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Original Article

Dentistry Section

Comparative Evaluation of Antimicrobial Efficacy, Depth of Penetration into Dentinal Tubules and Effect on Microhardness of Root Dentin by Sodium Hypochlorite, Neem Extract and Gau Ark as Root Canal Irrigants: An In-vitro Study

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ABSTRACT

Introduction: Disinfection of root canals can be done using intracanal irrigants. Sodium hypochlorite or NaOCL is amongst the most commonly used endodontic irrigants. Due to disadvantages of NaOCl, there is a need to identify a more biocompatible irrigant.

Aim: To evaluate the antibacterial efficacy, depth of penetration into dentinal tubules and effect on microhardness of root dentin when NaOCl, Neem extract, Gau ark and Normal saline used as an irrigant against *Enterococcus faecalis*.

Materials and Methods: Prepared Neem leaf extract, 3% NaOCl, Gau ark, Normal saline, Brain Heart Infusion (BHI) broth and BHI Agar were used in the present in-vitro study conducted from August-October 2019. Antimicrobial efficacy was measured by agar well diffusion method on 10 petriplates. After access opening and biomechanical preparation, each tooth sample was irrigated with 5 mL of irrigant. Samples were decoronated and roots were split along the longitudinal axis to check penetration depth of irrigants under stereomicroscope for 40 teeth samples. After preparation of 40 teeth sample, 2 mm dentin discs prepared were embedded in acrylic resin. Microhardness was checked under Vickers Microhardness (VHN) tester after samples were irrigated. Statistical Analysis of Variance and Tukey post-hoc test were used.

Results: Zones of inhibition were highest with NaOCl (7.66 mm). Irrigants penetration depth into dentinal tubules was significant with NaOCl with average value 0.35 mm. Microhardness of root dentin of saline was 145.90 VHN, followed by Neem 136.59 VHN which was then followed by Gau ark 123.05 VHN hence, Neem has lesser effect on microhardness value as compared to NaOCl and Gau ark. There was statistically significant difference among four groups regarding antimicrobial efficacy, depth of penetration into dentinal tubules and microhardness of root dentin.

Conclusion: Neem extract showed significant antimicrobial property as compared to Gau ark and normal saline, good penetration depth and less effect on microhardness of root dentin. Herbal irrigant like Neem extract can be used as a root canal irrigant.

Keywords: Brain heart infusion agar, Irrigation, Stereomicroscope, Vickers hardness test

INTRODUCTION

The success of endodontic treatment depends upon the elimination of microorganisms from the infected root canals. Most of the teeth undergo retreatment due to inefficient elimination of pathogens. *Enterococcus faecalis* (*E.faecalis*) is one of the most common organisms which can be cultured from the failed root canal treatment and considered to be the most resistant species to chemomechanical debridement. Haapasalo M et.al., showed that the bacteria present in the necrotic root canal can be found not only inside the root canal system but also in the dentinal tubules and lateral canals [1]. Chemomechanical preparation is very important in disinfection of root canals, but because of complexity it is very difficult to remove all the microorganisms. Along with instrumentation technology, irrigation plays an important role in removal of tissue remnants from the root canal system especially due to anatomical variations [1].

Sodium hypochlorite is the most widely used endodontic irrigant, because of properties like its unique ability to dissolve pulp tissue and an excellent antimicrobial activity [2]. Whereas, its disadvantages are, allergic reactions in susceptible patients, foul smell, bad taste and inability to remove smear layer and its cytotoxic effect, when it is penetrated into periapical tissues [3]. Hence, there is a need for new irrigating agents which will overcome these disadvantages.

Herbal medicines have been used as anti-inflammatory, analgesic and antibacterial agents with advantages that, they have few side effects, economical, better tolerated by patients and renewable in nature. Among various medicinal extracts studied, neem extract has shown promising results as an endodontic irrigant. Neem has several active constituents because it causes maximum reduction in adherence of *E.faecalis* to dentin [4].

