

Antimicrobial Effect and Surface Tension of Some Chelating Solutions with Added Surfactants

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This study assessed the antimicrobial efficacy and surface tension of established irrigating solutions with a new experimental chelating solution in infected dentin tubes. Twenty-five specimens were randomly assigned to each of the irrigating solutions. Twenty specimens were used as negative and positive controls. After 21 days of contamination with *E. faecalis*, the irrigating solutions MTAD, QMiX and Tetraclean NA were delivered into each infected root canal. The solutions were removed and dentin samples were withdrawn from the root canals with sterile low-speed round burs with increasing ISO diameters. The dentin powder samples obtained with each bur were immediately collected in separate test tubes containing 3 mL of BHI broth. After that, 100 µL from each test tube was cultured on blood agar. The grown colonies were counted and recorded as colony-forming units (CFU). The surface tension of the irrigants was measured using a Cahn DCA-322 Dynamic Contact Angle Analyzer. A Kruskal Wallis nonparametric ANOVA and a Friedman test were used ($p < 0.05$). Tetraclean NA showed lower surface tension and CFU values than MTAD and QMiX. Better antibacterial action and low surface tension were observed for Tetraclean NA, probably due to the improved penetration into the root canal and dentinal tubes

Introduction

Cleaning and shaping of the root canal is of utmost importance for removing pulp tissue, bacteria and debris from the complex root canal system (RCS) (1). *Ex vivo* studies and clinical evidence have shown that mechanical instrumentation leaves untouched significant portions of the root canal walls and complete elimination of bacteria by instrumentation alone is unlikely to occur (2). Therefore, irrigating solutions should use to improve cleaning and disinfection during root canal preparation (3). Sodium hypochlorite (NaOCl) has excellent antibacterial and dissolving abilities, as reported by several studies (4) and it is the solution of choice during root canal instrumentation. On the other hand, NaOCl cannot remove the smear layer formed on the canal walls during manual or rotary instrumentation (5). The smear layer may delay or prevent penetration of antimicrobial agents, such as endodontic irrigants. In an *ex vivo* histological study on human teeth, it has been found that the bactericidal effectiveness of endodontic irrigants depends on their ability to penetrate the infected dentinal tubules and this process is undoubtedly influenced by the presence or absence of smear layer (6). Fogel and Pashley (7) have previously shown that formation of smear layer reduced root dentin permeability after treatment with 5% NaOCl, but it increased many times after treatment with citric acid.

The use of chelating solutions, such as ethylenediaminetetraacetic acid (EDTA) or citric acid (8), is

required to remove the inorganic components of the smear layer. Since 1957, EDTA was recommended and frequently employed for its ability to remove the smear layer (9). Arias Moliz et al. (10) investigated the bactericidal activity of final rinse irrigating solutions against *Enterococcus faecalis* and found that 17% EDTA solution showed no bactericidal activity against *E. faecalis*, even after 60 min of contact. Although EDTA has a long-standing history because it is the most frequently recommended agent for smear layer removal in endodontics, its potential for irritation has been highlighted (11). Therefore, other chelating agents have been suggested to remove the smear layer from instrumented root dentin. The most important requirements for an ideal endodontic irrigant are to have an antibacterial effect and to penetrate deep into the dentinal tubules. In the last few years, new irrigant solutions for smear layer removal with added antimicrobial agents have been developed (12-14).

Surface tension is considered as one of the most important factors in determining the wettability of a solution (15). Wettability is the tendency of a fluid to spread over or adhere to a solid surface. This property is required for the chemical solution to penetrate the main and lateral canals, as well as the dentinal tubules and it depends on the surface tension (16). The aim of this study was to assess the antimicrobial efficacy and to compare the surface tension of MTAD, QMiX and Tetraclean NA irrigating solutions.

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Material and Methods

The method of this study was based on the procedure previously described by Haapasalo and Ørstavik (17).

Dentin Specimens Preparation

Bovine central incisors were selected for this study. The apical 5 mm and two-thirds of the crown were removed from each tooth with a water-cooled rotary diamond saw at 1000 rpm (Isomet Plus precision saw; Buehler, Lake Bluff, IL, USA). The roots were cut into 4-mm-thick slices with a diamond saw as above. The cementum was removed by polishing paper (Ecomet 3, variable-speed grinder-polisher; Buehler), which resulted in a center-holed piece of root dentin with a 6-mm outer diameter. The internal diameter was standardized by an ISO 023 slow speed round bur. The organic and inorganic debris were removed by treating each dentin tube with 5.25% sodium hypochlorite followed by 17% EDTA in an ultrasonic bath for 5 min and dipped in distilled water for 10 min. The specimens were then placed in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and autoclaved at 121 °C.

