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Bacteriological Study of Dental Caries Infection in Baquba

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Abstract

This Study was done from the period October 2016 Until June 2017 In the central laboratory of a hospital belonging to Diyala Health Governorate, to isolate and diagnoses *Streptococcus mutans* bacteria form dental caries infections, to detect the ability of isolate to biofilm formation, the sensitivity of isolates to antibiotics, and effect of plant extracts for Green tea and *Capsicum annuum* on the growth of the bacteria. The results showed that 68 Isolates belonged to Oral Streptococci were identify from 150 samples taken from dental caries infections, 20 isolates (13.3%) of *S. mutans* bacteria were obtained. The ability of isolate to form of biofilms by (80%), the isolates varied in their sensitivity to antibiotic, Where the percentages of sensitivity for Tetracycline (70%), Erythromycin (65%), Streptomycin (60%), Ciprofloxacin (50%), whereas resistance to antibiotics Amoxicillin, Ampicillin, Cefepime, Optochin (100%). The value of Minimum Inhibitory Concretion (MIC) of Tetracycline ranging from (256 - 1024) micrograms \ ml , Erythromycin ranged from (64 - 1024) micrograms \ ml. The inhibition zone of chili pepper and Green tea extracts for test isolates were (30)mm and (27) mm respectively, the comparison of inhibitory effect between plant extract and antibiotics the results indicate that the extracts of chili pepper and green tea have higher inhibitory effect than antibiotics against *S.mutans* Bacteria .This refers to the possibility of benefiting from those extracts in the manufacture of an alternative treatment, to minimizing the risks of resistance of bacterial as well as reduction the side effects that may be result from the use of s antibiotics , in addition, advantages such plants are easy to get and cheaply priced.

Keywords: *Streptococcus mutans*, Dental caries, Biofilms, Antibiotic, inhibition effect of Green tea and chili pepper extract Of bacteria.

Introduction

Dental caries are one of the most common oral diseases among people in the world and is an important public health problem. It is a microbial disease that occurs on the surface of the hard teeth in the dental plaque [1]. As a result, the elements on the teeth surface are disintegrated and a chronic infectious disease begins in a series of the complex chemical reactions and the microbial activity associated with the formation of biofilm on the plaque leading to the removal of metals from the calcified tissues of the teeth and the breakdown of organic components [2]. The carbohydrates in the diet are the main source of energy under the conditions leads to the production of organic acids, in turn reduce the pH to about

4.5, and causes the removal of minerals also [3]. *S. mutans* are a major cause of tooth decay, positive gram anaerobic bacteria, The higher pathogenicity is due to the ability of bacteria to biofilms formation, acid production, and tolerance of acids and salts [4]. *S.mutans* is one of the most common types associated with dental caries, survival in the natural environment. Biofilms formation begin to form when the bacteria bind to the surface of the teeth, so they can be affected When the concentration of substances reaches a critical threshold, cells. Begin to change their lifestyle and under the control of a variety of different genes, and vary from one species to another. Biofilm is not homogenous or is composed of a layer of microbial cells on a surface; it is a



varied structure that constantly changes on the temporal and spatial scales [5].

the development of resistance of bacteria in general and *S.mutans* in particular to many antibiotics as well as the side effects of these antibiotics on the human when used as a treatment, and has been conducted many studies and research to find natural alternatives with a disincentive effect of the growth of pathogens, including plant extracts from Fruits, leaves, seeds or legs of these plants, which can be used as an alternative to antibiotics in the treatment of infections caused by bacteria and other microorganisms .

Green tea, made from the leaves of *Camellia sinensis*, is one of the best antioxidants known to be effective as antibacterial activity and contains polyphenols such as Catechin and Epicatechin which have a lethal effect on many types of positive and negative bacteria , Recent studies suggest that green tea has significant medical applications and importance [6]. It may contribute to reducing the risk of cardiovascular disease and some forms of cancer, as well as promoting oral and dental health and other physiological functions such as anti-hypertensive effect and protection from ultraviolet [7].

Capsicum annum (Solonaceae) is characterized by containing alkaloid Capsicum, which used as a spice. Alkaloids are important compounds in the pharmaceutical and therapeutic industry and have clear physiological effects on nature and function of the cell membrane, though present in low volumes, makes it an antimicrobial agent [8]. Due to the fact that tooth decay is an important and complicated health problem and that there is no study in Baquba in this regard, the study was conducted with the aim of Isolation and diagnosis of *Streptococcus mutans* from dental caries infections, detection the ability of bacteria to biofilm formation, the sensitivity of the bacteria to antibiotics, to determination the minimum inhibitory concentration of a number of them, and Comparison the effect of different concentrations of some plants extracts on the growth of *Streptococcus mutants* with the effect of antibiotic.

