Antibiotic Susceptibility Testing and Antimicrobial Compound Production Potential Study of Bacteria Isolated from *Periplaneta americana*

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Abstract: ***Cockroaches live in dirty environment and they are possible vector to spread pathogenic disease and cause series illness. Another side there are one thought that microflora of cockroaches can also produce some antimicrobial compounds. This study was carried on the microorganisms isolated from the external surface and gut of the cockroaches. 26 cockroaches were collected from the different household areas of Valsad, Gujarat. They were frozen at 0ºC and treated with chloroform for immobilized purpose. They are treated with sterile normal saline for external surface and dissected aseptically for gut microorganisms. Nutrient agar and Tryptic Soy agar were used for isolation. 36 isolates were isolated in which 16 and 20 were obtained from surface and the gut of the cockroaches respectively. Antibiotic susceptibility test was performed by disc diffusion method in which Gentamycin was found 100% effectiveness against isolates. Antimicrobial compound production by isolates was also performed. Out of 9 tested isolates 4 isolates gave good results; confirmed by zone of inhibition against test organisms. Isolate $C_{28}G_{28}$ showed highest effectiveness. Some pigmented colonies were also isolated and pigments were extracted, pigments were used to assess antimicrobial activity. Pigment of $C_{14}S_{14}$ was most potent. This study gave overall idea about antibiotic susceptibility of cockroach Microflora. Moreover antimicrobial compounds, if obtained, may prove effective against pathogens.***

Key Words: Cockroaches surface and gut bacterial flora, Antibiotic Susceptibility, Antimicrobial compound

I. INTRODUCTION

Cockroaches live in the dump and warm places like Kitchen, hotels, restaurant, bakeries, sewage, ship, public latrine where plenty of food is available so mostly, they found at the polluted and dirty places. Cockroaches consume garbage, rotting food, and even fecal waste of other roaches. They then transmit disease to your food, eating utensils, kitchen surface and other area around your home. They can easily contaminate food by leaving droppings which may contain bacteria that can cause food poisoning, fungi and other pathogenic organisms [1-4]. Their feeding mechanisms and filthy breeding habits make them the ideal agents for harbouring and transmitting pathogenic bacteria [5, 6, 7]. Their nocturnal and filthy habits make them also ideal carriers of various pathogenic microorganisms [8,9]. Cockroaches can move freely between human/animal wastes and may play a significant role in the dissemination of drug resistant bacteria to the environment and human beings [10]. So they are the possible vector to spread the pathogenic diseases. Their presence is the sing of poor sanitation and they carry number of bacteria which could rise the series illness which is difficult to treat.

Nowdays the major problem is the multidrug resistant infection which is difficult to treat and cockroaches are potential vector which are responsible to spared MDR infection. Once the particular bacterium become resistant to the particular antibiotic it is difficult to treat that infection and sometime it is impossible to treat. Antibiotic resistant is a major public health global concern, with fears expressed that we shortly could run out of antibiotics [10]. There is need to identified novel antibiotic compounds which are used to treat infection. Cockroaches live in the dirty environment still they can survive in it. Cockroach microflora may have potential to produce some antimicrobial compounds. By extracting these antimicrobial compounds and testing them, we can use this against pathogens. Bacterial pigments also have some antimicrobial property which we can be used to cure infection. Overall goal of this study was to know about the drug sensitivity of bacteria isolated from the cockroaches which may also be responsible for the spread and transmission food borne diseases.

II. MATERIAL AND METHOD

A. Sample collection

Cockroaches leave in the dump and dirty areas. 36 cockroaches were collected from the different household areas of the Valsad district, Gujarat in the clean container. Collected cockroaches were transferred in the sterile test tube.

B. Sample preparation [10]

They were immobilized in sterile test tube by using chloroform. Complete immobilization of cockroaches were done by freezing at 0º C for 5 min. Normal saline was added in the test tubes having the immobilized cockroaches. Tubes were shaken vigorously for 2-3 min to suspend bacteria from its body surface. This was the homogenate suspension to isolate the bacteria. Subsequently, dislodge cockroaches treated with 90% ethanol for 5 min and dried to decontaminated their external surface. Then they were again washed with sterile normal saline to remove traces of ethanol. Then, cockroaches alimentary track was dissected aseptically using sterile dissecting equipment. Dissected gut material homogenized in sterile normal saline and use to isolate gut bacteria.

