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In vitro and in vivo antimicrobial effects of mastic chewing gum against *Streptococcus mutans* and mutans streptococci

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Summary

Background and objective: Dental caries is associated with oral pathogens and *Streptococcus mutans* (*S. mutans*) is one of the primary cariogenic organisms. Mastic gum, from *Pistacia lentiscus*, has been shown to have antibacterial properties. The objective of this study was to determine antibacterial activity of mastic chewing gum against *S. mutans* and mutans streptococci in vitro and in vivo conditions.
Setting: Cukurova University, Dental School, in 2002.
Materials and methods: Antimicrobial activity of mastic gum was evaluated using standard *S. mutans* strain by disc diffusion method in vitro. Cytotoxicity effect of mastic gum on HEp-2 cells was evaluated by conventional haemocytometer using the trypan blue exclusion method. Clinical studies were then performed on 25 periodontally healthy volunteers. The inhibitory effect of chewing mastic gum against mutans streptococci in saliva was compared to a placebo gum. Saliva samples were taken from the subjects immediately before and after chewing the mastic gum and the placebo gum for 15 min. Additional saliva samples were collected every 30 min. The samples were inoculated onto mitis salivarius-bacitracin agar and incubated for 48 h anaerobically at 37 °C. The total number of viable bacteria was then counted.
Results: Among tested solvents (chloroform, acetone, petrolium ether and ethanol), it was found that the acetone was found to be more convenient than the others to dissolve the mastic gum. In the cytotoxicity assay, concentrations up to 75 mg/ml of the mastic gum were not toxic for the replication of HEp-2 cells. Thus, lower

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concentrations of mastic gum (20 and 50 mg/ml) were used for the experiments. In vitro experiments, the diameters of growth inhibition zones of mastic gum were in the range 9.0—27.0 mm. In the clinical trials, the mean number of bacteria in samples taken after chewing the mastic gum and placebo gum were following; at minute 15 was $112 \times 10^4 \pm 268 \times 10^3$ and $175 \times 10^4 \pm 417 \times 10^3$ cfu/ml, for minute 45 was $85 \times 10^4 \pm 219 \times 10^3$ and $165 \times 10^4 \pm 329 \times 10^3$ cfu/ml, at minute 75 was $65 \times 10^4 \pm 100 \times 10^3$ and $160 \times 10^4 \pm 216 \times 10^3$ cfu/ml, at minute 105 was $60 \times 10^4 \pm 127 \times 10^3$ and $150 \times 10^4 \pm 138 \times 10^3$ cfu/ml, and at minute 135 was $55 \times 10^4 \pm 65 \times 10^3$ and $145 \times 10^4 \pm 354 \times 10^3$ cfu/ml, respectively. Significantly fewer bacteria was found in saliva samples collected after chewing mastic gum compared to those after chewing paraffin ($p < 0.001$).
Conclusions: This preliminary study showed that mastic gum had significant antibacterial activity against *S. mutans* and mutans streptococci and it may be a useful adjunct in the prevention of caries.

Introduction

Bacterial plaque accumulated on teeth surfaces and composed of native oral flora, is the primary aetiological agent for periodontal disease and dental caries which may result in teeth loss if left untreated.[1,2] Dental caries is destruction of dental structures by acid produced as a by-product of carbohydrate metabolism by cariogenic bacteria.[3] Mutans streptococci, commonly found in human dental plaque, are the primary species associated with dental caries.[4]

Numerous antimicrobials and antibiotics including chlorhexidine, spiramycin and vancomycin have been used for against *Streptococcus mutans* (*S. mutans*) to reduce plaque mediated diseases including dental caries.[5] However, antibotics have several adverse effects such as vomiting, diarrhoea and teeth staining.[6] In addition, the development of antimicrobial resistant strains is a growing cause of concern. These drawbacks justify further research and development of natural antimicrobial agents targeting specific oral pathogens while being safe for the host.[7,8]

Natural products have recently been investigated more thoroughly as promising agents to prevent oral diseases, especially plaque-related diseases such as dental caries.[9—11] Recent studies have demonstrated antimicrobial activity of natural products against selected oral pathogens. Mastic gum is a natural resin derived from the stem and the leaves of the mastic tree, *Pistacia lentiscus* Linn, native to Mediterranean areas.[12,13] It has been used by traditional healers for the relief of upper abdominal discomfort, stomachaches, dyspepsia and peptic ulcer.[14,15] It has also been shown in numerous studies to have impressive antibacterial and antimicrobial properties.[13,16] Studies performed in vitro conditions have demonstrated that a short treatment time is required for the bacteriostatic effect of mastic gum against *S. mutans*.[7,8] Therefore, it appears to be a potent antibacterial agent applicable for use in mouthwash preparations.