Materials and Methodologies

Collection of sample

One hundred fifty samples of dental caries infections were collected from patient admitted the specialized dental center and from some schools in the Baquba city, and transport by Brain Heart broth (Oxoid – England) for culture and identification.

Isolation of *Streptococci mutans* Bacteria

Samples were spread on the surface of Mitis Salivarius Bacitracin Agar(Himedia- India) plates using sterile swabs, Inoculated plates were incubated an aerobically for 48 hour at 37 °C, identification of bacteria on the base of Morphology, Cultural characteristic and, Biochemical tests [9].

Rapid Differentiation of Colonies

A differentiation between colonies belongs to *S.mutans* from that of other species like *S. sanguis* on MS-agar could be achieved by the inoculation of the MS-agar with the isolate and incubated anaerobically at 37 °C for two days. Then a test solution of (10%) mannitol and (4%) of 2,3,5-triphenyltetrazolium chloride (TTC) solution were added on the plates. A change in color to a dark pink due to hydrolysis of mannitol to acid by the enzyme mannitol-1-phosphate dehydrogenase and a reduction of (TTC) which could taken as an indicator for the presence of *S.mutans* [10].

Tolerance to (4 %) NaCl

The tolerance of *Streptococcus mutans* isolates to 4 % NaCl were tested by inoculation of bacteria in Brain Heart broth (BHI) medium , incubated under suitable after inoculation anaerobically at 37 °C for 48h. then Incubation under Suitable conditions, Growth was monitored visually as compared with a control tube which did not contain this concentration of sodium chloride. Turbidity is an indicator for the ability of the bacterial isolates to grow on this concentration of NaCl [11].

Utilization of Different Carbohydrates

Sources

To study the ability of bacterial isolates to utilize in different carbohydrate sources, (BHI) broth medium supplemented with (10%) of

each carbohydrate (Sucrose, Mannitol, Sorbitol and Inulin). Sucrose was used as positive control and brain heart infusion broth medium as negative control, carbohydrates solutions were sterilized by filtration and added aseptically to the autoclaved brain heart infusion broth medium which contains a (0.02%) of phenol red, then the suspended media was inoculated with the tested isolates and incubated anaerobically at 37°C for 72 hrs. The change in the color of media from red to yellow indicated the ability of isolates to utilize these carbohydrates sources [12].

Detection of the biofilm formation

The Micro Titer Plate (MTP) method was used based on [13], Bacterial isolates were culture on MSA for 48 h at 37 °C under the anaerobic conditions. And transferred 2-4 of the colonies to the (BHI) broth and comparison to Mac Farland tube . transfer of 150 microliters from the bacterial suspension of each isolates under study to the drilling of a 96-well , three replications of each isolated were performed, addition of the medium to several well and leaving some of the holes without bacterial inoculation and considered a negative control drilling, and incubated at 37 °C for 48 hours.

After incubation, contains of wells were removed by washing 2 - 3 times with distilled water. And added 200 µL of methanol for 10 Minutes to stabilized the Bacteria, the wells were stained with added 200 µL of the Crystal Violet solution for 15 min , the excess dye was removed by washing with distilled water 2-3 times. Add 200 µL of 95% ethanol for 10 min to remove the pigment attached to the cells. Finally microtiter plate read by enzyme-linked immunosorbent assay (ELISA) at a 630 nm wavelength. This assay was performed by triplicate and the mean biofilm absorbance value was determined. Biofilm degree was calculated as follows: $*2Ac \leq A$ high produce biofilm $*Ac \leq A < 2Ac$ middle produce biofilm $*A \leq Ac$ non- produce biofilm, Ac mean control wells.

Antibiotic Susceptibility Test

The sensitivity of *S.mutans* isolated were do against 13 Antibiotic according to Disc diffusion Method [14]. And determination the minimum inhibitory concentration (MIC) of Tetracycline and Erythromycin for ten *S.mutans* isolates, and compare the results with the [15].

Preparation of Plants Extracts

Green tea and Chili pepper they were naturally dried under the sunlight for 24 hours, then cut into small pieces, and crushed to obtain powdered by the electrical grinder and stored in sterile glass bottles for use. The alcoholic and hot and cold water extract of the plants were prepared by mixing 20 gm of dried plant powder with 1000 ml of (cold and hot distilled water, and 95% Ethyl alcohol) leaving for 24 h shaking incubator at 30 °C , layers of muslin clothes then divided in test tubes and separate Content by Centrifuge with 2500 cycles / min for 10 min. Then distributed in glass dishes and place in oven at 40 °C [16].

