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Isolation and Identification of Dental caries causing Actinomycetes and antimicrobial activity of *Punica granatum*

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Abstract

All cases were selected from the patients in and around Pudukkottai Dist. This study comprised of 58 cases, ranging in the age from 26 to 58 years having dental caries. Among the total patients 30 were females and 28 were males. The purified colonies grown on Kenknight Muniares medium plates subjected to gram staining to identify the Gram's nature and shape of the bacteria. Antimicrobial activity of leaf Extract of Pomegrante *(Punica granatum*). Assay of antibacterial activity of the leaf extracts were done by Disc diffusion technique. chemical preparation for SDS - PolyArylamide Gel Electrophoresis. Among the total 58 dental caries experienced subjects Actinomyces spp were isolated from 21subjects. High concentrations of pomegranate leaf extract (100,150,200,mg/ml) possessed inhibitory effect against all the 21 isolates of Actinomyces spp while 50mg concentration of the extract showed zone of inhibition less than 7mm in diameter. The protein profile study was conducted for the oral *Actinomyces* spp. isolated from different dental caries patients. There are twenty-one *Actinomyces us* samples were analysed for their protein-banding pattern (Fig. 6.1 to 6.4). All the twenty one organisms developed a single band as a common one with the M.wt. of 9.5 KD. Similarly twelve organisms developed a common band with the M.wt of 97.4 KD. In addition different bands were developed by different isolates of *Actinomyces* spp. with the M.wt. ranging from 32 to 43 KD. Some stains of *Actinomyces* spp.*i.e* strains 5, 11, 15and 18 developed two bands with the M.wt. between 35 and 42KD.

Keywords: *Actinomyces*, Pomegrante *(Punica granatum*) , protein profile.

Introduction

Dental caries is a chronic disease, a process that progresses very slowly in most individuals. The Latin word caries means rottenness. The disease can affect enamel, dentin and cementum. The disease is seldom self limiting and in the absence of treatment caries progresses until the tooth is destroyed. The destruction of the hard tissues often referred to as the 'lesion' is the sign of the disease.

Dental plaque is a general term for the diverse microbial community (Predominantly bacteria) found on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. Plaque develops naturally on teeth, and forms part of the defence systems of the host by helping to prevent colonization of enamel by exogenous (and often pathogenic) microorganism (colonization resistance). Plaque is an example of a biofilm; Current research in showing that the properties of bacteria associated with a surface in a biofilm can be markedly different than those of the same cells growing in liquid broth (planktonic cells). Plaque is found preferentially at protected and stagnant surfaces, and these are at the greatest risk of disease.

Mechanisms of Plaque formation:

The attachment, growth, removal and reattachment of bacteria to the tooth surface is a continuous and

dynamic process. However several distinct processes can be recognized. Adsorption of salivary proteins and glycoproteins together with same bacterial molecules, to the tooth surface to form a conditioning film (the acquired pellicle).

- Long-range (>50nm), non-specific interaction of microbial cell surfaced with the acquired pellicle via Vander walls attractive forces.
- Short range (10-20nm) interactions, in which the interplay of Vander walls attraction forces and electrostatic repulsion produces a weak area of attraction that can result in reversible adhesion to the surfaces.
- Irreversible adhesion can occur if specific inter – molecular inferactions take place between adhesions on the cell surface and reception in the acquired pellicle.
- Seconadary or late colonizers attach to primary colonizers (coaggregation) also by specific inter – molecular interaction.
- Cell division of the attached cells to produce confluent growth and a biofilm.

Plaque composition and structure:

Environmental conditions on a tooth are not uniform. Differences exist in the degree of protection from oral removal forces and in the gradients of many biological and chemical factors that influence the growth of the resident microflora.

These differences will be reflected in variations in the composition of the microbe's community, particularly at sites so obviously distinct as the gingival crevice, approximal regions, smooth surfaces, and pits and fissures. For eg: Fissure plaque will be influenced more by saliva than other sites, where as gingival crevicular fluid (GCF) has a greater impact on plaque in the gingival crevice. This latter site also has a lower redox potential (Eh) and is colonized by higher number of anaerobes, especially proteolytic species which obtain key growth factors from the catabolism of host proteins and glycol proteins in GCF. Plaque structure has been studied mainly by electron microscopy. A heterogenous and a colonial type of sub-structure have been observed in sections of smooth surface plaque.

