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Antimicrobial activity, toxicity and accumulated hard-tissue debris (AHTD) removal efficacy of several chelating agents

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Antimicrobial activity, toxicity and accumulated hard-tissue debris (AHTD) removal efficacy of several chelating agents

Abstract

Aim To evaluate the antimicrobial, toxicity, and cleaning effectiveness of ethylenediaminetetraacetic acid (EDTA) and Maleic acid (MA) alone and combined with cetrimide (CTR).

Methodology Cytotoxic and genotoxic effects were assessed on Chinese hamster cells V79 using the MTT, clonogenic, and micronucleus assays, respectively. The bacterial inhibitory and bactericidal concentrations (MIC and MBC, respectively) were determined on a strain of *E. faecalis*. Antimicrobial tests were performed on a biofilm model after treatment with the chelating agents by using a biofilm eradication concentration (MBEC) and confocal laser scanning microscope (CLSM) assays. Quantification of cell biomass and percentage of live and dead cells in the biomass was assessed for each group. The percentage reduction of accumulated hard tissue debris (AHTD) after root canal preparation and final irrigation protocols was evaluated by micro-CT. Statistical tests of one-way analysis of variance (ANOVA), Bonferroni test, Kruskal-Wallis test, Dunn's multiple comparison test, and Wilcoxon matched-pairs signed-rank tests were used.

Results CTR alone as well as in combination with EDTA and MA at dilutions of 1/10 and 1/100 were significantly toxic as compared to untreated control ($p<0.001$). All tested mixtures were nontoxic at a dilution of 1/1000. EDTA retained a weak inhibitory and bactericidal effect against planktonic cells, while MA inhibited cells growth and killed 99.9% of the cells when diluted. CTR revealed the most prominent effect, being inhibitory and bactericidal, also when diluted. Cetrimide alone or combined with EDTA was able to remove, respectively, 40% ($p<0.01$) and 60% ($p<0.001$) of the entire biomass after 1 min. Conversely, MA alone and in combination with CTR had not a significant effect on biomass reduction. After final irrigation, the AHTD volume was significantly decreased in all groups ($p<0.05$). EDTA + CTR and MA + CTR were associated with a significant reduction in the percentage of AHTD on the entire root canal compared to the same solutions without surfactant.

Conclusions 7% MA was less cytotoxic in comparison to 17% EDTA. The addition of CTR to EDTA and MA removed accumulated hard-tissue debris effectively from the canal walls and increased their antimicrobial activity when compared to the same solutions without detergents.

Keywords: biofilm; chelating agents; confocal laser scanning microscopy; irrigation; Micro-CT.

Introduction

The main goal of root canal treatment is to optimize root canal disinfection and to prevent re-infection of the root canal space after treatment (Zehnder 2006). Cleaning and shaping of the root canal system are considered the key requirements in eliminating microorganisms. Mechanical instrumentation of the root canal system produces an irregular, granular layer covering the canal walls known as the smear layer (McComb & Smith 1975), which consists of inorganic dentine debris, pulp tissues, severed odontoblastic processes, necrotic debris, microorganisms, and their metabolic products (Torabinejad *et al.* 2002). Studies have demonstrated that removal of smear layer improves the fluid-tight seal of filled root canals, allows the elimination of bacteria within the dentinal tubules and facilitates the penetration of intracanal medicaments, irrigating agents and sealers into the dentinal tubules, increases the bond strength of resin sealers to root canal dentine, resulting in a significantly better apical seal (White *et al.* 1984, Cergneux *et al.* 1987, Ørstavik & Haaapasalo 1990, Gettleman *et al.* 1991, Shahravan *et al.* 2007).

Currently, there is no single irrigating solution capable of killing bacteria, dissolving organic tissue, and removing the smear layer simultaneously (Giardino *et al.* 2018). Hence, various studies have suggested the use of a combination of irrigants (O'Connell *et al.* 2000, Teixeira *et al.* 2005). Combined application of ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite (NaOCl) is frequently recommended for effective removal of the smear layer from the root canal system (Zehnder *et al.* 2005). Also, irrigating agents intended for endodontic use should be biocompatible when applied as intended (Peters 2013).

Conflicting results on the biofilm disruption or antimicrobial effects of EDTA have been demonstrated (Heling *et al.* 1999, Arias-Moliz *et al.* 2008, Ballal *et al.* 2011, Ordinola-Zapata *et al.* 2012). The only antibacterial property associated with EDTA is to weaken the bacterial cell membrane without killing the cell and thus promoting biofilm detachment (de Almeida *et al.* 2016). EDTA *per se* is not an effective antimicrobial agent (Reidmiller *et al.* 2006). However, since it

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disperses biofilms (Cavaliere *et al.* 2014) and permeabilizes cell membranes (Chávez de Paz 2010), it could aid in sensitizing biofilms to antimicrobials.

Regarding its biocompatibility, it has been shown that extrusion of even a low concentration of EDTA solution through the apical constriction can not only lead to an irreversible decalcification of the periapical bone but can also have consequences for neuroimmunological regulatory mechanisms (inflammatory reactions and immune response) involved in periapical lesion even in lower concentrations than those used in endodontics (Segura *et al.* 1996). Also, many studies conducted using cytotoxicity assays on cell cultures have reported the cytotoxic effects of EDTA at various concentrations (Malheiros *et al.* 2005, Ballal *et al.* 2009a). Using cell viability – MTT assay and genotoxicity, it has been reported that EDTA was the least cytotoxic of irrigation solutions, used either individually or in combination (Botton *et al.* 2016), which is in disagreement with previous studies mentioned above.

Maleic acid (MA) is a recently proposed chelating agent for the removal of the smear layer from the root canal system. At 7% concentration, MA was associated with better smear layer removal (especially in the apical third) and less toxicity than 17% EDTA (Ballal *et al.* 2009a,b, Ballal *et al.* 2019a).

The high surface tension of chelating agents (Giardino *et al.* 2006), could represent a further drawback, because of preventing its smear layer removal ability in the less accessible areas of the root canal space. Due to these limitations, recently, new chelating solutions alone or combined with surface-active agents have been investigated (Dunavant *et al.* 2006, Liu *et al.* 2015, Ballal *et al.* 2018). It was reported that cetrimide (CTR) added to the EDTA decreases the surface tension of the solution (Yilmaz *et al.* 2011), increasing the penetration of the mixture into the dentinal tubules and even into the uninstrumented areas of the root canal system. EDTA+CTR also displayed antibacterial activity, decreasing the biofilm mechanical stability by destabilization of the cohesive forces of the biofilm (Arias-Moliz *et al.* 2010). Interestingly, 7% MA alone or in association with

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CTR also had antimicrobial activity against *E. faecalis* from 30 seconds of exposure onward (Ferrer-Luque *et al.* 2010). Conversely, in the study mentioned above (Ferrer-Luque *et al.* 2010), the combination of EDTA and CTR eradicated biofilm grown in the MBEC-HTP device after 1 minute of contact.

In order to investigate the suitability of these recently proposed irrigants for the disinfection of the root canal system, this study aimed to evaluate the antimicrobial activity, the toxicity, and cleaning effectiveness of EDTA and MA alone and in combination with a detergent (CTR).

The null hypothesis tested was that the addition of CTR to EDTA or MA does not affect their antimicrobial activity, toxicity, and cleaning efficiency.

