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ESTIMATION OF ELLAGIC ACID ACTIVITY WHEN MIXED WITH SOME TYPES OF CANDY AGAINST *Streptococcus mutans* ISOLATED FROM ADULT PATIENTS IN BAGHDAD CITY

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ABSTRACT

Microbial activity of Ellagic acid when mixed with some types of candy toward *Streptococcus mutans* microorganism was studied. The main purpose of carrying out this study is to produce a new type of candy that contains Ellagic acid in addition to xylitol instead of sucrose to prevent dental caries. The results show that the inhibitory action of Ellagic acid was more effective when mixed with this type of candy for the purpose of reducing *Streptococcus mutans* microorganisms, while sensory evaluation was applied in this study to 20 volunteers to that candy sample evaluated which contain (5 mg/ml) Ellagic acid with 100g xylitol to determine consumers acceptability of this sample of candy. The results were expressed as mean value, slandered deviation of the variables, in this in vitro and in vivo study which data processing and analysis were carried out by using SPSS program, as the limit of significance, when the analysis was accepted at P<0.05.

Key words: Ellagic acid, Candy, *Streptococcus mutans*, Xylitol.

تقدير نشاط حامض الاليجيك عند مزجه مع بعض انواع الحلوى ضد بكتريا العقدية الطافرة المعزولة من متبرعين في مدينة بغداد.

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الخلاصة

تم دراسة النشاط الميكروبي لحامض الإلجيك عند مزجه مع بعض انواع الحلوى لدرء الكائنات الميكروبية (بكتريا العقدية الطافرة) *Streptococci mutans*،وان الهدف الرئيس من اجراء هذه الدراسة هو انتاج نوع من الحلوى تحتوي على حامض الاليجيك فضلا عن الزايليتول (xylitol) بدلا من السكر لمنع تسوس الأسنان، واظهرت النتائج ان التأثير المثبط لحامض الاليجيك كان اكثر فعالية عند مزجه مع هذا النوع من الحلوى لغرض تقليل البكتيريا، إذ تم تطبيق التقييم الحسي في هذه الدراسة على عشرين من المتطوعين على عينة الحلوى التي تم تقييمها والتي تحتوي على (5 ملغم/مل) حامض الالجيك و100غم من الزايليتول لتحديد تقبل المستهلك في هذه العينة من الحلوى، وقد تم التعبير عن النتائج على القيمة المتوسطة والانحراف المعياري للمتغييرات في هذه الدراسة وتطبيقها داخل الفم وخارجه والتي تم معالجة البيانات وتحليلها بإستعمال برنامج SPSS للدلالة عند قبول التحليل عند P<0.05 .

الكلمات المفتاحية: حامض الاجيك، حلوى، بكتريا العقدية الطافرة، زايليتول.

INTRODUCTION:

Dental caries is a process in which the enamel layers of the teeth are demineralised by acids produced by bacterial fermentation of carbohydrates, It is the most common infectious oral diseases that affects all age groups of human being,that progress slowly which found in both primary and permanent teeth **(Matsui & Cvitkovitch, 2010; Trentesauxo *et al*., 2011)**.
The most essential causative agents of caries initiation are a group of Streptococcal species collectively referred to as the *mutans streptococci* in which *Streptococcus mutans* and

Streptococcus sobrinus are the famous agents associated with dental caries **(Kuramitsu & Wang, 2011).**

Numerous studies have shown Streptococcus mutans are the principal aetiological agents of dental caries, It showed positive relationship between Streptococcus mutans with the initiation of carious lesion because have several properties which are: acid producing ability, formation of insoluble extracellular polysaccharides, formation and utilization of storage polysaccharides and acid –uric **(Nicolas & Lavoie, 2011; Muslim, 2009)**.

Dental caries can be controlled by blocking the transference of Streptococcus mutans and control of carbohydrate composition **(Matsui & Cvitkovitch, 2010; Kidd & Bechel, 2002)**. Axelsson et al study showed dental caries can be prevented by good cleaning of the teeth which have been obtained from proper dental flossing and tooth brushing **(Axelsson, 1999**).

