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Anti-microbial activity of Green Tea Extracts and Nicotine on the Growth, Biofilm Formation of Salivary *Mutans Streptococci* (In-vitro study)

DOI: https://doi.org/10.32007/jfacmedbagdad.2024.

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Abstract:

Background: The green tea have antimicrobial activity against many types of bacteria It is considered a natural substance with few side effect.

Aim of the Study: An in vitro study was carried out to investigate the ability of green tea extract and nicotine to inhibit growth, biofilm formation by salivary *Mutans streptococci*.

Methods: This study included a convenient sample of 40 healthy Iraqi volunteers aged 18–23 years old from College of Dentistry / University of Baghdad. Green tea and nicotine aqueous extract were prepared in different concentration to use in agar diffusion method to detect the activity of extract, and ELISA reader in multi titer plate was used to determine the ability of salivary mutans Streptococci to form biofilm in the presence and absence of the extracts.to measure the biofilm inhibition rate.

Results: *Mutans Streptococci* were sensitive to green tea and nicotine in different concentrations the diameters of the inhibition zone were effective in a dose dependent manner significantly. There was a significant difference between the concentrations of each extracts, antibacterial activity was in a dose dependent manner for the extracts. The minimum bactericidal concentration of green tea was (280 mg/ml) and minimum bactericidal concentration of nicotine was (45mg/ml). The study found that biofilm formation by *Mutans Streptococci* was markedly decreased in the presence of 1/2 minimum bactericidal concentration of both green tea and nicotine with mean of O.D 590 nm = 0.54 in comparison with green tea extracts and nicotine alone O.D 590 nm = 0.15, 0.68 respectively.

Conclusions green tea and nicotine extracts in different concentration effectively reduced the biofilm formation of salivary *Mutans streptococci*. While the presence of nicotine has negatively impacted on the ability of green tea extracts in the inhibition of biofilm formation by *Mutans Streptococci in* vitro.

Keywords: *Mutans Streptococci*, Biofilm activity, Antimicrobial, Green tea, Nicotine.

J Fac Med Baghdad
2023; Vol.65, No. 2
Received: Nov., 2022
Accepted: May, 2023
Published: July 2023

Introduction:

Mutans Streptococci and other forms of biofilm-forming bacteria are major contributors to the prevalence of dental caries and plaque, two of the most common oral health problems in the world (1). Oral flora often includes these gram-positive, facultative anaerobic bacteria. *Streptococcus mutans* has been linked in several studies to the onset of a carious lesion, and isolation of this bacterium from dental plaque near a carious tooth surface has been shown to be possible (2, 3, 4). Biofilm, a slimy covering of bacterial cells, salivary polymers, and food detritus, coats the tooth surface. This biofilm lacks order and may quickly grow to be several hundred cells thick on the teeth's surfaces (5). Consequently, it would be preferable to use an antibacterial approach for caries species in the oral environment (6).

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The World Health Organization (WHO) supports this line of inquiry, and it has issued a call for the discovery of novel antibacterial agents and natural bioactive substances. Multiple drug resistance and unwanted side effects are constant concerns while treating these illnesses with traditional antimicrobials (7). The treatment of multi-drug-resistant patterns among clinical and environmental isolates has been greatly aided by the discovery and use of medicinal plants as medications. Medicines generated from plants have many advantages over their synthetic counterparts: lower risks, more effective therapy, and lower costs (8). Oral cavity is inevitably affected by smoking, since it is the first part exposed to tobacco smoke. The incidence of periodontal diseases and oral cancer is much higher in smokers than in non-smokers (9). In our previous studies, we also showed that nicotine can promote the formation of bacterial biofilms in multiple bacterial strains isolated from patients (10). Green tea directly inhibits the growth of these bacteria and has been shown to reduce its adhesion to oral areas. Additionally, green tea contains naturally occurring fluoride. (11). both

teeth and gums are affected, which is bad news for your dental health. Bacteria called Mutans Streptococci are primarily responsible for dental cavities (12). The aim of the study was to determine the ability of combination effects the green tea and nicotine extracts on the growth, biofilm formation of salivary *M.S.*

