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Research Article

Comparative Evaluation of the Antibacterial Activity of *Cinnamon zylanicum* (True Cinnamon) and *Azadirachta indica* (Neem) Herbal Extracts Versus Calcium Hydroxide Intracanal Medication against *Enterococcus faecalis* in Single Rooted Premolars Teeth: A Randomized *In Vitro* Study

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Abstract

Aim of the Work: This Comparative Randomized *in-vitro* study was conducted to compare the antibacterial effect of two different herbal extracts: *Cinnamon zylanicum* (True Cinnamon) and *Azadiracta Indica* (Neem) versus Calcium Hydroxide ($Ca(OH)_2$) as an intracanal medication against *Enterococcus Faecalis (E. faecalis)* in single-rooted premolars teeth.

Materials and Methods: Thirty nine single-rooted premolars teeth were randomly divided according to the intracanal medication used into three groups (n = 13), either True Cinnamon or Neem or $Ca(OH)_2$.

The teeth were decoronated, infected with *E. faecalis* and incubated for 21 days, then Pre-operative sample (S1) was taken using size 25 sterile paper points. The root canals were prepared using ProTaper Universal rotary system till size F4, then Post-Instrumentation sample (S2) was taken using F4 sterile ProTaper paper points; this was followed by the application of the intracanal medications for 7 days, then the Post-medication sample (S3) was taken using F4 sterile paper points. The antibacterial effect was assessed using Agar Diffusion Test to count the colony forming units.

Results: Showed that there was no difference in the antibacterial effect of True Cinnamon, Neem and $Ca(OH)_2$, however in each group there was a statistically significant difference in the antibacterial effect between the Pre-operative samples (S1), the Post-Instrumentation sample (S2) and the Post-medication sample (S3).

Conclusion: True Cinnamon, Neem and $Ca(OH)_2$ showed similar antibacterial activity against *E. faecalis*, when used as intracanal medications for 7 days.

Keywords: *Cinnamon zylanicum*; Cinnamon; *Azadiracta indica;* Neem; Calcium Hydroxide; Antibacterial; *Enterococcus faecalis*

Introduction

The main objective of root canal treatment is to eliminate bacteria from the infected root canal system and to prevent the re-infection, through cleaning the infected soft and hard tissues. Thus, providing sufficient access for irrigants, delivery of medicaments and the subsequent canal fillings allow the heal of the periapical legions [1]. The persistence of microorganisms in the root filled teeth particularly the apical part is considered the main factor affecting the success of endodontic therapy [2].

Enterococci are common inhabitants of the human body, which have been associated with infection in a variety of sites including the oral cavity [3]. *Enterococcus faecalis (E. faecalis)* appears to possess the ability to establish an endodontic infection and maintain

an inflammatory response especially in obturated root canals, in addition to exhibiting signs of chronic apical periodontitis [4]. Once established in the root canal, it has the ability to face several challenges for survival, as it can suppress the action of lymphocytes, activate the host protease plasminogen in a fashion that increases the local tissue destruction. In addition, it can form a biofilm, withstand high PH antimicrobial agents used during treatment, colonizing into dentinal tubules and reinfecting the obturated root canal [4,5]. Recent studies also showed that *E. faecalis* is highly resistant to commonly used intra canal medications such as calcium hydroxide Ca $(OH)_2$ [6-8], although Ca $(OH)_2$ has several advantageous properties including the high alkalinity, the ability to destroy the cytoplasmic membrane, denature bacterial proteins and damage bacterial DNA [9], in addition to the tissue-dissolving ability, inhibition of tooth resorption, hard tissue formation and promoting periradicular healing, [9] however, the incomplete removal from the root canal surface is considered the main dis-advantage of Ca $(OH)_2$, as it prevents the sealer from penetrating into the dentinal tubules and interferes with its normal setting reaction, which affects the seal of obturating material leading to microleakage and subsequent treatment failure [10].

