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Theobromine as a root canal irrigant – An in-vitro study

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ABSTRACT: Background: The key reason for failure of an endodontic treatment is incomplete biofilm removal. Some microbes like Enterococcus faecalis thrive in periapical lesions,in spite of the chemical and mechanical debridement of root canals triggering a failure of an endodontic treatment.Sodium hypochlorite is the most widely used irrigant in endodontic practice, but it has various disadvantages. Thus,considering the adverse effects and toxicity issues of synthetic medications, the herbal alternatives have been sought for endodontic irrigation.

Objectives: The aim of this study was to compare the antibacterial effect of Theobromine, Neem, Tulsi extract, Gow-Arkaand sodium hypochlorite (NaOCl) against Enterococcus faecalis as an intracanalirrigant.

Materials and Methods:A preliminary agar well diffusion test was done to determine the antibacterial activity of Theobromine, Neem and Tulsi at different concentration (25%,50%,75% and 100%) along with NaOCl and Gow-Arka. The prepared plant extracts (50 & 100 µl) were incorporated into the wells and the plates containing E. faecalis were incubated at 37 °C for 24 h and Zone of Inhibition (ZOI) was recorded in each plate.Twenty-five recently extracted human permanent single-rooted, fully formed teeth without dental caries, root fractures and root resorption were irrigated with Theobromine, Neem, Tulsi, NaOCl (positive control) and distilled water (negative control) by pour plate method and the colony forming units were calculated from each plates.

Statistical analysis: The obtained data were tabulated and subjected to statistical analysis using one-way ANOVA test and post hoc-Tukey test.

Results: Among the herbal experimental groups, Theobromine and Neem had the most effective antibacterial effect against E.faecalis. No statistical significant difference was detected between Theobromine and Neem extract when compared against NaOCl as an irrigant.

Conclusion: Even though NaOCl remains the gold standard as an irrigant, there are some disadvantages such as high toxicity and inability to remove smear layer from dentin. Hence, researchers are focusing on newer natural alternative irrigating solution and Theobromine can be considered as a safe and natural alternative endodontic irrigant.

Keywords: Antimicrobial activity, Enterococcus faecalis, herbal extracts.

I. INTRODUCTION:

Primary endodontic infections are caused by oral microorganisms, which are usually opportunistic pathogens that may invade a root canal containing necrotic tissue and establish an infectious process [1]. The number of facultative anaerobic bacteria increases when root canal remains infected for long periods [2].

Enterococcus faecalis, a facultative anaerobic gram-positive coccus, is the most common species cultured from nonhealing endodontic cases [3,4]. This microorganism can even survive in an environment with scant available nutrients and its mode of growth is through the formation of a biofilm, [5]an adaptive process that enables the microorganism to endure in severely harsh conditions like obturated root canals [6]. E. faecalis that invades the dentinal tubules [7] may survive chemo-mechanical instrumentation and intracanal medication. [8]

For many years, intracanalirrigants have been used as an adjunct to enhance the antimicrobial effect of cleaning and shaping in endodontics and the goal of endodontic therapy is the removal of all vital or necrotic tissue, microorganisms, and microbial by-products from the root canal system[9]. Instrumentation of the root canal system should be supported by irrigation, which is capable of removing necrotic debris, as well as microorganisms and without irrigation, accumulation of this debris makes biomechanical preparation ineffective[10].

Sodium hypochlorite (NaOCl) has become the most popular agent for endodontic irrigation even though its optimum working concentration has not been universally agreed[11]. It is a strong proteolytic substance and provides sufficient antimicrobial effect. But, adverse effects of NaOCl have been reported including unpleasant odour and taste, toxicity, possible paraesthesia of the mandibular nerve, allergy, and an increase in

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coronal micro leakage of adhesive restorations[12] [13].

Constant increase in antibiotic resistance and side effects caused by synthetic irrigantshave shifted the research towards developing herbal alternatives. Several natural extracts have been evaluated for endodontic purpose, with conflicting results.[14]

In the ancient science of Ayurveda, many herbs with potent therapeutic effects including antibacterial, antifungal, analgesic and anti-inflammatory properties have been demonstrated such as Neem (Azadirachtaindica)[15],Theobromine cacoa(3,7-dimethylxanthine)[16],Tulsi (Ocimum sanctum L)[17] and Gow-Arka[18].