Materials and Methods

This study included forty students aged 18 to 23 from the University of Baghdad/ College of Dentistry. Which stimulated saliva samples were obtained. The volunteers appeared healthy, had no his-tory of systemic disorders, and were not under a lot of stress. They had not taken antibiotics for three weeks prior to the saliva sample collection and were instructed not to eat or drink anything for two hours before the saliva Under standard conditions, according to Tenovuo and Lagerlof (1994) (13). Stimulated saliva samples were collected to obtain the microbial samples. First, each participant was given a piece of Arabic chewing gum (0.5g), and they were advised to chew it for five minutes to activate saliva production. Then, a vortex mixer was used to homogenize the saliva for two minutes. Next, ten-fold serial dilutions were prepared using phosphate buffer saline (14). In order to isolate *Mutans Streptococci*, 0.1ml of dilutions 10-3 and 10-5 was extracted and disseminated in duplicate on Mitis Salivarius bacitracin agar, a selective medium for isolation of *Mutans Streptococci*. Afterwards, the plates were incubated anaerobically for 48 hours at 37°C using a gas Pak provided in an anaerobic jar, followed by a 24-hour aerobic incubation at 37°C., a single colony of *Mutans Streptococci* was added to 10 ml sterile Brain Heart Infusion broth (BHI-B) and then incubated at 37C for 24 hours to obtain an activated inoculum. Also, to identify the microorganisms: the isolates were subjected to Grams 'stain, motility test, catalase test and mannitol fermentation test in Cystine Trypticase Agar. Green tea It prepared according to Cowans (1999) (15). Separate infusions of green tea, each containing z100 grams of dry leaves steeped in (500) milliliters of boiling distilled water, were allowed to cool to room temperature. The infusion has previously been agitated using a magnetic stirrer. The infusion was filtered via Wattman No. (1) Filter paper, and the waste was disposed of. The extract was allowed to dry at room temperature on a Petri plate, and the resulting powder was stored in a firmly sealed glass container in the refrigerator until it was utilized to make various concentrations. Nicotine was it the final concentration of (200) mg/ml. serial dilution with sterile water were prepared from tea extracts and nicotine and sterilized by Millipore filter (0.20 µm). Green tea and nicotine were tested for their antibacterial properties against M.S using agar diffusion method: the minimal bactericidal concentration (MBC) was determine as the lowest concentration of extracts kill the bacteria (16).

Biofilm formation by *Mutans Streptococci* and the biofilm inhibition rate of green tea and nicotine The Congo red agar strategy was used for distinguishing the capacity to produce biofilm by bacterial strains. Plates were inoculated with pure freshly single colony by streaking and incubated at (37 °C for 24 hr.). A positive result indicates either strong production of slime has a crude black colour or light black colonies denote to moderate slime production whereas no product of biofilm (negative) strains developed red or dark pink colonies (17).

Quantitative determination of biofilm formation by selected isolates was performed using ELISA reader on micro titter plate (MTP) assay of 96 wells flat bottom dish by use (1/2) MBC concentration. It was performed by a spectrophotometric method, and the biofilm inhibitory rate was measures by the following equation: (17, 18).

Rate of biofilm inhibition % = 1- (O.D After ÷ O.D before)

Statistical analysis:

Use SPSS .24 for statistical analysis and use Excel prog10. For fig to Descriptive Statistic and Mean, Standard Deviation (SD), Standard Error (SE), Min, Max and In vertical statistic-ANOVA One way by-P-value.

Results

In this study, *Mutans Streptococci* were isolated in mitis salivary bacitracin agar (MSBA) is appeared in the form of convex and rough colonies (Figure 1).

