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Research Article

COMPARISION OF ANTIMICROBIAL EFFICACY OF TRIPHALA, *WITHANIA SOMNIFERA* AND SODIUM HYPOCHLORITE AGAINST *ENTEROCOCCUS FAECALIS* BIOFILM-AN INVITRO STUDY

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ABSTRACT

Objectives: To compare the antimicrobial efficacy of sodium hypochlorite, Triphala, *Withania somnifera*, combination of Triphala & Withania somnifera against *Enterococcus faecalis (E faecalis)* biofilm in extracted human teeth.

Methods: Total of 40 human extracted teeth were collected & 10 were assigned under 4 groups (n=10 in each group). *E. faecalis* was cultured, inoculated in Brain Heart Infusion medium & incubated for 45days along with the teeth for biofilm formation. After incubation for 45days, teeth were subjected to irrigation. They were irrigated with sodium hypochlorite (Group I), Triphala (Group II), *Withania somnifera* (Group III) and Triphala + *Withania somnifera* (Group IV). After the irrigation, teeth were analyzed for *E faecalis* colony forming units. 2 teeth from each group were subjected for qualitative observation under laser scanning confocal microscopy.

Results: Teeth irrigated with sodium hypochlorite showed mean *E faecalis* colony count of 3.6± 0.193 lakhs which is significantly less when compared to colony counts in Group II and Group III which were 4.6±.003 (P<0.001) & 3.9±0.004 (P<0.001) lakhs respectively. However, combination of Triphala+*Withania somnifera* group showed mean *E faecalis* colony count of 3.7±0.004 lakhs which was not significantly different from that of group I. All the 4 experimental groups showed marked decrease in the *E faecalis* colony count when compared to the initial colony count of 10 ± 0.21 lakhs.

Conclusion: Sodium hypochlorite, Triphala and *Withania somnifera* failed to eliminate bacteria completely. But, considerable reduction in growth of *E. faecalis* was seen in herbal extract groups. Considering the non-toxic nature and other physiological benefits of these herbal extracts, further studies need to be carried out to consider them as an alternative for sodium hypochlorite at least in the initial stages of bacterial infection.

Keywords: herbal extracts, *Enterococcus faecalis,* Triphala, *Withania somnifera,* Sodium hypochlorite, Antimicrobial agents, Biofilm, Irrigants.

INTRODUCTION

Persistance of micro-organisms within the root canal system progresses to the development of periradicular disease and thus is the cause for failure of endodontically treated teeth [1]. It has been shown that *Enterococcus faecalis* (*E. faecalis*) is a predominant organism found in previously treated root canals [2]. The bacterium also has the ability to adapt to environmental changes and remain as pathogen in the root canal, which makes its elimination very difficult [3]. *E. faecalis* has the capacity to produce biofilms where they aggregate and co-aggregate with other micro-organisms and are embedded in extracellular matrix [4].

The biofilms are formed when any surface comes in contact with the natural liquid. The stages in the formation of biofilm include; 1) formation of conditioning film, 2) adhesion of the planktonic bacteria to the surface and stage of detachment [5]. The latter stage has been shown to be the cause of infection [6].

Within the root canal system, *E. faecalis* may undergo starvation, utilize serum as nutritional source through periodontal ligament and alveolar bone, thus makes it difficult to eradicate [7]. Bacterial cells within the biofilm develop resistance 1,500 times more than the planktonic form [8].

Mechanical preparation of the root canal system along with chemical irrigation is essential for success of the root canal treatment [9]. The irrigants must possess antibacterial property, should dissolve the necrotic tissue, and they should have low surface tension, substantivity, lubrication, harmless effect on microhardness and roughness of root canal dentin [10].

Dunavant et al. have showed that concentrations of 1% and 6% sodium hypochlorite were the most effective irrigant against *E faecalis* biofilm [11]. *E. faecalis* in starvation phase could produce biofilm with reduced efficiency when compared to that of exponential and stationary phases [12]. The starved cells possess more resistance to sodium hypochlorite [12].

Triphala is an ayurvedic herbal formulation consisting of dried and powdered fruits of three medicinal plants; *Terminalia bellerica*, *Terminalia chebula*, and *Emblica officinalis* [13]. It mainly consists of citric acid and has been found to be an antimicrobial agent [14]. *Withania somnifera* [Ashwaghandha, (Solanaceae)] is a medicinal plant, widely used as a home remedy for several diseases [15, 16]. *Withania somnifera* has been shown to have antimicrobial properties [17].

It exhibits little or no toxicity to cells. Antimicrobial efficacy of Withania somnifera has been proved by reducing bacterial loads in vital organ of animals [18]. However, there is no literature of antibacterial efficacy of Withania somnifera against E.faecalis. The aim of the study was to compare the antimicrobial efficacy of Withania somnifera, Triphala, combination of these two with the standard irrigant sodium hypochlorite against E. faecalis biofilm.

