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In Vitro Synergistic Effect of Xylitol with *Salvadora presica* L Extracts and Cephalexin on *Streptococcus mutans* Strains

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Abstract: Sixty six *Streptococcus mutans* isolates have been tested with cephalexin and *Salvadora presica* L extracts with or without addition of glucose, or xylitol to determine minimal inhibitory concentration (MICs), susceptibility of *S. mutans* isolates and time-killing curve. Result: All the strains were sensitive to cephalexin (12.8. ug/ml), *S. presica* aqueous extract (16.0 mg/ml), *S. presica* methanol extract (4.0 mg/ml), *S. presica* ethanol extract (12.0 mg/ml) and *S. presica* hexane extract (8.0 mg/ml). The addition of xylitol increased susceptibility of *S. mutans* isolates to the MIC values of cephalexin and *S. presica* extracts, whereas the addition of glucose decreased susceptibility of *S. mutans* to the MIC values of cephalexin and *S. presica* extracts. Time-killing curve of cephalexin combined with xylitol exhibit zero CFU/ml after 16 hrs. and xylitol combined with *S. presica* hexane extract exhibit zero CFU/ml after 18 hrs with highly significant values. Conclusion: The mix of xylitol with *S. presica* extracts or cephalexin may lead to inhibit microbial causative agents of dental decay, while addition of glucose has opposite effect.
Keywords: *Streptococcus mutans, Salvadora presica*, cephalexin.

1 Introduction

Dental decay is due to the dissolution of tooth mineral by acids derived from bacterial fermentation of sucrose and other dietary carbohydrates. *Streptococcus mutans* is naturally present in the human oral microbiota, along with at least 25 other species of oral streptococci and consider the main cause of dental decay [18,5]. *Streptococcus mutans* characterized by it's a Gram-positive, non-motile, non-spore forming, facultative anaerobic cocci bacterium commonly found in the human oral cavity [7]

Good oral and dental hygiene can be achieved by avoiding sugary sweets and regular brushing to avoid tooth decay.

Salvadora persica L, has important role in the oral hygiene [31], because it has many antimicrobial agents such as flavonoid [14], glycosides [15], fluoride [10], sulfur-containing organic substances [21], and several anionic components [14]

Sucrose is the only sugar that bacteria can use to form this sticky polysaccharide [25], while glucose sugar fermented by *S. mutans* strains to produce lactic acid [19]. On the other hand, fermentable sucrose sugar was substituted by non-fermentable xylitol sugar for growth inhibition of *Streptococcus mutans* [6]. Xylitol, a naturally in fruit, vegetables, and berries and is artificially manufactured from xylan-rich plant materials [22, 3].

This research aimed to determine the effect of antimicrobial activity of *Salvadora persica* L extracts and cephalexin with or without addition of xylitol against *Streptococcus mutans* isolates**.**

2 Materials and Methods

2.1 Streptococcus Isolation And Identification Method

Streptococcus sp were isolated from buccal surfaces of the caries teeth of patients of the general dental clinic (Faculty of dentistry, Al-Azhar University, Assiut branch, EGYPT) were grown on MS (mitis-salivarius agar) plates and incubated anaerobically, using anaerobic candle jar, for 48 hrs. at 37C. Count of more than 250 colonies (10^4cells/ml) was considered as positive samples [12,11]. Colonies grown on MS-agar medium was transferred and purified on the blood agar plates and incubated anaerobically for two days. The identification of *S. mutans* according to Bergeys Manual of Determinative Bacteriology 9th., 1994. Confirmation of the identification by using commercial kit ApI20 strep and Dextran Production Test [13].

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2.2 Cephalexin

Cephalexin Sigma-Aldrich -C4895 was used in this study.