Theobromine (Cocoa pod husk) is a natural ingredient that can be processed as an alternative material to replace sodium hypochlorite and chlorhexidine gluconate. Phytochemical analysis on chocolate fruit peel extract shows many active compounds, such as alkaloid, tannin, saponin, terpenoid, and flavonoid that have antibacterial activity and also rich source in copper, sulphur and vitamin C.[16]

Neem (Azadirachtaindica, A. Juss) is a tree which belongs to the Meliaceae family and is considered to be a holy medicinal tree found in India. It possesses a wide range of biological activities, such as anti-inflammatory, antimicrobial, antifungal, antioxidant, analgesic.Neemhas several active constituents like nimbidin, nimbin, nimbolide, and cyclictrisulfide which are responsible for its antibacterial action.[19]

Tulsi (Ocimum sanctum) is a holy plant of Indian origin. It is known as the mother medicine of nature. It is an easily available and economical material without side effects. It has antimicrobial properties and is most commonly used for treating variety of diseases such as arthritis, bronchitis, diabetes, and skin diseases.Antimicrobial activity of Tulsi is due to its constituents, ursolic acid and carvacrol.[20]

From the ancient period cow's urine has been used as a medicine. In India, drinking of cow urine hasbeen practiced for thousands of years. The Gow-Arka has been patented as activity enhancer and availability facilitator for bioactive molecules including anti- infective and anti-cancer agents (US Patent No 6410 059/2002). [21]

Thus, considering the beneficial effects ofPhytotherapy,the present in-vitrostudy was undertaken to evaluate the antimicrobial efficacy of Neem, Theobromine, Tulsi and Gow-Arka at various concentrations against E. faecalis in comparison to NaOCl.

II. MATERIAL AND METHODS:

Preparation of herbal extract (introduction)

In vitro microbiological study was conducted where Neem, Tulsi, Theobromine were taken in different concentrations (ie 25%, 50%, 75% &100%), along with Gow-Arka& Sodium hypochlorite to assess their antibacterial property against E-Faecalis in two loading quantities ie 50µL and 100µL

Cocoa bean husk was purchased from (The Campco, Ltd., Karnataka). whereasTulsi & Neem leaves were obtained freshly from local garden and washed in clear water. All the three samples were dried for 7 days and ground until a homogenous powder was obtained.

Ethanolic extract was prepared from the powder obtained using "cold extraction method." A total of 250 g of finely powdered leaf extract was macerated with 100% ethanol for 3 days. The alcoholic decoction was subjected to filtration with Whatman #1 filter paper to obtain a clear filtrate. The filtrate thus obtained was reduced at a low temperature of <60°C to obtain a solid residue and stored in refrigerator till further use. From the 250 g of powder dissolved in 1 L of ethanol, approximately 18 g of solid residue (extract) was obtained. Gow-Arka was purchased from an Ayurveda store in its pure distillate form for the study. (Figure-1)

The present study was conducted in two stages:

1. Well diffusion method to check the Antibacterial Activity against E-Feacalis:

Culture preparation

Luria Bertani (LB) broth was prepared by adding Tryptone 0.3g, Sodium chloride 0.3g, Yeast extract 0.18g, distilled water 30mL and autoclaved at 121°C for 15 mins.E. faecalis (ATCC 29212) was inoculated in 30mL of sterilized Luria Bertani (LB) broth and incubated at 37° C for 24h.

Sample preparation

50µL and 100µL of the sample were directly used to check the inhibitory concentration of E-Faecalis.