Goumutra (Gau ark) is used in traditional Indian medicinal system and its combination with herbal formulations have been used to treat various diseases like infections, cancer, diabetes and diseases of immune system. Yadav H, et al., stated that Gau ark (cows urine) alone has antibacterial property against Gram-positive and Gram-negative bacteria [5].

However, bacteria can remain inside the canal after the cleaning and shaping of the root canal system, because the penetration depth of irrigating solutions into dentinal tubules is limited, thus may lead to secondary infections and endodontic failure [6]. Therefore, newer

irrigants are required to increase the penetration depth of irrigating solution into dentinal tubules.

It has been shown that irrigating solutions can change the micro hardness of root dentin and hence, can alter the clinical performance of endodontically treated teeth, which further weakens the tooth structure [7]. As NaOCl has the disadvantages of cytotoxicity and severe tissue reactions, the need arose to overcome it by exploring herbal irrigants which are 100% natural and has no side-effects. (3). Therefore, the aim of the study was to explore such herbal irrigants with much lesser side effects, as root canal irrigants in endodontic procedures.

MATERIALS AND METHODS

This is an in-vitro study conducted during August-October 2019 for a period of 3 months and was undertaken at Department of Pedodontics and Preventive Dentistry, School of Dental Sciences, Karad, Maharashtra. The study was approved by the Institutional Ethics Committee under the protocol number 0256/2017-2018. Randomised sampling technique was carried out for sample size determination for minimum number of petriplates and teeth. The minimum sample size in each group for each parameter was calculated with 95% confidence level and 95% power. So, the sample size for evaluation of antimicrobial efficacy were 10 petri plates and 80 single-rooted premolar teeth samples for evaluation of depth of penetration and microhardness.

Inclusion criteria: Human single rooted premolar teeth which were indicated for orthodontic extraction purpose were included in the study.

Exclusion criteria: Teeth with caries, enamel fracture and previously done root canal treatment/restoration were excluded.

The study was divided into three parameters.

Evaluation of Antimicrobial Efficacy

Gau ark was commercially available in the market. A 0.2 mL of Gau ark was used to check antimicrobial efficacy on BHI agar plates. The source is from company Patanjali. Patanjali is a leading herbal brand, which claims it to be purified so microbial contamination test was not done and was not photo-activated.

Preparation of neem leaf extracts

Fresh mature neem leaves (Azadirachta Indica) were collected from the medicinal garden. Preparation of neem leaf extract was done in Department of Pharmaceutics, Krishna Institute of Pharmacy, Karad. Twenty-five grams of neem leaves were used. Leaves were washed in sterilised distilled water, dried and weighed in a sterile disposable cup, and two grams of neem leaves were added to 50 mL of absolute ethanol. (Shree Maruti Chem Enterprise Private Limited).

This mixture was macerated for two minutes, to remove coarse residue and the extract was filtered ready stored in an airtight container [8].

Agar diffusion test

To check the antimicrobial efficacy of 3% NaOCl (Prime Dental), Neem leaf extract, Gau ark (Patanjali) and Normal saline; agar well diffusion test was performed. BHI broth was used to grow *E.faecalis* cultures overnight at 37°C on a rotary shaker (Geindus Orbital; 0-210 RPM oscillator orbital rotator, 7 Lb) at 150 rpm, bacterial growth was checked by changes in turbidity after 24 hours. *E.faecalis* (200 µL) were streaked on 10 different BHI agar plates wells of 6 mm diameter, in the agar plates with sterile border.

A 0.2 mL of 3% NaOCl, Neem leaf extract, Gau ark (each 10 mL of Gau ark contains 10 mL of distilled purified cow's urine) commercially available by Patanjali company and Normal saline, were added and the plates were incubated for 24 hours at 37°C in an incubator (Kriday scientific solutions). Plates were removed and zones of inhibition were recorded after 24 hours of incubation, the inhibition zones against *E. faecalis* were recorded using the digital Vernier caliper (SSU Silver calliper) scale. The unit of measurement was in millimeter.