Materials and Methods

Preparation of the test agents

For this study, freshly prepared chelating solutions and the concentrations assayed were:

17% EDTA (Carlo Erba Reagents Srl, Milano, Italy)

17% EDTA + 0.5% CTR (Sigma-Aldrich, Milano, Italy)

7% MA (Carlo Erba Reagents Srl, Milano, Italy)

7% MA + 0.5% CTR

0.5% CTR

The 17% EDTA and 7% MA solutions were prepared individually and used as such, or combined adding 0.5 g of CTR to 100 mL of each chelating solution *(*(w/v %), and adjusted to pH 7.5 and 3.3, respectively. The solution of 0.5% CTR was obtained by adding 0.5 g of detergent to 100 mL of deionized water.

Cytotoxicity and genotoxicity assessment

Cell culture and maintenance

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Chinese hamster lung fibroblast (V79) cells were procured from NCCS, Pune, India, and

maintained as per ATCC guidelines. The cells were cultured in Dulbecco's Modified Eagle Medium

(DMEM; AT151, Himedia Laboratories, Mumbai, India) supplemented with 10 % foetal bovine

serum (RM9955, Himedia Laboratories) and 1× antibiotic antimycotic mix (A002, Himedia

Laboratories) and maintained at 37 °C in a 5% CO2 incubator (Galaxy 170S, Eppendorf, Hamburg,

Germany).

Preparation of test solutions & MTT assay

The test solutions were freshly diluted in DMEM on the day of experimentation. The effects of

various dilutions of the drugs, namely 1/10; 1/50; 1/100; 1/500 and 1/1000 were tested for its effect

on cell viability by MTT assay as described previously (Mosmann 1983). Briefly, 10^4 cells per 100

μL were seeded in 96-well cell culture grade plates and allowed to attach overnight in a 5% CO_2

incubator maintained at 37˚C. The next day, the cells were treated with various dilutions of the

drugs for 15 min that were prepared in pre-warmed DMEM. After 15 min of incubation with the

drugs, cells were incubated with 1mg/mL MTT reagent prepared in DMEM devoid of phenol red

and serum. Cells were incubated with MTT reagent for four hours following which the formazan

crystals was dissolved by adding 100 μL of DMSO. The optical density (OD) of each well was

obtained at 570 nm using a multi-well plate reader. Cell viability was determined by the following

formula:

% Viability = [(test OD- blank OD)/ (control OD- blank OD)] ×100 where, control corresponds to

untreated cells.

Clonogenic assay

This assay was carried out using the original method of Puck & Marcus (1955). A known number

of cells were seeded on 6-cm petri-dish and cultured overnight for attachment. Six hundred cells

were seeded for treatment groups receiving 1:10 and 1:100 dilutions of the drugs, whereas 200 cells

were seeded for control cell and 1:1000 dilutions. Next, cells were treated with the mentioned

dilutions of the test agents and incubated at 37 ˚C for 15 min. After treatment, the drug-containing DMEM was removed, and the cultures were further incubated at 37 ˚C for 12 days in DMEM to allow colony formation. Fixing and staining of the colonies were done using 0.1% crystal violet dissolved in methanol. Stained colonies were counted manually, and the surviving fraction in each treatment group was determined by the expression given below.

Plating efficiency; PE = (number of colonies counted/ number of cells seeded) ×100 where PE is calculated using the number of colonies obtained in the untreated or control cells.

Surviving fraction; SF = {[number of colonies counted]/[number of cells seeded×(PE/100)]}.

Assessment of Genotoxicity using a Micronucleus assay

Evaluation of genotoxicity was carried out by employing an *in vitro* micronucleus assay. For this, $6×10^5$ cells were seeded on 6-cm culture dishes and allowed to attach overnight. On the following day, cells were treated with the various test solutions (1:1000 dilution in DMEM) for 15 min. Next, the drug-containing media was removed and cells incubated in cytochalasin B (3 μg/mL) in DMEM for 24 h and further processed as described earlier (Ballal *et al*. 2019b). Briefly, after treatment, the cells were harvested, submitted to hypotonic shock in 0.075 M KCl and were fixed with ice-cold fixing solution (containing 3:1 methanol:glacial acetic acid). Fixed cells were smeared on clean glass slides and stained with acridine orange dye. For analysis, 1000 binucleated cells were scored under a fluorescence microscope (BX51, Olympus, Japan) for the presence of micronuclei.

Assessment of antimicrobial activity

Bacterial strains and culture media

Enterococcus faecalis (*E. faecalis*) strain ATCC 4083, stored at −80°C before analysis, was used because it was primarily isolated from the root canal of a pulpless tooth (Stuart *et al.* 2006). Then, before use, the strain was thawed and reconstituted in Tryptic Soy Agar (TSA, Biomérieux, Marci l'Etoile, France) for 24 hours at 37°C.

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Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the chelating agents under investigation were determined by the broth microdilution method, following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/MIC_testing/Edis5.1_broth_dilution.pdf). A microbial suspension at an optical density equal to 0.5 McFarland standard in Muller Hinton broth (MHB, Biolife Italiana S.r.l. Milano, Italy) was prepared from *E. faecalis* ATCC 4083. After obtaining a microbial load of 5×10^6 CFU/ml using appropriate dilutions, 10 μl of each suspension was inoculated in a 96-wells microplate containing 90 μL of a serial 2-fold dilution of the chelating solutions (17% EDTA, 17% EDTA + 0.5 CTR, 7% MA, 7% MA + 0.5 CTR, 0.5 CTR). Bacterial growth in the control group was performed by inoculating the *E. faecalis* suspension in MHB. MIC values were read after 24 h of incubation at 37°C. The MBC, defined as the lowest concentration of an antimicrobial substance able to kill 99.9% of the initial inoculum, was determined by subculturing 10 μL of microbial suspension from wells showing no visible growth in the MIC tests. Similarly, MBC values were read after 24 h of incubation at 37°C.

Determination of Minimum biofilm eradication concentrations (MBEC)

For biofilm susceptibility to chelating agents, the MBEC™ High-throughput (HTP) assay (Innovotech, Edmonton, Alberta, Canada) was used. The wells were filled with 150 μL of Brain Heart Infusion (BHI) broth (Biolife Italiana S.r.l. Milano, Italy) inoculated with 10^7 CFU/mL of *E. faecalis* resuspended from an overnight culture on Tryptic Soy Agar (TSA Biolife Italiana S.r.l.) and incubated for 24 h at 37 °C to establish a biofilm on the pegs located on the lid of the device. After the incubation, the lid was placed for approximately 30 seconds in a sterile 96 wells microplate containing 200 μL of sterile saline solution (rinse plate) to remove unattached cells. Afterward, the lid was placed on a "challenge plate" containing 200 μL of a serial 2-fold dilution of

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the five chelating solutions for 1 and 5 minutes while agitating on an orbital shaker and moved again in a new rinse plate for 30' to neutralize the test solutions.

Then, the lid was placed in a new 96 wells microplate containing 200 μL of BHI broth and placed in a dry stainless steel tray which sitting in a water bath and sonicated at maximum power for 10' to dislodge the remaining biofilm on the pegs. The lid was then removed, replaced with a non-pegged lid, and the plate incubated overnight at 37 °C. MBEC values were determined by visually checking the wells for turbidity. Clear wells indicated complete biofilm eradication.

