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Effects of Natural compound "Piperine" on the growth and Adhesive Properties of Streptoccus Mutans Bacterial Biofilm

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Abstract

Background: "Streptococcus mutans" bacterial biofilm is known to be a major causative organism for dental plaque which is acknowledged to be associated with biofilm. In this study we aim to evaluate the effectiveness of natural compound "Piperine" on the biofilm of "Streptococcus mutans" bacteria from One hundred cases of Dental unit Of Rajshahi Medical College.

Methods: This longitudinal study was conducted in the Molecular Pathology with the Insect Biotechnology Lab, IBSc of Rajshahi University and Dental Unit of Rajshahi Medical College from October 2017 to October 2019. A total of 100 cases were selected for this study from the same institution, collected dental plaque sample, identified, and isolated Streptococcus mutans bacteria by using MSB Agar (Mitis salivarius-bacitracin) medium, formation and detection of "Streptococcus mutans" bacterial biofilm using a Microtiter plate biofilm assay method. Then we collected Medicinal plant "Black pepper" from a standard medical food supply, extracted, isolated, purified and identified a specific compound named as "Piperine" by using Column chromatographic and Nuclear magnetic resonance spectroscopic (NMR) techniques and analysis their antimicrobial effectivities.

Result: Our result demonstrated that "Streptococcus mutans" bacterial biofilm represents a very strong microbial attachment with the tooth surface and after the application of 100ul ethyl acetate extract of piperine showed 77% growth of reduction with the value of P<0.001 in case of patient no 17, 75% growth inhibition with the value of P <0.003 in case of patients no 55 and 77% growth of inhibition with the value of p<0.005 in case of patients no 95 against the biofilm of streptococcus mutans bacteria.

Conclusion:After some investigations it was revealed the ethyl acetate extract of compound "Piperine" showed the highest antibiofilm activities.

Key words: Streptococcus mutans, Biofilm, Antibiofilm, Medicinal plant and Piperine.

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Introduction:

Streptococcus mutans bacteria is one of the most causative microorganisms for the initiation of dental caries[1] which can also be a source of infective endocarditis [2,3] Oral disease is a major health issue in the whole world [4] and biofilm-related infections created a major problem in the society.[5] *Streptococcus mutans* bacteria are highly acidic in nature and produce intracellular iodine-staining polysaccharides (IPS)[6] and extracellular glucan (EPS). These glucans play an important role in the accumulation, adhesion and matrix formation of microorganisms which lead to serious infections[1].Biofilm may be defined as aggregation of microorganisms in which cells are embedded within self-produced matrix of extracellular polymeric substances such as DNA, protein and long chain polysaccharides and the cells are stick to each other on a surface. *Streptococcus mutans* biofilm is a pale-yellow biofilm that develops naturally on the tooth surface and commonly referred to as microbial plaque biofilm or bacterial plaque biofilm. Scientifically the plaque was termed as biofilm because it is a community of living microbes surrounded by gluey polymer layer. *Streptococcus mutans* bacteria are also responsible for the formation of dental caries and causes demineralization of the hard tissues of tooth include enamel, dentin and cementum[7] There are four factors that cause dental caries including the host, substrate, bacteria and time[8]. The interaction of the four factors influences the formation of dental caries in oral cavity.[9]

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Recently, a human oral microbe project indicates that more than 764 kinds of bacteria and 102 kinds of fungi can causes the development of diseases in the oral cavity. These reports indicate that the dental microorganisms are not only responsible for various type of diseases in the oral cavity but also could be implicated in systemic disease. In case of these situations, it would be impossible to protect pathogenic bacteria of dental plaque biofilm from antibiotics. Although chlorohexidine gluconate and essential oil is widely used to prevent dental biofilm formation as an antimicrobial agent but it has some disadvantages like dental pigmentation, burning sensation in oral cavity and changes in the sensation of the taste[10]. For this reason, we need to give urgent attention for discovering some new drug molecules. Now-a-days natural products with their bioactive compounds are mainly used for treating various types of oral diseases.[11] These natural products have antibacterial, antibiofilm, antifungal and anti-inflammatory properties[12] and there is increased attention toward using natural, biologically active herbal compounds as an alternative medicine.[1] Antimicrobial studies of these plants are going on throughout the whole world in full swim. In this regard various compounds are very effective for the treatment of various types of microorganisms[13-18]. *Black pepper* is popularly known as the king of species and their biological active compounds have been used for treating various types of oral infections. *Piperine* is the natural compound which is the derivatives of Medicinal plant Black pepper and have been used as a traditional cure in Asian System of medicine. The aim and objectives of our study was to evaluate the effectiveness of natural compound *Piperine* on the biofilm of *Streptococcus mutans* bacteria from one hundred cases of Rajshahi region.