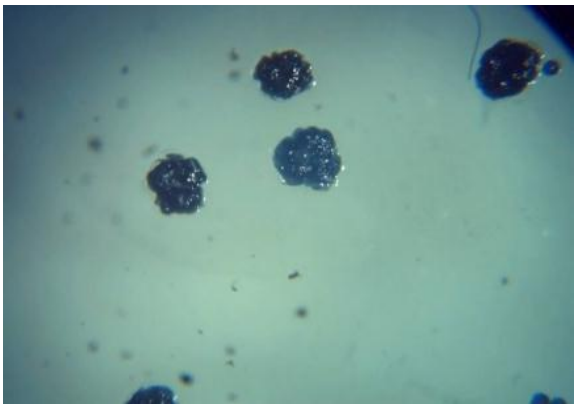

Figure 1: *Mutans Streptococci* colonies on MSBA (10X)

M.S isolates they appear as black colonies on Congo red agar Figure (2).

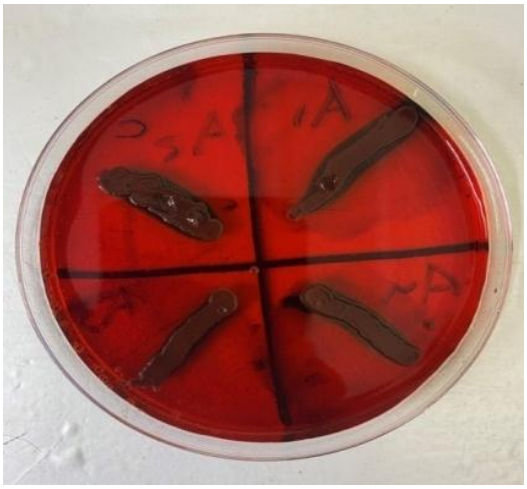

Figure (2): Biofilm formation by *mutans Streptococci* on Congo red agar

All isolates were gram positive cocci , M.S is catalase negative and ferment of mannitol by CTA (Figure 3).

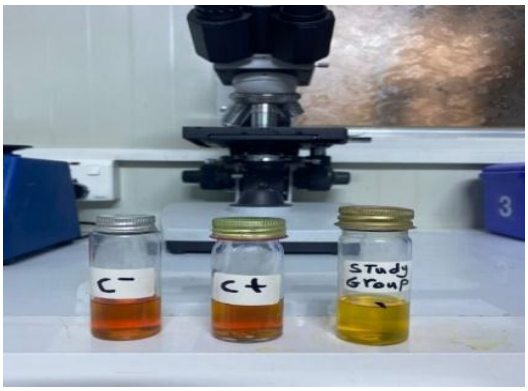

Figure (3): Cystine Trypticase Agar mannitol fermentation test by M.S

Determination the antimicrobial activity of Green Tea and Nicotine The results showed that Mutans Streptococci isolates were sensitive to the Green tea and Nicotine respectively and the diameters of the inhibition-zone was evaluate when the concentrations of the extracts increased table (1& 2) show the minimum, maximum and mean of inhibition zone.

Table 1: The minimum, maximum and mean of the diameter of inhibition zone in millimeter unit of the green tea against *mutans Streptococci*

Conc,	No. of strain	Min	Max	Mean (mm)	SE	SD
300 mg/ml	9	20.00	22.00	21.2778	.27778	.83333
250 mg/ml	9	18.00	21.00	19.3889	.32035	.96105
100 mg/ml	9	15.00	19.00	17.5000	.43301	1.29904
50 mg/ml	9	8.00	9.50	8.6111	.18215	.54645

Table (2): The minimum, maximum and mean of the diameter of inhibition zone of the nicotine against *mutans Streptococci*

Con.	N	Min	Max	Mean (mm)	SE	SD
45 mg/ml	9	8.00	9.50	8.6111	.16197	.48591
35 mg/ml	9	7.50	8.50	8.0000	.11785	.35355
25 mg/ml	9	7.00	8.50	7.5556	.15466	.46398
15 mg/ml	9	6.50	8.00	7.0556	.15466	.46398

ANOVA analysis shows there was highly significant difference between the diameter of inhibition zones of all concentrations for both Green tea and Nicotine table (3).

