Original Article

Comparative Evaluation of Antimicrobial Efficacy of Herbal Formulations of Septilin and Triphala with Conventional 2% Chlorhexidine on Root Canal and Oral Commensal Bacteria using Kirby Bauer Method: An *in-vitro* Study

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Abstract

Background: Endodontic flareups are always very difficult to treat because of reasons like drug resistance previously sodium hypochlorite and chlorhexidine have been used in the literature but with advancements newer materials like herbal products have been tried for this purpose. Thus, present vitro study was conducted to comparatively evaluate the efficacy of herbal formulations on commensals of oral cavity and root canal. **Materials and Methods:** In this *in vitro* study antimicrobial efficacy of chlorhexidine 2%, Septilin and Triphala wer evaluated with the help of Kirby Bauer test. The microorganisms used were *Staphylococcus aureus* and *Escherichia coli*. The samples were placed in standardized wells and were subjected to incubation. Subsequently zones of inhibition were measured with the help of a caliper. One-factor analysis of variance, Tukey's least significant difference *post hoc* test and Student's independent *t*-test were performed to find a significant difference ($P < 0.05$) in the two groups. **Results:** For chlorhexidine in *S. aureus* mean antibacterial efficacy was 11.10 ± 1.25 and in *E. coli* 10.0 ± 1.33. The value for *S. aureus* was lesser in Triphala with a value of 10.35 ± 1.63 and in *E. coli* mean value was 9.05 ± 1.27. For Septilin the mean for *S. aureus* was 10.40 ± 1.04, and the mean antibacterial efficacy in *E. coli* was 9.65 ± 1.38. **Conclusion:** Septilin and Triphala showed remarkable efficacy concerning zones of inhibition. These herbal formulations have tremendous potential to be used as adjuncts to traditional disinfection modalities though it has scope of further research.

Keywords: *Antimicrobial efficacy, escherichia coli, kirby bauer test, septilin, staphylococcus aureus, triphala*

Introduction

Primary endodontic infections are caused by a highly pathogenic oral microorganism that acts upon the necrotic tissue within the pulp and creates an invasive pathway that leads to further progression of the infection. Following a peri-radicular infection there is a rapid increase in the quantity of available anaerobic bacteria when the root canal is infected for prolonged periods. As the microorganisms thrive in this unfavourable environment evolution of genetic features takes place which leads to the formation of biofilms leading to the development of resistance to antimicrobials and persistence of species even after root canal therapy.[1-4]

Pathogenic oral microflora play important role in peri-radicular infection sequelae. One of the predominant species of oral cavity often found in endodontic flareups are staphylococcus and enterococcus species. Staphylococcus species are among those species which are commonly found in endodontic flare-ups, often overlooked in comparison to Enterococcus faecalis, they are one of the most persistent species which remain in areas that have not been disinfected appropriately. This can range from regularly used endodontic armamentarium as well as in cases of repeated flare-ups despite conventional cleaning and shaping.[4,5] Another counterpart of the Enterococcus species is the *Escherichia coli*. Although being a transient colonizer of the oral microflora it has frequently been discovered in cases of endodontic failures along with Enterococcus species.[6]

How to cite this article: Ahmed S, Shahnawaz K, Mandal TK, Ghafir M, Gummaluri SS, Vishal G. Comparative evaluation of antimicrobial efficacy of herbal formulations of septilin and triphala with conventional 2% chlorhexidine on root canal and oral commensal bacteria using kirby bauer method: An *in-vitro* study. Contemp Clin Dent 2022;13:383-8.

Submitted : 31-May-2021
Revised : 26-Oct-2021
Accepted : 26-Dec-2021
Published : 08-Nov-2022

Access this article online
Website: www.contempclindent.org
DOI: 10.4103/ccd.ccd_423_21
Quick Response Code:

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Sodium hypochlorite despite being one of the most frequently used endodontic irrigants with remarkable antibacterial effect still has several harmful drawbacks due to which many alternative options are also being researched. Two percent Chlorhexidine has been a time tested intracanal medicament and irrigant which has worked as a suitable adjunct in place of sodium hypochlorite.[7-10]

With advancement of diagnostic modalities, aberrant root canal anatomy and development of antibiotic resistant biofilms, susceptibility of dentin to chemicals and several other factors makes the existing treatment modalities challenging.[11-14] Thus, there is a scope to search and develop new alternatives that can act as adjuncts to existing standard materials.[7,8] One such field is that of phytotherapy which utilises a combination of plant based herbs to formulate an effective, natural and safer alternative.[15]

Septilin is a herbal formulation consisting of Glycyrrhiza glabra, Embelica officinalis and Rubia cordifolia. Phyllanthus emblica possess antioxidant and free radical scavenging activities and extract of Rubia cordifolia has a function to restore glutathione levels after oxidative stress. It acts to improve the defense mechanisms of the body as an immunomodulator. Studies have shown Septilin's efficacy in inhibiting a wide variety of microorganisms.

