

Original Article

Prevention of secondary caries by a new antibacterial compound

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ABSTRACT

Background: Since secondary caries is one of the main problems of dental composites. The creation of an antibacterial property in these composites is essential. The objective of this study was to synthesize 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one and check its biocompatibility and antibacterial properties in flowable dental composites.

Materials and Methods: In this animal study, the antibacterial activity of flowable resin composites containing 0–5 wt% 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one was investigated by using agar diffusion and direct contact tests on the cured resins. Statistical analysis was performed using one-way ANOVA test ($P < 0.001$). Thirty male albino Wistar rats were used, weighing 200–250 g. Animals were randomly divided into three groups of ten; each animal received three implants, 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one, penicillin V, and an empty polyethylene tube. A pathologist, without knowing the type of material tested and the timing of the test, examined the samples. Statistical analysis was performed using Kruskal–Wallis test ($P < 0.001$).

Results: According to our findings, although the agar diffusion test reveals no significant difference between the groups, the direct contact test demonstrates that, by increasing the 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one content, the bacterial growth was significantly diminished and the 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one has a good biocompatibility ($P < 0.05$).

Conclusion: Incorporation of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one into flowable resin composites can be useful to prevent *Streptococcus mutans* activity.

Key Words: Antibacterial agents, dental caries, dental materials, *Streptococcus mutans*

Received: September 2018
Accepted: January 2019

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INTRODUCTION

Usage of dental composites has been widely improved recently due to their advantages such as natural tooth color and easy handling over conventional material.^[1] According to previous *in vitro*^[2-5] and *in vivo*^[2,6] studies, aggregation of bacteria and dental plaque was more on the surface of dental composites comparing to other dental materials, so because of lack of antibacterial activity, these aggregations will cause

more secondary caries on tooth. This phenomenon decreases the life of tooth restoration and eventually makes the restoration of replacement necessary.^[7,8] Therefore, more recent studies are focused on the preparation of dental composites with antibacterial characteristics to prevent this kind of secondary caries. One of the methods for acquiring this purpose

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How to cite this article: Eskandarizadeh A, Mohammadzadeh I, Shahravan A, Bavafa M, Kakooei S, Torabi M. Prevention of secondary caries by a new antibacterial compound. Dent Res J 2020;17:40-7.

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is the use of antibacterial material in polymeric matrix. These methods called sustained release dosage forms (SR) and they considered to be one of the simplest and most efficient methods of novel drug delivery system (NDDS). Antibacterial component will slowly release from the matrix, providing a sustained dosage that prevent the bacterial growth. An example of this kind of material is chlorhexidine and fluoride.^[9-12] Despite having strong antibacterial activity, they lack proper prolonged drug release.^[13] Another method is to use metal oxides for increasing the antibacterial activity of dental materials.^[14-16] However, the usage of these oxides in dental materials is limited because most of them will change the color of tooth.^[17] *Streptococcus mutans* is a Gram-positive bacterium commonly found in the human oral cavity and is a major contributor in tooth decay.^[18-21] When formulating a composite material, it is necessary to consider the existence of this bacterium and its decay activity. There are some materials that have been used for reducing the effect of *S. mutans* such as pyrazoles. Pyrazoles are a group of organic compounds that have been studied widely because of their biological activities.^[22] They are the most important member of heterocyclic compounds that are used in pharmacological industries.^[22-25] Pyrazoles have some useful characteristics such as anti-arthritic, anticosuric, anti-inflammatory, and antibacterial properties.^[26] In this study, we synthesized pyrazole compounds, 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one. This compound has antibacterial properties and also its color is similar to the tooth color^[25] that preserves good tooth appearance. We used this compound to provide antibacterial properties in dental material. We also studied the biocompatibility of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one.

MATERIALS AND METHODS

Materials

In this animal study, lithium bis(trimethylsilyl) amide (LiHMDS) (CAS No: 4039-32-1) toluene (CAS Number: 108-88-3), 3-acetyl-2,5-dimethyl furan (CAS Number: 10599-70-9), ethyl chloroformate (CAS Number: 541-41-3), ethanol (CAS Number: 64-17-5), acetic acid (CAS Number: 64-19-7), ethyl acetate (CAS 141-78-6), hydrazine hydrate (CAS Number: 10217-52-4), and sodium sulfate (CAS Number: 7757-82-6) manufactured by Sigma-Aldrich (Sigma-Aldrich Corporation is an American chemical, life science and biotechnology company) were used.