Table (3): ANOVA test between different concentrations of Green Tea on S.M

Subject	F	P-value	Sig
Between Groups Of strain	313.824	0.000	HS P<0.001

Table (4): ANOVA test between different concentrations of Nicotine on S.M

Subject	F	P-value	Sig
Between Groups Of strain	19.883	0.000	HS P<0.001

The ranges of Minimum Bactericidal Concentration (MBC) of green tea and Nicotine were 100-280, 15-45 mg / ml respectively table (5).

Table 5: Minimum Bactericidal concentration (MBC) of green tea and Nicotine against M.S

	No. of isolates killed within the (MBC) of Tea and Nicotine					
	Subjects					
Nicotine	9	Concentration (mg\ml)				
		5	15	25	35	45
No. Killed strain				1	4	4
Green tea	9	50	100	200	250	280
		No. Killed strain	1	2	2	4

Detaction of anti-biofilm activity of green tea and Nicotine Extracts using microtiter plate assay A study using micro titer plates (MTP) revealed that nine mutans Streptococci isolates develop biofilm, and the anti-biofilm activity of ½ MBC of green tea extract and nicotine (140 mg\ml,17.5 mg\ml alone/ and in combination was determine by measure the O.D590 . Table (6) shows both green tea and nicotine alone was reduced the amount of biofilm with mean of O.D590 = 0.1567, 0.6822 respectively, in comparison to control (without extract or nicotine) O.D590 = 0.9233 with highly significant differences between these groups(green tea, nicotine and control) (F-value = 118.20) (P- value 0.001). Table (7). But the presence of nicotine has a negative effect on the ability of green tea extract to inhibit bacteria biofilm with mean of % of inhibition rate of the biofilm in the presence green tea, nicotine alone and in combination was 82, 25 and 40 , respectively in table (8) .

Table 6: Descriptive statistical analyses of the effect of the green tea and nicotine on biofilm formation by M.S on Multitier Plate method

Subject	N	Min	Max	Mean of O.D 590 nm	SE	SD
Control	9	.82	1.15	.9233	.03531	.10593
½ MBC Nico	9	.55	.80	.6822	.02681	.08043
½ MBC TEA	9	.09	.23	.1567	.01795	.05385
½ MBC TEA +Nicotine	9	.44	.68	.5467	.03149	.09447

Table 7: ANOVA show antimicrobial activity of Nicotine and tea on biofilm formation by M.S

	F	P-value	Sig
Between Groups Of strain	118.020	0.000	HS P<0.001

Table 8: Descriptive statistical analysis of mean of % of inhibition rate of *mutans Streptococci* biofilm in the presence of green tea, nicotine alone and in combination

Subject	N	Mini-mum	Maxi-mum	% of inhibi-tion rate	SE	SD
Broth	9	.00	.00	.0000	.00000	.00000
½ MBC Nico-tine	9	.16	.33	25	.02067	.06200
½ MBC TEA	9	.77	.89	82	.01553	.04658
½ MBC TEA +Nicotine	9	.21	.48	40	.02697	.08090