Herbal extracts such as *Morinda citrifolia* juice (MCJ) [11], *Salvldora persica* (Siwak) [12], Tea Tree oil (*Melaleuca altemifoia*) [13], Chamomile (*Marticaria Recutita*) extract [14], *Cinnamon zylanicum* (True Cinnamon), *Azadiracta Indica* (Neem) have been introduced as a new turning point in the dental field due to their biocompatibility, antimicrobial, anti-inflammatory and anti-oxidant properties [15].

Up to date, according to literature, there are few studies evaluated the antibacterial effect of True Cinnamon and Neem when used as intracanal medications after root canal preparation, thus the purpose of the present study was to compare the antibacterial effect of True cinnamon and Neem, with that of Ca $(OH)_2$ against *E. faecalis* when used as an intra-canal medication. The null hypothesis was that there would be a no statistically significant difference between the True Cinnamon, Neem extracts and Ca $(OH)_2$ regarding their antibacterial effect against *E. faecalis*.

Materials and Methods

Thirty-nine extracted single rooted premolar teeth were included in the study.

Medication preparation

True Cinnamon and Neem leaves were dried then ground by Spice mill to obtain a powder form. Forty milliliters (40 ml) of Monopropylene glycol solution was added to 20 grams of the powder to get a concentration 50%, then filtrated using a white cloth into a flask for 48-hrs at room temperature [16]. The infiltrate was collected and stored in air tight bottle until usage. The extract was mixed with methyl cellulose in ratio (1:1) to get the gel form [17] and then loaded in 25-gauge syringe.

Preservation of the samples

Teeth were thoroughly washed under running water, then the root surfaces were planned and any soft tissue remnant or calculus were removed from the root surfaces using the ultrasonic scaler. The teeth were then stored in saline at room temperature till the time of use. Root surfaces except the apical 3 mm were sealed by nail varnish to ensure a good bacterial seal.

Preparation of the samples

All the teeth were decoronated at cemento-enamel junction (CEJ) using a water cooled low speed diamond disk at speed of 8000 rpm under continuous irrigation to obtain a standard root with length of 15 mm. The canal patency was checked by placing size 15 K- file into the canals until visible at the apical foramen. Canals that didn't allow placement of a size 15 file into the apex or those wider than a size #20 file at the apex were excluded from the study.

The root canals were enlarged to the working length up to size 25 and then irrigated with 2.5% sodium hypochlorite (NaOCl) to remove debris from the canals and facilitate inoculation of the bacterial suspension.

The apical 3mm of the root of each sample was etched, bonded, and sealed with composite resin 3 in order to prevent bacterial leakage and to retain the irrigation solution within the canals to simulate the *in-vivo* apical counter pressure. The root samples were then autoclaved in a sterile falcon tubes containing phosphate buffered saline (PBS), to preserve the samples dehydration at temperature 121°C and pressure 2 bars for 20 minutes.

Root canal inoculation with *Enterococcus faecalis*

The reference strain of *E. faecalis*, American Culture collection 29212 (ATCC 29212), was supplied from a standard stock. A vial

of frozen bacteria (-20°C) was thawed and a sample was taken on a sterile wire loop. For bacterial reactivation, the sample was cultivated in sterile Brain Heart Infusion (BHI) broth and incubated at 37°C for 24-hrs. The bacteria was then seeded on a bile esculin agar plate and incubated overnight at 37°C under aerobic conditions.

Isolated colonies was harvested from the subculture and suspended in 4 ml of BHI in a sterile falcon glass tube. The bacterial culture was adjusted to 0.5 McFarland turbidity standards which is equivalent to 1.5 x 10^8 CFU/ml.

Each root specimen was inoculated with 30 microliters of the *E. faecalis* suspension under a laminar flow chamber using a sterile 1 ml insulin syringe with a 30-gauge needle, then each sample was placed in a sealed plastic tube containing 10 ml of BHI broth, and then incubated at temperature 37°C for 21 days and 100% humidity to allow colonization of the bacteria on the canal wall and into the dentinal tubules. The growth media was replenished every 48-hrs to ensure culture viability.