Dental restorative resin composite Heliomolar Flow was prepared by Ivoclar Vivadent, AG, FL-9494 schaan/Liechtenstein. *S. mutans* PTCC 1683 (Persian Type culture-collection) was received from IROST IRAN co.

Methods

Synthesis of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one
11 mmol of 1.0 M solution of LiHMDS in toluene was added to a solution of 3-acetyl-2,5-dimethyl furan (10 mmol in toluene [15 mL]) using a syringe at 0°C under stirring. It was stirred at this temperature for 10 min and then 11 mmol of ethyl chloroformate was added quickly. The resulted reaction mixture was brought to room temperature during 10 min and was stirred for another 10 min, and then 2 mL of acetic acid, 15 mL of ethanol, and hydrazine hydrate (30 mmol) were added and refluxed for 15 min. The mixture was concentrated to dryness under reduced pressure and dissolved in ethyl acetate. Organic impurities were washed by saturated NaCl solution dried over sodium sulfate (Na₂SO₄) and evaporated under reduced pressure. The final product was purified by recrystallization using ethanol.^[25] This process is shown in Figure 1.

Conditions of bacterial growth

Standard strains of *S. mutans* were used as the reference microorganism.

The bacteria were cultured overnight in 5 ml of brain–heart infusion broth (BHI) at 37°C. To avoid large bacterial aggregates or long streptococcal chains, the top 4 ml of the undisturbed bacterial culture were transferred to a fresh test tube and centrifuged for 10 min. The supernatant was discarded, and the bacteria were resuspended in 5 ml of phosphate-buffered saline.

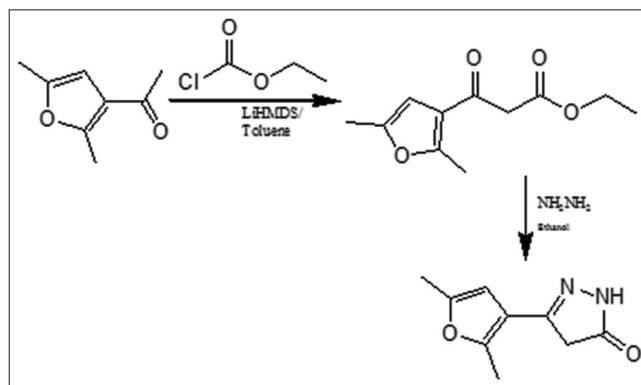


Figure 1: Synthesis of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one.

Preparation of specimens

Six groups of specimen were prepared by mixing of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one with resin composite (Tetric Flow, Ivoclar Vivadent, USA) in 1, 2, 3, 4, and 5 wt% and 0 wt% as the control group. Mixing was performed in a dark room for 15 min and in room condition using a spatula.

Agar diffusion test

This test was used to investigate the effect of antibacterial activity in the composites containing 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one on bacterial growth. Discs with 2 mm thickness and 8 mm diameter were prepared using each of the composite specimen groups. These discs were polymerized using a light-cured device (LED, DEML, SDS Kerr, USA, with an intensity of circa 800 mW/cm²) from two sides (bottom and top) for 40 s. In the six groups, 200 µL of bacterial suspension was spread on blood agar and then discs were placed on the surface of each of the plates. After 24 h of maintaining of plates in 37°C, inhibition zone diameter around each disc was measured. These tests were repeated three times each to ensure their accuracy.

Direct contact test

This test was performed to investigate the antibacterial properties of free surface of resins containing 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one. For this purpose, walls of 500 µl microplates were covered by 200 µl of unpolymerized resin. Then, resin layers were polymerized by a light-cured device for 40 s. 10 µl of 0.5 McFarland standard solutions of *S. mutans* (about 10⁶ bacteria) was added to each microplate, and the samples were kept in 37°C for 1 h. During this time (1 h), bacteria were in direct contact with the resin surface, and the solvent was evaporated. Then, 300 µl of BHI medium was added to each microplate. Caps were completely closed, and the samples were stored in 37°C in periods of 24, 48, 72, 96, and 120 h; 50 µl of the mixture (bacteria + BHI broth) was placed on the culture medium and after 24 h, the number of appeared bacterial colonies was counted by using colony counter apparatus. This test was repeated three times for each test group to ensure their accuracy.^[27,28] The results were expressed as log₁₀ (colony forming unit [CFU]) [Figure 2].

