Dental Research Journal

Original Article

Effect of hydroalcoholic extract of whole pomegranate fruit on cariogenic bacteria and its clinical effect on dental plaque formation in 8–10-year-old children

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ABSTRACT

Background: This study aimed to assess the effect of hydroalcoholic extract of the whole pomegranate fruit on *Streptococcus mutans* (*S. mutans*) and *Lactobacillus acidophilus* (*L. acidophilus*) and its clinical effect on dental plaque formation in 8–10-year-old children.

Materials and Methods: This study was conducted in two phases of *in vitro* and clinical trial. In the *in vitro* phase, the antibacterial effect of 50%–50% hydroalcoholic extract of whole pomegranate fruit on *S. mutans* and *L. acidophilus* was assessed by the disc diffusion test. In the double-blind cross-over clinical trial phase, 14 children between 8 and 10 years were randomly assigned to two groups of 38% pomegranate mouthwash and 0.12% chlorhexidine (CHX) after assessing their baseline dental plaque by oral hygiene index-simplified (OHI-S). The children were asked not to use any other plaque control measure during the study. Their OHI-S score was measured again after 5 and 14 days using disclosing tablets. Data were analyzed by *t*-test and paired *t*-test at 0.05 level of significance.

Results: The hydroalcoholic extract of pomegranate showed a positive antibacterial effect on *S. mutans* and *L. acidophilus*. However, its inhibitory effect was significantly lower than that of 0.12% CHX ($P < 0.05$). None of the tested mouthwashes inhibited plaque formation, but pomegranate mouthwash and CHX decreased the OHI-S score by 34% and 36%, respectively ($P < 0.05$), with no significant difference between them ($P > 0.05$).

Conclusion: The whole pomegranate fruit hydroalcoholic extract showed significant inhibitory effects on *S. mutans* and *L. acidophilus*. Furthermore, 38% pomegranate mouthwash had a comparable efficacy to CHX in the reduction of dental plaque.

Key Words: Dental plaque, lactobacillus, plant extracts, pomegranate, *Streptococcus mutans*

Received: 18-Mar-2023
Revised: 07-Oct-2023
Accepted: 10-Oct-2023
Published: 27-Nov-2023

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INTRODUCTION

Elimination of dental plaque-containing microbial biofilm is a challenge in pediatric dentistry. Biofilm is composed of a complex accumulation of microorganisms entrapped in a hydrated polymer matrix.[1] Dental plaque is a mixed biofilm formed on the tooth surface.[2] Microbial adhesion to tooth surface is probably the most important parameter responsible for biofilm formation.[3] In many children

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Website: www.drj.ir
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www.ncbi.nlm.nih.gov/pmc/journals/1480

How to cite this article: Alamdari Mahd M, Aref P, Emadi F, Javadi F, Fard MJ, Tavassoli-Hojjati S. Effect of hydroalcoholic extract of whole pomegranate fruit on cariogenic bacteria and its clinical effect on dental plaque formation in 8–10-year-old children. Dent Res J 2023;20:114.

and adolescents, as well as those with sensory and motor disabilities, plaque elimination may not occur efficiently with mechanical plaque removal measures alone. Resultantly, plaque accumulation leads to caries development, destruction of periodontium, bone resorption, and eventual tooth loss.[4]

Mouthwashes are among the most effective antimicrobial agents used for plaque control. Chlorhexidine (CHX) is the most commonly used antimicrobial mouthwash.[5] However, it has some side effects and complications such as tooth staining, altering the sense of taste, oral burning sensation, and interference with the oral microflora.[6,7]

Herbal extracts have long been used for the treatment of infectious diseases.[8,9] Nonetheless, research is still ongoing on the antimicrobial effects of plant extracts.[10] A large body of evidence exists regarding the optimal antimicrobial properties of pomegranate skin and juice.[11-13] Pomegranate with the scientific name *Punica granatum* has several favorable properties. The pomegranate fruit has antioxidant properties. It eliminates the free radicals and chelates the ferrous ions. It also has anti-inflammatory and antibacterial effects, which are attributed to the constituents of the pomegranate peel, seed, and juice. The whole fruit has superior effects compared with only one component of the plant.[14,15] It is rich in hydrolysable tannins, which are polyphenols with polyol as their central core such as punicalagin/ponicalin, ellagic acid, cyanidin-3-glucoside, cyanidin 3, 5-diglucoside, delphinidin, cyanidin, and pelargonidin (condensed tannins) as well as organic acids such as citric acid and ascorbic acid.[16]