Evaluation of Depth of Penetration into Dentinal Tubules

Sample preparation

Forty single-rooted teeth were selected, and stored in normal saline and disinfected by Chloramine T (HiMedia). Access opening was done and working length for each tooth specimen was determined with 10 no. K-file (Mani) manually. Biomechanical preparation was done by step-up method with #15 to #35 K-file. The working length was kept standardised to 12 mm for all specimens. Use of 1 mL of normal saline (Helios Pharmaceuticals Private Limited) as an irrigating solution was done in between each file, for removal of dentinal debris. Use of 0.5 gram of 17% EDTA (Prime dental) was done to remove the smear layer during biomechanical preparation. Nail varnish (Note Nail enamel) was applied on an outer surface of the root. All samples were immersed in Crystal violet (Anmol Laboratories private limited) dye for staining and incubated at 37°C for 12 hours [6]. Samples were then removed and rinsed under tap water; canals were dried with paper points (Waldent). Sample teeth were divided into four different groups:

Each specimen was irrigated with 5 mL of respective irrigant for five minutes while performing filing (#15 to #35 k file), followed by irrigation of canals with 1 mL of distilled water, to remove any residue of test irrigants. Smear layer removal was done with 17% EDTA, which was mentioned earlier.

Group 1: 3% NaOCl,

Group 2: Neem extract,

Group 3: Gau Ark,

Group 4: 0.9% Normal saline.

Each group contains 10 samples, so 50 mL of irrigant was used in each group with the help of needles and syringes while calculating the penetration depth and microhardness.

Following treatment, tooth specimen was decoronated and roots carefully splitted along the longitudinal axis using double-faced diamond disc (3MTM) to form root halves.

Image acquisition and data analysis

Root halves for all groups were examined under the stereomicroscope (Metlab equipment and engineering systems) at 100X magnification. The penetration depth into dentinal tubules was analysed using the in-built image analysis of software of stereomicroscope (Metlab equipment), appearing as horizontal line intensity profiles from root canal lumen towards the periphery. It is defined as the region devoid of crystal violet dye from the canal lumen of dentinal tubules, at coronal one third, middle one third and apical one third of root and their mean in millimetres [6].

Evaluation of Microhardness of Root Dentin

Preparation of dentin blocks

A total of 40 single-rooted teeth were used, access opening and biomechanical preparation were done till 35 no. K-file. Teeth were decoronated with a double-faced diamond disc, crown portion and apical one-third part of the teeth were discarded. Root portion of decoronated sample were used to prepare the dentin blocks of 2 mm which were sectioned horizontally under water cooling by using double-faced diamond disc. To maintain the 2 mm dentin disc, each block was embedded in acrylic resin (Dpi RR), taking care that acrylic should not flow inside the root canal and the specimens were polished with abrasive paper (matador abrasive papers 350, Germany) [9]. These dentin blocks were kept soaked in the airtight containers containing respective irrigant for five minutes. After five minutes dentinal blocks were checked for microhardness.

Microhardness test

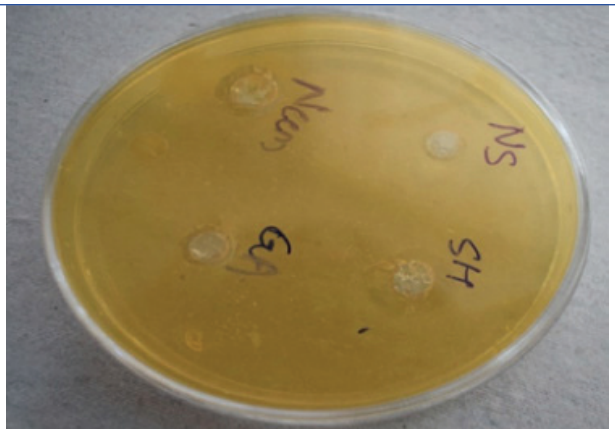
Microhardness was measured for each sample by Vickers hardness indentation machine (Mitutoyo HM-210, India), by the assigned, trained lab technician. Examiner caliberation was done by the Head of Mechanical Engineering Department of Rajarambapu Institute of Technology, Islampur. The indentations were made at three different points with a force of 200 gm for 15 seconds at 0.4 mm from root canal lumen and the mean value was calculated. The values were recorded in VHN unit and the data was recorded, tabulated and statistically analysed.

