MATERIALS

1. Petriplates
2. Tips (20, 200 and 1000 µl tips)
3. Micropipettes (0.2-2.5, 2-20, 20-200 and 200-1000 µl)
4. Test tubes
5. Gram staining kits
6. Hydrogen peroxide
7. Sterile water
8. Conical flask (100, 250, 500 and 1000 ml)
9. Non absorbent cotton
10. Cotton swabs
11. Brain heart infusion Broth (Himedia)
12. Mueller Hinton agar (Himedia)
13. Bile Esculin (Himedia)
14. Tri sodium tetrazolium salt (Hi Media)
15. Methyl cellulose
16. Fluorescence diacetate (sigma)
17. Propidium iodide (sigma)
18. Phosphate buffer saline
19. Dichloro Fluorescence di acetate (sigma)
20. 96 wells plate
21. Diamond Disc
22. K file – 10, 15, 20 size
23. Gates Gliden drill
Materials and Methods

4.1: Synthesis and Characterization of AgNps

AgNps were synthesized as described in our earlier work (91). Briefly, aqueous colloidal solution of silver nanoparticles was synthesized by reducing silver nitrate with sodium borohydride. However, for the present work, it was diluted in different concentrations i.e., 4 mM, 6 mM, 8 mM, 10 mM and 100 mM. The crystal structure, size and shape of the AgNps were investigated using ultra violet spectroscopy (UV spectroscopy) and high resolution transmission electron microscopy (HRTEM). UV
Materials and Methods

spectroscopy Lambda -19 was used to assess the presence of AgNps in suspension and the kinetic behavior of AgNps. In an earlier study, the authors have reported that the zeta potential for AgNps was -30 meV (91).

Sample preparation for UV Visible spectroscopy:

The prepared AgNPs solutions were centrifuged at 10000 rpm for 30 mins. The supernatant contains AgNPs and the pellet contains excess amount of CTAB, which was discarded. Few microliters of the supernatant was diluted to 3 mL of deionized water, so that there was enough transmittance in the colloidal solution. Then, it was transferred to quartz cuvettes and measured in the UV Vis spectrophotometer (Perkin Elmer Lambda 650 UV Visible/DRS Double beam spectrophotometer). For concentration dependent analysis, the supernatant was taken without dilution.

Sample preparation for HRTEM:

The centrifuged AgNPs solution was diluted in deionized water in the ratio of 1:9 (i.e., 1 part of sample in 9 parts of water or ethanol or acetone). Then, 10 to 20 microliters of the sample was drop coated on carbon coated copper grid over the carbon coated side and dried at room temperature for 4-5 h. Once the solvent was completely dried up, the grids were used for HRTEM (FEI Tecnai T30 G2) analysis.
4.2: Determination of zone of inhibition

Preparation of inoculums:

The antimicrobial activity of AgNps was tested against *E. faecalis* (ATCC 29212). Brain Heart Infusion agar (BHI) plates were used to maintain the culture of bacteria. The bacterial cultures were diluted and adjusted to cell suspension of $10^6$ colony forming units (CFU) per ml using spectrophotometer (600 nm). The *E. faecalis* strains were confirmed based on the biochemical tests such as gram staining reaction, catalase test and bile esculin.

The well diffusion method (92) was used to determine the antibacterial activity of AgNps against *E. faecalis* (ATCC 29212) on Brain Heart Infusion Agar (BHIA) plates. Diluted inoculum was spread on the surface of the plates and after 5 mins, the wells were drilled using well borer under aseptic conditions. The wells were loaded with 100 µl of AgNps (4 mM, 6 mM, 8 mM and 10 mM and 100 mM) from each concentration. Chlorhexidine was used as positive control. The plates were incubated for 24-48 hours at 37 °C. The width of the zone of the inhibition was measured.

4.3: Determination of Minimum Inhibitory Concentration (MIC)

MIC of AgNps (4 mM (0.0001 moles of Ag), 6 mM (0.00015 moles of Ag), 8 mM (0.0002 moles of Ag) and 10 mM (0.00025 moles of Ag)) against *E. faecalis* was determined by micro dilution method using Tri phenyl Tetrazolium Chloride (TTC) as an
Materials and Methods

indicator. BHI broth was used to culture the test organisms in 96 wells plate. From the stock, 100 µl of AgNps was serially diluted by two fold dilution to obtain the concentration range of 17 µg - 0.12 µg/ml. After dilution, 20 µl of culture was added and the plate was incubated at 37 °C for 24 hours. Following this incubation, 50 µl of 0.1% TTC was added and incubated for 3-4 hours. The color change (from yellow to pink) of broth indicated the growth of organisms. After incubation, the optical density (O.D) was measured at 600 nm using Thermoscan spectrophotometer (93).