2.3 Xylitol And Glucose

Sugar used in this study glucose and xylitol where purchased from Sigma-Aldrich Co**.**

2.4 Plant Samples

S. persica were purchased from local market at Assiut city, EGYPT. *S. persica* imported to Egypt from Saudi Arabia. (AlHaramin Company)

2.5 Preparation Of S Presica Extracts

The air-dried plant materials were ground into fine powder in grinder and extracted with distilled water, methanol 80%, ethanol 95% and hexane. A 100g sample of ground plant was soaked in 500 ml solvent. The extract was filtered through a Buchner funnel with Whatman filter paper, after filtration of total extracts; the extracts were evaporated under reducing pressure to dryness at 45 ◦C on a rota-evaporator (Büchi R114), all extracts were soluble in dimethylformaamid (DMF) with exception of the aqueous extracts dissolved in distilled water.

2.6 Determination Of Minimal Inhibitory Concentration (MIC) [4]

An agar dilution method used to determine the minimal inhibitory concentration (MIC) for cephalexin and all *S. persica* different extracts.. Serial concentrations of all tested extracts were achieved (% v/v) in plates containing BHI agar medium. Petri plates of BHI agar containing various concentrations of cephalexin or *S. persica* extracts were inoculated with 24 h culture of bacterial strain by spreading using glass rod in triplicates Each antibacterial test also included plates containing the culture medium plus solvent to obtain a control of the solvent antimicrobial effect. After inoculation procedures, using triplicates, test plates and control plate were then incubated at 37°C anaerobically using anaerobic candle jar in presence of 5% CO_2. Plates were evaluated for the presence or absence of visible growth of each strain after 24, 48 and 72 hrs of incubation. The absence of colonies on all plates tested was considered as an inhibitory effect. The lowest concentration of cephalexin or extracts required to inhibit the growth of the tested microorganism completely was designated as the MIC.

2.7 Synergistic Effect Of Xylitol With Cephalexin And S presica L Extracts [4]

The cells were cultured in 5ml Brain Heart Infusion (BHI) overnight at 37 °C. The cells were transferred to fresh BHI. Sets of plates contain 1% concentration of glucose with *S presica* L extracts, set of plates contain xylitol with *S presica* L extracts, set of plates contain glucose with cephalexin, and set of plates contain xylitol with cephalexin. After inoculation procedures, using triplicates, test plates and control plate were then incubated at 37°C anaerobically using anaerobic candle jar in presence of 5% CO_2. *In vitro* synergism assays of xylitol in combination with cephalexin and/ or with different *S. perisica* L extracts were carried out after evaluating the MIC of all test different extracts on BHI agar medium by agar dilution method.

2.8 Assessment Of Killing Curve Of Xylitol With Cephalexin And Hexane S Presica L Extract [4]

Selected strains of *Streptococcus mutans* isolates were grown overnight in BHI broth medium. 0.2 ml of inoculums was added to 20 ml BHI flasks containing antibacterial agent 4 MIC for cephalexin plus 1% xylitol, (1MIC) for *S. presica* L hexan extract, plus 1% xylitol in combination. Flasks were then incubated at 37°C using anaerobic candle jar in presence of 5% CO_2. After 2 hrs. the flasks were strongly agitated and a 0.1ml sample was diluted and plated; and the flasks were immediately returned to incubation.

2.9 Statistical Analysis

All experiments were carried out with three replicates. Statistical tests Analysis of variance (ANOVA) was performed.

3 Results

3.1 Determination Of Antibacterial Activities Of Cephalexin Against S. Mutans Isolates

According to the present results, the minimal inhibitor concentration of cephalexin on *Streptococcus mutans* ranged between 0.4 and 12.8 µg/ml where results exhibited 6 isolates sensitive for 0.4 µ g/ml, whereas MIC for 10 isolates was 0.8 µg/ml, 16 isolates MIC was 1.6 µ g/ml, 34 isolates MIC was 3.2 µg/ml, 47 isolates MIC was 6.4 µ g/ml, and 66 isolates was sensitive to 12.8 µg/ml of cephalexin compared with cephalexin plus glucose where number of susceptible isolates decreased. Highest increasing ratio of susceptibility by addition of xylitol was at 0.8 µ g /ml of cephalexin by 62.1% while addition of 1% glucose decreased susceptibility of isolates by 21.2% at 6.4

µg/ml of cephalexin (Table 1., Figure 1)