Microbial Interactions and succession in plaque :

In a biofilm such as dental plaque, microorganisms are in close proximity to one another and interact as a consequene. These interactions can be beneficial, while others can be antagonistic. Microbial metabolism with in plaque will produce gradients in factors affecting the growth of other species, including the depletion of essential nutrients with the simultaneous accumulation of toxic as inhibitory by products. These gradients lead to the development of vertical and horizontal stratification within the plaque biofilm. Such environmental heterogeneity enables organisms with widely differing requirements to grow, and ensures the coexistence of species that would be incompatible with one another in a homogenous habitat.

Beneficial interactions include the concerted action of two or more species to metabolize host macromolecules, such as mucin (individual species are unable to catabolise such molecules). The development of food chains (eg. Lactate consumption by *veillonella* spp)., and coaggregation . Antagonistic interactions include the production of inhibitary substances such as bacteriocins H_2O_2 and organic acids.

Early colonizers of the tooth surface are mainly *Neisseria spp* and *Streptococci*. The growth and metabolism of these pioneer species changes local environmental condition. (Eg. Eh, pH, Co aggregation, Substrate availability) there by enabling more fastidious organisms to colonize. E.g. : obligate anaerobes tend to be late colonizers in plaque. Only able to grow once favorable gradients in O_2 or Eh have developed in the biofilm.

Plaque develops naturally on teeth and gives benefit to the host by providing colonization resistance. Once established at a site, the plaque flora remains relatively stable with time despite regular environmental challenges. This stability (Microbial homeostatic) is not due to any metabolic indifference by the resident micro flora but is due to a dynamic balance being established among the resident member of this microbial community.

On occasions, homeostasis breaks down and imbalances in the micro flora can occur which predispose a site to disease for example the repeated intake of fermentable sugar in the diet produces frequent conditions of low pH in plaque which inhibits the growth of many of the species associated with dental health, and selects for the highly acidogenic (acid – producing) and aciduric (acid-loving) species, such as *Mutans sterptococci* and *Lactobacilli*, associated with dental caries.

In periodontal diseases, these is a shift in the competition on the plaque microflora to a more proteolytic gram negative anaerobic community, which can induce damage to tissues either directly via the "Side–effects" of an inflammatory host response or directly by the production of protease, cytotoxins and other virulence factors.

Cross -Sectional and longitudinal studies of a range of patient groups have shown a lesion in humans. This association is strongest for fissure and rampant caries; the evidence for approximal surface is less strong. Possibly due to problems of sampling plaque and diagnosing lesions at this inaccessible site. However, lesions can be detected at all sites in the apparent absence of *Mutans* streptococci, while these bacteria can be present at a site at relatively high levels without evidence of demineralization, suggesting that the disease doesnot have a absolute specificity interms of microbial aetiology.

Root surface caries was originally associated with *Actinomyces spp* but recent studies suggest a similar action to enamel caries, namely *Mutans streptococci* and *Lactobacilli*, with possibly a role for *A. naeslundii*.

Actinomyces species are classified as anaerobe, Gram positive and filamentous bacteria despite their fungal and bacterial characteristics. This microorganism possesses a low-grade virulence and develops a raprophytil infection programming into the tissues in susceptible hosts. Most of the species isolated from actinomycotic lesions have been identified as *A.israelli A. Viscosus, A. Odontolytisus, A. naesllundi or A.meyeri*. These microorganisms have been identified in dental plaque, dental or periodontal problems, trauma and following oral surgical procedures.

Actinomycosis is an infectious disease that frequently has chronic granulomatous and suppurative lesions caused by saprophytic actinomyces species that are part of oral flora. Intra oral infections are relatively rare and usually are accompanied by the cervicofacial type. Lesions in oral cavity frequently involve the mandible, tongue, lips and oral mucosa.

Measurement of Dental Caries :

The DMF index, which is a record of the number of Decayed, missing and filled teeth (DMFT) or surface (DMFS) in permanent teeth was first described by klewin and palmer (1937) and has now gained global acceptance. Fox deciduous teeth, because of the difficulty in distinguishing between teeth extracted for caries and natural exfoliation, especially in children aged over to years, variations such as the def (decayed, indicated for extraction, filled) and df (decayed filled) have been used (Burt and Eklund, 1992).