STATISTICAL ANALYSIS

The values obtained were tabulated and statistically analysed using Analysis of variance (ANOVA) test And Tukey Post-hoc test. Tukey Post-hoc test was applied to all the groups to counter check which group has significant results. All the tests were carried out using software SPSS for Windows (version 20.0). Descriptive statistics were expressed as mean±Standard Deviation (SD). The p-value was considered significant at (p<0.001).

RESULTS

One-way ANOVA test was used to find out the antimicrobial efficacy against *E.faecalis*, the depth of penetration into dentinal tubules and the effect on microhardness of root dentin when NaOCl, Neem extract, Gau ark, and Normal saline were used as root canal irrigants. Tukey post-hoc test was done to compare antimicrobial efficacy, the irrigants depth of penetration into dentinal tubules and their effect on microhardness of root dentin. It was analysed that there was statistically significant difference amongst all four groups with p<0.001. It was found that there was significant difference between all the irrigants with an average zone of inhibition of NaOCl against *E.faecalis* as 7.66 mm. [Table/Fig-1,2] followed by Neem 5.35 mm which was then followed by Gau ark 3.70 mm. The least zone of inhibition against *E.faecalis* was shown by Normal saline with a mean value of 0.04 mm. Tukey Post-hoc test concluded that NaOCL had the highest antimicrobial efficacy [Table/Fig-3], Neem had higher antimicrobial efficacy than Gau Ark. Depth of penetration into dentinal tubules of NaOCl was 0.35 mm [Table/Fig-4,5] followed by Neem 0.28 mm which was then followed by Gau ark 0.24 mm. The average depth of penetration into dentinal tubules for Normal saline was 0.04 mm. Tukey Post-hoc test confirmed that NaOCL had the highest penetration depth than Neem and Neem had more penetration depth than Gau-Ark [Table/Fig-6].



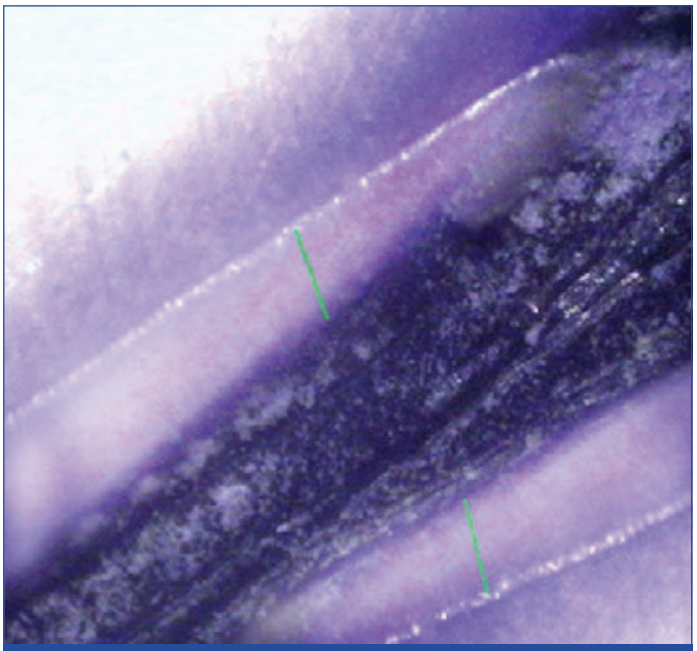
[Table/Fig-1]: Zone of inhibition against *Enterococcus Faecalis* (*E.Faecalis*) SH- Sodium hypochlorite, NS- Normal saline, GA- Gau ark.

