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The preventive effects of silymarine extract against *Streptococcus mutans* virulence and caries development in rat model and *in vitro* condition

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Streptococcus mutans have destructive effects on the development of caries. It is mainly found in biofilm and saliva, and can cause caries activity. Silymarin is an extract from the dried seeds of the milk thistle and has antibacterial activity. Seemingly, silymarine extract can prevent the destructive effects of *S. mutans*. Thus, this study was conducted to evaluate the effects of silymarine extract against *S. mutans* virulence and caries development in rat model and in vitro condition. Antibacterial activity, glucan adherence and synthesis, biofilm formation and acid production were investigated under in vitro condition. Gene expressions of relA, gtfC, brpA and comDE were assessed. Dental caries development was also evaluated. The results showed decreased biofilm formation, glucan synthesis and adherence and decreased acid production in higher doses of silymarine (P<0.05). The results also showed decreased gene expressions of relA, gtfC, brpA and comDE in the animals treated with silymarine than the control group (P<0.05). A significant decrease in caries development was found in the intervention groups compared with the control group (P<0.05). It can be concluded that use of silymarine, as a supplement, can inhibit caries development. It is recommended that silymarin can be used as a commercial supplement and/or in combination with other agents to prevent the development of dental caries.

KEYWORDS

Biofilm formation; caries development; comDE expression; silymarine; *Streptococcus mutans.*

Introduction

Dental caries is the most common infectious disease in the world [1]. It may occur for all the humans and induces demineralization and increases destruction of the dental enamel [2]. Microbes, especially *Streptococcus mutans*, induces dental caries [1]. Acidogenic and acid-tolerating species such as *S. mutans* and *Lactobacilli* are to be increased under dental caries [3]. *S. mutans* is a member of endogenous oral microflora and has significant roles in the pathogenesis of dental caries [4]. Its survival relies on biofilm formation in natural ecosystem [5]. Proliferation of bacterial biofilm increases during changed homeostasis of the oral cavity [6]. The formation of dental plaques increases dental persistence and inhibits penetration of agents that in turn increases resistance against antibiotics [7]. The biofilm production and glucan synthesis allow bacteria colonization in the oral cavity that results in the development of dental caries [8]. Another factor that can increase dental caries is glucan adherence. Glucans are formed from sucrose

through glucosyltransferases (GTFs) and play key roles in the adhesive interactions of *S. mutans* and increase dental plaque [9].

Some genes are involved in the development of dental caries. Gene relA plays an important role in increasing oxidative stress markers and acid tolerance mechanisms of *S. mutans*, but brpA is involved in biofilm formation and its structural integrity [10]. The gtfC catalyses the synthesis of water-soluble and water insoluble glucan from sucrose, but comDE has an important role in the quorum-sensing cascade of S. mutans [10].

Researchers are trying to find an alternative therapeutic agents with anti-cariogenic properties that have minimum side effects. Chemotherapeutic agents obtained from natural products have significant importance for the production of novel drugs [11]. Some studies have reported using medicinal plants as a source of chemotherapeutic agents to inhibit oral diseases [12, 13]. Silymarine, as one of the most known bioflavonoids, is extracted from *Silybum marianum* fruits, and has been shown to exert protective effects in different tissues, including liver [14]. As the most important advantage of silymarine, it not only scavenges reactive oxygen species but prevents free radical production at the same time, decreases inflammatory responses and amplifies the optimal redox balance in the cell [15,16]. The synergistic activity of silymarine combined with ampicillin and gentamicin against bacteria in the oral cavity It has been previously shown [17]. Seemingly, silymarine can have the preventory effects of silymarine extract against *S. mutans*. Silymarine properties motivated us to evaluate the effects of silymarine extract against *S. mutans*. This study was conducted for the first time to evaluate the preventive effects of silymarine extract against *Streptococcus mutans* virulence and caries development in rat model and *in vitro* condition.

Materials and methods

Materials

Silymarin was purchased from Goldaru Company (Isfahan-Iran). The bacterial strain of S. mutans was prepared and grown in a specified media culture at 37 ˚C in a 5% CO2 anaerobic atmosphere.

In vitro methods

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The antibacterial activities of MIC and MBC of fractions of silymarine against *S. mutans* were investigated by microdilution procedure [18]. We considered MIC as the lowest concentration that prevents visible bacterial growth and MBC as the highest dilution that produced no bacterial growth on solid medium. All the experiments were replicated in triplicate fashion.

Kinetic killing assay

The kinetics of the bacterial-killing effect of silymarine fractions were evaluated against *S. mutans* as reported by previous studies [19]. For this purpose, tubes containing *S. mutans* suspension (5×10^7 CFU mL^{-1}) and different levels of silymarine fractions (8, 16, 32, 64 and 128 µg mL^{-1}, final concentration) were incubated and evaluated for 24 h.