For viable cell counting, three pegs were removed from the MBEC™ – HTP lid biofilm treated devices to both established periods, washed three times with sterile saline solution to remove unattached bacteria then placed in sterile microtubes and sonicated (VWR International, Radnor, PA, USA) at maximum power for 10 minutes. Supplemental pegs treated with sterile saline solution serve as positive controls. The resulting suspensions were serially diluted, and 10 μL of each dilution was seeded in TSA plates and grown overnight at 37°C for colony counting.

Confocal Laser Scanning Microscopy (CLSM) analysis

E. faecalis biofilm was cultured on uncoated 10-mm diameter glass slides (VWR International Srl, Milano, Italy) placed in 24-well microplates for 48 h, by inoculating bacterial cells in 1mL of BHI broth to a final concentration of 10^7 CFU/mL. At the end of the incubation time, the culture medium was removed, and two washes with sterile saline were performed to remove non-adherent bacteria. Afterward, the biofilm was treated with each of the testing solutions for 1 and 5 minutes (controls were treated with sterile saline), then the solutions were removed, and the wells were washed twice with saline.

Glass slides were then stained with Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (Thermo Fisher Diagnostics SpA, Rodano, Italy), prepared according to manufacturer instructions. Each

sample was stained with 30 µL of staining solution by incubating for 15 min at room temperature in the dark.

Later, stained biofilms were thoroughly rinsed in physiological solution and the images acquired with a Confocal Laser Scanning Microscope TCS SP8 (Leica Microsystems CMS GmbH, Mannheim, Germany) using a 20× dry objective (HC PL FLUOTAR 20×/0.50 DRY). Sequential optical sections were gathered along the z-axis for the entire thickness of the biofilm. Images from at least three random areas were acquired for each sample. The selected images were processed with Las X (Leica Microsystems CMS GmbH) and analyzed with Fiji software (Fiji, ImageJ, Wayne Rasband, NIH Bethesda, Maryland, USA). The quantification of cells biomass was expressed in μm^3. The percentage of live and dead microorganisms in the biomass was assessed in each group.

Quantification of accumulated hard-tissue debris (AHTD) using Micro-CT

Sample size calculation

The effect size of AHTD removal for this study was 91%, based on a previous study with similar methodology (Silva *et al.* 2019), and was combined with a power β = 0.95, and α = 0.05 into an F test family for one-way ANOVA (G*Power 3.1.7 software for Windows; Heinrich Heine, Universität Düsseldorf, Düsseldorf, Germany). The minimum sample size to reveal statistical significance among groups amounted to twenty-six samples in total. Additional samples were used, in order to compensate for possible sample loss.

Root canal selection and preparation

The experimental protocols were approved by the institutional ethics committee for the use of human extracted teeth (IEC 519/2019). A total of fifty maxillary incisors with straight root, single relatively round canal in cross-section, as established preliminarily by using a micro-CT method (Peters *et al.* 2010, Versiani *et al.* 2019), and similar root length were selected from a pool of extracted teeth. Each tooth was radiographed in both mesiodistal and buccolingual directions to

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verify the presence of a single canal with closed apex, the absence of intra-radicular resorption, presence of caries, restoration, or fractures or root canal filling. The teeth were stored in 0.2% sodium azide (Millipore Sigma, St. Louis, MO, USA) at 4°C until use. The teeth were decoronated at the cementoenamel junction to obtain a standardized root length of 12 mm. Before instrumentation, the samples were subjected to a preoperative scanning procedure by using a micro-CT device (Skyscan 1176, Bruker, Kontich, Belgium). Each tooth was scanned separately at an isotropic resolution of 8.92 μm. The scanner parameters were set at 90 kV, 280 μA, 0.5° rotation around the vertical axis, and rotation step of 0.4°, using a 0.1-mm-thick copper filter.

The volume of interest was selected extending from the cemento-enamel junction to the apex of the root, resulting in the acquisition of 671-675 transverse cross-sections per tooth. Aiming to create a similar sample among the groups, ten anatomically pair-matched teeth, presenting a single oval-shaped canal, were selected based on similar morphological features of the root canal such as length, volume, and configuration to compose the experimental groups (De-Deus *et al.* 2019). The volume of the initial pre-shaping root canal for each element was calculated, and the five groups were compared to verify that the samples did not present anatomical or structural differences that could create disparities between groups. No statistically significant values between the groups, regarding the initial volume of each root canal, were found (One-way ANOVA, *P*=0.46); therefore, the samples were homogeneous for initial volume, then distributed among the five study groups and used for the shaping procedures.

The apical third of each tooth was sealed with a layer of cyanoacrylate adhesive to simulate an *ex-vivo* closed-end model (Giardino *et al.* 2017). The glide path in the samples of all the groups was accomplished with a 15 K-file (Dentsply Sirona, Ballaigues, Switzerland) until the apical foramen. Working length was established by inserting a size 10 K-file (Dentsply Sirona) into each root canal until it was just visible at the apical foramen (observed using magnifying loupes) then subtracting 1mm from the recorded length. Each sample, then, was shaped mechanically to 1mm before of the

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apical foramen (established working length) using the NiTi rotary system ProTaper Next (Dentsply Sirona) to a size 30,07 taper. At each instrument changeover, the canal was irrigated with 1 mL of 5.25% NaOCl (Ogna Laboratori Farmaceutici, Muggiò, Italy) for 1 min using a syringe with a 30-gauge side-vented needle (Max-i-Probe; Dentsply Rinn, Elgin, IL, USA) placed at 2 mm from the working length, for a total of 5 mL of 5.25% NaOCl. After root canal preparation and irrigation with 5.25% NaOCl was accomplished, the teeth were submitted to a second scan, applying the parameter settings above mentioned.

Final irrigation protocols

After root canal preparation, each specimen was randomly assigned to one of the five experimental groups (n=10), according to the final irrigation protocol:

Group 1-5.25% NaOCl followed by saline

Group 2- 5.25% NaOCl followed by 7% MA

Group 3- 5.25% NaOCl followed by 7% MA + 0.5% CTR

Group 4- 5.25% NaOCl followed by 17% EDTA

Group 5- 5.25% NaOCl followed by 17% EDTA + 0.5% CTR

For final irrigation, in all the groups, each specimen was irrigated with 2 mL of test agents assigned for 2 min. To remove chemical residues, the canals were flushed with 5 mL of distilled water and dried with size 30 absorbent paper points (Dentsply Sirona).

Then, a third scan was performed in all samples included in the five groups listed above after final irrigation with the assigned chelating agent to quantify the accumulation of hard-tissue debris. The images were reconstructed using a scanner software (NRecon 1.6.10.4, Skyscan, Bruker, Kontich, Belgium) with a beam hardening correction of 40% and ring artifact correction of 10.

To ensure that the three scans performed for each element could be analyzed with the same references, the Data Viewer software (Data Viewer v.1.5.1, Bruker) was used.

The segmentation threshold to separate dentine and debris from the root canal space and the

quantitative analyses were then performed by using CTAn software (CTAn v.1.14.4, Bruker) to

calculate quantitative parameters and CTVol (CTVol v.2.2.3.0, Bruker) to reconstruct visual 3D

models. By using CTAn software, it was also possible to obtain the volumetric values of the debris

present in the root canal by calculating in percentage the reduction of debris before and after the

final irrigation in each group. The total volume of AHTD was calculated in cubic millimeter (mm^3)

and expressed as the percentage of the entire canal system volume after preparation (vol %).