Although the antibacterial activity of mastic chewing gum has already been demonstrated, very few studies have been conducted on bacteria of clinical relevance in dentistry.[11,13—15,17] Hence, in this study, we aimed to evaluate the antibacterial activity of mastic chewing gum against *S. mutans* and mutans streptococci in vitro and in vivo conditions.

Materials and methods

Mastic gum

Mastic gum the concrete resinous exudate from the stem of the tree *P. lentiscus* Linn that is cultivated in Aegean and Mediterranean coasts of Turkey. Mastic gum samples were obtained from the grand bazaar of Istanbul. They have been collected from the Fethiye region, Turkey in June 2001.

Antibacterial effect

Preparation of bacteria

S. mutans (ATCC 27351) was incubated overnight at 37 °C on a mitis salivarius-bacitracin agar (Difco, USA) plate, and then washed twice after centrifugation at $3000 \times g$ in saline (0.9% NaCl in distilled water) for 10 min. The inoculum was prepared by suspension of bacterium in the saline solution. McFarland standards were used for standardization of numbers of bacteria for testing 10^5 cfu (colony forming units)/ml.

Disc diffusion method

The organisms to be tested were inoculated into mitis salivarius-bacitracin agar. After an incubation period of 24 h at 37 °C, the cultures were

adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 0.5 standard. Petri dishes containing mitis salivarius-bacitracin agar were impregnated with these microbial suspensions for bacterium.[17] Two con- centrations were prepared for each solvent: 20 and 50 mg/ml and blank discs of 6 mm diameter (Sterile Blank, Difco) were impregnated. Blank discs impregnated with chloroform, ethanol, acetone, petrolium ether were used as negative controls, and discs of Vancomycin (30 μg) as positive controls in the antibacterial assays. The plates were incubated overnight at 37 °C and the diameter of any resulting zones of inhibition (mm) measured. Each experiment was repeated at least three times and the mean of the diameter of the inhibition zones was calculated. Vancomycin was used as a control drug. Vancomycin discs were provided from oxoid firm (Oxoid, UK).

The bactericidal effects of different solvents were tested in tryptic soy broth with fecal calf serum (FCS). In order to test the effects of four solvents (chloroform, acetone, petrolium ether and ethanol), 1×10^6 *S. mutans* (ATCC 27351) were inoculated into each well of 12-well plates in tryptic soy broth with 10% (v/v) heat-inactivated FCS, and bacteria were allowed to grow for additional 48 h in presence of decreasing amounts of these solvents (50%, 25%, 12.5%, 6.25%, 3.125%). The non-toxic concentrations were determined up to 12.5%. Among these convenient solvent concentrations (12.5%, 6.25%, 3.125%), the lowest solvent concentration (3.125%) was chosen to dissolve mastic gum.

The effects of concentrations of 20 and 50 mg/ml of the mastic gum on the replication of *S. mutans* have been investigated. Antibacterial activity of the mastic gum was tested against *S. mutans* (ATCC 27351). Up to then, minimal inhibitory concentration (MIC) of mastic gum was determined by broth microdilution method following the procedures recommended by the National Committee for Clinical Laboratory Standards.[16]

Cytotoxicity effect

In order to test the effects of the mastic gum of the *P. lentiscus* on HEp-2 cells, 5×10^4 HEp-2 cells (in 1 ml EMEM, supplemented with 10% (v/v) FCS) were seeded into each well of flat-bottomed microplates, cultured for 6 h at 28 °C, and cells allowed to grow for additional 48 h in the presence of increasing amounts of mastic gum (10, 20, 50, 75, 100 mg/ml). The cytotoxicity of mastic gum was determined on a conventional haemocytometer using the trypan blue exclusion method.[15]

Clinical study

Twenty-five volunteers (11 males, 14 females, a mean age 25.9 years) were recruited for the study after informed consent had been obtained. All subjects had healthy periodontal condition and generally good oral hygiene standard. None of the subjects received antibiotics or topical antiseptics during the previous 30 days or had systemic disease that would have altered the amount or composition of the plaque or saliva.