4.4: Colony Forming Unit at 200 µm and 400 µm

Specimen Preparation for tooth study

The study protocol and design was approved by Sri Ramachandra University, Institutional Ethics Committee (Ref: IEC-NI/09/DEC/13/41). The model proposed by Haapasalo & Ørstavik (13) was modified. Thirty single-rooted human mandibular premolar teeth, freshly extracted for orthodontic reasons were selected for this study. A rotary diamond disc was used to decoronate the teeth below the cementoenamel junction and the apical part of the root to obtain middle third of the root (6mm). The cementum was removed from the root surface. The inner diameter of the root canals was standardised using Gates Glidden drills no. 3 (Mani Inc, Tachigiken, Japan) at 300 rpm under water cooling, in a slow-speed handpiece. To remove inorganic and organic debris, the specimens were placed in ultrasonic bath which contains 17% ethylene diamine
Materials and Methods

tetraacetic acid (Prime Dental Products, Mumbai, India) for 5 mins followed by 3% NaOCl (Prime Dental Products, Mumbai, India). Then, specimens were washed with PBS and immersed in an ultrasonic bath for 5 mins, which contained distilled water, to remove the traces of chemicals. All the specimens were sterilized in an autoclave for two cycles.

Specimen contamination

*E. faecalis* (ATCC 29212) cells were grown in Brain heart infusion broth (BHI) for overnight. After incubation, the culture was diluted in 5 mL of BHI broth and its turbidity was adjusted to 0.5 McFarland standards (1x10^6 CFU/ml). Each dentine block was immersed in pre-sterilized microcentrifuge tubes containing BHI broth. For contamination of the dentine block, 50µl of the overnight *E. faecalis* culture was transferred into each of the microcentrifuge tubes. Purity of the culture was checked by subculturing 5 µL of the broth from the incubated dentine blocks on BHI agar plates. Dentine blocks were contaminated with *E. faecalis* for 21 days and media was added every day.

Antibacterial Assessment at two different depths (200 µm and 400 µm)

After incubation (21 days), the tooth samples were washed with 5 ml of sterile saline to remove excess culture on the surface of the specimen. The thirty dentin blocks were randomly divided
into 3 groups with 10 specimens each: Group I - Saline (Control), Group II - 2% Chlorhexidine (CHX) (ASEP RC, Anabond - Stedman, India), Group III - AgNps (0.0002 moles of Ag). The compound (equal to their MICs) was mixed with Methyl cellulose and packed inside the root canal and sealed with paraffin wax at both ends. The samples were incubated at 37°C for 24 h. At the end of 24 h, an assessment of bacterial growth was carried out, by harvesting the dentine chips at 200 µm and 400 µm with Gates Glidden drills no 4 and 5 respectively. The collected dentine shavings were transferred into 1 mL of sterile BHI broth and incubated in an aerobic environment at 37°C for 24 hours. After 24 hrs, the contents of each tube were serially diluted; 100 µL of the broth in 100 µL of sterile saline five times. Fifty microlitres of the dilution was then plated on BHI agar plates and incubated for 24 h. The collected dentine shavings were followed up with the protocol used by Kandaswamy et al. (63). Colonies were counted and readings were tabulated.

4.5: Live and Dead (CLSM)

Sample preparation

Sixty single-rooted human mandibular premolar teeth, freshly extracted for orthodontic reasons were selected for this study. A rotary diamond disc was used to decoronate the teeth below the cementoenamel junction and the apical part of the root to obtain middle third of the root (6mm). The cementum was removed from
the root surface. The inner diameter of the root canals was standardised using Gates Glidden drills no. 3 (Mani Inc, Tachigiken, Japan) at 300 rpm under water cooling in a slow-speed handpiece (NSK, Tokyo, Japan). By using diamond disc, grooves were prepared on the root surfaces, which helped in splitting the root. Externally, roots were coated with two layers of nail varnish. To remove inorganic and organic debris, the specimens were placed in ultrasonic bath which contained 17% EDTA (Prime Dental Products, Mumbai, India) for 5 mins followed by 3% NaOCl (Prime Dental Products, Mumbai, India). Then, specimens were immersed in an ultrasonic bath for 5 mins which contained distilled water, to remove the traces of chemicals. All the specimens were sterilized in an autoclave for two cycles.

