those samples were transferred into Brilliant green agar (HiMedia, India) and incubated at 37ºC for 48 hours. Convex, pale red, translucent colonies were primarily identified on the basis of Gram's staining and biochemical tests like Catalase, Oxidase, Indole, Methyl red, Voges-Proskauer, Citrate and growth pattern in triple sugar iron agar (Quinn *et al*., 1994).

1.4. Isolation and identification of *Staphylococcus spp.*

Milk samples collected from the mastitic cattle were inoculated into peptone water followed by Mannitol salt agar (HiMedia, India) and incubated at 37^0C for overnight. Next day characteristic yellow coloured colonies were picked and streaked on nutrient agar (HiMedia, India) slant for further morphological and biochemical confirmation. The isolates were primarily identified on the basis of Gram's staining and biochemical tests like Catalase, Oxidase, Indole, Methyl red, Voges-Proskauer, Citrate (Quinn *et al*., 1994).

1.5. PCR based confirmation of *E. coli* and *Salmonella* isolates

The morphologically and biochemically verified *E. coli* isolates were subjected to PCR for molecular confirmation as described by Wang *et al*.(1996) with some modification. PCR tests were performed with 5 µl of extracted *E. coli* DNA samples which was directly added to 20 µl of PCR mixture containing 2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.25 mM each primer, and 1 U of Taq polymerase (Promega). The amplification conditions were: one cycle at 94°C for 5 min and followed by 35 cycles at 94 °C for 30 s, 58 °C, for 1 min, and 72°C for 1 min and one final cycle at 72°C for 10 min. The PCR product was visualized by gel

documentation system (UVP, UK) after electrophoresis in 2% (W/V) agarose (SRL, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India).

Salmonella isolates were confirmed by the presence of *invA* using oligonucleotide primers (Eurofins) as described by Oliviera *et al*. (2002). The PCR was carried out in a 25 µl mixture containing 2.5 µl 10X Taq buffer (500 mM KCl, 100 mM Tris, 0.1% gelatin), 0.20 µl dNTPs (100mM), 1 µl $MgCl_2$ (25 mM), 20 pmol of each primer,1 unit of Taq DNA polymerase (Promega) and 2 µl of DNA template of each isolate. The amplification was conducted in a thermal cycler of Eppendorf make. The cycle conditions were an initial denaturation at 94ºC for 60 sec followed by 35 cycles of denaturation at 94ºC for 60 sec, annealing at 64ºC for 30 sec and elongation at 72ºC for 30 sec. The cycles were completed by 7 minutes final extension at 72ºC. One each *Salmonella* Pullorum and *Escherichia coli* isolate was used as positive and negative control, respectively. The amplified PCR products were analyzed in agarose gel electrophoresis using 1.2% gel and visualized with documentation in Gel documentation system (UVP).

1.6. Characterization of *E. coli* isolates

1.6.1. Serogrouping

All the *Escherichia coli* isolates possessing antimicrobial resistance / biofilm genes were sent for O- serogrouping to National *Salmonella* & *Escherichia* Centre, Central Research Institute, Kasuli, HP, India. The results of the isolates are awaited.

1.7. Characterization of *Salmonella* isolates

1.7.1. Serotyping

All the duck *Salmonella* isolates possessing the studied virulence genes (*invA*, *sefA*) were sent for serotyping to National *Salmonella* & *Escherichia* Centre, Central Research Institute, Kasuli, HP, India. Results are awaited.

2. Results

2.1. Isolation and characterization of *E. coli* isolates

In total 217 *E. coli* were isolated, identified and confirmed by PCR from collected 370 cloacal swabs (cattle: 52%, poultry: 85%, ducks: 49%; Table 2; Figure 1). The samples collected from South Dinajpur are under process for isolation of bacteria. Majority of the duck *E. coli* isolates belonged to untypeable followed by O83, O84, O2, O88, O5, O119, O35, O8, O128 and O157 (serotyping results of North 24 Parganas and Hooghly duck isolates obtained so far). Isolation of O157 is zoonotically important finding due to its potentiality to cause haemolytic uraemic syndrome and haemorrhagic diarrhoea in human.

2.2. Isolation and characterization of *Salmonella* isolates

In total, 58 *Salmonella* were isolated, identified and confirmed by *invA*-PCR from collected 370 cloacal swabs (cattle: 30%, poultry: 25%, ducks: 6 %; Table 2; Figure 2).

2.3. Isolation and identification of *Staphylococcus* spp. from collected milk samples

Four *Staphylococcus* spp. were isolated and identified from the collected milk samples (4/4, 100%, Table 2) based on Gram's staining and biochemical tests.

Objective 2

To detect the correlation between biofilm-associated genes, virulence genes, antimicrobial resistance genes of the isolates

Timeline: 6-30 months

1. Materials and methods

1.1. Detection of *in vitro* biofilm production capacity of the isolates

Biofilm formation of the *Escherichia coli* and *Salmonella* isolates was measured by using microtitre plate assay with the crystal violet staining following the protocol of Mohamed et al. 2012 with minor modifications. All the viable isolates were grown in LB broth containing 0.25 % glucose overnight at 37°C. The culture was then diluted 1:100 in Dulbecco's Modified Eagles Medium (DMEM) containing 0.45 % glucose. Two hundred microlitre of the bacterial suspension was inoculated into individual wells of a sterile 96 well polystyrene plate (Tarson, India) and incubated for 18 h at 37°C. After incubation the biofilm was fixed with 200 µl Bouin fixative for 15 min and rinsed once with phosphate buffered saline (PBS). The fixed bacterial cells were then stained with 0.5 % crystal violet for 15 min and rinsed thoroughly with distilled water. After air drying, crystal violet was solubilized in 200 µl of ethanol–acetone (80:20, v/v) for 30 min. The optical density was measured at 570 nm by using ELISA reader (EIAQuant, Meril, India). Each assay was performed in duplicate and repeated at three different occasions. *Escherichia coli* ATCC 35218 served as positive control for the strong biofilm production, and the sterile broth served as negative control. The isolates were considered biofilm producer if, OD570 reading exceeded the mean plus two standard deviations of the negative control strain.

1.2. Screening of biofilm producing isolates for biofilm associated gene, virulence gene and antimicrobial resistance genes

PCR based screening of the *Escherichia coli* and *Salmonella* isolates for biofilm associated gene (*csgA*), virulence genes (*stx_1,stx_2, eaeA, ehxA, sefA*) and antimicrobial resistance genes (*bla_{TEM}*, *bla_{SHV}*, *bla_{CTX-M}*) was done by standard techniques.

1.2.1. PCR for detection of biofilm associated gene (*csgA, sdiA, rcsA, rpoS*) in *E. coli* isolates

All the *E. coli* isolates producing moderate or strong biofilm in phenotypical assay were subjected to PCR for detection of *csgA*. The PCR reaction was carried out in 25 µl reaction volume containing 5 µl of DNA template, 10 mM Tris-HCl (pH 8.3), 50 mM KCL, 2mM $Mgcl_2$, 0.01% Gelatin, 100 pmol of each primer, 0.2mM of each 2' Deoxynucleoside 5'-triphosphate and 1U of Taq DNA polymerase (Promega). The cycle condition was initial denaturation at 94°C for 4 minutes followed by 30 cycles consisting of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 4 minutes in a thermal cycler (Eppendorf) (Delicato et al., 2003). The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 2% (W/V) agarose (HiMedia, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India).

All the *E. coli* isolates producing moderate or strong biofilm in phenotypical assay were subjected to PCR for detection of *sdiA, rcsA, rpoS*. PCR was performed in a 25 µl reaction mixture containing 1U Taq DNA Polymerase, 2 mM $MgCl_2$, 0.4 mM each of dNTPs, 10 pmol concentrations of each primer. The cycle condition was denaturation at 95°C for 2 min followed by amplification for 30 cycles at 95°C for 1 min, annealing temperature (52°C for *sdiA*; 54°C for *rcsA*; 60°C for *rpoS*) for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min in a thermal cycler (Eppendorf) (Adamus-Białek et al., 2015).The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 2% (W/V) agarose (HiMedia, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India).

1.2.2. PCR for detection of virulence genes in *E. coli* (*stx_1,stx_2, eaeA, ehxA*) and *Salmonella* (*sefA*) isolates

All the duck originated *E. coli* isolates including positive control were subjected to m-PCR for detection of *stx_1, stx_2, eae* and *ehxA* genes considered for virulence factor of shiga-toxin producing *E. coli* (STEC) or enteropathogenic *E. coli* (EPEC). The STEC strain possessing all the four genes (*stx_1*, *stx_2*, *eaeA*, *ehxA*) provided by CAU, Aizawl was used as positive control and sterile distilled water was used as negative control. The PCR reaction was carried out in

25 µl reaction volume containing 5 µl of DNA template, 10 mM Tris-HCl (pH 8.3), 50 mM KCL, 2mM $Mgcl_2$, 0.01% Gelatin, 0.5 µM of each primer, 0.2mM of each 2' Deoxynucleoside 5' – triphosphate and 1U of Taq DNA polymerase (Promega). The samples were subjected to two régime of amplifications. First regime consisted of 15 cycles. Each cycle consisted of 95^0C for 1 min, 65^0C for 2 min and 72^0C for 1.5 min. Second regime consisted of 20 cycles of 95^0C for 2 min, 60^0C for 2 min, 72^0C for 2 min. This was followed by final extension of 5 min at 72^0C (Paton and Paton, 1998). The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 2% (W/V) agarose (HiMedia, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India).