3.2 Determination of antibacterial activities of extracts of S. presica L

According to the present results, the minimal inhibitory concentration of *Salvodora presica L* extracts on Streptococcus mutans isolates ranged between 6 and 16 mg /ml of aqueous extract, 2 and 4mg /ml of methanol extract , 6 and 12 mg /ml of ethyl alcohol extract , while hexane extract exhibited 4 and 8 mg /ml. Number of susceptible isolates varied according concentration of extracts and solvent types, where *S. presica L* aqueous extract exhibited 15 isolates sensitive for 4 mg/ml, whereas MIC for 20 isolates were 8 mg/ml, and all 66 isolates were sensitive to 16 mg/ml. *S. presica L* methanol extract exhibited five isolates sensitive for 2 mg/ml, whereas 33 isolates were sensitive to 3 mg/ml, and all 66 isolates were sensitive to 4 mg/ml. *S. presica L* ethanol extract exhibited twelve isolates were sensitive to 6 mg/ml, whereas 16 isolates were sensitive to 8 mg/ml, and all 66 isolates were sensitive to 12 mg/ml. *S. presica L* hexane extract exhibited eight isolates were sensitive to 4 mg/ml, whereas 17 isolates were sensitive to 6 mg/ml, and all 66 isolates were sensitive to 8 mg/ml.

By addition of 1% glucose into *Salvadora presica L* extracts in mixture exhibited decreasing *Streptococcus mutans* susceptibility numbers at all concentration, whereas by addition of 1% xylitol exhibited increasing of susceptibility numbers of *Streptococcus mutans*, where the number of susceptibility increased by 54.5% at 6mg/ml of SHE, followed by 25.8% at 8mg/ml SEE, 19.7 % at 8 mg/ml SEE and 16.7% at 3 mg/ml SME (Table 2, figures 2, 3, 4 & 5)

Table 1. Antibacterial activities of cephalexin, cephalexin with glucose & cephalexin with xylitol against 66 *Streptococcus mutans* isolates

Antibiotic	Susceptibility	Concentration (µ g /ml)					
		0.4	**0.8**	**1.6**	**3.2**	**6.4**	**12.8**
Ce	**No. of susceptible strain**	6	10	16	34	47	66
	% No. 0f susceptible strain	9.0	15.2	24.2	51.5	71.2	100
Ce -g 1%	**No. of susceptible strain**	2	6	8	27	33	59
	% No. 0f susceptible strain	3	9.1	12.1	40.9	50	89.4
Ce -X 1%	**No. of susceptible strain**	40	51	55	60	62	66
	% No. 0f susceptible strain	60.6	77.3	83.3	90.9	93.9	100

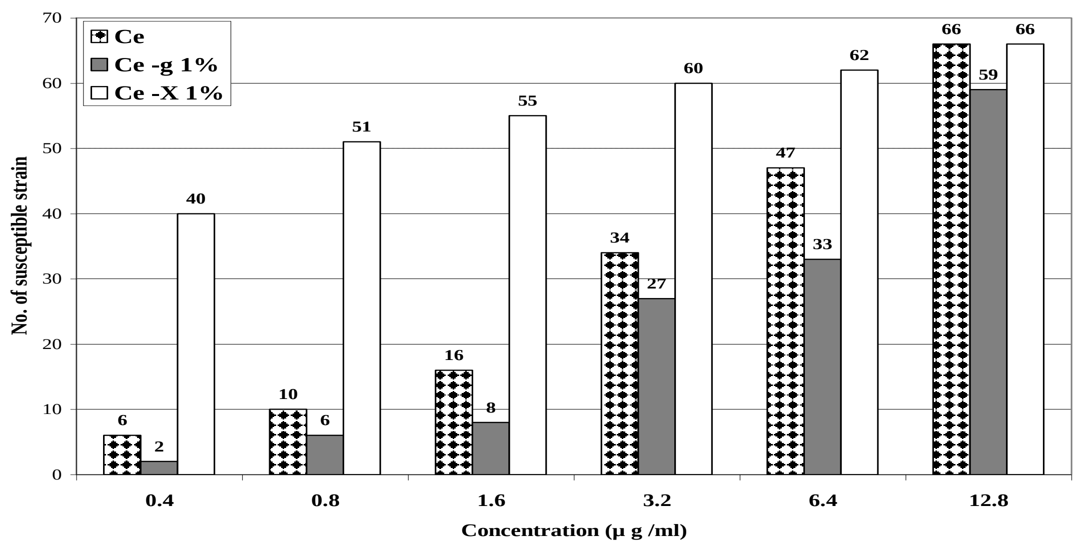

Fig1. Number of *Streptococcus mutans* susceptible stains to cephalexin Ce, Ce-g & Ce-x