Chemo prophylactic agents are used in the prevention of dental caries by antibacterial effects that reduced bacterial flora of the oral cavity and these agents were classified by Nolte (1982) to antibacterial and fluoridated mouth wash that aids in the prevention of dental caries,while Mandel (1988) classified them according to mechanism of action to antiseptics, antibiotics, single or combination of enzymes, agents that interfere with bacterial attachment and other groups including non-enzymatic or modifying agents **(Muslim, 2009)**.

Mouth rinses are the simplest vehicle for antiplaque agents like Listerine and Chlorhexidine have been recognized by the American Dental Association (ADA) as effective agent's plaque and gingivitis **(Harris *et al.*, 1995)**. Chlorhexidine effectiveness has proved in reducing cariogenic bacteria such as *Streptococcus mutans* in the plaque biofilm, it binds strongly to bacterial cell permeability, initiate leakage and/or precipitate intracellular components **(Jenkins *et al.*, 1988)**. A recent trend in processing mouth rinses is to avoid chemical preservative, thus the use of natural products from plants which have natural antimicrobial alternative are one of the most successful program for the discovery of new drugs especially used for prevention of dental caries and periodontal disease **(Kadhem *et al.*, 2010**; **Aneja *et al.*, 2009**; **Muslim, 2009)**.

Ellagic acid is a naturally poly phenolic compound found in different plants such as fruits of high amount in pomegranates that has strong antioxidant properties ,also has antiviral and antibacterial activities **(Kadhem *et al.*, 2010; Muslim, 2009)**.

Iraqi study was able to be estimated the antibacterial activity of Ellagic acid on *Streptococcus mutans* that may repress the growth and adherence of these microorganism bacteria in comparison to Chlorhexidine gluconate 0.2% therefore, Ellagic acid might be a favourable compound of future success as antibacterial agents averse to oral microorganism especially *Streptococuss mutans* and other bacteria causing dental caries **(Muslim, 2009)**. Anew study showed that Ellagic acid is a potentially useful when applied to oral hygiene regimens such as mouth rinse or chewing gum and candy **(Wings *et al.*, 2010).**

A sugar alcohol is a pleasant type of carbohydrate. Its texture is a form sweet to gum called “sugar alcohol”. It’s also sweet to the tongue and it is impervious to chemical break down of a substance by oral microorganism, meaning sugar-free gum manufacturers take on it discreetly to sweeten their products without causing cavities. Sugar alcohols are non-caloric, but all supply fewer calories than sucrose. Xylitol is a naturally occurring sugar alcohol found in most plant material, including many vegetables and fruits. Xylitol one of the more popular sugar alcohols, it tastes signally similar to sucrose, but it has about half the calories, we can find xylitol in corn husks, certain berries and mushroom fibres, it is extracted to make medicine **(Tanzer, 1995)**. Xylitol is widely used as a sugar substitute and in 'sugar free' chewing gums,

mints and others candies ,it is added to some chewing gum and other oral care products to avoid dry mouth and to prevent dental caries (**Hanson & Campbell, 2011**; **Assev & Rölla, 1986)**. Xylitol have been consider as the preferable choice of sugar-free chewing gum makers according to Its refreshing and cooling effect on the mouth and has good protective action and well-established preventive actions against dental plaque and then it can contribute in prevention of dental caries, Xylitol may be promote tooth remineralisation, in which positively affecting both tooth enamel and bone mineral density **(Hanson & Campbell, 2011; Tanzer, 1995).** It has been demonstrated that restriction of dietary sucrose reduces the level of *Streptococcus.*Sucrose is important for both glucan-mediated adhesion and acid production. Xylitol, sorbitol, saccharin, and aspartame have all been used as sugar substitutes for reducing dental caries in a wide variety of products including sweets, candies, chewing gum, oral hygiene products and pharmaceutical products, Xylitol is a five-carbon sugar that has the same sweetness as sucrose, but is not fermented by oral bacteria, it is also promotes remineralisation of the tooth, so early carious lesions can be arrested **(Hanson & Campbell, 2011; Tanzer, 1995; Assev & Rolla, 1986).**

MATERIAL AND METHODS:

Preparation of Ellagic acid and mixed with Candy

Ellagic acid powder was prepared, purified and characterized as reported in previous study of Ellagic acid which is prepared from a white flesh pomegranate after identified the prepared pure Ellagic acid with the standard pure Ellagic acid obtained from Sigma Company, Sigma E-2250 **(Muslim, 2009).**