C. Isolation of Bacteria

External body suspension was streaked on the Nutrient Agar and Tryptic Soy Agar plate. Plates were incubated at 37°C for 24-48 hours. Same procedure was followed for the gut sample of cockroaches. Primary characterization done by colony characteristics and Gram reaction. Different colonies were re-streaked on the Nutrient Agar plate and Tryptic Agar plates in order to purify the isolates.

D. Antibiotic Susceptibility Test

The test was performed for bacterial isolates by using disc diffusion method on Muller-Hinton Agar plates. Different suspensions of the different isolates were swabbed on the MH agar plate. Sterile antibiotic disc were placed on the MH agar plates and incubated at 37 ºC for 24 h. Antibiotic susceptibility testing performed with discs of: Ampicillin (10 mcg), Gentamicin (10 mcg), Ofloxacin (5 mcg), Chloramphenicol (30 mcg), Sulfamethoxazole (300 mcg), Penicillin (10 units), Streptomycin (10 mcg), Vancomycin (30 mcg), Amoxicillin (10 mcg), Erythromycin (15 mcg). After incubation results in the form of zone of inhibition were observed and measured.

E. Antimicrobial activity of produced antimicrobial compounds

Antimicrobial compound production was done using sterile Nutrient broth, which has depleted nutritional value. These broths were inoculated with isolates and incubated at 37ºC for 5-6 days. Then broth was centrifuged in cooling centrifuge at 10°Cat 7000 rpm for 15 min. Supernatant was used to check its antibacterial activity against test organisms like *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Bacillus cereus* by using the agar diffusion method on the Muller-Hinton Agar plate. Sterile MH agar plate were inoculated with test organisms and wells were made with cup borer and filled with appropriate amount (30 µl) of supernatant and plates were kept 4 °C for 30 minutes followed by incubated at 37 °C for 24 h and result in the form of zone of inhibition were observed and measured.

F. Antibacterial activity of Pigments

Sterile Nutrient agar broth containing glycerol was prepared and inoculated with pigmented isolates and incubated at 28 °C for 72 hours. Then broth was centrifuged in cooling centrifuge at 10°Cat 7000 rpm for 15 min. Depending on the water solubility either pellets or supernatant was used for further step. In case of colorful pellet; pellet was washed with solvent and centrifuge until the debris turned colorless. The supernatant containing pigment was allowed to become dry and re-dissolved in solvent to obtain pigment.[11]. Antibacterial activity of extracted pigment was tested by agar well diffusion method against the same test organisms as above. Sterile MH agar plates were inoculated with test organisms,

wells were made with the help of cup borer and filled with appropriate amount (30 µl) of supernatant containing pigment and plates were kept 4 °C for 30 minutes followed by incubation at 37 ºC for 24 h and results were recorded.

G. Isolate identification by 16s rDNA sequencing

DNA was isolated from the culture. Quality of DNA was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16s rRNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 357F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer. The 16s rRNA sequence was used to carry out BLAST against NCBI GenBank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple sequence alignment software program.

III. RESULT AND DISCUSSION

Total 26 cockroaches were collected from the different are of the house hold like: kitchen, toilet, sewage area of the houses from the Valsad district. 36 different colonies were obtained and isolates were purified. Primary characterization was done by the Gram reaction and colony characterization. Number of gram positive and negative isolates obtained from the gut and surface of cockroaches are shown in Table 1.

Table 1: Number of isolates purified from the surface and gut of the cockroaches

Type of Bacteria/ Gram reaction	Surface Bacteria	Gut Bacteria	Total
Gram positive	6	11	17
Gram Negative	10	9	19
Total	16	20	36

A. Results of Antibiotic Susceptibility Test

36 isolates were tested for antibiotic susceptibility test against 10 antibiotics by using disc diffusion method. Table 2 shows the effectiveness of the antibiotics on the isolates. Cockroaches are responsible for the spread of the food borne diseases. So it is important to know antibiotic susceptibility of isolates.