The first week, paraffin was given to the volunteers as placebo to chew for 15 min and then spit. Before the test, unstimulated saliva was collected by rinsing the mouth with 10 ml of phosphate buffered saline as a baseline sample. Then, either of the chewing gums was used (15 min). Saliva samples were collected at 30 min intervals at 45th, 75th, 105th and 135th minutes. After vortexing, a 10 ml aliquot of each saliva sample was diluted 1:10.000, which had been determined by our preliminary study for determining suitable dilution ratio of the saliva samples to count the cfu on agar plates. Aliquots (100 μl) of the dilution were plated on to mitis salivarius-bacitracin agar as triplicate, composed of mitis salivarius agar base (Difco, USA). Incubation of the samples was performed in an atmosphere of 5% CO_2 with at 37 °C 48 h. After incubation for 48 h at 37 °C, the total number of colony-forming cells on each plate was counted.

Statistical analysis

Data are reported as mean ± standard deviation (S.D.). Between-group comparisons were made using the Kruskal–Wallis and Mann–Whitney *U*-tests. $p < 0.05$ was considered significant. The statistical analyses were performed by using Statistical Package for Social Sciences (SPSS® for Windows V. 11.5, Chicago, USA) software.

Results

Cytotoxicity effect

In the cytotoxicity assay, concentrations up to 75 mg/ml of the mastic gum were not toxic for the replication HEp-2 cells. Thus, lower concentrations of mastic gum (20 and 50 mg/ml) were use for the experiments.

Antibacterial effect

In Fig. 1, the diameters of the inhibition zones of mastic gum in two different concentrations (20 and

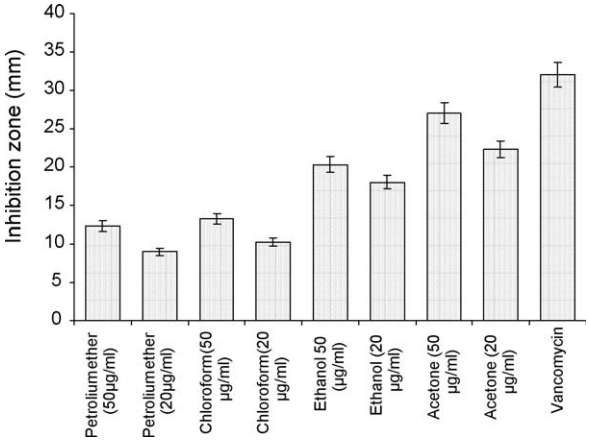

Figure 1 Evaluation of in vitro antibacterial activity of mastic gum.

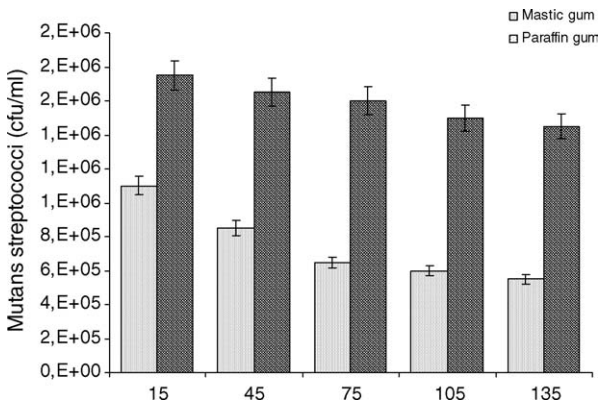

Figure 2 The antimicrobial activity of mastic gum against the total number of mutans streptococci in the oral cavity.

50 mg/ml) dissolved in four different solvents (chloroform, acetone, ethanol, petrolium ether) are seen. In the in vitro experiments, it was found that, the inhibition zones have been increased linearly with increasing mustic gum concentration. The most effective antimicrobial activity was obtained by dilution in acetone, ethanol, chloroform and petrolium ether, respectively. The most effective solvent for mastic gum was to be acetone.