Stock Solution

The stock solution prepare by 1gm of each of the hot and cold water and alcoholic extract of the (green tea and chili pepper) with 10 ml of distilled water to obtain 100 mg/ml concentration. Then sterilized by Millipore filter was with a diameter of 0.22mm. four concentrations from each extracts, (25, 50, 75, 100) mg / ml were prepared and used in antimicrobial test [17].

Effect Of plants Extracts on The Growth of *S.mutans*

The agar well diffusion method were used, surface of the nutrient gar dish sprayed by spreading 0.1 mL of bacterial suspension using the standard turbidity. The dishes were then left at room temperature for 15 minutes .7 mm wells were drilled in the culture medium, transferred 50 micro liters by means of a micropipette from each concentration (25, 50, 75, 100) mg/ml for the cold, hot and alcoholic water plant extracts and place in the hole.

Control dishes were also made by placing 50 microliters of distilled water in the pit instead of the plant extracts, incubated the dishes in 37 °C temperature for 48 hours, the result recorded by measuring the diameter of inhibition zone [18].

Statistical Analysis

Statistical analyzes were carried out using the (Chi-square) to analyze the data statistically and determine the statistical differences on the base SPSS program [19].

Results and Discussion

The study involved 150 samples of Dental caries for isolation and diagnosis of *S.mutans*, the samples were inoculated on the optional medium Mitis Salivarius Agar medium to promotes the growth of Streptococcus and some other oral bacteria, as well as the optional high-dose Mitis Salivarius Bacitracin Agar (MSBA) by added (20%) of sucrose and (0.2) g / mL of Bacitracin, which inhibits the growth of most bacteria except *S.mutans* and *S.sobriuns* 20 isolates were obtained from *S.mutans* bacteria. The growth of isolates on the MSA medium after incubation in anaerobic conditions at 37 °C for 48 h show Small, high, irregular colonies were attached to each other. Microscopic examination reveal gram positive, spherical shape bacteria arrangement in chain or as pairs. The developing isolates were marked on the center of the MSBA with 1mm diameters and give positive result for rapid diagnosis with (10%) TTC and (4%) Mannitol due to the change of colony color from blue to red Figure. Table 1 shows the results of biochemical test of growth isolates which are consistent with [20].

Table 1: Biochemical tests for 20 isolates of *S.mutans* bacteria

Test	Result	
Ability to Ferment carbohydrate	Sucrose	+
	Mannitol	+
	Inulin	+
	Sorbitol	+
Colony color after sprayed with 10% TTC and 4 mannitol	Pink color	
Catalase test	-	
Growth in 4% NaCl	+	

Biofilm formation

The Micro Titer Plate method (MTP) was used to detect the ability of 10 isolates of *S.mutans* to biofilms formation, Measurement was made at a wavelength of 630 nm. The results show that (80%) of the isolates produce biofilms. (20%) of the isolates are form strong level for biofilm formation with a absorbance value of (0.1286 - 0.175 nm) , (60%) have the ability to form biofilm in medium degree with a absorbance value of (0.068 -0.094 nm) ,and (20%) non-biofilm formation Table 2 The researcher AL-Kazrige [21] concluded that most of the isolates of *S.mutans* form biofilms. The results are also similar to those obtained by Zezhang *et al* [22].

Epidemiology of Dental caries

The percentage and number of dental caries caused by were (52%) female higher than those of males (48%) Table 3, The results were consistent with the findings of Khamise [23] in Najaf governorate, which found that males (25.2%) in permanent teeth and higher in females (31.2%), the results did not agree with the findings of the researcher AL-Mosawi [24] in Baghdad. The statistical analysis using the (Chi-square) showed no significant difference between the sexes in the number and percentage of tooth decay. There was also no significant difference between the sexes and the number of isolates. For age groups, the age group was (1-10) years Most vulnerable groups (35%), where the number was 34 (36%), and the lowest age group more 50 years (5.4%). The reason for the high rate of dental caries in children is the weakness of the levels of prevention of teeth in addition to the availability of sugars in foods and liquids and even In medications they eat especially if they are prolonged and continuously, they also practice returning children as well as low rates of breastfeeding by mothers and the spread of artificial feeding, which has a significant impact on the appearance of caries in children at an early age ,These results are similar to those found by AL-Sultani *et. al.* [25] in Babel governorate when they found that the infection was higher in children with aged (5-7) years, Statistical analysis showed a significant

difference between age groups of tooth decay and no significant difference in relation to the

number of isolates and age groups Table 4.