Sucrose-dependent and sucrose-independent adherence assay

Adherence to glass surface assessment was evaluated as reported by previous studies [20]. In summary, the bacteria were cultured at 37˚C at an angle of 30˚ for 24 h in a glass tube having 10 mL of BHI with and lack of 5% (w/v) sucrose and sub-MIC concentrations of the silymarine. DMSO and ethanol were used as solvent controls.

Biofilm formation assay

The inhibitory effect of silymarine on biofilm formation by S. mutans was assessed as reported by others [21]. Briefly, 50 µL of an overnight culture of *S. mutans* (10^5-10^6 CFU mL^{-1}) was inoculated into 150 µL of BHI with 5% (w/v) sucrose having the different concentrations of silymarine fractions with controls. Finally, separate biofilms were produced in the presence of silymarine for time-dependent effect in times of 6, 12, 20 and 24 h.

Inhibition of water-insoluble and soluble glucan formation

To evaluate the inhibitory effect on glucan production by *S. mutans*, sub-MIC concentrations of silymarine was included into the inoculum in BHI having 5% sucrose and incubated at 37 ˚C. We provided crude GTFs as reported by others [22]. We also evaluated amounts of water-soluble and insoluble glucan through the phenol-sulphuric acid procedure [23]. The experiments were repeated triplicate for per concentration and ethanol was considered as control.

Effect on glycolytic pH drop

The glycolytic pH drop of *S. mutans* was assessed as reported by previous studies [24]. In summary, *S. mutans* cells from the suspension cultures were obtained, washed a time with salt solution having 50 mM KCl and 1 mM MgCl2 and suspended in a salt solution containing sub-MIC concentration (128 µg mL^{-1}) of the silymarine and/or the vehicle control. The pH was kept at a range of 7.2-7.4 with 0.2 M KOH solution. The initial rate of pH drop was investigated by the pH values in the linear portion (0-60 min).

RNA isolation and real-time quantitative PCR (qRT-PCR)

To evaluate the effect of expressions of virulence genes of *S. mutans*, the organism was cultured in BHI medium provided with concentrations of the silymarine. Bacterial culture (OD_{600}=0.8) were diluted in a ratio of 1:50 that was followed by injection in a BHI media and incubated in 37 ˚C for an overnight growth. Isolation ad purification of RNA were conducted and qRT- PCR was conducted by others.[22] Primers were as follows; relA, forward (5'-ACAAAAAGGGTATCGTCCGTACAT-3') and reverse (5'-AATCACGCTTGGTATTGCTAATTG-3'), brpA, forward (5′- GGAGGAGCTGCATCAGGATTC-3′) and reverse (5′-AACTCCAGCACATCCAGCAAG-3′), gtfC, forward (5′-GGTTTAACGTCAAAATTAGCTGTATTAGC-3′) and reverse (5′ CTCAACCAACCGCCACTGTT-3′), comDE, forward (5′-ACAATTCCTTGAGTTCCATCCATCCAAG-3′) and reverse (5′ TGGTCTGCTGCCTGTTGC-3′).

In vivo methods

Pilot study

A total number of 20 Wistar rats with an initial weight of 120±10 g were divided into 4 groups and given the levels of 30, 60, 90 and 120 mg/kg of body weight. The animals were monitored for 24 h for observation of behavioral changes and mortality. We observed no behavioral changes and mortality.

Induction of caries in the animals

To assess the effects of the silymarie on oral colonization and cariogenic potential of *S. mutans*, 20 rats were prepared and divided into 2 groups (10 rats per group) and grouped as control and silymarine (SMN). To minimize microbial load, the animals were

fed with erythromycin water (100 µg mL^{-1}) and a regular diet for 1 wk. To confirm lack of *S. mutans* colonization in the oral cavity, oral swab was used on MSB plates. To increase the infection by *S. mutans*, the rats freely received 5% sucrose diet in all the trials. To colonize, the rats were inoculated with 1.4×10^{10} CFU of streptomycin-resistant strain of *S. mutans* (MT8148R), in molars surfaces once time a day for 1 wk, from day 8th. Animals in the SMN group received SMN two times a day (120 mg/kg body weight daily) by topical administration. In day 28, the samples were collected and caries scores were calculated as reported by previous studies.[13]

Data analysis

Results are expressed as mean±SD. The data were analyzed two-way ANOVA using SPSS software which followed by Tukey post hoc test for multiple comparisons. The accepted level of significance for all tests was $p<0.05$. The data for in vivo part were analyzed by T-test.

Results

The data for MIC and MBC

The results for MIC and MBC of the silymarine against S. mutans was observed to be 128 µg mL^{-1} for the both.