Contamination of Dentin Specimens

Isolated 24-h colonies of pure cultures of *E. faecalis* (ATCC 29212) were suspended in 5 mL of BHI broth (Oxoid). The bottles containing each specimen were opened in a laminar flow chamber. Two milliliters of sterile BHI were removed with sterile pipettes and replaced with 2 mL of bacterial inoculum. The bottles were closed and kept at 37 °C for 21 days, with replacement of 1 mL of contaminated BHI by 1 mL of freshly prepared BHI every 2 days, to avoid medium saturation. After the contamination period, the specimens were irrigated with 5 mL of sterile saline solution to remove the incubation broth. The bacterial growth was verified on three additional contaminated samples with a histological technique and Gram stain (Brown and Brenn staining method), and examined with an Olympus CX41 microscope (Olympus Europe Co GmbH, Hamburg, Germany) at 400× magnification.

Root Canal Irrigants

Three root canal irrigants were used: QMiX, a mixture of EDTA, Chlorhexidine (CHX) and a detergent (cetrimide) (Dentsply Tulsa, OK, USA), BioPure MTAD, a mixture of citric acid, tetracycline isomer (doxycycline) and detergent (Tween 80) (Dentsply), and Tetraclean NA, a mixture of citric acid and detergents (cetrimide + polypropylene glycol) (patent pending).

Antimicrobial Assessment

Ninety-five specimens, 85 contaminated with *E. faecalis* and 10 not contaminated, were randomly divided into five

groups as follows: QMiX, (n=25); BioPure MTAD, (n=25); Tetraclean NA, (n=25); positive control (infected dentin tubes) (n=10); negative control (sterile saline solution) (n=10). Then, each specimen was removed from its bottle under aseptic conditions, and the root canal was irrigated with 5 mL sterile saline and dried with sterile paper points. The outer surface of each specimen was covered with two layers of nail polish to prevent contact of the irrigant with the external surface. After that, using decontaminated sticky wax, the specimens were fixed at the bottom of the wells of 24-well cell culture plates, which also obliterated the apical surface of the root canal. Finally, the irrigating solutions were instilled into the canal lumen with sterile 3-mL plastic syringes and #27 gauge needles until the dentin tubes were filled. The solutions were removed using sterile paper points after 5 min (BioPure MTAD), 90 s (QMiX) and 60 s (Tetraclean NA), according to the manufacturers' recommendations, after placement into the root canal lumen. The specimens were then incubated at 37 °C for 28 days. At the experimental times of 0, 7, 14, 21 and 28 days, the dentin chips were removed from the root canals using sequential sterile low-speed round burs with increasing ISO diameters: 025, 027, 029, 031 and 033. Each bur removed approximately 0.1 mm (100 µm) of dentin around the canal. The dentin shavings obtained with each bur were carefully collected on sterile aluminum foil to avoid losing dentin chips during the sampling procedure. They were suspended in separate test tubes, each containing 3 mL of freshly prepared BHI broth followed by 10-fold serial dilutions in sterile saline solution. After that, 100 µL from each test tube was cultured on blood agar (Oxoid). Grown colonies were counted and recorded as CFU (colony-forming units).

Surface Tension Measurement

The surface tension of the tested liquids was measured using the Wilhelmy plate technique, with a Cahn DCA-322 Dynamic Contact Angle Analyzer (Gibertini Elettronica, Novate (MI), Italy) (16). The calculations of this technique are based on the geometry of the fully wetted glass slide in contact with the liquid. Using this method, a glass slide was immersed in 5 mL of the test liquid in a beaker cleaned with hot chromic acid, rinsed with MilliQ water and finally air plasma-cleaned in a glow-discharge reactor. The force on the glass slide was recorded continuously by the instrument software as the beaker was raised and withdrawn at the constant speed of 40 µm/s, until at least 1 cm of the glass slide was immersed. For each sample, 15 measurements were taken and mean values calculated.