Triphala is a plant-derived composition of three dried plants known as Terminalia Bellirica, Terminalia chebula, and Emblica Officinalis, with one of its key components being tannic acid along with various acids such as gallic acid, ellagic acid, and chebulinic acid, which are suitable antioxidants helping as immunomodulators. The bacteriostatic or bactericidal effects of tannic acid on gram-positive and negative microorganisms have been demonstrated in studies. It also has properties that are antioxidant and anti-inflammatory in nature.

Triphala and Septilin has numerous flavonoid derivatives, with oxides such as Nitric Oxide which show good antimicrobial and anti-inflammatory activity in various cardioprotective and neuroprotective mechanisms. They also help in decreasing the levels of cyclooxygenase-2 enzymes which aid in anti-inflammatory action.

Hence these formulations were comparatively evaluated with 2% Chlorhexidine to test antimicrobial efficacy using kirby bauer well diffusion method to formulate a basis for further research.[16,17]

Materials and Methods

Sample size estimation and ethical clearance

Based on G Power software version 3.0, when power of the study was kept as 80%, effect size of 0.25 and alpha value of 5% a sample of 18 was sufficient for the conduction of study. So, required was 108 in total. For better outcome of results 20 sample size was considered. Thus 120 samples were taken for the completion of study (two bacteria and three materials). Prior to the conduction of study, ethical clearance was obtained from institutional review board of Institute of Dental Sciences, Bareilly with number IDS/ETHCC/17/16 (November 15, 2017). Study was performed according to Helsinki Declaration 1975 revised in 2000. As it was an *in-vitro* study which not involves any patient, there was no requirement of written informed consent from patients.

Material and bacteria procurement and culture media used

E. coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 35668) strains were procured from the stock cultures of the Department of Microbiology, Rohilkhand Medical College and Hospital, Bareilly International University, Bareilly. The culture media utilized in this research was Mueller Hinton Agar (Hi media). Kirby Bauer disk diffusion method has been extensively used as a preliminary assessment to evaluate the antimicrobial efficacy of several materials.

Before a medication or formulation can be manufactured or prescribed it has to be determined if the medication works against the host pathogens. To do this, basic research modalities are used including phenotypic methods such as Kirby-Bauer susceptibility testing. It is a diffusion test which utilises a suitable culture media to grow the microorganisms and streak it on culture plates following which small concentrations of test materials are placed in small circular wells prepared on the culture plate. After overnight incubation circular zones are observed around the wells which are known as zones of inhibition. These zones are measured and data is tabulated and analysed statistically.

Culturing procedure and measuring the inhibition zones

A disinfected circular loop was used to procure the colonies for inoculum preparation and a direct colony suspension of each test isolate was prepared using peptone water and the turbidity was adjusted accordingly to 0.5 ml McFarland standardized scale. The culture plates were retained at room temperature for 2 h to permit the dispersal of the contents throughout. Under a pertinent ambiance, the specimens were placed in an incubator for 24–48 h with a temperature of 37°C (degree centigrade).(Yorco Bacteriological Incubator, yorco sales Pvt. Ltd).

After drying of the agar plate a sterile cotton swab stick was immersed into the adjusted suspension and this swab

was applied onto the agar surface in a streaking fashion with repetitions to evenly coat the agar surface and ensure proper distribution of the inoculum, this procedure is referred to as lawn culturing.

The culture plates were incubated at 37°C for 24 h to impart a suitable environment for the cultivation of the microorganisms, following which the culture plates were transferred to a laminar air flow chamber to prevent contamination and maintain an aseptic environment. Five wells of standardized diameter were prepared with help of Durham's tube, systematically placed equidistantly from each other to avoid conjoining of subsequent zones of surrounding wells upon each other. Three wells were designated for the test materials and two for control in which normal saline and distilled water was placed.

Minimum inhibitory concentration and minimum bactericidal concentration values were regulated by performing triplicate test tube dilution methods for the test samples. The test solution used was <0.1% concentration which was regarded as the lowest concentration allowed to cultivate on the medium.

Triphala (The Himalaya Drug Company) solutions were prepared from powder form by utilizing 10% dimethyl sulfoxide for dissolution and filtration process. Septilin (The Himalaya Drug Company) was available in form of solutions and tablets. The solutions were stored in airtight sample collection tubes. Two percentage Chlorhexidine (Ammdent) solution along with other test materials were then transferred to their respective wells using circular loops and pipettes.