After 21 days the Pre-operative sample (S1) was taken by introducing a size 25 sterile-pyogen free paper point for 1 minute into the full working length of the canal, then immediately transferred to sterile falcon glass tube containing BHI broth and stored in the incubator at temperature -20°C. This procedure was repeated using 4 paper points.

Root canal disinfection

Root canals were mechanically prepared by crown down technique using Protaper Universal NiTi rotary instruments rotated at a speed of 250 rpm and torque was adjusted as SX: 3 - 4 N.cm, S1: 2 - 3 N.cm, S2: 1 - 1.5 N.cm, F1: 1.5 - 2 N.cm, and F2-F3-F4: 2 - 3 N.cm. The sequence of the root canal preparation started with SX and S1 files to the coronal two thirds of the canal working length and then the canal was prepared to the full working length using S1, S2, F1, F2 then F3 and F4.

The irrigation was done using freshly prepared 2.5% NaOCl to maintain chlorine stability using side vented plastic syringe with a 30 gauge needle. Irrigation was performed 2 mm short of the working length which was verified by the rubber stops. Two milliliters of 2.5% NaOCl were expressed over 30 seconds between each successive file, this is followed by 5% sodium thiosulfate to neutralize the effect of NaOCl solution.

Three ml of 17% EDTA were used for 1 minutes to remove the smear layer followed by 10 ml of distilled water as a final flush to prevent erosion of dentinal tubules.

Post-instrumentation sample (S2) was taken by introducing F4 sterile-pyogen free Protaper absorbent paper points for 1 min to the full working length of the canal, then immediately transferred to a sterile falcon glass tube containing BHI broth and stored in the incubator at temperature -20°C. This procedure was repeated using 4 paper points.

Application of the intracanal medication

In Group A (True Cinnamon) and Group B (Neem), the extracted gels was injected into the canals after canal dryness using 25-gauge needle, inserted up to 2 mm from the root apex with continuous upward slow withdrawal of the needle till reaching the canal orifice, then the excess medication was removed by using sterile cotton rolls.

In Group C, Ca $(OH)_2$ powder was mixed with normal saline (1:1), then a Lentulo spiral was used to introduce the paste into the canals up to 2 mm from the root apex using low speed hand piece in a clockwise movement at a speed of 1000 rpm, and finally the paste was condensed using size 30 and 35 hand plugurs.

All the samples were sealed with temporary cement, applied directly to the canal orifice and incubated at temperature 37°C for 7 days. The canals were then re-opened and the medications were removed using size 40, 45 K-files and 5 ml sterile saline solution, while in Group C samples were irrigated by 1 ml of 0.5% citric acid, followed by 2 ml of sterile saline to emulsify and neutralize the Ca $(OH)_2$ allowing better bacterial sampling.

The Post-medication sample (S3) was taken by introducing F4 sterile-pyogen free Protaper absorbent paper points for 1 min to the full working length of the canal, then immediately transferred to sterile-pyogen free falcon glass tube containing BHI broth and stored in the incubator at temperature -20°C. This procedure was repeated using 4 paper points.

Evaluation of the antibacterial effect using agar diffusion test

The Pre-operative, Post-instrumentation and Post-medication samples, each were transferred to sterile test tube containing 1 ml of sterile BHI broth, then it was manually shacked for 3 minutes. Hundred microliters was taken from the infected broth and added to another test tube containing 900 microliter of sterile BHI broth to get 0.1 dilutions. Thirty microliter was taken from the diluted broth using sterile micropipette and smeared on the surface of the agar plates by using sterile L-shaped glass rod, then the agar plate was incubated at temperature 37°C for 48-hrs. Bacterial count of the colony forming unites was then done by visualization of the individual white pin point colonies on the agar plate.