PCR for detection of *sef-A* gene in *Salmonella* isolates was carried out in a 25 µl mixture containing 2.5 µl 10X Taq buffer (500 mM KCl, 100 mM Tris, 0.1% gelatin), 0.20 µl dNTPs (100mM), 1 µl $MgCl_2$ (25 mM), 20 pmol of each primer,1 unit of Taq DNA polymerase (Promega) and 2 µl of DNA template of each isolate. The amplification was conducted in a thermal cycler of Eppendorf make. The cycle conditions were an initial denaturation at 94ºC for 60 sec followed by 35 cycles of denaturation at 94ºC for 60 sec, annealing at 64ºC for 30 sec and elongation at 72ºC for 30 sec. The cycles were completed by 7 minutes final extension at 72ºC (Oliviera *et al*., 2002). One each *Salmonella* Pullorum and *Escherichia coli* isolate was used as positive and negative control, respectively. The amplified PCR products were analyzed in agarose gel electrophoresis using 1.2% gel and visualized with documentation in Gel documentation system (UVP).

1.2.3. Detection of antimicrobial resistance in biofilm producing *E. coli* and *Salmonella* isolates

1.2.3.1. Detection of ESBL producing *E. coli* / *Salmonella* isolates by phenotypic method

E. coli / Salmonella isolates were subjected to double disc test for phenotypic confirmation of ESBL (CTX-M) production. Double disc test was carried out in an agar plate with a disk containing cefotaxime (30 µg, HiMedia) and a disk containing cefotaxime /clavulanate (30 µg/10 µg, HiMedia), placed 30 mm apart (center to center) as described earlier (Brenwald *et al*., 2003). Similarly, for detection of TEM/SHV production double disc test with ceftazidime (30 µg, HiMedia) and ceftazidime/clavulanate (30 µg/10 µg, HiMedia) was carried out as described previously (Bedenic *et al*., 2007).

1.2.3.2. Detection of ESBL producing *E. coli* / *Salmonella* isolates by genotypic method

All the *E. coli* and *Salmonella* isolates including controls were subjected to PCR for detection of $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} genes using the primers and the cycle conditions as described earlier (Weill *et al*., 2004 and Weill *et al*., 2004a). The primers were procured from Eurofins. The PCR was carried out in a 25 µl master mixture containing 2.5µl DNA, 50 pmol of each primers, 200µM dNTPs , 1.25 U Taq polymerase, 2 mM $MgCl_2$ (Promega). The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 2% (W/V) agarose gel containing ethidium bromide (0.5µg/ml) (SRL, India) (Sambrook and Russel, 2001).

1.2.3.3. Detection of ampC-beta lactamase producing *E. coli* / *Salmonella* isolates by phenotypic method

All the *E. coli* / *Salmonella* isolates were subjected to cefoxitin-cloxacillin double disc synergy (CC-DDS) test for phenotypic confirmation of ACBL production (Tan *et al*., 2009).

1.2.3.4. Detection of ampC-beta lactamase producing *E. coli* / *Salmonella* isolates by genotypic method

All *E. coli* / *Salmonella* isolates were subjected to PCR amplification for bla_{AmpC} using the reaction mixture containing 5 µL DNA, 10 pmol each of the primer, 0.2 mM of each

deoxynucleoside triphosphate, 10 mM Tris–HCl pH 8.8, 50 mM KCl, 5% (v/v) dimethyl sulphoxide, 2.0 mM $MgCl_2$ and 1.25 U *Taq* DNA polymerase (Promega). PCR amplification comprised a first cycle of 7 min denaturation at 94°C, 5 min annealing at 60°C and 60 s extension at 72°C, followed by 30 cycles of 60 s at 94°C, 2 min at 60°C and 60 s at 72°C and a final extension step of 5 min at 72°C (Féria *et al*., 2002).

2. Results

2.1. Detection of *in vitro* biofilm production capacity of *E. coli* and *Salmonella* isolates

Among the studied *E. coli* isolates from duck and poultry, strong biofilm production was detected in two isolates (2/40, 5%) whereas majority of them (15/40, 37.5%) were moderate biofilm producers (Table 3).

Among the studied *Salmonella* isolates from cattle and poultry, moderate biofilm production was detected in three isolates (3/21, 14.2%) whereas majority of them (17/21, 80.9 %) were weak biofilm producers (Table 4).

2.2. Screening of biofilm producing isolates for biofilm associated gene, virulence gene and antimicrobial resistance genes

2.2.1. PCR for detection of biofilm associated gene (*csgA, sdiA, rcsA, rpoS*) in *E. coli* isolates

Among the studied 69 *E. coli* isolates (strong and moderate biofilm producing) from cattle and poultry, 33 isolates (33/69, 47.82%) possessed *csgA* gene; 68 isolates (68/69, 98.55%) possessed *sdiA*; 67 isolates (67/69, 97.1%) possessed *rcsA;* 69 isolates (69/69, 100%) possessed *rpoS* in PCR (Table 5; Figure 3, 12).

2.2.2. Detection of virulence genes in *E. coli* (*stx_1,stx_2, eaeA, ehxA*) and *Salmonella* (*sefA*) isolates

Multiplex-PCR for detection of shiga-toxins (*stx_1,stx_2*), intimin (*eaeA*) and enterohaemolysin (*ehxA*) genes revealed that majority of the duck *E. coli* isolates possessed *stx_1* with few strains possessing different combinations (Figure 4, 5). The isolate O157 possessed *stx_1*, *stx_2* and *eaeA* (Figure 6). Eight *Salmonella* isolates from ducks (8/13, 61%) possessed *sefA* gene (Figure 7).

2.2.3. Detection of antimicrobial resistance in biofilm producing *E. coli* and *Salmonella* isolates

Among studied 69 *E. coli* isolated from cattle and poultry, 16 *E. coli* isolates (16/69, 23.18%) were phenotypically found CTX-M producers and majority of the isolates possessed *$bla_{CTX\text{-}M}$* (Table 5). Only two *E. coli* isolates (2/69, 2.89%) were phenotypically found positive in double disc assay with ceftazidime and ceftazidime/clavulanate. However, 23 *E. coli* (23/69, 33.33%) and 8 *E. coli* isolates (8/69, 11.59%) possessed *bla_{TEM}* and *bla_{SHV}*, respectively (Table 5). Whereas, 44 *E. coli* isolates (44/69, 63.76%) were detected to possess *bla_{AmpC}* (Table 5; Figure 8, 9, 10, 11). Moreover, majority of the *E. coli* isolates (55/69, 79.7%) possessing biofilm associated gene (*csgA, sdiA, rcsA, rpoS*) were detected to possess any of the studied antimicrobial resistance gene (Table 5).

Among the *E. coli* isolates from ducks (n=102), 11 *E. coli* isolates (11/102, 10.7%) were detected phenotypically CTX-M producers and all of them possessed *$bla_{CTX\text{-}M}$*. None of the *E. coli* isolates from ducks possessed *bla_{TEM}* and *bla_{SHV}* and were detected positive in double disc assay with ceftazidime and ceftazidime/clavulanate. Whereas, 12 *E. coli* isolates from ducks (12/102, 11.7%) possessed *bla_{AmpC}* (Table 6).

Among the studied 35 *Salmonella* isolated from cattle and poultry, 17 *Salmonella* isolates (17/35, 48.5%) were phenotypically found CTX-M producers and majority of them (14/17, 82.3%) possessed *$bla_{CTX\text{-}M}$*. Only five *Salmonella* isolates (5/35, 14.2%) were

phenotypically found positive in double disc assay with ceftazidime and ceftazidime/clavulanate. However, 3 (3/35, 8.5%) and 13 (13/35, 37.1%) numbers of isolates possessed bla_{SHV} and bla_{TEM}, respectively. Whereas, 26 *Salmonella* isolates (26 /35, 74.2 %) were detected to possess bla_{AmpC} (Table 7).

Among the *Salmonella* isolates from ducks (n=13), 6 isolates (6/13, 46.1%) were detected phenotypically as CTX-M producers and all of them possessed bla_{CTX-M}. None of the *Salmonella* isolates from ducks possessed bla_{TEM} and bla_{SHV} and were detected positive in double disc assay with ceftazidime and ceftazidime/clavulanate. Further, none of the *Salmonella* isolates from ducks possessed bla_{AmpC} (Table 8).