Previous studies on the effects of pomegranate extract on plaque index evaluated only the effect of one component of *P. granatum* plant and not the whole fruit extract.[17-19] Thus, considering the gap of information regarding the antibacterial effects of the whole pomegranate fruit extract, this study aimed to assess the effect of hydroalcoholic extract of whole pomegranate fruit on *Streptococcus mutans* (*S. mutans*) and *Lactobacillus acidophilus* (*L. acidophilus*) *in vitro* and also the effect of this extract on dental plaque formation in children between 8 and 10 years. The first null hypothesis of the study was that the effect of hydroalcoholic extract of the whole pomegranate fruit on *S. mutans* and *L. acidophilus* would have no significant difference with the effect of CHX. The second null hypothesis of the study was that the effect of hydroalcoholic extract of the whole pomegranate fruit on dental plaque of 8–10-year-old children would have no significant difference with the effect of CHX.

MATERIALS AND METHODS

This study was conducted in two phases: an *in vitro* study and a clinical trial.

In vitro phase

Sample size

All tests for the assessment of the effect of hydroalcoholic extract of the pomegranate whole fruit were conducted in triplicate according to previous studies.[20-22]

Extract preparation

To prepare the hydroalcoholic extract of the whole pomegranate fruit, 10 medium-size pomegranates were diced into small pieces and crushed in a mixer. Water/ethanol (50:50 ratio) was then added as solvent, and the mixture was filtered after 72 h and heated at 60°C to obtain a honey-like consistency. Discs containing 100% concentrated extract were then prepared.

Assessment of the antibacterial effect of the hydroalcoholic extract of the whole pomegranate fruit

Standard-strain *S. mutans* (ATCC 35668) and *L. acidophilus* (ATCC 4356) were obtained from the Iranian industrial bacteria and fungi culture collection. Viable bacteria were cultured in brain–heart infusion nutrient medium in a Gas-Pak jar under anaerobic conditions for 24 h at 37°C. They were then streak cultured on blood agar plate and subcultured three times a week. The disc diffusion test was used for the assessment of the antibacterial activity according to the guidelines of the Clinical and Laboratory Standards Institute.[23] The blood agar culture medium was incubated for 24 h, and then microbial suspension was prepared with 0.5 McFarland standard concentration containing 1.5×10^8 colony-forming units/mL. A sterile swab was then used to lawn culture the bacteria on blood agar culture medium. One disc dipped in 0.12% CHX (Iran Najo, Iran) as the positive control group, one disc containing the hydroalcoholic extract of the pomegranate fruit as the test group, and a blank disc as a negative control were placed on the culture medium in a Petri dish. The Petri dish was capped and incubated at 37°C for 24 h (Memmert, Germany). The diameter of the growth inhibition

zone around each disc was measured by a ruler in millimeters. Zones with a diameter smaller than 1 mm were considered zero. To ensure the accuracy of the results, the experiment was repeated by the same examiner under aseptic conditions in triplicate. The antibacterial activity of CHX and the extract was determined according to the diameter of the growth inhibition zones formed in millimeters.[24-26]

Statistical analysis

Data were analyzed using the SPSS software version 15.5 (SPSS Inc., Chicago, IL, USA) using *t*-test and paired *t*-test at 0.05 level of significance.

Clinical trial phase

This study was conducted at the Pediatric Dentistry Department of School of Dentistry, Islamic Azad University of Medical Sciences in 2022. The study protocol was approved by the ethics committee of the university (IR.IAU.PS.REC.1401.424) and registered in the Iranian Registry of Clinical Trials (IRCT20230110057104N1).

Trial design

A double-blind crossover clinical trial was designed, in which one group used 0.12% CHX mouthwash (Nazhu Co, Iran) first and pomegranate mouthwash after a 7-day washout period, whereas this order was reverse for the second group. The results were reported in accordance with the Consolidated Standards of Reporting Trials.

