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Effect of Eugenol on *Streptococcus mutans* Adhesion on NiTi Orthodontic Wires: In Vitro and In Vivo Conditions

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Abstract

Objective The purpose of this study was to compare the antibacterial effect of two types of Eugenol against *Streptococcus mutans* and then assess the effect of different concentrations of two types of Eugenol on *S. mutans* adhesion on Nickel-Titanium (NiTi) orthodontic wires in comparison to in vivo wires with fluoride-based hygiene regimen.

Materials and Methods Culture of *Streptococcus mutans* with NiTi orthodontic wires was done. Different media were prepared by adding 100%, 50%, 25%, and 12.5% of two origins of Eugenol (one biological obtained by hydrodistillation of *Syzygium aromaticum* and one chemical already prepared available in drugstores for dental use (Idental, MOROCCO, lot number: UAN/17–211/1). Three sizes of NiTi wires (0.016 inch, 0.016×0.022 inch, 0.017×0.025 inch) were retrieved from adult patients undergoing orthodontic treatment after 1 month of setting them up in the mouth. After incubation, colony forming unites were calculated and a SEM analysis was done to the surface of each wire. ANOVA test was done between all groups to find statistical differences and post-hoc *t*-test with Bonferroni analysis was performed to elucidate differences between all groups with $\alpha = 0.05$.

Results Eugenol has an anti-bacterial effect against *S. mutans*. The biological Eugenol has greater effect than the chemical one. The same observations were done for anti-adherent effect, the biological Eugenol demonstrated the highest anti-adherent effect at all concentrations while the effect of the chemical Eugenol was the lowest.

Conclusions The origin and the extraction mode of Eugenol have a crucial role in its antimicrobial and anti-adherent effect. Eugenol might constitute an alternative to Fluoride because it has an anti-adherent effect, limiting the incidence of white spot lesions.

Keywords
- Nickel-Titanium
- *Streptococcus mutans*
- bacterial adhesion
- Eugenol
- Orthodontic wires

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Introduction

Over the last decades, there has been a tremendous progress in the composition of orthodontic wires from stainless steel to Nickel-Titanium.[1] Nickel-Titanium wires have great mechanical properties such as superelasticity, thermal shape memory, good corrosion resistance, and good biocompatibility that help orthodontists solve malocclusions easily.[2] The Titanium oxide layer covering the surface of Nickel-Titanium orthodontic wires during galvanic corrosion is responsible for its biocompatibility in the oral cavity[3] and this layer determine the corrosion resistance of the wire.

Bacterial adhesion to orthodontic wires leads to a biodegradation of the wire's surface and might lead to a decrease in corrosion resistance.[4] *Streptococcus mutans* is present in great amount in the plaques of orthodontic patients with fixed appliances[5] because fixed orthodontic appliances have an effect on the oral microbiota.[6] Acids produced by *S. mutans*, cause enamel demineralization.[7,8] Therefore, *S. mutans* adhesion to orthodontic materials could be regarded as a key factor in the pathogenesis of enamel demineralization during orthodontic treatment.[7]

Because white spot lesions are the major complications of a fixed orthodontic treatment, patients must undertake a good oral hygiene regimen. All orthodontists recommend to their patients a daily use of fluoridated toothpastes and mouthwashes because fluoride is responsible for the formation of calcium fluoride complex that adheres to the teeth and leads to its remineralization protecting enamel against acid attack.[1] Nevertheless, it has been widely proven that the increase in the concentration of fluoride leads to a decrease in the corrosion resistance of Nickel-Titanium wires.[9] The corrosion amount of Nickel-Titanium alloy in the presence of fluoride ions increases up to 1,000 times and a decrease in the passive film breakdown potential is also observed.[10]

The incidence of enamel demineralization after fixed orthodontic treatment can involve up to 50% of patients. The incidence of such white spot lesions around orthodontic brackets has been demonstrated within 1 month of the treatment.[11,12] Thus, an anti-bacterial and anti-adherent agent is of a major concern to limit the white spot lesion occurrence during orthodontic treatment.