The Previous study of **Muslim (2009)** has proved that the effectiveness of 5mg/ml Ellagic acid concentration was the optimum inhibition for *Streptococcus mutans*. To compare the inhibition of pure Ellagic acid antibacterial solution with Candy have the same value of Ellagic acid (5mg/ml), we made the Candy in the normal way but there are some points will be included which are:

1.100 gm. of xylitol + 200 ml water + 5ml acetic acid + 0.5 gm. flavour mint and color, this is the acceptable concentration after using different concentration of xylitol between four selection amount which are (50gm. ,100gm., 150gm. and 200gm.) after the application of sensory analysis in the present study and shown in (Table, 1)

2. Mix the above quantity in a pot, boiling for 5 minute and before solidation of the mixture, add the active ingredient agent which is Ellagic acid (5mg/ml) to each cast of silicon and then cool in cold water bath the mixture in cast of silicon to for 20min. get solid candy sample from each cast of silicon ready for use. Sensory analysis of the tested ready candy samples was carried out using (20) member volunteers consisting of staff of chemical Department of Ministry of Science and Technology. The sensory attributes evaluated were, Taste, Flavour, Mouth feel, Color /Appearance and General acceptability. The variously treated candy samples were served in clean way to individual volunteers.

The order of presentation of samples to the volunteers was randomized, potable water was provided for them to rinse their mouth between evaluations to avoid transfer of sensory attributes from one candy sample to the other. Each sensory attribute was scored on a 7-point hedonic scale as described by **Iwe, (2010)** with 1 and 7 representing the least and the highest scores, respectively as shown in (Table, 1**)**.

The present study involved collection of stimulated salivary samples from ten-male volunteer patients; they are 24-38 years old. The procedures were performed under the conditions following the criteria described by **Tenovuo & Lagerlof (1994**), then isolation of

Streptococcus mutans were diagnosed according to their morphological characteristics on Mitis Salivarius Agar (MSA), and maintenance of bacterial isolates that checked for purity by inoculation on Mitis Salivarius Bacitracin (MSB) agar plates incubated anaerobically for 48 hour at 37C. They Followed by incubation aerobically for 24 hour and continue the procedure to estimate the viability counts of SM in vitro, in vivo study to the Ellagic acid mixed with candy the volunteers involved in this study were male persons ,they were study group, it was conducted in Chemistry laboratory of Ministry of Science and Technology, average ages was ranging between (30-38)years the total number of volunteers were (30) and they were divided into three groups, (each group consist of 10 volunteers), the first group is the experimental group instructed each person to sucking on one study candy sample which contain(5mg/ml) Ellagic acid as explain its contents which have been made in this study while the second group instructed each person rinsing with pure (5mg/ml) Ellagic acid mouth rinse as control positive and the third group used de-ionized water mouth rinse as control negative, each group of volunteers has been instructed to use these three sample for one minute, then expectorate, stimulated salivary samples were recollected after one minute, 30 minutes, one hour and two hours, during this time volunteers were asked not to eat or drink anything except water. Sample of saliva were processed immediately, they were dispersed for one minute by vortex mixer, and then 0.1ml of saliva transferred to 0.9ml of Phosphate buffer saline (PBS), and tenfold dilutions were performed. From the dilution 10^{-3}, 0.1ml was taken and spread in duplicate on the surface of MSA and MSB agar plates, then incubated an aerobically for 48 hour at 37 C, and aerobically for 24 hour at room temperature. Following identifications, colonies of *Strepto cocci* on MSA and mutans streptococci on MSB agar plates were counted by the use of colony counter. The counts were expressed as the CFU colony forming unit taking in consideration dilution factor x10(to be)/ ml of saliva (CFUlml) **(Muslim, 2009).** We examine the inhibition of *Strepto coccus mutans* by (5mg/ml)Ellagic acid with that candy consist of sugar free which are 100 gm xylitol. They compared with pure 5 mg/ml Ellagic acid and deionized water on Mueller Hinton agar (MHA), then estimated the activity of the viability counts of MS in vitro by agar well diffusion technique of two sample of Ellagic acid**.**

Table (1) sensory properties of Candy with Ellagic acid (5mg/ml) mixed with (xylitol) with different concentration (50gm, 100gm, 150gm and 200gm.).