Participants, eligibility criteria, and settings

The inclusion criteria were age between 8 and 10 years, systemic health, no antibiotic intake within the past 3 weeks before the study onset, absence of oral habits, no use of orthodontic or prosthetic appliances, no xerostomia, no periodontal disease, and absence of active caries in the buccal and lingual surfaces of maxillary right first molar, mandibular left first molar, maxillary left lateral incisor, mandibular right lateral incisor, maxillary right central incisor, and mandibular left central incisor teeth.

The sample consisted of 14 children between 8 and 10 years who were selected by targeted sampling.

Interventions

Preparation of mouthwash

The hydroalcoholic extract obtained in the *in vitro* phase of the study was used for the preparation of the mouthwash along with the mint extract in 7:2 ratio and edible dye. The mint extract and edible dye were also added to the CHX mouthwash for the purpose of standardization of the taste and color of both mouthwashes. Furthermore, both mouthwashes were delivered to patients in identical bottles.

Intervention

After obtaining written informed consent from the parents, all children underwent a clinical oral examination using a dental explorer and a dental mirror (Fatahteb, Iran) to ensure meeting the eligibility criteria. All participants underwent prophylaxis and scaling and polishing, if required. Interdental areas were cleaned by an unwaxed dental floss (Essential Floss, Oral B, Ireland) to ensure the absence of dental plaque, stains, and calculus. A disclosing tablet was used to ensure complete cleaning of the teeth. All sessions were scheduled for children between 9:30 and 10:30 am, and the next session was scheduled 2 days later. The children were requested not to use any oral hygiene measure (no toothbrushing, mouthwash, dental floss, or chewing gum) during this period. In the second treatment session, a disclosing tablet was used to reveal the baseline dental plaque of patients, which was quantified using the oral hygiene index-simplified (OHI-S) by a trained dental assistant. Accordingly, six teeth including four posterior teeth (#16, 26, 36, and 46) and two anterior teeth (#11 and 31) were evaluated. The lingual surfaces of the mandibular posterior teeth and the buccal surfaces of other teeth were examined. The scoring system was as follows:

- Score 0: A surface free from dental plaque
- Score 1: Presence of plaque in less than one-third of the tooth surface
- Score 2: Presence of plaque in more than one-third and less than two-thirds of the tooth surface
- Score 3: Presence of plaque in more than two-thirds of the tooth surface.

The total OHI-S score was calculated by summing the scores of the examined buccal and lingual surfaces divided by the number of all tested surfaces.[27]

All children underwent prophylaxis again, and the quality of cleaning was assessed using disclosing tablets (Eviplac, Biodinamica Co., Brazil) to ensure clean teeth free from dental plaque, stains, and calculus. Each child was allocated a code, and half of the children randomly received 40 mL labeled bottles containing CHX, whereas the other half received 40 mL nonlabeled bottles containing the extract

mouthwash. The children and the examiner were not aware of the contents of the bottles. Each bottle was graded (4 grades) to indicate the volume to be used at each time of use. The participants were requested to rinse the mouthwash twice a day, once in the morning and once in the evening. They were asked to rinse 10 mL of the mouthwash for 30 s each time and then spit it out. Their next session was scheduled 2 days later, and they were asked not to use any other oral hygiene measures (toothbrush, dental floss, or chewing gum) during this 48 h period. In the third session, disclosing tablets were used to calculate the OHI-S score of the patients. Next, a 7-day washout period was considered,[28] and then the first group received the other mouthwash and vice versa. The same process was repeated with respect to recording the baseline and secondary OHI-S score for patients as explained for the first mouthwash (crossover design).

Outcomes (primary and secondary)

The main objective of this study was to assess the effect of hydroalcoholic extract of the whole pomegranate fruit mouthwash on OHI-S score of children.

Sample size calculation

The minimum sample size was calculated to be 14 patients according to a previous study[17] assuming alpha = 0.05, beta = 0.2, mean standard deviation of 35%, and effect size of 39% using one-way ANOVA power analysis feature of PASS 11.

Interim analyses and stopping guidelines

No interim analyses were performed, and no stopping guidelines were established.

Randomization

In this crossover study, children were randomly assigned to receive either 40 mL of pomegranate mouthwash (B) or CHX (A) in two different sequences (AB and BA) using a table of random numbers with the Rand option of Excel software.