Several antimicrobial agents have been described in the literature, among them *Syzygium aromaticum* essential oil, which mainly constitute Eugenol.[13] Eugenol (4-allyl-2-methoxyphenol) is an aromatic molecule found in essential oils and hydrosols of a wild range of plants.[14] Several researchers have reported that this molecule has antibacterial, antiviral, antioxidant, anti-inflammatory and analgesic effects.[15,16] Furthermore, Eugenol is widely used in dentistry to treat toothache and pulpitis[17] but its anti-adherent effect on orthodontic wires has never been studied.

It has also been reported that Eugenol has an anticorrosive effect against Nickel-Titanium orthodontic wires.[18] Because adding fluoride to hygiene regimen of orthodontic patients wearing fixed orthodontic appliance have a negative effect on corrosion resistance of wires, Eugenol may constitute an alternative.

The purpose of this study was to compare the antibacterial effect of two types of Eugenol against *S. mutans* and then assess the effect of different concentrations of two types of Eugenol on *S. mutans* adhesion on NiTi orthodontic wires in comparison to in vivo wires with fluoride-based hygiene regimen.

Materials and Methods

Orthodontic Wires

This study was done in two parts, the first was conducted in vitro on as received Nickel-Titanium orthodontic wires and the second was done on retrieved wires from orthodontic patients with in vivo conditions.

NiTi wire with equiatomic composition (NiTi, 3M) was used for the in vitro study. Samples were cut into segments of 20 mm from the straight part of as received wires. Before testing, wires were rinsed thrice in water and immersed in acetone for 5 minutes.

Regarding retrieved wires, three dimensions of NiTi orthodontic wires were selected representing different stages of the orthodontic treatment: 0.016, 0.016×0.022, and 0.017×0.025 inch. Patients were given hygiene instructions and they brushed their teeth thrice a day with their usual toothpaste containing fluoride (1,240 ppm). After one month, arch wires were retrieved cautiously with a weingart plier to avoid any contact with oral mucosa and were stored separately in phosphate buffered solution (PBS) in a 50 mL of sterile falcon tube until further use. They were conducted immediately to the laboratory and in sterile conditions 20 mm from the straight part of each wire were cut.

Bacterial Culture

Saliva was obtained from adult patients with caries. Samples were collected in sterile recipients and were immediately transferred to the laboratory. They were diluted at 1/100 in sterile physiologic serum. Next, 50 µL of aliquots were spread in Mitis Salivarius Agar (MSA). After 48 hours of anaerobic incubation at 37°C, the isolated bacteria were transferred and spread in another MSA medium and incubated for 24 hours. After 2 weeks of serial cultures, the bacteria isolated were identified using Rapid ID 32 strep galleries biochemical and enzymatic tests (BioMerieux, SA). The bacteria were added to Ringer solution enriched with tryptic soy broth (TSB) medium to obtain active cultures.

Growth Inhibition Produced by Well-Diffusion Method

Streptococcus mutans was inoculated in the brain heart infusion (BHI) broth and incubated for 24 hours, to the point when growth was considered to be in the logarithmic phase. The density of the bacterial suspension was adjusted with sterile PBS to match the density of McFarland's standard 0.5. The bacterial broth suspension was streaked evenly onto the BHI agar plates with a cotton swab.

After the inoculum had dried, 8-mm single wells were made using a cork borer. Two types of Eugenol were studied, the biological Eugenol made by hydrodistillation of *S. aromaticum* and the Eugenol sold by manufacturers and used in dentistry