(xylitol in gram	Color	Taste	Flavour	Mouth fell	General acceptability
50 gm.	5.2	3.6	5.0	5.1	4.8
100gm.	6.1	4.8	5.8	6.2	6.6
150 gm.	6.0	4.9	5.8	6.1	6.6
200 gm.	6.1	4.7	5.9	6.1	6.5

RESULTS AND DISCUSSION:

Colonies of Streptococci were identified, as they grew on mitis salivarius agar plates. They were light blue or violet in colour and about 1mm in diameter. They were gram-positive spherical or ovoid cells arranged in short or medium in length chains. Streptococci colonies were examined and diagnosed according to their morphological characteristics on Mitis Salivarius Bacitracin agar plates Bacitracin. They appeared as spherical or ovoid in shape with raised or convex surface, Under microscopic examination showed that *streptococcus mutans* cells were gram positive, spherical or ovoid in shape arranged in short or medium length none-spore forming chains when stained specimen were taken from agar plate (**Kadhem *et al.*, 2010**; **Muslim, 2009)**. Sensitivities of *Streptococcus mutans* to (5 mg/ml) Ellagic acid with candy, pure(5 mg/ml) Ellagic acid and deionized water in vitro was determined by using agar

well diffusion method, the diameter of inhibition zones were measured, which had been increased in case of (5mg/ml) Ellagic, in which (5 mg/ml) Ellagic acid with study candy show higher zone of inhibition compared to the same concentration of pure Ellagic acid with highly significant differences ($p<0.001$) as in (Table, 2). While De-ionized water shows no zone of inhibition. Statistical tests by using t-test were performed between two different sample of Ellagic acid and de-ionized water. The analysis was accepted as $p<0.05$ as the limit of significance when $p<0.001$ were regarded as highly significant.

Table (2): Inhibition in (millimetres) mean and SD (standard deviation) Statically test between acid (5mg/ ml) Ellagic &candy contain5mg/ ml Ellagic and Deionized water.

Variable	Mean	SD	T-test	Sig
Deionized water	0.0	0.0		
Ellagic acid 5mg/ml	22.60	0.862	7.415	0.00169
Ellagic acid 5mg/ml with candy	26.80	0.992		

Salivary viable counts of *Streptococcus mutans* were estimated for the three groups, after single rinse with (5mg/ml) pure Ellagic acid, deionized water for five time intervals and after sucking on that candy of (5mg/ml) Ellagic acid for five time intervals among group of the volunteers. For both study and control samples with (5mg/ml) pure Ellagic acid, deionized water, reduction in the viability counts of were noticed at one minute immediately after rinsing in 5mg/ml pure Ellagic acid as a mouth rinse and taken candy contain 5mg/ml Ellagic acid by sucking on, reduction in the counts of *Strepto coccus mutans* continued after 30 minutes, but there were increased in the counts in the following two hours. (5mg/ml) Ellagic acid with candy sample had the maximum reduction in the bacterial counts followed by (5mg/ml) pure Ellagic acid, while deionized water had the least reduction of the bacterial counts *Strepto coccus mutans* as seen in (Table, 3) and (Figure, 1) .

The differences among the counts of *Streptococcus mutans* for the three groups were examined by ANOVA test between groups in (Table, 3). We noticed that there was non-significant difference ($P>0.05$) before rinsing and, significant difference ($P<0.05$) at 1 minute and highly significant difference ($p<0.001$) after 30 minutes, 1 and 2 hour. When each two groups compared by using t-test as seen in **(**Table 4**),** The results showed that significant difference ($P<0.05$) found between (5mg/ml) pure Ellagic acid and deionized water at one minute, while highly significant differences ($p<0.001$) were found between each pair of the three groups at 30 minutes and the following time intervals (1 and 2 hour) as seen in (Table, 5)

Table (3): *Streptococcus mutan*s Count Forming Unit/ Millimetre (CFU) for pure Ellagic acid (5mg/ml) Ellagic acid (5mg/ml) with candy and deionized water**.**