Major Findings of the reported 1st and 2nd year (WBUAFS centre, Kolkata)

- Occurrence of *E. coli* was higher in poultry than cattle, ducks in studied samples collected from West Bengal
- Occurrence of *Salmonella* was higher in cattle and poultry than the ducks in studied samples collected from West Bengal
- EHEC O157 serotype was detected in duck in West Bengal
- Strong and moderate biofilm producing *E. coli* and *Salmonella* isolates were detected mostly in poultry and ducks
- *AmpC* gene was detected as most prevalent antimicrobial resistance genes among the studied ESBL and AmpC genes in *E. coli* and *Salmonella* isolates followed by CTX-M in cattle and poultry in West Bengal
- A positive correlation between biofilm associated gene (*csgA*) and antimicrobial resistance gene (bla_{AmpC}) was observed in *E. coli* isolated from cattle and poultry in West Bengal

Table 1. Collection of samples from cattle, poultry and ducks in West Bengal

District / Gram Panchayet	Species	Numbers of samples
Kolkata	Poultry (backyard)	10
	Ducks (*Deshi*)	02
Nadia(Kalyani SLF)	Ducks (Khaki Campbell)	40
Nadia(Nabadwip)	Ducks (*Deshi*)	33
Hooghly (Haripal / Rajbalhat)	Cattle (rectal swabs)	30
	Cattle (mastitic milk)	04
	Poultry (organized)	19
	Ducks (*Deshi*)	14
Howrah (Shyampur)	Cattle	37
	Poultry	07
Howrah (Bargachhia)	Ducks (*Deshi*)	33
North 24 pgs (Gobardaqnga)	Ducks (Khaki Campbell)	52
Burdwan(Debipur)	Ducks (*Deshi*)	33
Jalpaiguri (Malbazar)	Poultry (Broilers)	14
North 24 Parganas (Agarpara)	Poultry (Backyard)	04
North 24 Parganas (Barasat)	Poultry (Backyard)	36
	Cattle	04
South Dinajpur (Balurghat)	Poultry (Farmed Layers)	50
South Dinajpur (Kushnmandi)	Cattle	40
	Ducks *(Deshi)*	40
	Total	**502**

Table 2. Isolation and identification of *Escherichia coli*, *Salmonella* and *Staphylococcus* from collected samples

Species	*Escherichia coli*	*Salmonella* spp.	*Staphylococcus* spp.
Cattle (rectal swabs, n=73)	38 (38/73, 52.05%)	22 (22/73, 30.13%)	-
Cattle (mastitic milk, n =4)	-	-	04 (4/4, 100%)
Poultry (n=90)	77 (77/90, 85.55%)	23 (23/90, 25.55%)	-
Ducks (n = 207)	102 (102/207, 49.2%)	13 (13 / 207, 6.2%)	-
Total (n=374) (cloacal swabs / rectal swabs = 370; milk: 04)	**217 (217/370, 58.6%)**	**58 (58/370, 15.6%)**	**04 (4/4, 100%)**

Table 3. Phenotypical biofilm production of *E. coli* isolates

Sample	source	Spices	Biofilm Production
S-27 (C)	Hooghly	Cattle	Weak
S-4(P)	Hooghly	Poultry	Weak
S-5(C)	Hooghly	Cattle	Moderate
S-13 (C)	Hooghly	Cattle	Moderate
S-3 (P)	Hooghly	Poultry	Non
S-29 (C)	Hooghly	Cattle	Moderate
S-19 (P)	Hooghly	Poultry	Weak
S- 8 (P)	Hooghly	Poultry	Moderate
S-7 (C)	Hooghly	Cattle	Weak
S- 5 (P)	Hooghly	Poultry	Weak
S-11 (C)	Hooghly	Cattle	Weak
S-9 (C)	Hooghly	Cattle	Moderate
S-U 3	Hooghly	Poultry	Non
S-10 (D)	Hooghly	Duck	Moderate
S-U2	Hooghly	Poultry	Weak
S-B	Hooghly	Poultry	Strong
S-17 (P)	Hooghly	Poultry	Weak
S- 15 (P)	Hooghly	Poultry	Weak
S- 6 (P)	Hooghly	Poultry	Moderate
S-14 (P)	Hooghly	Poultry	Moderate
GD E 4	North 24 PGS	Duck	Weak
GD E 46	North 24 PGS	Duck	Moderate
GD E 25	North 24 PGS	Duck	Weak
GD E 2	North 24 PGS	Duck	Moderate
B	North 24 PGS	Poultry	Weak
HD E 2	Hooghly	Duck	Weak
GD E 31	North 24 PGS	Duck	Weak
GD E 11	North 24 PGS	Duck	Strong
HD E 6	Hooghly	Duck	Weak
GD E 32	North 24 PGS	Duck	Moderate
GD E 24	North 24 PGS	Duck	Weak
GD E 12	North 24 PGS	Duck	Moderate
GD E 14	North 24 PGS	Duck	Weak
GD E B	North 24 PGS	Duck	Weak
GD E 3	North 24 PGS	Duck	Weak
GD E 1	North 24 PGS	Duck	Moderate
GD E 5	North 24 PGS	Duck	Weak
HD E 3	Hooghly	Duck	Moderate
GD E 9	North 24 PGS	Duck	Weak
GD E 7	North 24 PGS	Duck	Moderate

Table 4. Phenotypical biofilm production of *Salmonella* isolates

Sample	Sources	Biofilm Production
S-29 (C)	Hooghly	Weak
S-17 (C)	Hooghly	Weak
S-9 (C)	Hooghly	Weak
S-21 (C)	Hooghly	non
S-14 (C)	Hooghly	Moderate
S-4 (P)	Hooghly	Weak
S-4 (C)	Hooghly	Weak
S-1 (C)	Hooghly	Weak
S-13	Hooghly	Weak
S-13 (D)	Hooghly	Weak
S-7 (P)	Hooghly	Weak
S-4	Hooghly	Weak
S-18 (P)	Hooghly	Weak
S-7 (P)	Hooghly	Moderate
S-5 (C)	Hooghly	Weak
S- 24 (C)	Hooghly	Weak
S-14	Hooghly	Moderate
S-6 (C)	Hooghly	Weak
S-B	Hooghly	Weak
S-24	Hooghly	Weak
S-37	Hooghly	Weak

Table 5. Detection of antimicrobial resistance (genotypical and phenotypical) and biofilm associated genes in *E. coli* isolates from cattle and poultry

Source	Sample ID	Double disc test		Antimicrobial resistance genes				Biofilm associated gene			
		CEC-CTX	CAC-CAZ	bla_{CTX-M}	bla_{TEM}	bla_{SHV}	bla_{ampC}	*csgA*	*sdiA*	*rcsA*	*rpoS*
Belgachia	S4 D	+	+	+	+	+	-	+	+	+	+
	S10	+	-	-	-	-	+	-	+	+	+
	SI2B	-	-	-	-	-	+	+	+	+	+
	S12A	-	-	-	-	-	+	-	+	+	+
	S2A	+	-	-	-	+	-	-	+	+	+
	S4B	-	-	-	-	-	-	+	+	+	+
	S4C	-	-	-	-	-	-	+	+	+	+
	S4A	-	-	-	+	-	-	-	+	+	+
Shyampur	S1	-	-	+	-	-	+	-	+	+	+
	S35	-	-	-	+	+	+	-	+	+	+
	S21	-	-	-	-	-	+	+	+	+	+
	S26	-	-	-	+	-	+	+	+	+	+
	S28	-	-	+	+	+	+	+	+	+	+
	S24	+	-	+	-	-	+	-	+	+	+
	S23	+	-	+	+	-	+	-	+	+	+
	S25	-	-	-	-	-	+	-	+	+	+
	S32	-	-	-	-	+	+	-	+	+	+
	S20	-	-	-	+	-	+	+	+	+	+
	S31	-	+	-	+	-	+	-	+	+	+
	S21	+	-	-	-	-	+	-	+	+	+
Haripal	S-15 (P)	-	-	-	-	-	-	-	+	+	+
	S-U1	+	-	-	-	-	-	-	+	+	+
	S-16(C)	+	-	-	-	-	-	-	+	+	+
	S-27 (C)	-	-	-	+	-	+	-	+	+	+
	S-U	+	-	+	+	-	-	+	-	+	+
	S-25 (C)	+	-	-	+	-	-	-	+	-	+
	S-20(C)	-	-	+	-	-	+	-	+	+	+
	S-11(C)	+	-	+	-	-	+	-	+	+	+
	S-15(P)	-	-	-	-	-	+	-	+	+	+
	S-B	+	-	-	-	-	-	+	+	-	+
	S-4(P)	-	-	-	+	-	+	-	+	+	+
	S-10 (D)	-	-	-	-	-	+	+	+	+	+

Haripal	S-U3 (C)	-	-	-	+	-	+	-	+	+	+
	S-18 (P)	-	-	-	-	-	+	+	+	+	+
	S-12 (P)	-	-	-	+	-	-	-	+	+	+
	S-9 (C)	-	-	-	-	-	-	+	+	+	+
	S-14 (C)	+	-	-	-	-	-	-	+	+	+
	S-19 (P)	-	-	-	+	-	+	-	+	+	+
	S-5 (P)	-	-	+	-	-	+	-	+	+	+
	S-13 (C)	-	-	-	+	-	+	+	+	+	+
	S-29 (C)	-	-	-	+	-	+	+	+	+	+
	S-7 (C)	-	-	-	-	-	-	-	+	+	+
	S-U2(C)	-	-	+	+	+	+	-	+	+	+
	S-6 (P)	-	-	-	-	-	+	+	+	+	+
	S-22 (C)	-	-	-	-	-	-	+	+	+	+
	S-4 (C)	-	-	+	+	-	-	-	+	+	+
	S-14 (D)	-	-	-	-	-	-	+	+	+	+
	S-15 (C)	-	-	-	-	-	-	-	+	+	+
	S-4 (P)	-	-	-	-	+	+	-	+	+	+
	S-1(C)	-	-	-	+	-	-	-	+	+	+
	S-17 (P)	-	-	+	-	-	-	+	+	+	+
	S-18 (C)	-	-	-	+	-	-	-	+	+	+
	S-3 (P)	-	-	-	-	+	-	-	+	+	+
	S-24 (C)	-	-	-	-	-	-	-	+	+	+
	S-5 (C)	-	-	-	-	-	-	+	+	+	+
	S-25 (C)	-	-	+	+	-	+	-	+	+	+
	S-10 (D)	-	-	-	-	-	+	+	+	+	+
Malbazar	S-1A	-	-	-	-	-	+	+	+	+	+
	S-11B	-	-	-	-	-	+	+	+	+	+
	S-8B	-	-	-	-	-	+	+	+	+	+
	S-2A	+	-	+	-	-	+	+	+	+	+
	S-3B	-	-	-	+	-	+	+	+	+	+
	S-11A	+	-	+	-	-	+	+	+	+	+
	S-9A	-	-	-	-	-	+	+	+	+	+
	S-1B	-	-	-	-	-	+	+	+	+	+
	S-7A	-	-	-	-	-	+	+	+	+	+
	S-7B	+	-	-	-	-	+	+	+	+	+
	S-8A	-	-	-	-	-	+	+	+	+	+
	S-3A	-	-	-	-	-	+	+	+	+	+
Total	**69**	**16**	**2**		**23**	**8**	**44**	**33**	**68**	**67**	**69**