Statistical analysis

The data were analyzed by one-way ANOVA, and the Tukey's *post hoc* honestly significant difference multiple comparison test. The level of significance was determined as $P = 0.001$.

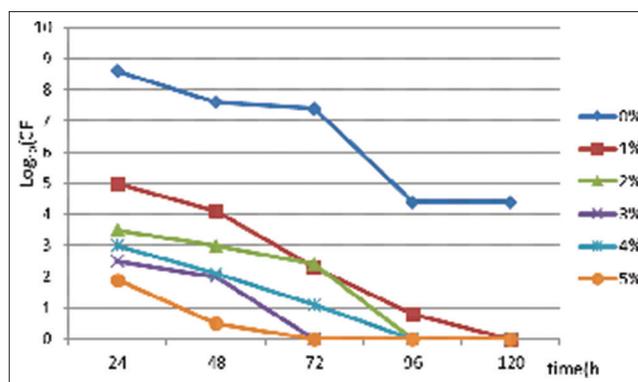


Figure 2: Colony-forming unit following direct contact between *Streptococcus mutans* and resin composites containing 0–5 wt% 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one.

Biocompatibility

The research protocol was approved by the Research Ethics Committee of Kerman University of Medical Science (approval number IR.KMU.REC.1395.199). The experiment was performed in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington DC, revised 1996.

Thirty 6–7-month-old male albino Wistar rats weighing 200–250 g were used. The animals were anesthetized for about 40 min by intraperitoneal administration of 47.5 mg/kg of ketamine hydrochloride and 0.01 mg/kg of Rompun 2%.^[29] For each animal, three areas (two anterior regions and one posterior region) were scrubbed by brown iodine and then shaved. All areas were disinfected using green iodine, and in each region, a small incision with a length of approximately 12 mm was made by a razor scalpel number 20. Then, using the backside of a blunt dissecting scalpel, a 20-mm-deep pocket was created in the subcutaneous tissue for implantation of tubes.

Animals were randomly divided into three groups of ten, each animal receiving three implants (sterile polyethylene tubes with 1.1-mm inner diameter and 10-mm length) of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one, penicillin V, and empty polyethylene tube. Then, it was sutured plainly and individually with nylon yarn. The use of penicillin V (capsule 500 mg) was due to the fact that it is currently used as a common antibiotic. One week, 1 month, and 2 months^[28] after that the tubes were implanted, the animals were sacrificed by injection of an overdose of ketamine hydrochloride. The hair extensions of the

tubes were re-shaved, and the skin and connective tissue surrounding the implant were brought out of the block section and kept in the formalin 10% for at least 48 h. After fixing, parallel sections with longitudinal axis of tissue were prepared for staining with hematoxylin and eosin. A pathologist, without knowing the type of tested material and timing, examined the samples. To evaluate the histological response, studies of Yaltirik *et al.*,^[28,30,31] Derakhshan *et al.*, and also Parioikh *et al.* were used.^[29]

Histological criteria

The tissue reaction at both ends of the tubes was studied according to the following histological criteria:

The size of the subcutaneous tissue around the tube was measured at $\times 100$ and measured with micrometers and the grading was as follows:^[29]

- 0: No capsules
- 1: Thickness of the capsule is $<150 \mu\text{m}$
- 2: Thickness of the capsule is $>150 \mu\text{m}$.

Severity of inflammation

By observing the number of inflammatory cells in or around the subcutaneous tissue capsule with a $\times 40$ fold, the following were defined:^[29]

- 0: No inflammation
- 1: <25 cells
- 2: Between 25 and 50 cells
- 3: Between 50 and 75 cells
- 4: More than 75 cells.

The extent of inflammation

The amount of expansion of inflammatory cells at both ends of the tube was observed at $\times 40$.^[29]

- 1: Inflammatory cells only appear in the surface layer of the capsule
- 2: Inflammatory cells are limited to fibrous capsules
- 3: Inflammatory cells can be seen around the capsule.