Statistical analysis

Numerical data were explored for normality by checking the data distribution, calculating the mean and median values and using Kalmogrov-smimov test and Shapiro-wilk test. Data showed parametric distribution so it was represented by mean and standard deviation (SD) values. One-way Analysis of Variance (ANOVA) was used to study the effect of different tested variables and pairwise comparisons were done using Tukey's post-hoc test. The significance level was set at P ≤ 0.05 within all tests. Statistical analysis was performed with IBM® SPSS®17 Statistics Version 25 for Windows.

Results

Antibacterial effect of each intracanal medication at different observation points

Group A: *Cinnamon zylanicum* (True Cinnamon)

The mean and standard deviation (SD) values of the *E. faecalis* count (CFU/ml) in Group A were summarized in table 1 and presented in figure 1 and 2. There was statistically significant difference in *E. faecalis* count at different observation points (P = 0.001, P ≤ 0.05), there was statistically significant difference between the Pre-operative and the Post-instrumentation samples (P = 0.001, P ≤ 0.05) and between the Pre-operative and the Post-medication samples (P = 0.001, P ≤ 0.05), as well as between the Post-instrumentation and the Post-medication samples (P = 0.001, P ≤ 0.05). The Pre-operative sample showed the highest CFUs, followed by the Post-instrumentation and then the Post-medication samples.

Group B: *Azadiracta indica* (Neem)

The mean and standard deviation (SD) values of the *E. faecalis* count (CFU/ml) in Group B were summarized in table 2 and presented in figure 3 and 4. There was statistically significant difference in *E. faecalis* count at different observation points (P = 0.001, P ≤ 0.05), there was statistically significant difference between the Pre-operative and the Post-instrumentation samples (P = 0.001, P ≤ 0.05) and between the Pre-operative and the Post-medication samples (P = 0.001, P ≤ 0.05), as well as between the Post-instrumentation and the Post-medication samples (P = 0.001, P ≤ 0.05). The Pre-operative sample showed the highest CFUs, followed by the Post-instrumentation and then the Post-medication samples.

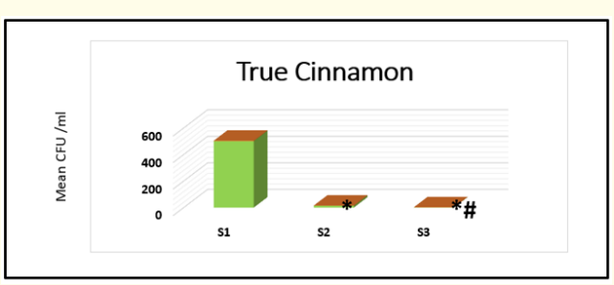

Figure 1: Bar chart representing the mean values of E. faecalis counts CFU/ml in Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group A (True Cinnamon).

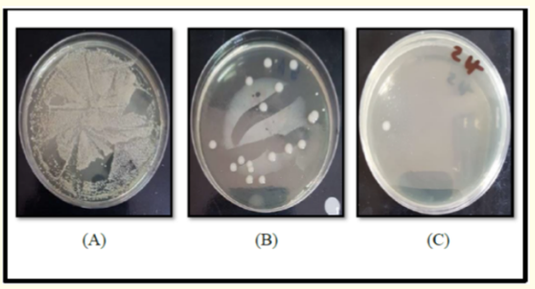

Figure 2: (A) Photograph of the viable colony of E. faecalis on BHI agar in Pre-operative sample, (B) Post-instrumentation sample and (C) Post-medication sample in Group A (True Cinnamon).

	Pre-operative (S1) n = 13 Mean (SD)	Post-instrumentation (S2) n = 13 Mean (SD)	Post-medication (S3) n = 13 Mean (SD)	P- value
Bacterial colonies (CFU/ml)	501+3.05a	13.9+1.8b	0.7+0.24c	0.001*

Table 1: The mean, standard deviation (SD) values and results of Tukey's post hoc test of the E. faecalis counts (CFU/ml) in the Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group A (True Cinnamon).