Plate preparation

Approximately 40mL of LB agar media was poured into the sterilized petriplates and allowed it to solidify, later 24h cultured 200µL inoculum of E-Faecalis was added on the agar plates respectively and spread throughout the plate using spreader.Two wells measuring 0.5cm was made using the well borer in respective plates, 50µL and 100µL of sample were loaded into the respective wells and incubated at 37°C for 24hrs. (Figure-2)

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2. Determination the CFU:

Twenty-fiverecently extracted human permanent single-rooted, fully formedteeth without dental caries, root fractures and root resorption were selected for the study. The teeth were cleaned of superficial debris and tissue tags and is stored in normal saline to prevent dehydration. Each tooth was radiographically assessed to confirm the presence of a single patent canal. The root canals were then instrumented using the crown-down technique with rotary instrument (ProTaper), and the canals were enlarged to an apical size F3 ProTaper. 2ml of 3% NaOCl was used between each instrument during the procedure, which is followed by a final rinse with 17% EDTA. All the specimens were sterilized by autoclaving and stored aseptically until use. allthe teeth were taken in a sterile tube respectively and were inoculated with 200 µL of E. faecalis and incubated at 37˚ C for 48hours.

Specimen preparation

The teeth were then divided intofive groups, according to the irrigant used.

Group 1: Negative control- distilled water (n = 5)

Group 2: Positive control - 5.25% NaOCl (n = 5)

Group 3: Neem extract 100%(n=5)

Group 4: Tulsi extract 100% (n=5)

Group 5: Theobromine extract 100% (n=5)

The infected specimens were washed with 2 mL of PBS (Di-sodium hydrogen phosphate 1.44g, Potassium dihydrogen phosphate 0.24, Sodium chloride 8g, Potassium chloride 0.2g, and Distilled water 1000mL, pH 7). 200µL of the respective irrigant were introduced into the root canal and incubated for 24 hours at 37˚C. After 24hours, 200µL of irrigant sample was reloaded into the specimens and incubated for another 24 hours at 37˚C. 200µL of PBS was poured and sonicated for 5mins. 100µL of suspension was plated (pour plate method) respectively, the plates were incubated at 37˚ C for 48hours. Later, the colony forming units were calculated from each plates.(Figure-3)

III. RESULTS:

The present study showed that the antimicrobial activity of herbal irrigants was greater in 100µL loading dose than in 50µL loading dose.

Table-1represents the zone of inhibition of Theobromine, Neem, Tulsi at various concentration along with NaOCl and Gow-Arka. NaOCl (23.5+/-1.5) had the maximum zoneof inhibitionfollowed by Neem 100% extract (23+/0), Theobromine 100% extract (21.5+/-0.5) and Tulsi extract (19.5+/-0.5). However, no zone of inhibition was showed by Gow-Arka.

Intergroup comparison was done using Tukeys Post Hoc test, revealed that the mean ZOI of N75%, N100%, TB100%at 100µL loading had no statistical significant difference with sodium hypochlorite as shown intable-2

Table-3showedthat the mean CFU in Negative control (1635.2±644.2) was found to be highest following Tulsi extract (212.8±10.8), Theobromine (98.8±9.0), Neem (98.0±10.8) and Positive control (43.2 ±11.6)which had least CFU and the difference was found to be statistically significant ($p \leq 0.0001$).

Table-4 revealed the intergroupcomparisons, which showed that mean CFU of Negative control had significant difference ($p \leq 0.001$*) with Positive control, Neem, Theobromine and Tulsi and thus, the study concluded that there was no significant difference between herbal irrigants when compared against sodium hypochlorite.

IV. DISCUSSION:

The root canal of infected teeth is the harbour of various colonies of micro-organisms[22]. Out of all the inhabitant species, E. fecalis is the major cause of infection and reinfection of the root canal.The persistence of E. faecalis in treated root canals has been attributed to its ability to resist the high pH of the antimicrobial agents used during the root canal treatment [23].E. faecalis has been demonstrated to synthesize a variety of stress proteins when exposed to acids and alkali. Acid resistance to E. faecalis is the result of activity of the cell membrane-bound proton-translocating ATPase, which maintains pH by excreting protons from the cells [24]. Elimination of E. fecalis from the root canal has always been a challenge for complete disinfection of the root canal due to its deeper penetration in the dentinal tubules[8].