Necrosis

The disappearance of cells or the observation of the ghost of cells without a nucleus, that is, its presence or absence was observed to be $\times 40$.^[29] The grading was as follows:

- 0: Lack of necrosis
- 1: Necrosis.

Inflammation type

Inflammation type has been defined with various types of inflammatory cells as follows:^[29]

- Acute inflammation: Overcoming acute inflammation cells (polymorphonuclear)

- Chronic inflammation: Overcoming chronic inflammatory cells such as macrophages, plasma cells, and lymphocytes
 - 0: No inflammation
 - 1: Chronic inflammation
 - 2: Acute inflammation.

Based on previous articles in this field, ten samples were used for each period.^[32-35]

Statistical analysis

After describing the findings, nonparametric methods such as Kruskal–Wallis were used based on the rank of the variables. Mann–Whitney U-test was used for comparing the two methods, and the significant correlation was modified with Bonferroni method.

RESULTS

Agar diffusion test

There was no inhibition zone around the samples in agar medium containing *S. mutans* strain.

Direct contact test

The results of bacterial colony count (CFU) are shown in Figure 2. It is clear that when the w% of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one was increased, the antibacterial activity of composite increases significantly ($P < 0.001$) and also time has a meaningful effect on the antibacterial properties of resin.

Evaluation of histopathologic observations in each of the studied groups

Empty polyethylene tube group

In this group, the capsule thickness was increased over time, so that during 7 days, the thickness of the capsule was $<30 \mu\text{m}$. After 60 days, the thickness of the capsule had a significant difference with the 7-day capsule ($P < 0.05$).

The severity of inflammation, the extent of inflammation, and the presence of chronic inflammation were significantly higher in the 7-day interval than the 30-day and 60-day periods ($P < 0.05$). However, no significant difference was observed between 30 and 60 days ($P > 0.05$).

Acute necrosis and inflammation were not observed in any of the periods.

3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one group

The thickness of the capsule increases over time in this group. However, this difference was not statistically significant ($P > 0.05$).

The extent of inflammation decreased by 7, 30, and 60 days, but there was no significant difference between the groups.

The severity of inflammation decreased by 7, 30, and 60 days, with a significant decrease of 60 days comparing with the first 7 days ($P < 0.05$).

Acute necrosis and inflammation were not observed in any group.

Penicillin V group

The thickness of the capsule in the 30- and 60-day periods was significantly higher than that of the 7-day period.

However, no significant difference was observed ($P > 0.05$).

The severity of inflammation and the extent of inflammation after 60 days were <30 and 7 days, but this difference was not statistically significant ($P > 0.05$).

Like other groups, acute necrosis and inflammation were not established in any of the samples.

Histological evaluation and comparison between the groups studied at different time periods

After 7 days

There was no significant difference in the amount of capsule thickness, intensity of inflammation, and extent of inflammation between different groups during this time period ($P > 0.05$). The results are shown in Figures 3-7.

After 30 days

During this period, there was no significant difference in the thickness of the capsule, severity of inflammation, extent of inflammation, and type of inflammation between the three different groups ($P > 0.05$). The results are shown in Figures 3-5.

After 60 days

In this period, there was no significant difference in any of the cases of capsule thickness, intensity of inflammation, extent of inflammation, and type of inflammation between the three different groups ($P > 0.05$). The results are shown in Figures 3-7.

DISCUSSION

The agar diffusion test and the minimum inhibitory concentration are important traditional tests for evaluating and investigating the antibacterial properties and behavior of many pharmaceutical materials.^[36]

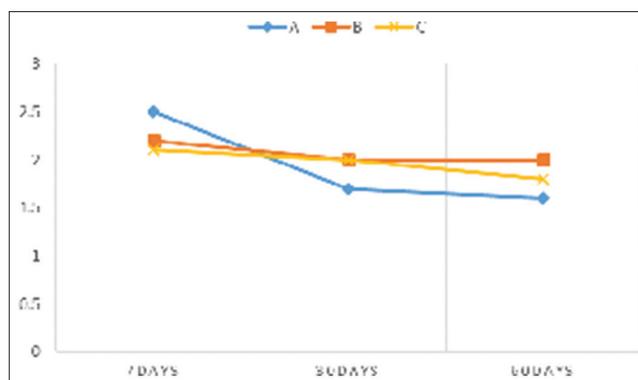


Figure 3: Extent of inflammation by time intervals. A: Empty polyethylene tube. B: 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one. C: Penicillin V.