Blinding

The mouthwashes were delivered to children in identical bottles, and the children and the examiner were not aware of the contents of the bottles.

Statistical analysis

Data were analyzed using SPSS version 15.5 (SPSS Inc., Chicago, IL, USA) by *t*-test and paired *t*-test at 0.05 level of significance.

RESULTS

Participant flow

The sample consisted of 8–10-year-old boys. Figure 1 shows the CONSORT flow diagram of patient selection and allocation to the study groups.

In vitro results

Table 1 presents the mean diameter of the growth inhibition zones of *S. mutans* and *L. acidophilus* caused by the hydroalcoholic extract of pomegranate and CHX. The paired *t*-test showed that CHX created significantly larger growth inhibition zones in both bacterial cultures than the pomegranate extract ($P = 0.0001$).

Clinical results

Table 2 presents the mean OHI-S score of the two groups at baseline and after the intervention. The results showed a significant reduction in OHI-S score after the intervention compared with baseline ($P < 0.05$); the percentage of reduction was 34% in the pomegranate extract group and 36% in the CHX group. Paired *t*-test showed no significant difference in OHI-S score between the two groups of pomegranate extract and CHX after the intervention ($P > 0.05$).

DISCUSSION

This study assessed the effect of hydroalcoholic extract of the whole pomegranate fruit on *S. mutans* and *L. acidophilus in vitro* and also the effect of

Table 1: Mean diameter of growth inhibition zones (mm) of *Streptococcus mutans* and *Lactobacillus acidophilus* caused by the hydroalcoholic extract of pomegranate and chlorhexidine

Mouthwash	Mean±SD	
	Growth of inhibition zone for *Streptococcus mutans*	Growth of inhibition zone for *Lactobacillus acidophilus*
Hydroalcoholic extract of pomegranate	12.000±0.500	17.500±0.5
CHX	16.167±0.289	25.667±0.289
P (using paired *t*-test)	0.0001	0.0001