Table 6. Comparative distribution of antimicrobial resistance genes in *E. coli* isolated from different species of animals and birds

Species	bla_{CTX-M}	bla_{TEM}	bla_{SHV}	bla_{AmpC}
Poultry (n=43)	7/43, (16.27%)	5 (5/43, 11.6%)	5 (5/43, 11.6%)	20 (20/43, 46.5%)
Cattle (n=46)	13 (13/46, 28.26%)	13 (13/46, 28.26%)	15 (15/46, 32.6%)	17 (17/46, 36.9%)
Ducks (n=102)	11 (11/102, 10.7%)	0	0	12 (12/102, 11.7%)
Total (n=191)	31 (31/191, 16.23%)	18 (18/191, 9.42%)	20 (20/191, 10.4%)	49 (49/191, 25.6%)

Table 7. Detection of antimicrobial resistance (genotypical and phenotypical) and biofilm associated genes in *Salmonella* isolates

Source	Sample No	Double disc test		Antimicrobial resistance genes			
Belgachia		CEC-CTX	CAC-CAZ	bla_{CTXM}	bla_{SHV}	bla_{TEM}	amp^C
	S1	-	+	-	-	-	-
	S2	-	-	-	-	-	+
	S3	-	-	-	-	-	+
	S4	-	+	-	-	-	-
Shyampur	S13	-	-	+	-	+	+
	S7 (P)	-	-	+	-	+	+

	S14	+	-	**+**	-	**+**	+
	S18	+	-	**-**	-	**+**	+
	S37	+	-	**+**	-	**+**	+
	S6 (P)	+	-	**+**	-	**+**	+
	S 15	-	-	**-**	-	**+**	+
	S 4	+	-	**+**	-	+	+
Haripal	S-14 (U)	**+**	-	**-**	-	-	+
	S-1 (C)	**+**	-	**-**	-	-	-
	S-17 (C)	**+**	-	**-**	-	-	+
	S-5 (C)	**+**	-	**-**	-	-	+
	S-21 (C)	**-**	-	**-**	-	-	+
	S-13 (D)	**-**	-	**-**	-	-	-
	S-4 (C)	**+**	-	**-**	-	-	-
	S-6 (C)	**-**	-	**-**	-	-	-
	S-4 (C)	**+**	-	**+**	-	-	-
	S-29 (C)	**-**	-	**+**	-	-	+
	S-18 (P)	**+**	-	**-**	-	+	+
	S-8 (C)	**-**	-	**-**	-	+	+
	S-4 (P)	**+**	-	**-**	-	+	-
	S-9 (C)	**+**	**+**	**-**	-	+	+
Total	**26**	**14**	**3**	**8**	**0**	**12**	**18**

Table 8. Comparative distribution of antimicrobial resistance genes in *Salmonella* isolated from different species of animals and birds

Species	***bla*$_{CTX-M}$**	***bla*$_{TEM}$**	***bla*$_{SHV}$**	***bla*$_{AmpC}$**
Poultry (n=09)	2 (2/9, 22.2%)	4 (4/9, 44.4%)	0	5(5/9, 55.5%)
Cattle (n=17)	6 (6/17, 35.2%)	8 (8/17, 47.0%)	0	13 (13/17, 76.4%)
Ducks (n=13)	6 (6/13, 46.1%)	0	0	0
Total (n=39)	14 (14/39, 35.8%)	12 (12/39, 30.7%)	0	18 (18/39, 46.1%)

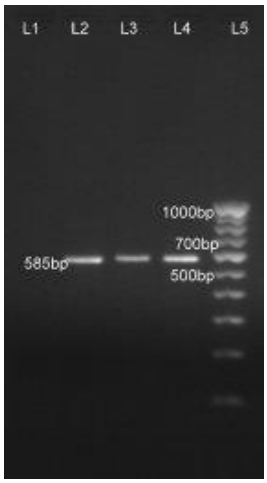


Figure 1. Gel documentation system photograph of PCR products for 16S rRNA gene of *Escherichia coli* isolated from ducks. L1: negative control; L2; L3: representative *E. coli* isolated from ducks; L4: positive control; L5: 100bp ladder

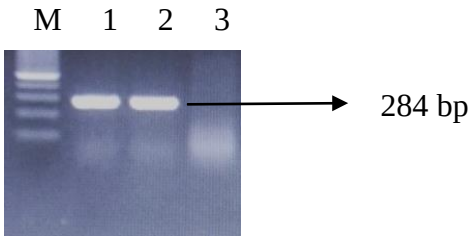


Figure 2. Confirmation of *Salmonella* isolates by *invA* specific PCR. M, 100 bp ladder; lane 1; representative *Salmonella* isolates from ducks; lane 2, positive control; lane 3, negative control

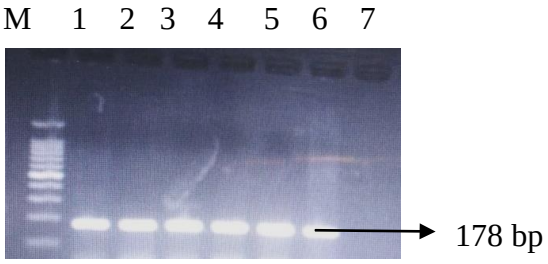


Figure 3. PCR gel documentation image of *E. coli* isolates possessing *csgA*. M: 100 bp ladder; lane 1-5: representative *E. coli* isolates possessing *csgA*; lane 6: positive control; lane 7: negative control

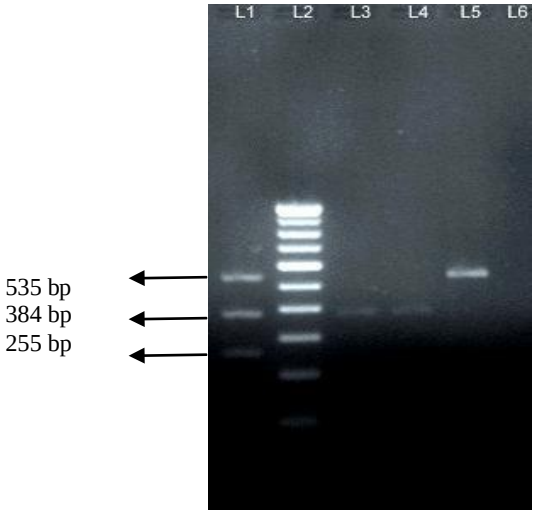


Figure 4 PCR gel documentation photo showing amplification of stx_1, stx_2, *eaeA* in representative *E. coli* isolates from ducks. L1: duck *E. coli* isolate possessing stx_2, *eaeA*, *ehxA*; L2: 100 bp ladder; L3, L4, L5: duck isolates positive for *eaeA* and *ehxA* gene; L6: negative control

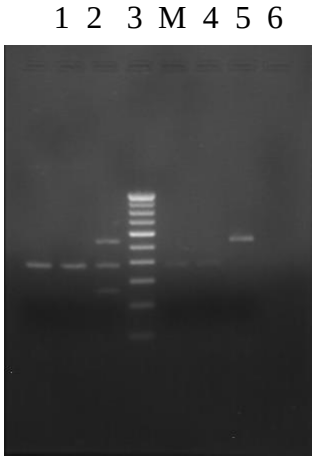


Figure 5. PCR gel documentation photo showing amplification of *eaeA, ehxA* in representative *E. coli* isolates from ducks. L1, L2: duck *E. coli* isolates possessing *eaeA*; L3: duck *E. coli* isolate possessing *stx*$_2$, *eaeA*, *ehxA*; L4: 100 bp ladder; L5, L6: duck *E. coli* isolates possessing *eaeA*; L7: duck *E. coli* isolate possessing *ehxA* gene; L6: negative control

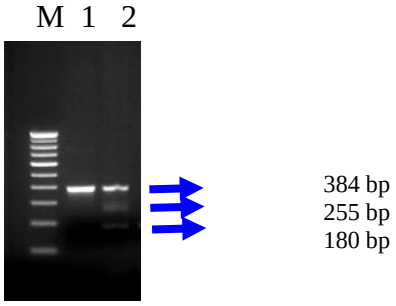


Figure 6. PCR gel documentation photo showing amplification of *stx*$_1$, *stx*$_2$, *eaeA* in *E. coli* isolates from ducks. Lane M, 100 bp DNA ladder; Lane 1, positive control possessing *eaeA* gene, Lane 3, studied O157 *E. coli* isolate from duck

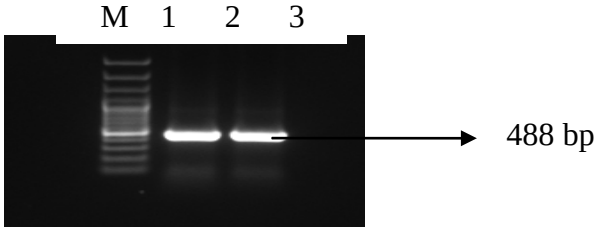


Figure 7. Gel showing PCR amplicon of *sefA* gene of *Salmonella* isolated from ducks. Lane M, 100 bp Ladder, lane 1, representative sample; lane 2, positive control; lane 3, negative control.