Discussion

Concentration-dependent inhibitory Effects of green tea and nicotine on M.S Inhibitory effects of tested extracts on *mutans Streptococcus* growth were measured via serial dilution. Regarding, green tea it has possibility of antibacterial activity, in this study results found the growth of M.S defected and inhibition zone increase when the concentration of green tea extract increased and with highly significant difference (*p*- value <0.001). In addition, several studies were indicated that the polyphenols included in green tea extracts have antibacterial effects against bacteria that cause human and animal disease, as well as phytopathogenic bacteria and bacteria that are present in the food (19). The green tea extract of was greatly active exhibiting the highest potency with MBC from (280 mg/ml) to kill all isolate of *mutans Streptococci*. The MBC values obtained showed the green tea extracts has the most potent effect against Mutans Streptococci. This activity may be attributed to the rich plant contents of active components such as tannins, saponins, alkaloids and flavone glycine (20) . On other hands the nicotine extract had an antibacterial effect on all nine isolates at high concentrations (15–45 mg/ml). The concentration (45 mg/ml) of the nicotine has a antibacterial effect against M.S with a mean diameter of inhibition zone (8.6111 mm) compared with other concentrations (15 mg, 25mg, and 35 mg/ml), with highly statistically significant difference between different concentration (*p*- value 0.0001). The nicotine in low concentration is increases the adhesion and growth of M.S but in high concentration its toxic to bacterial cells, with MBC (45 mg/ml) inhibit the growth of 9 isolates of mutans Streptococci, so, a high concentration of nicotine to be has toxic effect on the bacteria. Our result disagreement with several studies for example, these studies noticed the MBC of nicotine is (32 mg/ml) effect on the gram-positive bacterial cells (21). This may be depending on the type of nicotine used, the manufacturer of nicotine, and the type of M.S isolate. Effect of Green tea and Nicotine Extracts on *mutans Streptococcus* Biofilm Formation *mutans Streptococcus* is the most important bacterium in the formation of dental plaque and dental caries. This study evaluating the antibacterial rate and antibiofilm activity of green tea, and nicotine against M.S. which have the ability to biofilm formation. Isolates capable of producing biofilm were identified by cango red agar where it changed the colour to black as a result of the presence of secondary metabolites product, as confirmed by other studies (22) and in other studies by freeman the mechanism of action of the dye is unknown (16). A recent study found that biofilm formation was markedly decreased in the presence of green tea extracts in the Brain hart infusion broth BHIB, compared to control. Furthermore, green tea revealed a greatly decrease in biofilm development than nicotine. Green tea contains four major flavonoids which are: catechins epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (25). Targeting the glucosyltransferase enzymes that convert sucrose in the diet into glucan, the building-block of the exopolysaccharide matrix, EGCG plays a crucial role in limiting the establishment and spread of biofilms (26). The results are line with the other studies indicated low concentrations of nicotine (1, 2, and 4 mg/ml) , Enhanced and increased the growth and metabolic activity of M.S (22). Since the amount of nicotine in human saliva varies from around (0.02) to about (0.08) micrograms per millilitre, our data showed that low concentration of nicotine enhances the development of M.S growth and biofilm formation. Also, The result confirms the previous study that high concentration of nicotine was toxic to bacteria (24). The results showed high concentration of nicotine (45 mg/ml) killed M.S isolates. findings line with study in (2012) conducted by Huang indicated the effects of nicotine on M.S strains and demonstrated that (0.25–8 mg/ml) of nicotine increases M.S biofilm formation significantly while higher concentrations killed the bacteria (27).

Moreover, the addition of nicotine extract decreased the effectiveness of tea extracts in reducing biofilm formation and has negative effect on the effectiveness of other extracts in inhibition of biofilm formation by M.S. Nicotine was shown to increase both the quantity of *S. mutans* and the rate of synthesis of extracellular polysaccharide (EPS), the primary component of the biofilm matrix. Up to (4.0-fold) increased expression of glucan binding protein A (GbpA) and up to (2.2-fold) increased expression of glucosyltransferase (Gtfs) have been linked to nicotine's evolutionary history (24).

Conclusion:

Green tea and nicotine at different concentrations have antimicrobial activity against salivary mutans Streptococci, and their activity increased was increased in a dose dependent manner. The MBC was 280 mg/ml and (45 mg/ml) respectively. Green tea and high concentration of nicotine have anti-biofilm activity by mutans Streptococci. Nicotine increased biofilm formation and metabolic activity of mutans Streptococci in the presence of green tea. Therefore, smokers with a high consumption of tea should consider shifting to other soft drinks and sweeteners in order to minimize their chance of developing dental caries by reducing biofilm formation from mutans Streptococci.

Authors' Declaration:

We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given permission for re-publication attached with the manuscript.-Authors sign on ethical consideration's approval-

Ethical Clearance: The project was approved by the college's in-house ethics committee of Dentistry, University of Baghdad .according to the code number project (No 403821 Date 27-12-2021)

Authors` contribution:

Each of the authors contributed significantly, directly, and intellectually to the work, and they all gave their consent for it to be published.Funding: This research received no external funding

Conflict of interest: The authors have disclosed no potential conflicts of interest.