The primary objective of root canal treatment is complete disinfection of root canals and elimination of microbes. [25] The biomechanical preparation involves instrumentation which aids in the removal of infected dentin and use of irrigants to aid in complete removal of debris and disinfection. [26]The efficacy of irrigation depends on the ability to bring the irrigant in contact with those elements, materials and structures within the canal system that have to be removed.[27] Root

canal irrigants ideally should have a broad antimicrobial spectrum, especially against anaerobic and facultative microorganisms. [28]

Even though, NaOCl is used as the popular irrigant till date, it has limited use in paediatric dentistry due to its strong proteolytic potential which leads to sufficient tissue lysis. [29]The use of higher concentrations increases its ability to dissolve the necrotic tissue and shorten the time needed for the inhibition of bacterial growth, but it causes damage to periapical tissues. [30]The main disadvantages of NaOCl are high toxicity, corrosive to instruments, inability to remove smear layer and reduction in elastic modulus, and flexural strength of dentin,[31]Hence, researchers are focusing on newer natural alternative irrigating solution.

Indian traditional medicine was dependent on herbs and herbal products, and recently it has gained importance. The major advantages of herbal alternatives are easily available, inexpensive, less side-effects and lack of microbial resistance.[32] Several natural extracts with proven antimicrobial efficacy against E. faecalis liketriphala[12], green tea polyphenols,[33] liquorice[34]etc. have been tested as endodontic irrigants, with conflicting results. Also, literature shows that many herbal extracts in cow urine distillates have been found to be effective as antimicrobials.[35]

In the present study, three potent antimicrobial herbs were used in four concentrations (25%, 50%,75% and 100%) to be tested as a root canal irrigant against E. faecalis by agar-diffusion method in 50µL and 100µL loading doses.The antimicrobial efficacy of any natural or synthetic agent can be evaluated using broth dilution method, agar dilution method, disc diffusion method, agar well-diffusion method, and ditch-plate method. However, agar well-diffusion method was used in the present study as it depends on the diffusion of the tested material to such an extent that growth of the added microorganism is prevented entirely in a zone around the hole containing the test material. [36]

The antimicrobial efficacy of all the irrigants were assessed in 50µL and 100µL loading doses and the current study revealed that the ZOI was greater among 100µL loading dose. In our study, the mean zone of inhibition of Neem 100% (23mm) and Theobromine (21.5+/- 0.5mm) was almost equivalent to the ZOI of sodium hypochlorite (23.5mm).

Neem exhibits a significant antibacterial activity against both Gram-positive and Gram-negative bacterial species. [37] The presence of active constituents such as nimbidin, nimbin, nimbolide, gedunin, azadirachtinand cyclic trisulfide contributes to the antibacterial activity of neem. These active constituents uncouple mitochondrial oxidative phosphorylation, thus inhibiting the respiratory chain. This resulted in its anti-adherence activity by altering the bacterial adhesion and the ability of the microorganism to colonize thereby causing maximum reduction in adherence of E.faecalis to dentin.[38]Besides the antimicrobial action, this group of compounds also demonstrates anti-inflammatory function (ability to prevent the production of prostaglandins, especially prostaglandin E1 and 5-hydroxytryptamine) which is a desirable characteristic of the irrigant. [39]. The results of our antimicrobial research is similar to the study results by Pulipparambil et al [40] where a comparative study exhibited the significant result of Neem extract to E. Faecalis and the ZOI similar to NaOCl. Also, other studies byVinothkumaret al[41], Dutta and Kundabala[15], Babaji et al. [42]have found comparable effectiveness of neem irrigant with sodium hypochlorite irrigation.

Flavonoid is an antibiofilm material contained in the cocoa bean pod husk (Theobromine) extract responsible for the inhibition of E. faecalis biofilm formation in the dentinal walls.[16] Wai-Leung &Bassler[43] and Li & Tian [44] stated that flavone can act as an inhibitor of biofilm formation., by interfering with signalling quorum sensing pathway. Other study by Yunita et al [16] showed that theobromine eliminatesE.faecalisby inhibiting glucosyltransferase enzyme activity which decreasesglucan formation, resulting in reduced biofilm formation of E. faecalis.However not many literatures are present comparing theobromine as a potential root canal irrigant.