MATERIALS AND METHODS

The study was approved by the ethical committee of Manipal University. Forty extracted single rooted human mandibular premolars were collected from the dental clinics of Manipal College of Dental Sciences. Teeth were cleaned ultrasonically and were then decoronated at cement- enamel junction with the help of diamond disc. Teeth were catogarised into 4 groups; Group I; Sodium hypochlorite group (n=10), Group II; Triphal group (n=10), Group III; *Withania somnifera* group (n=10) and Group IV; Triphal + *Withania somnifera* group (n=10).

The teeth from all the groups were dipped in sodium hypochlorite for one minute and washed with saline. Following this, working length was determined and canals were enlarged upto F2 size protaper file (Dentsply, USA), followed by irrigation with EDTA for 1min to remove the smear layer produced during the procedure. The teeth were autoclaved at 121^{0}C for 15minutes.

Bacterial inoculation

All the teeth were suspended in separate eppendorf tubes containing nutrient rich Brain Heart Infusion broth as medium (BHI). ATCC (American type cell culture) strain of E. faecalis (29112) was cultured and was innoculated into the Eppendorf tube containing the blocks. It was adjusted to contain $1x10^6$ CFU (Colony Forming Units) /ml, corresponding to 0.5 McFerlands tube. This suspension was incubated at 370ºC in air orbital shaker at 100 rotations / min (MTS 2, IKA, Staufen, Germany). Teeth within the BHI broth were kept in the orbital shaker for12 hrs. The teeth were innoculated with bacteria and incubated for 45 days. Optical density of the test agents with bacteria with distilled water as medium was measured at 405nm using photospectrometer (Bio-Rad laboratories, India) .All the teeth were then subjected to the irrigation with irrigants under four groups. Group I was irrigated with 5.25% of sodium hypochlorite and was considered as control group. Group II was irrigated with 80mg/ml of Triphala extract dissolved in distilled water (Manufactured by Manipal Pharmacy). Group III was irrigated with 80mg/ml *Withania somnifera* dissolved in dimethyl sulphoxide (supplied by Natural Remedies, Bangalore). As *Withania somnifera* was being used as irrigant in endodontics for the first time, agar diffusion test was performed to observe zone of inhibition and was found to be 3cm.

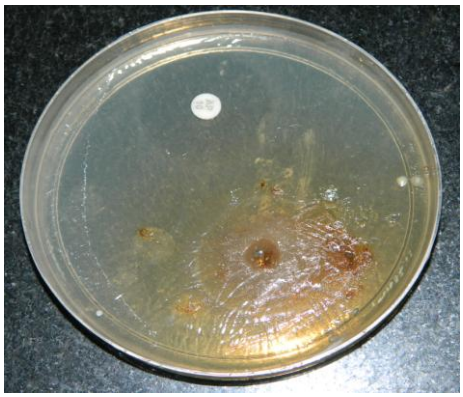

Fig. 1: Agar diffusion test result to observe the zone of inhibition for *Withania somnifera* which is 3cm.

Group IV was first irrigated with 80mg/ml of Triphala followed irrigation with by 80mg/ml *Withania somnifera*.

In all the groups, the irrigation was done 3times continuously without any time interval using 5ml syringe. Following this, the 8 teeth from each group were washed with phosphate buffered saline and dentinal shavings were collected. Colony forming units of viable cells were determined on BHI agar plates in triplicates in 1:10 dilution (phosphate buffered saline).

BHI plates were incubated in 5% CO_2 at 37ºC in carbon dioxide incubator and the number of bacterial colony forming units was counted at 24hrs. Plates containing 30 and 300 bacterial colonies were used preferentially for data analysis.

Optical density measurement

Optical density is the optical thickness which measures the total light blocking power of a certain medium with certain thickness. The effect of drugs against bacterial multiplication can be measured by optical density. If the optical density increases, the drug is not effective. The optical density of different irrigating solution with bacteria using distilled water as medium was measured at 405nm using photospectrometer (Bio-Rad laboratories India)

Tooth preparation for laser scanning confocal microscopic observation (LSCM)

Two teeth from each group were selected for laser scanning confocal microscopic observation. Longitudinal grooves were made on selected teeth before the inoculation of *E faecalis* and were washed with phosphate buffered saline and split opened. They were then fixed with 4% glutaraldehyde for 8hrs at 4 to 6ºC. Teeth were dehydrated with ascending concentrations of ethanol (30%, 50%, 70%, 90%, 100%). Teeth were stained with acridine orange before observation. The observation was at a magnification of 400X on LSCM (Carl Zeiss, Germany). The photomicrographs were captured and qualitative assessment of the density of *E faecalis* was performed by comparing between different groups.