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How to Cite this Article

alshamary abbas, Al-Mizraqchi AS. Anti-microbial activity of Green Tea Extracts and Nicotine on the Growth, Biofilm Formation of Sali-vary Mutans Streptococci (in-vitro study). JFacMedBagdad [Internet]. 2023 Jul. 1 [cited 2023 Jul. 4];65(2). Available from: https://iqjmc.uobaghdad.edu.iq/index.php/19JFacMedBaghdad36/article/view/2024

النشاط المضاد للميكروبات لمستخلصات الشاي الأخضر والنيكوتين على النمو ، وتشكيل البيوفيلم للمكورات العقدية اللعابية. (دراسة في المختبر)

البكتريولوجي الاختصاص عباس نصيف عباس الشمري /مدينة الطب/مستشفى بغداد التعليمي

الاستاذ الدكتورعباس صبري عبد الرزاق المزرقجي/جامعة بغداد/كلية طب الاسنان

الخلفية: الشاي الأخضر له نشاط مضاد للميكروبات ضد أنواع عديدة من البكتيريا ، ويعتبر مادة طبيعية ذات آثار جانبية قليلة.

الهدف من الدراسة: أجريت دراسة في المختبر للتحقق من قدرة مستخلص الشاي الأخضر والنيكوتين على تثبيط النمو وتكوين الأغشية الحيوية بواسطة المكورات العقدية اللعابية.

الطريقة العمل: تضمنت هذه الدراسة عينة ملائمة من 40 متطوعا عراقيا أصحاء تتراوح أعمارهم بين 18 و 23 عاما من كلية طب الأسنان / جامعة بغداد. تم تحضير الشاي الأخضر ومستخلص النيكوتين المائي بتركيز مختلف لاستخدامه في طريقة انتشار الآجار للكشف عن نشاط المستخلص ، وتم استخدام قارئ ELISA في لوحة متعددة العيار لتحديد قدرة المكورات العقدية اللعابية على تكوين غشاء حيوي في وجود وغياب المستخلصات لقياس معدل تثبيط الغشاء الحيوي.

النتائج: كانت المكورات العقدية الطافرة حساسة للشاي الأخضر والنيكوتين بتركيزات مختلفة كانت أقطار منطقة التثبيط فعالة بطريقة تعتمد على الجرعة بشكل كبير. كان هناك فرق معنوي بين تراكيز كل مستخلص ، وكان النشاط المضاد للبكتيريا في طريقة تعتمد على الجرعة بالنسبة للمستخلصات. كان الحد الأدنى لتركيز مبيد الجراثيم للشاي الأخضر (280 مجم / مل) وأقل تركيز مبيد للجراثيم من النيكوتين (45 مجم / مل). ووجدت الدراسة أن تكوين الأغشية الحيوية بواسطة بكتيريا Mutans Streptococci انخفض بشكل ملحوظ في وجود 2/1 الحد الأدنى من تركيز مبيد الجراثيم لكل من الشاي الأخضر والنيكوتين بمتوسط 590 نانومتر = 0.54 مقارنة بمستخلصات الشاي الأخضر والنيكوتين وحده O.D 590 نانومتر = 0.15 ، 0.68 على التوالي.

الاستنتاجات: مستخلصات الشاي الأخضر والنيكوتين بتركيزات مختلفة قللت بشكل فعال من تكوين الأغشية الحيوية للمكورات العقدية اللعابية. بينما أثر وجود النيكوتين سلبًا على قدرة مستخلصات الشاي الأخضر في تثبيط تكوين البيوفيلم بواسطة بكتيريا Mutans Streptococci في المختبر.

الكلمات المفتاحية: العقديات الطافرة ، نشاط الأغشية الحيوية ، مضادات الميكروبات ، الشاي الأخضر ، النيكوتين.