The major advantage of DMF index is that, because of its widespread use world wide over the past 60 years, it provides a reasonably accurate historical account of charges in the prevalence of dental caries. The limitations of the DMF index have been widely recognized. The disadvantage is that the DMF index does not take account of the number of teeth or surfaces at risk in difference subjects.

The missing (M) component also present problems amongst adults, for whom the reason for missing teeth is not easy to establish. Perhaps the major difficulty with the DMF index new – a- days is the fact that in many studies. The f component deminate the stores, filling have been inserted by dental professionals who vary enormously in their decisions on when to intervence in the caries process and place a filling. Despite these other limitations, however the traditional DMF index is recommended for continued use in epidemiological studies of dental caries (pine 1997).

Antibacterial activity of Pomegrante leaves:

The antibacterial activity of leaf of *Punica granatum* was investigated. Different solvents used were water, ethanol, methanol, acetone, propanol,1-4-dioxan,N, N-Dimethylformamide(DMF) and Benzyl alcohol. The selection of solvents was on the basis of their polarity. The antibacterial activity of six clinical strains (*S.paratyphi,S.aureus, S.epidermis, E.aerognes*, and *B.subtilis*) was determined by Growth inhibition using Agar ditch diffusion assay. The aqueous extract was able to inhibit only *B.subtilis* and *S.aureus* and was ineffective against all the other four bacterial strains. On the other hand organic solvents proved much better in inhibiting the studied bacterial strains except benzyl alcohol extract which was ineffective against all tested bacterial strains. Among the various solvents, 1-4, Dixon proved to be best while propanol was least effective. Finally, *E.aerogenes* was found to be most resistant bacteria while *S.aureus, S.epidermidis* and *B. subtilis* were most susceptible.

Pomegranate Food Value and Uses

The pomegranate is known to be a powerful antioxidant, natural phytoestrogen, and rare plant based sources of omega 5 fatty medicinal properties of the Pomegranate, and in particular Pomegranate seed oil, have resulted in a number of clinical studies:

Antioxidant and eicosanoid enzyme inhibition properties of Pomegranate seed oil and fermented juice flavonoids. The antioxidant and eicosanoid enzyme inhibition properties of pomegranate (*Punica granatum*) fermented juice and seed oil in.

Chemo preventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer pomegranate. Seeds contain an oil of which about 80% is a rare trans 18 carbon fatty acid.

Materials and Methods

Collection of samples:

All cases were selected from the patients in and around Pudukkottai Dist. This study comprised of 58 cases, ranging in the age from 26 to 58 years having dental caries. Among the total patients 30 were females and 28 were males.

Caries was recorded at present when there was a cavity, undermined enamel or detectably softened floor or wall. Qualified subjects had no chronic disease and not received any antibiotic therapy for at least 3 weeks. Dental data of each subject was recorded.

Plaque samples were collected from each patient with the tips of sterile dental probes. The sample was collected from sites nearer to the carious lesions. Care was taken that plaque samples were not contaminated by saliva. Plaque samples sites varied depending on the conditions of the oral cavity.

Media preparation for the isolation of Actinomycetes:

Kenknight Munaier's medium is used for the isolation and enumeration of oral Actinomycetes. The medium was frequently agitated and boiled for 5 minutes for complete dissolving of the powder. Then the media was autoclaved at 121 °C for 15 minutes.

Inoculation of samples:

The plaque samples collected from patients were immediately transferred to the prepared plates by making a single streak across the center by using a sterile inoculation loop. The inoculum was then spread evently at right angle to the primary streak (Koneman et al.,1997). All the plates were incubated at 28° C for 72 hours.

Morphological identification:

Actinomycetes were identified from plaque samples on the basis of colony morphology and by microscopic examination(Brown,1973).

Purification of Actinomycetes:

Each single colony was transferred to 5ml of nutrient broth(Hi-media) and allowed to grow for 72 hours. A portion was used Gram's staining. After confirmation of the culture purity the second portion of broth was streaked on Kenknight Munaier's medium plates and were incubated at 28°C for 72 hours (Fure *et al*.,1987).