The antimicrobial activity of tulsi can be attributed to essential oils like eugenol, chavicollinalol, carvacrol. Eugenol (1-hydroxyl-2 methoxy– 4 allybenzene), is the active constituent present is largely responsible for therapeutic potential of tulsi.[45]Prabhakar et al [13] and Shenoy et al[46] showed that Tulsihad a better antimicrobial efficacy against periapical pathogens isolated from the root canals of primary molars when compared against conventional irrigants. Similarly, our study also showed that Tulsi possessed antimicrobial activity against E-faecalis with ZOI (19.5+/-0.5).

Gow-Arka showed no inhibition in our present study which is controversial to studies done by Kumar et al[47], Anami J[48], Edwin J[49]and Fariha P[50] et al who showed antimicrobial activity against common endodontic pathogens.

CFU were calculated after irrigation with various irrigants [Neem, Theobromine,Tulsi,

NaOCl (positive control) and distilled water (negative control)] on E.faecalis.The mean bacterial counts were least in NaOCl irrigated group (43.2+/-11.6) followed by Neem 100% extract (98+/-10.8) < Theobromine 100% extract (98.8+/-9) < Tulsi 100% extract (21.28+/-37.1) < distilled water (1653.5 +/- 644.2). These results are in accordance with Bohra et al.[51], Nayak et al.[52], Chandrappa et al.[21], Sundaram et al.[53] and Afzal et al.[54] who concluded that the herbal irrigants showed antibacterial efficacy against conventional irrigants and suggested its potential role in the future of endodontics.

This study also showed that the antimicrobial action of Neem, Theobromine and Tulsiwas higher than the negative control group and did not possess any significant difference when compared against sodium hypochlorite. The results of this study concludes that antimicrobial activity of Theobromine and Neem were equivalent to sodium hypochlorite.

Theobromine, due to its bioactive and antimicrobial compounds, such as alkaloids, flavonoids, polyphenols, and tannins can be used as an endonticirrigant. It is non-toxic, non irritatnt and non-carcinogenic on pdl cells.[55] Hence, Theobromine can be considered as a safe and natural alternative in the treatment of endodontic diseases and could serve as lead compound for the development of novel irrigants and medicaments.

V. CONCLUSION:

The use of Phytotherapycontinues to expand rapidly across the world as they areeffective as well as biocompatible to human tissues. It has advantages of ease of availability, reduced cost, reduced side effects and antimicrobial resistance when compared to conventional counterparts. This in-vitro study concludes that antimicrobial property of Neem and Theobromine in 100% concentration are equivalent to NaOCl and did not possess any statistically significant difference (p=0.05) thus,they have a potential to be used as an herbal alternative in root canal irrigation. However, future in-vivo research on these extracts should be aimed in the direction of finding its efficacy against various endodontic biofilm.

TABLES:

Table 1: Comparison of Mean Zone of Inhibition using one-way ANOVA (100 µL)

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum	p
				Lower Bound	Upper Bound			
N 25%	3	11.5	0.5	10.2579	12.7421	11	12	
N 50%	3	16.8	0.7	14.936	18.7306	16	17.5	
N 75%	3	21	0	21	21	21	21	
N 100%	3	23	0	23	23	23	23	
T 25%	3	0	0	0	0	0	0	
T 50%	3	11.5	0.5	10.2579	12.7421	11	12	
T 75%	3	17.5	0.5	16.2579	18.7421	17	18	
T 100%	3	19.5	0.5	18.2579	20.7421	19	20	
TB 25%	3	10.5	0.5	9.2579	11.7421	10	11	
TB 50%	3	15.5	0.5	14.2579	16.7421	15	16	
TB 75%	3	19.5	0.5	18.2579	20.7421	19	20	0.0001*
TB 100%	3	21.5	0.5	20.2579	22.7421	21	22	
Go. Arka	3	0	0	0	0	0	0	
Sodium hypochlorite	3	23.5	1.5	19.7738	27.2262	22	25	

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Table 2: Tukey Post Hoc pair wise comparison test (100 µL)