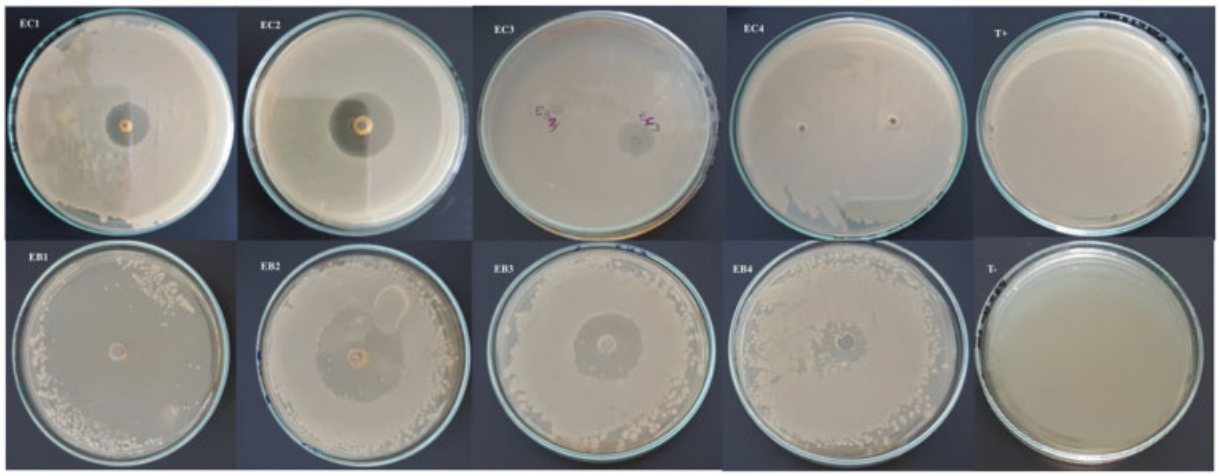

Fig. 1 Growth inhibition of eugenol against *Streptococcus mutans* produced by well-diffusion method. EC1, EC2, EC3, and EC4 correspond to chemical Eugenol with 100%, 50%, 25%, and 12.5%, respectively. EB1, EB2, EB3, and EB4 correspond to biological Eugenol with 100%, 50%, 25%, and 12.5%, respectively. T−: negative control. T+: positive control.

to prepare zinc oxide eugenol paste. The two types of Eugenol were diluted to four concentrations: 100%, 50%, 25%, and 12.5%. Then, 50 µL of each concentration of the two types of Eugenol was added and the plates were kept incubated at 37°C for 24 hours. After incubation, diameters of the growth inhibition zones were measured in mm at three different points in the circle zones.

Assessment of the Bacterial Adhesion of *Streptococcus mutans* on Wire's Surface

Artificial saliva (Ringer solution) was used as base and as the previous study, the same types of Eugenol and the same dilutions were used in the anti-adherent study. Dilutions were prepared using sterile distilled water and dimethyl sulfoxide (DMSO) was used to solubilize Eugenol.

Each wire (in vitro and in vivo retrieved wires) was cultured separately to avoid cross contamination in a tube containing 5 mL of artificial saliva with 50 µL of bacterial suspension to obtain a 1/100 concentration. The different concentrations of the two types of Eugenol were then added and all tubes were incubated at 37°C with agitation during 48 hours.

After incubation, wires were removed from the solutions to assess the adhesion of *S. mutans*. Each wire was rinsed thrice with a saline solution and transferred to a new microtube containing 1 mL of sterile distilled water. Then, microtubes were sonicated to detach adherent bacteria using four pulses of 30 seconds with intermittent cooling as described by Yang et al.[19] Serial dilution with sterile distilled water was then done for each microtube. Next, 100 mL of the two dilutions 10^{-3} and 10^{-5} after vortex were seeded in the BHI single agar plate. Positive (with wires and bacteria and without Eugenol) and negative (wires without bacteria) controls were used for each dilution. Petri dishes were then incubated at 37°C during 24 hours. Each group consists of three samples.

Colony counting was done by colony forming units to unitary surface (CFU/mL).

SEM Analysis

A SEM analysis was done to each wire to observe whether bacteria have been removed from the wire surface or not. Images were taken at magnification ×250 and ×2,500.

Statistical Analysis

The collected data were analyzed with the RStudio software. Results were presented in means and standard deviations. Because the test for normality showed that the data were normal to find the significant differences between the simples. ANOVA test was performed along with post-hoc *t*-test and Bonferroni comparison were used to elucidate statistical differences.

Results

Inhibitory Effect of Eugenol on *Streptococcus mutans*

►**Fig. 1** shows diameters of inhibition zones in the presence of each dilution of the two types of Eugenol. The width of halos increases by increasing the concentration of Eugenol, 42 mm with 100% of biological Eugenol and 16 mm with 12.5% of the same Eugenol. Halos are bigger in biological Eugenol (42 mm at 100%) than in chemical Eugenol (23 mm at 100%).