Statistical analysis

The normality of data distributions was assessed using the D'Agostino & Pearson's test and the

Shapiro–Wilk test. For data distributed normally, results were presented as means and standard

deviations (SD), and one-way analysis of variance (ANOVA) was used together with the post-hoc

Tukey Multiple comparison test for between-group comparisons. For toxicity assays, between-

group comparisons were made using a one-way ANOVA followed by Bonferroni's correction. For

assessing differences in root canal volume before and after preparation, and in AHTD removal,

within-group comparisons were performed using the paired Student t-test. For data not following a

normal distribution, among-group comparisons were assessed by using the Kruskal-Wallis test and

the Dunn's multiple comparison test, and the Wilcoxon matched-pairs signed-rank test was used for

within-group comparisons. Statistical analysis was undertaken using the software GraphPad Prism

5.0 (GraphPad Software, La Jolla, CA, USA). The significance threshold was set at $P = 0.05$.

Results

Toxicity assessment

Changes in the cell viability in V79 cells in the presence of different dilutions of the test compounds

were determined by MTT Assay. The viability of cells in the presence of the test compounds is

shown in Figure 1. A group-wise statistical comparison for the effect of different dilutions of the

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drugs on cell viability is shown in Table 1. Cells treated with each of the test agents at a dilution of

1:10 exhibited profound toxicity showing the lowest viability when compared to the other dilutions.

Apart from EDTA, cells receiving the test agents at a dilution of 1:50 also reduced cell viability. On

the other hand, cells had a gradual increase in viability with increasing dilutions of the test

compounds with 1:100, 1:500 and 1:1000 dilution. Treated cells had a significant (p< 0.001)

increase in cell viability when compared to the group of cells receiving 1:10 dilution of each of the

test agents (Fig. 1).

The PE of the V79 cells in the present study was found to be 92.7±3.51. The SF of V79 cells upon

treatment with the various test solutions is summarized in Table 2. Representative crystal violet-

stained colonies are shown in Figure 2. CTR alone, as well as in combination with EDTA and MA

at dilutions of 1/10 and 1/100, were significantly toxic (p< 0.001 when compared to controls), as

these did not allow colony formation. Similarly, EDTA and MA at dilution 1/10, too, resulted in a

significant decrease in SF (Table 2). All tested mixtures were nontoxic and showed no significant

difference at a dilution of 1/1000 (Table 2).

The genotoxic potential of CTR, EDTA, MA alone, or in combination, was assessed by

micronucleus assay. A 1/1000 dilution (dose selected based on viability studies) of each of the test

agents was investigated for their genotoxic potential. Data indicated that the test agents did not

exhibit a significant increase in micronucleus frequency as compared to control cells showing their

non-genotoxic nature at this tested dilution (Fig. 3).

Antimicrobial activity

Employing the broth microdilution method (MIC and MBC), EDTA retained a weak inhibitory and

bactericidal effect against planktonic cells, while MA inhibited cells growth even when diluted 1:16

and killed 99.9% of the cells when diluted 1:8 (Table 3). CTR revealed the most prominent effect,

being inhibitory, also when diluted 1:1024 and bactericidal at 1:512. While the addition of EDTA

did not improve neither MIC nor MBC values of CTR, the addition of MA improved its bactericidal

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activity of one dilution (Table 3). When MBEC assay was employed to assess the biofilm removal

activity of the surfactant agents, CTR was once again the most active agent, being able to remove

completely *E. faecalis* biofilm from the MBEC pegs diluted 1:8 and 1:32, respectively when applied

for 1' and 5' (Table 3). Only the addition of EDTA slightly improved CTR removal activity (Fig. 4,

5).

To assess the irrigating solutions ability to eradicate *enterococcal* sessile cells encased in an

exopolymeric matrix, mature biofilm was challenged for 1 and 5 minutes and analyzed using

CLSM.

Compared to the control, a significant biomass reduction was observed when EDTA was present in

the solution. CTR alone was able to remove 30-40% of the whole biomass and when added to

EDTA, was not able to improve the removal of the sessile cells. However, the percentage of dead

cells significantly increased when CTR was added to the irrigant solutions (Fig. 4, 6). Conversely,

MA displayed a lower bactericidal activity on biofilm cells (Fig. 4) and was the only molecule that

had a time-dependent activity, both when used alone or in combination.

Accumulated hard tissue debris estimation

By using micro-CT, it was possible to identify and measure AHTD after preparation and final

irrigation in the root canals for all the tested protocols. The data were distributed normally. Table 4

summarizes the mean values and standard deviations for the main parameters assessed. There was

no significant difference among groups both for pre-operative root canal volume ($P = 0.46$) and for

volume after root canal preparation ($P = 0.41$). The root canal volume was significantly increased in

all groups after preparation ($P < 0.01$). After preparation, there was no significant difference among

groups for AHTD volume ($P = 0.13$). After final irrigation, the AHTD volume was significantly

decreased in all groups ($P < 0.01$). The results were confirmed also expressing the AHTD data as %

of the total root canal volume (Table 4). Figure 7 shows the micro-CT images of the removal of

accumulated hard-tissue debris in the experimental groups, considering the whole canal.

The mean percentage of hard tissue debris removed by the different experimental solutions is shown in Table 4. All the irrigating solutions were significantly superior to saline (control) in the overall removal of AHTD. Significances for AHTD removal, and *P*-values of the among-group (ANOVA) and between-group comparisons (unpaired Student's t-test) are listed in Table 5.

Discussion

The present study investigated the toxicity, antimicrobial activity, and AHTD removal of EDTA and MA alone or in combination with CTR. Since the results of this study revealed that the addition of CTR to EDTA or MA improved its antimicrobial activity and also AHTD removal efficiency, the null hypothesis has to be rejected.

Cytotoxicity is one of the most important factors for assessing any adverse effect of the anti-biofilm molecules before using them commercially for the prevention and removal of biofilm (Roy *et al.* 2018). The present work investigated various agents, namely, CTR, EDTA and MA alone, and CTR in combination with EDTA and MA, for their cytotoxic potential. Viability analysis using MTT assay showed that cells treated with the test agents alone or in combination at dilution 1/10 had the highest cell killing rate. This could be attributed to their chemical property. CTR being a surfactant, would readily disrupt cell membrane lipids resulting in the lysis of cells. EDTA is a strong metal chelator and can readily remove essential divalent metal ions such as Ca^{2+} and Mg^{2+}, causing detachment of cells, and resulting in MTT reduction. The acidic nature of MA would contribute in a major way to killing cells at the 1/10 dilution. With an increase in dilution of each of the test agents, their toxic effect on cell viability, as shown by MTT assay, vanished.

The colony-forming assay is a gold standard assay (Franken *et al.* 2006) that has found profound use in estimating the cytotoxicity of agents as it measures the reproductive capacity of cells after they have undergone cellular insult. In the present investigation, V79 cells were tested for clonogenicity after treatment with the test agents. Interestingly, CTR alone and in combination with

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EDTA and MA at dilutions 1/10 and 1/100 did not allow colony formation. Although the MTT data indicated the viability of greater than 60% for CTR alone and in combination with EDTA and MA, the lack of colonies indicates their long term impact on cell reproducibility. EDTA and MA treatment also reduced the clonal survival of V79 cells at 1/10 and 1/100 dilution but was less severe when compared to CTR and its combination with EDTA and MA. All the test agents at dilution 1/1000 were associated with colony formation similar to that of untreated/ control cells, indicating their non-toxic nature at this particular dilution.