Materials and Methods:

This longitudinal study was conducted in the Molecular pathology with the Insect Biotechnology Lab, IBSc of Rajshahi University and Denal Unit of Rajshahi Medical College from October 2017 to November 2019. One hundred cases aged between 19 to 45 years were enrolled through purposive and convenient sampling method. After that we collected dental plaque with saliva sample from those 100 cases from Dental unit of Rajshahi Medical College (RMC) with the history, clinical examination and laboratory investigations were recorded. Then we identified long chain purple colored *Stretococcus mutans* bacterial colony and produced *Streptococcus mutans* bacterial biofilm by using Mitis salivarious-bacitracin agar (MSB) medium, detected their adhesion strength and determined their antibiofilm activities through the applications of Microtiter plate assay method. After that we collected the medicinal plant *Black pepper* (seeds portion) from a Standard Medical food supply, prepared crude extract by Soxhlet extraction method, isolated, purified and identified of these plant extracts using Column chromatographic and Nuclear Magnetic Resonance Spectroscopic (NMR) techniques. finally, we had got an active compound named as *Piperine* and added solvent ethyl acetate with it and the effect of ethyl acetate extract of compound *Piperine* in different concentrations were evaluated. For this study we selected another two bacteria named as *Pseudomonas aeruginosa* and *Escherichia coli* which acted as a control group along with *Streptococcus mutans* bacteria and analyzed their antibiofilm activities.

Procedure of biofilm formation

At first, we cultured the bacterial cells by adding 5ml liquid broth medium in to each test tubes. Then 1ml bacteria were added to the liquid broth medium and Shaked it overnight (at 37°C) for 24 hours and transferred it in to triplicate wells of a fresh 96-well microtiter (MTP) plate and filled with row A (top) to row H (bottom) with 100 ml fresh liquid broth medium Incubate the MTP plate and remove the supernatant or nonadherent cells carefully by using a pipette of 100 ml, then add 150 ml of 1% crystal violet solution to each well and incubate the MTP at room temperature for 30 minutes. Remove the crystal violet by extensive washing twice with 175ml de ionized water and added 175 ml dimethyl sulfoxide (DMSO) to each well in order to release bound crystal violet with the plate. After that the plate was read spectrophotometrically, Compared and quantified the biofilm control.

Adhesion strength of the biofilm *Streptococcus mutans, Pseudomonas aeruginosa, Escherichia coli* and % of their growth of inhibition with the mean and P value were calculated by the following formula: The laboratory findings were stated as mean±standard error. The results obtained by utilizing a more quantitative and reliable method named as Tissue culture plate method for the detection of all the bacterial biofilm and Microtiter plate biofilm assay method for monitoring microbial attachment to an antibiotic surface. At first we calculated the mean which was evaluated by total sum of all sample and control groups are divided by the number of sample and control by using Microsoft excel software for analyzing. We also calculated standard error by dividing the standard deviation. We obtained standard deviation

from calculating each measurements deviation from mean value. divide the sum from all the sample and control sizes and take the square root of the numbers which gives us standard deviation.

Result:

At first we were identified a long chain purple colored streptococcus mutans bacterial colonies (figure-1) and detected the adhesion strength of biofilm developed by streptococcus mutans bacteria of 100 people of Rajshahi region (figure-11).Our result demonstrated that treatment with 100µl ethyl acetate extract of piperine showed 77% growth of inhibition (mean is reduced from 4.12±0.244 to 0.933±0.289 with the value of P<0.001) in case of patient no 17, 75% growth inhibition (mean is reduced from 4.32±2.59 to 0.97±0.294 with the value of P <0.003) in case of patients no 55 and 77% growth of inhibition (mean is reduced from 3.61±.225 to 0.84±.298 with the value of p<0.005) in case of patients no 95 (Table-1, 11 and 111) in comparism with the other two bacteria such as 11%, 59% and 58% growth of inhibition on the biofilm of *Pseudomonas aeruginosa* in case of patients no 17 and 35%, 26% and 49% growth of inhibition in case patient no 17, 55 and 95 (Table-1V).