►**Table 1** shows the analysis of the inhibitory effect regarding biological Eugenol that has a more inhibitory effect on *S. mutans* than the chemical one in all concentrations. For the two types of Eugenol, decreasing the concentration of the

Table 1 Zone of inhibition of *Streptococcus mutans* following different concentrations of the two types of Eugenol (mm)

	Biological Eugenol	Chemical Eugenol
100%	42 mm	23 mm
50%	41 mm	22 mm
25%	24 mm	19 mm
12.5%	16 mm	0 mm

Table 2 Mean and standard deviation of colony forming units of *Streptoccocus mutans* in the presence of the different concentrations of biological and chemical Eugenol in vitro

	Biological Eugenol		Chemical Eugenol	
	Mean ($\times 10^5$) (CFU/mL)	SD	Mean ($\times 10^5$) (CFU/mL)	SD
100%	0.2^{ABC**}	0.035	0.25^{HJ**}	0.212
50%	5.25^{D*}	1.06	10^{IK*}	0.989
25%	10.1^{BF*}	0.424	14.5^{L*}	5.939
12.5%	$15.15^{ADEG***}$	1.626	30.35^{HI**}	6.434
Positive control	$37.65^{CEFG*** JKL}$		± 3.747	
Negative control	0		0	
F value	117.1		25.3	

Note: Within a column, values with the same capital superscript letter indicate statistically significant difference based on the Bonferroni multiple comparison tests at $\alpha = 0.05$.
No statistically significant difference was present between the positive control and the chemical Eugenol at 12.5%.
$^{*}p < 0.05$.
$^{**}p < 0.01$.
$^{***}p < 0.001$.

molecule decreases the zone of inhibition of *S. mutans*. At concentration of 12.5%, the chemical Eugenol has no antibacterial effect on *S. mutans*. In the absence of Eugenol, a bacterial growth has been noted and no halo has been detected (0 mm).

Colony Forming Units Regarding the Different Concentrations of the Two Origins of Eugenol

►**Table 2** shows that biological Eugenol has a more anti-adherent effect than the chemical one. The more the concentration of the two types of Eugenol decreases, the more the anti-adherent effect decreases too.

Wires with In Vivo Conditions

The mean of colony forming units regarding *in vivo* wires is higher than the one in the presence of Eugenol. The third wire with the 0.017×0.025 size shows the higher amount of adherent bacteria (►**Table 3**).

SEM Wires Surface Analysis

►**Fig. 2** shows SEM images of NiTi wires in the presence of *S. mutans* after immersion in different concentrations of the two types of Eugenol. For the chemical Eugenol, the 100% concentration (EC1) shows an image with scratches and black depositions on the surface of wire while in biological Eugenol (EB1), and at the same concentration, the surface of the wire is similar to the control with deposition of white crystals on its surface. In both wires, there are no bacteria in the surface.

With 50% of Eugenol concentration, NiTi wire with chemical Eugenol shows white irregular surfaces all over the wire (EC2) and the biological Eugenol has a surface similar to the control with some pitting in the surface of the wire (EB2).

With 25% of Eugenol concentration, NiTi wires with chemical Eugenol demonstrate a surface with white appositions (EC3) while with biological Eugenol, there is a large white surface on the wire (EB3).

With 12.5%, white crystals have been observed on the surface of the wire with chemical Eugenol and (EC4) with biological Eugenol, there are black points on the surface of the wire (EB4).

With in vivo wire N°1, there is a lot of pitting and deep scratches on the surface of the wire. In the in vivo wire N°2 and 3, cocci and bacilli bacteria have been observed on the surface both wires forming complex units.