Statistical Analysis

Statistical analysis was performed with Stata 12.0 software (Stata, College Station, TX, USA) both for data

regarding antibacterial effects and for surface tension values. Descriptive statistics, including mean, standard deviation, median, minimum and maximum values were calculated as CFU for antibacterial effects of all irrigating solutions. The normality of the data was calculated using the Shapiro-Wilk test. The Kruskal-Wallis non-parametric ANOVA was applied to determine whether there were significant differences in antibacterial effects among the irrigating solutions. A Dunn test was used *post-hoc* for multiple comparisons to assess significant differences between each irrigating solution after 28 days. Surface tension values were analyzed initially with a Shapiro-Wilk test. Kruskal-Wallis ANOVA and Dunn tests were then applied to assess the differences among chelating agents. Friedman test was applied to assess the differences over time for each tested irrigating solution. Significance for all statistical tests was predetermined at $p < 0.05$.

Results

The positive control group showed viable bacteria at all experimental times, confirming the efficiency of the methodology. In contrast, the negative control group

showed no viable bacteria at all experimental times.

Table 1 shows the average CFU for each irrigating solution. The Kruskal-Wallis ANOVA followed by the Dunn test showed that MTAD had significantly higher CFU values ($p < 0.05$) than Tetraclean NA and QMiX, which were comparable. Figure 1 shows the CFU average values recorded at each time point for each irrigant. The Friedman test showed for all irrigating solutions a significant increase in CFU values over time ($p < 0.0001$).

Average surface tension values for each irrigant are in Table 2. Tetraclean NA showed significantly lower surface tension values compared to MTAD and QMiX ($p < 0.05$).

Discussion

Removal of the smear layer produced after root canal instrumentation has been recommended. The bacteria with their by-products and necrotic debris in smear layers compromise the disinfection process (5) and decrease dentin permeability, hampering the diffusion of antimicrobial agents like irrigants into root dentin (6).

EDTA has been suggested and widely used as an irrigation solution because it chelates calcium ions and removes the mineralized portion of smear layers (5,6,8). Although EDTA has a long history as an endodontic chelating agent, it has limited or no antibacterial activity (10) and a high surface tension value (16). In order to overcome these problems, several ways have been suggested like increasing the antibacterial activity of root canal irrigants for the final rinse. One of these ways is to add surfactants and antibiotic (tetracycline) to irrigating solutions. In an attempt to produce an irrigant that could remove the smear layer by its antimicrobial properties, Torabinejad et al. (12) developed a new solution containing a mixture of tetracycline, citric acid and Polysorbate 80 detergent (BioPure MTAD), able to remove both the smear layer and disinfecting the root canal system. Their results showed that BioPure MTAD was useful as a final rinse to remove the smear layer and eradicate bacteria from infected root canals. However, some of these findings have been challenged by other researchers, who found a lower antibacterial activity of BioPure MTAD compared with NaOCl (18). These results could be correlated with the activity of doxycycline, which has a bacteriostatic

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Table 1. Descriptive statistics of the CFU data obtained for each irrigating solution. Different capital letters indicate significant differences among irrigating solutions when overall data are tested ($p < 0.05$), according to Kruskal-Wallis followed by Dunn tests. Different numerical indexes indicate significant differences over time for each irrigating solutions ($p < 0.05$)

Time	Irrigating solution	Obs	Median	Min	Max	Sig.
Day 0	Tetraclean NA	125	0.22	0.04	0.46	1
	MTAD	125	2.35	0	4.5	5
	Qmix	125	0.9	0.66	1.34	9
Day 7	Tetraclean NA	125	1	0.48	1.92	2
	MTAD	125	4.37	2	6.7	6
	Qmix	125	3.46	2.76	4.32	6
Day 14	Tetraclean NA	125	4.82	3.28	5.9	3
	MTAD	125	8.34	5.99	11.49	7
	Qmix	125	7.38	4.69	11.17	7
Day 21	Tetraclean NA	125	11.5	8.21	13.57	4
	MTAD	125	19.59	15.79	22.61	8
	Qmix	125	12.45	8.23	14.69	10
Overall	Tetraclean NA	125	4.82	0.04	24.08	A
	MTAD	125	8.34	0	44.98	B
	Qmix	125	7.38	0.66	32.96	B