The culture plates (n = 120) [Figure 1] were divided into a total of three groups (twenty/group) according to division of standard comparison group of 2% Chlorhexidine (Ammdent) and 2 test groups comprising of herbal formulations, Triphala (The Himalaya Drug Company) and Septilin (The Himalaya Drug Company).

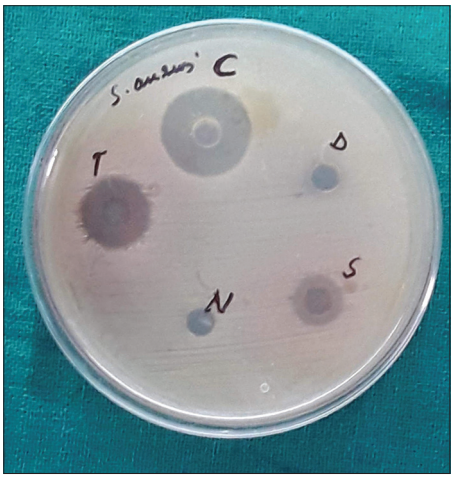

Figure 1: Depicts the image of culture plates containing the inhibition zones of all the groups

Culture plates were incubated at 37°C temperature under required humidity and gaseous environment for 24 h, and subsequently 48 h.

The inhibitory zone is contemplated to be the shortest measurement of length from the exterior circumference of the circular well to the preliminary site of microbial growth. Regions depicting the suppression of microbial expansion around the circular well depicts the zone of inhibition. Measurements of zones of inhibition were recorded with help of an electronic caliper and readings were subjected to statistical analysis.

Statistical analysis

Entire data gathered was transferred to Microsoft excel spread sheet. Data were summarised as Mean ± (standard deviation). The analysis was performed on Statistical Package for Social Sciences (SPSS) version 22.0, IBM, Pvt Ltd, Chicago, USA. One-factor analysis of variance (ANOVA) was used to compare the groups and the significance of the mean difference between (inter) the groups was done by Tukey's (Least significant difference) *post hoc* test. Further Student'S independent *t*-test was performed to find a significant difference in the two groups. $P < 0.05$ was considered statistically significant.

Results

In the group one consisting of Chlorhexidine, zones of inhibition were observed in both *S. aureus* and *E. coli*. In *S. aureus* mean antibacterial efficacy was 11.10 ± 1.25 and in *E. coli* 10.0 ± 1.33. In comparison to group two consisting of Triphala which was known for its antibacterial properties showed notable zones of inhibition with more amount of diffusion among microorganisms. The mean antibacterial efficacy in *S. aureus* was lesser in Triphala with a mean value of 10.35 ± 1.63 and in *E. coli* mean value of 9.05 ± 1.27 [Figure 1]. In group three, Septilin was considered, where the mean antibacterial efficacy in *S. aureus* was 10.40 ± 1.04, and the mean antibacterial efficacy in *E. coli* was 9.65 ± 1.38 [Table 1].

During the intergroup comparison there was no significant difference between the two groups with one way ANOVA

Table 1: The comparison of mean and difference of antibacterial efficacy in *Staphylococcus aureus* and *Escherichia coli*

Bacteria	Analysis	Sum of squares	df	Mean square	*F*	*P*
Staphylococcus	Between groups	7.033	2	3.517	1.982#	0.147
	Within groups	101.15	57	1.775		
	Total	108.18	59	-		
Escherichia coli	Between groups	9.233	2	4.617	2.593#	0.084
	Within groups	101.50	57	1.781		
	Total	110.73	59	-		

*P<0.05 consider statistically significant. #P>0.05 consider statistically non-significant

(*S. aureus* and *E. coli*) [Table 2]. However, there was a statistically significant difference in mean antibacterial efficacy recorded in *S. aureus* and *E. coli* for Triphala and Chlorhexidine, with a $P = 0.08$ in Triphala and 0.011 in Chlorhexidine respectively using student independent *t*-test [Table 3].

Discussion

The present study comparatively evaluates the efficacy of herbal formulations of Septilin and Triphala with 2% Chlorhexidine on commensals of oral cavity and root canal. In the first group consisting of 2% Chlorhexidine, culture testing against *S. aureus* and *E. coli* was performed. In *S. aureus* mean antibacterial efficacy was 11.10 ± 1.25 and in *E. coli* 10.0 ± 1.33.