Table 2: Zone of inhibition given by various isolates against the selected antibiotics

Isolates/ Antibiotics	SF	AMP	AMX	P	OF	GEN	E	VA	C	S
	Zone of Inhibition in mm									
C_1S_1	26	24	23	17	32	26	28	16	30	27
C_2S_2	32	37	38	36	31	27	33	21	28	20
C_3S_3	-	-	-	-	-	13	16	25	35	-
C_4S_4	35	39	39	39	39	21	-	24	30	12
C_5S_5	15	-	-	-	19	18	18	15	20	19
C_7S_7	10	-	-	-	17	17	19	15	20	18
C_8G_8	25	40	40	33	27	23	-	27	33	21
C_9G_9	22	19	17	10	33	21	16	-	27	19
$C_{10}G_{10}$	31	33	34	31	36	22	-	33	22	18
$C_{11}S_{11}$	24	18	18	-	22	19	16	-	24	16
$C_{12}G_{12}$	30	22	21	11	28	25	17	-	31	18
$C_{13}G_{13}$	24	-	18	-	-	17	-	-	-	25
$C_{14}S_{14}$	-	-	-	-	30	26	15	-	14	23
$C_{14}G_{14}$	22	30	31	21	33	29	25	-	13	22
$C_{15}G_{15}$	39	33	33	31	33	24	31	23	28	19
$C_{15}S_{15}$	31	28	29	27	30	22	30	18	27	10
$C_{16}S_{16}$	30	23	24	20	24	25	-	13	29	26
$C_{17}S_{17}$	-	16	18	-	17	20	-	26	29	16
$C_{25}G_{25}$	21	16	16	-	35	27	16	-	26	17
$C_{25}S_{25}$	33	39	40	37	-	24	31	20	27	20
$C_{16}G_{16}$	29	-	-	-	31	21	29	-	28	17
$C_{26}G_{26}$	-	13	11	20	23	26	-	10	33	20
$C_{27}G_{27}$	36	27	31	17	40	34	30	33	29	31
$C_{27}S_{27}$	28	32	34	27	29	29	40	33	-	30
$C_{28}S_{28}$	-	22	12	-	18	36	35	33	40	32
$C_{28}G_{28}$	30	29	28	28	28	25	29	18	28	22
$C_{29}S_{29}$	31	22	20	14	29	22	19	-	31	21
$C_{30}S_{30}$	28	21	24	18	30	33	30	28	34	24
$C_{30}G_{30}$	28	28	30	24	34	29	31	34	34	18
$C_{31}G_{31}$	34	29	30	29	31	28	35	21	29	24
$C_{32}G_{32}$	11	32	27	23	15	24	-	23	27	13
$C_{33}G_{33}$	17	27	28	23	34	27	14	23	14	23
$C_{34}G_{34}$	-	-	-	-	-	14	-	-	-	-
$C_{35}G_{35}$	-	31	33	28	18	27	-	28	30	25
$C_{36}G_{36}$	37	15	-	13	27	28	-	-	37	16

[Sulphafurazole- (300mcg), AMP- Ampicillin(10mcg), AMX- Amoxicillin(10mcg), P- Penicillin-G(10 units), OG- Ofloxacin(5mcg), E- Erythromycin(15mcg), C- (30mcg), GEN- Gentamicin(10mcg), VA- Vancomycin(30mcg), S- streptomycin(10mcg)]

As shown in the Table 2 isolate no. C_3S_3, $C_{13}G_{13}$, $C_{14}S_{14}$ and $C_{34}G_{34}$ are resistant to some antibiotics. $C_{34}G_{34}$ isolate was sensitive towards the GEN and gave 14 mm of the zone size and resistant to the rest of the antibiotics. Isolate C_3S_3 was resistant to the SF, AMP, AMX, P, OF and S. In case of $C_{13}G_{13}$ isolate was resistant to the AMP, P, OF, E, VA, C. Isolate C14S14 was resistant to the SF, AMP, AMX, P and VA. These 4 isolates show the maximum resistance towards the antibiotics. Some isolates were

sensitive to the antibiotics but showed few colonies in inhibition zone. This may be due to presence of antibiotic resistant cells in population.