Obs: Number of observations. Min: Minimum. Max: Maximum; Sig.: Significance

Table 2. Mean surface tension (mJ/m^2) for irrigating solutions

Irrigating solution	Obs	Mean \pm Std. Dev.
Tetraclean NA	15	28.91 \pm 0.95*
MTAD	15	34.54 \pm 0.76
Q-mix	15	36.43 \pm 0.48

Kruskal-Wallis ANOVA followed by Dunn test. * $p < 0.05$ vs MTAD and Q-mix. Std. Dev.: standard deviation. Obs= Number of observations.

action and cannot kill bacteria. The addition of cetrimide (CTR) to replace Tween 80 may increase the antibacterial activity of solutions like EDTA, citric acid and BioPure MTAD (22). CTR is a cationic surfactant, which combined with chelating agents, has greater antimicrobial activity than the chelating irrigants alone (20).

QMIX, a non-antibiotic single solution used as a final rinse after NaOCl for one-step smear layer removal and disinfection, was introduced in 2011. This is a premixed and ready to use irrigating solution that contains EDTA, chlorhexidine (CHX) and a detergent (cetrimide), with pH slightly above neutral. This irrigant has demonstrated antibacterial activity comparable to NaOCl and superior activity against planktonic and biofilm bacteria compared to 2% CHX and MTAD (13). Contrarily, Ordinola-Zapata et al. (21) observed the poor antibacterial activity when QMiX was applied for 5 min on multispecies biofilm from oral microbiota. Tetraclean NA is a new combination that contains citric acid, cetrimide and polypropylene glycol without antibiotic. Recently, a comparative study (22) indicated satisfactory antibacterial activity of Tetraclean NA on root canals infected with *E. faecalis* for 60 days. The current *in vitro* study compared the antimicrobial activity of different low surface tension chelating agents with on root canals infected with *E. faecalis* after variable time intervals and their surface tension values.

The results of the present study showed that Tetraclean NA had the lowest CFU values, while the highest CFU counts resulted from MTAD (Fig. 1, Table 1). These findings

are not a surprise, because Tween 80 (Polysorbate 80), a detergent present in MTAD, has limited or no activity as an antibacterial agent (23) and it may neutralize various substances (24). According to Pappen et al. (19), modifications of MTAD where CTR replaced Tween 80 were more effective against *E. faecalis* and polymicrobial biofilm than MTAD, confirming that CTR improved the antimicrobial properties of the solutions, whereas Tween 80 seemed to have a neutral or negative impact on their antimicrobial effectiveness. The lower antibacterial activity of QMiX compared to Tetraclean NA, as observed in the present study, is due to the different composition and concentration of detergents and chelating agents in the two irrigating solutions. QMiX has a lower content of CHX and cetrimide, while Tetraclean NA is an irrigant mixture that contains more cetrimide and citric acid than QMiX. Citric acid as well as cetrimide has antibacterial efficacy and previous studies highlighted that citric acid shows antimicrobial activity against anaerobic bacteria of the infected root canals, in particular against cocci (25).

In a recent study, Poggio et al. (14) compared the decalcifying capacity of different irrigating solutions at different contact times and observed a significantly higher release of Ca^{2+} in samples exposed to citric acid-based agents, including Tetraclean NA, than EDTA solutions in the presence or absence of cetrimide. The higher release reported in that study may be due to the lower pH of the citric acid solutions ($\text{pH} < 2$), thus increasing the removal of inorganic elements such as calcium from the hydroxyapatite