Table 2. Antibacterial activities of extracts of *S. presica* , *S. presica* extracts with glucose and extracts of *S. presica* with xylitol against 66 *Streptococcus mutans* strains

***S. presica* aqueous extract (SEE)**				
SAE	**Concentration (mg/ml)**	**4**	**8**	**16**
	No. of susceptible strain	15	20	66
	(%) susceptible strain	22.7	30.3	100
SAE-G 1%	**No. of susceptible strain**	12	14	55
	(%) susceptible strain	18.2	21.2	83.4
SAE-X 1%	**No. of susceptible strain**	27	33	66
	(%) susceptible strain	40.9	50	100
***S. presica* methanol extract (SME)**				
SME	**Concentration (mg/ml)**	**2**	**3**	**4**
	No. of susceptible strain	5	33	66
	(%) susceptible strain	7.8	50	100
SME-G 1%	**No. of susceptible strain**	2	12	50
	(%) susceptible strain	3	18.2	75.7
SME-X 1%	**No. of susceptible strain**	9	44	66
	(%) susceptible strain	13.6	66.7	100
***S. presica* Ethanol extract (SEE)**				
SEE	**Concentration (mg/ml)**	**6**	**8**	**12**
	No. of susceptible strain	12	16	66
	(%) susceptible strain	18.2	24.2	100
SEE-G 1%	**No. of susceptible strain**	8	14	56
	(%) susceptible strain	12.1	21.2	84.8
SEE-X 1%	**No. of susceptible strain**	25	33	66
	(%) susceptible strain	37.9	50	100
***S. presica* Hexane extract (SHE)**				
SHE	**Concentration (mg/ml)**	**4**	**6**	**8**
	No. of susceptible strain	8	17	66
	(%) susceptible strain	12.1	27.3	100
SHE-g 1%	**No. of susceptible strain**	5	12	55
	(%) susceptible strain	7.6	18.2	83.3
SHE-X 1%	**No. of susceptible strain**	29	53	66
	(%) susceptible strain	43.9	80.3	100