In our study, for 20 mg/ml dilution of mastic gum; the inhibition zone diameter for acetone was found 22.3 ± 2.0 mm whereas diameter of inhibition zones was 18.0 ± 1.0 mm for ethanol, 10.3 ± 2.5 mm for chloroform and 9.0 ± 2.0 mm for petrolium ether. For 50 mg/ml concentration, the inhibition zone diameters of acetone, ethanol, chloroform and petrolium ether were found 27.0 ± 1.0, 20.3 ± 3.5, 13.3 ± 2.5 and 12.3 ± 0.5 mm, respectively.

Regarding the inhibition zones of acetone, ethanol and chloroform dilution there had been found a difference statistically significant ($p < 0.05$) whereas no significance was seen on the inhibition zones of chloroform and petrolium ether ($p > 0.05$).

Clinical study

It is determined that mastic gum had also an antibacterial effect in the oral cavity. The total number of bacterial colonies was significantly reduced during the five sampling times at 15th minute just after spitting the gum, and on the 45th, 75th, 105th, 135th minutes following. The total number of bacterial colonies of mutans streptococci by chewing mastic gum compared to the paraffin was also found significant statistically ($p < 0.001$).

The mean quantitative bacterial data about in vivo studies are presented in Fig. 2. The mean number of bacteria from cultures (mastic gum groups) at minute 15 was $112 \times 10^4 \pm 268 \times 10^3$ cfu/ml, for minute 45 was $85 \times 10^4 \pm 219 \times 10^3$ cfu/ml, at minute 75 was $65 \times 10^4 \pm 100 \times 10^3$ cfu/ml, at minute 105 was $60 \times 10^4 \pm 127 \times 10^3$ cfu/ml, and at minute 135 was $55 \times 10^4 \pm 65 \times 10^3$ cfu/ml. In paraffin groups, the mean number of bacteria at minute 15 was $175 \times 10^4 \pm 417 \times 10^3$ cfu/ml, for minute 45 was $165 \times 10^4 \pm 329 \times 10^3$ cfu/ml, at minute 75 was $160 \times 10^4 \pm 216 \times 10^3$ cfu/ml, at minute 105 was $150 \times 10^4 \pm 138 \times 10^3$ cfu/ml, and at minute 135 was $145 \times 10^4 \pm 354 \times 10^3$ cfu/ml. There were significantly fewer bacteria in oral cavity chewing mastic gum than in those chewing paraffin ($p < 0.001$).

The 37% reduction in mutans streptococci counts (cfu/ml) were found just after application of mastic gum. This was the inital effect of the gum. And 48.5%, 56.7%, 62.5%, 62.1% reduction was found at 45th, 75th, 105th and 135th minutes after spiting mastic gum subsequently. All values obtained after application of mastic gum except 105th and 135th minutes applications were found statistically significant ($p < 0.05$). In contrast, paraffin gum used as placebo showed no antibacterial effect.

Discussion

Mutans streptococci are considered to be predominant species isolated from human saliva and dental plaque,[2] and have been identified as the major aetiological agent for caries. Individuals heavily colonised by mutans streptococci were thought to be at high risk for caries. Hence, eradication of these cariogenic bacteria is off importance for the treatment of human dental caries. Several antiseptic agents including chlorhexidine, cetyl pyridinium chloride, fluorides and phenol derivatives have been used widely in dentistry to inhibit bacterial growth. Nevertheless, dental scientists have still been searching for new applications of

therapeutic drugs to prevent or treat dental plaque-related diseases.[4,18–20]

Lately, mastic gum has attracted much attention as a natural useful substance in folk medicine to treat a variety of ailments for its antibacterial, anti-inflammatory and antiulcer activities.[11,21–23] Because of this broad spectrum of biological activities, mastic gum has attracted much attention as a natural useful substance in medicine, health food, and cosmetic industries. These results support the possibility that mastic gum also has potential antibacterial activity against oral bacteria. Although mastic gum appears promising as a potential antibacterial agent against oral bacteria, it has not been explored widely in dentistry.