Gram's Staining:

The purified colonies grown on Kenknight Muniares medium plates subjected to gram staining to identify the Gram's nature and shape of the bacteria.

Bio- Chemical tests:

Catalase test:

Catalase test was performed on all the purified isolates to identify the genus Actinomyces. With the help of sterile wooden stick, an isolated colony was transferred to class slide and few drops of 3 % H_2O_2 was added to observe the presence or absence of effervescence.

Lactose fermentation:

Fermentation medium was prepared and the isolated colonies were inoculated in the fermentation medium. All the tubes were incubated at 37°C for 24-48 hours.

Antimicrobial activity of leaf Extract of Pomegrante *(Punica granatum)*

Collection of leaves:

The infection free leaves of Pomegranate were collected from the campus of J.J.College of Arts and Science, Pudukkottai, Tamilnadu.

Preparation of leaf extract by Soxhlet apparatus

The collected leaves were washed and shade dried.

Shade dried leaves were powdered by using morter and pestle.
60g of powdered sample was used for extraction with 100ml of deionized distilled water in a soxhlet apparatus.
Thus collected extracts were concentrated and stored at 4°C until further use (Caceres *et al*., 1995).

Preparation of Sterile antibiotic disc:

The extracts of leaf samples were incorporated into sterile disc, which were prepared 5mm in diameter of whattman No.1 filter paper by using punching machine.
Each sterile disc was incorporated individually with 50,100 and 150 µl of the extract using micropipette.
This can be achieved by adding small quantity of extracts and the disc were allowed to dry in laminar airflow chamber.
Another dose of the extract was applied to already prepared disc.
Control was maintained by adding distilled water on the discs.

Preparation of cultures for the assay:

10ml of nutrient broth was prepared in the different test tubes.
These were cotton plugged and sterilized.
The tubes were labeled according to the type of Actinomycetes culture to be inoculated.
The nutrient broth was inoculated with the Actinomycetes under aseptic conditions.
The tubes were incubated at 37°C for 24 hours.

Assay of antibacterial activity :

Assay of antibacterial activity of the leaf extracts were done by Disc diffusion technique.

Disc diffusion method: (Kirby- Bauer *et al*.,1960)

Sterile Muller Hinton agar medium was prepared in petriplates
After solidification, the test Actimnomycets cultures were inoculated by means of swab method using sterile cotton swab.
The prepared leaf extract discs were placed on the surface of the plates of seeded bacterial cultures.
Control (distilled water)was also maintained.
The plates were incubated at 37°C for 24 hours.
After incubation the zone of inhibition was measured and expressed as mm in diameter (Astal *et al*., 2005; Shihchin *et al*., 1990 and Clark *et al*., 1981).

Procedure and chemical preparation for SDS - PolyArylamide Gel Electrophoresis:

Electrophoresis is the movement of net charged molecules in an electric field. When proteins are separated on polyacrylamide, the procedure is called polyacrylamide gel electrophoresis. When SDS is used with the process, the procedure is abbreviated as SDS-PAGE.

SDE-PAGE first described by Shapiro *et al*., (1967) and Weber and Osborn in 1969 is one of the most widely used techniques for the analysis of proteins based on their molecular weights. Electrophoresis of proteins in polycrylamide gel has proved to be one of the most useful analytical and preparative techniques. A polycrylamide gel electrophoresis technique has the advantage of

High resolution
Sensitivity and
Is simple to carry out

A gel is formed by free radical polymerization of acrylamide (CH_2=CH-CO-NH_2) and a suitable cross linking agent normally, N, N- metheylene bisacrylamide (CH_2 (NHCOCH-CH_2)$_2$ in the presence of an initiator ammonium per sulfate (APS) and catalyst TEMED (Tetra Ethyl Methylene Ethylene Diamine) A chain reaction in initiated in the presence of free radicals generated by APS where monomers of acryl amide are polymerized into long chains in a head to tail fashion. Occasionally, a cross linker is incorporated into the growing chain providing additional sites for polymerization, The chains become cross-linked to feem a gel whose porosity is determined by the degree of cross-linking. Thus, the gels are neutral, hydrophilic, three-dimensional network of acryl amide and bisacrylamide.