- **Equation for the Antibiotic effectiveness**:

$$\text{Effectiveness of Antibiotic in \%} = \frac{\text{No of isolates which are Sensitive or resistant}}{\text{Total no of isolates}} \times 100$$

Table 3: Results of the comparison of Antibiotic Susceptibility Test of the references studies and study carried out in form of the percentage (%)

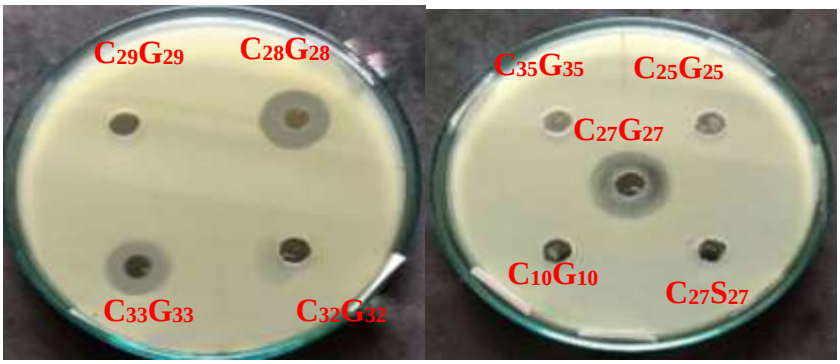
Effectiveness/ Antibiotics	SF (%)	AMP (%)	AMX (%)	P (%)	OF (%)	GEN (%)	E (%)	VA (%)	C (%)	S (%)
SS	80.55	80.55	80.55	64.44	88.88	100	69.44	69.44	91.66	94.44
RS	19.44	19.44	19.44	30.55	11.11	-	30.55	30.55	8.33	5.55
Ref S	78.45	43.83	36.25	29.93	88.50	47.60	66.52	74.52	80.77	74.17
Ref R	21.55	56.16	63.75	70.93	11.50	40.50	33.48	25.96	19.23	25.83

In Table 3: SS- Sensitivity of study carried out, RS- Resistant of study carried out, Ref S- Sensitivity of references data, Ref RS- Resistant of the references data (Collection of references data: [4, 10, 13, 14, 15, 16]

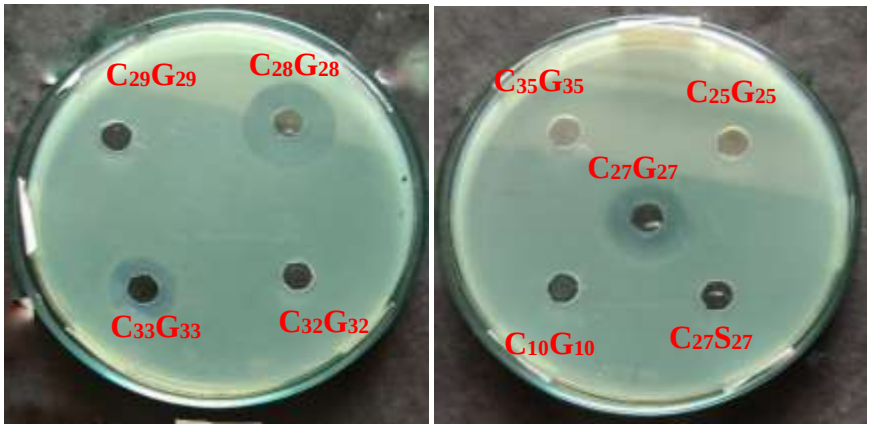
Table 3 shows the comparison of the results between study carried out and the reference studies. As shown in Table 3 results of the study carried out, GEN was effective against all isolates. Another antibiotics C and S both gave 91.44% and 94.44% effectiveness towards isolates respectively. SF, AMP, AMX gave 80.55% effectiveness towards isolates. E and VA were 69.44% effective towards isolates. In the references effectiveness of antibiotics were in series of OF> C > SF > VA > S > E > GEN > AMP > AMX > P. For the study carried out the effectiveness of antibiotics were in series of GEN > S > C > OF > SF = AMP = AMX > E = VA > P. This shows that Antibiotics were more effective and most of the isolates are well sensitive to antibiotics in compare to reference study. So antibiotics are still effective against the microorganisms and infection may be cured by this type of antibiotics. Isolates have not become much more drug resistant. This study provides the information about effectiveness of antibiotic towards microorganisms.