Table 2: Biofilm Formation of (10) *S.mutans* isolates.

Isolate number	Absorption value at wavelength 630nm	The level of biofilm that compared with the control drill	Isolate number	Absorption value at wavelength 630nm	The level of biofilm that compared with the control drill
1	0.094	Moderate	6	0.1286	High
2	0.0832	Moderate	7	0.0923	Moderate
3	0.0686	Moderate	8	0.0866	Moderate
4	0.059	None	9	0.175	High
5	0.057	None	10	0.074	Moderate

Table 3: Percentage of Dental caries & Number of isolates of *S.mutans* bacteria on the base f sex.

Sex	Dental caries		<i>S.mutans</i>	
	Number	%	Number	%
Male	72	48	Λ	ε .
Female	78	52	12	60
Total	150	100	20	100
X ²	0.62		0.8	
P value	NS, γ ε		NS γ γ	

Table 4: Number of Dental caries & isolates of *S.mutans* bacteria by age.

Age group year	Dental caries		<i>S.mutans</i>	
	Number	%	Number	%
1-10	53	35.3	γ	35
11-20	15	10	ε	20
21-30	37	24.7	γ	10
31-40	26	17.5	γ	15
41-50	11	7.5	γ	15
More than 50	8	5.40	γ	ο
Total	150	100	20	100

The results showed a distribution of dental caries by urban areas more than rural areas where the percentage (58%) in urban and (42%) in the countryside, The results were consistent with the study presented by [23] which concluded that the infection was higher in the city than in the countryside, The statistical analysis indicated that there was a significant difference between the site of residence, the number and percentage of tooth decay, and the absence of significant difference between the location of the dwelling and the number and percentage of *S. mutans* bacteria isolates. Among the causes of high dental caries in urban areas may be attributed to the fact that the number of individuals in the sample of the study was most of the city dwellers as well as the consumption of high-sugar materials for the members of these areas

or air pollution bacteria and the small proportion in the countryside due to the use of rural people to health food of the plant products, such as vegetables and fruits, which strengthens the growth of teeth and prevents the spread of bacteria in the mouth, while in terms of link to diabetes patients were very few and by (4.7%), While the researcher Al-Khayoun [26] in Baghdad, the proportion of the injury was (100%) among diabetic patients explained the increase of these patients because of the high proportion of glucose and proteins in the saliva and their impact on the level of presence of bacteria and the presence of decay, The statistical analysis revealed a significant difference (P 0.001) among diabetics, tooth decay and number of isolates with *S.mutans*. and the study revealed the distribution of dental caries in women who have a number of

Children were the highest among women who had between 3-4 reach 55.17%, The results were inconsistent with the study presented by AL-Sultani *et al* [27], where the incidence was among pregnant women (74%), Statistical analysis using the chi square test showed a significant difference (0.002) among women with large number of children and the rate of dental caries , and there was no significant difference with the number of isolates.

Sensitivity Of *S.mutans* To Antibiotics

Check the sensitivity of 13 antibiotics of all bacterial isolates. All *S.mutans* isolates showed 100% complete resistance to Amoxicillin, Ampicillin, Cefepime, and Optochin. Most bacterial isolates showed high resistance to more than one antibiotic, due to the ability of bacteria to produce enzymes , and the sensitivity of Gentamycin (45%), Tetracycline (70%), Erythromycin (65%), Streptomycin (60%), Ciprofloxacin (50%), Cephalothin (60%) and Chloramphenicol (45%), and medium resistance for Amikacin (50%), Trimethoprim-Sulfamethoxazole and (45%) respectively, the findings of the study AL-mosawi [24]. The isolates showed sensitivity to Gentamycin, Tetracycline, Erythromycin.

Table 5. The higher resistance of bacterial isolate may be due to the wide and random usage of several antibiotics by patients, and resistant factors with genetic and non genetics origins that isolates possess them.

Determination of Minimal Inhibitory Concentration

The Minimal inhibitory concentration of Tetracycline and Erythromycin for 10 isolates of *S.mutans* were determined ranged between 256-1024 μ g/mL, The researcher AL-Kazrighy [21] obtain similar result For Erythromycin. when the minimum inhibitory concentration values of *S.mutans* isolates ranged from 64 to 1024 μ g/ml Table 6.