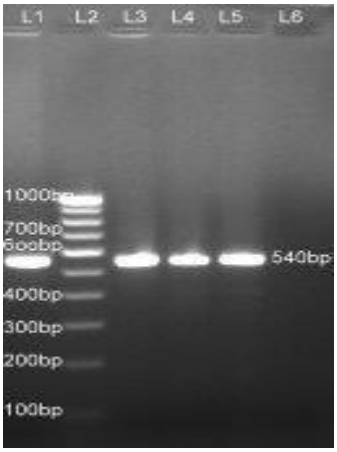


Figure 8. Gel showing PCR amplicon of *bla*$_{CTX-M}$ gene of *E. coli* isolated from cattle. Lane 1: positive control; Lane 2: 100 bp Ladder; lane 3, 4, 5: representative *E. coli* isolates; lane 6: negative control.

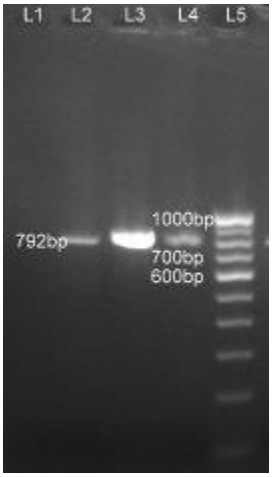


Figure 9. Gel showing PCR amplicon of *bla*$_{SHV}$ gene of *E. coli* isolated from cattle. Lane 1: negative control; Lane 2, 4: representative *E. coli* isolates; lane 3: positive control; lane 5: 100 bp DNA ladder

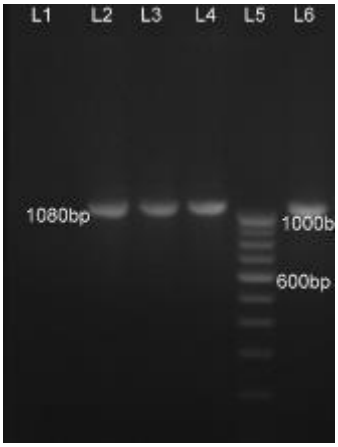


Figure 10. Gel showing PCR amplicon of *bla*$_{TEM}$ gene of *E. coli* isolated from poultry. Lane 1: negative control; Lane 2, 3, 4: representative *E. coli* isolates; lane 5: 100 bp DNA ladder; lane 6: positive control

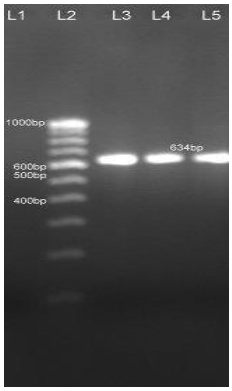


Figure 11. Gel showing PCR amplicon of *bla*$_{AmpC}$ gene of *E. coli* isolated from ducks. Lane 1: negative control; Lane 2: 100 bp DNA ladder; Lane 3, 4: representative *E. coli* isolates; lane 5: positive control

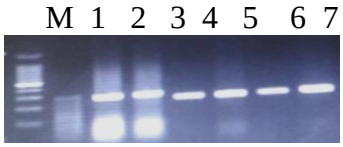


Figure 12. Gel showing PCR amplicon of *sdiA* gene of *E. coli* isolated from ducks. Lane 1: negative control; Lane 2, 3: positive control; Lane 4, 5, 6, 7: representative *E. coli* isolates

C3. Details of Recurring Expenditure: Expenditure statement enclosed.

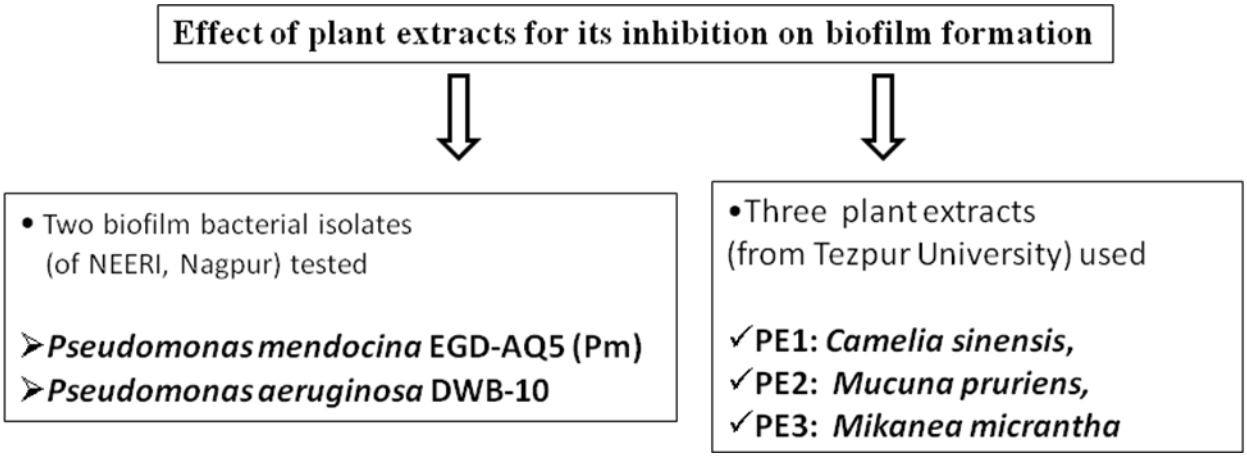
Collaborating Centre-2 at NEERI, Nagpur

Objective fulfilled:

- To study the mechanism of action of bioactive molecule on quorum sensing inhibition and/or anti-biofilm property (NEERI, Nagpur & Tezpur University)
- To study the gene expression analysis of targeted biofilm forming genes (*pel, alg, bdlA*) and transcriptional profile in model biofilm bacterial culture in presence of bioactive molecules (NEERI).

Biofilm INHIBITION activity of Plant Extracts (PE1, PE2, PE3) on *Pseudomonas mendocina* and *Pseudomonas aeruginosa*

At NEERI Nagpur, two biofilm forming bacterial isolates (model bacteria) were tested with three plant extracts (sent by Tezpur University to NEERI Nagpur) for studying antibiofilm property. Bacteria (*Pseudomonas mendocina* and *Pseudomonas aeruginosa*) were grown in 96 well plate in presence of plant extract till 48 hours. After 48 hours, biofilm forming ability of bacteria were analysed by Crystal violet assay using multiplate reader at OD590nm.



Different dilutions of plant extracts (1:1, 1:10, 1:100) were tested with constant size of inoculums (0.01 OD600nm) of model bacteria at 48 hours of incubation. PE 1: *Camelia sinensis* and PE 2: *Mucuna pruriens* were found to be active on increased dilutions in *Pseudomonas mendocina*, while PE 3: *Mikanea micrantha* was effective in 1:1 ratio in both the strains. Effect of PE 2: *Mucuna pruriens* was different in *Pseudomonas mendocina* and *Pseudomonas aeruginosa*. Control used was biofilm bacteria without plant extract.

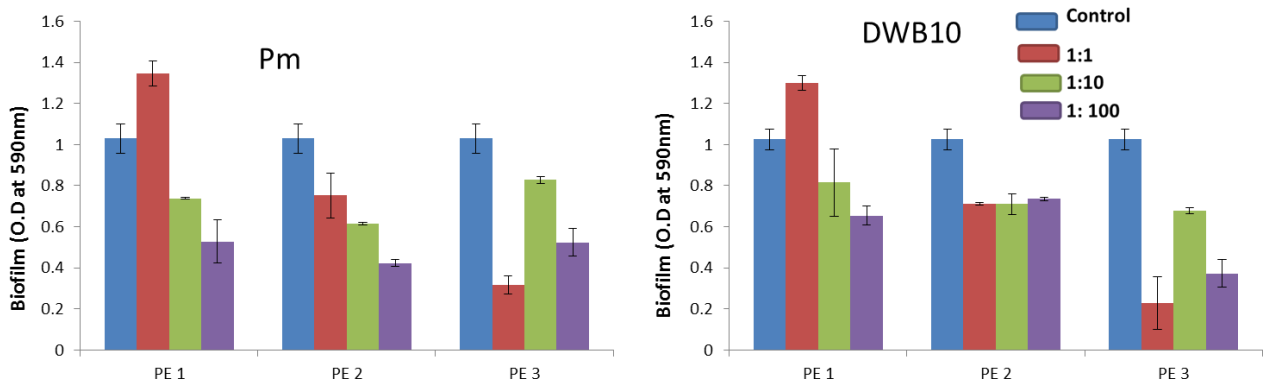


Fig.1: Effect of different concentration of plant extracts on biofilm formation ability by *Pseudomonas* sp PE 1: *Camelia sinensis*, PE 2: *Mucuna pruriens*, PE 3: *Mikanea micrantha*

Biofilm DISPERSIVE activity of Plant Extracts (PE1, PE2, PE3) on *Pseudomonas mendocina* and *Pseudomonas aeruginosa* biofilms

Plant Extracts were also tested for dispersive activity on the preformed biofilms of *Pseudomonas* strains. The strains were allowed to form biofilm in the microtitre plates till 48 hours and then plant extracts of different dilutions (1:1, 1:10, 1:100) were added and allowed to interact for further 24hrs, after which crystal violet assay were carried out. All the three plant extracts (PE1, PE2, PE3) showed dispersion of biofilms formed by Pseudomonas isolates. Dispersion of preformed *P. aeruginosa* biofilms were comparatively significant to preformed biofilm of *P.mendocina* in presence of all the three plant extracts (Fig.2). Control used was biofilm bacteria without plant extract.