Materials	Mean (mm)	STD (mm)	F-test	p-value
NaOCL	7.66	1.57	95.6049	**0.0001**
Neem	5.35	1.02		
Gau ark	3.7	0.89		
Saline	0.04	0.07		

[Table/Fig-2]: Descriptive statistics for mean Zone of inhibition (Antimicrobial efficacy) among four groups (ANOVA).
STD: Standard deviation; p-value <0.001 to be considered significant; n=10, for each group

Material	Group	T-diff	p-value
NaOCL	Neem	-2.310	**0.0001**
	Gau ark	-3.960	**0.0001**
	Saline	-7.6200	**0.0001**
Neem	Gau ark	-1.6500	0.0057
	Saline	-5.3100	**0.0001**
Gau ark	Saline	-3.660	**0.0001**

[Table/Fig-3]: Tukey Post-hoc Test for comparing the antimicrobial efficacy among four groups.



[Table/Fig-4]: Depth of penetration into dentinal tubules. Seen under stereomicroscope at 100X magnification. Green arrow indicates penetration depth of irrigant in millimeter, which is devoid of crystal violet dye.

Materials	Mean (mm)	STD (mm)	F-test	p-value
NaOCL	0.35	0.01	4727.6	**0.0001**
Neem	0.28	0.01		
Gau ark	0.24	0.01		
Saline	0.04	0.00		

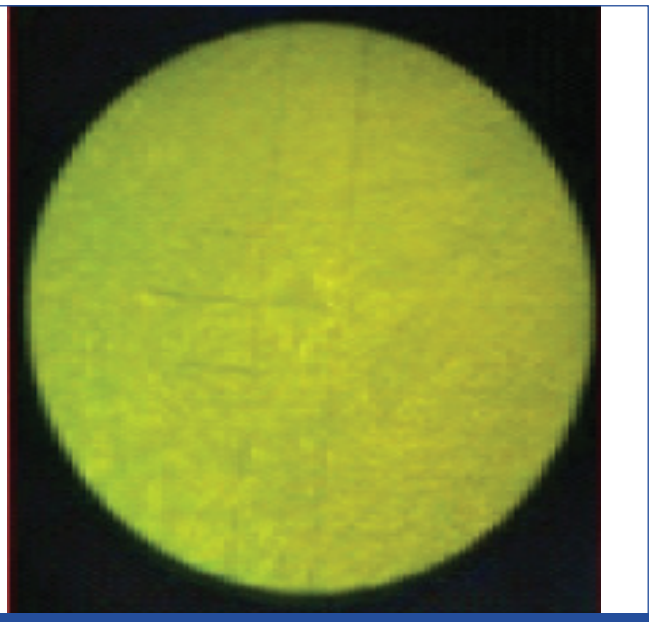
[Table/Fig-5]: Descriptive statistics for mean depth of penetration into dentinal tubules among four groups (ANOVA).
p-value <0.001 to be considered significant; STD: Standard deviation, n=10 for each group

Material	Group	T-diff	p-value
NaOCL	Neem	-0.0700	**0.0001**
	Gau ark	-0.110	0.4793
	Saline	-0.310	0.9375
Neem	Gau ark	-0.0400	**0.0001**
	Saline	-0.2400	0.9375
Gau ark	Saline	-0.2000	0.9375

[Table/Fig-6]: Tukey Post-hoc Test for comparing the depth of penetration among the groups.
p-value <0.001 to be considered significant

It was found that there was significant difference between all the root canal irrigants with an average effect on microhardness of root dentin with saline 145.90 VHN, followed by Neem 136.59 VHN which was then followed by Gau ark 123.05 VHN. NaOCl showed least average effect on microhardness of root dentin which was 80.05 VHN [Table/Fig-7,8]. NaOCL had the lowest VHN as compared to Saline and Neem extract [Table/Fig-9]. Neem extract showed higher microhardness value as compared

to NaOCl and Gau ark. That means when we use neem as an irrigant it has less tendency to reduce the microhardness value whereas NaOCl tends to reduce the microhardness, hence, it concluded that neem has least effect on microhardness value as compared to NaOCl and Gau ark.