* Indicates significance at P ≤ 0.05.

	Pre-operative (S1) n = 13 Mean (SD)	Post-insrumenttion (S2) n = 13 Mean (SD)	Post-medication (S3) n = 13 Mean (SD)	P- value
Bacterial colonies (CFU/ml)	503+2.07a	14.3+1.7b	1.2+0.15c	0.001*

Table 2: The mean, standard deviation (SD) values and results of Tukey's post hoc test of the E. faecalis counts (CFU/ml) in the Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group B (Neem).

* Indicates significance at P ≤ 0.05.

Group C: Calcium hydroxide ($Ca(OH)_2$)

The mean and standard deviation (SD) values of the *E. faecalis* count (CFU/ml) in Group C were summarized in table 3 and presented in figure 5 and 6. There was statistically significant difference in *E. faecalis* count at different observation points (P = 0.001, P ≤ 0.05), there was statistically significant difference between the Pre-operative and the Post-instrumentation samples (P = 0.001,

P ≤ 0.05) and between the Pre-operative and the Post-medication samples (P = 0.001, P ≤ 0.05), as well as between the Post-instrumentation and the Post-medication samples (P = 0.001, P ≤ 0. 05). The Pre-operative sample showed the highest CFUs, followed by the Post-instrumentation and then the Post-medication samples.

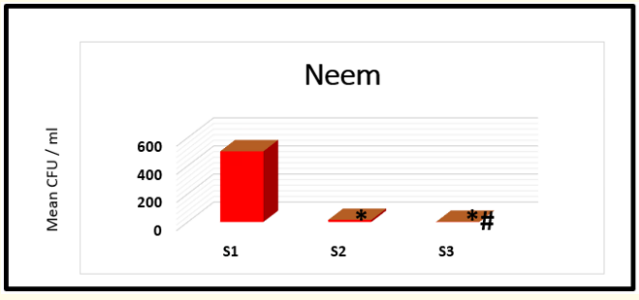

Figure 3: Bar chart representing the mean values of E. faecalis counts CFU/ml in Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group B (Neem).

Comparison of *Enterococcus faecalis* count at each observation point in different groups

The mean and standard deviation (SD) values of the *E. faecalis* count (CFU/ml) in Group A (True Cinnamon), Group B (Neem) and Group C (Ca $(OH)_2$) were summarized in table 4 and presented in figure 7.

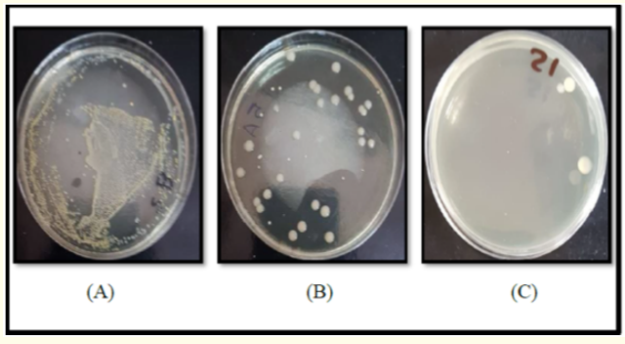

Figure 4: Bar chart representing the mean values of E. faecalis counts CFU/ml in Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group B (Neem).

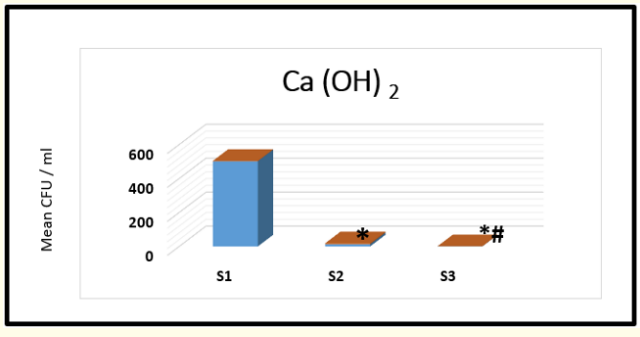

Figure 5: Bar chart representing the mean values of E. faecalis counts CFU/ml in Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group C (Ca $(OH)_2$).