Table 2: Comparisons of mean antibacterial efficacy in *Staphylococcus aureus* and *Escherichia coli* by student independent *t*-test

Group	*n*	Mean±SD	*P*
Triphala (S)	20	10.35±1.63	0.008*
Triphala (E)	20	9.05±1.27	
Septilin (S)	20	10.40±1.04	0.061
Septilin (E)	20	9.65±1.38	
CHX (S)	20	11.10±1.25	0.011*
CHX (E)	20	10.00±1.33	

*$P<0.05$ consider statistically significant. SD: Standard deviation; CHX: Chlorhexidine

Table 3: The multiple comparisons with *post hoc* least significant difference in all three groups with two bacteria

Dependent variable	Mean difference (I−J)	SE	*P*#	95% CI (lower bound-upper bound)
Staphylococcus aureus				
Triphala				
Septilin	0.200	0.531	0.709	−0.87-1.27
CHX	−0.533	0.531	0.321	−1.61-0.54
Septilin				
Triphala	−0.200	0.531	0.709	−1.27-0.87
CHX	−0.733	0.531	0.175	−1.81-0.34
CHX				
Triphala	0.533	0.531	0.321	−0.54-1.61
Septilin	0.733	0.531	0.175	−0.34-1.81
Escherichia coli				
Triphala				
Septilin	−0.667	0.509	0.198	−1.69-0.36
CHX	−0.867	0.509	0.096	−1.89-0.16
Septilin				
Triphala	0.667	0.509	0.198	−0.36-1.69
CHX	−0.200	0.509	0.697	−1.23-0.83
CHX				
Triphala	0.867	0.509	0.096	−0.16-1.89
Septilin	0.200	0.509	0.697	−0.83-1.23

*$P<0.05$ consider statistically significant. CI: Confidence interval; CHX: Chlorhexidine; SE: Standard error. #$P>0.05$ considered statistically non-significant

Consistent results of chlorhexidine were in accordance to study conducted by Singh M *et al*. where chlorhexidine was compared with sodium hypochlorite and other herbal formulations on *Enterococcus feacalis* and *Candida albicans*.[18] Stewart HL *et al*. evaluated antimicrobial properties and safety of 0.0005% chlorhexidine solution in upper respiratory tract of normal horses.[19] This is in co-occurrence with its efficacy against *E. coli*. A study by Inoue BS *et al*. on chlorhexidine with bacterial cellulose membrane on *S. aureus* and *E. coli* showed effective inhibition of microbial growth in concurrence with the findings in this study.[20]

In the second group there was a decrease in mean antibacterial efficacy in triphala on comparison of *S. aureus* 10.35 ± 1.63 to *E. coli* 9.05 ± 1.27 [Figure 1].

In vitro studies conducted by Susan AC *et al*. on intra-radicular smear removal efficacy of triphala as a final rinse solution in curved canals showed promising efficacy which is in accordance with this study.[21] In a study using agar diffusion by Al-Sabawi *et al*., it was reported that Residual antibacterial effect of calcium hydroxide combined with chlorhexidine gel as an intracanal medicament showed good antimicrobial efficacy in combination.[22]

It acts as an effective standard group to compare with due to its time tested antimicrobial properties and routine use in endodontics Studies by Prabhakar *et al*. have shown the effectiveness of Triphala as a potential irrigant in inhibiting Enterococcus faecalis biofilms of three and 6 weeks in comparison with sodium hypochlorite. Studies have shown the effectiveness of Triphala as a potential irrigant in inhibiting Enterococcus faecalis biofilms of three and 6 weeks in comparison with sodium hypochlorite.[23]

Satti P *et al*. compared cytotoxic activity of triphala and liquorice which is a component of septilin on human periodontal ligament cell fibroblasts showing no cytotoxic effects in comparison with sodium hypochlorite. In the present study the remarkable antimicrobial efficacy demonstrated by triphala is in concurrence with the studies.[24]

The third group comprised of septilin was considered, where the mean antibacterial efficacy in *S. aureus* was 10.40 ± 1.04, and the mean antibacterial efficacy in *E. coli* was 9.65 ± 1.38 [Table 1]. Septilin which is a polyherbal formulation consisting of balsamodendron powders, Tinospora Cordifolia, Rubbia Cordifolia, Emblica Officinalis, Moringa Pterigosperma, and Glycyrrhiza Glabra. It acts to improve the defense mechanisms of the body as an immunomodulator.