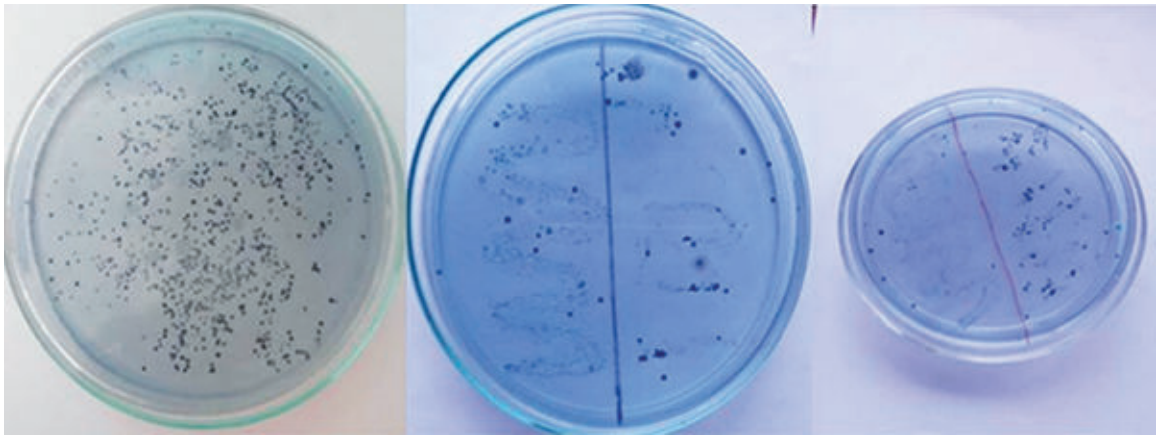

Fig.-1: *Identification of a long chain purple colored Streptococcus mutans bacterial biofilm colonies on Mitis salivarius bacitracin agar media.*

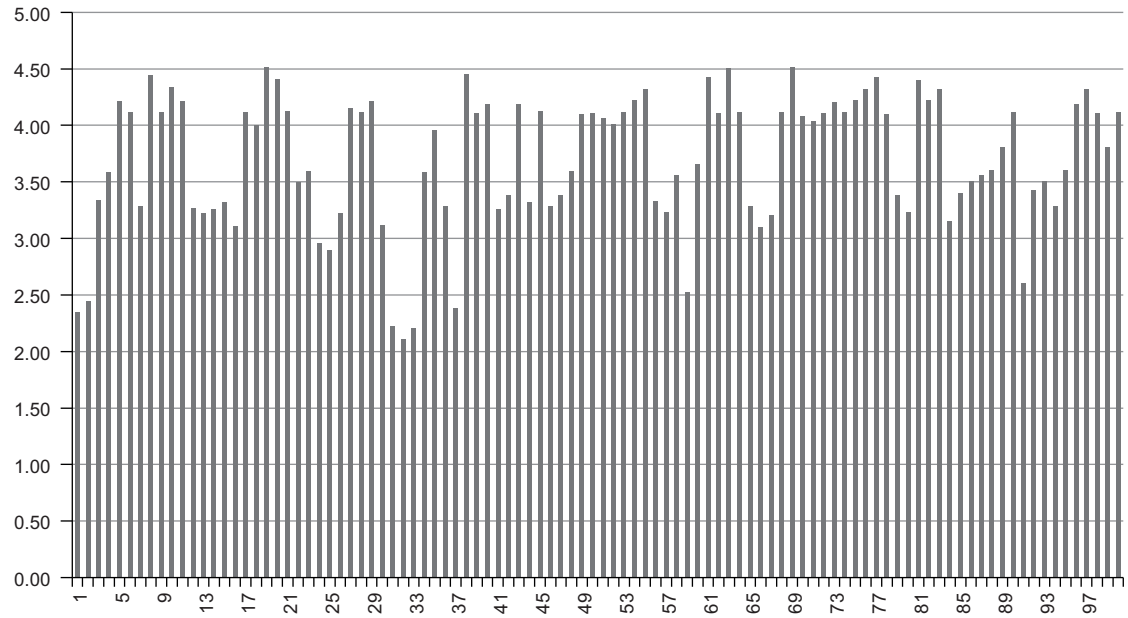

Fig.-2: *Detection of adhesion strength of S. Mutans Bacterial Biofilm from the 100 Patients of the Dental Unit of Rajshahi Medical College & Hospital.*