In the study, the mastic gum showed significantly reduced bacterial growth in saliva compared with placebo gum. We have confirmed the antibacterial effect of mastic gum on mutans streptococci. The results of the antimicrobial activity by the disc diffusion method were presented in Fig. 1. It could be observed that the mastic gum dissolved in all four of the solvents (chloroform, ethanol, acetone and petrolium ether) studied showed a good antimicrobial activity against *S. mutans*. Among tested solvents (chloroform, acetone, petrolium ether and ethanol), it was found that the acetone was found to be more convenient than the others to dissolve the mastic gum. The diameters of growth inhibition zones of mastic gum were in the range 9.0–27.0 mm.

We found that the antimicrobial activity of the mastic gum varied depending on the sample, dosage of mastic gum, and the solvent used (i.e., chloroform, ethanol, petrolium ether or acetone). The inhibitory effect of the mastic gum has been increased with increasing mustic gum concentration. The diameters of the maximum inhibitory zones at the highest mastic gum dosage (50 mg/ml) for the acetone and ethanol extracts were as large as 27.0 and 20.3 mm for *S. mutans*. Although mastic gum dissolved in all four of the solvents (chloroform, ethanol, acetone and petrolium ether) had a good antimicrobial activity against *S. mutans*, mastic gum dissolved in acetone was found to be the most effective. Compared with the antibiotic (vancomycin; as a control drug) tested, mastic gum had a similar antimicrobial activity against *S. mutans*.

In routine clinical works, saliva samples are often preferred owing to easier handling.[24] Therefore, we investigated the effect of mastic gum on the salivary bacterial numbers in vivo. In the present study, the saliva samples collected in 30 min intervals on 15th, 45th, 75th, 105th and 135th minutes, after chewing gum, there had been found significant reduction in the total number of mutans streptococci compared with substantial samples taken after paraffin chewing as placebo ($p < 0.001$).

Generally gum chewing has a mechanical effect washing out the bacteria by increasing the salivary flow rate. The placebo gum was used in the present study had identical amounts of xylitol, sorbitol, mannitol, mint flavour and wax. Previous studies have shown that chewing gums containing xylitol- and sorbitol had antibacterial effects.[25,26] In our study, mastic gum was more effective reducing the number of mutans streptococci in saliva compared to placebo chewing gum (Fig. 2). In this study, our results indicate that mastic gum had significant antimicrobial activity and support the possibility that mastic may be a useful ingredient to aid oral health.

When analysed the mastic gum is seen to have the main constituents of leaves of mastic tree (*P. lentiscus*) which are terpinen-4-ol and α-terpineol. These constituents are believed to be active compounds of many essential oils, and particularly tea-tree oil.[21] In the study of Magiatis et al.,[12] the in vitro antimicrobial activity of the three essential oils of *P. lentiscus* and of the resin (total, acid and neutral fraction) against six bacteria and three fungi was reported.[12] In our study parallel to the study of Magiatis et al., it was found that mastic gum obtained from *P. lentiscus* had serious antibacterial effect on *S. mutans* in vitro conditions.

The number of in vivo studies on this subject is limited in dentistry. In the study of Takahashi et al.[13] which was published on 2003, mastic gum was found to inhibit the plaque accumulation compared with placebo gum, indicating that mastic itself has anti-dental plaque formation activity which is an important factor for total streptococccci counts in the oral environment. In agreement with the results of the studies of Takahashi et al.,[13] our results suggest that regular use of mastic gum may be useful to control dental caries via its antibacterial effect and anti-plaque formation activity.

The idea of adding additional mechanical hygiene measures to patients' oral hygiene methods has significant appeal from a convenience and compliance perspective. Routine usage of mastic gum may also be useful where mechanical hygiene measures or mouthrinses would be impractical but where a chewable anti-plaque agent would be desirable.

Considering its antibacterial effect; our results suggest that mastic gum has an antibacterial activity against one of the most important cariogenic oral bacteria (*S. mutans*), which yields to decalcification of enamel and surface caries, and it may be useful for maintaining oral hygiene by reducing the bacterial growth (mutans streptococci) in saliva. Nevertheless further studies are needed to identify and

purify its active ingredients for future use in trials using toothpastes and mouthrinse formulas. In addition longer term studies will be required to evaluate the usefulness of this material more exactly. What's more, its bacteriostatic mechanism and specificity against this cariogenic bacterium need to be further researched.

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