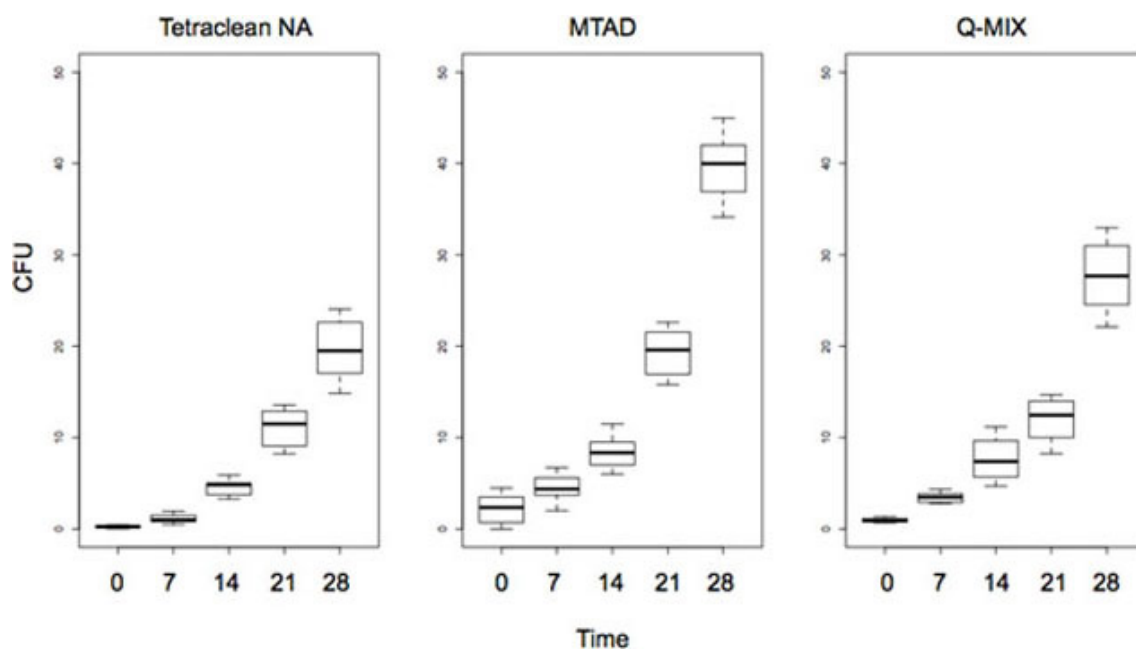


Figure 1. Box-plot of CFU values and antibacterial effects for each irrigating solution at different times. For each employed irrigant, the median value, the minimum and the maximum values were indicated.

crystals, agreeing with previous results (8).

The present study also compared the surface tension of three chelating solutions modified with detergents and demonstrated that Tetraclean NA had significantly lower surface tension values compared to MTAD and QMiX (Table 2). In view of what has been observed in this investigation, the better antibacterial action of Tetraclean NA may be explained by its low pH (unpublished laboratory results), low surface tension and better removal of the smear layer (data not published), all of which improve its penetration into the root canal and the dentinal tubules. Further studies should be performed to confirm the promising results observed in this *in vitro* study.

In conclusion, Tetraclean NA and QMiX showed significantly better antibacterial activity and lower surface tension values compared to MTAD.

Resumo

Este estudo avaliou a eficácia antimicrobiana e tensão superficial de soluções irrigadoras e uma nova solução quelante em tubos de dentina infectada. Vinte e cinco espécimes foram aleatoriamente distribuídos conforme as soluções irrigantes. Decorridos 21 dias de contaminação com *E. faecalis*, a soluções de irrigação MTAD, QMiX e Tetraclean NA foram distribuídas em cada canal radicular infectado. As soluções foram removidas e as amostras de dentina foram retiradas dos canais radiculares com brocas esféricas de baixa velocidade com diâmetros ISO sucessivamente maiores. As amostras do pó de dentina obtidas com cada broca foram imediatamente colocadas em tubos de ensaio separados contendo 3 mL de caldo BHI. A seguir, 100 µL de cada amostra do tubo de teste foi cultivada em agar de sangue. As colônias crescidas foram contadas e registradas como unidades formadoras de colônias (UFC). A tensão superficial das soluções irrigantes foi medida utilizando o método de Wilhelmy. A análise não paramétrica de Kruskal-Wallis e o teste de Friedman foram utilizados ($p < 0,05$). Tetraclean NA apresentou menor tensão de superfície e menores valores de UFC do que MTAD e QMiX. A melhor ação antibacteriana e baixa tensão superficial foram observadas para Tetraclean NA, provavelmente devido à melhor penetração no canal radicular e túbulos dentinários.