SD: Standard deviation; CHX: Chlorhexidine

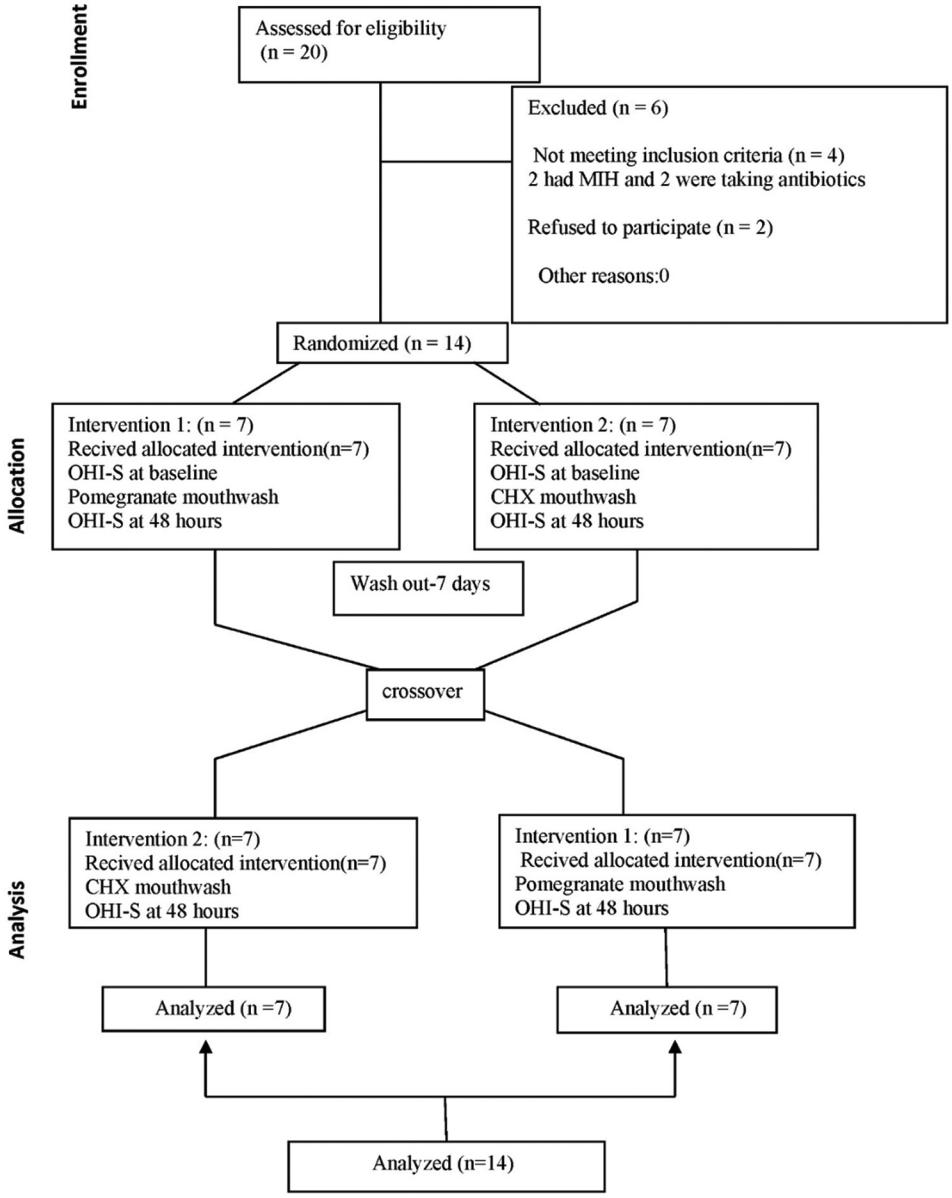

Figure 1: CONSORT flow diagram of patient selection and allocation to the study groups. OHI-S: Oral hygiene index-simplified, CHX: Chlorhexidine, MIH: Molar Incisor Hypomineralisation.

Table 2: Mean oral hygiene index-simplified score of the two groups at baseline and after the intervention

Mouthwash	Mean±SD		*P***
	Initial OHI-S score	Final OHI-S score	
Pomegranate extract	2.37±0.6	1.53±0.5	<0.001
CHX	2.30±0.5	1.47±0.9	<0.001
*P**	0.64	0.88	

*Using *t*-test (comparison between groups), **Using paired *t*-test (comparison between before and after using the mouthwash). SD: Standard deviation; CHX: Chlorhexidine; OHS-S: Oral hygiene index-simplified

this extract on dental plaque in children between 8 and 10 years. The first phase of the study which had an *in vitro* design showed that the hydroalcoholic extract of the pomegranate fruit had inhibitory effects on both *S. mutans* and *L. acidophilus*; however, its effects were significantly lower than those of CHX. Thus, the first null hypothesis of the study was rejected. Consistent with the present results, Naz *et al*.[29] chemically analyzed the methanolic extract of the pomegranate fruit. They fractioned different parts of the fruit and found that all components had antibacterial effects against different species such as *Staphylococcus aureus* (with a growth inhibition zone diameter of 20.4 mm), *Streptococcus pneumoniae* (with a growth inhibition zone diameter

of 20.4 mm), and *Escherichia coli* (with a growth inhibition zone diameter of 12.2 mm). They had a greater effect on Gram-positive bacteria. Among the isolated components, gallic acid showed the highest antibacterial activity due to its phenolic structure. Differences in the results can be attributed to the use of different types of solvents and method of extraction. El-Sharkawy[30] evaluated the antimicrobial effect of the pomegranate extract on *S. mutans* in 45 children aged 5–10 years. The children were randomly divided into three groups: Group A (consumed fresh pomegranate juice), Group B (used pomegranate peel extract), and Group C (used 0.2% CHX mouthwash). Distilled water was used to make the pomegranate mouthwash, and the participants were instructed to use 35 mL of the mouthwash for 2 min. Saliva samples were collected before and 60 min after using the mouthwash. The results showed that rinsing with pomegranate peel extract led to a significant reduction (100%) in *S. mutans* colony count, followed by CHX (99.96%) and fresh pomegranate juice (99.75%). The differences in methodology and mouthwash preparation were the main reasons for the different results compared with the present findings. Nair *et al*.[31] conducted an *in vitro* study about the effect of pomegranate extract on *S. mutans*. They heated 400 mL of pomegranate juice for 1 h to prepare 1200, 900, 600, 300, 150, and 75 mg/mL concentrations. Antimicrobial activity was measured by the diameter of the zone of inhibition of *S. mutans* using the agar well-diffusion method. The results showed that only 600 mg/mL (1.94 ± 0.19 cm) and 300 mg/mL (1.42 ± 0.13 cm) concentrations created inhibition zones compared with CHX (2.4 ± 0.07 cm). They also demonstrated that CHX had a significantly larger inhibition zone than the pomegranate mouthwash. Reddy *et al*.[32] found that the antibacterial activity of the pomegranate fruit is due to its ellagic acid, gallagic acid, ponicalin, punicalagin, and tannin contents, which are effective against *Pseudomonas aeruginosa*, *Candida albicans*, methicillin-resistant *S. aureus*, *Aspergillus fumigatus*, and *Mycobacterium intracellulare*. Another study[33] evaluated the efficacy of pomegranate fruit for periodontal therapy. The results showed that the pomegranate extract had healing properties by increasing the migration of fibroblasts, collagen synthesis, and angiogenesis. They demonstrated that 5%, 10%, and 15% methanolic extract of the pomegranate peel in the form of an ointment enhanced healing due to the presence of tannin and polyphenol in its composition.