[Table/Fig-7]: Microhardness tester indentation for Vickers microhardness test.

Materials	n	Mean (VHN)	STD	F-test	p-value
NaOCL	10	80.05	6.21	650.9931	**0.0001**
Neem	10	136.59	1.62		
Gau ark	10	123.05	2.30		
Saline	10	145.90	2.37		

[Table/Fig-8]: Descriptive statistics for mean Microhardness among four groups *Enterococcus Faecalis* (ANOVA).
STD: Standard deviation; n=10 for each group

Material	Group	T-diff	p-value
NaOCL	Neem	56.540	0.8919
	Gau ark	43.000	0.2785
	Saline	65.8500	0.9350
Neem	Gau ark	-13.540	0.0001
	Saline	9.3100	0.0001
Gau ark	Saline	22.8500	0.0001

[Table/Fig-9]: Tukey Post-hoc Test for comparing the hardness numbers among groups.

DISCUSSION

E.faecalis is one of the most resistant bacteria that is found inside the root canal causing reinfection of the root canal, thus eradication of microorganism from the root canal system is very important which depends on biomechanical preparation, irrigation and intracanal medicaments. Sodium hypochlorite is the most commonly used irrigant but due to its limitations as an endodontic irrigant, there is a need of agents that are both antibacterial and exerts minimal tissue irritating effects [1]. In recent years, attention has been diverted towards the search for the new novel compounds, due to the increasing trend of multidrug resistance. Therefore, this study has been very important which concentrated on newer antimicrobial compounds of plants and animal origin. Bohora A et al., compared the antibacterial efficacy of the neem medicaments, leaf extract and 2% NaOCl against *E. faecalis* and *C.albicans* and found that there was a significant difference between the zone of inhibition of neem leaf extract and the 2% NaOCl against *E. faecalis* [8].

As a part of the growing trend to seek natural remedies for dental treatment, Neem extract can be used as an endodontic irrigant. Antimicrobial activity of Gau ark (Cow urine) is well known therefore we used Gau ark as a root canal irrigant in the present study. In this study, it was revealed that the highest zone of inhibition was observed with group I (NaOCl) with a mean value of 7.6 mm. The reason could be, the presence of water containing active chlorine, hypochlorite acid is formed due to antimicrobial effect by irreversible oxidation of hydrosulphuric groups of essential enzymes, which disturbs the metabolic functions of the cell. Chlorine can also adhere to bacterial cytoplasm components forming highly toxic N-chloro composites that destroy the microorganisms thus, illustrate the bactericidal mode of action [10]. Previous study of Mustafa M showed that the highest antimicrobial efficacy was shown by 2% chlorhexidine followed by 3% NaOCl and Neem extract [11] which was in agreement with the present study. Group 2 (Neem extract) showed the next highest zone of inhibition with a mean value of 5.35 mm, this could be explained because Neem has several active constituents like nimbidin, nimbin etc., which are responsible for its antimicrobial action. The study done by Sundaram D et al., indicated that 5.25% NaOCl is more effective as root canal irrigant followed by the 100% neem leaf extract and then 40% honey [12]. Group 3 (Gau ark) showed a zone of inhibition with a mean value of 3.70 mm. Group 4 acted as a control. All the other three groups proved better antimicrobial products than the control group. Results of the present study illustrate that the use of 3% NaOCl as an irrigant provides more effective antimicrobial efficacy as compared to Neem extract, Gau ark and Normal saline.