The formation of micronuclei is an indicator of DNA damage, and this is a sensitive method to analyze the genotoxicity potential of any chemical agent (Nersesyan *et al.* 2016). Concerning to genotoxicity, the drugs did not induce a significant rise in the frequency of micronuclei formation in V79 cells, indicating its non-genotoxic nature at this tested concentration.

However, the assays used have several limitations when compared to *in vivo* conditions. Cell cultures are generally more sensitive to the toxic effects of materials than "*in vivo*" tissues (Bajrami *et al.* 2014); under *in vivo* conditions, substances are diluted with body fluids and their concentration decreases (Labban *et al.* 2014); they are carried away from the lymph, blood, and phagocytes of the vascular and lymphatic systems (Ballal *et al*. 2009a); in equal concentrations, the cytotoxicity of materials decreases over time in the clinical setting compared to *in vitro* observation (Malheiros *et al.* 2005). Therefore, the present results may not be directly generalizable to the *in vivo* settings.

As already mentioned above, conflicting results on the biofilm disruption or antimicrobial effect of EDTA have been reported. The only antibacterial property associated with EDTA was to weaken the bacterial cell membrane without killing the cell and promoting biofilm detachment (de Almeida *et al*. 2016). These data were herein confirmed as EDTA was not inhibitory when diluted 1:1 and not bactericidal even when not diluted and exposed to *E. faecalis* cells in planktonic killing tests (MIC, MBC). Also, in MBEC assay, EDTA was not able to dislodge the biofilms formed on the

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4pegs. A significant biomass reduction was observed when mature biofilm was analyzed employing

CLSM, in agreement with previous reports (Cavaliere *et al*. 2014, de Almeida *et al*. 2016).

MA has recently been suggested as an alternative chelator to EDTA (Ballal *et al.* 2009b). The

highly acidic pH of MA, which can alter cell membrane permeability, could explain its substantial

antimicrobial properties (Ferrer-Luque *et al*. 2012). In the present study, it was also observed that it

could inhibit cell growth even when diluted 1:16 and kill 99.9% of cells when diluted 1:8 (Table 3).

Conversely, the present results revealed that MA was associated with a lower bactericidal activity

on biofilm cells (Fig 4), and it was able to dislodge the biofilms formed on the pegs only undiluted

to 1' and diluted 1: 2 to 5' by using MBEC assay in both treatments time.

Various ingredients such as detergents added to irrigants, reduce the surface tension (Giardino *et al*.

2018) and enhance the antibacterial effect of the solutions by a residual antibacterial activity,

increasing the resistance to microbial regrowth in the root canal system (Baca *et al*. 2012). Some

studies stated that CTR, a cationic surfactant, in addition to a proven bactericidal activity and the

ability to reduce the surface tension of irrigants (Wang *et al.* 2012), can decrease the mechanical

stability of the biofilm by destabilization of the cohesive forces of the biofilm. This probably

depends on the interaction between the chemical structure of the molecule and the anionic

properties of the extracellular polymeric substance (EPS) surrounding the biofilm (Simões *et al.*

2005). All these features can contribute to greater efficiency of irrigating solutions where detergents

are added, as highlighted in the current investigation, and explain the best results obtained

compared to both surfactant-free solution combinations.

The results of the current study showed that CTR alone had the most prominent effect, being

inhibitory also when diluted 1:1024 and bactericidal at 1:512. Moreover, it was able to dislodge *E.*

faecalis biofilm completely from the MBEC pegs, respectively, when applied for 1 and 5'. This

removal activity improved when mixed with EDTA.

Some researchers (De-Deus *et al*. 2011) formerly highlighted that scanning electron microscopic (SEM) evaluation for AHTD in a laboratory setting has a limited utility to consider a side effect of the chemomechanical preparation procedures in the intricate root canal anatomy. The status of the dentinal surface before and after the application of the chelators is unknown, so the evaluation is not performed in the very same dentine area of the same sample (De-Deus *et al*. 2011).

The assessment of hard-tissue debris accumulation has been made possible through the combination of nondestructive micro-computed tomography (micro-CT) imaging and the development of robust image analysis and processing software (Paqué *et al*. 2009).

Therefore, to verify the debris removal ability of the chelators used in the present study, the 3D non-destructive micro-CT imaging was carried out because it currently represents a gold standard technique to evaluate the AHTD inside anatomic complexities of the root canal system of shaped and irrigated human teeth while preserving sample integrity (Keleş *et al.* 2016).

The present investigation showed that the percentage volume of AHTD removal after final irrigation was lower in the canals irrigated with saline solution (30.65%) in comparison with the EDTA and MA irrigation protocols, which markedly showed less remaining debris (AHTD removal of 59.66% and 75.57% respectively), as reported in Table 5. In previous studies (Ballal *et al.* 2009b, Ulusoy & Görgül 2013), MA had a better ability to remove the smear layer in comparison to EDTA. These findings were not in agreement with the present study. Currently, when MA and EDTA with or without CTR added were compared with each other, there was no significant difference between them in removal AHTD, respectively. The discrepancy with the present results might be explained by the differences in the experimental models and methods of investigation used. Firstly, in the present study, the apical end of each sample was covered with a layer of cyanoacrylate to create a closed environment (Giardino *et al.* 2017) and to simulate an *ex vivo* closed-end model, contrary to the previous reports where the apical end of each tooth was not closed during irrigation procedures, then not reproducing the dynamics and impact on the

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replenishments of irrigants which does not mimic the clinical setting. As stated by Tay *et al*. (2010), the root is enclosed by the bone socket during *in vivo* irrigation; then, the root canal behaves as a closed-end channel, which results in gas entrapment at its closed-end, producing a vapor lock effect during irrigant delivery. For the closed system, the effect of a vapor lock was most conspicuous along the apical portion of the canal, with retention of debris and smear layer remnants, contrary to the open system, where complete smear layer removal and debris clearance were seen. Secondly, in the present study, a micro-CT assay was used to investigate the hard debris removal ability of the irrigants in place of SEM evaluation, a scientifically questionable tool to study the real effect of the chelating solutions because the lack of solid and reliable experimental features, where the results may not be precisely determined (De-Deus *et al.* 2011). In line with the present findings, it has been reported that there is no significant difference found between MA and EDTA group in smear layer removal (Jaiswal *et al.* 2018). However, MA has shown better smear layer removing ability than EDTA, experimentally.

The addition of CTR to EDTA and MA produced similar outcomes in AHTD removal (80.83% and 82.07% respectively), resulting in a significant improvement over both solutions used without detergent. This suggests that the addition of surfactant improves the properties of the chelators, in line with the existing literature, which recommends the use of chelators enriched with surfactants (Yilmaz *et al.* 2011, Ballal *et al.* 2018). These results reconfirm the correlation between wettability of irrigants and effectiveness in removing debris since a greater diffusion is obtained both inside the canal and in its ramifications, and microscopically at the level of the dentinal tubules.

Conclusions

Both final irrigation protocols tested (MA+CTR and EDTA+CTR) had similar effectiveness in the reduction of AHTD from the canal walls and increased their antimicrobial activity when compared to the same solutions without detergents. It was also shown that 7% MA was less cytotoxic in

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comparison to 17% EDTA. However, further *in vivo* studies need to be performed to confirm the

results.

Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this

article.

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Figure Legends

Figure 1. Graph showing changes in the viability of V79 cells in the presence of different dilutions of test agents. ns= Not significant; * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$ when compared to its respective 1: 10 dilution for each of the test compound. Control= cells with DMEM alone. Data are shown as Mean ± SD, obtained from three independent experiments. Statistical analysis was done by 1-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Figure 2. Representative photomicrograph showing the clonogenicity of V79 cells in the presence of different dilutions of the indicated test agents.