Studies by Daswani and Yegnanarayan evaluated the Immunomodulatory activity of septilin and efficacy in inhibiting a wide variety of microorganisms which is in accordance with mean antibacterial efficacy shown by septilin.[16] Rai A *et al*. evaluated the efficacy of Tinospora

cordifolia (Guduchi), and Glycyrrhiza glabra (Licorice) extracts on *streptococcus mutans* and *Lactobacillus acidophilus*, they are among the chief components present in septilin. The antibacterial efficacy shown by these two components are in concurrence with the study.[25]

Research on root canal microflora has a historical significance W. D. Miller who was regarded as the father of oral microbiology collaborated with the findings of Van Leeuwenhoek and investigated the association of microorganisms with pulpal diseases.[2,3]

Recent emergence of the term biofilm which was conceptualized to describe aggregations of various microorganisms has formed the basis for several research studies.[14] Staphylococcus has persevered as one of the prominent community pathogen with recent findings showing heterogeneous populations, within the biofilms with a subset of cells contributing to the development of antibiotic resistance.[5] *E. coli* are among the transient colonizers of the oral cavity which are more common in the Asian population. Strains of Enterococcus and *E. coli* species have been frequently discovered in cases of endodontic failures and periodontal disease conditions.[6]

Chemo mechanical measures along with intracanal irrigants were recommended as adjuncts to the other canal preparatory methods though successful they came with drawbacks.[8,11] One such alternative field to overcome drawbacks of therapeutic agents is that of phytomedicine or phytotherapy which is a science-based therapy utilizing a single herb or a synergistic mixture of several herbs. Herbal therapy has been an ancient traditional practice, recently there has been a renewed interest in the study of medicinal plants and herbs in search of alternatives.[15]

Triphala is a plant-derived composition of three dried plants known as Terminalia Bellirica, Terminalia chebula, and Emblica Officinalis, with one of its key components being tannic acid. The bacteriostatic or bactericidal effects of tannic acid on gram-positive and negative microorganisms have been demonstrated in studies. It also has properties that are antioxidant and anti-inflammatory in nature.[17,21] In a study conducted by Penmetsa *et al*. triphala was found to be effective in the reduction of plaque, gingival inflammation, and bleeding and almost at par with chlorhexidine.[26] The success of this material is in accordance to its selection in this study.

Septilin is a multi-herbal formulation commonly used for upper and lower respiratory tract infections it has immunomodulatory, antioxidant, anti-inflammatory, anti-allergic, antimicrobial actions. Its Components such as Glycyrrhizin potentiates the reticuloendothelial system, enhances immunostimulation, and acts on macrophage function *in vitro*, leading to stimulation of macrophages de novo, Beta-glycyrrhetinic acid is a potent inhibitor of the classical complement pathway and antiviral activity Daswani and Yegnanarayan evaluated the Immunomodulatory activity of septilin.[16] Shetty *et al*. conducted a clinico-biochemical evaluation of the role of septilin as an adjunct immunomodulator in chronic periodontal disease. It was found to be a safe and effective immunomodulator as an adjunct to routine periodontal therapy, however further studies on larger population was recommended. Which is in accordance with purpose and limitations of this study that septilin being a newer material in dentistry is being researched and has potential.[27]

Singh M *et al*. conducted an *in vitro* comparative evaluation of antimicrobial efficacy of propolis, morinda citrifolia juice, sodium hypochlorite and chlorhexidine on enterococcus faecalis and *C. albicans* using a culture dependent method which is in accordance to the methodology used in this study.[18]

Limitations of the present study are that limited number of microorganisms were selected for the study. Culture based methods are often unable to simulate the growth conditions required by fastidious bacteria that thrive on specific environmental conditions and nutritional requirements, because of this several bacterial species are difficult or impossible to culture and there is an emergence of uncultivable species involved in endodontic infections. With advancements in diagnostic modalities in microbiology such as polymerase chain reaction help in identifying the species more accurately. The root canal microbiota is different from lab conditions comprising of several species of microorganisms, further *in-vivo* trials and research would give a better exposure to the antimicrobial properties of these formulations.

Conclusion

Thus within the limitations, the present study its concluded that commercially available Polyherbal preparations of Septilin and Triphala have shown good antimicrobial properties on root canal and other oral commensal microflora. There is a tremendous scope for development of safer and cost effective plant derived adjuncts to be used along with conventional therapy. The present study has provided the preliminary research data for these relatively new materials for further research and development.

Clinical Relevance: Dentistry is a rapidly evolving field where there is always the scope of development of suitable alternatives or adjuncts to currently available materials. Herbal formulations are being researched extensively all over the world as they are naturally derived from plant based extracts, relatively cost effective and with less harmful adverse reactions. Triphala And Septilin have proven their clinical significance for a wide variety of medical disorders. Several dental research papers have shown the effectiveness of these potential herbal formulations as alternative irrigants and medicaments every

material has to go through a series of research studies to study its efficacy, which is the purpose of this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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