The porosity of the gel can be adjusted by varying the concentration of acryl amide and the degree of cross-linking. Electrophoresis on polyacrylamide gels in the anionic detergent SDS (Sodium dodecyl sulfate (CH_3 (CH_2) $10CH_2OSO3Na$) is a rapid and often employed technique you the determination of molecular weights of proteins. The usefulness of this procedure for accurate molecular weight determinations depends upon two factors.

Proteins in general bind constant amounts of SDS per gram (1.4g SDS/g protein) when saturated. The protein then has an overall negative charge that masks its intrinsic charge, resulting in a constant charge to mans ratio of proteins.
Proteins saturated with SDS take on a rod like configuration, the length of the structure being proportional to its polypeptide chain length, and thus its molecular weight.

Procedure:

Cleaned and air dried glass plate and spacers were assembled together with bulldog clips. While petroleum jelly or 2% agar was applied around the edges of the plates to prevent any leakage of liquid.

Sufficient volume of separating gel (30ml) was prepared by mixing the following

30% Acryl amide – 10ml
Lower Tris – 7.5ml
Distilled water – 12.3ml
APS - 150µl
TEMED - 20µl were added.

The mixture was gently mixed and poured immediately into the chamber between the glass plates.
A layer of distilled water were added to obtain a leveled gel and allowed the gel to set for 30-60 minutes.

The water was decanted from the top of the gel and washed with a little volume of stacking gel solution. The stacking gel mixture was poured and the comb was placed and it is allowed to set.

After polymerization, the comb was removed carefully and the lower spacer was removed. The slab was placed carefully with the well slide yacing the to buffer tank after removing the clips. It was connected to the power pack.

Sample preparation:

The Sample with the required protein content was taken and mixed with equal volume of 1X sample buffer corhalf the volume of 2X sample buffer in sterilized microfuge tubes and boiled for s minutes in a boiling water bath. It is cooled at room and then used for loading.

After loading the power pack was connected to the electrodes. When the sample had moved through the stacking gel the power supply was increased to 50 volts to complete the run.

After the run was completed, the gel was carefully removed between the plates and were placed in a staining solution for 4 hours to overnight with intermittent shaking. The proteins absorb coomassive brilliant blue.

The gel was transferred to a suitable destainer with at least 200-300ml of destaining solution I and slowly shacked for 30 minutes. Then the gel was transferred to destaining solution II. This solution was changed in time and again till the background of the gel was clear.

Storage of gel was done by adding 1% glycerol with destaining solution.

Estimation of protein by Lowry's method:

Lowry's method is used to estimate the amount protein present in the given sample.

Procedure:

Extraction of proteins:

5ml of 10% TCA was added in 1ml of sample in a centrifuge tube.
It was centrifuged at 2000 rpm for 10 minutes.
The pellet was decanted and the supernatant was collected and stored at 4 °C.
From the supernatant o.2ml to 1 ml of sample was taken in a series of test tube (concentration ranging from 50- 250µg) and made up to 4ml with distilled water and 5.5ml of alkaline $CuSO_4$ was added.
It was kept in boiling water bath for 10 minutes.
Then 0.5ml of Folin's phenol reagent was added in all the tubes and incubated for 30 minutes at room temperature.
Then the O.D is taken at 650nm.

Calculation:

Amount of protein present in per ml of sample.

= Test O.D / Std O.D X Conc. Std

The O.D values were plotted in the graph. From this, the amount of protein can be calculated.

Results and Discussion

Total recovery of *Actinomyces* spp

Among the total 58 dental caries experienced subjects Actinomyces spp were isolated from 21subjects.Therefore the total percentage of isolation from plaque samples were 36.20%and were illustrated in Table 1.

Measurement of Decayed,Missing and Filled Teeth (DMFT)

The average DMFT value in males were found to be 4.6 and in case of females were 5.Table1 represents the DMFT value of each of each subject.

Colony Morphology

After 72 hours of incubation Actinomyces spp were identified based on the colony morphologies. White mucoid colonies and branched colonies appeared on Kenknight Munaier's medium which were illustrated in fig 1and fig 2.