Figure 3. Representative photomicrograph of V79 cells stained with acridine orange exhibiting mono-nucleated and binucleated states with micronucleus. Images were taken using 200× magnification using fluorescence filters with FITC-TRITC dual-band fluorescence filter. Scale = 80 μm. **(B)** Graph showing the effect of different drugs tested at a dilution of 1/1000 for the induction of micronuclei (MN) in V79 cells. No symbol indicated that the changes in the MN frequency was not significant when compared to control as well as between the groups.

Figure 4. Biofilm removal efficacy of the tested solutions on pre-formed *E. faecalis* biofilm analyzed by CLSM**.** Residual biomass is represented in percentage upon treatment with respect to the control. Whole bars represent the total biomass; the green fraction is viable cells, in red the dead cells still encased in the biofilm. Ctrl = control; MA = maleic acid; CTR = cetrimide. * P<0.05, **P<0.01, ***P<0.001.

Figure 5. Exposure time effect of the tested solution on preformed biofilm. Black bars, 1' treatment; white bars, 5' minutes treatment. Ctrl = control; MA = maleic acid; CTR = cetrimide. * P<0.05.

Figure 6. CLSM 3D images of *E. faecalis* biofilms treated with the tested solutions**.** Ctrl = control; MA = maleic acid; CTR = cetrimide.

Figure 7. Representative micro-CT reconstructions of the root canal system of the treated teeth before and after the final irrigation with the solutions assigned. Accumulated hard tissue debris (AHTD) is depicted in black.

Table 1.Summary of the statistical comparison between different test agents for each of the dilutions used for assessment of cell viability by MTT assay. Statistical analysis was done by 1-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Dilutions	Cetrimide vs EDTA	Cetrimide vs MA	Cetrimide vs Cetrimide +EDTA	Cetrimide vs Cetrimide +MA	EDTA vs MA	EDTA vs Cetrimide +EDTA	EDTA vs Cetrimide +MA	MA vs Cetrimide +EDTA	MA vs Cetrimide +MA	Cetrimide +EDTA vs Cetrimide +MA
1:10	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
1:50	p < 0.001	ns	ns	ns	p < 0.001	p < 0.001	p < 0.001	ns	ns	ns
1:100	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
1:500	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
1:1000	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Ns: Not significant.

Table 2. Surviving Fraction (SF) of V79 cells treated with the different test agents.

Solutions	1:10	1:100	1:1000
Control	1.0±0.00	1.0±0.00	1.0±0.00
Cetrimide	0.00±0.00[#]	0.00±0.00[#]	0.86±0.15[A]
EDTA	0.18±0.22[#]	0.50±0.20[#, A]	0.90±0.07[A]
MA	0.03±0.03[#]	0.50±0.20[#, A]	1.04±0.06[A]
Cetrimide+EDTA	0.00±0.00[#]	0.00±0.00[#]	0.97±0.06[A]
Cetrimide+MA	0.00±0.00[#]	0.00±0.00[#]	0.96±0.03[A]

[#] $p < 0.001$ indicated the statistical comparison between untreated /control cells with various test agents. [A] $p < 0.001$ indicates the statistical comparison between the various dilutions of each test agent (n=6). Statistical analysis was done by 1-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the tested solutions.

	dilutions				concentrations			
	MIC	MBC	MBEC 1'	MBEC 5'	MIC	MBC	MBEC 1'	MBEC 5'
MA	1:16	1:8	nd	1:2	2.1875 g/L	4.375 g/L	70g/L	17.5g/L
EDTA	1:1	nd	nd	1:1	85 g/L	170 g/L	170 g/L	85 g/L
CTR	1:1024	1:512	1:8	1:32	0.0024 g/L	0.0048 g/L	0.3125 g/L	0.078125 g/L
MA + CTR	1:1024	1:256	1:8	1:32	0.0342 g/l + 0.0024 g/L	0.1367 g/l + 0.0098 g/L	4.375 g/L + 0.3125 g/L	1.09375 g/L + 0.078125 g/L
EDTA + CTR	1:1024	1:512	1:16	1:32	0.083 g/L + 0.0024 g/L	0.166 g/L + 0.0048 g/L	5.3125 g/L + 0.15625	2.65625 g/L + 0.078125 g/L

MA = maleic acid; CTR = cetrimide; nd = not diluted.

Table 4. Mean values and standard deviations for the main parameters assessed with micro-CT.

Parameters			EDTA	EDTA+ Cetrimide	Maleic acid	Maleic acid +Cetrimide	saline solution	P-value*
Root canal space	Volume, mm^3	Before preparation	3.11±2.24	4.25±1.33	3.89±1.55	3.88±0.89	3.09±1.47	0.46
		After preparation	5.81±1.80	5.88±0.82	6.11±1.47	6.31±0.79	5.18±0.99	0.41
		P-value**	<0.01	<0.01	<0.01	<0.01	<0.01	
AHTD	Volume, mm^3	After preparation	0.24±0.16	0.11±0.13	0.12±0.11	0.29±0.22	0.16±0.16	0.13
		After irrigation	0.09±0.06	0.02±0.03	0.04±0.09	0.05±0.05	0.10±0.10	0.52
		P-value**	<0.01	<0.01	<0.01	<0.01	<0.01	
AHTD	Volume, % of total root canal volume	After preparation	3.55±3.04	2.23±3.05	1.92±2.05	3.62±3.22	2.62±2.21	0.53
		After irrigation	1.30±1.29	0.60±1.08	0.76±1.66	1.14±1.41	1.54±1.59	0.50
		P-value**	<0.01	<0.01	<0.01	<0.01	<0.01	

AHTD= accumulated hard tissue debris; *=among-group comparisons (Kruskal-Wallis test); **=within-group comparisons (Wilcoxon matched-pairs signed-rank test)

Table 5. Mean values and standard deviations for the percentage of removed accumulated hard tissue debris (AHTD), and P-values of the among-group (ANOVA) and between-group comparisons (unpaired Student's t-test).

EDTA	EDTA+ Cetrimide	Maleic acid	Maleic acid +Cetrimide	saline solution	
59.7±20.0	80.85±7.4	75.6±21.1	82.1±14.2	30.6±20.6	P=0.049*
	0.01**	0.14	0.02**	0.01**	**EDTA**
		0.52	0.83	<0.001**	**EDTA+ Cetrimide**
			0.48	0.001**	**Maleic acid**
				<0.001**	**Maleic acid +Cetrimide**