					95% Confidence Interval	
(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
N 75%	N 100%	-2.00000*	0.46348	0.011	-3.7079	-0.2921
	T 100%	1.5	0.46348	0.133	-0.2079	3.2079
	TB 75%	1.5	0.46348	0.133	-0.2079	3.2079
	TB 100%	-0.5	0.46348	0.998	-2.2079	1.2079
	Sodium hypochlorite	-2.50000*	0.46348	0.001	-4.2079	-0.7921
N 100%	TB 100%	1.5	0.46348	0.133	-0.2079	3.2079
	Sodium hypochlorite	-0.5	0.46348	0.998	-2.2079	1.2079
TB 75%	TB 100%	-2.00000*	0.46348	0.011	-3.7079	-0.2921
TB 100%	Sodium hypochlorite	-2.00000*	0.46348	0.011	-3.7079	-0.2921

Table 3: Comparison of Mean CFU using ANOVA

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum	p
				Lower Bound	Upper Bound			
Negative Control	5	1635.2	644.2	835.2	2435.1	872	2164	0.0001*
Positive Control	5	43.2	11.6	28.7	57.6	30	56	
Neem extract	5	98	10.8	84.5	111.4	82	112	
Tulsi extract	5	212.8	37.1	166.6	258.9	164	252	
Theobromine extract	5	98.8	9	87.6	109.9	86	110	

Table 4: Tukey Post Hoc pair wise comparison test

(I) Grp2	(J) Grp2	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Negative Control	Positive control	1592	0.00*	1045.56	2138.44
	Neem	1537.2	0.00*	990.76	2083.64
	Tulasi	1422.4	0.00*	875.96	1968.84
	Theobromine	1536.4	0.00*	989.96	2082.84
Positive control	Neem	-54.8	1	-601.24	491.64
	Tulasi	-169.6	0.88	-716.04	376.84
	Theobromine	-55.6	1	-602.04	490.84
Neem	Tulasi	-114.8	0.97	-661.24	431.64
	Theobromine	-0.8	1	-547.24	545.64
Tulasi	Theobromine	114	0.97	-432.44	660.44

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FIGURES:

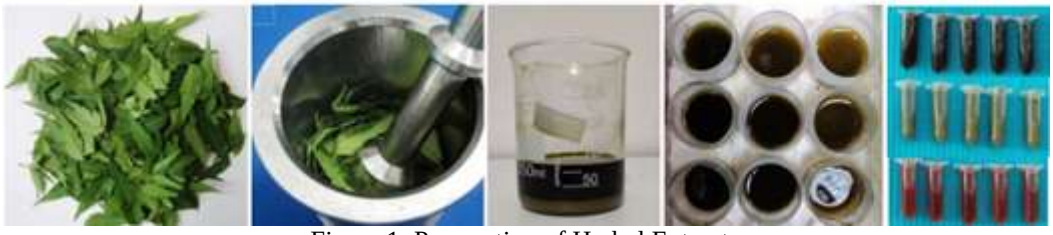

Figure 1: Preparation of Herbal Extracts

Figure 2: ZOI of Herbal extracts, NaOCl and Gow-Arka

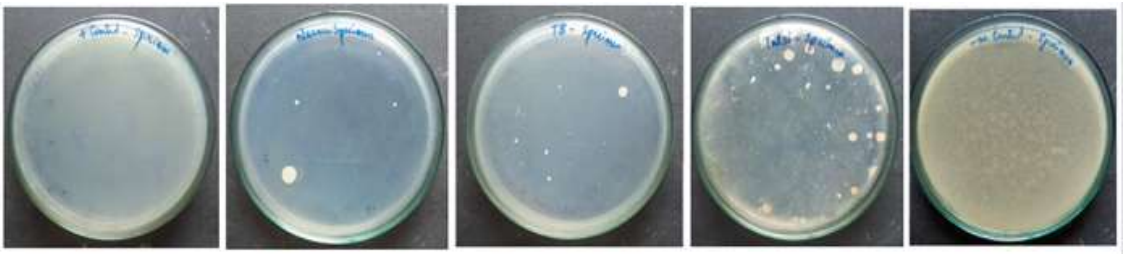

Figure 3: Colony forming units (CFU) of NaOCl, Neem, Theobromine, Tulsi and Distilled water