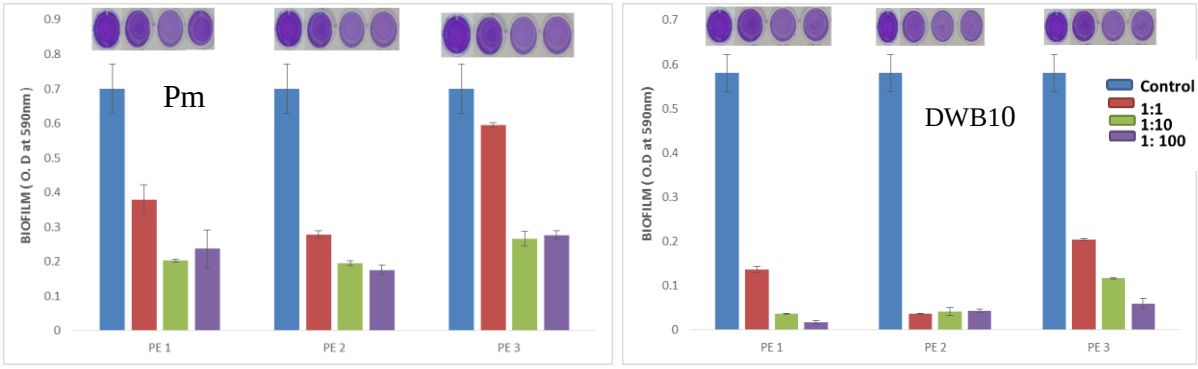


Fig.2: Effect of biofilm dispersive activity of plant extracts on *Pseudomonas* sp
PE 1: *Camelia sinensis*
PE 2: *Mucuna pruriens*
PE 3: *Mikanea micrantha*

Design of Primers for Polymerase Chain Reaction (PCR) for biofilm forming genes:
Primers were designed for analysing biofilm forming genes (*algA*, *pelA*, *bdlA, lasI*) (Table:1)

Table 1: List of primers for biofilm genes analysis

Genes	Primer sequence(5'-3')	Pdt size
icaA (intercellular adhesion gene)	TTTCGGGTGTCTTCACTCTAT CGTAGTAATACTTCGTGTCCC	234bp
lasI(quorum sensing gene)	GCGTGCTCAAGTGTTCAAGG ATTCGCCAGCAACCGAAAAC	246bp
bdlA (biofilm dispersion gene)	CGTATATGCCCGTTTTCGCC TATTCTCCAGCTCTTGCGGC	298bp
pelA (biofilm forming gene) in *Pseudomonas*	CGGCGGCGGTGAAGGAGTT CGGGCACACGCGACCATAGT	243bp

Optimization of PCR using designed primers and Pseudomonas genomic DNA as template:

Gene specific PCR was performed using model bacterial genomic DNA as template. Selected genes were amplified as shown in Fig.3.

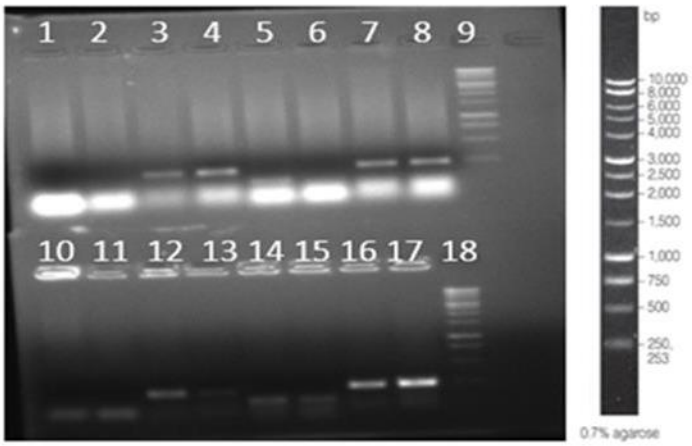


Fig.3: Gene specific PCR of *alg, pelA, bdlA, lasI* in *Pseudomonas mendocina* and *Pseudomonas aeruginosa*

1 kb DNA ladder

LANES	SAMPLES
1	Pm bdlA
2	Pm bdlA
3	Pm pelA
4	Pm pel A
5	Pm las I
6	Pm las I
7	Pm alg
8	Pm alg
9	DNA ladder
10	DWB 10 bdlA
11	DWB 10 bdlA
12	DWB 10 pel A
13	DWB 10 pel A
14	DWB 10 las I
15	DWB 10 las I
16	DWB 10 alg
17	DWB 10 alg
18	DNA ladder

B2. Summary and Conclusions of the Progress made so far (minimum 100 words, maximum 200 words):

- All the three Plant Extracts (PE1, PE2, PE3) could inhibit biofilms in both *Pseudomonas mendocina* and *Pseudomonas aeruginosa.*
- Plant Extract (PE3) could inhibit biofilm formation at 1:1 ratio.
- All the three plant extracts could disperse the preformed biofilms of *Pseudomonas* sp. Biofilm dispersion effect was relatively more on *Pseudomonas aeruginosa* than *Pseudomonas mendocina*
- Biofilm forming genes viz; *pel*A and *alg* genes were amplified in the isolates.

- Expression of genes by real time PCR and transcriptome sequencing of *Pseudomonas aeruginosa* in presence of methanol extract of plant *Mikanea micrantha* are going-on.

B3. Details of New Leads Obtained, if any: Nil

C4. Financial Requirements for the Next Year with Justifications:

Fund may be sanctioned as per the sanction order No. BT/PR16149/NER/95/85/2015 dated 19/01/2017 for smooth execution of the project work.

Publications:

- Rather, M. A., Deori, P. J., Gupta, K., Daimary, N., Deka, D., **Qureshi, A., Dutta, T. K., Joardar, S. N. & Mandal, M.** (2022). Ecofriendly phytofabrication of silver nanoparticles using aqueous extract of *Cuphea carthagenensis* and their antioxidant potential and antibacterial activity against clinically important human pathogens. *Chemosphere*, *300*, 134497. https://doi.org/10.1016/j.chemosphere.2022.134497 (IF 7.086)
- **Rather, M. A.,** Gupta, K., Gupta, A. K., Mishra, P., **Qureshi, A., Dutta, T. K., Joardar, S. N., & Mandal, M**. (2023). Phytochemical Analysis and Demonstration of Antioxidant, Antibacterial, and Antibiofilm Activities of Ethnomedicinal Plants of North East India. *Applied Biochemistry and Biotechnology*, 195, 3257-3294. https://doi.org/10.1007/s12010-022-04273-0 (IF 3.0)
- Rather, M. A., Saha, D., Bhuyan, S., Jha, A. N., & **Mandal, M.** (2022). Quorum quenching: A drug discovery approach against Pseudomonas aeruginosa. *Microbiological Research*, 264 127173. https://doi.org/10.1016/j.micres.2022.127173
- Rather, M. A., Gupta, K., & **Mandal, M**. (2021). Inhibition of biofilm and quorum sensing-regulated virulence factors in *Pseudomonas aeruginosa* by *Cuphea carthagenensis* (Jacq.) JF Macbr. Leaf extract: An in vitro study. *Journal of Ethnopharmacology*, *269*, 113699. https://doi.org/10.1016/j.jep.2020.113699 (IF 5.2)
- Rather, M. A., Gupta, K., Bardhan, P., Borah, M., Sarkar, A., Eldiehy, K. S., ... & **Mandal, M**. (2021). Microbial biofilm: A matter of grave concern for human health and food industry. *Journal of Basic Microbiology*, 61(5), 380-395. https://doi.org/10.1002/jobm.202000678 (IF 3.1)
- Rather, M. A., Gupta, K., & **Mandal, M**. (2021). Microbial biofilm: formation, architecture, antibiotic resistance, and control strategies. *Brazilian Journal of Microbiology*, 52, 1701–1718. https://doi.org/10.1007/s42770-021-00624-x (IF 2.2)
- Rather, M. A., Gupta, K., & **Mandal, M**. (2023). Scope of Plant-Based Nanoparticles as Antibacterial and Antibiofilm Agents. Nanotechnology Horizons in *Food Process Engineering: Food Preservation, Food Packaging, and Sustainable Agriculture*, 257.
- Ghosh S, **Qureshi A**, Purohit HJ (**2019**). D-Tryptophan governs biofilm formation rates and bacterial interaction in *P. mendocina* and *S. aureus*. *J. of Biosciences.* 44 : 3 (ImpactFactor : 1.528)
- S. Chakarborty, P. Roychoudhury, I. Samanta, P.K. Subudhi, Lalhruaipuii M. Das, A. De, S. Bandyopadhayay, **S. N. Joardar, M. Mandal, A. Qureshi and T.K. Dutta** Molecular detection of biofilm virulence and antimicrobial resistance associated genes of Salmonella serovars isolated from pig and chicken of Mizoram, India., Indian Journal of Animal Research, 2019, DOI: 10.18805/ijar.B-3817
- Ghosh S, **Qureshi A**, Purohit HJ (**2018**) Aromatic compounds and Biofilms: regulation and interlinking of metabolic pathways in bacteria. Book Chapter in Springer International Publishing AG
- Mahanti, A., Ghosh, P., Samanta, I., Joardar, S.N., Bandyopadhyay, S., Bhattacharyya, D., Banerjee, J., Batabyal, S., Sar, T.K. and Dutta, T.K., (**2018**) Prevalence of CTX-M- producing *Klebsiella* spp. in broiler, kuroiler, and indigenous poultry in West Bengal state,India. *Microbial Drug Resistance* 24(3): 299-306 (IF: 2.34; NAAS score: 8.34)
- Samanta, A., Mahanti, A., Chatterjee, S., Joardar, S.N., Bandyopadhyay, S., Sar, T.K., Mandal, G.P.,