Gram staining

All Actinomyces spp were subjected to gram staining showed typical filamented Gram-positive cells. They were illustrated in fig 3

Biochemical results of Actinomyces spp

All the samples were subjected to lactose fermentation and catalase test, which showed positive result for lactose fermentation and negative result for catalase test which were illustrated in Table 2and fig 4 and 5

Protein profile

The protein profile study was conducted for the oral *Actinomyces* spp. isolated from different dental caries patients. There are twenty-one *Actinomyces us* samples were analysed for their protein-banding pattern (Fig. 6.1 to 6.4). All the twenty one organisms developed a single band as a common one with the M.wt. of 9.5 KD. Similarly twelve organisms developed a common band with the M.wt of 97.4 KD. In addition different bands were developed by different isolates of *Actinomyces* spp. with the M.wt. ranging from 32 to 43 KD. Some stains of *Actinomyces* spp.*i.e* strains 5, 11, 15and 18 developed two bands with the M.wt. between 35 and 42KD.

The study indicates that each *Actinomyces* spp. developed its own protein-banding pattern on SDS polymerase gel electrophoresis. The difference in banding pattern may be due to the difference in the strain specificity of the individual organisms. *Actinomyces* strains one, three, seven, nine, ten, nineteen and twenty developed the same banding pattern, indicates that all the seven organisms were the same strains. Similarly *Lactobacillus* strains 2, 8, 12, 13, 17 and 21 developed the same banding protein pattern in SDS-PAGE gel electrophoresis indicates that these six falls into the same *Actinomyces* strain type. Similarly strain no. 5 and 18 and 11 and 15 also developed a same banding pattern. All the other strains produced some new bands of their own or lack of some bands present in other strains. Though the banding pattern was same in some strains, the difference in the intensity was also observed in this study. This may be due to the difference in the protein content of different isolates of *Actinomyces* spp. from different dental caries patients.

From this study it is evident that different strains of *Actinomyces* spp. are involved in the caries. The difference in the strain specificity may be due to the environmental influences of the local habit and were illustrated in Table .3

Antibiotic sensitivity test

All the isolates were sensitive to the tested antibiotics such as Penicillin, Cefazolin, Cephotaxime, Ampicillin, Erythromycin, Vancomycin.Among them Penicillin showed the highest zone of inhibition while, vancomycin was the lowest. The susceptibility of Actinomyces spp to the tested antibiotics were illustrated in Table 4.

Antibacterial activity of leaf extract of Pomegranate (Punica granatum)

High concentrations of pomegranate leaf extract (100,150,200,mg/ml) possessed inhibitory effect against all the 21 isolates of Actinomyces spp while 50mg concentration of the extract showed zone of inhibition less than 7mm in diameter. The results were illustrated in Table 5.

Table: 1 Determination of Actinomyces spp. in plaque sample

Sample	Gender	Age (Year)	DMFT	Plaque
1	F	26	5	+
2	F	24	8	+
3	F	28	3	-
4	M	30	2	+
5	F	46	5	+
6	F	47	4	-
7	M	36	6	+
8	M	39	7	-
9	F	44	4	+
10	M	28	5	-
11	M	32	4	+
12	F	35	4	+
13	M	38	5	+
14	M	42	4	-
15	F	51	4	+
16	M	50	3	-
17	F	35	3	+
18	M	55	2	+
19	F	42	8	-
20	F	53	5	-
21	F	54	6	+
22	M	55	4	-
23	M	35	3	-
24	F	28	2	+
25	F	53	8	-
26	F	41	5	-
27	M	34	4	-
28	F	30	2	-
29	F	31	3	-
30	M	42	8	+
31	F	45	6	-
32	M	52	6	+
33	F	38	4	-
34	F	56	7	-
35	M	44	5	+
36	F	28	4	-
37	M	26	4	-
38	F	29	3	-
39	M	41	3	-
40	F	45	6	+
41	M	33	8	-
42	M	38	4	-
43	F	42	5	+
44	M	51	4	+
45	F	48	4	-
46	M	49	3	-
47	M	55	6	-
48	F	38	7	+
49	M	56	6	-