Time	Deionized water		Ellagic acid 5mg/ml		Ellagic acid 5mg/ml with candy	
	Mean	SD	Mean	SD	Mean	SD
Base line	713.3	5.64	705.4	5.45	720.8	7.29
One min.	716.1	9.62	617.3	19.8	554.0	24.8
30min.	721.9	6.09	389.2	18.56	331.2	30.77
One hour	723.1	9.41	532.4	25.4	446.4	27.4
Two hour	723.2	6.25	560 .1	34,7	566.2	11.48

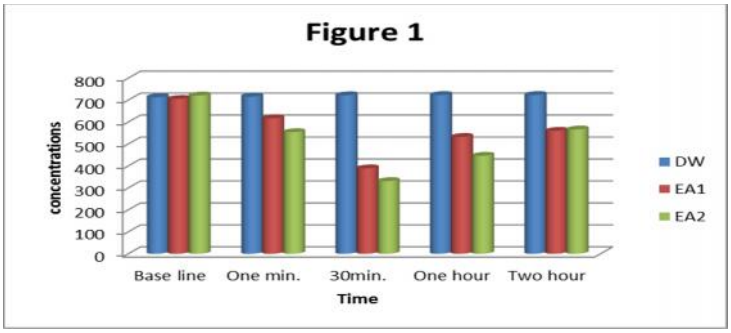

Figure (1): CFU/ml for three groups' samples (5 mg/ml) pure Ellagic (EA1),(5mg/ml) Ellagic acid (EA2) with candy and deionized water (DW) on viability count of MS $X10^3$

Table (4): Least Significance Differences (LSD) between groups of table (3).

Time	D.water & pure Ellagic acid		D.water & Ellagic acid with Candy		pure Ellagic acid & Ellagic acid with Candy	
	Mean difference	P	Mean difference	P	Mean difference	F
Base line	5.23	0.349	8.3	0.53	13.8	0.041
One min.	103.5	0.00	166.3	0.00	554.0	0.003
30min.	332.4	0.00	395.2	0.00	331.2	0.001
One hour	189.9	0.00	266.4	0.00	446.4	0.011
Two hour	156.3	0.001	148.9	0.00	566.2	0.155

Table (5): A NOVA test between groups of table (3).

Time	F-test	P-value	Sign.
Base line	2.04	0.416	NS
One min.	10.68	0.00	HS
30 min.	48.56	0.00	HS
One hr.	21.68	0.00	HS
Two hrs.	82.1 9	0.00	HS

The three sample study (EA2), control positive (EA1) and control negative (DW), in this study produce immediate increase in salivary flow rates which continue to increase after 30 minutes, then began to drop down slowly until it approximate the base line after 120 minutes. The possible explanation is that any mechanical stimulation can increase the salivary flow rates (**Sreebny, 2000**). The effects of study sample (EA2) lead to slightly increase in the salivary flow rates than control positive (EA1) and control negative (DW) as shown in **(**Table, 6**)** and (Figure, 2).

Table (6) Mean and standard deviation of salivary flow rate before and after taking of types of pure, Ellagic acid and with candy and deionized water. Two types of pure Ellagic

Time	Deionized water flow rate		Pure Ellagic acid flow rate		Ellagic acid with candy flow rate	
	Mean	SD	Mean	SD	Mean	SD
Base line	3.40	0.21	3.42	0.32	3.05	0.19
One min.	3.42	0.24	3.45	0.25	3.36	0.14
30min.	3.60	0.22	4.02	0.26	3.72	0.18
One hour	3.30	0.27	3.36	0.21	3.42	0.22
Two hour	3.02	0.24	3.10	0.22	3.20	0.23