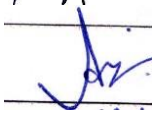
Dutta, T.K., Samanta, I. (**2018**) Pig farm environment as a source of beta- lactamase or AmpC-producing *Klebsiella pneumoniae* and *Escherichia coli*. *Annals of Microbiology* 68(11): 781-791 (IF: 1.4; NAAS score: 7.4)

- Dutta TK, Chakraborty S, Das M, Mandakini R, Vanramhlimpuii, Roychoudhury P, Ghorai S and Behera SK (**2018**) Multidrug-resistant *Staphylococcus pettenkoferi* isolated from cat in India, *Veterinary World*, 11(10): 1380-1384.
- Chakraborty S, Dutta TK, De A, Das M and Ghosh S. (**2018**) Impact of bacterial biofilm in veterinary medicine: An overview. *International Journal of Current Microbiology and Applied Sciences*. 7(4): 3228-3239

(T. K. Dutta, PI, CAU, Aizawl) 

(Manabendra Mandal, PI, Tezpur University, Assam) 

(S. N. Joardar, PI, WBUAFS, Kolkata) 

(A. Qureshi, PI, NEERI, Nagpur) 

[Signature(s) of the Investigator(s)]

Appendix-B

Utilization Certificate

(For the financial year 19th January 2017 to 31st March 2017)

(Rs. in Lakhs)

1.	**Title of the Project/Scheme:**	"Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts obtained from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India; and cattle, poultry and ducks of West Bengal"
2.	**Name of the Organisation:**	Tezpur University, Assam.
3.	**Principal Investigator:**	Prof. Manabendra Mandal.
4.	**Deptt. of Biotechnology sanction order No. & date of sanctioning the project:**	Order No.BT/PR16149/NER/95/85/2015 dated January 19, 2017
5.	**Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given:**	Nil
6.	**Amount received from DBT during the financial year** (*please give No. and dates of sanction orders showing the amounts paid*)**:**	31.28 lakhs No. - BT/PR16149/NER/95/85/2015 dated January 19, 2017
7.	**Other receipts/interest earned, if any, on the DBT grants:**	Nil
8.	**Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):**	**31.28 lakhs**
9.	**Actual expenditure (excluding commitments) incurred during the financial year 19th January 2017 to 31st March 2017 (statement of expenditure is enclosed):**	**Nil**
10.	**Unspent balance refunded, if any** *(Please give details of cheque No. etc.):*	Not Applicable

11.	**Balance amount available at the end of the financial year:**	**31.28 lakhs**
12.	**Amount allowed to be carried forward to the next financial year vide letter No. & date:**	**31.28 lakhs**

1. Certified that the amount of **Rs. Nil lakhs** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 31.28 lakhs** remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1.

2.

3.

4.

5.

(PROJECT INVESTIGATOR)

1.1.4

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix-C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the period from **19th January 2017 to 31st March 2017**

(Rs. in Lakhs)

Item	Unspent balance carried forward from previous year (Rs.)	Grant received from DBT during the year 2016-2017	Other receipt/ interest earned, if any, on the DBT grants	Total of Col. (2+3+4) (Rs.)	Expenditure (excluding commitments) incurred Jan. 2017 to 31st Mar. 2017	Balance (5-6-) (Rs.)	Rema--rks
1	2	3	4	5	6	7	8
(1)Non- recurring							
(i)Equipments	0.00	19.93	0.00	19.93	0.00	19.93	
(2) Recurring							
(i)Human resource	0.00	8.05	0.00	8.05	0.00	8.05	
(ii)Consumables	0.00	2.00	0.00	2.00	0.00	2.00	
(iii)Travel	0.00	0.50	0.00	0.50	0.00	0.50	
(iv)Contingency	0.00	0.50	0.00	0.50	0.00	0.50	
(v)Overhead	0.00	0.30	0.00	0.30	0.00	0.30	
Total	0.00	**31.28**	**0.00**	**31.28**	**0.00**	**31.28**	

(PROJECT INVESTIGATOR)

13.1.21

(FINANCE OFFICER

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Appendix-B

Utilization Certificate

(For the financial year 1st April 2017 to March 31, 2018)

(Rs. in Lakhs)

1.	**Title of the Project/Scheme**:	**"Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts obtained from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India; and cattle, poultry and ducks of West Bengal"**
2.	**Name of the Organisation:**	Tezpur University, Assam.
3.	**Principal Investigator**:	Prof. Manabendra Mandal.
4.	**Deptt. of Biotechnology sanction order No. & date of sanctioning the project**:	Order No.BT/PR16149/NER/95/85/2015 dated January 19, 2017
5.	**Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given**	31.28 lakhs No. - BT/PR16149/NER/95/85/2015 dated January 19, 2017
6.	**Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid)*:**	**Nil**
7.	**Other receipts/interest earned, if any, on the DBT grants:**	**0.72023 lakhs**
8.	**Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):**	**32.00023 lakhs**
9.	**Actual expenditure (excluding commitments) incurred during the financial year 1st April 2017 to 31st March, 2018 (statement of expenditure is enclosed):**	**7.27241 lakhs**
10.	**Unspent balance refunded, if any *(Please give details of cheque No. etc.):***	Not Applicable

11.	**Balance amount available at the end of the financial year:**	**Rs. 24.72782 lakhs**
12.	**Amount allowed to be carried forward to the next financial year vide letter No. & date:**	**Rs. 24.72782 lakhs**

1. Certified that the amount of **Rs. 7.27241 lakhs** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 24.72782 lakhs** remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1.

2.

3.

4.

5.

(PROJECT INVESTIGATOR)

13.1.21

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix-C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the period from **1st April 2017 to 31st March, 2018**

(Rs. in Lakhs)

Item	Unspent balance carried forward from previous year (Rs.)	Grant received from DBT during the year 2017-2018	Other receipt/ interest earned, if any, on the DBT grants	Total of Col. (2+3+4) (Rs.)	Expenditure (excluding commitments) incurred **1st April 2017 to 31st March 2018** (Rs.)	Balance (5-6) (Rs.)	Rema--rks
1	2	3	4	5	6	7	8
(1)Non- recurring							
(i)Equipments	19.93			19.93	0.00	19.93	
(2) Recurring							
(i)Human resource	8.05	0.00	0.00	8.05	4.07147	3.97853	
(ii)Consumables	2.00	0.00	0.00	2.00	1.99283	0.00717	
(iii)Travel	0.50	0.00	0.00	0.50	0.42222	0.07778	
(iv)Contingency	0.50	0.00	0.00	0.50	0.48589	0.01411	
(v)Overhead	0.30	0.00	0.00	0.30	0.30	00	
(vi) Interest	0.00	0.00	0.72023	0.72023	0.00	0.72023	
Total	**31.28**			**32.00023**	**7.27241**	**24.72782**	

(PROJECT INVESTIGATOR)

13.1.24

(FINANCE OFFICER)
Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

Appendix-B

Utilization Certificate

(For the financial year 1st April 2018 to 31st March, 2019)

(Rs. in Lakhs)

1.	**Title of the Project/Scheme**:	**"Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts obtained from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India; and cattle, poultry and ducks of West Bengal"**
2.	**Name of the Organisation:**	Tezpur University, Assam.
2.	**Principal Investigator**:	Prof. Manabendra Mandal.
3.	**Deptt. of Biotechnology sanction order No. & date of sanctioning the project**:	Order No.BT/PR16149/NER/95/85/2015 dated January 19, 2017
4.	**Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given**	24.72782 lakhs No. - BT/PR16149/NER/95/85/2015 dated January 19, 2017
5.	**Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid*):**	6.50 lakhs order no BT/PR16149/NER/95/85/2015 Dated Feb 20, 2019
6.	**Other receipts/interest earned, if any, on the DBT grants:**	**0.53922 lakhs**
7.	**Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):**	**31.76704lakhs**
8.	**Actual expenditure (excluding commitments) incurred during the financial year** 1st April 2018 to 31st March, 2019 **(statement of expenditure is enclosed):**	**6.75382 lakhs**

9.	**Unspent balance refunded, if any** *(Please give details of cheque No. etc.):*	Not Applicable
10.	**Balance amount available at the end of the financial year:**	**Rs. 25.01322 lakhs**
11.	**Amount allowed to be carried forward to the next financial year vide letter No. & date:**	**Rs. 25.01322 lakhs**

1. Certified that the amount of **Rs. 6.75382 lakhs** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 25.01322 lakhs** remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1.

2.

3.

4.

5.