Assessment of kinetic killing

The results for kinetics of the antimicrobial effect of the silyamrine against *S. mutans* is illustrated in Figure 1. The results showed that the silymarine could kill *S. mutans* in a time and dose-dependent manner. The best response was observed in 24 h and 128 µg mL^{-1}.

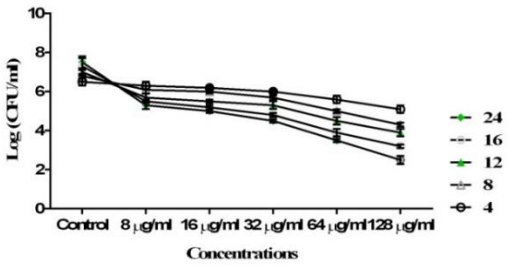

FIGURE 1 The bacteriostatic activity of the silymarine fraction at different sub- MIC levels on *S. mutans* cell cultures during 24h incubation. Treatment with silymarine in higher doses and more time killed bacteria.

Virulence and biofilm production

The results for virulence and biofilm production are illustrated in Figure 2. The results showed that silymarine in higher concentrations (64 and 128) decreased the types of glucan and sucrose-dependent and independent ($P<0.05$). Virulence properties were progressively decreased from 8 to 128 µg/mL ($P<0.05$). The results for biofilm production also showed that biofilm formation was significantly decreased with increasing time and concentration ($P<0.05$). The results showed that the highest biofilm production was observed in the control group, while the lowest biofilm formation was observed in the concentration of 128 µg and time of 6 h.

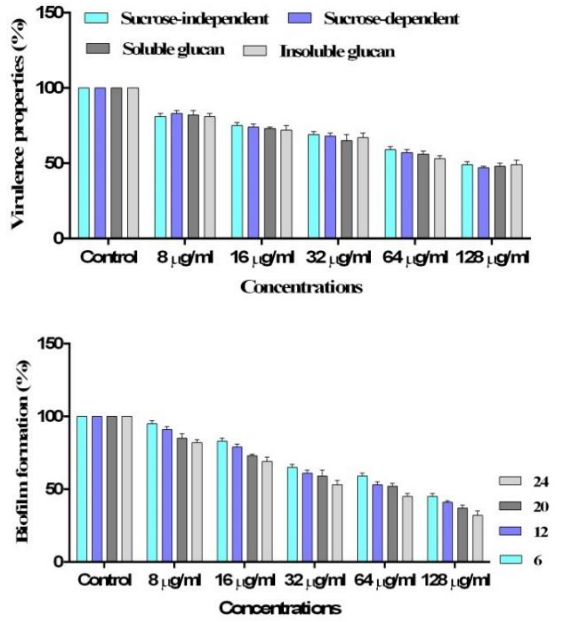

FIGURE 2 The results for virulence and biofilm production. The results showed that silymarine in higher concentrations decreased the types of glucan and sucrose-dependent and independent. The results for biofilm production showed that silymarine in higher concentrations and more times decreased biofilm production.

Glycolytic pH drop

The results for glycolytic pH showed that acid production progressively increased in the control group (pH=7.00 in time of 0 and pH=4.00 in time of 60), while silymarine prevented acid production (pH=7.20 in time of 0 and pH=6.20 in time of 60).

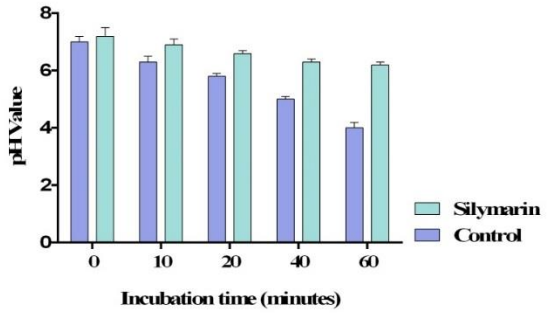

FIGURE 3 Inhibitory effect on acid production. The results showed that acid production was prevented in the silymarine group, while it progressively increased in the control group.

Gene expression

The results for gene expression is shown in Figure 4. The results showed that gene expressions of relA, gtfC, brpA and comDE significantly decreased in silymarine group compared with the control group (P<0.05).

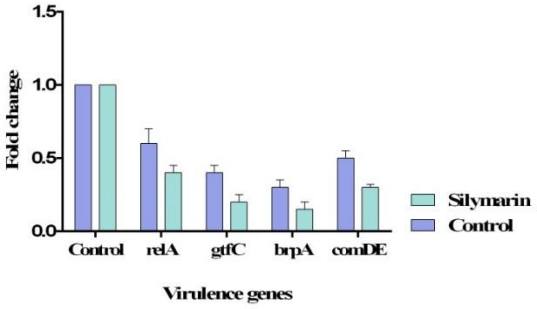

FIGURE 4 The effects of silymarine on the gene expression. The results showed that silymarine significantly decreased gene expression compared with the control group.