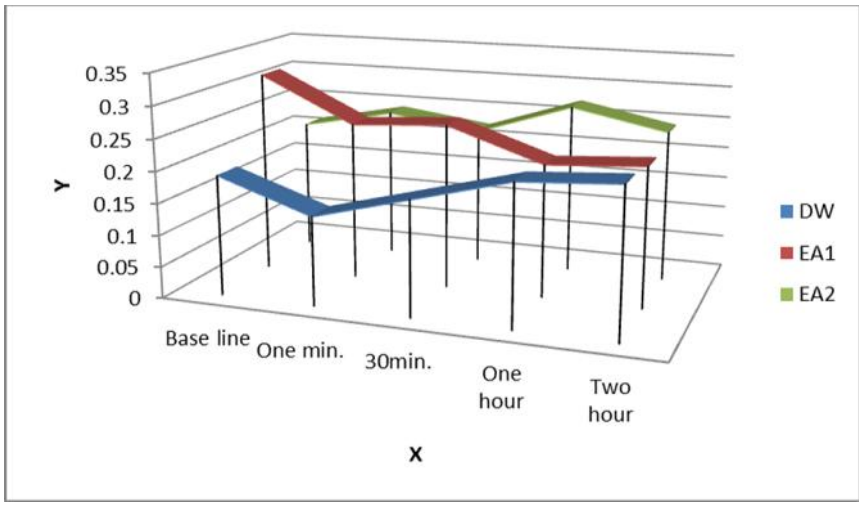

Figure (2): salivary flow rates ml/min of (EA1), pure Ellagic (5mg/ml), Ellagic (5mg/ml) With candy and deionized water, X=time and Y=concentration.

The results showed that(5mg/ml) pure Ellagic acid were able to inhibit the growth of *Streptococci mutans*, this fact had been predicted that Ellagic acid had antibacterial effects against *Streptococci mutans* and inhibit its growth this finding were in coincidence with (**Muslim, 2009)** On the other hand (5mg/ml) Ellagic acid with candy sample showed more effective in reduction of viable counts of *Streptococcus mutans* than of (5mg/ml) pure Ellagic acid in vitro and in vivo on the plaque bacteria in general, The zone of inhibition of MS at (5mg/ml) Ellagic acid with candy sample was higher than that at 5mg/ml pure Ellagic acid with significant differences. this fact may be explained by the activity of combination of Ellagic acid and the mixture of sugar free used which is xylitol this finding were in coincidence with (**Tanzer, 1995 ; Assev & Rölla, 1986**).

Previous studies showed Ellagic acid that may have the ability to interfere with growth, metabolism and/or enzymatic activity of bacteria (**Muslim, 2009**; **Lesso *et al.*, 2004)**.

When aqueous extract of 5 mg/ml Ellagic acid were tested for its effects on salivary MS colony forming unit counts among group of volunteers in comparison to (5 mg/ml) Ellagic acid mixed with candy sample and deionized water in this study,bacterial counts were estimated at different time intervals including as a base line, (1,30,60 and 120min). The results of this study

indicate that (5 mg/ml) Ellagic acid mixed with candy sample had highly significant antimicrobial activity against *Streptpcocci mutans*, it can reduce the viable count of bacteria in comparison to (5 mg/ml) Ellagic acid after 30 and 60min. and following times. The immediate slight reduction in the viable counts of bacteria after administration by volunteers may be explained by mechanical removal of different types of oral flora (**Peter**, **2003; Wyler & Miller, 1990**) and for these results, we can estimate that Ellagic acid may affect the progression of caries because it can affect the viability counts, adherence, and retard acid production of MS, been contributed in prevention of caries, therefore it may be preferable to use (5 mg/ml) Ellagic acid mixed with candy sample for several times daily, so reduction of caries activity will occur. We can estimate the effects of (5 mg/ml) Ellagic acid mixed with candy sample, 5 mg/ml Ellagic acid and de-ionized water on salivary flow rates (ml/min). They produced immediate increase in salivary flow rates which continue to increase after 30min. then began to drop down slowly until it approximate the base line after 60min. The possible explanation is that any mechanical stimulation in the administration can increase the salivary flow rates **(Dawes, 1987)**, the effect of candy sample with (5mg/ml) Ellagic acid lead to slightly increase in salivary flow rates than the two other samples after 30 min. from start using in the mouth.

CONCLUSION:

We can concluded that the study sample of candy when taken orally yield oral health benefits that include reduction in oral dryness, increase of biofilm PH and reminalization of enamel. The candy sample with active ingredient aim to increase these effects and lead to inhibition of oral microorganisms especially *Streptococcus mutans* which represent the main cause of initiation of dental caries and periodontal disease.

The results show that the inhibitory action of Ellagic acid more effective when mixed with this type of candy in addition to Xylitol instead of sucrose for the purpose of reducing *Streptococcus mutans* microorganisms, and contribute in prevention of dental caries and periodontal disease, then play essential role in oral health care in the future. It has been considered that Ellagic acid is useful as an easy-good way upon application to candy with xylitol in which facilitate prevention and removal of oral biofilm.

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