The inhibitory effect of plants extracts

The effect of Alcoholic and aquatic extracts (hot and cold water) on green tea and chili pepper plant extract on the isolates (4 and 7) of *S.mutans* isolates from dental caries was studied for being the most common among the infected and for its high resistance to antibiotics. The current study showed effectiveness of plant extracts on the bacterial isolates. The inhibition effect of alcohol extract for green tea and chili pepper were the highest.

Table 5: Sensitivity of (20) *S.mutans* isolate to antibiotics.

Antibiotics	Resistant		Inter medium		Sensitive	
	Number	%	Number	%	Number	%
Gentamycin	5	25	6	30	9	45
Streptomycin	8	40	-	0	12	60
Chloramphenicol	4	20	7	35	9	45
Trimethoprim-Sulfamethoxazole	5	25	9		6	30
Amoxicillin	20	100	-	0	-	0
Ampicillin	20	100	-	0	-	0
Optochin	20	100	-	0	-	0
Cephalothin	2	10	6	30	12	60
Tetracycline	2	10	4	20	14	70
Erythromycin	2	10	5	25	13	65
Ciprofloxacin	3	15	7	35	10	50
Amikacin	6	30	10	50	4	20
Cefepime	20	100	-	0	-	0

Table 6: Values of (MIC) for Tetracycline and Erythromycin.

Isolate number	Tetracycline	Erythromycin	Isolate number	Tetracycline	Erythromycin
1	206	128	6	012	206
2	012	128	7	1.24	1.24
3	012	206	8	012	012
4	012	1.24	9	012	012
5	1.24	64	10	206	64
P value			0.001***		

The diameters of inhibition zone for 4, 7 isolates reach (27 and 26) mm for green tea and (30) mm for chili pepper respectively at 100 mg/ml, The hot water extract came with a similar effect to the alcohol extract at the high concentration of the isolates, 25 and 26 mm diameter for green tea, 26 and 28 mm For chili peppers at 100 mg/ml concentration . The hot water extract came with a similar approach to the alcohol extract at the high concentration of the isolates, 25 and 26 mm diameter for green tea, 26 and 28 mm For chili peppers at 100 mg/ml concentration, followed by the extract Aqueous cold diameter and (24 and 25) mm green tea and (25, 25) mm by hot pepper Figure 1. The two high concentrations of 75 and 100 mg/ml were more effective in inhibiting the growth of the isolates than the

two low concentrations (25 and 50 mg/ml. Table 7 The results of current study agreed with finding of Abdolmehdi *et. al.* [27], they found that green tea inhibits *S.mutans* and can be used as mouthwash to prevent tooth decay. The results are also close to that of a group of researchers Taketo *et al* [4], who concluded that vegetable tea has the ability to inhibit *S.mutans* by preventing the adhesion to the surface of the teeth and inhibit the formation of biofilms, the results came in line with the findings of a group of researchers (Moema Mocaiber [8]. Who concluded that capsaicin and dihydrocapsaicin present in chili pepper have a high effectiveness against *S. mutans* that the main causes of tooth decay and, prevent the formation of biofilms by bacteria.

Table 7: Effect of green tea and chili pepper extract on the growth of *S.mutans*.

Type of extract	Isolate number	Diameters of inhibition zones in millimeters of green tea				Diameters of inhibition zones in millimeters of chili pepper			
		Concentration mg/ml				Concentration mg/ml			
		25	50	75	100	25	50	75	100
Alcoholic extract	4	15	19	23	27	17	19	25	30
	7	18	20	22	26	17	19	24	30
Hot water extract	4	10	13	24	25	15	18	23	28
	7	13	16	24	26	13	17	20	26
Cold water extract	4	13	16	21	25	12	17	20	25
	7	15	18	20	24	13	15	22	25



Figure 1: Inhibition zone of *S.mutans* by alcohol extract of green tea and chili pepper.

Conclusion

Therefore we conclude that the alcoholic and aquatic extracts of two plants under study had inhibitory effect The presence of, effective

compounds, and the ability of the last to penetrate and in tissues, high efficiency and the ability to penetrate the cell wall and inhibit

growth of bacteria. This study may be useful for the development of oral care products such as dental cleaning materials study and can be used plant extracts as a mouthwash and treatment of tooth decay diseases and can be used as alternative treatment of antibiotics to overcome the problem of resistance to those antidepressants and prevent the side effects that may be result from antibiotics usage as a treatment.

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