Table 3 Mean and standard deviation of colony forming units of bacteria from wires with in vivo conditions

	Mean ($\times 10^5$) (CFU/mL)	SD
Wire 1	33.05	4.03
Wire 2	39.7	6.646
Wire 3	50.5	5.656

Discussion

The aim of this study was to compare the antibacterial effect of two types of Eugenol (a Biological one made by hydrodistilation of *S. aromaticum* and the commercial one used in dentistry) against *S. mutans*. Results of this study confirm that Eugenol has an anti-bacterial effect on *S. mutans*. Biological Eugenol has more antibacterial effects than the one sold in commerce. Xu (2013) reported in his study that Eugenol demonstrated significant inhibitory effects against acid production by *S. mutans*. The synthesis of water-insoluble glucans by glucosyltransferases was reduced by eugenol.[20] At 12.5% of chemical Eugenol, there is no antibacterial effect on *S. mutans* while with the biological Eugenol, an inhibition zone of 16 mm was observed. This result confirms that the biological Eugenol is more efficient on *S. mutans* ++.

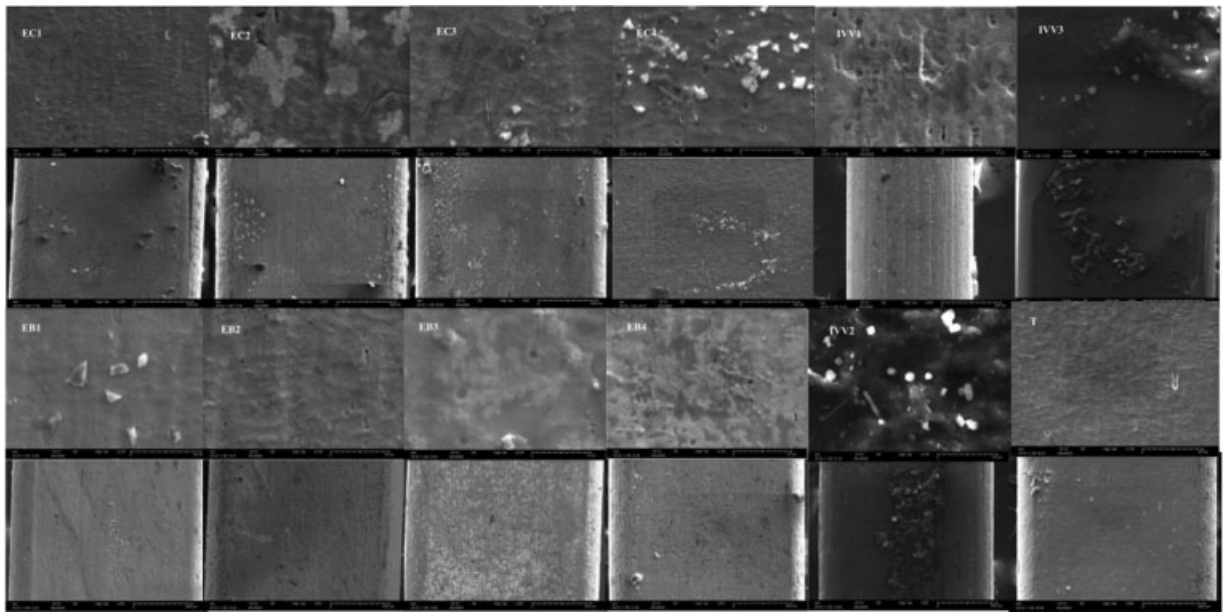

Fig. 2 SEM analysis of NiTi wires in different solutions at the end of the experiment. EC1, EC2, EC3, and EC4 correspond to chemical Eugenol with 100%, 50%, 25%, and 12.5%, respectively. EB1, EB2, EB3, and EB4 correspond to biological Eugenol with 100%, 50%, 25%, and 12.5%, respectively. IVV1, IVV2, and IVV3 represent wires with in vivo conditions at 016 inch, 0.016 × 0.022 inch, and 0.017 × 0.025 inch, respectively. T: control. Each wire is represented by two images one on the top with ×2,500 magnification and other on the bottom with ×250 magnification. White surfaces and crystals correspond to appositions on the surface of the wire indicating a corrosion inhibition, and black pitting matches with the degradation of the wire surface. IVV2 and IVV3 show the amount of adherent bacteria on their surfaces.