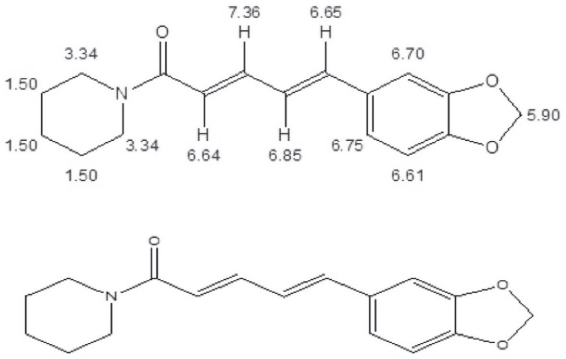

Fig.-3: *a) Chemical structure of Compound Piperine with the chemical formula C17H19NO3 and With the IUPAC name 1-(5-{1,3-benzodioxol-5-yl}-1-oxo-2,4pentadienyl) Piperine*

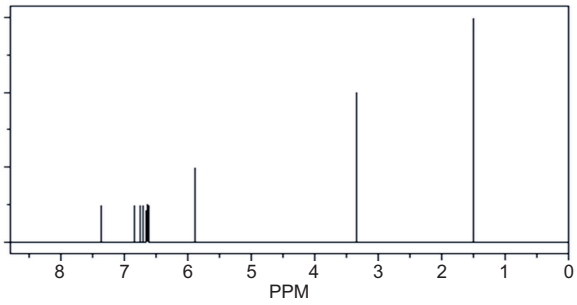

Fig.-4: *1H NMR of Piperine from CHEMDRAW*

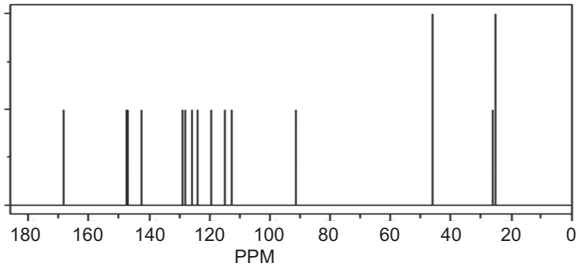

Fig.-5: *C-13 NMR of Piperine from CHEMDRAW*

Table-I

Growth of inhibition of the biofilm of Pseudomonas aeruginosa, Escherichia coli and Streptococcus mutans after the application of ethyl acetate extract of Piperine (Patients no 17).

Name of the bacteria	No treatment	25 ml	50 ml	100 ml
P. aeruginosa	3.48±0.382	3.21±0.332	2.85±0.325	3.10±0.289
Escherichia coli	1.43±0.257	1.21±0.307	1.07±0.318	0.92±0.321
Streptococcus mutans	4.12±0.244	2.54±0.326	1.47±0.331	0.933±0.289

1Table-II

Growth of inhibition of the biofilm of Pseudomonas aeruginosa, Escherichia coli and Streptococcus mutans after the application of ethyl acetate extract of Piperine (Patients no 55).

Name of the bacteria	No treatment	25 ml	50 ml	100 ml
P. aeruginosa	3.48±0.382	3.08±0.010	2.26±0.320	1.43±0.260
Escherichia coli	1.43±0.246	1.28±0.272	1.06±0.300	1.21±0.356
Streptococcus mutans	4.32±0.239	2.65±0.305	1.74±0.298	0.97±0.294

Table-III

Growth of inhibition of the biofilm of Pseudomonas aeruginosa, Escherichia coli and Streptococcus mutans after the application of ethyl acetate extract of Piperine (Patients no 95).

Name of the bacteria	No treatment	25 ml	50 ml	100 ml
P. aeruginosa	2.62±0.388	1.89±0.328	1.23±0.301	1.10±0.265
Escherichia coli	1.12±0.275	1.0±0.312	0.64±0.309	0.57±0.29
Streptococcus mutans	3.61±0.225	1.50±0.327	0.96±0.337	0.84±0.296

Table -IV

Percentages of growth of inhibition of the biofilm of Pseudomonas aeruginosa, Escherichia coli and Streptococcus mutans after the application of ethyl acetate extract of Piperine in different concentrations in cases of patients no 17,55 and 95.

Compounds	*P.acaroginosa*			pvalue	*E. coli*			p Value	*S. mutans*			*pvalue*
with solvent	25mL	50mL	100mL		25mL	50mL	100mL		25mL	50mL	100mL	
T_1	.07%	18%	11%	.05	15%	25%	35%	.044	38%	64%	77%	.001
T_2	11%	35%	59%	.01	10%	15%	26%	.011	39%	55%	75%	.003
T_3	28%	53%	58%	.06	10%	43	49%	.073	50%	76%	77%	.005

$T_{1=}$ *Ethyl acetate* extract of *Piperine* in case patients no (17),
$T_{2=}$ Ethylacetate extract of *Piperine* in case patients no (55),
T_3= Ethylacetate extract of *Piperine* in case patients no (95).