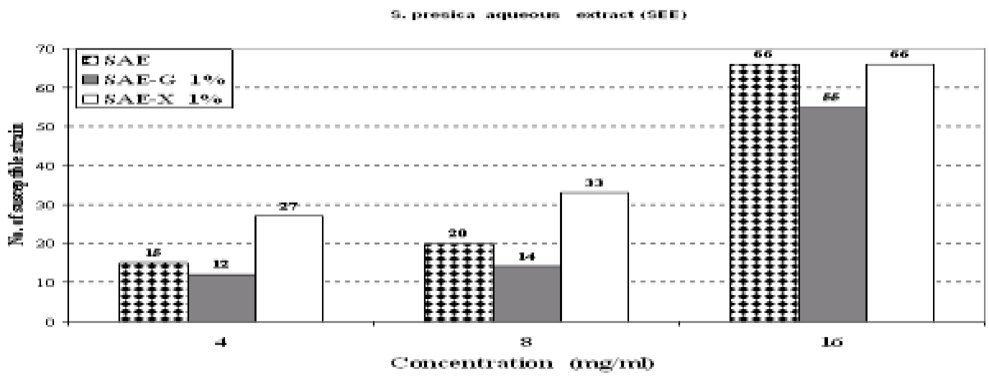

Fig 2. Number of *Streptococcus mutans* susceptible stains to SAE, SAE-g & SAE-x

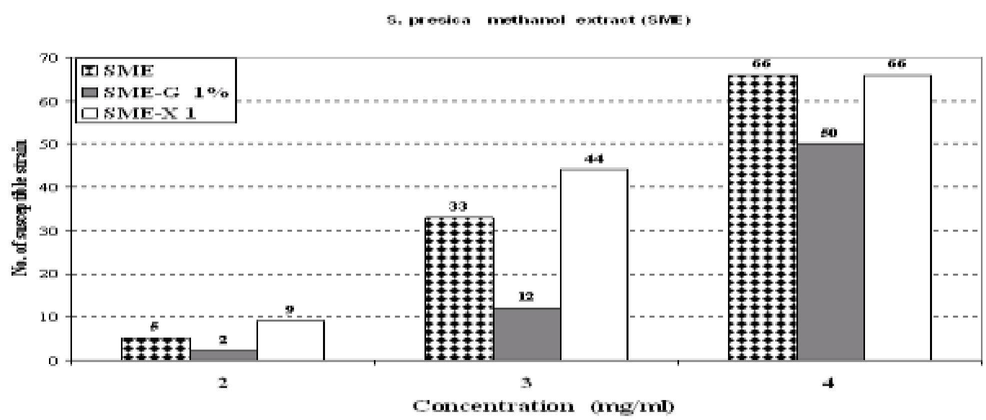

Fig 3. Number of *Streptococcus mutans* susceptible stains to SME, SME-g & SME-x

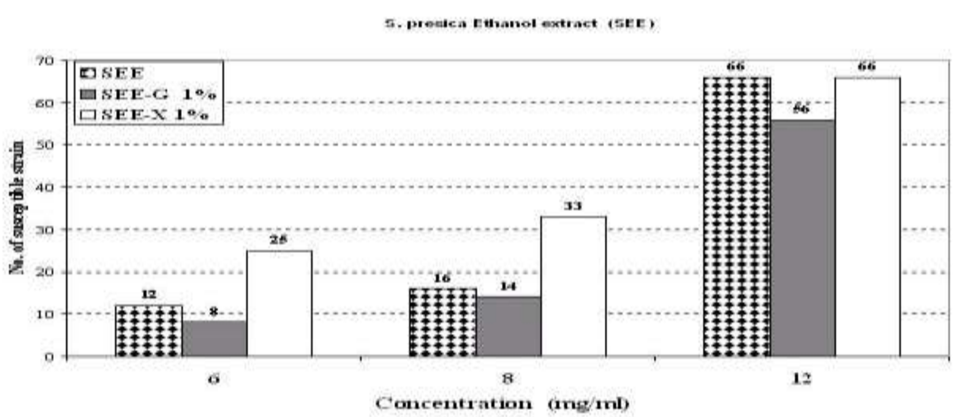

Fig 4. Number of *Streptococcus mutans* susceptible stains to SEE, SEE-g & SEE-x

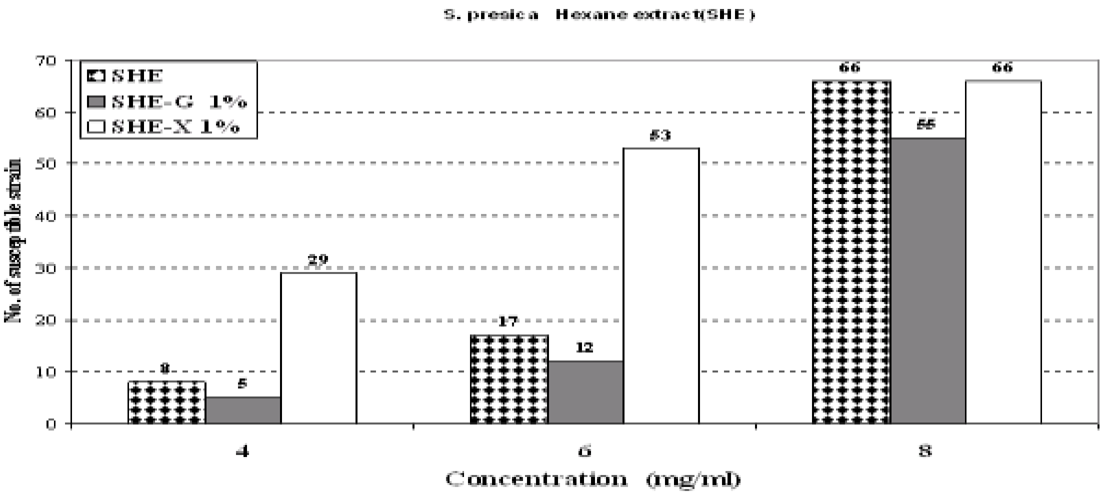

Fig 5. Number of *Streptococcus mutans* susceptible stains to SHE, SHE-g & SHE-x

3.3. Time-Killing Curve Of Cephalexin And S. presica Hexane Extract (SHE) With Or Without Addition Of 1% Glucose Or 1% Xylitol Against S. Mutans Strains.

This is research study has only focused on time-killing curve of cephalexin and *S. presica* hexane extract (SHE) with or without addition of 1% glucose or 1% xylitol challenged against selected *S. mutans* strains.

Cephalexin exhibit zero CFU/ml at 20 hrs of incubation time while cephalexin with xylitol exhibited zero CFU/ml after 16 hrs of incubation period whereas cephalexin with 1% glucose, *S. mutans* exhibited resistance to cephalexin (Table 3. Fig 6)