B. Results of Antimicrobial Compound Production

In this total 9 isolates were selected based on the primary study to check their antimicrobial compound production. 4 isolates gave the positive results for the antimicrobial compound production. Zone of inhibition was measured, shown in Figure 2.



Bacillus cereus



Pseudomonas aeruginosa

Figure 1: Results of effect of Antimicrobial compound against test organisms *Bacillus cereus* and *Pseudomonas aeruginosa*

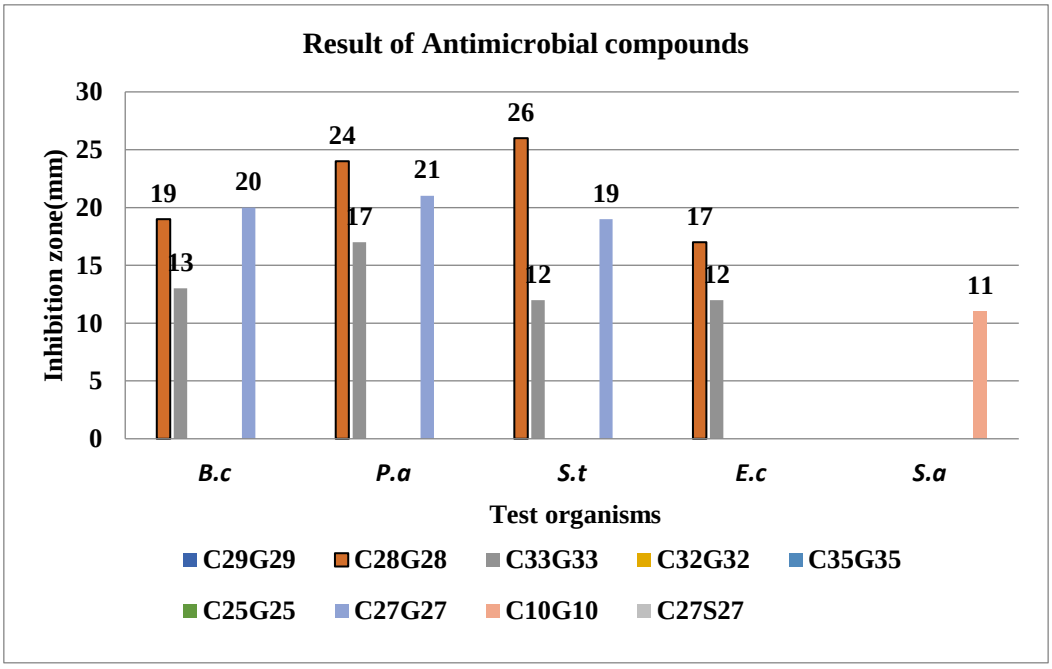


Figure 2: Results of Antimicrobial compound activity against test organisms

From Figure 2 it can be observed that isolates no $C_{28}G_{28}$, $C_{33}G_{33}$, $C_{27}G_{27}$ and $C_{10}G_{10}$ gave positive results for Antimicrobial compound production. In which specially $C_{18}G_{28}$, $C_{33}G_{33}$ and $C_{27}G_{27}$ were effective against

Bacillus subtilis, Pseudomonas aeruginosa, Salmonella typhi and *Escherichia coli*. No isolate except one was able to produce such compounds that were effective against *Staphylococcus aureus*. The isolate $C_{10}G_{10}$ gave inhibition zone against *Staphylococcus aureus* which was 11mm in size. In case of *Bacillus cereus* inhibition zone were given by extracted antibiotic compound from $C_{28}G_{28,}$ $C_{33}G_{33}$ and $C_{27}G_{27}$ which were 19mm, 13mm and 20mm in size respectively. In case of *Pseudomonas aeruginosa* inhibition zone were given by extracted antimicrobial compound from $C_{28}G_{28}$, $C_{33}G_{33}$ and $C_{27}G_{27}$ which were 24mm, 17mm and 21mm in size respectively. In case of *Salmonella typhi* zones were given by extracted antimicrobial compound from $C_{28}G_{28,}$ $C_{33}G_{33}$ and $C_{27}G_{27}$ which were 26mm, 12mm and 19mm in size respectively. In case of *Escherichia coli* zones were given by extracted antimicrobial compound from $C_{28}G_{28}$ and $C_{33}G_{33}$ which were 17mm and 12mm in size respectively. Out of four only one isolate $C_{28}G_{28}$ gave highest effectiveness for antimicrobial compound activity against test organisms. This isolate was further send for the microbial identification using 16s rDNA sequencing in Gene Xpore Diagnostics & Research Center at Ahmedabad.