*=among-group comparison (ANOVA); **=statistically significant difference

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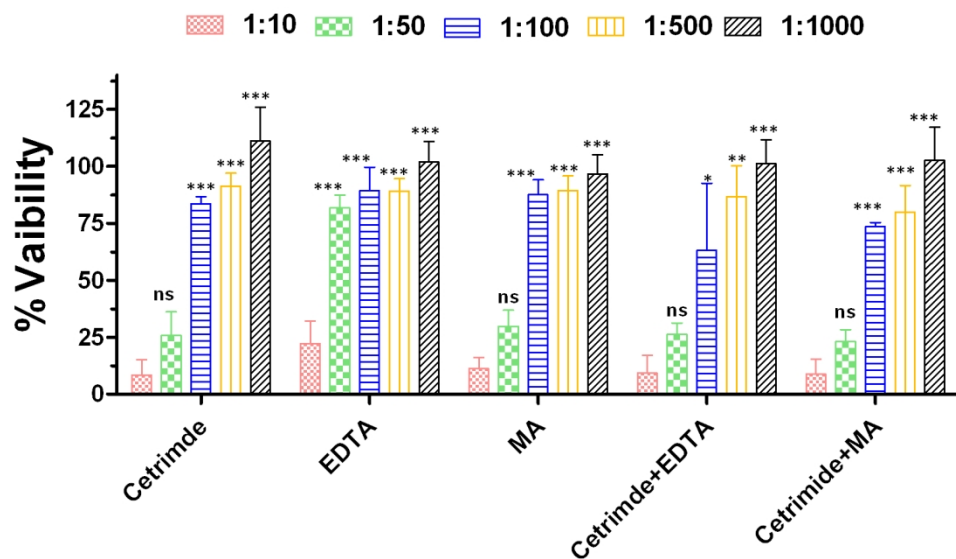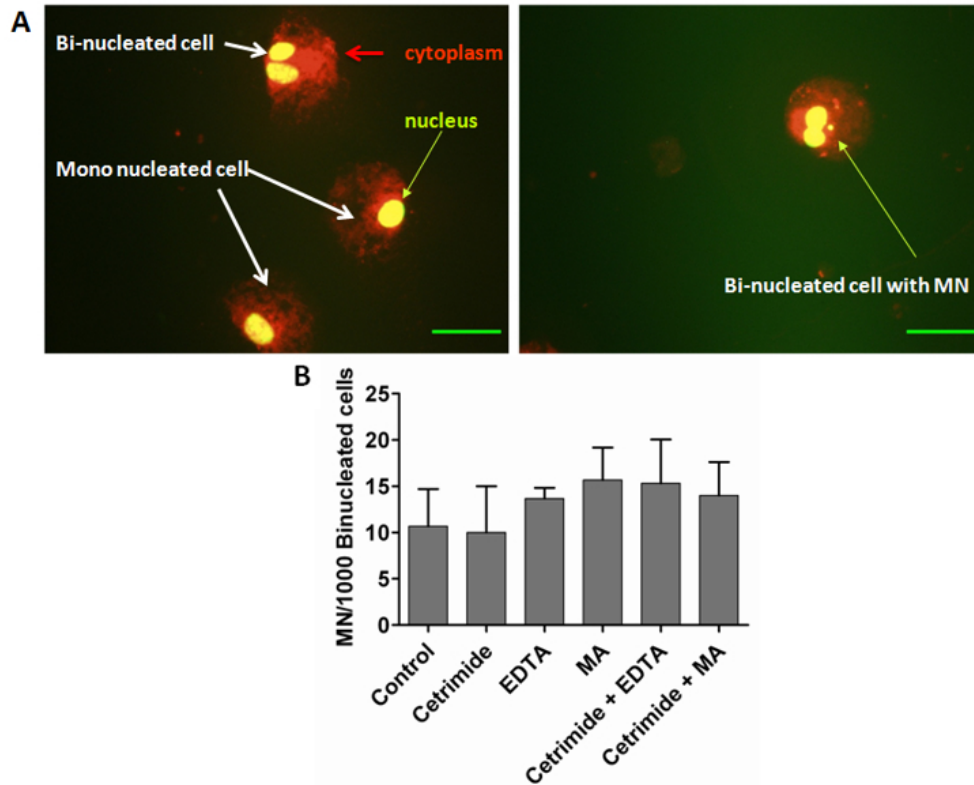6
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Figure 1. Graph showing changes in the viability of V79 cells in the presence of different dilutions of test agents. ns= Not significant; * p < 0.05, **p< 0.01, *** p < 0.001 when compared to its respective 1: 10 dilution for each of the test compound. Control= cells with DMEM alone.
Data are shown as Mean ± SD, obtained from three independent experiments. Statistical analysis was done by 1-way ANOVA followed by Bonferroni's Multiple Comparison Test.

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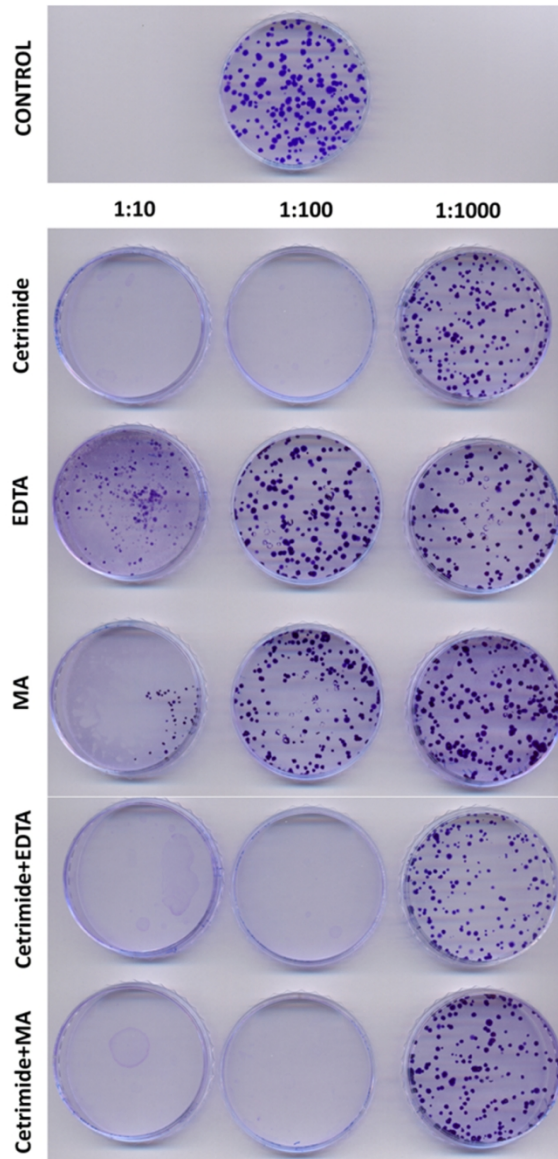
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Figure 2. Representative photomicrograph showing the clonogenicity of V79 cells in the presence of different dilutions of the indicated test agents.

93x190mm (300 x 300 DPI)