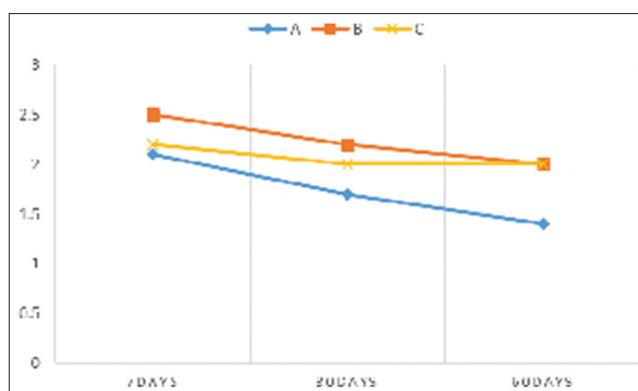


Figure 4: Severity of inflammation by time intervals. A: Empty polyethylene tube. B: 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one. C: Penicillin V.

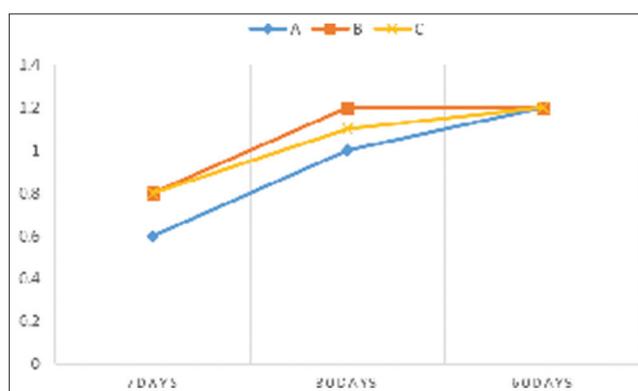


Figure 5: The thickness of subcutaneous tissue capsule by time intervals. A: Empty polyethylene tube. B: 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one. C: Penicillin V.

Agar diffusion test works based on the solvability of materials as the tested material diffuses from the bulk surface and eliminates microorganisms. Hence, this method cannot be used for the materials with low solvability in water. Giving that one of the most important and necessary properties of proper dental

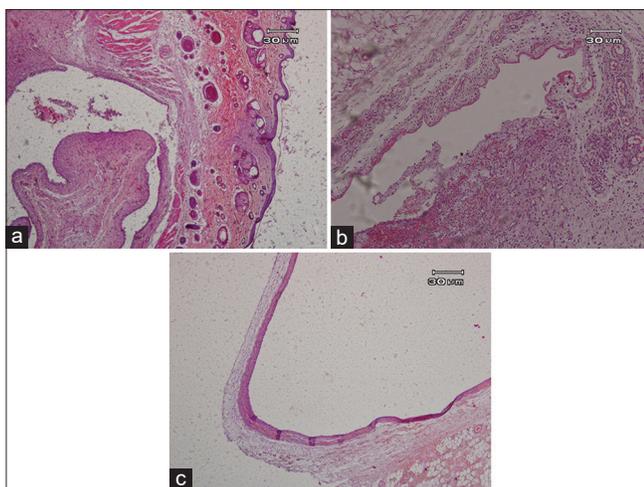


Figure 6: (a) 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one after 7 days ($\times 40$). (b) Penicillin after 7 days ($\times 40$). (c) Empty tube after 7 days ($\times 40$).