Table 3. Time of killing rate (log 10 CFU/ 0.1 ml**)** of cephalexin (**c**) against *Streptococcus mutans* isolates compared with cephalexin with 1% glucose (**cg**) & cephalexin with 1% xylitol (cx) compared with growth curve (G)

Time	G	C	Cg	Cx	F-test	L.S.D.
0	**6.64**	**6.64**	**6.64**	**6.64**	**n.s**	**----**
4	**7.75**	**5.54**	**6.81**	**4.64**	**	**0.065**
6	**7.90**	**4.52**	**6.83**	**3.99**	**	**0.058**
8	**8.29**	**3.82**	**6.85**	**2.54**	**	**0.095**
10	**8.44**	**3.74**	**6.85**	**1.99**	**	**0.121**
12	**8.99**	**2.54**	**6.81**	**1.74**	**	**0.115**
16	**8.98**	**2.39**	**6.71**	**0**	**	**0.175**
18	**8.94**	**1.54**	**6.65**	**0**	**	**0.191**
20	**7.88**	**0**	**6.58**	**0**	**	**0.123**
22	**6.81**	**0**	**6.51**	**0**	**	**0.107**
24	**6.79**	**0**	**5.88**	**0**	**	**0.121**

**=Significant ** =highly significant ns= no significant*

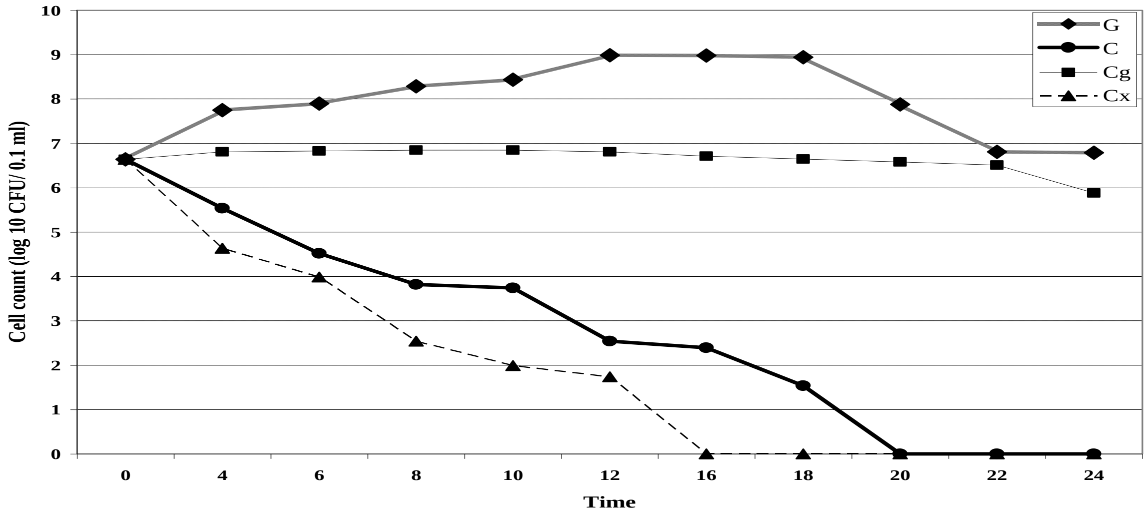

Fig 6. Time of killing rate (log 10 CFU/ 0.1 ml) of cephalexin (c) against *Streptococcus mutans* isolates compared with **Cg, Cx** & growth curve **(G)**