**Specimen contamination**

*E. faecalis* (ATCC 29212) cells were grown overnight in Brain heart infusion broth (BHI). After incubation, the culture was diluted in 5 mL of BHI broth and its turbidity was adjusted to 0.5 McFarland standards. Each dentine block was immersed in pre-sterilized microcentrifuge tubes containing BHI broth. For contamination of the dentine block, 50µl of the overnight *E. faecalis* culture was transferred into each of the microcentrifuge tubes. Purity of the culture was checked by subculturing 5 µL of the broth from the incubated dentine blocks on BHI agar plates. Dentine blocks were contaminated with *E. faecalis* for 21 days.
Antimicrobial assessment

The specimens were irrigated with 5 mL of sterile saline to remove the incubation broth at the end of 21 days. The dentin blocks were randomly divided into 3 groups with 20 specimens each: Group I- Saline (Control), Group II - 2% Chlorhexidine (CHX) (ASEP RC, Anabond - Stedman, India), Group III- AgNps. Methyl cellulose was used as a thickening agent for Groups 1, 2 and 3. The medicaments were placed inside the canals and sealed at both ends with paraffin wax. They were incubated in an anaerobic environment for 37 °C. At the end of Days 1 and 3, an assessment of live and dead cells was carried out with 10 specimens at each time interval, for each medicament. The specimens were rinsed thoroughly in PBS buffer for 1 minute and the fractured vertically through the root canal into 2 halves, thereby exposing fresh longitudinally fractured dentin canals for Confocal laser scanning microscopy (CLSM) examination.

CLSM Examination (94)

The Confocal microscopy was performed using a LSM 710 Meta laser scanning Confocal microscope (Carl Zeiss) having a 63x oil immersion objective. The images were acquired and the AIM software was used for analysis. For the Confocal microscopy, the washed dentin specimens were stained in dark, at room temperature with two stains. Live and dead cells in the bacterial biofilm were differentiated by staining with Fluorescein Diacetate (green
Materials and Methods

fluorescence) and Propidium iodide (red fluorescence) (83, 85). The specimens were washed twice in PBS buffer to removes excess of the dye. The specimens were placed onto glass cover slips covered with immersion oil before image acquisition. Confocal microscopy illumination was performed using an argon laser with excitation wavelength of 543nm for red and 488nm green fluorescence, respectively. Simultaneous dual-channel imaging mode was used to display both green and red fluorescence. For each group, 10 dentin blocks were used for image analysis and each sample was scanned in 5 different areas. The images were processed and analyzed to remove the background fluorescence and to quantify live/dead cells. The acquired images from CLSM were stacked and analyzed.

4.6: Increase antibacterial efficacy of AgNps - CLSM

Sample preparation

The forty one non carious single rooted mandibular teeth without fractures were selected for this study. All the teeth were decoronated 12 mm from the root end to standardize the root length. The apical patency has been checked by using 10 size hand K file (Dentsply Maillefer, Switzerland). Then, the root was shaped upto 20 size hand K file (Dentsply Maillefer, Switzerland). Saline was used between each instrument. Canals were then enlarged with protaper rotary system (Dentsply Maillefer, Switzerland) upto F 3. Irrigation was done using 5.25% NaOCl with a needle placed, 1 mm
short of the apical terminus, followed by 1 ml of 17 % EDTA and saline to remove the debris. By using diamond disc, grooves were prepared on the root surface, which helped in splitting the root. Externally, roots were coated with two layers of nail varnish. Apical patency was verified (95). Then, specimens were washed with PBS and immersed in an ultrasonic bath for 5 mins which contained distilled water, to remove the traces of chemicals. All the specimens were sterilized in an autoclave for two cycles.

**Specimen contamination**

*E. faecalis* (ATCC 29212) cells were grown overnight in Brain heart infusion broth (BHI). After incubation, the culture was diluted in 5 mL of BHI broth and its turbidity was adjusted to 0.5 McFarland standards (1x10^6 CFU/ml). Each dentine block was immersed in pre-sterilized microcentrifuge tubes containing BHI broth. Under aseptic conditions, *E. faecalis* was introduced into the root canal. For contamination of the dentine block, 50µl of the overnight *E. faecalis* culture was transferred into each of the microcentrifuge tubes. Purity of the culture was checked by subculturing 5 µL of the broth from the incubated dentine blocks on BHI agar plates. Dentine blocks were contaminated with *E. faecalis* for 21 days; media was added every day. Randomly, one tooth was selected and split longitudinally for Scanning Electron Microscope observation, to check the presence of *E. faecalis* biofilm.
Antimicrobial assessment

The specimens were irrigated with 5 mL of sterile saline to remove the incubation broth at the end of 21 days. The dentin blocks were randomly divided into 4 groups with 10 specimens each: Group I: Replenishing – AgNps mixed with methylcellulose, placed inside the canal. At the end of 24 hrs medicament (AgNps mixed with methylcellulose) was removed and irrigated with saline. Then, freshly mixed medicament (AgNps mixed with methylcellulose) was placed inside the canal and kept for another 24 hrs; Group II: AgNps mixed with lutrol was inserted into the canal and placed for 24 hrs; Group III: AgNps mixed with methycellulose was placed for 24 hrs and Group IV: AgNps mixed with lutrol was inserted into the canal followed by ultrasonic agitation. The ultrasonic agitation (79, 80) was carried out by ultrasonic unit (Satelec, Suprasson NewtronP5, France) connected with 15 size file for 1 min, applying vibrations to shake only the solution, (AgNps and Lutrol) without affecting the dentin walls. Then, samples were not disturbed for 24 hrs. The specimens were rinsed thoroughly in PBS buffer for 1 minute and then fractured vertically through the root canal into 2 halves, thereby exposing fresh longitudinally fractured dentin canals for Confocal laser scanning microscopy (CLSM) examination. For CLSM examination, our previous protocol has been followed.
4.7: MECHANISM BEHIND ANTIBACTERIAL EFFICACY