C. Isolate Identification by 16s rDNA sequencing

Based on the 16s rRNA sequencing the Blast analysis and Phylogenetic analysis were also performed. From the Phylogenetic analysis by using Neighbor-Joining method highest matching was found with NR_117946.1_*Bacillus_amyloliquefaciens*_ strain_MPA_1034_16s_ribosomal_RNA_partial_sequence and NR_112685.1_ *Bacillus_amyloliquefaciens*_strain_NBRC_15535_16s_ribosomal_RNA_partial_sequence which are in Figure 3. From all this results isolate no $C_{28}G_{28}$ wasn found to be *Bacillus amyloliquefaciens.*

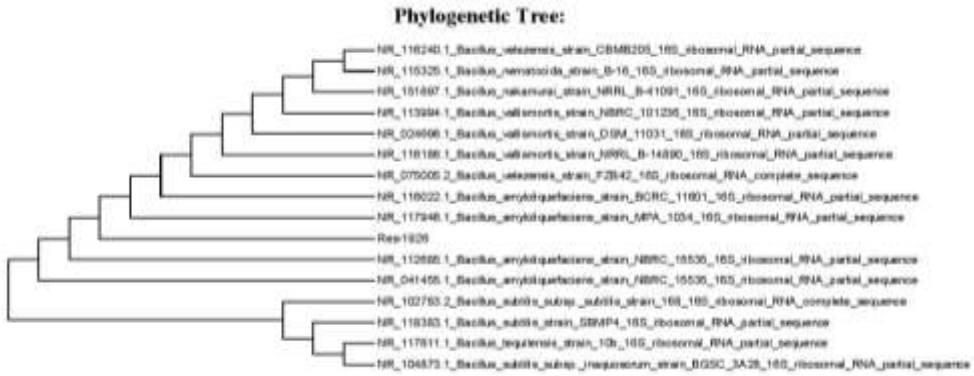
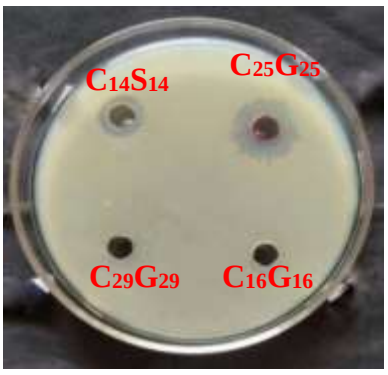


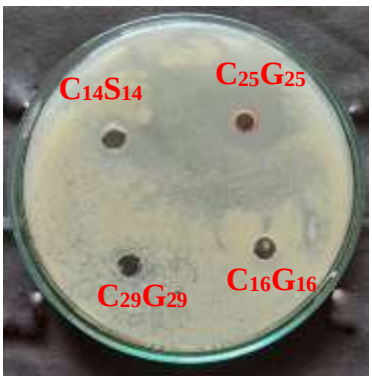
Figure 3: Result of Phylogenetic Analysis of Isolate $C_{28}G_{28}$

D. Results of Antibacterial activity of Pigments

There were 4 isolates which were able to produce pigment. One out of four isolates isolated from the surface and rest three isolated from the gut of the cockroaches. These 4 isolates were: $C_{25}G_{25,}$ $C_{14}S_{14}$, $C_{29}G_{29}$ and $C_{16}G_{16}$. Extracted pigments were used to check antibacterial activity against test organisms.