The effect of xylitol against *S. mutans* indicated number of *S. mutans* inoculant not increased. i.e. bacteriostatic effect comparing with growth curve without any treatment. *S. presica* hexane extract (SHE) exhibited zero CFU after 22 hrs. of incubation time whereas by addition of 1% xylitol to SHE exhibited zero CFU after 18 hrs. of incubation period with highly significant values., while glucose addition to SHE exhibit resistance effect to activity of the extract (Table 4, Fig. 7)

Table 4. Time of killing rate **(**log 10 CFU/ 0.1 ml**)** of *S. presica* hexane extract (**SH**) against *Streptococcus mutans* isolates compared with **SHE** with 1% glucose (**SH-g**), **SH** with 1% xylitol (**SH-x**) & xylitol 1% (**X**)

Time	X	SH	SHg	SHx	F-test	L.S.D.
0	6.64	6.64	6.64	6.64	ns	----
4	6.49	6.54	6.85	5.99	*	0.045
6	5.43	6.52	6.85	4.52	**	0.048
8	5.20	6.46	6.74	3.82	**	0.105
10	5.07	5.99	6.85	2.39	**	0.117
12	5.07	5.94	6.81	2.20	**	0.174
16	5.06	4.46	6.71	1.65	**	0.165
18	5.07	3.94	6.65	0	**	0.095
20	5.06	1.98	6.58	0	**	0.185
22	5.06	0	6.51	0	**	0.164
24	5.07	0	5.88	0	**	0.054

**=Significant ** =highly significant ns= no significant*

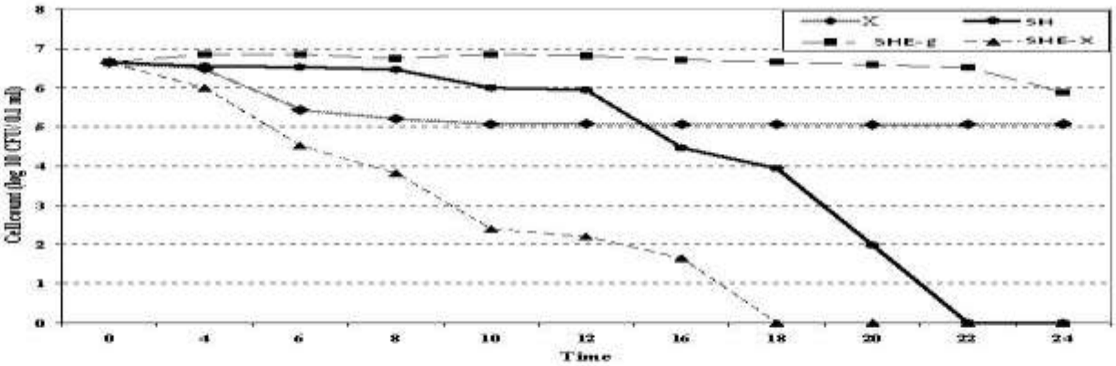

Fig 7. Time of killing rate (log 10 CFU/ 0.1 ml) of SH against *Streptococcus mutans* isolates compared with SHg, SHx and X

4 Discussion

Streptococcus mutans is the primary bacterium involved in plaque formation and the initiation of dental caries and the most cariogenic of all the oral Streptococci responsible for dental caries [18]. Many antimicrobial agents have been used to control effect of *S mutans* involve antibiotics and medicinal plants.

Salvadora presica is considering one of very important traditional tool for oral hygiene. On the other hand xylitol has been used to reduce growth of *S mutans* works as bacteriostatic.