Caries reduction in vivo

The results for dental caries are shown in Figure 5. The results showed that the administration of silymarine decreased caries scores in terms of smooth surface caries and sulcal surface caries compared with the control group (P<0.05).

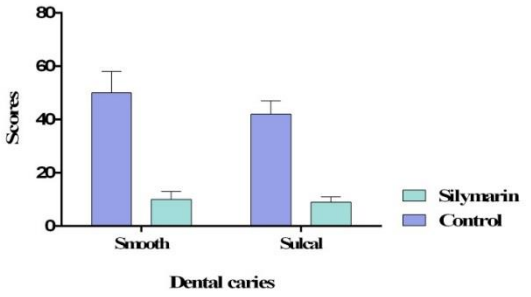

FIGURE 5 The effects of silymarine on dental caries in the rats. The results showed that silymarine significantly decreased dental caries development.

Discussion

This study was conducted to evaluate the effects of silymarine extract against *S. mutans* virulence and caries development in rat

model and *in vitro* condition. The results showed that silymarine significantly decreased *S. mutans* counts from lowest to highest doses. It means that silymarine has antibacterial properties. Silymarin is an extract that is achieved from the seeds of *S. marianum* that has the silymarin flavonolignans and approximately 20 to 30% undefined fraction, including polymeric and oxidized polyphenolics compounds [25]. Its antibacterial properties are attributed to phenolic compounds. Phenolic compounds create complexes with extracellular soluble proteins and bind with the bacterial cell walls [26]. In addition, some natural compounds change the permeability of the cell membrane and penetrate into bacteria [27]. Flavonoids also show preventive effects on DNA topoisomerase activity through complexes that change enzyme binding [28]. In sum, silymarine show antibacterial activity by the mentioned mechanisms.

The results also showed decreased adherence in the silymarine groups, especially in higher levels. Adherence is known as one of the important stages for establishment of dental caries and its prevention can be considered as a strong step for inhibition of dental caries [29]. Hydrophobic interactions between the cells and the adhering surface cause sucrose-independent adherence [13]. Seemingly, silymarine increases hydrophobic interactions and results in decreased adherence. Decreased adherence and increased hydrophobic interactions prevent bacteria for adhering. GTFase increases conversion of sucrose to adherence glucans that result in an increase to stick *S. mutans* to the surface of the tooth. It seems that silymarine has anti-GTFase activity and prevents adherence to sucrose. It is well known anti-GTFase activity of flavonoids [30]. Additionally, glucans mediate the formation of biofilm [13]. Decreased glucans decrease biofilm formation and modulate with the pathogenesis through damaging physical integrity and stability, decreasing the availability of binding sites for *S. mutans* [31]. The results showed that silymarine decreased glucan adherence that results in the reduction of biofilm formation.

Our findings for glycolytic pH drop indicated preventive activity of the silymarine against acidogenicity that was observed as it decreased the initial and final rate of the pH. Decreased acidic value of pH is associated with the prevention of the bacterial glycolytic enzymes [13]. In fact, *S. mutans* is an aciduric bacteria that performs glycolytic activity at very low pH values. Our findings showed that silymarine prevented increased acidity and thus prevented *S. mutans* activity.

The results also showed decreased expression of genes involved in the development of dental caries. Gene relA plays an important role in increasing oxidative stress and acid tolerance mechanisms of *S. mutans*, but brpA is involved in formation of biofilm and its structural integrity [10]. The gtfC catalyses the synthesis of water-soluble and water-insoluble glucan from sucrose, but comDE has an important role in the quorum-sensing cascade of *S. mutans* [10]. The downregulation of the genes decreases the internal association system used through the bacteria for changing their gene expression in a critical cell density that results in cell death. The results show that silymarine decreases the expression of the genes and thus confirms our findings for dental caries.

In vivo studies showed that the topical administration of silymarine progressively decreased the smooth surface caries and sulcal surface carious lesions. It means that active compounds are available in the efficacious concentrations in oral cavity and can influence caries development. The persistence of topical administration in the oral cavity can increase approaches against dental caries [32]. Decreased lesions in silymarine group may be associated with prevention of GTF activity and bacterial

glycolytic pathway will decrease the pathogenicity of *S. mutans* in vivo [13].

Conclusion

This study was conducted to evaluate the effects of silymarine extract against *S. mutans* virulence and caries development in rat model and *in vitro* condition. Silymarine is a bactericide and suggest using the silymarine as an oral agent and/or in combination with other agents for the prevention of dental caries.

Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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