	Pre-operative (S1) n = 13 Mean (SD)	**Post-instrumentation (S2) n = 13 Mean (SD)**	**Post-Medication (S3) n = 13 Mean (SD)**	**P- value**
Bacterial colonies (CFU/ml)	502+2.08a	13.4+1.4b	1.07+0.1c	0.001*

Table 3: The mean, standard deviation (SD) values and results of Tukey's post hoc test of the E. faecalis counts (CFU/ml) in the Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group C (Ca $(OH)_2$).

* Indicates significance at P ≤ 0.05.

	Group A True Cinnamon n = 13 Mean (SD)	Group B Neem n = 13 Mean (SD)	Group C Calcium Hydroxide n = 13 Mean (SD)	P- value
S1	502 (2.08)	503 (2.07)	501 (3.05)	0.964
S2	13.4 (1.4)	14.3 (1.7)	13.9 (1.8)	0.928
S3	1.07 (0.1)	1.2 (0.15)	0.7 (0.24)	0.928

Table 4: The mean, standard deviation (SD) values of E. faecalis count (CFU/ml) and results of ANOVA for comparison between the *E. faecalis* counts in Group (A) True Cinnamon, Group (B) Neem and Group (C) Ca $(OH)_2$ intracanal medications at different observation point.

* Indicates significance at P ≤ 0.05.

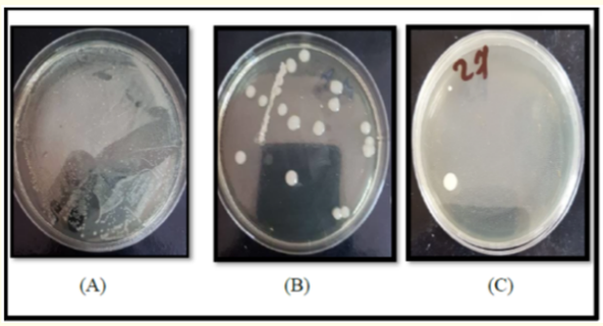

Figure 6: (A) Photograph of the viable colony of E. faecalis on BHI agar in Pre-operative sample, (B) Post-instrumentation sample and (C) Post-medication sample in Group C (Ca $(OH)_2$.

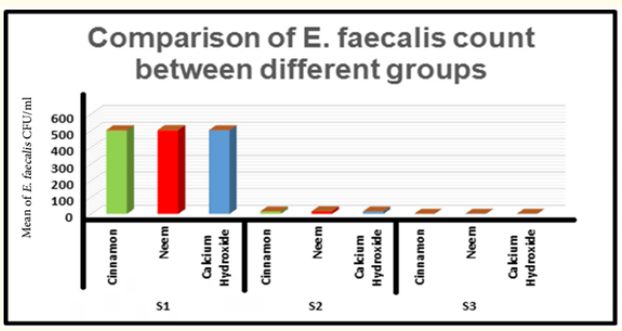

Figure 7: Bar Chart showing the mean values of *E. faecalis* CFU/ml in Pre-operative (S1), Post-instrumentation (S2) and Post-medication (S3) samples in Group A (True Cinnamon), Group B (Neem) and Group C (Ca $(OH)_2$).

Pre-operative sample (S1): The mean and standard deviation (SD) values of *E. faecalis* count were 501+3.05 CFU/ml in Group A, 503+2.07 CFU/ml in Group B and 502+2.08 CFU/ml in Group C. There was no statistically significant difference between the groups (P = 0.964, P > 0.05).

Post-instrumentation sample (S2): The mean and standard deviation (SD) values of *E. faecalis* count were 13.9+1.8 CFU/ml in Group A, 14.3+1.7 CFU/ml in Group B and 13.4+1.4 CFU/ml in Group C. There was no statistically significant difference between the groups (P = 0.928, P > 0.05).