The pomegranate fruit has been used for centuries with no toxic effects.[34] Pomegranate is a strong antioxidant with anticancer and anti-inflammatory properties. Several studies have confirmed its antimicrobial effects. Pomegranate has several antimicrobial ingredients and exerts antibacterial effects through several mechanisms. Punicalagin and ellagitannin impair the synthesis of polyglycans and prevent the adhesion of microorganisms to the tooth surface. Furthermore, tannins pass through the cell wall, adhere to the cell membrane, and cause protein deposition and suppression of enzymes such as glycosyltransferase. Moreover, phenolic compounds bind to the surface of substrates such as carbohydrates and minerals and make them unavailable to microorganisms, resulting in cell wall disintegration.[35]

The second phase of the study assessed the effect of 38% hydroalcoholic extract of the pomegranate fruit on dental plaque in children between 8 and 10 years. The results showed that both mouthwashes significantly decreased the OHI-S score with no significant difference between them. Thus, the second null hypothesis of the study was accepted. Clinical studies on the effects of pomegranate mouthwash on plaque index are limited. Consistent with the present study, Bhadbhade *et al*.[36] evaluated the effect of pomegranate mouthwash on dental plaque and found that pomegranate mouthwash decreased dental plaque in patients. They evaluated 30 periodontally healthy participants and asked them to refrain from oral hygiene measures for 4 days. The plaque index was measured at baseline and after 5 days, and the results showed no significant difference between the CHX and mouthwash groups regarding plaque index.

In contrast to the present findings, Ahuja *et al*.[17] compared the effects of pomegranate mouthwash and CHX on plaque index and gingivitis. The patients had moderate gingivitis and were evaluated at 0, 7, and 15 days while adhering to plaque control measures such as toothbrushing. They showed that the pomegranate mouthwash had no significant effect on plaque index. Salgado *et al*.[37] assessed the antiplaque and antigingivitis effects of a gel containing 10% pomegranate extract in a double-blind crossover clinical trial. The participants randomly received the extract or the placebo gel. No significant difference was noted between the two groups in plaque index or gingival bleeding index. Their methodology was

different from the present study. Thus, comparison of the results was not possible.

Conduction of the study in two phases of *in vitro* and *in vivo* was the main strength of this study. However, uncertainty about regular use of mouthwashes by children as instructed was a limitation of this study which might have affected the results. Furthermore, the constituents of the pomegranate fruit were not isolated to assess their individual effect in the present study. Future studies are recommended to assess the isolated fractions of the pomegranate fruit and evaluate their antibacterial effects on a wider range of oral pathogens, especially Gram-negative microorganisms. Moreover, the long-term cariostatic and anti-inflammatory effects of the pomegranate mouthwash in conjunction with mechanical plaque control measures should be investigated in both children and adults.

CONCLUSION

The whole pomegranate fruit hydroalcoholic extract showed significant inhibitory effects on *S. mutans* and *L. acidophilus*. Furthermore, 38% pomegranate mouthwash had a comparable efficacy to CHX in reduction of dental plaque.

Financial support and sponsorship

This study was derived from a research project approved and funded by Islamic Azad University of Medical Sciences, Tehran, Iran.

Conflicts of interest

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

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