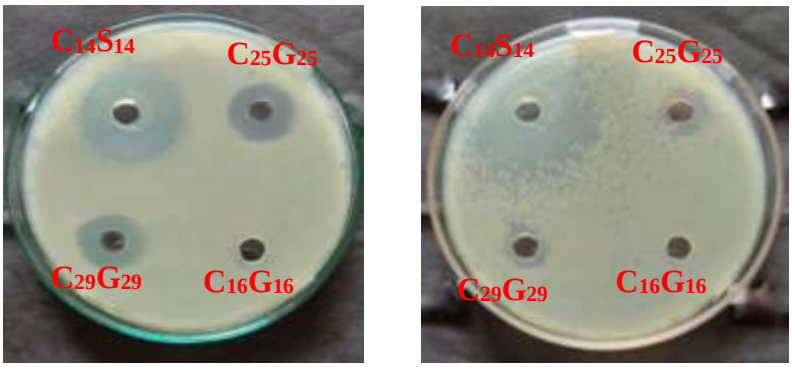


Bacillus cereus



Staphylococcus aureus

Pseudomonas aeruginosa



Salmonella typhi ***Escherichia coli***

Figure 4: Result of Antibacterial actibvity of extracted pigments against test organisms

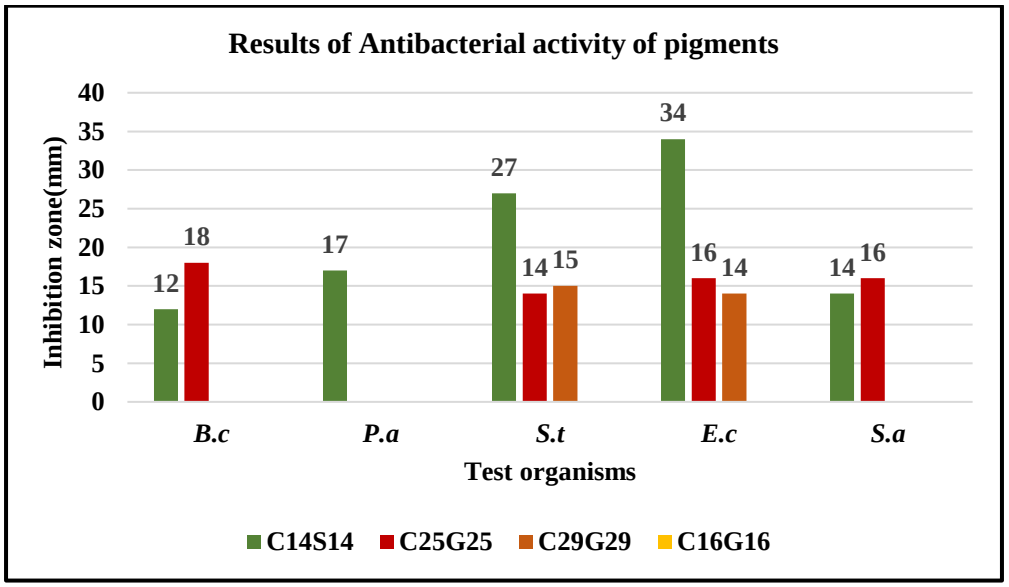


Figure 5: Graphical representation of Antibacterial activity of extracted pigments against test organisms

Figure 5 represents antibacterial activity of extracted pigments against test organisms and the results of zone of inhibition. Out of 4 there were 3 organisms gave the antibacterial activity against test organisms. These 3 isolates were $C_{14}S_{14}$, $C_{25}G_{25}$, $C_{29}G_{29}$ which were effective against test organisms. $C_{16}G_{16}$ was ineffective against test organisms. $C_{14}S_{14}$ was highly effective against all test organisms and gave highest zone against *Escherichia coli* which was 34mm. In case of $C_{25}G_{25}$ the extracted pigment was not effective against *Pseudomonas aeruginosa* test organism and effective against rest 4 test organisms. In case of $C_{29}G_{29}$ extracted pigment effective against *Salmonella typhi* and *Escherichia coli* and gave zone of inhibition 15mm and 14mm respectively.

IV. CONCLUSION

From the above study it can be concluded that antibiotics are effective against isolates isolated from the cockroaches. Gentamycin was 100% effectiveness towards the isolates. Study was compared with references which also shows that most of the selected antibiotics are effective against isolates. Although cockroaches are considered one of the major reasons for spread of food borne infections, infections are capable to be treated with antibiotics. Antimicrobial compounds were successfully tested and highest zone was given by the $C_{28}G_{28}$ isolate. Pigments also have the antibacterial activity and Bacterial pigments are the natural compounds so it can be used as the antibacterial components. This study gave overall idea about antibiotic susceptibility of cockroach Microflora. Moreover antimicrobial compounds, if obtained, may prove effective against pathogens.

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