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References

- Schilder H. Cleaning and shaping the root canal. *Dent Clin North Am* 1974;18:269-296.
- Peters OA, Laib A, Gohring TN, Barbakow F. Changes in root canal geometry after preparation assessed by high-resolution computed tomography. *J Endod* 2001;27:1-6.
- Siqueira JF, Lima KC, Magalhaes FAC, Lopes HP, de Uzeda M. Mechanical reduction of the bacterial population in the root canal by three instrumentation technique. *J Endod* 1999;25:332.
- Mohammadi Z. Sodium hypochlorite in endodontics: an update review. *Int Dent J* 2008;58:329-341.
- McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. *J Endod* 1975;1:238-242.
- Berutti E, Marini R, Angeretti A. Penetration ability of different

- irrigants into dentinal tubules. *J Endod* 1997;23:725-727.
- Fogel HM, Pashley DH. Dentin permeability: effects of endodontic procedures on root slabs. *J Endod* 1990;16:442-445.
- Scelza MF, Teixeira AM, Scelza P. Decalcifying effect of EDTA-T, 10% citric acid, and 17% EDTA on root canal dentin. *Oral Surg Oral Med Oral Pathol* 2003;95:234-236.
- Torabinejad M, Handysides R, Khademi AA, Bakland LK. Clinical implications of the smear layer in Endodontics: a review. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2002;94:658-666.
- Arias-Moliz MT, Ferrer-Luque CM, Espigares-Rodríguez E, Liébana-Ureña J, Espigares-García M. Bactericidal activity of phosphoric acid, citric acid, and EDTA solutions against *Enterococcus faecalis*. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008;106:e84-e89.
- Scelza MF, Daniel RL, Santos EM, Jaeger MM. Cytotoxic effects of 10% citric acid and EDTA-T used as root canal irrigants: an *in vitro* analysis. *J Endod* 2001;27:741-743.
- Torabinejad M, Shabahang S, Apécio RM, Kettering JD. The antimicrobial effect of MTAD: an *in vitro* investigation. *J Endod* 2003;29:400-403.
- Stojčić S, Shen Y, Qian W, Johnson B, Haapasalo M. Antibacterial and smear layer removal ability of a novel irrigant, QMiX. *Int Endod J* 2012;45:363-371.
- Poggio C, Dagna A, Vinci A, Beltrami R, Cucca L, Giardino L. Decalcifying capability of irrigating solutions on root canal dentin mineral content. *Contemp Clin Dent* 2015;6:201-205.
- Lopes HP, De Faria AR, Alves FRF, Elias CN. Wettability of irrigants used in root canal treatment. *Dentistry* 2015;5:283.
- Giardino L, Ambu E, Becce C, Rimondini L, Morra M. Surface tension comparison of four common root canal irrigants and two new irrigants containing antibiotic. *J Endod* 2006; 32:1091-1093.
- Haapasalo M, Ørstavik D. *In vitro* infection and disinfection of dentinal tubules. *J Dent Res* 1987;66:1375-1379.
- Dunavant TR, Regan JD, Glickman GN, Solomon ES. Comparative evaluation of endodontic irrigants against *Enterococcus faecalis* biofilms. *J Endod* 2006;32:527-531.
- Pappen FG, Shen Y, Qian W, Leonardo MR, Giardino L, Haapasalo M. *In vitro* antibacterial action of Tetraclean, MTAD, and five experimental irrigation solutions. *Int Endod J* 2010;43:528-535.
- Ferrer-Luque CM, Conde-Ortiz A, Arias-Moliz MT, Valderrama MJ, Baca P. Residual activity of chelating agents and their combinations with cetrimide on root canals infected with *Enterococcus faecalis*. *J Endod* 2012;38:826-828.
- Ordinola-Zapata R, Bramante CM, Garcia RB, Andrade FB, Bernardineli N, Moraes IG, et al.. The antimicrobial effect of new and conventional endodontic irrigants on intraorally infected dentin. *Acta Odontol Scand* 2013;71:424-431.
- Giardino L, Estrela C, Generali L, Mohammadi Z, Asgary S. The *in vitro* effect of irrigants with low surface tension on *Enterococcus faecalis*. *Iran Endod J* 2015;10:174-178.
- Dawson, R. M. C., D. P. Elliott, W. H. Elliott, K. M. Jones. 1986. Data for biochemical research, 3rd ed. Oxford University Press, New York, NY. p. 289.
- Metzger Z, Basrani B, Goodis HE. Instruments, Materials, Devices. in: Cohen's Pathways of the Pulp. 10th ed. Mosby Inc., St Louis; 2011: 251-252.
- Georgopoulou M, Kontakiotis E, Nakou M. Evaluation of the antimicrobial effectiveness of citric acid and sodium hypochlorite on the anaerobic flora of the infected root canal. *Int Endod J* 1994;27:139-143.

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