Statistical Analysis

Statistical analysis was performed by using SPSS software version 16.0 (USA). One-way ANOVA was applied to analyze the data and Bonferroni's post hoc test was used to see the significance between different groups.

RESULTS

Colony forming units

In the present study, teeth of all the groups were inoculated with about 10^6 or 10 ± 0.21 lakhs of *E faecalis* colonies.

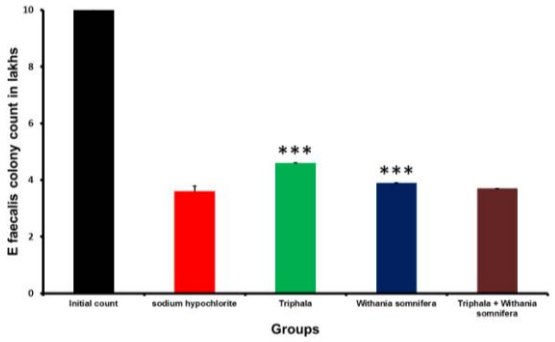

Fig. 2: *Enterococcus faecalis* colony counts after irrigating with different antimicrobial agents.

Group I: Teeth treated with sodium hypochlorite showed mean colony counts of 3.6 ± 0.198 lakhs, which is considered as control group. (Figure 2)

Group II: Teeth irrigated with Triphala showed mean colony count of 4.6 ±0.003 lakhs which is significantly (P<0.001), more when compared to the sodium hypochlorite irrigated group. (Figure 2)

Group III: Teeth irrigated with *Withania somnifera* showed mean colony count of 4.1 ± 0.004 lakhs which is significantly (P<0.001), more when compared to the sodium hypochlorite irrigated group. (Figure 2)

Group IV: Teeth irrigated with combination of Triphala and *Withania somnifera* showed no significant difference in mean colony count when compared to the sodium hypochlorite irrigated group (3.70 ± 0.004 lakhs).

Optical density reading

Optical density reading showed decline in growth curve when *E faecalis* was treated with sodium hypochlorite. Reduced bacterial growth was also seen when *E faecalis* is treated with combination of *Withania somnifera* and Triphala. A slight decrease in bacterial count was also observed when *E faecalis* was treated with *Withania somnifera* when compared to that of Triphala.

Laser Confocal Scanning Microscope (LCSM) Observation

LCSM photomicrographs of two samples from each group were taken. An untreated sample was also taken and observed for *E faecalis* biofilm, which revealed 80µm thick biofilm formation. In the samples irrigated with the different irrigants, a qualitative assessment of areas of biofilm left behind after the irrigation was carried out. Areas of biofilm were compared between Group I, Group II, Group III and Group IV. Sodium hypochlorite treated samples (Group I) showed minimal areas of biofilm. Samples treated with

Withania somnifera (Group III) showed more areas of biofilm than that of hypochlorite group (Group I), but less than that treated with

Triphala(Group II). Samples treated with combination of *Withania somnifera* and triphala (Group IV) showed fewer areas of biofilm than that of Group II and Group III.

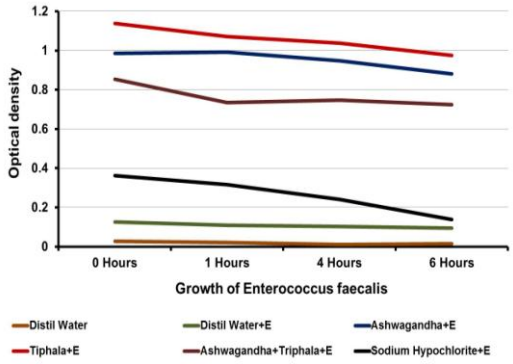

Fig. 3: Optical density reading showing growth curve of Enterococcus faecalis in various solutions.

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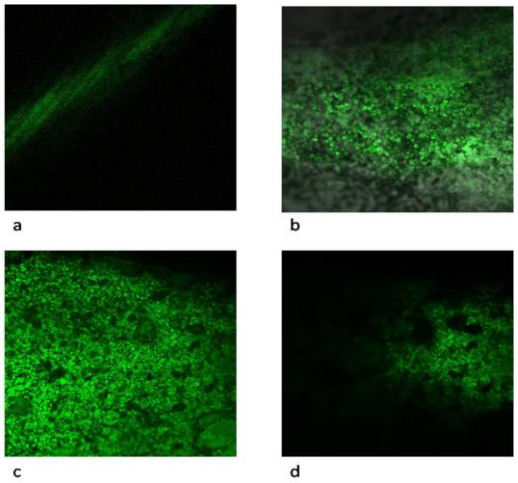