Figure 3. Representative photomicrograph of V79 cells stained with acridine orange exhibiting mono-nucleated and binucleated states with micronucleus. Images were taken using 200× magnification using fluorescence filters with FITC-TRITC dual-band fluorescence filter. Scale = 80 μm. (B) Graph showing the effect of different drugs tested at a dilution of 1/1000 for the induction of micronuclei (MN) in V79 cells.
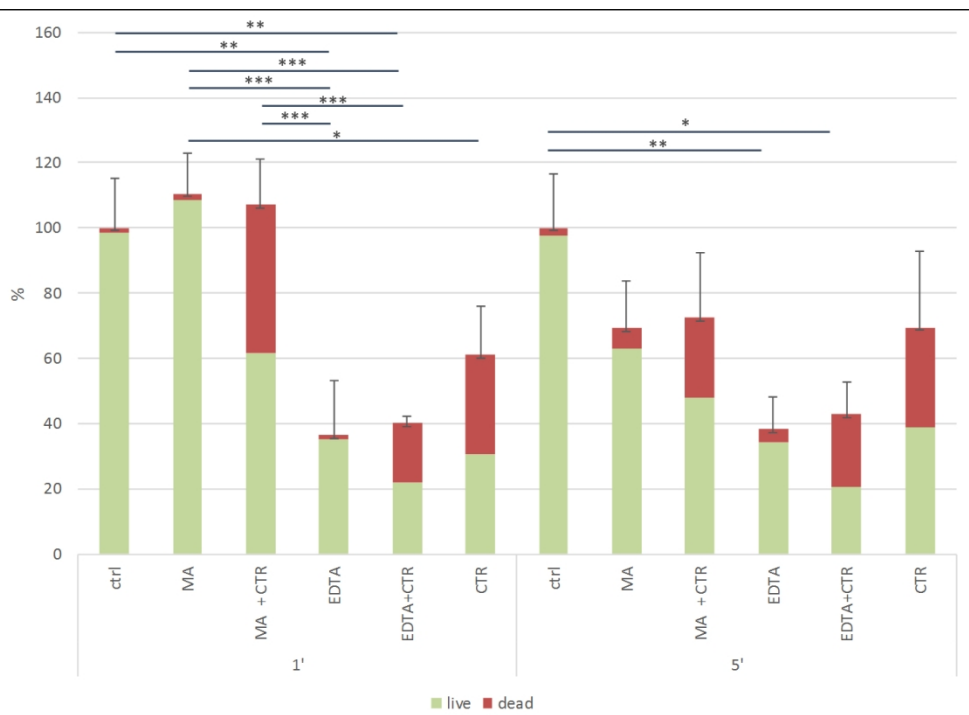
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Figure 4. Biofilm removal efficacy of the tested solutions on pre-formed E. faecalis biofilm analyzed by CLSM. Residual biomass is represented in percentage upon treatment with respect to the control. Whole bars represent the total biomass; the green fraction is viable cells, in red the dead cells still encased in the biofilm. Ctrl = control; MA = maleic acid; CTR = cetrimide.   * P<0.05, **P<0.01, ***P<0.001.
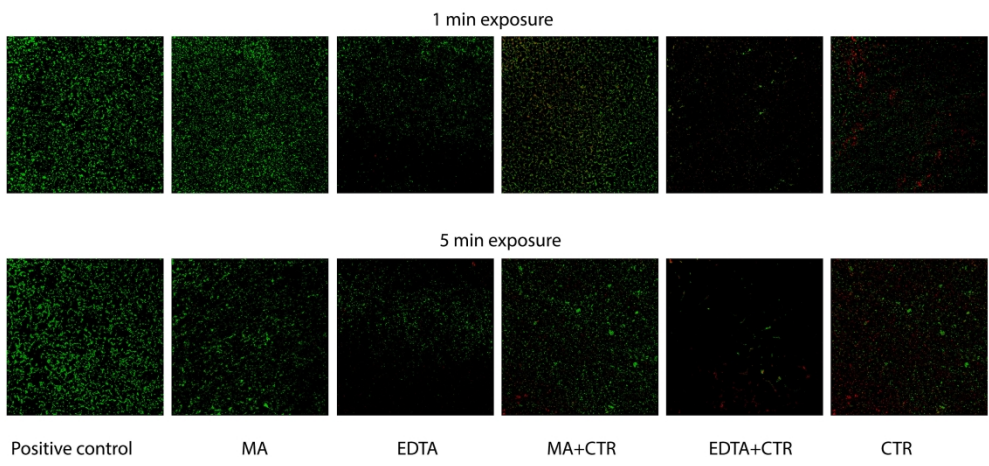
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Figure 5. Exposure time effect of the tested solution on preformed biofilm. Black bars, 1' treatment; white bars, 5' minutes treatment. Ctrl = control; MA = maleic acid; CTR = cetrimide. * P<0.05.

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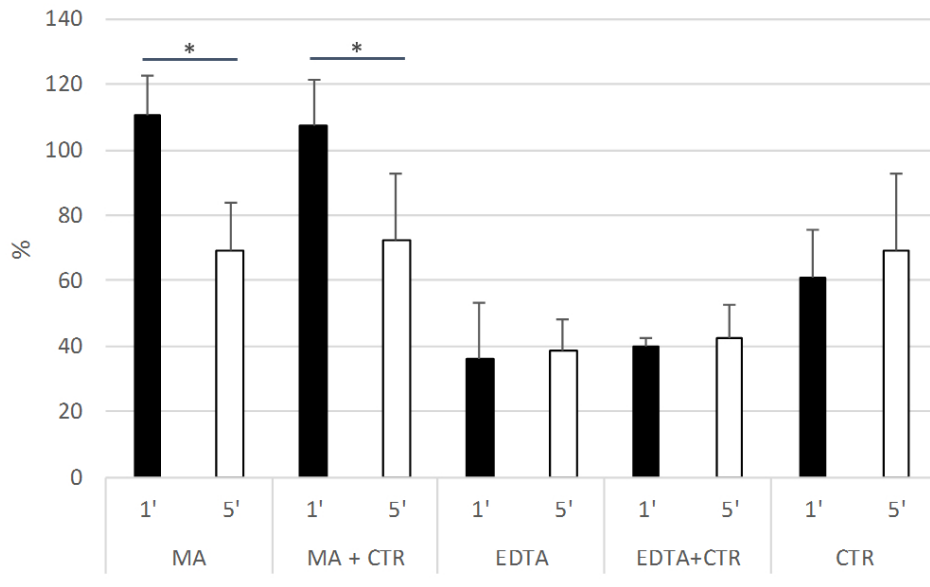42
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Figure 6. CLSM 3D images of E. faecalis biofilms treated with the tested solutions. Ctrl = control; MA = maleic acid; CTR = cetrimide.

178x107mm (150 x 150 DPI)

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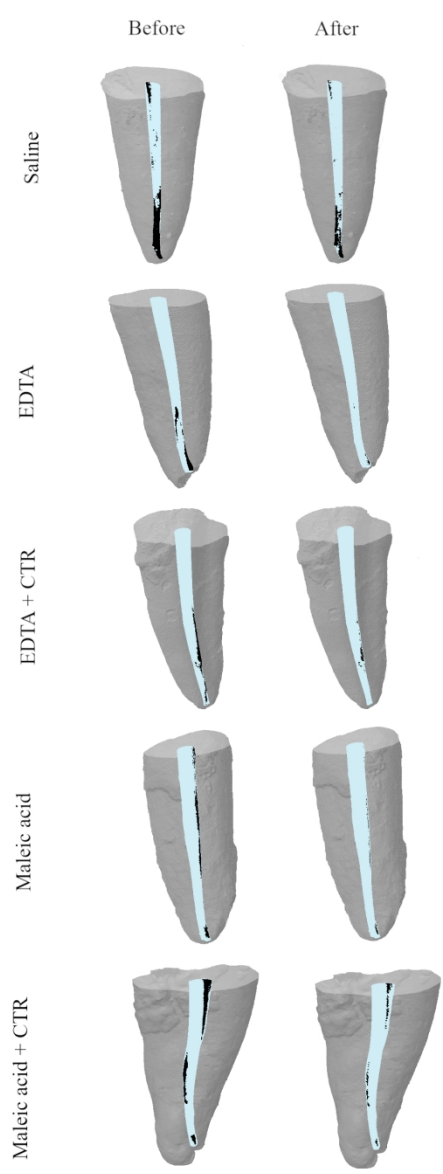51
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Figure 7. Representative micro-CT reconstructions of the root canal system of the treated teeth before and after the final irrigation with the solutions assigned. Accumulated hard tissue debris (AHTD) is depicted in black.

93x207mm (300 x 300 DPI)