i) 4.7.1 - Morphological changes - scanning electron microscopy

Morphological changes and biofilm assays described by Chai WL et al (96) were adopted in this study. *E. faecalis* was grown on sterile Whatman No: 1 filter paper strips and incubated overnight at 37°C. After incubation, the stripes were treated with CHX and AgNps at their MICs for 24 hrs and followed by immersion in neutralizing broth to stop the action of CHX and AgNps. The filter paper discs without any treatment were used as the controls. The strips were dried and processed for Scanning electron microscopy. The observations were made on scanning electron microscope (94) (Supra 55, Carl Zeiss).

ii) 4.7.2 - Propidium Iodide uptake assay (or) Membrane permeabilization assay by FACS analysis

Overnight *E. faecalis* cells were sub-cultured in BHI broth, adjusted to 1x10^6 cells /ml and were harvested by centrifugation at 6000 rpm for 10 min. The pellet was washed and re-suspended in HEPES buffer (5mM glucose, 5mM HEPES). The cells were treated with AgNps and CHX for 45 min at 37°C. Untreated cells served as negative controls. After incubation, 5 µl of PI (1mg/ml) was added and incubated for 15 min at room temperature. Following this incubation, the cells were subjected to Fluorescence Activated Cell Sorter (FACS) analysis (97).
iii) 4.7.3 - Measurement of Reactive Oxygen Species (ROS) production

The ROS generation was measured by flow cytometry analysis with 2', 7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, overnight *E. faecalis* cells were sub-cultured in BHI broth and adjusted to 1x10^6 cells/ml. The cells were harvested at 6000 rpm for 10 min. The cell pellet was then re-suspended in PBS and treated with AgNps and CHX for 1h at 37 °C. Untreated cells served as negative controls. Hydrogen peroxide (H_2O_2) was used as positive control. After incubation, 10 µM of DCFH-DA in PBS was added and incubated for 10 min at room temperature. Following this incubation, the cells were subjected to Fluorescence Activated Cell Sorter (FACS) analysis (87, 98).

4.8: Biocompatibility: Hemolytic assay

Hemolytic assay was performed using the heparinized whole human blood collected from volunteers. Following the centrifugation of blood samples at 3000rpm for 5 min, the RBC pellet was obtained. Supernatant was discarded and RBC pellet was washed with PBS. RBC suspension (1 %) was prepared and 100 µl of RBC suspension was incubated with CHX and AgNps for 2 hours. After the incubation, the samples were spun for 5 min at 3000 rpm. Supernatant was collected and O.D was measured at 545 nm to determine the RBC lysis. Triton X-100 was used as positive control and untreated blood with PBS served as negative control.
Percentage of lysis was calculated using the formula mentioned below (99).

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\text{Percentage of haemolysis} = \frac{O.D \text{ of test sample} - O.D \text{ of negative control}}{O.D \text{ of positive control} - O.D \text{ of negative control}} \times 100
\]

4.9: Microhardness Test

Twenty extracted human single rooted mandibular premolar teeth were used. The teeth were stored in distilled water for up to 4 weeks prior to use. Debris and soft tissue remnants on the root surfaces were removed with the help of scaler. The crowns of teeth were removed and pulp tissue extirpated. Cleaning and shaping was done with protaper rotary instruments (Dentsply – Mallifer, Switzerland) up to F2. The roots were fixed in acrylic resin blocks and cut transversally in 2 mm sections. The specimens were polished with 2500 grit abrasive paper (Hermes, Hamburg, Germany) using a microgrinding system. Sections were obtained from the middle third of each root. The sections were checked for baseline microhardness test by using a micro vicker hardness tester (Wilson Wolpert – Germany). All indentations were made with 100g loading for 15 s contact time. Each disc received indentations at points around the pulp space, 1 mm from canal wall. Hardness numbers were calculated for each specimen. Then, the medicament Group 1: AgNps (n-10) and Group II: CHX (n-10) were packed inside the canal and kept for one day. After the treatment period (1
Materials and Methods

day), the specimens were rinsed with distilled water and dried before measuring microhardness. Then, the dentin disc received indentation, for post treatment hardness values (83).