Increasing the penetration depth of irrigation solutions inside dentinal tubules may be effective in promoting the success rate of endodontic treatment [13]. Hence, for deeply penetrated bacteria, an irrigant penetrating deep into the dentinal tubules will be beneficial. In this study, the average penetration depth of studied irrigant was different in coronal, middle and apical third. The highest penetration depth was seen in the NaOCl group. According to the results of the present study, the depth of irrigant penetration into dentinal tubules was highest with 3% NaOCl at the middle third.

In the present study, all the root canals were prepared to a size of 35, 2% taper to achieve a balance between the adequate volume of irrigant and preservation of radicular dentin. Removal of the smear layer is one of the important criteria for achieving better penetration of irrigants. Hence, 0.5 gram of 17% EDTA was used in the present study to remove the smear layer in all the samples to aid in deeper penetration of irrigant. Samples were immersed in crystal violet dye for staining purpose and to evaluate the penetration depth of irrigant. All the irrigants were used into the root canal, to simulate the clinical scenario. All the samples were sectioned and were seen under the stereomicroscope, typically using light reflected from the surface of an object rather than the one transmitted.

Apart from the benefits, irrigants may exhibit detrimental effects on root dentin. Saha SG et al., concluded that irrigating solutions can affect the microhardness of root dentin, that consequently affects the clinical performance of endodontically treated teeth. Although the reduced microhardness helps in instrumentation throughout the root canal, but it can make the root structure weak [7]. As it is more sensitive to measurement errors, less sensitive to surface conditions and small specimens can be tested with good accuracy, Vickers microhardness testing was employed, since action of irrigating solutions may lead to alteration in the physical and chemical properties of root canal dentin including hardness, hence the present study evaluated the effect of various endodontic irrigating solutions i.e., 3% NaOCl, Neem extract, Gau ark and Saline on the microhardness of root canal dentin.

The observations showed that Group 2 (Neem extract) had less effect on microhardness which was followed by Group 3 (Gau ark) which then followed by Group 1 (NaOCl). In the present study, 3% NaOCl was found to reduce the dentin microhardness. Neem has

less tendency to reduce the micro hardness, whereas NaOCl has more tendency to reduce the microhardness value. The reduction in the dentinal microhardness could be due to the lack of organic phase (type I collagen) of dentin caused by NaOCl. Kruzic JJ and Ritchie RO reported that the destruction of the collagen matrix in dental hard mineralised tissues leads to a substrate which is less tough, more brittle which precipitates fatigue crack propagation during cyclic stresses [14]. Dayal C et al., stated that instrumentation and irrigation with NaOCl changes the biomechanical properties of dentin [15].

In this study, root dentin disk of 2 mm was embedded in acrylic resin. Ground polishing of the samples removed surface irregularities and provided a mirror-like finish. This enables the reflection of light and clear visibility of the indentations when using the VHN testing machine [16]. The samples were immersed for five minutes and was kept the same for all the four groups.

There was a statistically significant difference among four groups i.e., NaOCl, Neem Extract, Gau ark and Normal Saline regarding antimicrobial efficacy, depth of penetration into dentinal tubules and microhardness of root dentin when used as root canal irrigants.

Limitation(s)

Firstly, smell and taste of herbal irrigants need to be improvised for better patient compliance. Also, the in-vitro studies have the limitation of the laboratory and clinical set-up errors like subjective or manual errors by the examiner.

CONCLUSION(S)

Herbal irrigant like Neem extract and Gau ark can be used as a root canal irrigant. Neem extract showed significant antimicrobial property as compared to Gau ark and normal saline, good penetration depth and less effect on reducing the microhardness of root dentin. The use of neem as an endodontic irrigant may be advantageous because neem is an excellent antioxidant with a very high biocompatibility and thus, there is no risk of tissue toxicity with its use. Therefore, further clinical studies are indicated to evaluate the efficacy of herbal irrigants as a root canal irrigant as they have no side effects.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: May 11, 2020
- Manual Googling: Aug 19, 2020
- iThenticate Software: Sep 29, 2020 (21%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

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