Fig. 4: Photomicrographs of laser confocal microscope after irrigation

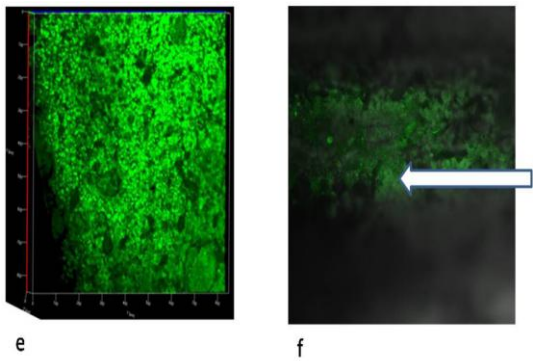

Fig. 5: Photomicrographs of laser confocal microscope prior to irrigation.

DISCUSSION

The purpose of present study was to investigate whether triphala and *Withania somnifera* could be used as alternative or more beneficial antimicrobial agents than sodium hypochlorite against E. *faecalis*. Sodium hypochlorite is considered to be an ideal irrigant, but it causes severe inflammatory reaction when extruded into the periapical tissues [19]. In the present study, after 45 days of incubation, a biofilm matrix of 80µm thickness was observed. Distel et al. in their study estimated the thickness of biofilm formed in root canal as 28 to 30µm after 160 days of incubation and 21µm after 86 days of incubation [20]. Wood et al. reported 75 to 220µm thickness of biofilm on tooth surface after 4 days [21]. Therefore, *E. faecalis* biofilm formation and growth are dependent on the type of bacterial isolate, environmental and nutritional conditions [22]. It was shown that *E. faecalis* was isolated from root canals, even after the treatment with calcium hydroxide for 77 days [20]. In our study, irrigation with sodium hypochlorite has significantly decreased the *E. faecalis* count which is in agreement with previous reports [21, 22].

In the present study, irrigation with Triphala extract also has shown its antibacterial property by decreasing the *E. faecalis* count though it is not as significant as sodium hypochlorite group. Antibacterial activity of Triphala is due to the presence of tannic acid which is bacteriostatic and bacteriocidal [14]. In this in-vitro study, Triphala was least effective when compared to other irrigant groups. This is in agreement with a previous study where Triphala was effective against three week old biofilm, but was ineffective against six week old biofilm [14]. This may be due to development of bacterial resistance to the drug [14].

In the present study, irrigation with *Withania somnifera* extract has shown its antibacterial property by decreasing the *E. faecalis* count though it is not as significant as sodium hypochlorite group. *Withania somnifera* has been shown to be effective against micro-organisms and reported to possess anti-inflammatory properties thereby preventing protein denaturation [23]. Its antibacterial property may be due to presence of alkaloids in it [18]. Other components like ethyl acetate, methanol, tetracycline and chloramphenicol present in the plant extract might also help in bacterial eradication [22]. Ingredients like steroidal alkaloids and lactones known as withanolides might also be responsible for antibacterial activity. An earlier study has reported that minimum inhibitory concentration of *Withania somnifera* is 40mg/ml [24]. However, since bacteria in biofilms have higher degree of resistance, 80mg/ml leaf extract of *Withania somnifera* was used in the present study.

When the combination of Triphala and *Withania somnifera* were used in equal concentrations, there was significant reduction in *E faecalis* counts as compared to that of group treated with Triphala alone and the group treated with *Withania somnifera* alone. This may be due to their synergistic activity. The antimicrobial activity of *Withania somnifera* assisted by similar property of Triphala might have helped to reduce the *E faecalis* count.

The inability of combination of Triphala and *Withania somnifera* to decrease the *E faecalis* count as effective as sodium hypochlorite may be due to longer incubation period, where the bacteria might have produced serine protease, gelatinase and collagen binding protein which help them to bind to dentin and penetrate deep inside the tubules [25, 26]. The above combination of antimicrobial agents may not have penetrated deep inside the tubules. Though use of sodium hypochlorite as an antimicrobial agent is well established, precautions need to be taken to prevent its extrusion into the periapical tissues and their inflammation [19]. On the other hand, Triphala and *Withania somnifera* are proven to be non toxic and are also known to contain active constituents that have beneficial physiological effect, antioxidant, anti-inflammatory and radical scavenging activity [13, 27].

CONCLUSION

Sodium hypochlorite, Triphala and *Withania somnifera* failed to eliminate bacteria completely. But, considerable reduction in growth of *E. faecalis* was seen in the herbal extract groups though it was not as effective as sodium hypochlorite group. Considering the non-toxic nature and other physiological benefits of these herbal extracts, further studies need to be carried out to consider them as an alternative for sodium hypochlorite at least in the initial stages of bacterial infection.

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