Post-medication sample (S3): The mean and standard deviation (SD) values of *E. faecalis* count were 0.7+0.24 CFU/ml in Group A, 1.2+0.15 CFU/ml in Group B and 1.07+0.1 CFU/ml in Group C. There was no statistically significant difference between the groups (P = 0.928, P > 0.05).

Discussions

The present study showed observed reduction in bacterial count in all tested groups after irrigation using 2.5% NaOCl, this was in accordance with Siqueira., *et al*. (2007) [18] who reported that chemomechanical preparation using NiTi files and irrigation with 2.5% NaOCl was highly effective in reducing the bacterial population within the root canal, Marion., *et al*. (2012) [19] who reported that both 2.5% NaOCl and 5.25% NaOCl have similar antibacterial properties, but the first is less cytotoxic and more indicated for root canals endodontic treatment, moreover Reyhani., *et al*. (2016) [20] reported that 2.5% and 5% NaOCl completely eliminated *E. faecalis* biofilms.

Results showed that the True Cinnamon had observed reduction in the bacterial count after 7 days, this was in agreement with Veerale., *et al*. (2018) [21] and Abbaszadegan., *et al*. (2016) [22], which may be related to its bio-compatibility, anti-inflammatory, anti-oxidant properties and antibacterial effect against *E. faecalis*, as it contains several oils including cinnamaldehyde, eugenol, safrole, which have the ability to disrupt the bacterial cell membrane and its structures leading to ion leakage and bacterial cell death [23].

Regarding the Neem, results showed bacterial reduction after 7 days, which was in consistent with Muktishree., *et al*. (2016) [24]. This may be attributed to the presence of isoprenoid group in the neem oil, which has anti-inflammatory, antibacterial, antifungal, and immunomodulatory properties. In addition, it contains triterpenoids, phenolic compounds, carotenoids, steroids, valavinoids, ketones, tetratriterpenoids and azadirachtin that render its antibacterial effect [25]. Moreover, it contains various active phytoconstituents such as alkaloids, glycosides and tannins that may alter bacterial adhesion and colonization [26].

In the present study Ca $(OH)_2$ group showed observed reduction in the bacterial count after 7 days [27-31], this may be attributed to its alkalinity and ability to destroy the cytoplasmic membrane, denature bacterial proteins, damage bacterial DNA and tissue-dissolving ability [32-34].

Results of the present study showed no difference in the antibacterial effect of True Cinnamon, Neem and Ca $(OH)_2$ after 7 days of application as they commonly affect the integrity of the bacterial cell membrane, causing protein denaturation and subsequent cell death [23,25,33]. This was in contrast with Abbaszadegan., *et al*. (2016) [22] who reported that True Cinnamon and TAP had more antibacterial effect against *E. faecalis* than Ca $(OH)_2$, Muktishree., *et al*. (2016) [24] who reported that Neem showed higher antibacterial effect than that of Ca $(OH)_2$ and Veerale., *et al*. (2018) [21] who reported that Cinnamon extract had better antibacterial efficacy compared to Ca $(OH)_2$. This may be attributed to the difference in methodology as the root canal preparation was done prior to bacterial inoculation, in addition to the different in the way of sample taking (dentinal shaving), however in the present study, root canal inoculation was done prior to the root canal preparation in according to Siqueira., *et al*. (2007) [18] and Valera., *et al*. (2016) [35] to simulate the clinical condition.

Conclusion

Within the limitation of this *in-vitro* study, the following can be concluded that each intracanal medication used (True Cinnamon, Neem and Ca $(OH)_2$) reduced the bacterial count after 7 days and they seemed to have similar antibacterial effect. Bacterial reduction was observed after chemo-mechanical preparation and irrigation using 2.5% NaOCl, however further reduction was observed after 7 days of application of the intracanal medications

Conflict of Interest

The authors deny any conflicts of interest in this study.

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