Several aromatic plants has been used in dentistry for their beneficial effects, they are an interesting source of eco-friendly, inexpensive, and available new large spectrum antimicrobials with low levels of cutaneous cytotoxicity and environmental toxicity.[21–23] Besides, because sodium fluoride added to toothpastes increases the corrosion process of Nickel-Titanium orthodontic wires, providing a prosperous environment for bacterial colonization, it might be interesting to search for another alternative that can have anti-bacterial and anti-corrosive effects at the same time.

Hydrodistillation of *S. aromaticum* yield clove essential oil, which is an aromatic and volatile substance. It has multiple properties such as antimicrobial, antioxidant, and anti-inflammatory effects that make it widely used in different areas. The US Food and Drug Administration listed it like "generally regarded as safe" chemical.[24] The main constituent of this essential oil is Eugenol (4-allyl-2-methoxyphenol). It has a large spectrum antimicrobial ability against several bacteria species. Eugenol displays also an essential activity against anaerobic oral bacteria like *S. mutans*.[25,26] Other essential oils have been described and showed an antibacterial effect. Among them, Origanum dubium oil and Cinnamomum cassia oil with a mean zone of inhibition of 31 mm and 38.67 mm, respectively,[27] which are minor than the results found with Eugenol in this study (42 mm), suggesting that the biological eugenol is more effective on *S. mutans*. The second purpose of this study was to evaluate the effect of different concentrations of these two types of Eugenol on *S. mutans* adhesion on NiTi orthodontic wires. Results show that biological Eugenol has more anti-adherent effects than the chemical one and increasing the concentration of Eugenol leads to an increase in the anti-adherent effect on *S. mutans*. The difference in results between the two types of Eugenol might be due to the mechanism of preparation of the solution and the molecule purification.

In fact, white spot lesions (WSL) are the most common side effects of a fixed orthodontic treatment. Studies have demonstrated that the amount of *S. mutans* in biofilm over WSL is high.[28] Plaque accumulation around orthodontic brackets often results in enamel white spot formation adjacent to the brackets. This plaque is composed of various microorganisms of which *S. mutans* is the most cariogenic. Its adherence to the fixed appliance is largely contributed to the bracket material. *S. mutans* along with glycosyltransferase degrades sucrose to make insoluble glucans that also attach to the tooth surface, providing ideal sites for oral bacteria to inhabit. The resulting complex of glucan and various bacteria then creates an oral biofilm that is the mature stage of dental plaque. As plaque accumulates, acidic compounds such as fructose and other fatty acids degrade the enamel surface of the teeth through a process known as dental caries.[29]

Mechanical removal of biofilm by brushing is very effective. This is why orthodontists prescribe fluoridated toothpastes and mouthwashes to help patients controlling plaque formation.[30,31] Nevertheless, as reported in this study, the highest amount of adherent bacteria was observed with wires in vivo among patients who brushed their teeth with fluoridated toothpaste. This suggests that Eugenol might be considered as alternative to fluoride. It might constitute an adjuvant element to hygiene regimen because it has an

antibacterial effect along with anti-adherent effect against *S. mutans*. Eugenol can also be used in toothpastes and mouthwashes for patients wearing fixed appliances from the beginning of their treatment. Further studies have to be done to determine the minimum and the maximum dose to avoid the side effects of Eugenol.

Other substances described in the literature such as *Rhus coriaria* L. water extract had significant antibacterial properties against *S. mutans* and is able to inhibit bacterial biofilm formation on orthodontic wires.[32] Furthermore, Dias has confirmed in her study that *S. mutans* biofilms adhesion to brackets were susceptible to CHX treatment.

Conclusions

This study confirmed that Eugenol has an antibacterial effect against *S. mutans*. The biological Eugenol is more effective than the one sold in commerce. Furthermore, Eugenol obtained in laboratory by hydrodistillation of *S. aromaticum* has more anti-adherent activity against *S. mutans* on Nickel-Titanium orthodontic wires than the Eugenol used in dentistry and found in commerce. Orthodontists should be aware of *S. mutans* adhesion because it causes white spot lesions, especially when using the same arch wire for a long period of time.

Conflict of Interest
None declared.

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