This research paper mainly aimed to test presence of synergistic effect between xylitol and *S presica*, furthermore xylitol with cephalexin antibiotic, which compared with addition effect of glucose only. The beginning of this research aimed to clarify differences in the results of the previous researches regarding antimicrobial activity of *S. presica* L plant against *S. mutans.* The result of this study showed that all *S. presica* extraction solvents (water, methanol, ethanol and hexane) exhibit antimicrobial activity against *S. mutans* with relatively differences according to isolated strains, solvent and concentration of the extract.

Other research results reported that there is no effect of any *Salvadora persica* alcoholic extracts against *S. mutans* [2], otherwise other research study exhibits alcohol is relatively a more efficient solvent than water for the extraction of bioactive compounds of *Salvadora persica* than water [27]. On the other hand, other research study exhibited that the aqueous extract was more potent than alcoholic form in inhibiting microorganis[1].Those differences in potency of extracted compounds may due to plant from different regions and may due to time of harvesting. Tiwari indicated that botanical source, time of harvesting stage of development, and method of extraction in addition to the composition, structure, and functional groups of the natural compounds affecting on the antimicrobial activity of natural compounds [29]

The result of this research paper exhibited that addition of glucose to cephalexin and extracts of *S presica* led to reduce susceptibility of *Streptococcus mutans* isolates, that because glucose sugar enhanced isolates ability to grow and because the growth environment is favorable. High environmental glucose concentration led to a decrease in the culture pH and an increase in *S. mutans* growth [32]. Therefore, the MICs can fail to clear higher density infections [17]. Previous study reported that 21% of *Streptococcus mutans* strains were sensitive in the presence of sucrose and resistant in the presence of glucose to cephalosporins [24].

Sugar alcohols such as xylitol were not utilized by oral organisms and may be used as sugar substitutes to reduce dental caries incidence [28]. From the results of this research study, in case of addition of xylitol only within inoculated medium showed that there are reductions of *S. mutans* average numbers.

Streptococcus mutans cannot use alcoholic sugars such as xylitol and sorbitol. Xylitol only absorbed and accumulated in *M. streptococcus* with no energy yield [20]. According the results of this research, addition of xylitol to cephalexin and *S. presica* extracts increased susceptibility of *Streptococcus mutans* isolates at different tested MICs. Wåler and Rølla, reported that xylitol is recommended as a sugar substitute and it has a certain bacteriostatic effect against *S. mutans* and *S. sanguis* [30]. El-Sherbiny indicated that inhibited growth of *S. mutans* depend on xylitol concentration [9]

Other research study exhibited that the cholrhexidin, cationic peptides and xylitol combinations was efficient and superior to single treatments in suppressing *S. mutans* [23], [16]. Other research results indicate that synergistic effect of xylitol and ursolic acid combination on oral bio films [33], and there are significant reductions in the scores of *S. mutans* were found after the four week use of 20% xylitol mouthrinse [8]**.** Combination between xylitol with other dental therapies can reduce the incidence of new tooth decay and arrest existing dental caries [22]. According to the results of killing curve of xylitol and *Salvadora persica* hexane extract or with cephalexin combination exhibited clear synergistic effect. Synergy to be present if the number of CFU was ≥ 2 $\log_{10}$ lower in the presence of the combination than with the single while antagonism to be present if CFU/0.1ml were ≥ 2 $\log_{10}$ higher after incubation with the combination than with the single [26]. According to the obtained results, we recommended that addition of xylitol (bacteriostatic substance) to *S. presica* extracts (bactericidal substances) is considering an important route to overcome tooth decay.

5 Conclusion

In conclusion, both *Salvadora persica* and xylitol have evidence to control *Streptococcus mutans* as synergistic effect, so the addition of xylitol to *Salvadora persica* extract can be a useful method to control and minimize tooth decay by *Streptococcus mutans*. However, it should be one of the choices to increase susceptibility to oral pathogen, to minimize this plant extract concentration and short time for recovery from *Streptococcus mutans* infection. To complete this research study, implementation the final result of this research *in vivo* using xylitol- *Salvadora presica* extracts mixtures necessarily required to make sure that mixtures can be used as a new product of oral hygiene.

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