50	F	27	5	-
51	M	58	6	-
52	F	46	5	-
53	F	32	5	-
54	M	28	4	-
55	M	35	3	+
56	F	42	3	-
57	M	51	2	-
58	M	29	4	-

Table : 2 Biochemical results of Actinomyces spp isolated from plaque sample

S.No	Plaque sample	Presence of colonies on agar	Lactose fermentation	Catalase test
1		Detected	+	-
2		Detected	+	-
3		Not detected	-	
4		Detected	+	-
5		Detected	+	-
6		Not detected		
7		Detected	+	-
8		Not detected		-
9		Detected	+	-
10		Not detected		
11		Detected	+	-
12		Detected	+	-
13		Detected	+	-
14		Not detected		
15		Detected	+	-
16		Not detected		
17		Detected	+	-
18		Detected	+	-
19		Not detected		
20		Not detected		
21		Not detected	+	-
22		Not detected		
23		Not detected		
24		Detected	+	-
25		Not detected		
26		Not detected		
27		Not detected		
28		Not detected		
29		Not detected		
30		Detected	+	-
31		Not detected		
32		Detected	+	-
33		Not detected		
34		Not detected		
35		Detected	+	
36		Not detected		
37		Not detected		

38		Not detected		
39		Not detected		
40		Detected	+	-
41		Not detected		
42		Not detected		
43		Detected	+	-
44		Detected	+	-
45		Not detected		
46		Not detected		
47		Not detected		
48		Detected	+	-
49		Not detected		
50		Not detected		
51		Not detected		
52		Not detected		
53		Not detected		
54		Not detected		
55		Detected		
56		Not detected		
57		Not detected		
58		Not detected		

Table –3 Extraction of protein by Lowry's method

Sample No.	Optical density at 650nm	Amount of protein mg/ml
1	0.026	0.185
2	0.025	0.178
4	0.031	0.221
5	0.027	0.192
7	0.026	0.221
9	0.024	0.171
11	0.032	0.228
12	0.028	0.2
13	0.027	0.192
15	0.024	0.171
18	0.025	0.178
21	0.034	0.242
24	0.027	0.192
30	0.026	0.185
32	0.028	0.2
35	0.031	0.221
40	0.034	0.242
43	0.028	0.2
44	0.027	0192
48	0.031	0.221
55	0.028	0.2

Table – 4 Antibiotic sensitivity testing

S. No	Sample No	Diameters of inhibition zone in mm						Interpretation
		P	A	E	Ce	Am	At	
1	1	14	10	13	13	12	11	Sensitive
2	2	16	12	17	16	14	13	Sensitive
3	4	18	14	17	16	16	14	Sensitive
4	5	15	10	14	13	14	14	Sensitive
5	7	14	12	13	13	13	12	Sensitive
6	9	15	10	14	14	14	12	Sensitive
7	11	14	10	13	13	13	13	Sensitive
8	12	14	11	13	13	13	12	Sensitive
9	13	18	15	17	16	15	15	Sensitive
10	15	18	16	17	16	15	14	Sensitive
11	17	15	14	14	13	14	13	Sensitive
12	18	17	16	16	16	15	14	Sensitive
13	21	15	14	14	14	14	12	Sensitive
14	24	16	15	15	15	14	3	Sensitive
15	30	14	13	13	13	12	10	Sensitive
16	32	18	17	17	16	16	14	Sensitive
17	35	16	15	15	15	14	13	Sensitive
18	40	15	14	14	14	13	12	Sensitive
19	43	14	13	13	13	12	10	Sensitive
20	44	16	15	15	15	15	13	Sensitive
21	48	14	13	13	13	13	12	Sensitive
22	55	16	15	15	15	15	10	Sensitive

P – Penicillin; A- Ampicillin; E-Erythromycin; C-Cephotaxime; A-Amoxyllin; At-Azithromycin

Table. 5 Inhibition of aqueous extract of pomegranate at various concentrations on Actinomyces spp.

Sample No.	Diameter of inhibition zone (mm)at various concentrations mg/ml			
	50	100	150	200
1	-	10	13	15
2	-	11	14	16
4	-	9	12	14
5	-	8	10	12
7	-	10	11	13

9	-	12	13	14
11	-	11	12	13
12	-	10	12	13
13	-	9	10	12
14	-	10	11	13
15	-	11	13	14
16	-	9	10	12
17	-	11	12	14
18	-	10	12	15
19	-	12	14	15
20	-	9	11	13
21	-	8	10	12
22	-	10	12	14
23	-	12	13	15
24	-	11	12	14
25	-	10	12	13

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