(PROJECT INVESTIGATOR)

13.1.24

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix-C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the period from **1st April 2018 to 31st March, 2019**

(Rs. in Lakhs)

Item	Unspent balance carried forward from previous year (Rs.)	Re-appropriated from previous year amount as per DBT	Grant received from DBT during the year 2018-2019	Other receipt/ interest earned, if any, on the DBT grants	Total of Col. (2+3+4+5) (Rs.)	Expenditure (excluding commitments) incurred **1st April 2018 to 31st March 2019** (Rs.)	Balance (6-7) (Rs.)	Rema--rks
1	2	3	4	5	6	7	8	9
(1)Non- recurring								
(i) Equipment	19.93				19.93	0.00	19.93	
(2) Recurring								
(i)Human resource	3.97853		4.07		8.04853	6.19200	1.85653	
(ii)Consumables	0.00717		1.99		1.99717	0.00	1.99717	
(iii)Travel	0.07778		0.42		0.49778	0.19703	0.30075	
(iv)Contingency	0.01411	0.47	0.02		0.50411	0.36479	0.13932	
(v)Overhead	00	0.25	0.00		0.25	0.00	0.25	
(vi) Interest	0.72023	-0.72		0.53922	0.53945	0.00	0.53945	
Total	**24.72782**	**0.0**	**6.5**	**0.53922**	**31.76704**	**6.75382**	**25.01322**	

(PROJECT INVESTIGATOR)

(FINANCE OFFICER)
Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

Appendix-B

Utilization Certificate

(For the financial year **1st April 2019 to 31st March, 2020**)

(Rs. in Lakhs)

1.	**Title of the Project/Scheme:**	"Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts obtained from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India; and cattle, poultry and ducks of West Bengal"
2.	**Name of the Organisation:**	Tezpur University, Assam.
2.	**Principal Investigator:**	Prof. Manabendra Mandal.
3.	**Deptt. of Biotechnology sanction order No. & date of sanctioning the project:**	Order No.BT/PR16149/NER/95/85/2015 dated January 19, 2017
4.	**Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given**	**25.01322** lakhs No. - BT/PR16149/NER/95/85/2015 dated January 19, 2017
5.	**Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid*):**	Nil
6.	**Other receipts/interest earned, if any, on the DBT grants:**	**0.02309**
7.	**Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):**	**25.03631 lakhs**
8.	**Actual expenditure (excluding commitments) incurred during the financial year 1st April 2019 to 31st March, 2020 (statement of expenditure is enclosed):**	**24.24340 lakhs**

9.	**Unspent balance refunded, if any** ***(Please give details of cheque No. etc.):***	Not Applicable
10.	**Balance amount available at the end of the financial year:**	**0.79291 lakhs**
11.	**Amount allowed to be carried forward to the next financial year vide letter No. & date:**	**0.79291 lakhs**

1. Certified that the amount of **Rs. 24.24340 lakhs** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 0.79291 lakhs** remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1.

2.

3.

4.

5.

(PROJECT INVESTIGATOR)

3.1.24

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix-C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the period from **1st April 2019 to 31st March, 2020**

(Rs. in Lakhs)

Item	Unspent balance carried forward from previous year (Rs.)	Re-appropriated from previous year amount as per DBT	Grant received from DBT during the year 2019-2020	Other receipt/ interest earned, if any, on the DBT grants	Total of Col. (2+3+4+5) (Rs.)	Expenditure (excluding commitments) incurred **1st April 2019 to 31st Mar 2020** (Rs.)	Balance (6-7) (Rs.)	Rema-rks
1	2	3	4	5	6	7	8	9
(1)Non- recurring								
(i) Equipment	19.93	0.00	0.00		19.93	19.90510	0.02490	
(2) Recurring								
(i)Human resource	1.85653	0.00	0.00		1.85653	1.75090	0.10563	
(ii)Consumables	1.99717	0.00	0.00		1.99717	1.98610	0.01107	
(iii)Travel	0.30075	0.00	0.00		0.30075	0.32008	-0.01933	
(iv)Contingency	0.13932	0.00	0.00		0.13932	0.12497	0.01435	
(v)Overhead	0.25	0.00	0.00		0.25	0.15625	0.09375	
(vi) Interest	0.53945	0.00	0.00	0.02309	0.56254	0.00	0.56254	
Total	**25.01322**	**0.0**	0.00	**0.02309**	**25.03631**	**24.24340**	**0.79291**	

(PROJECT INVESTIGATOR)

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Appendix-B

Utilization Certificate

(For the financial year 1st April 2020 to 18th July, 2020)

(Rs. in Lakhs)

1.	**Title of the Project/Scheme:**	**"Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts obtained from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India; and cattle, poultry and ducks of West Bengal"**
2.	**Name of the Organisation:**	Tezpur University, Assam.
2.	**Principal Investigator:**	Prof. Manabendra Mandal.
3.	**Deptt. of Biotechnology sanction order No. & date of sanctioning the project:**	Order No.BT/PR16149/NER/95/85/2015 dated January 19, 2017
4.	**Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given**	**Rs. 0.79291 lakhs** No. - BT/PR16149/NER/95/85/2015 dated January 19, 2017
5.	**Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid*):**	Nil
6.	**Other receipts/interest earned, if any, on the DBT grants:**	**Nil**
7.	**Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):**	**0.79291 lakhs**
8.	**Actual expenditure (excluding commitments) incurred during the financial year 1st April 2020 to 18th July, 2020 (statement of expenditure is enclosed):**	**0.0 lakhs**

9.	**Unspent balance refunded, if any** *(Please give details of cheque No. etc.):*	Not Applicable
10.	**Balance amount available at the end of the financial year:**	**Rs. 0.79291 lakhs**
11.	**Amount allowed to be carried forward to the next financial year vide letter No. & date:**	**Rs. 0.79291 lakhs**

1. Certified that the amount of **Rs. 0.0 lakhs** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 0.79291 lakhs** remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1.

2.

3.

4.

5.

(PROJECT INVESTIGATOR)

13.1.24

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix-C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the period from **1st April 2020 to 18th July, 2020**

(Rs. in Lakhs)

Item	Unspent balance carried forward from previous year (Rs.)	Re-appropriated from previous year amount as per DBT	Grant received from DBT during the year 2020-2021	Other receipt/ interest earned, if any, on the DBT grants	Total of Col. (2+3+4+5) (Rs.)	Expenditure (excluding commitments) incurred **1st April 2020 to 18th July, 2020** (Rs.)	Balance (6-7) (Rs.)	Rema-rks
1	2	3	4	5	6	7	8	9
(1)Non- recurring								
(i) Equipment	0.02490	0.00	0.00		0.02490	0.0	0.02490	
(2) Recurring								
(i)Human resource	0.10563	0.00	0.00		0.10563	0.0	0.10563	
(ii)Consumables	0.01107	0.00	0.00		0.01107	0.0	0.01107	
(iii)Travel	-0.01933	0.00	0.00		-0.01933	0.0	-0.01933	
(iv)Contingency	0.01435	0.00	0.00		0.01435	0.0	0.01435	
(v)Overhead	0.09375	0.00	0.00		0.09375	0.0	0.09375	
(vi) Interest	0.56254	0.00	0.00	0.00	0.56254	0.00	0.56254	
Total	**0.79291**	**0.0**	0.00		**0.79291**	**0.0**	**0.79291**	

(PROJECT INVESTIGATOR)

(FINANCE OFFICER)
Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

FINALCONSOLIDATED STATEMENT OF EXPENDITURE
(FOR FINAL SETTLEMENT OF ACCOUNTS)

1. Title of the Project : **Studies on antimicrobial and anti-biofilm activities of medicinal plant extracts obtained from North Eastern India against pathogenic bacteria isolated from pigs, cattle and poultry of NER India; and cattle, poultry and ducks of West Bengal"**

2. Sanctioned Project Cost : **52.97 lakhs**

3. Revised cost, if any : **Nil**

4. Duration of the project : **3 Y**

5. Sanction Order No. & Date BT/PR16149/NER/95/85/2015 dated January 19, 2017

6. Date of commencement of Project : January 19, 2017

7. Extension, if any : **6 M**

8. Date of completion of project :**18th July, 2020**

Details of grant, expenditure and balance

S. No.	Heads	Sanctioned Cost	Year-wise Releases made					Year-wise Expenditure incurred					
			1st yr	2nd yr	3rd Yr	4th Yr	Total	1st yr	2nd yr	3rd yr	4th yr	Total	Balance
A.	**Non-recurring**												
	Equipments	19.93	19.93	0.00	0.00	0.00	19.93	0.00	0.00	19.90510	0.00	19.90510	0.02490
B.	**Recurring**												
1.	Manpower	24.54	8.05	4.07	0.00	0.00	12.12	4.07147	6.19200	1.75090	0.00	12.01437	0.10563
2.	Consumables	5.00	2.00	1.99	0.00	0.00	3.99	1.99283	0.00	1.98610	0.00	3.97893	0.01107
3.	Travel	1.25	0.50	0.42	0.00	0.00	0.92	0.42222	0.19703	0.32008	0.00	0.93933	-0.01933
4.	Contingency	1.50	0.50	0.02+0.47[a]	0.00	0.00	0.99	0.48589	0.36479	0.12497	0.00	0.97565	0.01435
5.	Overhead	0.75	0.30	0.0+0.25[a]	0.00	0.00	0.55	0.30	0.00	0.15625	0.00	0.45625	0.09375
	Total	**33.04**	**11.35**	**6.5+0.72[a]**	**0.00**	**0.00**	**18.57**	**7.27241**	**6.75382**	**4.3383**	**0.00**	**18.36453**	**0.20547**

10.01.23

	Grand Total (A+B)	**52.97**	**31.28**	**7.22**	**0.00**	**0.00**	**38.5**	**7.27241**	**6.75382**	**24.24340**	**0.00**	**38.26963**	**0.23037[b]**

Note: [a]- amount (0.72) reappropriated from interest generated during 2017-2018; **b**- add an amount of 0.56254 (interest generated during 2018-2019 & 2019-2020) to the balance amount i,e 0.23037+0.56254=0.79291. **So, total unspent balance =0.79291 lakh**

Note: The unspent balance amounting 0.79291 lakhs has been refunded in the account of Refund of Unspent Grant (PAO DBT) through bharatkosh.gov.in with transaction Ref. No. 2807220007549 (INR 23037.00) and 2807220007664 (INR 56254.00) dated Jul 30, 2022

(PROJECT INVESTIGATOR)

25/11/23

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(FINANCE OFFICER) 25/11/23

Finance Officer
Tezpur University