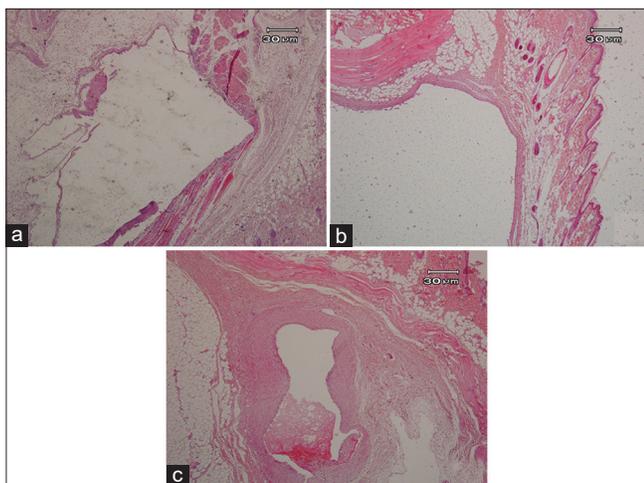


Figure 7: (a) 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one after 60 days ($\times 40$). (b) Penicillin after 60 days ($\times 40$). (c) Empty tube after 60 days ($\times 40$).

material is low water solvability, the agar diffusion test cannot be used here, and it is not a suitable test method for dental material. In another words, direct contact test (DCT) has very low sensitivity toward the test subject with solvability properties. Knowing that it is an efficient method to evaluate antibacterial properties, we can use it for investigating antibacterial properties in materials with very low water solvability. As shown in Figure 2, the results of DCT indicated that 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one compound gives excellent antibacterial properties to the resin that increase with time and in higher concentration. The antibacterial property of 3-(2, 5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one reaches its maximum

at 5% w and after 24 h. Its nontoxic properties and also its very low aqueous solvability make 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one a very suitable material to be used in oral environment. In addition to the mentioned advantages, 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one is white and it has the same color as tooth enamel. Hence, it can improve the antibacterial properties of the dental composites and at the same time preserving the esthetic characteristics of them.

According to previous researches, 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one probably uses the following mechanism to prevent bacterial growth.^[25] This compound is a derivative form of pyrazoles, one of the most important nitrogenated heterocycle compound groups. Pyrazols prevent bacterial activity by possessing electron-rich property and having noncovalent interaction with microorganisms. Considering that the success of antibacterial tests alone did not indicate the use of these substances for clinical use, its biocompatibility was also considered.

In histopathologic studies, tissue responses depend on the size and shape of the implanted material.^[32] In some studies, materials have been directly applied to the subcutaneous tissue. However, many agree to implant materials in the tube.^[33] Tubes that are commonly used are silicone tubes, polyethylene tubes, Teflon tubes, or dentine tubes.^[34] The use of materials in the tubes is similar to that of the clinical situation.^[35] When compared with the direct use of materials, this method helps to stabilize the material in place and to achieve a better standard of the material–tissue interface.^[31,37] In this work, we used polyethylene tubes with internal diameter 1.1 mm. The empty tubes' reaction in this study was similar to those of Torneck and Makkes *et al.* They found that the use of a polyethylene tubes caused a lack of reaction or low response in subcutaneous connective tissues.^[38-40]

There are several methods to compare the severity of the inflammatory response around the tubes containing the observing substance. One of these is the Stanford method.^[28] In this method, the number of inflammatory cells is counted in different regions of the microscopic section.^[41] However, because the inflammatory response of the connective tissue has different aspects, focusing solely on the number of inflammatory cells cannot reflect all aspects of the connective tissue response and does not appear to be comprehensive.^[42]

Therefore, we used the Yaltirik *et al.*'s. method in addition to the number and extent of the spread of inflammatory cells, including other aspects of inflammation, such as the formation of fibrous capsules and the presence of necrosis.^[31] Because in some similar studies, the type of inflammatory cells was studied, also this variable was considered in this study.^[29,42-44]

According to the results of this study, in the 30- and 60-day periods, in most samples of each of the three tested materials, a fibrous capsule was created around the tube, which suggests good tissue tolerance of these materials. Formation of fibrous capsule around the material can keep it away from the surrounding tissue and prevents damage of the material in the tissue.^[28,45,46] The formation of a fibrous capsule has been reported in many similar studies.^[28,31,45]

The present study showed that the 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one has a good biocompatibility.

CONCLUSION

Within the limitation of this study, it can be concluded that incorporation of 3-(2,5-dimethylfuran-3-yl)-1H-pyrazole-5(4H)-one into flowable resin composites can reduce the activity of *S. mutans*.

Acknowledgment

We would like to thank Kerman University of Medical Sciences for their financial supports in fundamental research funds.

Financial support and sponsorship

The study was financially supported by Kerman University of Medical Sciences in fundamental research funds.

Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial, in this article.

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