Discussion:

Natural compound alkaloid is derived from the Medicinal plant piper nigrum or the "the Black gold" (another name of Black pepper) and are of the most widely used species belonging to the family of Piperaceae, Piperine and its role in chronic disease. Study done by Hikal[19] who used piperine and Black pepper in different concentration to evaluate their antimicrobial activity against *Staphylococcus aureus, Bacillus substiles,, Salmonella sp and E.coli* through agar well method which had some similarities with our study. Their study revealed that the antimicrobial activity of piperine and Black pepper oil increases when their concentrations increase. On the other hand, the maximum zone of inhibition was recorded with both *Piperine* and Black pepper oil at the concentration of 5% against all gram-positive bacteria with zone of inhibition 18, 14, 15, 10.43 and 8.3 mm. Finally Their study concluded that *Piperine and* Black pepper oil have the potent activity as an antimicrobial agent especially piperine with both gram-positive and negative microorganisms. Another study was done by Hakim[20] where he demonstrated the effect of Black pepper(*Pipper nigrum*) were made by using maceration method on muller Hinton agar media. The concentrations of black pepper extract used in this study were 6.25%, 12.5%, 25%, 50% and 75%. The results of this study were analyzed by using One-way ANOVA test showed that significance of Black pepper extract in inhibition of *S. mutans* bacterial growth. Finally, this study concluded that the effectivity of Black pepper extract for the inhibition of *S. mutans* growth with the concentrations of 75% is the optimum concentration which value 16.8mm. Another study done by Shweta *etal* [12] where they revealed that clove and Black pepper extract had a very effective bactericidal action, and their effectivity on *S. mutans* bacteria were studied by using disc diffusion method. Their study demonstrated that Black pepper and clove extract declared a very powerful antibacterial activity against *S. mutans*. Not only Black pepper but also several medicinal plants have been used for the prevention of *Streptococcus mutans* bacterial biofilm which was confirmed by a study of N. dedryl *etal* [21]in those they observed that the chloroform extract of *dor sera peltate* plants showed the broad-spectrum antibacterial activity against *S. mutans* and *S. sobrinus* (MIC = 31.25 and 15.25 ml gm $L1^{-1}$ resp). Smullen [22] determined the ability of commercially available fresh aquas propane extract of foods with high polyphenol content inhibit the growth of S. mutans and other oral

pathogens. All the extracts showed the antimicrobial activity but the plant extract of red grapes seeds exhibiting the greatest activity against *S. mutans* (MIC = 500 mg Ll^{-1}). These data suggest that the extract of polyphenol containing foods may have a preventive role against dental caries. The effectivity of the piperine and Black pepper which showed all the above mentioned studies had some similarities with our study. In our study we also tried to identify a new active compound which have both antibiofilm and antimicrobial properties and after finishing some investigations we got a specific compound from the Medicinal plant Black pepper named as- "Piperine",analyzed the effect of ethyl acetate extract of compound piperine on the biofilm of *S. mutans* bacteria, collected dental plaque sample from 100 patients of RMC and screened for biofilm formation and finally among these 100 patients we selected17,55 and 95 no patients to analysis their inhibitory effect of ethyl acetate extract of compound piperine. Finally in our research work we revealed that after the application of 100 mL of ethyl acetate extract of piperine showed the highest antibiofilm activity (77% and 75% growth of inhibition with the p value $p < .0001$) which was much better than other two control group such as P. aeruginosa (59 and 58% growth of inhibition) and E. coli showed (20% growth of inhibition) in case of patient no 17,55 and 95. Similar types of study was done by Dwibedi[1] wher analyzed the effect of natural compound embelin and piperine on the biofilm of *streptococcus mutans* bacteria. A total 30 isolates were identified as a streptococcus mutans and screened for the biofilm formation. The strongest biofilm producer (SHO3) was used to identify both minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentrations of natural compounds are analyzed. The MIC of embelin was 0.55±0.02 where that of piperine was 0.33±0.02. (MIC, piperine < embelin). The MBIC of embelin was (0.0620±0.03 where that of piperine was 0.0407±0.03 which was lower than Embelin. These results confirmed that piperine is the most active compound which had both antimicrobial and antibiofilm activities (Piperine > embelin). The MIC and MBIC value indicate that piperine has better ability to inhibit the biofilm formation rather than embelin.

Conclusion:

In the present study activity of *Piperine* was evaluated at different concentrations and revealed that 100µl ethyl acetate extract of compound *piperine* showed the highest antimicrobial activities. '

Compliance with ethical standards

Acknowledgments

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The authors declare that there is no conflict of interest exist.

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